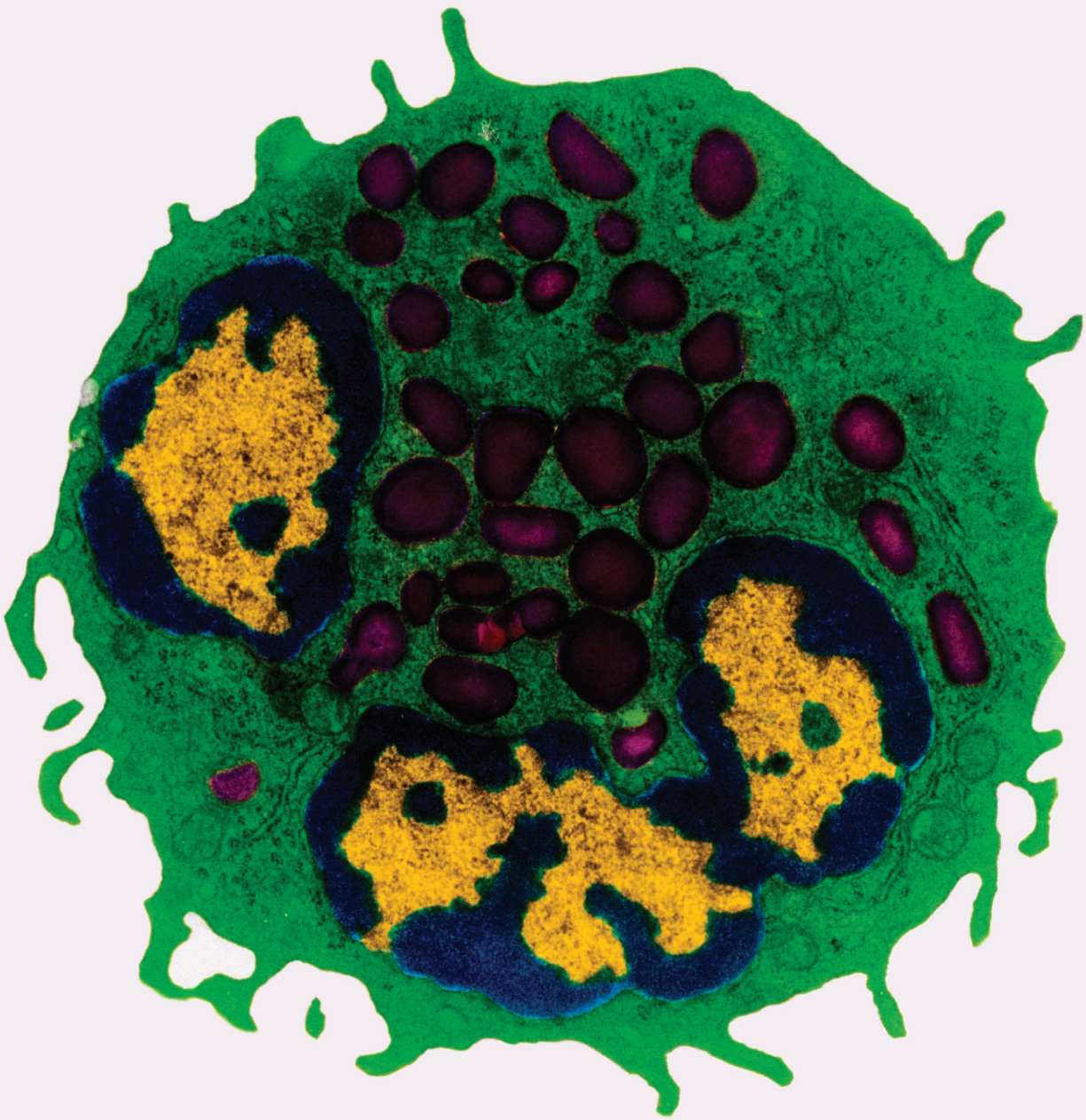
Mediators of Inflammation

Inflammatory Mediators in Periodontal Pathogenesis

Lead Guest Editor: Olivier Huck
Guest Editors: Nurcan Buduneli and Denisse Bravo



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Editorial Board

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Editorial

Inflammatory Mediators in Periodontal Pathogenesis

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This special issue is dedicated to the periodontal pathogenesis and emphasizes the complexity of the disease. This topic has received interesting original studies or review articles, and a total of 12 manuscripts were finally accepted out of the 21 manuscripts received. The selected articles address different aspects of periodontal disease, from the implication of selected cytokines or gene polymorphism on periodontitis. Among the others, the role of receptors such as TLR-4 and CXCR2 and inflammatory and immune-related markers such as CD14, OPG, RANKL, or IL-18 was demonstrated. Identification of specific inflammatory patterns may help to better understand the different phases of periodontitis onset and progression and may also lead to the development of new clinical approaches for the diagnosis and monitoring of periodontitis patients.

Systemic influence of periodontitis is also discussed in this special issue. Periodontal diseases are regarded as a significant risk factor for various systemic diseases such as cardiovascular diseases, diabetes, or adverse pregnancy outcomes. Here, some of the suggested links are discussed with innovative approaches. Indeed, F. Ceccarelli et al. detailed the potential molecular mechanisms underlying the association between periodontitis and rheumatoid arthritis from genetic factors and autoantibodies to inflammatory biomarkers, while S. Schulz et al. emphasize the association of genetic variations in proinflammatory cytokines (TNF-α, IFN-γ) and cytokine receptor (IL4Rα) in rheumatoid arthritis and periodontal diseases. Another article by A. Hoare et al. presented the role of chronic inflammation driven by periodontitis-associated bacteria on the development of oral and extraoral carcinogenesis. These data clearly emphasize the importance of periodontal diagnosis and appropriate treatment not only for a healthy dentition but also for a systemic health.

Different treatment procedures are also investigated in an attempt to correlate periodontal treatment outcomes and systemic levels of inflammatory biomarkers. The effect of Er,Cr:YSGG and diode lasers on IL-37 and IL-1β levels was analyzed by A. C. Talmaç et al. in the context of aggressive periodontitis treatment. The role of CEMP-1 during the early phase of healing was analyzed by C. Dellavia et al., and C. Petit et al. reviewed the pleiotropic effects of statins and their potenital interest in the management of periodontal diseases.

Periodontal diseases are among the most common chronic inflammatory and infectious diseases worldwide, and we hope that this special issue brings new insights into the complex mechanisms driving the inflammatory processes associated with such bacteria-elicited disease. These valuable data may help to develop new diagnostic tools and therapeutic strategies based on the control of the inflammatory and immune responses acting in the

pathogenesis of periodontal diseases. Moreover, it can be suggested that a closer collaboration between dental professionals and physicians may help to improve the health as a whole.

Conflicts of Interest

There is no conflict of interest related to this editorial.

Olivier Huck
Nurcan Buduneli
Denisse Bravo

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Research Article

Effects of Er,Cr:YSGG and Diode Lasers on Clinical Parameters and Gingival Crevicular Fluid IL-1β and IL-37 Levels in Generalized Aggressive Periodontitis

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Guest Editor: Denisse Bravo

Aim. The objective of the current study is to analyze the correlation between cytokine levels and periodontal parameters in aggressive periodontitis patients before and after periodontal treatment that was performed by using two different laser therapies. *Materials and Methods*. Twenty-six generalized aggressive periodontitis patients were treated with three different methods (SRP, SRP+diode laser, and SRP+Er,Cr:YSGG laser) applied to three different half-jaws in the same patients. Pre- and posttreatment clinical periodontal parameters and GCF IL-1β and IL-37 levels were measured. *Results*. There was a statistically significant decrease ($p < 0.05$) between pretreatment and posttreatment clinical periodontal parameters and IL-1β and IL-37 levels. When the reduction rates of IL-37 and IL-1β levels after treatment were evaluated, the decrease in IL-37 and IL-1β levels after treatment was lowest in the SRP group and highest in the SRP+Er,Cr:YSGG group. In addition, the amount of decrease in IL-1β in SRP+diode and SRP+Er,Cr:YSGG groups was found to be higher than that in IL-37. Furthermore, there was a positive correlation between IL-37 and IL-1β in all groups ($p < 0.01$). *Conclusion*. Er,Cr:YSGG laser is more effective than diode laser for the treatment of aggressive periodontitis. IL-37 and IL-1β are cytokines that function together and thus must be evaluated together.

1. Introduction

Aggressive periodontitis (AgP) is a periodontal disease that is mostly observed in young individuals and characterized by rapidly advancing periodontal tissue destruction and shows genetic predisposition [1]. In a recently published periodontal disease classification criteria, "Classification of Periodontal and Peri-Implant Diseases and Conditions 2017," aggressive periodontitis was combined with chronic periodontitis to form a single periodontitis category. In this classification, periodontitis characterization is based on the multidimensional staging and grading system [2]. Maintenance of oral hygiene by the patient and scaling and root planing (SRP) processes are the gold standards during treatment [3]. However, since the pathogens have the ability to invade soft tissue, they can persist even after the mechanical treatment. The presence of pathogens in the tissue can reduce the success rate of the treatment and could result in recurrence of the disease [4]. Thus, new approaches are developed for the treatment of aggressive periodontitis, one of which is laser-based therapy.

Use of lasers in periodontology have several advantages such as less pain, less edema, and faster wound healing compared with periodontal surgery. In addition, the laser has bactericidal activity in the application area [5, 6]. Thus, it is advantageous compared to the antibiotic treatment that is performed in addition to the periodontitis treatment, since laser use does not cause bacterial resistance to antibiotics [7]. Therefore, soft tissue lasers such as Erbium, Chromium: Yttrium Scandium Gallium Garnet (Er,Cr:YSGG) laser and

diode laser are widely used in different periodontal operations, including the treatment of aggressive periodontitis [6, 8]. The efficacy of Er,Cr:YSGG and diode laser in the treatment of aggressive periodontitis has been previously demonstrated [8–10]. In these studies, one of the parameters to evaluate the treatment success is cytokine levels in the gingival crevicular fluid (GCF) [11, 12], since periodontopathogens and virulence factors result in fast inflammatory and immune responses [13].

The initial response of periodontal tissues to the attack of periodontopathogens is the release of some mediators such as cytokines, kinins, and matrix metalloproteinases (MMPs). This tissue response determines the course of the disease [13, 14]. Cytokines play important roles during the inflammatory response after the tissue destruction and during the initiation, regulation, and continuation of the immune response in periodontal diseases [15]. The cellular responses against proinflammatory cytokines whose effects are restrained by anti-inflammatory cytokines and the equilibrium between these two cytokine types are important in the formation of the inflammatory response [16, 17]. In the GCF samples obtained from the periodontal tissues that showed inflammatory responses, the proinflammatory cytokine levels are higher than the levels in the GCF from the healthy regions [18]. In addition, cytokines are known to have direct and indirect roles in tissue destruction [19, 20]. Therefore, the cytokine response has been suggested to be an important parameter for the pathogenesis of periodontal diseases [21]. Cytokines that are known as innate immunity cytokines such as IL-1, IL-6, and TNF-α and IFN-γ, IL-4, IL-10, IL-12, IL-17, IL-18, and IL-37 are some of the known proinflammatory and anti-inflammatory cytokines that have been studied in relation to periodontal diseases [22–32].

One of the proinflammatory cytokines causing periodontal tissue destruction is interleukin-1β (IL-1β). IL-1β is an important mediator of the inflammatory response and the pathophysiology of periodontitis and is associated with cell proliferation, differentiation, and apoptosis. It is regarded as a strong gingival crevicular fluid (GCF) biomarker for many parameters, such as severe clinical inflammation, bone destruction, and the progression of periodontal disease. Studies have shown a strong relationship between the severity of periodontal disease and IL-1β levels in the gingiva and GCF [33–37]. Another cytokine that is currently widely researched in relation to the inflammatory diseases is IL-37. IL-37, also known as IL-1F7, is one of the 6 new members of the IL-1 family. Although IL-37 is known to function in the inflammation response, its role in different tissues is not fully known [38, 39]. IL-37 was demonstrated to be an anti-inflammatory cytokine consisting of 5 subgroups and acts as a regulatory element during the inflammation response. These findings suggest that IL-37 might be an indicator of several diseases [39–41]. Although IL-37 was recently shown to be associated with inflammatory diseases and could be used as an important parameter in the prognosis of these diseases by reducing proinflammatory cytokine levels [31], the relationship between the expression and function of IL-37 and aggressive periodontitis is limited. Offenbacher et al. [39] reported that the IL-37 variants are associated not only with high inflammatory response but also with more severe clinical findings of the periodontal diseases. IL-37 has also been reported to have broad inhibitory effects on many mediators of the natural immune response, including IL-1β [41].

The aim of this study was to determine the efficacy of two different lasers applied in addition to periodontal treatment in generalized aggressive periodontitis patients and to investigate their effects on GCF cytokine levels before and after treatment.

2. Materials and Methods

2.1. Study Population. A total of 30 subjects, who were treated at the Yuzuncu Yil University Faculty of Dentistry, Department of Periodontology Clinics in 2014-2015, were enrolled in this study; however, 4 patients were excluded due to poor oral hygiene and the lack of compliance with the recommendations. All procedures were approved by the Human Ethics Research Committee of Yuzuncu Yil University (no. B.30.2.YYU.0.01.00.00/44-100912). Diagnoses were made after their clinical and radiographic examinations. It was ensured that the individuals who were included in the study did not have any systemic diseases; were not menopausal, pregnant, or lactating; had not used antibiotics or any other medication affecting the immune system in the previous six months; were nonsmokers; and had not received any periodontal treatment in the previous 6 months. All subjects were informed about the aim and content of the study by a clinician and signed an informed consent form stating that they voluntarily participated in the survey. Each individual read the Helsinki Declaration before joining the study.

2.2. Criteria for the Diagnosis of Generalized Aggressive Periodontitis. 26 patients (12 female and 14 male) aged between 18 and 35 years (average 31.23 ± 7.4 years), with a clinical diagnosis of generalized aggressive periodontitis, were selected for the study. The selected patients had a minimum of 16 teeth with at least one tooth in each posterior sextant and at least one posterior sextant with a minimum of three natural teeth. The subjects also presented with ≥ 5 mm of attachment loss around at least seven teeth involved, excluding first molars and central incisors. Patients who had body mass indexes (BMI) that were higher than 25 were not included in the study. BMI values of the patients were between 18.5 and 24.5. According to "Classification of Periodontal and Peri-Implant Diseases and Conditions 2017" (Caton 2018), our patients can be included in the "Stage III and IV, Generalized, Grade C" group, based on the clinical findings, the age of onset, and the clinical course of the disease.

2.3. Clinical Measurements. Measurement of clinical parameters was performed by a calibrated clinician. Plaque index (PI) [42], gingival index (GI) [42], bleeding on probing (BOP), probing pocket depth (PPD) (mm), and clinical attachment level (CAL) (mm) were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual/distopalatinal, lingual/palatinal, and mesiolingual/mesiopalatinal) in all teeth, excluding third molars. BOP was recorded

Table 1: Diode laser and Er,Cr:YSGG laser specifications.

	Diode laser	Er,Cr:YSGG laser
Type	MZ6-14 mm	14 mm Z-6
Irradiation times	20 s	30 s
Duration of treatment session	2	2
Energy density	13.5 J/cm^2	15 J/cm^2
Laser wavelength	940 ± 15 nm	2780 μm
Methods	10 Hz, 1.5 W (150 mJ), 65% air, 55% water with H mode, 140 μs pulse length	1.5 W with a pulse interval of 20 ms and pulse length of 20 ms delivering 20 s/cm^2

as present or absent if there were signs of bleeding within 30 s after PPD and CAL measurements. Subsequently, the PPD and CAL measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA). The cementoenamel junction was detected by probing the cervical area of each tooth and was used to calculate the CAL.

2.4. Calibration of the Examiner. Calibration of the examiner was performed through a calibration exercise where the examiner measured one quadrant per subject among a group of 10 nonstudy subjects with periodontitis. Each chosen quadrant contained at least six teeth. The examiner measured PPD and CAL in the same quadrant twice with 60 min between measurements, where the same patient was probed twice during the same visit. The variability between measurements was assessed in order to analyze the intraexaminer variability, and calibration was performed through a protocol described previously [43]. The standard error of measurement was calculated to be 0.11 mm for PPD and 0.21 mm for CAL measurements.

2.5. Study Design. The study was designed as a "split-mouth" study, and all individuals received nonsurgical initial periodontal treatment. At the baseline of the study, all quadrants were shown the similarity of periodontal disease. All participants were treated with the same procedure described below, and three half jaws were randomly selected for the treatment.

(1) Only SRP group (SRP-control)

(2) SRP+Er,Cr:YSGG laser group (SRP+Er,Cr:YSGG)

(3) SRP+940±15 nm diode laser group (SRP+diode)

2.6. The Sampling of the Gingival Crevicular Fluid. Before collection of the GCF samples, the supragingival plaque around the probing site was removed and the area was cleaned of saliva through sterile cotton rolls and buffer, after which the tissue was dried with air blowing. The GCF samples were collected from the deepest pocket that was identified after probing measurements. The samples were obtained through paper strips (PerioPaper, Oraflow, NY, USA) both at the beginning of the study and at 3 months after treatment. The uniformly cut paper strips were introduced into the tissue until a light resistance was felt in the sulcus and were kept in the area for 30 seconds. The strips that had any blood on them were excluded from the evaluation. The amount of fluid on the strips was measured by using a Periotron device (Periotron 8000, Oraflow, NY, USA), and the GCF volume was calculated by using these values. Four strips were obtained from each patient and were put into individual 1.5 mL tubes containing 500 μL of phosphate-buffered saline at pH 7.4 and were kept at -80°C.

2.7. Er,Cr:YSGG Laser and Diode Laser Application. SRP was first performed to the control quadrants of the individuals diagnosed with aggressive periodontitis, and following the SRP procedure, the Er,Cr:YSGG laser (Waterlase, Biolase, Irvine, CA, USA) was applied. Unlike the Er,Cr:YSGG laser, the 940 ± 15 nm diode laser (iLase, Biolase, Irvine, CA, USA) was applied prior in order to SRP to prevent changing the diode laser efficiency following bleeding. For the Er,Cr:YSGG laser, a 14 mm Z-6 tip (600 μm fiberoptic tip, suitable for periodontal use) marked to the depth of the pocket was used at a setting of 10 Hz, 1.5 W (150 mJ), 65% air, 55% water with H mode, and 140 μs pulse length. The total irradiation time was 30 s. The 940 ± 15 nm diode laser with MZ6-14 mm standard tip was used at a setting in continuous wave mode. The irradiation duration for 940 ± 15 nm diode laser was adjusted to 20 s (Table 1).

2.8. ELISA Measurements of IL-37 and IL-1β Levels. The IL-37 and IL-1β analysis in the GCF was conducted through the ELISA method by using commercial ELISA kits, Human IL-37 ELISA Kit (Hangzhou Eastbiopharm Co. Ltd, Hangzhou, China), and Human IL-1β ELISA Kit (AssayMax Human ELISA Kit, Assaypro, Missouri, USA). The evaluation of IL-37 and IL-1β was carried out according to the manufacturers' instructions.

Tehe test samples were placed into the wells of the ELISA plate with 100 μL of the standard solutions. The plate was incubated at room temperature for 1 h and was washed 4 times. Afterwards, IL-37 and IL-1β detection antibodies were added to each well at a volume of 100 μL. The plate was incubated at room temperature for 30 min and washed 4 times. After adding 100 μL of the color-reactive agent to each well, the plate was incubated at room temperature for 30 min and the reaction was stopped by adding 100 μL of stop solution to each well. The plate was read at 450 nm wavelength by using a microplate reader (Microplate Reader BioTek, VT, USA). GCF IL-37 and IL-1β concentrations (pg/mL) were calculated by using the dilution ratio (500 μL) divided by the GCF volume.

Table 2: Comparison of mean values and standard deviations of clinical periodontal indices at the baseline (day 0) and after the treatment (3rd month).

	Diode laser		Er,Cr:YSGG laser		SRP (control)		p values
	Day 0	3rd month	Day 0	3rd month	Day 0	3rd month	—
PI (M ± SD)	1.54 ± 0.19	1.23 ± 0.2*	1.53 ± 0.25	1.13 ± 0.1*	1.51 ± 0.2	1.26 ± 0.1*	<0.05
GI (M ± SD)	1.52 ± 0.22	1.22 ± 0.2*	1.53 ± 0.23	1.15 ± 0.1*	1.46 ± 0.19	1.35 ± 0.2*	<0.05
PPD (mm) (M ± SD)	3.99 ± 0.76	3.28 ± 0.6*	3.96 ± 0.59	3.15 ± 0.4*	3.91 ± 0.6	3.34 ± 0.4*	<0.05
CAL (mm) (M ± SD)	4.4 ± 0.76	3.69 ± 0.6*	4.56 ± 1.04	3.71 ± 0.7*	4.5 ± 0.93	3.9 ± 0.86*	<0.05
BOP% (M ± SD)	51.92 ± 19.9	26.92 ± 19*	53.84 ± 23.12	15.38 ± 15*	50 ± 18.7	28.84 ± 13*	<0.05

PI: plaque index, GI: gingival index, PPD: probing pocket depth, CAL: clinical attachment level, BOP: bleeding on probing.*Statistically different from day 0 (for each group) ($p < 0.05$).

2.9. Data Analysis. The statistical analysis was carried out using the SPSS 16 package program (SPSS Inc., Chicago, IL, USA). Descriptive statistics, such as the arithmetic means and standard deviation values, were used during the presentation and evaluation of clinical and laboratory data. The comparisons of the pre- and posttreatment values within the group were realized by the Wilcoxon test. The statistical significance of the results was assessed in a 95% reliability interval at the level of $p < 0.05$. A log transformation was performed to normalize the data, and Pearson correlation was done to compare the GCF IL-37 and IL-1β levels with a significance set at $p < 0.01$.

3. Results

3.1. Clinical Findings. 26 patients with GAgP, of whom 12 are female and 14 are males, aged 31.23 ± 7.4 years and with no systemic diseases were included in our study. Clinical periodontal indices for all of the individuals included in the study and mean values and standard deviation values regarding pretreatment (day 0) and posttreatment (3rd month) are given in Table 2. According to the results of the statistical evaluation, a decrease of the PI, GI, PPD, CAL, and BOP mean values was observed after the treatment in all three groups ($p < 0.05$) (Table 2).

3.2. Cytokine Levels. When IL-1β and IL-37 levels in GCF were evaluated, there was a statistically significant decrease in cytokine levels after treatment in all three groups ($p < 0.05$) (Figure 1) (Table 3). In the SRP group, the level of decrease in IL-1β concentration was in the range of 2-45 Pg/30 s between the baseline and 3rd month after treatment. In the SRP+diode group, the decrease level of IL-1β was in the range of 10-100 Pg/30 s, whereas in the SRP+Er,Cr:YSGG group, the decrease level of IL-1β was in the range of 22-98 Pg/30 s (Figure 2). On the other hand, the reduction level of IL-37 in the SRP group was concentrated in the range of 2-35 Ng/30 s. In the SRP+diode group, the reduction level of IL-37 was observed in the range of 7-67 Ng/30 s, whereas in the SRP+Er,Cr:YSGG group, the reduction level of IL-37 was observed in the range of 11-95 Ng/30 s (Figure 2).

Overall, after 3 months, the reduction rate of the IL-1β level was higher compared to the reduction rate of the IL-37 level in the SRP+Er,Cr:YSGG group. In addition, there was a weak positive correlation between the reduction rate of IL-1β and that of the IL-37 level. In the SRP group, the decrease in the IL-1β level at 3 months after periodontal treatment was enhanced compared to the IL-37 level. In addition, there was a strong positive correlation between the reduction rates of IL-1β and IL-37 levels. In the SRP+diode group, the decrease rate of the IL-1β level was again higher compared to the reduction rate of the IL-37 level at 3 months after periodontal treatment. In addition, there was a strong negative correlation between the reduction rates of IL-1β and IL-37 levels after treatment (Figure 2).

4. Discussions

The present study analyzes the clinical periodontal parameters and the IL-1β and IL-37 levels in the GCF in generalized aggressive periodontitis patients before and after treatment with SRP and with or without additional diode or Er,Cr:YSGG laser therapy. To the best of our knowledge, this is the first study reporting the levels of GCF IL-37 after SRP, SRP+diode laser, and SRP+Er,Cr:YSGG laser therapy in generalized aggressive periodontitis patients.

Different lasers have been used in combination with periodontal therapy [5, 44], such as the Er,Cr:YSGG and diode lasers used in this study. It has been reported that laser treatment reduces the number of periodontopathogens by focusing on the biofilm layer, removing tartar and bacterial toxins from the root surface, decreasing pocket depth by removing the sulcular epithelium and inducing the formation of new connecting epithelia, and stimulating wound healing promoting healing [45]. There are many studies on the use of diode laser in periodontology in the literature [9, 46–48]. Controlled clinical trials evaluating the treatment of moderate and deep pockets in patients with aggressive periodontitis have shown that the diode laser application in addition to SRP improves the clinical outcome of the periodontal treatment [9, 49, 50]. Although several studies have investigated the efficacy of Er,Cr:YSGG laser use in periodontology [10, 51–54], there are very few controlled clinical studies analyzing the efficacy of Er,Cr:YSGG laser in aggressive periodontitis patients [8, 55]. According to a study investigating the effects of Er,Cr:YSGG laser in patients with chronic and aggressive periodontitis, the use of this laser in addition to periodontal treatment led to a significant decrease in the number of periodontal pathogens and thus

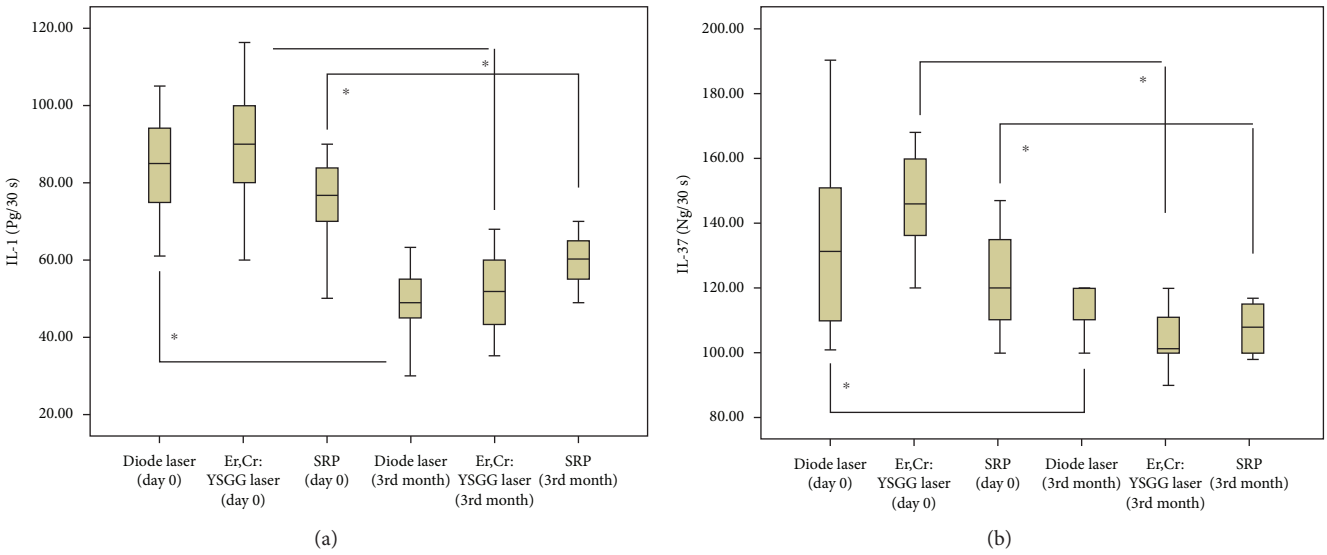

Figure 1: Comparison of cytokine levels at the baseline (day 0) and after the treatment (3rd month) between all groups.*Statistically different from day 0 (for each group) ($p < 0.05$).

Table 3: Comparison of mean values and standard deviations of cytokine levels at the baseline (day 0) and after the treatment (3rd month).

	Diode laser		Er,Cr:YSGG laser		SRP (control)		Variation between day 0 and 3rd month (p values)
	Day 0	3rd month	Day 0	3rd month	Day 0	3rd month	
IL-1β (M ± SD) Pg/30 s	84.20 ± 43.60	50.69 ± 33*	92.61 ± 56.15	53.7 ± 38*	77.5 ± 46.15	57.9 ± 41*	<0.05
IL-37 (M ± SD) Ng/30 s	131.66 ± 34.71	109.36 ± 3*	136.42 ± 34.33	100.6 ± 27.1*	125.43 ± 35.03	114.5 ± 33*	<0.05

*Statistically different from day 0 (for each group) ($p < 0.05$).

helped to maintain periodontal health [55]. Similarly, our study showed that the application of both of these lasers in addition to SRP resulted in an improvement of the clinical parameters and GCF cytokine levels.

The number of studies that have compared the efficacy of these lasers when they are used in combination with periodontal therapy is limited. In a study evaluating the effects of Er,Cr:YSGG and diode laser in aggressive and chronic periodontitis patients, Ertugrul et al. found that both lasers decreased the human β-defensin and IL-1β levels more than the SRP-alone treatment group. They also reported that Er,Cr:YSGG laser is more effective in the treatment of generalized aggressive periodontitis and chronic periodontitis compared to diode laser [8]. Similar to this study, we found that diode and Er,Cr:YSGG laser application was beneficial on the clinical periodontal parameters and GCF cytokine levels when used in addition to the classical periodontal treatment.

Cytokines play an important role in the pathogenesis of periodontal diseases [14, 56, 57]. Inflammatory cytokines are induced throughout the inflammatory response in periodontal diseases and are closely related to the onset and/or progression of periodontal disease [37, 58]. The cytokine levels were similar in the inflamed periodontal tissues of individuals with both chronic periodontitis and aggressive periodontitis. On the other hand, T-cell levels were higher and macrophage counts were lower in aggressive periodontitis patients compared to chronic periodontitis patients. However, it is very difficult to compare the cytokine responses between these diseases, because, as the disease progresses from an early stage to a more advanced stage, it is possible that there would be temporary changes in the cytokine profiles. Since only the chronic stages of chronic periodontitis are evaluated, it is possible that there is a change in the cellular or cytokine profiles of the early stages of the disease. In addition, other issues, including genetic diversity, the presence or absence of certain microorganisms, and the severity and duration of the disease, may also affect cellular populations [59, 60].

Proinflammatory cytokines increase inflammation and osteolysis in the periodontal tissues, while anti-inflammatory cytokines repress the synthesis of proinflammatory cytokines preventing or at least slowing the tissue destruction [61]. Some of these cytokines are present in the GCF and are used as disease indicators [62]. In the present study, we analyzed the levels of IL-1β, and an anti-inflammatory cytokine IL-37 in the GCF samples of periodontitis patients before and after treatment, and their association with other clinical parameters.

IL-1 is a multifactorial cytokine which exhibits strong inflammatory properties and has the ability to activate many cell types. IL-1 is mostly released by macrophages in addition

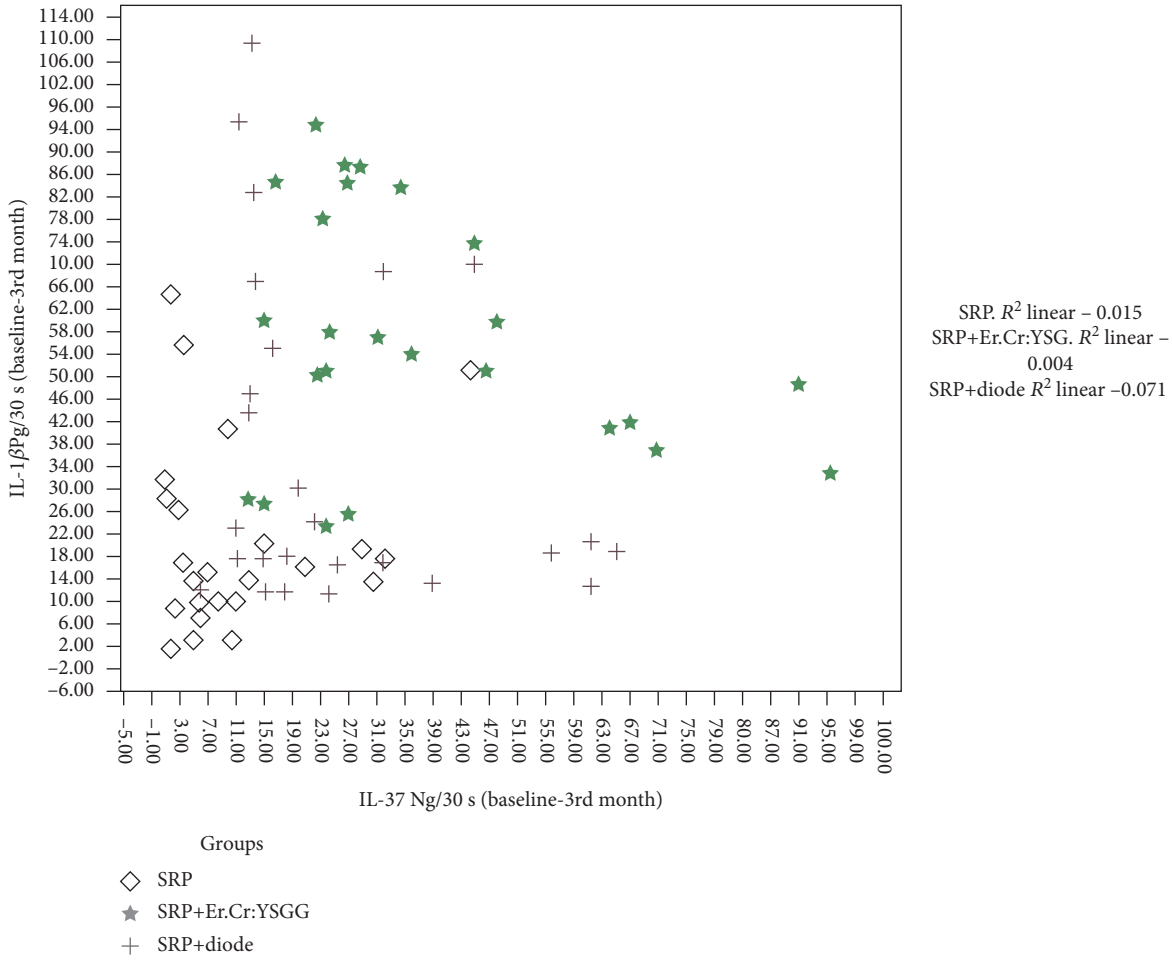

Figure 2: Correlation between the IL-37 (baseline-3rd month) and IL-1β (baseline-3rd month) levels in the gingival crevicular fluid of generalized aggressive periodontitis patients. Generalized aggressive periodontitis patients were treated with three different methods (SRP, SRP+Er,Cr:YSGG, and SRP+diode). The relationship between variables was evaluated using the Pearson correlation test ($p < 0.01$).

to monocytes [63]. IL-1 is a polypeptide that is involved in the tissue destruction and hemostasis [64] and has local and systemic effects on immune and inflammatory systems [64, 65]. While IL-1 has 11 subtypes [64], IL-1β is the most common form [66]. Although it has similar biological properties with IL-1α, IL-1β expression is 10-50 times more than IL-1α and it is much more potent [67, 68]. In their study evaluating the efficacy of Er,Cr:YSGG and diode lasers in aggressive and chronic periodontitis patients, Ertugrul et al.[8] reported that both lasers decrease the levels of GCF IL-1β. Similarly, in our study, a significant reduction in the level of GCF IL-1β was observed after treatment with both lasers.

IL-37 is the newest member of the IL-1 cytokine family. Although most of the 11 members of the IL-1 family are proinflammatory, IL-37 is anti-inflammatory [69]. IL-37 can be upregulated by proinflammatory cytokines, and its levels increase during the disease progression and decrease during healing [70]. IL-37 has 5 isoforms found in different tissues. In one study, it was found that the IL-37b (isoform1) is the dominant IL-37 isoform in the human gingival tissue. In the same study, it was also found that IL-37b levels were significantly higher in gingival tissues with periodontitis, and immunohistochemistry experiments have shown that IL-37 expression and localization was increased in gingival tissue with periodontitis, especially in the infiltrate in the connective tissue and epithelium [41]. Similarly, in our study, GCF IL-37 levels in samples from inflamed areas in aggressive periodontitis patients were found to be high before treatment, while a significant decrease was observed in the level of GCF IL-37 after treatment.

IL-37 is usually released in inflamed tissues [71]. The presence of cytokines such as IL-1β, IL-18, TNF-α, IFN-γ, and TGF-β can increase the synthesis of IL-37 in inflammatory diseases [72], which in turn suppresses the release of proinflammatory cytokines such as IL-1β, IL-16, IL-18, and TNF-α [31, 41]. These results clearly show the anti-inflammatory properties of IL-37. In the present study, GCF IL-1β and IL-37 cytokine levels were also found to be increased during inflammation. IL-37 probably increased due to elevated IL-1β cytokine levels in the inflamed periodontal tissues prior to aggressive periodontitis treatment. After the treatment, however, we observed a reduction in IL-37 levels for all 3 treatment groups, likely due to the clinical healing of periodontal tissues and the decrease of elevated proinflammatory cytokine levels.

There are many studies on the possible roles of IL-37 in inflammatory response; however, there are very few studies examining the role of IL-37 in the area of periodontology. Sağlam and colleagues [73] analyzed the correlation between the levels of IL-37 in the GCF, saliva, and blood with clinical periodontal parameters. The study consisted of 20 periodontally healthy subjects, 20 gingivitis patients, and 20 chronic periodontitis patients. IL-37 was reported to be present in the GCF, saliva, and blood; thus, this biomarker is not exclusive to periodontal tissues. In comparison among groups, it was suggested that IL-37 levels were not correlated with the GCF volume. In addition, there was no correlation between the clinical periodontal parameters and IL-37 levels in the GCF, saliva, and blood. These results suggested that IL-37 is not an effective parameter for the diagnosis of or for determining the progression of periodontal disease. In contrast to these results, our results suggest that IL-37 levels correlate with clinical periodontal parameters and IL-1β levels. In all three groups, a statistically significant decrease was observed in the posttreatment levels of IL-1β and IL-37 compared to their levels before treatment. The decrease in the levels of IL-37 in addition to the levels of other cytokines (IL-1β) in association with the clinical parameters was found to be statistically significant. We propose that there is a correlation between IL-37 and IL-1β levels and periodontal disease, and levels of IL-37 and IL-1β correlation may be an important parameter to evaluate the efficiency of the treatment of aggressive periodontal disease.

5. Conclusion

The levels of proinflammatory cytokine IL-1β, which plays a role in the pathogenesis of aggressive periodontitis, were shown to be positively correlated with the IL-37 levels. These data indicate that IL-37 may play an important role in protecting periodontal tissues from excessive inflammatory response. Therefore, IL-37 may be used as a novel treatment biomarker for generalized aggressive periodontitis diagnosis. However, further studies are needed to elucidate the regulatory mechanisms of IL-37 in the pathogenesis of generalized aggressive periodontitis.

Data Availability

All the generated or analysed data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors state that there are no conflicts of interest in connection with this article.

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Research Article

The Influence of *TLR4*, *CD14*, *OPG*, and *RANKL* Polymorphisms in Periodontitis: A Case-Control Study

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The pathogenesis of periodontitis involves a complex interaction between the microbial challenge and the host immune response. The individual immunoinflammatory response has a great contribution in the pathogenesis of the disease and becomes a trigger in the process of bone remodeling which is a characteristic of the disease. Thus, the aim of this study was to evaluate the influence of the *TLR4* A896G (rs4986790), *TLR4* C1196T (rs4986791), *CD14* C-260T (rs2569190), *RANKL* (*TNFSF11*, rs2277438), and *OPG* (*TNFSF11B* C163T, rs3102735) polymorphisms in periodontitis. A case-control study was conducted on patients with periodontitis ($N = 203$) and controls ($N = 213$) over 30 years of age, without diabetes mellitus, acute infections, and osteoarthritis, and patients without aggressive periodontitis, i.e., stage IV and C degree of periodontitis, and any periodontal treatment performed in the last 6 months. Genotypes were determined by the PCR-RFLP and sequencing method. The frequency comparisons between case and controls were performed using the chi-square test and logistic regression (OpenEpi and SNPStats software). The risk (OR) was evaluated for values of $P < 0.05$. Differences in *TLR4*, *CD14*, *RANKL*, and *OPG* genotype and allele frequency distributions were not observed between patients and controls. However, some variants were a risk factor for the development of periodontitis when considering gender and smoking habits. The *TLR4* 896 A/G genotype was a risk factor for periodontitis in males (OR = 2.86), and the *TLR4* 1196C/C genotype was a risk factor for nonsmoking males (OR = 1.85) when compared to women. The *RANKL* A/A and the *OPG* T/C genotype was associated with the risk of the disease in nonsmoking men compared to nonsmoking women with the same genotype (OR = 1.96 and OR = 2.9, respectively). In conclusion, *TLR4*, *CD14*, *RANKL*, and *OPG* variants were not associated with periodontitis. However, *TLR4*, *RANKL*, and *OPG* polymorphisms could be a risk for periodontitis in males regardless of smoking habits.

1. Introduction

The pathogenesis of periodontitis involves a complex interaction between microbial challenge in the oral cavity and the immune response of the host [1]. The immune inflammatory response in periodontitis includes the innate and the acquired mechanisms that work together modulating the magnitude of the response. Activation of innate immunity mechanisms occurs as a response to initial bacterial recognition. Antigens and bacterial biofilm products, such as lipopolysaccharides (LPS), are recognized by Toll-like receptors (TLRs) expressed on the immune cell membrane surfaces, the first receptors activated during pathogen and host interaction. Another molecule that interacts with the LPS is the cluster of differentiation antigen 14 (CD14) [2]. The persistence of infection amplifies the immune response,

resulting in the release of inflammatory mediators leading to damage of the connective tissue and in the process of bone resorption that occurs through the RANK (receptor activator of nuclear factor-kappaB)- RANKL-OPG (osteoprotegerin) dependent mechanism [3].

TLR2 and TLR4 have been identified as the principal receptors for bacterial cell wall components [4]. TLR4 recognizes LPS from Gram-negative bacteria. TLR2 recognizes LPS and lipoteichoic acid from Gram-positive bacteria as well as lipoproteins and peptidoglycans from both Gram-positive and Gram-negative bacteria [5, 6]. Although TLR2 and TLR4 have been shown to be important for the progression of inflammation and related bone metabolism in periodontitis [7, 8], little is known about their contributions to the disease. In TLR2, TLR4 and TLR2-TLR4 knockout mice are periodontally infected with *Porphyromonas gingivalis*; periodontal bone resorption was found as TLR4-dependent [9].

CD14 interacts with TLR4 and favors the delivery of LPS to TLR4-LBP-MD-2 complex [2]. This molecule is found as an anchored membrane protein (mCD14) and in a soluble form (sCD14). The main biological function of mCD14 is to act as a receptor to recognize and link to LPS or LPS/LBP (LPS-binding protein) complexes and mediate cell inflammatory reactions [10, 11]. The sCD14 may have an important role in potentiating the immune responses to LPS in cells lacking mCD14 surface [12]. CD14 variant was found to be associated with the severity of periodontitis [13].

Receptor activator of nuclear factor-kappaB ligand (RANKL) binds to the RANK and provides signaling for osteoclast differentiation from hematopoietic progenitor cells. The OPG is a molecule that negatively regulates the RANKL-RANK binding and inhibits bone turnover by osteoclasts [3]. The OPG molecule has three structural domains that specifically influence its biological activities [14]. This protein is a member of the TNF receptor superfamily [15]. Periodontal ligament cells can produce RANKL and OPG, and these molecules have been found in the gingival crevicular fluid of periodontitis patients [16].

As TLR4 and CD14 were previously associated with LPS hyporesponsiveness [17–19] and RANKL and OPG to the regulation of bone mass [3, 20–22], we hypothesized that the polymorphisms in the genes that codified these proteins could influence the pathogenesis of periodontitis. Thus, the aim of this study was to evaluate the influence of the polymorphisms in *TLR4* (rs4986790 and rs4986791), *CD14* (rs2569190), RANKL (*TNFSF11*, rs2277438), and OPG (*TNFSF11B*, rs3102735) in the development of periodontitis.

2. Material and Methods

2.1. Sample Selection. This case-control study was approved by the Human Research and Ethics Committee of the State University of Maringá (UEM-No. 719/2011, 2011 and 1.866.509, 2016). The studied populations were from the North and Northwest regions of the state of Paraná (22°29'30"-26°42'59"S and 48°02'24"-54°37'38"W), Southern Brazil. All the individuals voluntarily sought dental clinics at the State University of Maringá (DOD-UEM) and the Inga University Center (UNINGÁ) from January 2012 to September 2017. These participants underwent a clinical periodontal examination, and the diagnostic was confirmed by an experienced Periodontist (COS). The subjects who met all eligibility criteria of this study (patients and controls) and agreed to participate were informed about its nature and signed an informed consent form.

The selection criteria was defined according to the International Workshop for a Classification of Periodontal Diseases and Conditions of 1999 [23]. Clinical parameters were evaluated in order to classify the subjects into patients or control groups. Probing depth (PD), bleeding on probing (BOP), and clinical attachment level (CAL) were examined at six sites (mesiovestibular, vestibular, distovestibular, mesiolingual, lingual, and distolingual) of each tooth. However, it was considered only as the site with the higher clinical attachment loss in the mesial face (between mesiobuccal and mesiolingual) and distal face (between discobuccal and distolingual). In that way, for inclusion criteria, four measurements for each tooth were taken into account. The patients had to have at least 5 sites in different teeth with PD ≥5 mm, CAL ≥3 mm, and more than 25% of BOP. Moderate periodontitis was defined according to the CDC (Centers for Disease Control and Prevention) criteria as ≥2 interproximal sites with CAL ≥4 mm (not on the same tooth) and OR ≥2 interproximal sites with PD ≥5 mm (not on the same tooth). According to the new classification of periodontal diseases [24], periodontitis was defined as interdental CAL detectable at ≥2 nonadjacent teeth or buccal or oral CAL ≥3 mm with pocketing ≥3 mm detectable at ≥2 teeth. However, the observed CAL cannot be ascribed to nonperiodontitis-related causes. The control group was formed by individuals having no pocket ≥4 mm and exhibiting less than 25% of BOP. The noninclusion criteria for both groups were diabetes mellitus, acute infections, osteoarthritis, pregnancy, patients without aggressive periodontitis, i.e., stage IV and C degree of periodontitis according to the classification of periodontal diseases of 2017 [24], and those who had periodontal treatment within the last 6 months or taking antibiotics during this same time. All subjects were over 30 years of age and with at least 20 teeth in the oral cavity. Due to the great miscegenation in Brazilians, the population was classified according to a previous study by Probst et al. [25] and confirmed for our region [26]. Information on the patient's smoking history was obtained by anamnesis.

2.2. Blood Sample Collection and DNA Extraction. Peripheral blood samples were collected by venipuncture in a tube containing EDTA. The salting-out method [27] with some modifications [28] and the QIAamp® DNA blood mini kit (Qiagen, Valencia, CA) were used in accordance with the manufacturer's instructions to perform DNA extraction from whole blood or buffy coat. The concentration and quality of the DNA were analyzed by optical density in a Thermo Scientific NanoDrop 2000® apparatus (Wilmington, USA).

2.3. Genotyping of TLR4 A896G (rs4986790) and TLR4 C1196T (rs4986791). *TLR4* A896G (Asp299Gly, rs4986790) and *TLR4* C1196T (Thr399Ile, rs4986791) polymorphisms were performed according to Folwaczny et al. [29] with

modifications. The polymerase chain reaction (PCR) was performed in 10 μL final volume with 10 ng of DNA, 1.0 ng of each primer, 3.33 mM of $MgCl_2$, 0.16 mM of dNTP (Invitrogen®, Frederick, MD, USA), 1X PCR buffer (5X Green GoTaq® Flexi Buffer, Promega, USA), and 0.75 U of *Taq* Polymerase (GoTaq® DNA Polymerase, Promega, USA). Cycles were performed in the Veriti thermal cycler (Applied Biosystems): one minute at 95°C; 35 cycles of 30 seconds at 95°C, 45 seconds at 60°C (to *TLR4* A896G) or 62° (to *TLR4* C1196T), and one minute at 72°C; at the end, 10 minutes at 72°C. PCR products were digested for 10 minutes at 37°C with *Nco*I (Fermentas, Canada) for *TLR4* A896G and for 60 minutes at 37°C with *Hinf*I (Fermentas, Canada) for *TLR4* C1196T. The results were observed by electrophoresis on 3.5% agarose gel with SYBR Safe (Invitrogen Life Technologies, Grand Island, NY, USA) and visualized under UV light.

2.4. Genotyping of CD14 C-260T (rs2569190). *CD14* C-260TT (rs2569190) polymorphism was performed according to Ito et al. [30] with modifications. The PCR reaction was performed in 10 μL final volume with 5 ng of DNA, 1.5 ng of each primer, 1.5 mM of $MgCl_2$, 0.15 mM of dNTP (Invitrogen®, Frederick, MD, USA), 1X PCR buffer (5X Green GoTaq® Flexi Buffer, Promega, USA), and 0.5 U of *Taq* Polymerase (GoTaq® DNA Polymerase, Promega, USA). The PCR product was cleaved with *Hae*III restriction enzyme (New England Biolabs) according to the manufacturer. The results were observed through electrophoresis on 3% agarose gel with SYBR Safe (Invitrogen Life Technologies, Grand Island, NY, USA) and visualized under UV light.

2.5. Genotyping of OPG C163T (rs3102735). The *OPG* polymorphism (*TNFRSF11B* C163T, rs3102735) was evaluated by PCR-RFLP according to Langdahl et al. [31], with modifications. The PCR reaction was performed in 10 μL final volume with 5 ng of DNA, 1.2 ng of each primer, 2.0 mM of $MgCl_2$, 0.12 mM of dNTP (Invitrogen®, Frederick, MD, USA), 1X PCR buffer (5X Green GoTaq® Flexi Buffer, Promega, USA), and 0.5 U of *Taq* Polymerase (GoTaq® DNA Polymerase, Promega, USA). Cycles were performed in the Veriti thermal cycler (Applied Biosystems): one minute at 95°C; 35 cycles of 30 seconds at 95°C, 45 seconds at 60°C, and one minute at 72°C; at the end, 10 minutes at 72°C. The PCR product was cleaved with using the *Vsp*I restriction enzyme (Invitrogen, USA) according to the manufacturer for three hours at 37°C. The results were observed by electrophoresis on 3% agarose gel with SYBR Safe (Invitrogen Life Technologies, Grand Island, NY, USA) and visualized under UV light. The genotyping was performed only in nonsmokers.

2.6. Genotyping of RANKL (rs2277438). The polymorphic region of *RANKL* (*TNFSF1*, rs2277438) was amplified using the PCR with specific primers, and their sequences were determined by sequencing reaction according to Eun et al. [21] with some modifications. The PCR reaction was performed in 20 μL final volume with 5 ng of DNA, 1.5 ng of each primer, 1.5 mM of $MgCl_2$, 0.15 mM of dNTP (Invitrogen®, Frederick, MD, USA), 1X PCR buffer (10X buffer 50 mM Invitrogen®, USA), and 0.1 U of *Taq* Polymerase Platinum® (Invitrogen®, USA). Cycles were performed in the Veriti thermal cycler (Applied Biosystems): one minute at 95°C; 35 cycles of 30 seconds at 95°C, 45 seconds at 62°C, and one minute at 72°C; at the end, 10 minutes at 72°C. The sequencing reactions were performed with the BigDye™ terminator v3.1 cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific, USA) according to the manufacturer in an automated DNA sequencer (Applied Biosystems 3500xL). The amplified and sequenced regions were edited and evaluated using the Chromas program version 2.6.4 (https://www.technelysium.com.au) and aligned in the EMBL-EBI web program, available at https://www.ebi.ac.uk/. The nucleotide sequence used as the basis for alignment corresponding to *TNFSF11* rs2277438 is available on NCBI's website (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2277438). The genotyping was performed only in nonsmokers.

2.7. Statistical Analysis. Statistical analysis was done using SNPStats software [32] (https://www.snpstats.net/start.htm) and OpenEpi program Version 3.01 (https://www.openepi.com/Menu/OE_Menu.htm). Allele and genotype frequencies for *TLR4*, *CD14*, *RANKL*, and *OPG* were obtained, and the association between genetic polymorphisms and periodontitis was evaluated using the Chi-square test with the Yates correction and logistic regression for multivariate analyses. The Student *t*-test was used to compare the differences in age, and Fisher's exact test was used to compare the differences in gender between groups. The association tests were performed for codominant, dominant, recessive, overdominant, and log-additive genetic inheritance models, and the better inheritance model was chosen according to the minor Akaike Information Criteria (AIC) [32]. The association was determined after correcting for confounding factors using multivariate logistic regression analysis including age, gender, and smoking status covariates. Haplotype frequency estimates were carried out using the expectation-maximization algorithms. Odds ratio with 95% confidence intervals was deemed only for significant *P* values. All tests were carried out using a significance level of 5%. Genotype frequency distributions were evaluated to ensure the Hardy-Weinberg equilibrium for all genes in the populations. Quanto (http://biostats.usc.edu/software) was used to calculate the sample size from the minor allele frequency (frequency of 0.03 for *TLR4* C1196T), with a population risk of 50% and a genetic effect of 2.0, with statistical power of 80%.

3. Results

In this study, allele and genotype frequency distributions of *TLR4* A896G (rs4986790), *TLR4* C1196T (rs4986791), *CD14* C-260T (rs2569190), *RANKL* (rs2277438), and *OPG* C163T (rs3102735) polymorphisms were analyzed in a total of 203 patients with periodontitis and 213 subjects without the disease. Among the participants, women were 56.3%, nonsmokers were 67.3%, and the age group was between 30 and 77 years. *TLR4* and *CD14* polymorphisms were evaluated in all subjects and in nonsmoking groups. *RANKL* and

Table 1: Characteristics of patients with periodontitis and controls.

	Patients	Controls	*P*	OR (95%CI)
All subjects	*N* = 203	*N* = 213		
Gender (*n* and %)[a]				
Male	99 (48.8)	83 (39.0)		
Female	104 (51.2)	130 (61.0)	0.06	
Age mean ± SD (year)[b]	47.8 ± 9.0	46.0 ± 8.8	0.04	
Smoking habit (*n* and %)[a]				
Nonsmokers	121 (59.6)	159 (74.7)		
Smokers	82 (40.4)	54 (25.3)	<0.01	2.0 (1.31-3.03)
Nonsmokers	*N* = 121	*N* = 159		
Gender (*n* and %)[a]				
Male	56 (46.3)	54 (34.0)		
Female	65 (53.7)	105 (66.0)	0.05	
Age mean ± SD (year)[b]	48.2 ± 9.1	46.1 ± 8.6	0.04	

n: number of individuals; *N*: population size; %: percentage; SD: standard deviation; *P*: *P* value; OR: odds ratio; CI: confidence interval. [a]Comparison by Fisher's exact test; [b]comparison by the Student *t*-test.

OPG polymorphisms were analyzed only in nonsmoking groups. These groups were independently analyzed to avoid bias because smoking is a risk factor for periodontitis. Subjects were matched by gender ($P = 0.06$), and nonpairing was observed for age ($P = 0.04$). The characteristics of patients and controls are described in Table 1.

The distribution of the genotype frequencies for all analyzed genes was consistent with the Hardy-Weinberg equilibrium ($P > 0.05$). The *TLR4*, *CD14*, *RANKL*, and *OPG* genotype and allele frequency distributions are summarized in Table 2. Differences in the allele and genotype frequency distributions were not observed between patients and controls in linear analyses in the recessive, dominant, or codominant inheritance models.

After multivariate analysis (considering age and smoking habit adjustment) and stratification by gender, significant differences were observed for the TLR4, RANKL, and OPG (Table 3). In a cross-classification interaction, considering polymorphisms and gender, *TLR4* 896 A/G genotype was associated with periodontitis in men when compared to the A/A wild genotype in females (OR = 2.86, 95%CI: 1.02-8.00; all subjects), and *TLR4* 1196 C/C wild genotype and *RANKL* A/A genotype were associated with periodontitis in nonsmoking men when compared to the same genotype in nonsmoking women (OR = 1.85, 95%CI: 1.03-3.28, and OR = 1.96, 95%CI: 1.01-3.78). Still, after stratification and considering gender within polymorphism, *OPG* T/C genotype was associated with the risk of developing periodontitis in nonsmoking men compared to nonsmoking women (OR = 2.9; 95%IC: 1.03-8.14).

4. Discussion

The genetic factors influencing the pathogenesis of periodontal disease and single nucleotide polymorphisms (SNPs) in genes involved in the host inflammatory immune response have been studied [33–37]. Therefore, this case-control study was conducted to evaluate the possible influence of immune innate and bone resorption gene polymorphisms, *TLR4*, *CD14*, *RANKL*, and *OPG*, in periodontitis, in carefully selected patients and controls based on clinical parameters and especially in nonsmokers.

We found that these polymorphisms were not associated with the disease. However, the *TLR4*, *OPG*, and *RANKL* polymorphisms were involved in the pathogenesis of periodontitis when considering gender and smoking habits. In multivariate analyzes, nonsmoking men carrying the *TLR4* 896 A/G and 1196 C/C genotypes showed approximately 200% risk of periodontitis development compared to women carrying the wild genotypes. Added to this, those men carrying the *RANKL* A/A and *OPG* C/T genotype had 100% to 200% risk of developing the disease, compared to women carrying the same genotypes. Thus, it is possible to infer that genetic variants affecting the innate response and the process of bone resorption were factors predisposing to the disease in men, regardless of the use of cigarettes.

It is known that smokers and males are at risk for the development of periodontitis [38–46]. Smoking tobacco was an epigenetic factor and may change the transcription and methylation states of extracellular matrix organization-related genes, which exacerbate the periodontal condition [47]. In our study, smokers with periodontitis were more frequent (40.4%) than the total population of Brazilian smokers, which accounts for 17.2% [48]. Men have been considered to be more susceptible to periodontitis due to hormonal factors, personal hygiene habits, and poor health prevention habits [49]. However, our results showed that polymorphisms in immune response genes might also influence the pathogenesis of periodontitis in men, regardless of smoking habits.

As for the *TLR4* polymorphisms, our results are in concordance with others, though we found such risk only in men. In meta-analysis studies, *TLR4* 896G allele was associated with the risk of developing periodontitis [1], in a

Table 2: Genotype and allele frequency distributions for *TLR4*, *CD14*, *RANKL*, and *OPG* polymorphisms in patients with periodontitis and controls (all subjects and nonsmoking individuals).

Genotypes and alleles	Nonsmokers		*P*	All subjects		*P*
	Patients $N = 100$ n (%)	Controls $N = 144$ n (%)		Patients $N = 182$ n (%)	Controls $N = 198$ n (%)	
TLR4 A896G						
A/A	84 (84.0)	129 (90.0)		151 (83.0)	176 (89.0)	
A/G	16 (16.0)	15 (10.0)	0.28	31 (17.0)	22 (11.0)	0.13
A	184 (92.0)	273 (95.0)		333 (91.5)	374 (94.0)	
G	16 (8.0)	15 (5.0)	0.29	31 (8.5)	22 (6.0)	0.15
TLR4 C1196T						
C/C	85 (85.0)	129 (90.0)		155 (85.2)	176 (89.0)	
C/T	15 (15.0)	15 (10.0)	0.38	27 (14.8)	22 (11.0)	0.35
C	185 (92.0)	273 (95.0)		337 (92.6)	374 (94.0)	
T	15 (8.0)	15 (5.0)	0.40	27 (7.4)	22 (6.0)	0.37
CD14 -C260T						
C/C	29 (29.0)	39 (27.0)		48 (26.4)	55 (28.0)	
C/T	47 (47.0)	77 (53.0)	0.63	91 (50.0)	102 (52.0)	0.97
T/T	24 (24.0)	28 (19.0)	0.84	43 (23.6)	41 (21.0)	0.63
C	105 (52.0)	155 (54.0)		187 (51.4)	212 (54.0)	
T	95 (48.0)	133 (46.0)	0.85	177 (48.6)	184 (46.0)	0.62
RANKL rs2277438*	$N = 108$	$N = 134$				
A/A	69 (63.9)	83 (61.9)		—	—	
G/A	37 (34.3)	48 (35.8)	0.89	—	—	
G/G	2 (1.8)	3 (2.3)	0.83	—	—	
A	175 (81.0)	214 (79.9)		—	—	
G	41 (19.0)	54 (20.1)	0.84	—	—	
OPG C163T*	$N = 111$	$N = 145$				
T/T	80 (72.1)	99 (68.3)		—	—	
C/T	28 (25.2)	39 (26.9)	0.79	—	—	
C/C	3 (2.7)	7 (4.8)	0.56	—	—	
T	188 (84.7)	237 (81.7)		—	—	
C	34 (15.3)	53 (18.3)	0.45	—	—	

*Polymorphism evaluated only in nonsmoking individuals. The nonpairing of the number of individuals in the studied polymorphisms occurred due to lack of some samples during the course of the study. *N*: population size; *n*: number of individuals with the allele or genotype; %: allele and genotype frequencies ×100. *P*: *P* value.

recessive model [50] and after stratification by ethnicity, only in Caucasians [13, 51]. Regarding *TLR4* 1196 C>T polymorphism, the T allele was associated with an increased risk of developing periodontitis in Caucasians in an additive model of inheritance [13]. For *CD14* polymorphisms, we did not find an association between *CD14* and periodontitis. On the other hand, a meta-analysis conducted by Han et al. [13] showed that the *CD14* -260C allele was a risk factor to the severity of periodontitis. Folwaczny et al. [52] suggested that this same allele was a risk factor for disease only in female patients. However, many reports found no association between *TLR4* and *CD14* polymorphisms and patients with periodontitis in different populations [29, 52–61].

Regarding *RANKL* polymorphism, different from that was observed in our results, no differences were observed in a study with Iranian patients with periodontitis [62] as well as in another study with adolescents with periodontitis [63]. As for *OPG* polymorphisms, studies have reported higher levels of OPG in the gingival crevicular fluid, saliva, and gingival tissues of healthy individuals than in patients with periodontitis [64–66], and Mogi et al. [66] observed higher levels of OPG in the gingival crevicular fluid in mild periodontitis compared to the moderate or severe forms. This same study also showed that the proportion of RANKL to OPG concentration in the gingival crevicular fluid was significantly higher in patients with periodontitis disease than in healthy individuals. No association study with *OPG* C163T polymorphism and periodontitis has been performed before. However, Lucena et al. [67] evaluated the influence of this polymorphism in patients with periodontitis and diabetes and no association was found. The *OPG* 163C allele was observed less frequently in this study, which corroborates

Table 3: *TLR4*, *RANKL*, and *OPG* genotype frequency distributions between patients with periodontitis and control after stratification according to gender.

Genotype (*n* and %)	Female			Male		*P*	OR (95%CI)
	Patients	Controls		Patients	Controls		
*All subjects**							
TLR4 A896G rs4986790	*N* = 223			*N* = 156			
A/A	79 (35.4)	110 (49.3)	Ref.	71 (45.5)	66 (42.3)	0.01	2.86 (1.02-8.00)
A/G	18 (8.1)	16 (7.2)		13 (8.4)	6 (3.8)		
*Nonsmokers***							
TLR4 C1196T rs4986791	*N* = 159			*N* = 85			
C/C	47 (29.6)	90 (56.6)	Ref.	38 (44.7)	39 (45.9)	0.02	1.85 (1.05-3.28)
C/T	11 (6.9)	11 (6.9)		4 (4.7)	4 (4.7)		
RANKL rs2277438	*N* = 144			*N* = 99			
A/A	37 (25.7)	57 (39.6)	Ref.	33 (33.3)	26 (26.3)	0.03	1.96 (1.01-3.78)
G/A	18 (12.5)	28 (19.4)		19 (19.2)	20 (20.2)		
G/G	2 (1.4)	2 (1.4)		0	1 (1.0)		
		Patients	Controls				
OPG C163T rs3102735		*N* = 111	*N* = 145				
T/C	Female	14 (12.6)	29 (20.0)	Ref.			
	Male	14 (12.6)	10 (6.9)			0.04	2.9 (1.03-8.14)

*Multivariate analyses adjusted by age and smoking habits. **Multivariate analyses adjusted by age. Only significant results are shown. Ref.: genotype used as reference; *n*: number of individuals with the allele or genotype; %: allele and genotype frequencies ×100; *N*: sample size; *P*: *P* value; OR: odds ratio (adjusted values); CI: confidence interval.

with the described frequencies of this polymorphism in different populations (https://www.ncbi.nlm.nih.gov/snp/rs3102735#frequency_tab).

The exact mechanism involved in the susceptibility to periodontitis is unclear. We hypothesized that the polymorphisms in genes that codified proteins related to receptors of the immune cell membranes as well as mediators related to bone resorption were involved in periodontitis immunopathogenesis. Our results showed that *TLR4* polymorphism could be associated with disease in men. The human *TLR4* gene is located on chromosome 9q33.1 and encodes a type I transmembrane protein that has an extracellular leucine-rich repeat domain and Toll/IL-1 receptor (TIR) domain [68] that is very important for signal transduction. The *TLR4* wild-allele genotype is associated with the normal expression of the membrane protein, which is able to recognize the LPS in the complex formation of LPS-LPB-TLR4 [69–71]. A single point mutation in this domain can interfere in the response of LPS [72]. The *TLR4* A896G polymorphism results in the replacement of a conserved aspartic acid residue by a glycine at position 299; in *TLR4* C1196T, a threonine was replaced with an isoleucine in position 399. Both variants are related to the leucine-rich repeat (LRR) region, mainly in the ligand-binding (*TLR4* A896G) and in the coreceptor-binding (*TLR4* C1196T) regions [73]. The wild type has been associated with better LPS response [17].

As to bone resorption, we found that *RANKL* and *OPG* polymorphisms were a risk factor for the disease. RANKL encoded by the gene *TNFSF11* located on chromosome 13q14.11 is an anchored membrane protein expressed on osteoblasts, T and B lymphocytes, and fibroblasts in low levels; it is also cleaved in the soluble form [74]. *RANKL* (*TNFSF11* rs2277438) polymorphism is a variant in the intron 2 [21]. *RANKL* alleles were associated with susceptibility to bone involvement disease [75–77]. OPG is encoded by the *TNFRSF11B* gene located on chromosome 8q24.12. It is produced by dendritic and endothelial cells, fibroblasts, and T and B lymphocytes [20]. *OPG* polymorphism (*TNFRSF11B* C163T, rs3102735) is located in the promoter region of the gene and can influence its transcription. The C allele was associated with a lower bone mineral density or higher frequency of fractures at different bone sites [78].

The complex interaction between genetic, biological, and environmental factors increases periodontitis susceptibility, and thus, case-control studies conducted in different ethnic groups are important because some polymorphisms have differences in the frequency distributions among populations. The positive points of this study were the judicious selection of patients and controls, who had the diagnostic confirmed by sole and experienced Periodontist, and did not present predisposing factors for periodontitis-like infections and inflammatory diseases, diabetes, and bone diseases [79–84]. Age was also considered, and no one younger than 30 years old was added in the groups. Nonsmoking individuals were separately analyzed. A limitation of the study was the low statistical power obtained when analyzing subgroups, such as nonsmokers and gender. Also, the possible influence of covariate age, gender, and smoking habit on the composition of cases and controls in our sample should be considered, given their relevance in oral health and the profile of periodontitis.

5. Conclusion

In conclusion, *TLR4*, *CD14*, *RANKL*, and *OPG* variants were not associated with periodontitis. However, *TLR4*, *RANKL*, and *OPG* polymorphisms could be a risk for periodontitis in males regardless of smoking habits.

Data Availability

The genotype and haplotype data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Review Article

Periodontitis and Rheumatoid Arthritis: The Same Inflammatory Mediators?

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The strict link between periodontitis (PD) and rheumatoid arthritis (RA) has been widely demonstrated by several studies. PD is significantly more frequent in RA patients in comparison with healthy subjects: this prevalence is higher in individuals at the earliest stages of disease and in seropositive patients. This is probably related to the role of *P. gingivalis* in inducing citrullination and leading to the development of the new antigens. Despite the many studies conducted on this topic, there is very little data available concerning the possibility to use the same biomarkers to evaluate both RA and PD patients. The aim of the review is to summarize this issue. Starting from genetic factors, data from literature demonstrated the association between HLA-DRB1 alleles and PD susceptibility, similar to RA patients; moreover, SE-positive patients showed simultaneously structural damage to the wrist and periodontal sites. Contrasting results are available concerning other genetic polymorphisms. Moreover, the possible role of proinflammatory cytokines, such as TNF and IL6 and autoantibodies, specifically anticyclic citrullinated peptide antibodies, has been examined, suggesting the need to perform further studies to better define this issue.

1. Introduction

Abundant evidences recorded in literature demonstrated that rheumatoid arthritis (RA) and periodontitis (PD) are frequently associated with each other and share several pathogenic and clinical features [1].

RA is a systemic inflammatory autoimmune disease characterized by chronic inflammation and joint tissue destruction, leading to functional disability. Similarly, PD patients experience chronic inflammatory diseases [1–3]. In particular, PD are dysbiotic conditions characterized by an imbalance between subgingival communities and host immune response. The transition from health to PD is characterized by shifts in the community structure of the complete subgingival microbiome [4–6].

PD is currently considered a risk factor for RA: the first link between these two conditions was identified in *P. gingivalis*, a gram-negative anaerobe bacteria characterized by the presence of peptidylarginine deiminase (PAD). This enzyme contributes to RA development by catalyzing citrullination, a posttranslational modification playing a crucial role in the production of anticyclic citrullinated peptide antibodies (ACPA), widely recognized as diagnostic and prognostic biomarkers for RA patients [7, 8]. Recently, our group observed a significant association between the percentages of *P. gingivalis*, assessed by real-time PCR, on the total tongue biofilm and RA disease activity (evaluated as disease activity score on 28 joints—DAS28) [9]. This result suggests

that the oral cavity microbiological status could play a role in the pathogenic mechanisms of inflammation, leading to more active diseases [9, 10].

More recently, the role of *Aggregatibacter actinomycetemcomitans* has been suggested. This oral pathogen could induce hypercitrullination at neutrophil level by secreting leukotoxin A that is able to change neutrophil morphology, mimicking extracellular trap formation. Finally, this process results in the hypercitrullinated autoantigen release, triggering autoimmune response in RA patients [11]. From an epidemiological point of view, several studies have been conducted which remarked the association between RA and PD. The main data is summarized in Table 1. Specifically, case-control studies underline a higher prevalence of PD in RA patients in comparison with healthy controls [12–24]. PD prevalence is higher in early RA patients at disease onset, despite the young age and paucity of smoking history [14–18]. In addition to higher prevalence, RA patients show a more aggressive PD compared to HS (healthy subjects) [15, 16, 18–21]. Furthermore, some studies have carried out a comparison with OA patients. Dissick and his colleagues observed that PD was more common and severe in RA patients compared to OA. Moreover, in RA, the presence of PD was significantly associated with positivity for rheumatoid factor (RF) and ACPA [13]. More recently, Gonzalez and coauthors observed that ACPA-positive RA patients showed significantly higher mean percentage of sites with alveolar bone loss (ABL) greater than 20% in comparison with OA patients. Moreover, ABL substantially connected to ACPA titers and disease activity in terms of DAS28 [25]. The same cohort was evaluated in terms of HLA-DRB1 and anti-*P. gingivalis* antibodies, showing higher ACPA levels in patients with subgingival *P. gingivalis* and in those with higher anti-P. gingivalis antibody levels [26].

Moving passed the strict association between RA and PD, from an epidemiological and pathogenic point of view, it is possible to hypothesize a common genetic background and the sharing of inflammatory mediators between RA and PD. The review thoroughly covers this topic.

1.1. Genetic Biomarkers. Several studies have confirmed the role of genetic factors in the RA development: according to a multifactorial model, the interaction between genetic background and environmental factors leads to the development of an autoimmune inflammatory condition, resulting in autoantibodies production. The highly polymorphic HLA-DRB1 locus (the so-called shared epitope—SE) represents the strongest genetic factor involved in disease development. Particularly, all HLA-DRB alleles with the SE provide RA-prone antigen recognition: this leads not only to an increased risk of developing RA but also to the progression into a more erosive, deforming disease. Furthermore, a gene–environment interaction between smoking and SE genes seems to be crucial in the development of seropositive RA. Nonetheless, the contribution of other genetic polymorphisms on RA susceptibility has been also investigated: among these, SNPs in signal transducer and activator of transcription 4 (STAT4), Fc gamma receptor (FCGR), protein tyrosine phosphatase nonreceptor type 22 (PTPN22), PADI-4, tumor necrosis factor (TNF), and interleukin 6 (IL6) genes have been associated with disease development in several case-control studies [27]. It is important to note that genetic polymorphisms could be also associated with different disease phenotypes, in terms of radiographic damage progression [28–30]. Moreover, in 2011, we suggested the possible role of TGF-β 869C/T and IL6-174G/C polymorphisms in determining erosive damage evaluated by ultrasonographic assessment in a cohort of RA patients [31].

Some of these genetic factors have been also associated with PD susceptibility, reinforcing the hypothesis of common pathogenic mechanism with RA. Specifically, SE positivity has been widely linked to PD development.

In 2006, Marotte and colleagues investigated the presence of an association between bone destruction at the joint and periodontal level in a wide RA cohort. The analysis of 147 subjects—56.5% of whom with PD—demonstrated a strong association between PD and wrist destruction, assessed by the radiographic Larsen score. Specifically, the authors identified a significant association between SE positivity and bone destruction in wrist and periodontal sites. In fact, SE+ patients showed 2.5 times greater risk of having wrist joint destruction than SE- (OR = 2.5). In the same way, SE+ patients had a 2.2 times greater risk to have periodontal destruction compared to SE- (OR = 2.2). The comparison between patients with both site destruction and those without any destruction demonstrated the association with SE positivity (OR = 3.9). This evidence underlines the possible role of SE in bone destruction at both sites, suggesting a simultaneous action [32].

Data from Marotte and colleagues agreed with a previous study conducted by Bonfil and colleagues in 1999, suggesting the role of SE as a prognostic factor for PD susceptibility [33].

The possible role of SE-coding DRB1 alleles has been recently underlined by Gehlot and colleagues: the authors observed that transgenic SE+ mice, but not SE- mice, spontaneously developed PD, associated with IL17 overexpression and periostin disruption. Moreover, SE-positive mice showed significantly lower mandibular bone volumetric and mineralization parameters, together with increased alveolar bone resorption [34].

In addition to SE, the possible role of other RA-related genetic polymorphism has been investigated to analyze the association with bone destruction at periodontal level [35]. The studies conducted so far did not produce conclusive results, mainly due to small size cohorts (generally less than 100 patients enrolled), leading to a lack of statistical power to properly detect an association. Hereupon, genotype and allele frequencies could widely vary between different ethnic groups and the same genetic variants could play a different role in different populations. Data from literature provides some evidences to support the association between an aggressive PD and SNPs in interleukin 1 beta (IL1β), interleukin 1 receptor antagonist (IL1RN), FCGR IIIb, vitamin D receptor (VDR), and Toll-like receptor 4 (TLR4) genes. Moreover, a chronic PD was associated with polymorphisms in IL1B, IL1RN, IL6, IL10, VDR, CD14, TLR4, and matrix metalloproteinase-1 (MMP1) genes [34]. The low statistical

Table 1: Main study evaluating prevalence of periodontitis in rheumatoid arthritis patients.

Study	RA group (*N*)	Control group	Mean age (years)	Female sex (%)	Smokers in RA (%)	Smokers in controls (%)	RA duration	RF (%)	ACPA (%)	PD prevalence in RA	Results
Pischon et al. 2008 [12]	57	HS 52	RA 52.1	RA 95%	59.7%	40.4%	10 years	NR	NR	8.05-fold increased odds of PD in RA compared with HS	Higher prevalence of PD in RA versus HS
Dissick et al. 2010 [13]	91	OA 41	RA 62 OA 58	RA 12 OA 5	65%	46%	14 years	81%	87%	RA 51% OA 26%	PD more common and severe in RA pt in comparison with OA. Association between PD and RF/ACPA
Scher et al. 2012 [14]	eRA 31 lRA 34	18	eRA 42.2 lRA 47.7	eRA 68% lRA 79% HS 65%	eRA 32% lRA 30%	22%	eRA 3.4 mts lRA 62.9 mts	eRA 92% lRA 78%	eRA 96% lRA 88%	eRA 88% lRA 91% HS 44%	High PD prevalence in eRA at disease onset
de Smit et al. 2012 [15]	95	Non-RA 44 HS 36	RA 56 Non-RA 54 HS 34	RA 68 Non-RA 57 HS 57	24.2%	Non-RA 61.4% HS 38.9%	7.4 years	RA 53%	RA 71%	RA 43% moderate and 27% severe Non-RA 18% HS 12%	Higher prevalence of severe PD in RA pt in comparison with controls. Association between severe PD and higher DAS28
Ranade and Doiphode 2012 [16]	RA 40	Non-RA 40	RA 45	RA 80%	NR	NR	2.15 years	NR	NR	RA 97.5%	High prevalence of mild-to-moderate PD in patients with RA
Reichert et al. 2013 [17]	RA 42	Non-RD 114	RA 56.1	RA 52.4%	26.2%	25%	NR	NR	NR	RA 34.3% Non-RD 49.1%	In patients with RA, DNA of P. gingivalis was detected in synovial fluid more often than in controls
Wolff et al. 2014 [18]	eRA 22	HS 22	RA 51.7	RA 68%	19%	19%	5.9 mts	37%	41%	100%	More severe PD detected in eRA pts
Joseph et al. 2013 [19]	RA 100	HS 112	RA 46.5	RA 76%	0%	0%	NR	NR	NR	RA 58% HS 7.1%	Higher prevalence and severity of PD in RA
Chen et al. 2013 [20]	RA 13779	Non-RA 137790	RA 52.6	RA 77.4%	NR	NR	NR	NR	NR	RA 39% Non-RA 35.1%	Association between periodontitis and incident RA
Mikuls et al. 2014 [26]	287	OA 330	RA 59 OA 59	RA 37% OA 30%	62%	46%	12.6 years	77%	83.6%	RA 34.8% OA 26%	Higher PD prevalence in RA versus OA. PD significantly associated with higher disease activity, radiographic damage, and ACPA levels. Higher ACPA in pts with subgingival P gingivalis and in those with higher levels of anti-P gingivalis antibodies. No differences between RA and OA in the levels of anti-PG

Table 1: Continued.

Study	RA group (*N*)	Control group	Mean age (years)	Female sex (%)	Smokers in RA (%)	Smokers in controls (%)	RA duration	RF (%)	ACPA (%)	PD prevalence in RA	Results
Gonzalez et al. 2015 [25]	287	OA 330	RA 59 OA 59	RA 37% OA 40%	62%	46%	Not specified	Not specified	80.5%	100%	ACPA-positive RA patients with significantly higher mean percentage of sites with ABL >20% compared with OA pts
Potikuri et al. 2012 [22]	91	93	RA 43.9 HS 41.7	RA 76% HS 69%	0	0	PD 17.1 mts Non-PD 12.9 mts	63%	41%	RA 64.8% HS 28%	Strict association between PD and RA in nonsmoking subjects and DMARD-naïve pts
Eriksson et al. 2016 [21]	RA 2740	HS 3942	18-70 years	RA 73% HS 73%	25%	18%	9.6 years	64%	63%	RA 33% HS 32%	No evidence of an increased prevalence of PD in patients with lRA compared to HS and no differences based on ACPA or RF status among RA subjects
Choi et al. 2016 [23]	RA 264	HS 88	58.2	RA 87.5% HS 87.5%	6.4%	8%	13.8 years	68.5%	69.1%	RA 63.6 HS 34.1%	Prevalence of moderate or severe PD increased in RA patients compared to HS. Periodontal inflammation was correlated with RA duration, ESR, and ACPA
Äyräväinen et al. 2017 [24]	eRA 53 lRA 28	HS 43	eRA 51 lRA 52	eRA 85% lRA 82% HS 88%	eRA 21% lRA 11%	14%	eRA 10.4 mts lRA 176 mts	eRA 79.2% lRA 69.2%	NR	eRA 67.3% lRA 64.3% HS 39.5%	Moderate PD more frequent in RA patients than HS

RA: rheumatoid arthritis; PD: periodontics; HS: healthy subjects; OA: osteoarthritis; eRA: early RA; lRA: long-standing RA; ACPA: anticyclic citrullinated peptide antibodies; ABL: alveolar bone loss; ESR: erythrocyte sedimentation rate; mts: months; NR: not reported.

power of these studies was also demonstrated by the results of the meta-analysis conducted by Nikolopoulos and colleagues in 2008, confirming exclusively a moderate and weak positive association between the IL1 composite and IL1B-511 genotypes and the occurrence of chronic PD [36]. More recently, the SNP rs2237892 of KCNQ1 gene resulted in a significant association with the coexistence of RA and chronic PD, confirmed in the multiple logistic regression. These results suggest that individuals carrying rs2237892 T allele are likely to have both diseases [37]. The specific role of KCNQ1 gene in RA and PD pathogenesis has not been completely defined: the associated SNP is located in intron 15 of the KCNQ1 gene on chromosome 11p 15.5, encoding the pore-forming α subunit of a voltage-gated K^+ channel, crucial for the repolarization phase in the cardiac muscle. Moreover, this channel is also expressed at the plasma membrane of fibroblast-like synoviocytes from RA patients and could play a role on cell proliferation and adhesion and secretion of proinflammatory cytokines [38].

1.2. Inflammatory Biomarkers. As widely demonstrated, both RA and PD are characterized by an imbalance between proinflammatory and anti-inflammatory cytokines. In general, high levels of IL1, IL6, and TNF have been demonstrated both in patients with RA and PD. This increased expression of proinflammatory cytokines could stimulate STAT3 activation, playing a key role in the pathophysiology of RA and PD [39].

Particularly, an increased expression of IL1 and TNF has been demonstrated in RA synovium and PD gingival tissues [40]. The central role of inflammatory cytokines in RA pathogenesis has been confirmed by the introduction of biological drugs more than 20 years ago. These drugs are characterized by an innovative mechanism of action, based on the targeted inhibition of specific molecular or cellular targets directly involved in the disease pathogenesis: proinflammatory cytokines (TNF, IL1, and IL6), CTLA-4, and molecules involved in the activation, differentiation, and maturation of B cells. Their use is associated with better prognosis and the possibility to obtain a clinical remission [41].

Moving on a PD scenario, increased IL1 and TNF levels in periapical exudates were identified in these patients [42]. Moreover, PD progression was reduced by IL1 and TNF inhibitors in experimental models: specifically, histomorphometric analysis indicates that IL1 and TNF antagonists significantly reduced the loss of connective tissue attachment by approximately 51% and the loss of alveolar bone height by almost 91% [43]. In 2013, Cetinkaya and colleagues aimed to assess whether PD and RA patients share similar proinflammatory and anti-inflammatory cytokine profiles at a serum and gingival crevicular fluid (GCF) level. The study included 17 RA patients, 16 PD patients, and 16 HS. The authors did not obtain consistent results regarding proinflammatory and anti-inflammatory cytokine levels. Specifically, the total amount and GCF concentration of IL1b, IL4, IL10, and TNF were similar in RA and PD patients. However, the authors underlined the possible influence of the treatment in RA patients [44].

The salivary levels of matrix metalloproteinase-8 (MMP8) and IL1B were also evaluated in RA patients in comparison with PD and HS. The PD group showed significantly higher salivary levels of MMP-8 and IL1B in comparison with other groups; nevertheless, IL1B was the only biomarker significantly higher in RA compared to controls. Interestingly, RA patients treated by anti-TNF showed lower IL1B and TNF levels compared to nontreated patients [45].

Despite these nonconclusive results, some studies suggest that anticytokine treatment could improve PD. In 2016, Kobayashi and colleagues demonstrated a significant reduction of periodontal inflammation (assessed in terms of gingival index, bleeding on probing, and probing depth) in RA patients treated with tocilizumab and TNF inhibitors. As expected, treatment induced also decreased significantly in RA disease activity parameters, including DAS28-CRP, number of tender and swollen joints, and serum levels of ACPA and RF [46]. This treatment relationship was confirmed also by the evidence that nonsurgical treatment for PD seems to be able to improve the RA activity status: growing evidences demonstrated significant improvement of ESR, CRP, and DAS28 during PD treatment in RA patients [47, 48].

2. Autoantibodies

Some studies have investigated the presence of RA-related autoantibodies in PD patients. Taken together, PD patients demonstrated a high frequency of ACPA compared to controls; moreover, these antibodies showed a significantly higher titer in PD [3, 49].

However, these studies enrolled small populations and differences between patients and controls were not statistically significant. In 2014, De Pablo and colleagues tested sera from 194 patients with and without PD, none of whom with RA, to assess the presence of different antibodies. PD was associated with a normal frequency of ACPA and antimutated citrullinated vimentin (about 1%), but a significantly higher frequency of positive anticitrullinated α-enolase peptide-1 (anti-CEP-1; 12% versus 3%) and its uncitrullinated form (16% versus 2%; $p < 0.001$). Moreover, positive antibodies against uncitrullinated fibrinogen and uncitrullinated equivalent of CCP were more common in PD compared to non-PD patients (26% versus 3%; 9% versus 3%). The presence of these autoantibodies was not associated with smoking status, confirming that the PD autoantibody response was not exclusively due to smoking [50]. Moreover, the study conducted by Gonzales in 2015 underlines the possible role of ACPA, observing that ACPA-positive RA patients had a significantly higher mean percentage of sites with ABL greater than 20% compared to OA controls [51]. More recently, the presence of anticitrullinated histone H3 autoantibodies was investigated: these biomarkers were found in 39% of RA patients compared to 8% in HC and 10% in PD patients. No associations were found between anticitrullinated histone H3 levels and periodontal status in RA patients [52].

3. Conclusions

Despite the widely demonstrated connection between RA and PD from an epidemiological and pathogenetic point of

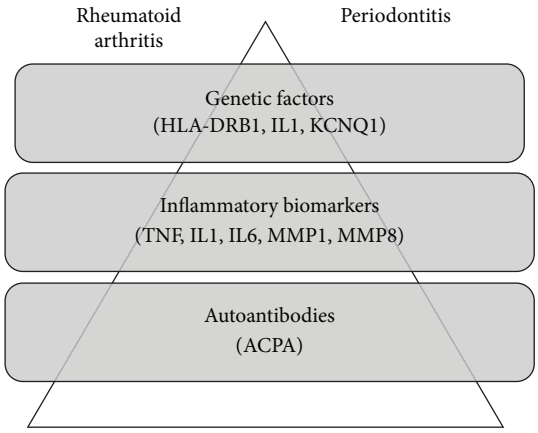

Figure 1: Suggested genetic factors, inflammatory biomarkers, and autoantibodies shared by rheumatoid arthritis and periodontitis. IL1: interleukin-1; TNF: tumor necrosis factor; MMP1: matrix metalloproteinase-1; MMP8: matrix metalloproteinase-8; ACPA: anticyclic citrullinated peptide antibodies.

view, data from literature does not seem to support the sharing of the same mediators. Regarding genetic factors, the most consistent data are related to HLA-DRB1 alleles: especially the presence of SE is associated with susceptibility and severity in both diseases. Conversely, contrasting results are available concerning other genetic polymorphisms or proinflammatory cytokines such as TNF, IL1, and IL6. Finally, there are few cases in which RA-related antibodies are found in PD patients. These evidences are graphically represented in Figure 1. Altogether, the paucity of data on this topic suggests that deeper studies, including wider populations and with a longitudinal design, are required to better clarify this issue.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Fulvia Ceccarelli, Matteo Saccucci, and Gabriele Di Carlo contributed equally to this work.

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Research Article

Soluble CD14 Enhances the Response of Periodontal Ligament Stem Cells to Toll-Like Receptor 2 Agonists

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Human periodontal ligament stem cells (hPDLSCs) do not express membrane-bound CD14, and their responsiveness to bacterial lipopolysaccharide (LPS) is drastically enhanced by soluble CD14 (sCD14), which is due to the facilitation of the interaction between LPS and Toll-like receptor- (TLR-) 4. Several studies also show that sCD14 enhances the responsiveness of different immune cells to TLR-2, but such effect in hPDLSCs has not been studied so far. In the present study, we investigated for the first time the potential effect of sCD14 on the hPDLSC response to two different TLR-2 agonists, *in vitro*. Primary hPDLSCs were stimulated with synthetic lipopeptide Pam3CSK4 or lipoteichoic acid (LTA) in concentrations 1-1000 ng/ml in the presence/absence of sCD14 (250 ng/ml). Additionally, the effect of different sCD14 concentrations (2.5-250 ng/ml) on the TLR-2 response was determined in Pam3CSK4- or LTA-triggered hPDLSCs. The resulting expression of interleukin- (IL-) 6, chemokine C-X-C motif ligand 8 (CXCL8), and chemokine C-C motif ligand 2 (CCL2) was measured by qPCR and ELISA. The production of IL-6, CXCL8, and CCL2 was gradually increased by both TLR-2 agonists and was significantly enhanced by sCD14. The response of hPDLSCs to low and submaximal concentrations of TLR-2 agonists (1-100 ng/ml) was most effectively enhanced by sCD14. The effect of sCD14 on TLR-2 response in hPDLSCs was concentration-dependent and was already detectable at low sCD14 levels. Our data showed that exogenous sCD14 significantly enhanced the responsiveness of hPDLSCs to TLR-2 agonists and enabled the detection of their small amounts. This effect was already detectable at low sCD14 levels, which are comparable to those in saliva and gingival crevicular fluid. Changes in the local sCD14 level may be considered as a crucial factor influencing the susceptibility of hPDLSCs to different pathogens and thus may contribute to the progression of periodontitis.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitor cells, exhibiting self-renewal potential and an ability to differentiate *in vitro* into multiple cell types [1]. MSCs reside in various dental tissues [2–4], including the periodontal ligament [5]. Human periodontal ligament stem cells (hPDLSCs) are a heterogenous population of fibroblast-like cells [6], which fulfils the minimal criteria for MSCs such as the expression of characteristic surface markers and the multilineage differentiation potential [6, 7]. Similar to other MSCs, hPDLSCs possess immunomodulatory ability and modulate the activity of immune cells by either paracrine mechanisms or direct cell-to-cell contact. hPDLSCs are involved in regulating the processes implicated in periodontal tissue homeostasis, regeneration, and periodontal disease progression [6, 8, 9].

Periodontitis is an inflammatory, multifactorial disease, leading to periodontal tissue destruction and may result in tooth loss in severe cases [10, 11]. It is associated with an impairment of host-microbial homeostasis, leading to an inappropriate, overwhelming immune response [12], and is affected by several risk factors including genetic predisposition [13] and smoking habits [14]. The Gram-negative bacterium *Porphyromonas gingivalis* is most often associated with periodontitis [15, 16] and is currently considered as a

keystone pathogen [17]. *P. gingivalis* and its virulence factors, like lipopolysaccharide (LPS) [18, 19] and various lipoproteins [20, 21], induce an inflammatory response in hPDLSCs and influence their immunomodulatory potential.

Bacterial components are recognized by TLRs, a family of pattern recognition receptors of the host cells [21–26]. The activation of TLRs in hPDLSCs results in the production of different inflammatory mediators and is assumed to regulate their immunomodulatory ability [27]. hPDLSCs express different types of TLRs including TLR-2 and TLR-4, which sense lipoproteins and bacterial LPS, respectively [28–30]. Previous studies show that activation of hPDLSCs by *P. gingivalis* LPS leads to the secretion of several potent proinflammatory mediators, like interleukin- (IL-) 1β, IL-6, chemokine C-X-X motif ligand 8 (CXCL8), and chemokine C-C motif ligand 2 (CCL2) [18, 19]. In contrast to specialized immune cells, hPDLSCs exhibit a very low reactivity to *P. gingivalis* LPS in concentrations up to 1 μg/ml, reaching a strong activation with only a quite high *P. gingivalis* LPS concentration (10 μg/ml) [18]. The low sensitivity of hPDLSCs to *P. gingivalis* LPS is explained by the lack of membrane-bound CD14 on the hPDLSC surface [9, 19], an important coreceptor of TLR-4 [31, 32]. Our previous study shows that the soluble form of CD14 (sCD14) significantly enhances the response of hPDLSCs to *P. gingivalis* and *Escherichia coli* LPS and allows sensing even low LPS levels in the range of ng/ml [19, 33].

Several studies indicate an association between periodontitis progression and TLR-2 (reviewed in [34]). Lipoproteins of different bacteria, including *P. gingivalis*, are the main ligands of TLR-2 [20, 21, 35]. A study of Morandini et al. shows that lipoprotein-induced expression of IL-6 and CXCL8 is significantly decreased in TLR-2-silenced periodontal ligament fibroblasts [21]. A recent study of our group shows that the synthetic TLR-2 agonist Pam3CSK4, a triacylated lipoprotein, induces a significantly higher inflammatory response in hPDLSCs than bacterial LPS [19].

Previous studies on distinct immune cells suggest that sCD14 might enhance the TLR-2 activation in the presence of an appropriate agonist [36–39]. Ranoa et al. demonstrates an involvement of sCD14 in the interaction of TLR-2 with synthetic triacylated lipopeptides, making cells susceptible for lipopeptides even on the nanogram range [38]. Further, Nakata et al. shows that CD14 directly binds triacylated lipopeptides, without binding TLR-2 [36]. Additionally, other studies also show a CD14-dependent induction of the cytokine synthesis in T cells and monocytes after stimulation with TLR-2 agonist lipoteichoic acid (LTA) [37, 40]. To the best of our knowledge, the potential of sCD14 to modulate the activation of TLR-2 in hPDLSCs is not known to date. Therefore, in the present study, we investigated the effect of sCD14 on the response of hPDLSCs to TLR-2 agonists. Particularly, we measured the expression of IL-6, CXCL8, and CCL2 in hPDLSCs upon stimulation with different concentrations of the synthetic triacylated lipoprotein Pam3CSK4 or LTA in the presence/absence of a single sCD14 concentration. Further, we tested the dependency of the hPDLSC response to a submaximal response to TLR-2 agonists on different sCD14 concentrations.

2. Material and Methods

2.1. Cell Culture. Third molars from five different periodontally healthy patients were extracted due to orthodontic reasons and used to isolate primary hPDLSCs, as described in our previous study [19]. All patients were informed and gave their written consent before the surgical procedure. The study protocol was approved by the Ethics Committee of the Medical University of Vienna. All procedures were performed according to the "Good Scientific Practice" guidelines of the Medical University of Vienna and the Declaration of Helsinki. Primary hPDLSCs were cultured under humidified conditions in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, USA), 1% penicillin, and streptomycin (P/S, Gibco, Carlsbad, USA). Cells between the 3rd and 7th culture passages were used for the experiments. Cell surface marker expression of mesenchymal stem cells (CD29, CD90, CD105, and CD146) and of hematopoietic cells (CD14, CD31, CD34, and CD45) was analysed to verify minimal MSC criteria in isolated hPDLSCs [19].

2.2. Stimulation Protocol. Primary hPDLSCs were seeded in 24-well plates at a density of 5×10^4 cells per well, in 0.5 ml DMEM, supplemented with 1% P/S and 10% FBS. In the first series of experiments, the effect of sCD14 in a constant concentration of 250 ng/ml on the response of hPDLSCs to different concentrations of TLR-2 agonists was investigated. The sCD14 concentration was chosen due to our experience from a previous study [19]. Cells were stimulated with either TLR-2/1 agonist Pam3CSK4 (InvivoGen, San Diego, USA) or TLR-2 agonist lipoteichoic acid (LTA, InvivoGen, San Diego, USA) 24 hours after seeding. Stimulation was done with different concentrations (1, 10, 100, and 1000 ng/ml) of TLR-2 agonists in the presence/absence of exogenous sCD14 (Sigma-Aldrich, St. Louis, USA). In the second series of experiments, the impact of different sCD14 concentrations on the hPDLSC response to constant concentrations of TLR-2 agonists was examined. In these experiments, the cells were treated with submaximal concentrations (10 ng/ml) of either Pam3CSK4 or LTA in the presence of sCD14 at concentrations 2.5, 10, 25, 100, and 250 ng/ml. All stimulations were performed in duplicates and in FBS-free DMEM, supplemented with 1% P/S. After 24 hours of stimulation, IL-6, CXCL8, and CCL2 gene expression levels were measured by quantitative polymerase chain reaction (qPCR), and the levels of corresponding proteins in the conditioned media were determined using the enzyme-linked immunosorbent assay (ELISA).

2.3. Quantitative PCR. The TaqMan Gene Expression Cells-to-CT kit (Applied Biosystems, Foster City, USA) was used for cell lysis, mRNA extraction, reverse transcription into cDNA, and qPCR according to the manufacturer's protocol. Reverse transcription was conducted on the Primus 96 advanced thermocycler (PeqLab/VWR, Darmstadt, Germany) using the following settings: 37°C for 1 hour and 95°C for 5 minutes for enzyme deactivation followed

by 4°C. qPCR was performed using the ABI StepOnePlus device (Applied Biosystems, Foster City, USA) with the following thermocycler settings: 10 minutes at 1×95°C followed by 50×15 seconds at 95°C and 1 minute at 60°C. For amplifying the target genes, the following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, USA) were used: IL-6, Hs00985639_m1; CXCL8, Hs00174103_m1; CCL2, Hs00234140_m1; and GAPDH Hs99999905. qPCR was performed in paired reactions, followed by specifying the Ct value for each sample. Gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method by the formula

$$\Delta\Delta Ct = \left(Ct^{\text{target}} - Ct^{\text{GAPDH}}\right)_{\text{sample}} - \left(Ct^{\text{target}} - Ct^{\text{GAPDH}}\right)_{\text{control}}. \quad (1)$$

The n-fold expression of the target genes compared to the corresponding untreated control was determined. GAPDH served as endogenous reference.

2.4. Enzyme-Linked Immunosorbent Assay. For determining IL-6, CXCL8, and CCL2 protein levels in the conditioned media, ELISA Ready-Set-Go! Kits (eBioscience, Waltham, USA) were used, according to the manufacturer's protocol. ELISAs were performed in duplicates, followed by measuring optical density at 450 nm. Measured absorbance values were plotted against the corresponding standard curves, determining the appropriate protein concentrations.

2.5. Statistical Analysis. The statistical program SPSS 24.0 (IBM, Armonk, USA) was used for all statistical analysis. The Friedman test followed by the Wilcoxon test for pairwise comparison was used. Differences with p values < 0.05 were considered as statistically significant.

3. Results

3.1. Effect of sCD14 on the hPDLSC Response to TLR-2 Agonist Pam3CSK4. Gene and protein expression levels of IL-6, CXCL8, and CCL2 in primary hPDLSCs, stimulated with different Pam3CSK4 concentrations in the presence/absence of sCD14 (250 ng/ml), are shown in Figure 1. In the absence of sCD14, Pam3CSK4 induced a concentration-dependent increase in IL-6, CXCL8, and CCL2 gene expression levels. The highest response was observed at 1000 ng/ml Pam3CSK4. Stimulation with 10 to 1000 ng/ml Pam3CSK4 led to a significant increase in IL-6, CXCL8, and CCL2 gene expressions. Exogenous sCD14 significantly enhanced the expressions of IL-6, CXCL8, and CCL2 at submaximal Pam3CSK4 concentrations (1-100 ng/ml). The response to the highest Pam3CSK4 concentration (1000 ng/ml) was not significantly affected by exogenous sCD14.

Protein measurements with ELISA showed that in the absence of sCD14, IL-6, CXCL8, and CCL2 protein levels were significantly increased after stimulation with Pam3CSK4 starting from 10 ng/ml in a concentration-dependent manner. The presence of sCD14 during stimulation resulted in significantly enhanced levels of all proteins in response to all tested Pam3CSK4 concentrations excepting the highest one (1000 ng/ml).

3.2. Effect of sCD14 on the hPDLSC Response to TLR-2 Agonist LTA. Gene and protein expression levels of IL-6, CXCL8, and CCL2 in primary hPDLSCs, stimulated with different LTA concentrations in the presence/absence of sCD14 (250 ng/ml), are shown in Figure 2. In the absence of sCD14, LTA induced a concentration-dependent increase in IL-6, CXCL8, and CCL2 gene expression levels. The highest response was observed at 1000 ng/ml LTA. A significant increase in the expression of all three target genes was observed after stimulation from 10 to 1000 ng/ml LTA. Adding the exogenous sCD14 during stimulation significantly enhanced the expression levels of all three target genes in response to all tested LTA concentrations.

As measured by ELISA, in the absence of sCD14, IL-6, CXCL8, and CCL2 protein levels were significantly increased after stimulation with LTA in a dose-dependent manner. A significant increase started at 100 ng/ml for IL-6 and CXCL8 and at 1 ng/ml for CCL2. The presence of exogenous sCD14 during stimulation resulted in significantly enhanced levels of IL-6 and CCL2 in response to all tested LTA concentrations excepting the highest one. Additionally, sCD14 significantly increased CXCL8 protein production induced by all tested LTA concentrations.

3.3. Effect of Different sCD14 Concentrations on the hPDLSC Response to Submaximal Concentration of Pam3CSK4. The dependency of the hPDLSC response to submaximal Pam3CSK4 concentration (10 ng/ml) on different sCD14 levels is shown in Figure 3. Stimulation of hPDLSCs with Pam3CSK4 in the absence of sCD14 resulted in increased IL-6, CXCL8, and CCL2 gene expression levels. This response to Pam3CSK4 was increased by sCD14 in a concentration-dependent manner. A significant increase was observed starting from 10 ng/ml sCD14 for IL-6 and from 2.5 ng/ml for CXCL8 and CCL2.

In the absence of sCD14, 10 ng/ml Pam3CSK4 already induced protein expression of IL-6, CXCL8, and CCL2 in a concentration-dependent manner. A significant increase of all three investigated proteins was observed starting from sCD14 concentration as low as 25 ng/ml.

3.4. Effect of Different sCD14 Concentrations on the hPDLSC Response to Submaximal Concentration of LTA. The dependency of hPDLSC response to submaximal LTA concentration (10 ng/ml) on different sCD14 levels is shown in Figure 4. LTA treatment of hPDLSCs in the absence of sCD14 caused an increase in gene expressions of IL-6, CXCL8, and CCL2. This response to LTA was increased by sCD14 in a concentration-dependent manner, showing significances at higher sCD14 levels for all three genes. Gene expression of CCL2 was already significantly increased at the lowest tested sCD14 level (2.5 ng/ml).

Protein expressions of all three targets were increased upon LTA stimulation and were further enhanced by exogenous sCD14 in a concentration-dependent manner. Statistically significant differences were observed starting

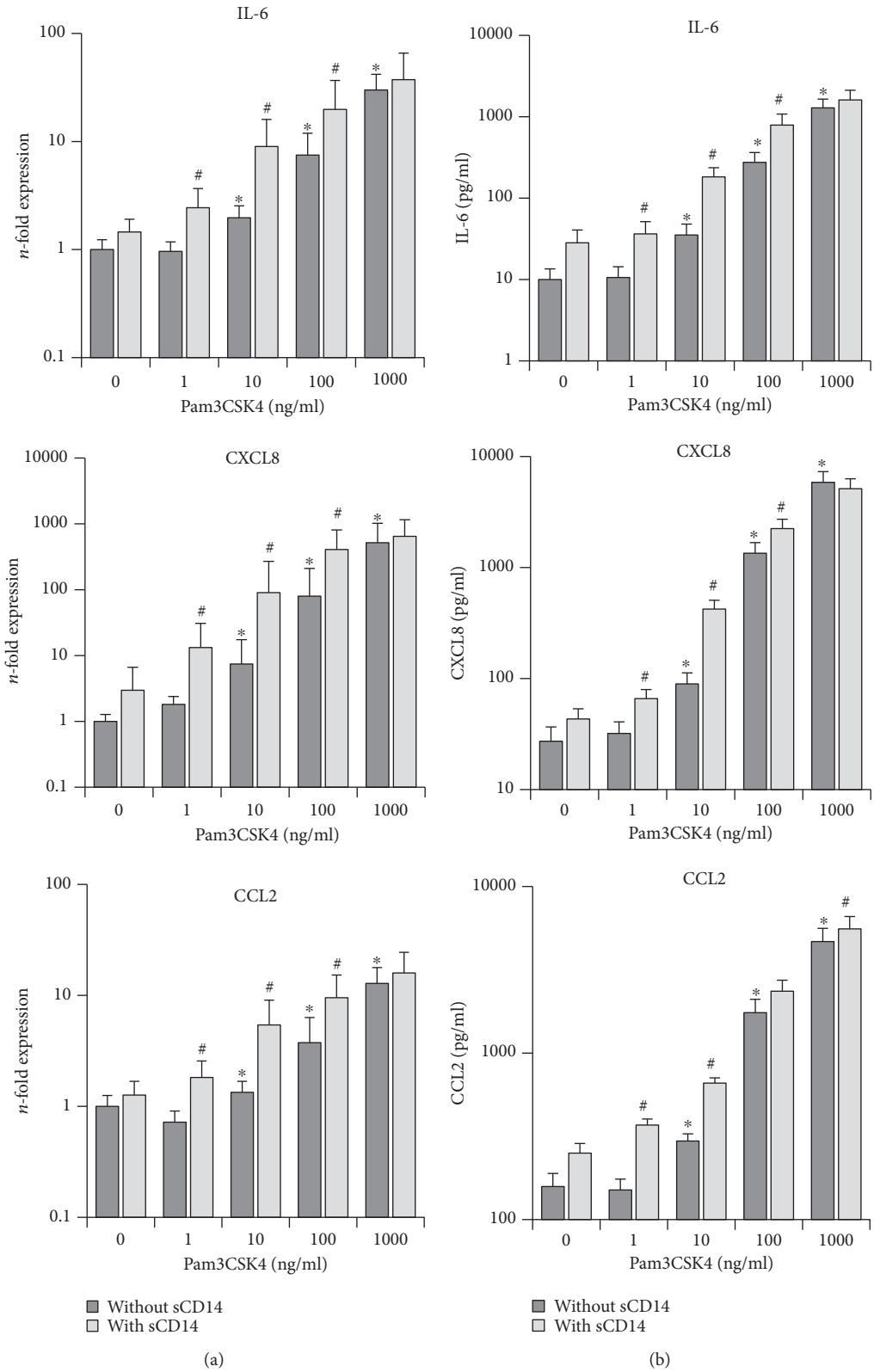

Figure 1: Effect of sCD14 on the hPDLSC response to TLR-2 agonist Pam3CSK4. Primary hPDLSCs were stimulated with different Pam3CSK4 concentrations (1-1000 ng/ml), in the presence/absence of sCD14 (250 ng/ml). Untreated cells served as the control. After 24 hours of incubation, IL-6, CXCL8, and CCL2 gene expression levels (a) were measured using qPCR. The *y*-axis shows the *n*-fold expression of the target genes compared to the unstimulated control. The corresponding protein levels in conditioned media (b) were determined by ELISA. All data are presented as mean ± s.e.m. from five independent experiments using cells from five different donors. $^{*}p$ value < 0.05 compared to control. #p value < 0.05 compared to the appropriate group stimulated in the absence of sCD14.

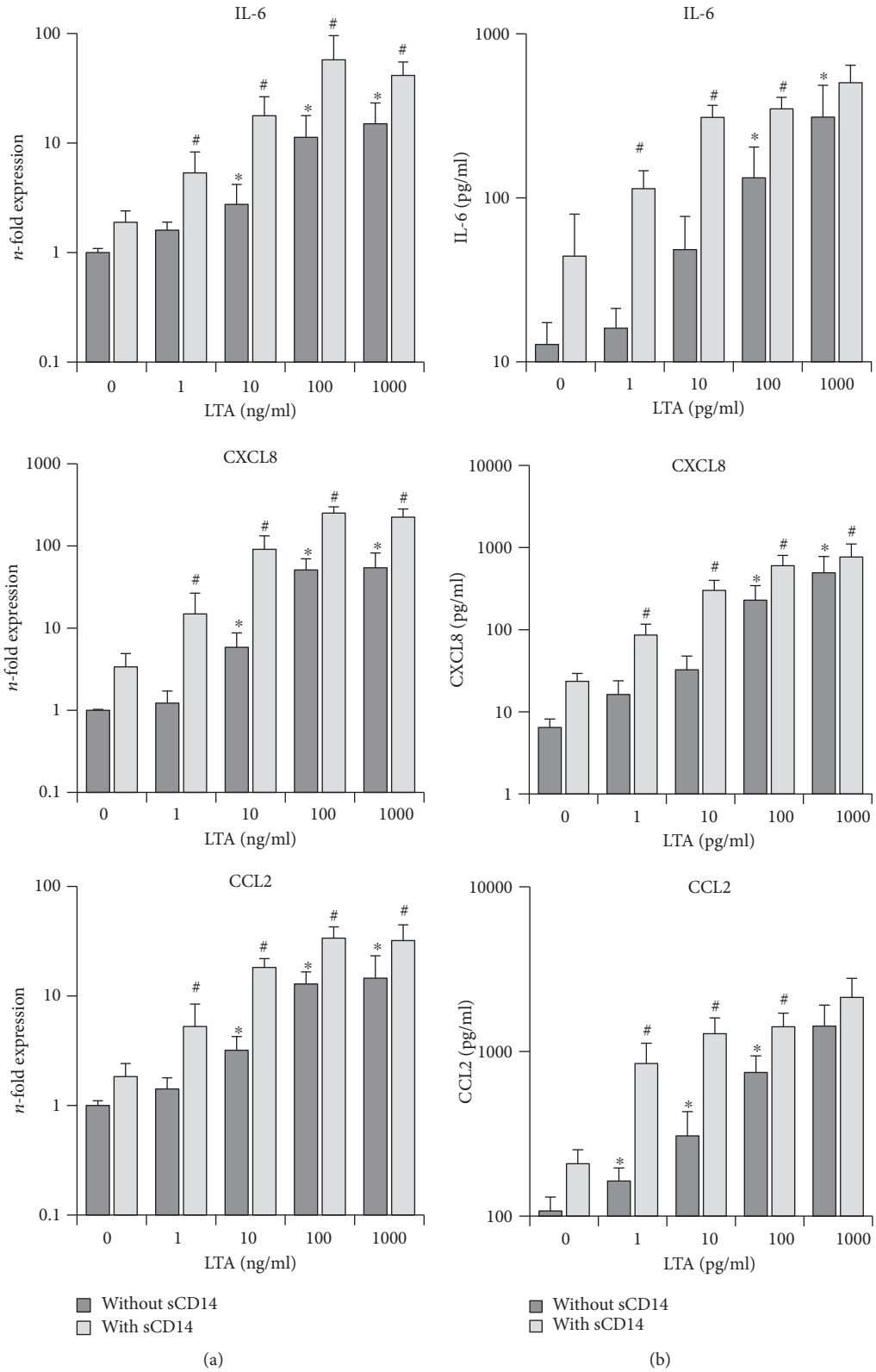

Figure 2: Effect of sCD14 on the hPDLSC response to TLR-2 agonist LTA. Primary hPDLSCs were stimulated with different LTA concentrations (1-1000 ng/ml), in the presence/absence of sCD14 (250 ng/ml). Untreated cells served as the control. After 24 hours of incubation, IL-6, CXCL8, and CCL2 gene expression levels (a) were measured using qPCR. The *y*-axis shows the *n*-fold expression of the target genes compared to the unstimulated control. The corresponding protein levels in conditioned media (b) were determined by ELISA. All data are presented as mean ± s.e.m. from five independent experiments using cells from five different donors. $^{*}p$ value < 0.05 compared to the control. #p value < 0.05 compared to the appropriate group stimulated in the absence of sCD14.

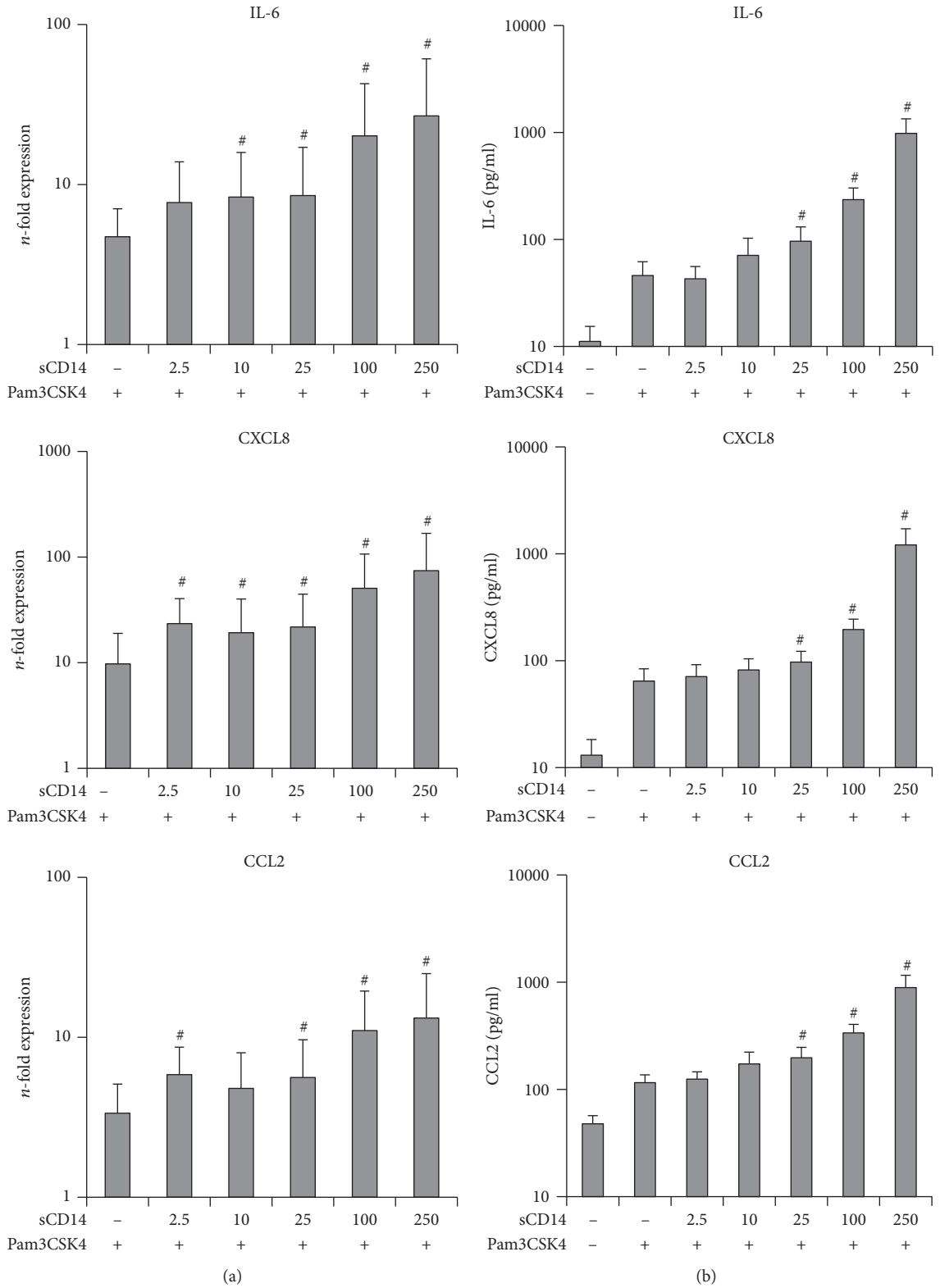

Figure 3: Effect of different sCD14 concentrations on the hPDLSC response to submaximal Pam3CSK4 concentration. Primary hPDLSCs were stimulated with Pam3CSK4 at submaximal concentration (10 ng/ml) and different concentrations of sCD14 (2.5–250 ng/ml). Untreated cells served as the control. After 24 hours of incubation, IL-6, CXCL8, and CCL2 gene expression levels (a) were determined using qPCR. The *y*-axis shows the *n*-fold expression of the target genes compared to the unstimulated control. The corresponding protein levels in conditioned media (b) were determined by ELISA. All data are presented as mean ± s.e.m. from five independent experiments using cells from five different donors. #p value < 0.05 compared to cells stimulated with Pam3CSK4 in the absence of sCD14.

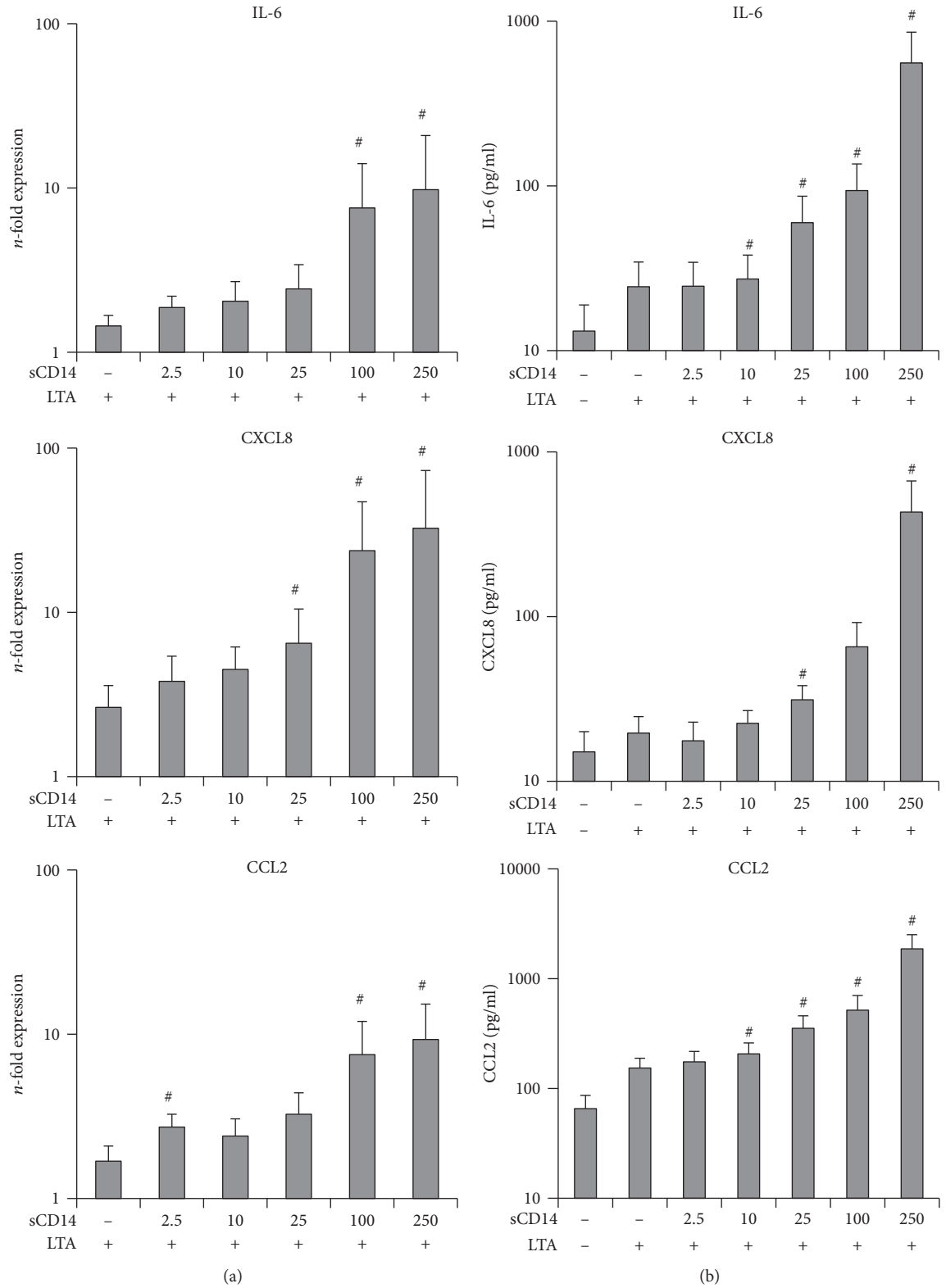

Figure 4: Effect of different sCD14 levels on the hPDLSC response to submaximal LTA concentration. Primary hPDLSCs were stimulated with LTA at submaximal concentration (10 ng/ml) and different concentrations of sCD14 (2.5-250 ng/ml). Untreated cells served as the control. After 24 hours of incubation, IL-6, CXCL8, and CCL2 gene expression levels (a) were measured using qPCR. The *y*-axis shows the *n*-fold expression of the target genes compared to the unstimulated control. The corresponding protein levels in conditioned media (b) were determined by ELISA. All data are presented as mean ± s.e.m. from five independent experiments using cells from five different donors. #p value < 0.05 compared to cells stimulated with LTA in the absence of sCD14.

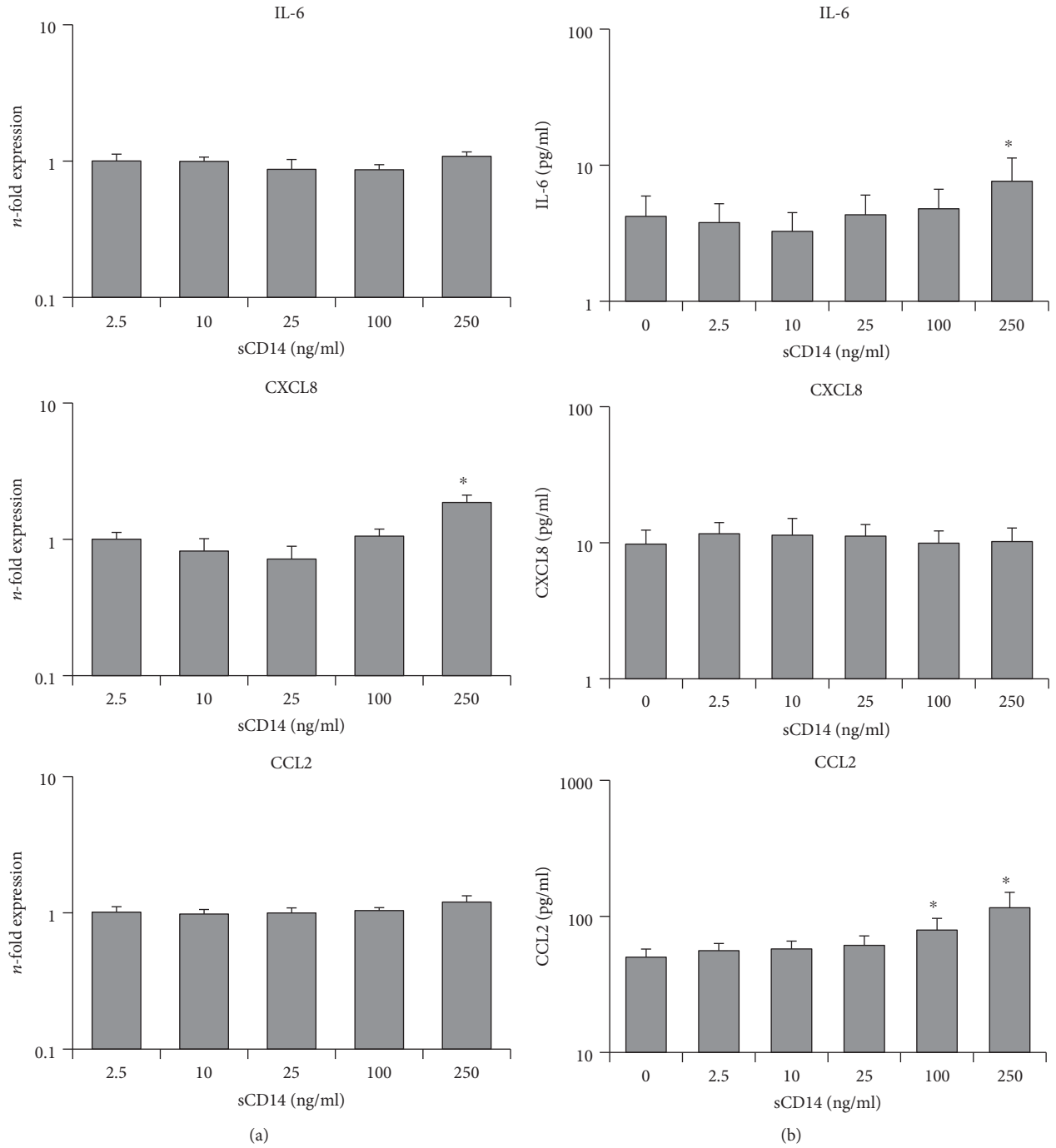

Figure 5: Response of hPDLSCs to different sCD14 concentrations. Primary hPDLSCs were stimulated for 24 hours with different sCD14 concentrations (2.5 ng/ml–250 ng/ml) in the absence of TLR-2 agonists. Untreated cells served as the control. After 24 hours of incubation, IL-6, CXCL8, and CCL2 gene expression levels (a) were measured using qPCR. The y-axis shows the n-fold expression of the target genes compared to the unstimulated control. The corresponding protein levels in conditioned media (b) were determined by ELISA. All data are presented as mean ± s.e.m. from five independent experiments using cells from five different donors. $^{*}p$ value < 0.05 compared to the control.

from 10 ng/ml sCD14 for IL-6 and CCL2 and from 25 ng/ml sCD14 for CXCL8.

3.5. Response of hPDLSCs to Exogenous sCD14. Figure 5 shows the response of hPDLSCs to exogenous sCD14 without any TLR-2 agonist. Under these conditions, low concentrations of exogenous sCD14 have no significant effect on IL-6, CXCL8, and CCL2 expressions, on gene and protein levels. However, significances were observed only for the highest tested sCD14 levels (250 ng/ml) for IL-6 protein and CXCL8 gene expressions as wells as starting at 100-250 ng/ml for CCL2 protein expression.

4. Discussion

In our previous study, we showed a significant sCD14-mediated increase in the bacterial LPS- induced TLR-4 response in hPDLSCs [19]. Although several studies on different immune cells showed an impact of sCD14 on TLR-2 [36–39], the contribution of sCD14 on the TLR-2 response in hPDLSCs has not been investigated so far. Hence, we investigated for the first time *in vitro* the potential impact of sCD14 on Pam3CSK4- or LTA-induced TLR-2 response in hPDLSCs. Our data demonstrated that exogenous sCD14 substantially enhances the production of inflammatory mediators by hPDLSCs in response to stimulation even with small amounts of TLR-2 agonists. This finding further indicates an important role of sCD14 in the host inflammatory response to different bacterial components.

The production of the inflammatory mediators IL-6, CXCL8, and CCL2 was substantially increased by exogenous sCD14 in response to both tested TLR-2 agonists. The enhancement of the response was observed for all tested Pam3CSK4 and LTA concentrations excepting the highest tested Pam3CSK4 concentration of 1000 ng/ml. The presence of exogenous sCD14 allowed hPDLSCs to sense different TLR-2 agonists at very low concentrations, starting from 1 ng/ml, and thus, might sense very low amounts of bacterial pathogens. Such situation is imaginable immediately after epithelial barrier destruction and bacterial invasion into periodontal tissue. Production of different inflammatory mediators by hPDLSCs under these conditions will substantially contribute to the further host inflammatory response and disease progression.

In our study, we focused on the expression of three proteins, IL-6, CXCL8, and CCL2, which are thought to play an essential role in periodontal tissue inflammation and are usually regarded as the factors which enhance the inflammatory response in periodontitis [41, 42]. IL-6 is an important proinflammatory cytokine, involved in the acute inflammation phase and bone resorption [41]. CXCL8 and CCL2 are both chemokines, attracting neutrophils and macrophages, respectively, to the site of inflammation, promoting acute inflammation [42, 43]. However, studies of the last years suggest that besides classical proinflammatory effects, these proteins play some anti-inflammatory roles and/or contribute to periodontal tissue homeostasis. Particularly, there are increasing evidences that IL-6 is involved in MSC-dependent suppression of T cell proliferation. There are also evidences that IL-6 suppresses neutrophil apoptosis and respiratory burst and facilitates regulatory dendritic cell and anti-inflammatory macrophage formation [44–47]. Lower CXCL8 levels are thought to diminish neutrophil recruitment to the periodontal pocket, which may lead to overgrowth of some pathogenic microorganisms.

Based on this dual role of all investigated mediators, it is difficult to assess the exact role of local sCD14 levels and the enhancement of TLR-2-induced response by sCD14. It might on the one hand promote the inflammation response and on the other hand contribute to the maintenance of periodontal tissue homeostasis. The dual role of the immune response should be also noted, which on the one hand is aimed at eliminating pathogens and on the other hand leads to collateral tissue damages [48]. Hence, the physiological functions and the exact role in periodontal tissue inflammation of IL-6, CXCL8, and CCL2, produced by hPDLSCs in response to different virulence factors, need to be further investigated.

Our data showed a significant effect of sCD14 on the TLR-2 response in hPDLSCs regardless of the TLR-2 agonist. This may occur obviously by facilitating the interaction between lipoproteins and TLR-2 on the hPDLSC surface by sCD14 [36–38, 40]. However, the hPDLSC response to 1000 ng/ml Pam3CSK4 was not further enhanced by sCD14, which was also observed in our previous study [19]. This finding can be explained by the fact that Pam3CSK4 at this concentration induces a maximal TLR-2 response and that TLR-2 signalling seems to be fully activated even in the absence of sCD14. This observation is in line with other studies, exhibiting CD14 as critical for the response to low LPS doses and less important for high LPS doses [49] and that sCD14 makes immune cells more susceptible to triacetylated lipopeptides on the nanogram level [38].

In contrast to Pam3CSK4, sCD14 significantly enhanced the hPDLSC response to LTA at 1000 ng/ml. However, it should be noted that in the absence of sCD14, the production of different proinflammatory mediators by hPDLSCs in response to 1000 ng/ml LTA was up to 90% lower than that induced by similar concentration of Pam3CSK4. Therefore, it can be assumed that LTA at 1000 ng/ml did not induce a maximal activation of TLR-2-dependent response, which can be further increased by adding exogenous sCD14. The differences in the hPDLSC response might be explained by the distinct chemical nature of the two used TLR-2 agonists and the resulting different activation mechanisms [36–38, 40, 50].

The molecular mechanisms of the cell response activation by TLR-2 agonists are differently discussed in the literature. Triacylated lipoprotein Pam3CSK4 coordinately binds to TLR-2 as well as to TLR-1, leading to a heterodimerization of these two receptors and resulting in the formation of a stable ternary signalling complex, consisting of TLR-2, TLR-1, and triacetylated lipoprotein [36, 38, 50]. sCD14 as well as lipopolysaccharide-binding protein (LBP) sensitize cells to lipoproteins [51, 52] by disaggregating lipoproteins and delivering monomeric lipoproteins to the receptors [36, 38]. In contrast to the membrane-bound CD14, which is physically associated with the TLR heterodimer [53], sCD14 facilitates the formation of the ternary signalling complex without being a part of the complex itself. A study of Ranoa et al. suggests that during agonist delivery to the receptors, sCD14 stably interacts with TLR-1 but is replaced by TLR-2 during ternary complex formation [38]. Concerning LTA, several functional studies show an LTA-activated cellular response through TLR-2 recognition without the involvement of other TLRs but in the presence of sCD14 [37, 40, 54]. However, the knowledge about the precise interactions of involved proteins is limited. One study demonstrates the complex formation of LTA with LBP and catalytic transfer of LTA to sCD14 through LBP, resulting in the formation of a LTA-sCD14 complex [37]. Although both TLR-2 agonists seem to differ in the TLR activation mechanism,

they cause NF-κB translocation into the nucleus and further the expression of proinflammatory cytokines [37, 55].

We further investigated the dependency of the hPDLSC response to submaximal concentrations of TLR-2 agonists on different local sCD14 levels. We found a clear dose-dependent increase of the TLR-2 response from the lowest to the highest sCD14 concentrations. On the protein level, a significant increase in hPDLSC response to submaximal concentrations of TLR-2 agonists was induced by 25 ng/ml of sCD14, which is a rather low level compared to those detected in physiological fluids. Particularly, the local sCD14 concentration in gingival crevicular fluid and blood serum is in μg/ml [56, 57]. Since the effect of sCD14 levels on the hPDLSC response to both TLR-2 agonists and bacterial LPS [19] is concentration-dependent, we suggest that alterations of local sCD14 levels might play an important role in periodontitis through sensitizing hPDLSCs to periodontal pathogens. For example, an increase in sCD14 will facilitate the recognition of periodontal bacteria-associated lipoproteins of LPS by hPDLSCs. This enhances the production of diverse inflammatory mediators, including IL-6, CXCL8, and CCL2, which may contribute to the overwhelming immune response and consequently to progression of periodontitis. This assumption is supported by different clinical studies, which mostly show a positive association between sCD14 levels and periodontal disease [57–59]. Particularly, Isaza-Guzmán et al. detected significant higher sCD14 levels in the saliva of periodontitis patients compared to healthy controls [58], whereas other studies also showed a significantly higher sCD14 concentrations in the serum of subjects with chronic periodontitis [57, 59]. Additionally, a positive correlation between sCD14 saliva levels and different clinical parameters of periodontitis severity was demonstrated [58]. Interestingly, Jin and Darveau exhibited a negative correlation between sCD14 levels in gingival crevicular fluids and the number and depth of periodontal pockets in patients suffering from periodontitis [56]. These inconsistencies, concerning the relationship between local sCD14 levels and periodontitis severity, may be explained by the dual nature of the immunomodulatory properties of hPDLSCs. On the one hand, hPDLSCs produce proinflammatory mediators under certain conditions, promoting the inflammatory response. On the other hand, hPDLSCs are known to produce immunosuppressive proteins, which diminish the local inflammatory response [60]. Regulation of the immunomodulatory activity of hPDLSCs by TLR activation may effect periodontal disease progression [19, 27]. This assumption is supported by studies, showing an impaired immunomodulation of hPDLSCs in periodontitis patients [61, 62].

It seems that there are some differences in the affinity of sCD14 to different TLRs in hPDLSCs. In our previous study, we show that the maximal effect of sCD14 on the TLR-4 response to bacterial LPS is already achieved at concentrations of 25 ng/ml [19]. In contrast, in the present study, a gradual increase in hPDLSC response to both TLR-2 agonists was observed with a gradual increase of the sCD14 concentration from 2.5 to 250 ng/ml. Although sCD14 facilitates the formation of a stable ternary signalling complex for both TLRs, without being a part of the complex itself [36, 38, 63], the activation mechanisms of both TLRs differ from each other, due to the different natures of their agonists or the involvement of the MD-2 protein in the TLR-4 signalling complex [64]. These differences may possibly explain the higher sensitivity of the TLR-4 response to sCD14 than TLR-2.

We further investigated a potential influence of sCD14 on the expression of inflammatory markers in hPDLSCs in the absence of TLR-2 agonists. Surprisingly, we found a significant increase in the secretion of IL-6 and CCL2 and a significant increase in the expression of CXCL8 at the highest used sCD14 concentrations. However, the expression levels, triggered solely by sCD14, were rather negligible compared with its effect on Pam3CSK4- or LTA-induced responses. Therefore, we conclude that the enhancement of the TLR-2 responses by sCD14 is due to its interaction with the TLR-2 receptor. Additionally, the stable interaction of sCD14 with TLR-1 until the heterodimerization with TLR-2 [38] may influence the expression of inflammatory markers, at least at high sCD14 concentrations.

5. Conclusion

In conclusion, our study shows that sCD14 enhances the response of hPDLSCs to submaximal concentrations of TLR-2 agonists in a concentration-dependent manner. The stimulating effect was detectable at sCD14 levels, which are comparable to those in saliva and gingival crevicular fluid. The presence of sCD14 sensitizes hPDLSCs to bacterial pathogens and enables their response even to low amounts of TLR-2 agonists in the range of few nanograms per millilitres. We suggest that different local sCD14 levels may affect the strength of TLR-2-mediated immune response in hPDLSCs, leading to a stronger IL-6, CXCL8, and CCL2 production at higher sCD14 levels. These higher proinflammatory cytokine levels may contribute to the overwhelming immune response and consequently affect the development of periodontitis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

IL18 Polymorphism and Periodontitis Susceptibility, Regardless of *IL12B*, *MMP9*, and Smoking Habits

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Genetic variations contribute to the susceptibility in the development of periodontitis. The aim of this study was to investigate the influence of *IL18*, *IL12*, and *MMP9* polymorphisms in the chronic periodontitis. This case-control study involved 381 individuals matched by gender and age. Genotyping of *IL18* (rs187238 and rs1946518) and *IL12B* (rs3212227) was performed by PCR-SSP and PCR-RFLP was used for *MMP9* (rs3918242). IL-18 and MMP-9 were quantified in the serum by ELISA. SNPStats and OpenEpi software were used for statistical analysis and, in order to eliminate smoking as a confounding factor, the analyses were also performed in nonsmoking subjects. The *IL18*-137G/C genotype was associated with the risk of chronic periodontitis in nonsmokers ($P_c = 0.03$; OR = 1.99; overdominant inherence model). In the multivariate analyses, homozygous *IL18*-137G/G and *IL18*-607C/C were more frequent in males compared to women with these same genotypes (OR = 2.51 and OR = 3.30, respectively). The serum levels of the IL-18 in patients were higher than those in healthy controls ($P = 0.005$). *IL12B* and *MMP9* polymorphisms and MMP-9 serum concentration were similar in patients and controls. In this study, *IL18* was associated with chronic periodontitis susceptibility. Men had greater risk than women for developing the disease when *IL18* polymorphism was considered and the susceptibility was independent of the smoking status.

1. Introduction

Chronic periodontitis (CP) is a complex and common oral disease of microbial origin, characterized by inflammatory responses that affect the supporting tissue of the tooth, resulting in the formation of a periodontal pocket and alveolar bone resorption [1, 2]. Eventually, it leads to tooth loss in adult humans impacting their quality of life [3]. Despite the presence of bacteria, immune response is involved in the pathogenesis of CP and genetic polymorphisms in the mediators of immunity have been associated with the susceptibility and severity of periodontitis [4–6]. Among the immune mediators, interleukins (ILs) and matrix metalloproteinases (MMPs) are related to the development of inflammatory response, remodeling of periodontal tissue and bone resorption [7].

IL-18 is a proinflammatory cytokine that stimulates the migration of neutrophils, amplifies IFN-γ secretion through NK cells, and activates osteoclast [8]. Studies have indicated that IL-18 induces the release of matrix metalloproteinase 9 (MMP-9) and IL-1β, both with proinflammatory activity, resulting in tissue degradation [9]. The IL-18, belonging to the IL-1 cytokine superfamily, is involved in a wide variety of inflammatory diseases [10–12]. It is mainly produced by

monocytes, active macrophages, and dendritic cells in response to antigenic stimuli such as lipopolysaccharide of Gram-negative bacteria [13].

The interleukin-12 (IL-12) is expressed through activated macrophages and acts on T and NK cells [14]. This is an immunoregulatory cytokine which affects the Th1 response contributing to the production of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) inducing bone and cartilage resorption by osteoclasts. IL-12 also positively regulates the expression of IL-18 functional receptor and synergizes with IL-18 to improve the production of IFN-γ and IL-1β [15].

MMPs are a structural and functional family of proteolytic enzymes responsible for the degradation of collagen fibers and extracellular matrix components. These enzymes are produced mainly by polymorphonuclear leukocytes, keratinocytes, monocytes, fibroblasts, and mesenchymal cells. MMPs may play an important role in tissue remodeling and repair associated with the development of inflammatory response [16].

The biological mechanisms involved in CP pathogenesis mediated by IL-18, IL-12, and MMP-9 are described in Figure 1.

Polymorphism in the *IL12B*, *IL18*, and *MMP9* genes could affect the transcriptional activity, the production of proteins, and their serum and crevicular fluid levels in the CP. Therefore, we hypothesize that there was an association between the polymorphisms in the *IL12B*, *IL18*, and *MMP9* regions and the development of periodontitis. Thus, the aim of this study was to investigate the influence of *IL18* (rs187238 and rs1946518), *IL12B* (rs3212227), and *MMP9* (rs3918242) polymorphisms and the IL-18 and MMP-9 serum levels on the immunopathogenesis of CP in individuals from the North/Northwest regions of the state of Paraná, Southern Brazil.

2. Material and Methods

2.1. Sample Selection. This case-control study was approved by the Human Research and Ethics Committee of the State University of Maringá (COPEP–UEM–number: 719/2011 and 1.866.509/2016). In total, 381 individuals were recruited from the dental clinics of the State University of Maringá (UEM) and Inga University Center (UNINGA) from January 2012 to August 2017. All individuals who agreed to participate in this research were informed about the nature of the study and signed an informed consent form. Clinical parameters of probing depth (PD) and clinical attachment level (CAL) were examined at four sites (mesial, buccal, distal, and lingual) of each tooth, as well as bleeding on probing (BOP), which were realized by the dentist responsible for the clinic, based on the classification of the 1999 workshop [1]. The participants were divided into two different groups: (i) the chronic periodontitis group (CP) was composed by individuals who had at least 5 sites in different teeth with PD $\geq$ 5 mm, CAL $\geq$ 3 mm, and more than 25% of BOP; and (ii) the control group was formed by individuals who displayed a PD of less than 4 mm and exhibited less than 25% of BOP. CP patients were classified for their type of extension (localized and generalized) and degree of severity (light, moderate, and severe). According to the classification of periodontal diseases of 2017 [17], the patients in this study can be included in the following categories: stages II, III, and IV based on severity, complexity, extension, and distribution; and grades B (moderate rate of progression) and C (rapid rate of progression). All individuals were from the North and Northwest regions of the state of Paraná (22°29′30″-26°42′59″S and 48°02′24″-54°37′38″W), Southern Brazil, and were defined as mixed ethnicity with predominantly European origin [18, 19]. Due to the great Brazilian miscegenation, individuals were classified as previously described by Probst et al. [18] who provided a better picture of Paraná's ethnic constitution, and based on this criterion, descendants of Asians were excluded from the sample. The groups were matched by sex and age. The information about smoking habits was obtained by interviewing the individual (anamnesis).

The inclusion criteria had individuals aged between 30 and 65 years and dental arch with at least 20 teeth. For the determination of serum concentrations for IL-18 and MMP-9, patients and controls were not using antibiotic or anti-inflammatory drugs. The noninclusion criteria included individuals with aggressive periodontal disease, acute infection, diabetes, and rheumatic diseases and individuals who had been treated for periodontitis in the last 6 months. The characteristics of patients with CP and controls are shown in Table 1.

2.2. Blood Collection and DNA Extraction. From each individual, 10 ml of peripheral blood was collected in two tubes: one tube without anticoagulant to measure the serum level of cytokines and the other with EDTA for genotyping. DNA was extracted from peripheral blood collected in EDTA using the salting-out method [20]. The concentration and quality of the DNA were analyzed by optical density in Thermo Scientific Nanodrop 2000 apparatus® (Wilmington, USA).

2.3. Genotyping of IL18. *IL18*-137G>C (rs187238) and *IL18*-607A>C (rs1946518) genotyping was performed by the PCR-SSP according to previous standardization [11]. The DNA concentration used was 50 to 100 ng. For the *IL18*-137 position, we used 0.5 μM of a specific primer sequence and a common reverse primer, 0.3 μM of control primer, $MgCl_2$ 1.5 mM, dNTPs 200 μM, and 1.0 U of *Taq* DNA polymerase. For the *IL18*-607 position, we used 0.4 μM of a common reverse primer and 0.4 μM of specific forward primer sequences; in addition, we used 0.13 μM of forward control primer, $MgCl_2$ 1.5 mM, dNTPs 200 μM, and 1.0 U of *Taq* DNA polymerase. Amplification cycles were used in a GeneAmp PCR System 9700 (Applied Biosystems™) thermocycler. Amplification product analysis was done on SYBR Safe stained agarose gel (Invitrogen®, Life Technologies, Grand Island, NY).

2.4. Genotyping of IL12B. *IL12*+1188A>C (rs3212227) genotyping was performed by PCR-SSP using specific genotyping kits (Invitrogen®, Carlsbad, CA, USA) according

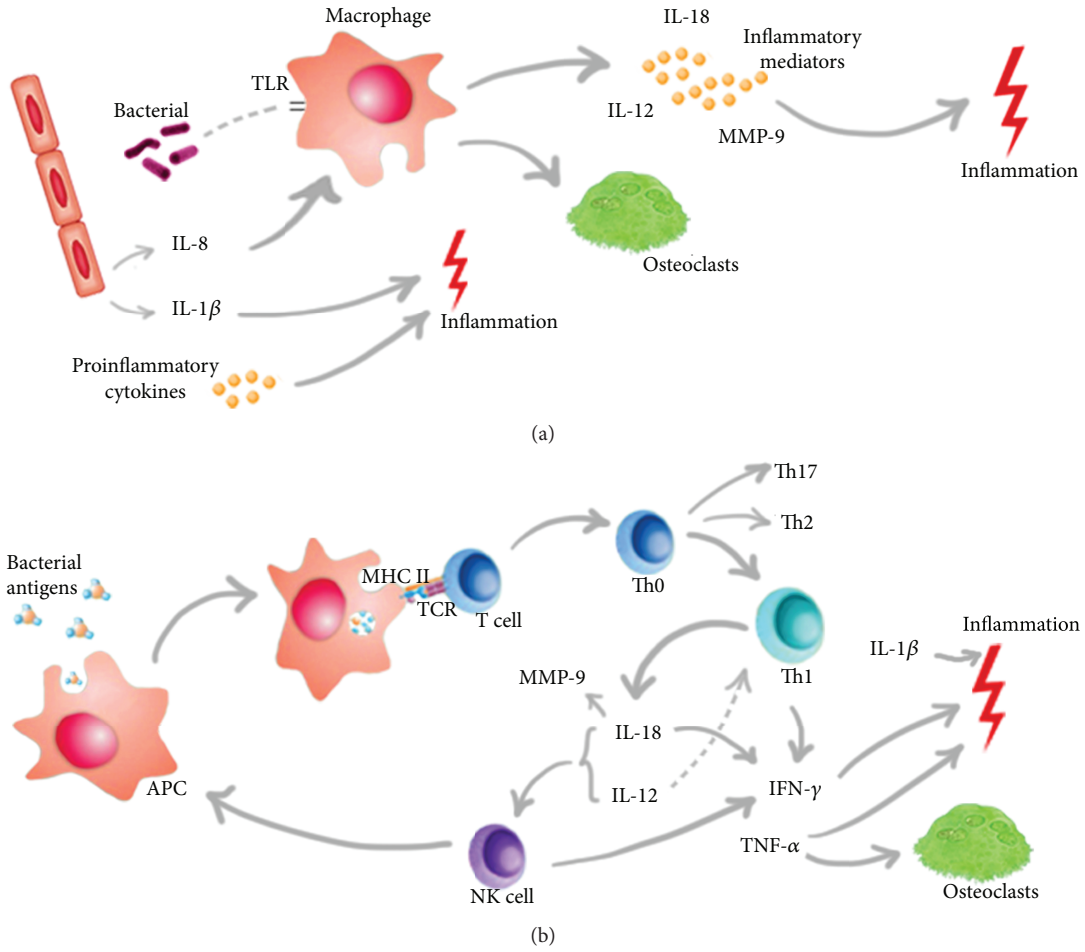

Figure 1: Representation of the biological mechanisms of the cytokines and MMP-9 in the immunopathogenesis of CP. (a) The initial trigger for the immune response is the recognition of components of periodontopathogens, as LPS, by TLRs (Toll-like receptor). This recognition generates an intracellular signaling cascade leading to increased secretion of proinflammatory cytokines, MMPs, and recruitment of osteoclasts by macrophages. (b) This innate immune mechanism of defense may not be sufficient to eliminate the pathogen and with this the adaptive immune response is activated. APCs (antigen-presenting cells) internalize and process bacterial antigens, which bind to MHC II and is transported to the cell surface to be recognized by specific T cell. The Th1 immune response is the main response activated. IL-18 is expressed by macrophages, osteoblasts, fibroblasts, and Kupffer cells, being the main cytokine inducing IFN-γ. This cytokine acts synergistically with IL-12 in NK cells to induce IFN-γ production and activation of macrophages and dendritic cells that direct the Th1 response. TNF-α, IL-1β, and proinflammatory cytokines, such as IL-12 and IL-18, orchestrate enzyme-producing events such as MMP-9 and recruitment of osteoclasts, macrophages, and NK cells, causing greater inflammation and destruction of periodontal tissues.

to the manufacturer's specifications. Visualization of fragment size was performed on 3% agarose gel electrophoresis stained with SYBR Safe DNA Gel Stain (Invitrogen®, Life Technologies, Grand Island, NY).

2.5. Genotyping of MMP9. *MMP9*-1562C>T (rs3918242) genotyping was performed using PCR-RFLP technique according to Nelissen et al. [21]. For the reaction, 100 ng of DNA, 5 pmol of each primer forward and reverse, 200 μM of each nucleotide, 25 mM $MgCl_2$ buffer (PCR Amplification Buffer, Promega), and 0.5 U of *Taq* DNA polymerase were used. Amplification cycles were performed on a GeneAmp PCR System 9700 (Applied Biosystems ™) thermocycler. The amplification products were digested with 5.0 U of the *Sph*I enzyme (Fermentas® Life Science) for 3 hours at 37°C. Analysis of the fragments was performed by electrophoresis on a SYBR Safe stained agarose gel (Invitrogen® Life Technologies, Grand Island, NY).

2.6. Determination of the IL-18 and MMP-9 Serum Concentration. Eighteen nonsmoker patients and six nonsmoker controls were selected for IL-18 and MMP-9 serum concentration assays. The CP patients were classified according to disease extension form and on degree of severity (light, moderate, and severe).

The IL-18 and MMP-9 serum concentration was determined using a Human IL-18 ELISA kit (Medical & Biological Laboratories Co. Ltd., code no.7620 Ltd., Nagoya, Aichi, Japan) and Invitrogen Human MMP-9 ELISA kit (Corporation Invitrogen, Catalog #KHC3061, Frederick, Maryland,

Table 1: Characteristics of patients with CP and controls.

	CP patients $N = 192$ n (%)	Controls $N = 189$ n (%)	P	OR (95% CI)
Gender				
Female	96 (50)	116 (61)		
Male	96 (50)	73 (39)		
Age				
Mean ± sd (year)	47.5 ± 9.1	46.3 ± 8.4		
Smoking				
Smokers+ex-smokers	82 (43)	47 (25)		
Nonsmokers	110 (57)	142 (75)	0.0002	2.25 (1.45-3.49)
Nonsmokers	$N = 110$	$N = 142$		
Gender				
Female	57 (52)	98 (69)		
Male	53 (48)	44 (31)		
Age				
Mean ± sd (year)	47.8 ± 9.2	46.5 ± 8.2		

n: number; sd: standard deviation; OR: odds ratio; P: P value—only significant P values are shown; CP: chronic periodontitis.

USA) in accordance with the manufacturer's instructions. Absorbance of each well was read on the ELISA reader (ASYS HITECH GMBH–Eugendorf, Austria) using 450 nm and the reference at 620 nm. Concentrations in the tested sample were estimated using the standard curve. Reactions were done in duplicate.

2.7. Statistical Analysis. To evaluate if the estimate genotype distribution between the observed and expected frequencies is found in the Hardy-Weinberg equilibrium, the SNPStats (https://www.snpstats.net/start.htm) statistical program [22] was used. The association between genetic polymorphisms and chronic periodontal disease was assessed using the chi-square test with Yates correction and logistic regression, and the risk was assessed by odds ratio with a 95% confidence interval only for significant P value using the SNPstats [22] and OpenEpi Version 2.3 program (https://www.openepi.com/Menu/OE_Menu.htm). Covariate analysis included age, gender, and smoking status. The association tests were realized for codominant, dominant, recessive, overdominant, and log-additive genetic inheritance models where the best inheritance model was defined by the minor Akaike information criteria (AIC) [22]. To eliminate smoking as a confounding factor, the analyses also were done in the nonsmoking patients versus nonsmoking controls. The Bonferroni adjustment for multiple testing was applied and the corrected value (P_c) for a truly significant value was obtained after the multiplication of the P value by the number of analyzed SNPs (three SNPs, because of the linkage disequilibrium between *IL18*-137 and *IL18*-607: $\Delta' = 0.98$, $P < 10^{-16}$). The values for cytokine levels were expressed as mean ± SEM (standard error of mean), and for the differences between the groups, the Student t-test was used (https://www.graphpad.com/quickcalcs/ttest2/). The Mann-Whitney U test was used to analyze the correlation between concentration and genotype (https://www.socscistatistics.com/tests/). All tests were performed at a significance level of 5.0%. Quanto (http://biostats.usc.edu/software) was used to calculate the sample size using the less frequent allele (0.14 for *MMP9*), population risk (50% and OR = 1.9), for statistical power of 80%, and considering the codominant inheritance genetic model.

3. Results

The allele and genotype frequency distributions of *IL12B*, *MMP9*, and *IL18* in CP patients and controls are shown in Table 2. The genotype frequency distributions for all genes were consistent with the Hardy-Weinberg equilibrium ($P > 0.05$).

For *IL18*-137, a higher frequency of the G/C genotype was found in patients (50%) when compared to controls (35%) in the nonsmoking group. When analyzing the models of inheritance, significance was observed for codominant and overdominant models, being the overdominant model of choice according to the minor Akaike information criteria ($P_c = 0.03$, OR = 1.99, 95% CI = 1.17-3.36) (Table 2). In the analysis of interaction with the covariant gender, *IL18*-137G/G and *IL18*-607C/C genotypes were higher in nonsmoking men ($P_c = 0.03$, OR = 2.51, 95% CI = 1.19 -5.30 and $P_c = 0.03$, OR = 3.30, 95% CI = 1.29-8.40, respectively) when compared with nonsmoking women carrying the same homozygous genotypes (Table 3).

The serum levels for IL-18 in CP patients were higher (164.8 ± 66.4 pg/ml) than those in healthy controls (82.3 ± 43.3 pg/ml; $P = 0.005$). IL-18 concentrations were also higher in different extension forms of CP and in

Table 2: Genotype and allele frequency distributions of the *IL12, IL18* and *MMP9* in the CP and nonsmoking CP patients compared to their respective controls.

		All subjects		Nonsmokers					
		CP patients *n* (%)	Controls *n* (%)	CP patients *n* (%)	Controls *n* (%)	*P*	P_c	OR (95% CI)	AIC
IL18									
137G>C		*N* = 191	*N* = 185	*N* = 109	*N* = 138				
(rs187238)	G/G	91 (48)	97 (52)	48 (44)	75 (54)				
Genotype	G/C*	85 (44)	70 (38)	54 (50)	48 (35)				
	C/C	15 (8)	18 (10)	7 (6)	15 (11)				
	G/G+C/C*	106 (56)	115 (62)	55 (50)*	90 (65)*	ref			
	G/C*	85 (44)	70 (38)	54 (50)*	48 (35)*	0.01	0.03	1.99 (1.17-3.36)	330.7
Allele	G	267 (70)	264 (71)	150 (69)	198 (72)				
	C	115 (30)	106 (29)	68 (31)	78 (28)				
IL18									
607A>C		*N* = 192	*N* = 187	*N* = 110	*N* = 140				
(rs1946518)	A/A	43 (22)	35 (19)	29 (26)	27 (19)				
Genotype	A/C	93 (49)	82 (44)	50 (46)	61 (44)				
	C/C	56 (29)	70 (37)	31 (28)	52 (37)				
Allele	A	179 (47)	152 (41)	108 (49)	115 (41)				
	C	205 (53)	222 (59)	112 (51)	165 (59)				
IL12B									
1188A>C		*N* = 128	*N* = 131	*N* = 78	*N* = 114				
(rs3212227)	A/A	63 (49)	66 (51)	40 (51)	59 (52)				
Genotype	A/C	49 (38)	53 (40)	27 (35)	46 (40)				
	C/C	16 (12)	12 (9)	11 (14)	9 (8)				
Allele	A	175 (68)	185 (71)	107 (69)	164 (72)				
	C	81 (32)	77 (29)	49 (31)	64 (28)				
MMP9									
1562C>T		*N* = 188	*N* = 185	*N* = 101	*N* = 142				
(rs3918242)	C/C	150 (80)	142 (77)	78 (77)	111 (78)				
Genotype	C/T	37 (20)	40 (21)	23 (23)	29 (20)				
	T/T	1 (1)	3 (2)	0 (0)	2 (2)				
Allele	C	337 (90)	324 (88)	179 (89)	251 (88)				
	T	39 (10)	46 (12)	23 (11)	33 (12)				

n: number; ref: reference; OR: odds ratio; *P*: *P* value; CP: chronic periodontitis; AIC: Akaike information criteria. **IL18-137*G/C genotype: CP patient vs. control in the nonsmoking group.

Table 3: *IL18* genotype frequency distributions between nonsmoker CP and controls considering the interaction analysis with the covariant gender.

	CP patients *n* (%)	Controls *n* (%)	*P*	P_c	OR (95% CI)
IL18-137G/G (rs187238)					
Female	22 (48)	51 (68)	ref		
Male	26 (52)	24 (32)	0.01	0.03	2.51 (1.19-5.30)
IL18-607C/C (rs1946518)					
Female	14 (45)	38 (73)	ref		
Male	17 (55)	14 (27)	0.01	0.03	3.30 (1.29-8.40)

n: number; ref: reference; *P*: *P* value; OR: odds ratio; CI: confidence interval; CP: chronic periodontitis.

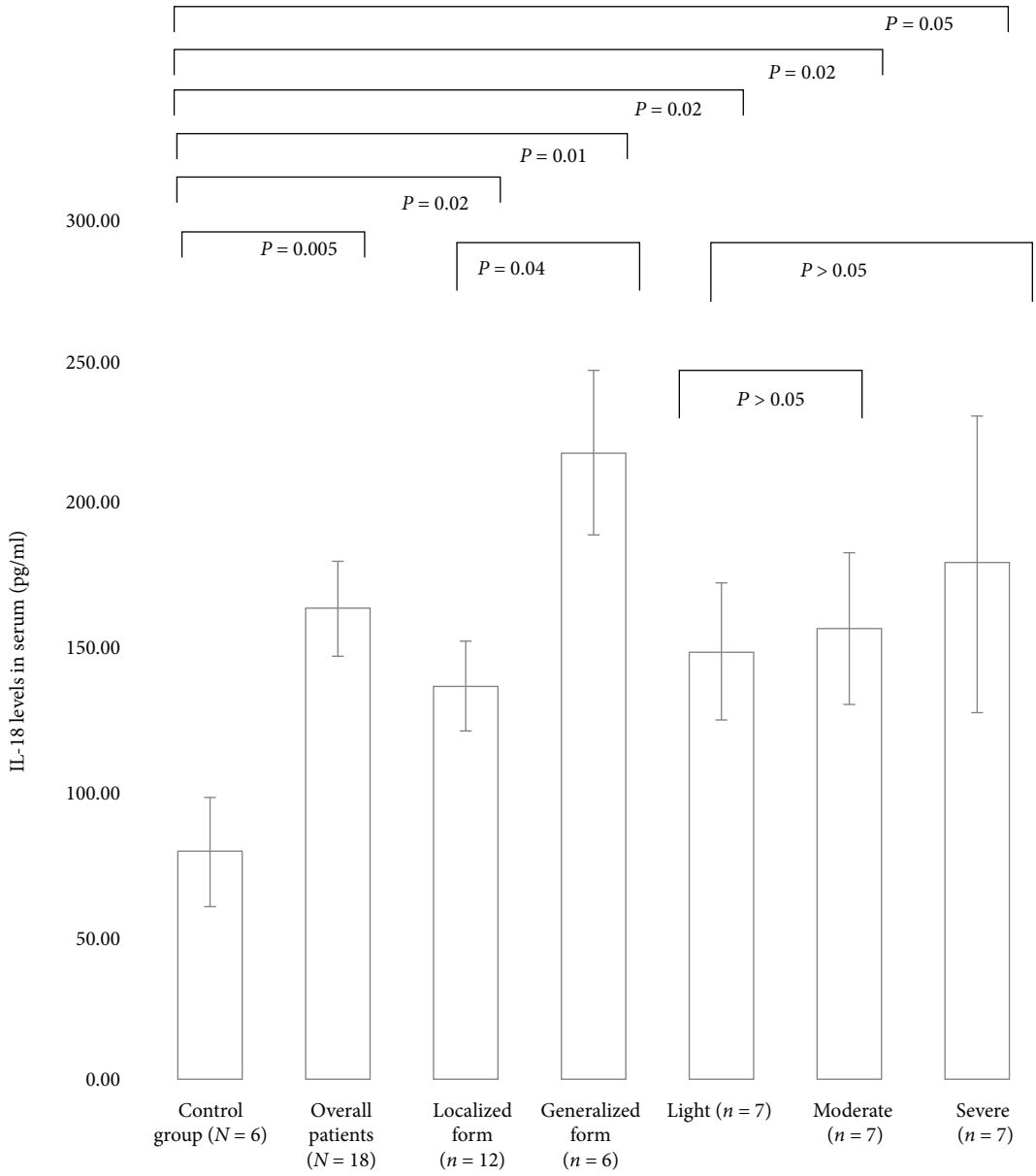

Figure 2: IL-18 serum levels in nonsmoking CP patients ($N = 18$) and controls ($N = 6$). Comparisons were done between the control group and overall patients, CP extension (localized and generalized), and degree of disease severity (light, moderate, and severe); between CP extension (localized x generalized) and light degree x moderate and severe degree. The results are shown as mean ± SEM. Student's t-test was used; $P \leq 0.05$ was considered significant.

diverse degrees of severity of the disease than those in the controls (Figure 2). The *IL18*-137G/C+G/G and *IL18*-607A/C+C/C genotypes were related to good cytokine production ($P = 0.012$, Z-score = 2.50, Uvalue = 10 for critical = 17 and $P = 0.011$, Z-score = 2.53, Uvalue = 8 for critical = 14, respectively).

After analyzing the *IL12B* and *MMP9* polymorphisms, no significant differences in the allele and genotype frequency distributions were observed between CP patients and controls (total sample and nonsmokers). There were no significant differences for the serum levels of MMP-9 between the control group and the several extension forms and severity of CP patients.

4. Discussion

In order to evaluate a possible influence of *IL18*, *IL12*, and *MMP9* polymorphisms and IL-18 and MMP-9 serum levels in the immunopathogenesis of the CP, a careful selection of patients and controls was performed in this study. Subjects were matched according to age and gender, and all individuals did not exhibit disease-related disorders, which may influence the course of the disease, such as diabetes, arthritis, and other inflammatory disorders [23–25]. Smoking habits are a risk factor for CP [26, 27]; thus, analyses were also separately done in nonsmoking patients versus nonsmoking controls.

The best results were that *IL18* contributes to the risk for disease independently of the smoking habits and that men had a greater risk than women when these *IL18* polymorphisms were considered. The serum levels of IL-18 were higher in CP patients than in the controls and in patients with more severe and extensive form of the disease.

In this study, the *IL18*-137G/C genotype was found more frequent in the nonsmoking patients and was associated with the risk of CP development. The choice inheritance model was the overdominant that compared the heterozygous genotype versus both homozygous genotypes. This association was observed in nonsmoking individuals and highlights the independency of this confounding factor. The risk was also observed in men carrying the *IL18*-137G/G and the *IL18*-607C/C genotypes when compared with these same genotypes in women. Up to this point, men were considered more susceptive to CP due to the hormonal factors [28] and mainly the personal hygiene habits [29]; however, here it was observed that they had a genotype related to better IL-18 cytokine production which could exacerbate periodontal destruction. *IL18*-137G and *IL18*-607C alleles have been involved in the development of different diseases. The allele *IL18*-607C has been linked to hepatitis C susceptibility [30] and chronic obstructive pulmonary disease [31], and the genotype C/C has been associated with higher IL-18 production in multiple sclerosis [32]; *IL18*-137G was associated with the risk of arthritis [10] and *IL18*-137G/C genotype with oral cancer [12]. More specifically with regard to CP, Li et al. [33] showed in a meta-analysis study, involving nine case-control studies and a total of 576 patients with periodontitis and 458 healthy controls, that the *IL18*-607C and *IL18*-137G alleles were associated with an increased risk of periodontitis. We found no allelic association in our study, possibly due to the small sample of patients.

When the IL-18 serum levels were measured, the IL-18 concentration was statistically significantly higher in CP than in controls and highest concentration was found in the generalized form of the disease. High levels of IL-18 in the serum of the patients with chronic periodontitis were previously documented [33, 34]. Previously study found that *IL18*-137G and *IL18*-607C alleles, located on chromosome 11q22, increase the gene transcription and lead to a higher level of IL-18 protein synthesis [32]. We found that *IL18*-137G/C+G/G and *IL18*-607A/C+C/C genotypes were related to good cytokine production. These facts point to a potential functional role of these variants and their influence on cytokine levels of periodontal tissue and plasma in patients.

In vivo, when using IL-18 transgenic (Tg) mice, IL-18 overexpression was related to periodontal disease [35]. When IL-18Tg and wild-type mice were inoculated intraorally with *Porphyromonas gingivalis*, after seventy days of infection, there was periodontal bone loss in IL-18Tg mice but not in wild-type. RT-PCR analysis showed elevated expressions of mRNAs for the receptor activator of nuclear factor kappa-B ligand (a key stimulator for the development and activation of osteoclast) and CD40 ligand (a marker of T cell activation) in the gingiva of IL-18Tg-infected mice [35]. Considering this fact as well as the polymorphisms in IL-18 promoter regions and the increased levels of IL-18 in the plasma of CP patients, which we observed in our and other studies, *IL18* polymorphisms and serum concentration may be useful biomarkers for predicting the development of periodontitis.

IL-18 is the major inducer of IFN-γ and acts synergistically with IL-12 on NK cells. IL-18 induces the production of IFN-γ that activates macrophages, dendritic cells, and Th1 cells directing immune response to the cellular response. Activated macrophages are a potent cell producing TNF-α and other chemical mediators, such prostaglandins-E2, which induce bone and cartilage resorption by activation of the osteoclasts. When in the absence of IL-12, IL-18 induces the immune response of Th2 [36] that could deregulate the specific immune response. This biological mechanism may be responsible for initiation and progression of periodontal tissue destruction, by inducing the genesis of osteoclast and increase secretion of matrix metalloproteinases.

In this studied population, *IL12* and *MMP9* polymorphisms and MMP-9 serum levels were not associated with CP. With regard to IL-12, several studies related the highest level of IL-12 in serum, gingival tissue, and crevicular fluid in periodontal disease [15, 34, 37]. Other investigations including IL-12 determination of serum and crevicular fluid should be done for best conclusions regarding the association of these mediators with CP. As for *MMP9* polymorphism, specifically *MMP9*-1562C>T, a meta-analysis published in 2016 related a reduced risk between the T allele and periodontal disease susceptibility in both Caucasian and Asian populations [38]. Different from our results, Silosi et al. [39] showed that MMP-9 levels in the serum and in the gingival crevicular fluid were significantly higher in CP patients when compared to controls.

The present study had some limitations, such as the nonpairing of the number of individuals in the three studied genes and their polymorphisms. This occurred due to the lack of some samples in the course of the study. The serum level of IL-12 was also not determined.

5. Conclusion

IL-18 may confer susceptibility to CP independently of smoking habits and *IL12B* and *MMP9* polymorphisms. Men had a greater risk than women for developing the disease when *IL18* polymorphism was considered. *IL18*-137G>C (rs187238) and *IL18*-607A>C (rs1946518) polymorphisms might influence cytokine levels in the plasma of CP patients.

Data Availability

The data used to support the findings of this are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Review Article

Chronic Inflammation as a Link between Periodontitis and Carcinogenesis

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Periodontitis is characterized by a chronic inflammation produced in response to a disease-associated multispecies bacterial community in the subgingival region. Although the inflammatory processes occur locally in the oral cavity, several studies have determined that inflammatory mediators produced during periodontitis, as well as subgingival species and bacterial components, can disseminate from the oral cavity, contributing therefore, to various extraoral diseases like cancer. Interestingly, carcinogenesis associated with periodontal species has been observed in both the oral cavity and in extra oral sites. In this review, several studies were summarized showing a strong association between orodigestive cancers and poor oral health, presence of periodontitis-associated bacteria, tooth loss, and clinical signs of periodontitis. Proinflammatory pathways were also summarized. Such pathways are activated either by mono- or polymicrobial infections, resulting in an increase in the expression of proinflammatory molecules such as IL-6, IL-8, IL-1β, and TNF-α. In addition, it has been shown that several periodontitis-associated species induce the expression of genes related to cell proliferation, cell cycle, apoptosis, transport, and immune and inflammatory responses. Intriguingly, many of these pathways are linked to carcinogenesis. Among them, the activation of Toll-like receptors (TLRs) and antiapoptotic pathways (such as the PI3K/Akt, JAK/STAT, and MAPK pathways), the reduction of proapoptotic protein expression, the increase in cell migration and invasion, and the enhancement in metastasis are addressed. Considering that periodontitis is a polymicrobial disease, it is likely that mixed species promote carcinogenesis both in the oral cavity and in extra oral tissues and probably—as observed in periodontitis—synergistic and/or antagonistic interactions occur between microbes in the community. To date, a good amount of studies has allowed us to understand how monospecies infections activate pathways involved in tumorigenesis; however, more studies are needed to determine the combined effect of oral species in carcinogenesis.

1. Introduction

Periodontal diseases are dysbiotic conditions in the gingival margin, which are characterized by an imbalance between subgingival communities and the host immune response [1]. Such diseases include gingivitis, which is a reversible condition characterized by the inflammation of the gingiva driven by the combined effect of specific microbial taxa. If not treated, gingivitis could progress to periodontitis, characterized by the destruction of supporting tissues of the teeth. From health to gingivitis, to periodontitis, several ecological successions occur in the subgingival microbiome, leading to both an increased biomass and the establishment of distinct dysbiotic communities. Interestingly, not only local effects in the oral cavity have been associated with such disorders but also periodontitis has been largely considered as a risk factor for a number of both oral and systemic diseases [2–5]. Among these, orodigestive cancers are highly influenced by both a direct carcinogenic effect of periodontitis-associated bacteria in either oral cells or in other body sites and inflammatory mediators migrating from the oral cavity [6, 7]. Either way, there is extensive evidence showing that species such as *Porphyromonas gingivalis* (highly abundant and prevalent in periodontitis) and *Fusobacterium nucleatum* (closely interacting with periodontitis-associated species in the disease) directly

activate transduction pathways leading to cell transformation [7–12]. Comparatively, less information exists about other periodontitis-associated bacteria.

However, although increasing evidence links periodontitis and carcinogenesis, the fact that periodontitis is a polymicrobial disease has not been well addressed in the context of cancer. This is especially relevant when evaluating the direct carcinogenic effect exerted by oral bacteria, since combined species act locally in oral cells and also migrate from the oral cavity. Thus, more studies evaluating how interbacterial interactions affect carcinogenesis process are needed.

2. Periodontal Diseases

Periodontal diseases are associated with chronic inflammation, which affects the supporting tissues of the teeth including the gums or gingival tissue, as well as the periodontal ligament and the alveolar bone in more severe forms of the diseases [13]. Gingivitis is a periodontal disease characterized by local inflammatory processes driven by subgingival bacteria that in most cases do not promote destruction of the tissues and can be reversible. However, clinically, it is considered as the starting point of other periodontal diseases, such as periodontitis [14]. Periodontitis is triggered by an imbalance between resident subgingival microbiota and the inflammatory response of the host that leads to destruction of the supporting tissues of the teeth, even producing the loss of teeth [13]. According to the World Health Organization, between 35% and 50% of the world population are affected by periodontitis [15]. In the United States, the prevalence of gingivitis in children aged between 3 and 11 years is 9-17%, while at puberty, prevalence rises to 70-90% [16] and corresponds to 47% of adult population [17].

2.1. Role of Subgingival Communities in the Etiology of Periodontal Diseases. Both gingivitis and periodontitis are driven by bacterial communities interacting with the host immune system and therefore contributing to the inflammation of tissues. Because of the relevance of the bacterial component, different theories have been proposed in order to establish the importance of these subgingival bacterial communities in the etiology of periodontitis. In 1954, it was proposed that the accumulation of microorganisms promotes the release of compounds that produce inflammation in the gingival tissue [18, 19]. This idea eventually evolved into researchers demonstrating that the colonization of certain anaerobic subgingival bacteria, including *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, promoted both the onset and the development of periodontitis [20]. However, different studies that sought to determine the composition of the bacterial community associated with periodontitis managed to determine that these bacteria were not only present in patients with periodontitis but also in periodontally healthy individuals [1].

This was a key point in supporting current theories establishing that it is not the colonization of specific bacteria what triggers the disease, but rather the changes in the relative abundances of specific taxa in the subgingival communities due to dysbiotic processes occurring in subgingival areas that would determine the development of periodontitis. In this context, Marsh [21] created the concept of "ecological catastrophe," which establishes that the environmental and host factors, such as poor hygiene, inappropriate diets, and use of tobacco and drugs that produce side effects in the immune defense of the patient, select and enrich pathogenic bacteria, and a disease state. The authors described that an increase in bacterial plaque increases local inflammation, which in turn increases the flow of crevicular gingival fluid (CGF), produces bleeding, and provides proteinaceous nutrients, which increase the proliferation of Gram-negative anaerobes [21, 22].

This theory has been supported by several studies aimed at characterizing the microbiome of periodontally healthy individuals and patients with periodontitis [1, 23–25]. Diaz et al. [26] reviewed these studies concluding that different subgingival microbiomes are characteristic of healthy individuals, as well as patients with gingivitis and periodontitis. While most health-associated bacteria are early colonizers of the subgingival biofilm, periodontitis-associated bacteria are mainly late colonizers. In the periodontitis-associated group of bacteria, species such as *Filifactor alocis*, *P. gingivalis*, *Porphyromonas endodontalis*, *T. forsythia*, and *T. denticola* are found in all the 4 studies reviewed. *P. gingivalis* was proposed as a key player among such species ("keystone pathogen"), since Hajishengallis et al. [27] demonstrated that even when it is found in low abundance in healthy individuals, it can promote changes in homeostasis of the normal microbiota, remodeling it towards a harmful microbiota that promotes destruction of tissues and inflammation in *in vivo* models. This concept was refined by Hajishengallis himself in 2012, proposing the polymicrobial synergy and dysbiosis theory (PSD). This theory adds the fact that every component of a symbiotic and synergistic microbiota is relevant in the onset of the disease and not only the periodontitis-associated bacteria. Thus, the whole dysbiotic community will synergistically initiate processes of tissue inflammation, activate production of cytokines, and initiate the recruitment of immune cells [28].

Interestingly, besides having determined both periodontitis and health-associated bacteria, a third group called "core species," which are equally prevalent and found in the same proportion both in health and periodontitis individuals, was characterized, being *F. nucleatum* the most abundant in this group [1, 24]. *F. nucleatum* plays a central role in the subgingival biofilm, since it physically interacts with other microorganisms in the subgingiva [29]: *P. gingivalis* [30], *Aggregatibacter actinomycetemcomitans* [31], *Prevotella* spp. [32], *Streptococcus gordonii* [33], *Candida albicans* [34], and others [29, 35]. Such close interactions with several species in the biofilm are reflected in the fact that *F. nucleatum* acts as a bridge attaching early colonizers like *Streptococcus* spp. and other facultative species and late colonizers such as *P. gingivalis* [36, 37]. This process is essential for the ecological successions that establish the subgingival plaque and determine the progression of periodontitis [35], in which thousands of species colonize the subgingival area in an ordered manner.

These successions include specific modification of the local environment in the biofilm [38, 39] which select specific groups of bacteria and eventually induce changes in the subgingival bacterial communities that lead to a dysbiotic community able to induce a deregulation of the host inflammatory response and eventually cause chronic inflammation.

2.2. Chronic Inflammation Driven by Periodontitis-Associated Bacteria. In the periodontal pocket, the first host responses to the dysbiotic subgingival community are characterized by the infiltration of natural killer (NK) cells, neutrophils, and granulocytes (polymorphonuclear cells) that promote the initial inflammatory response and the subsequent infiltration of lymphocytes to present antigens to dendritic cells [40]. The neutrophils are overwhelmed with the abundance and persistence of microorganisms, being destroyed or undergoing either apoptosis or necrosis as they interact with bacteria within the gingival crevice.

T cells promote a profile characterized by CD8+ and CD4+ cells that generate a proinflammatory medium rich in cytokines such as tumor necrosis factor alpha (TNF-α), interleukin- (IL-) 1, IL-4, IL-10, interferon-γ (IFN-γ), and transforming growth factor β (TGF-β) [41]. In addition, T CD4+ lymphocytes produce RANK-L, a cytokine that promotes bone resorption [42]. It was also described that T cells contribute to cell-mediated immune responses by stimulating T helper cells such as Th1, Th2, Th9, Th17, and Th22 and the deregulation of this response could be related to the appearance of the disease and its chronicity [43, 44]. On the other hand, B cells produce antibodies against the microorganisms present in the subgingival pocket in order to eliminate them and decrease the local inflammation [44].

In addition to the inflammatory mediators produced by the immune cells, the gingival epithelium also releases other cytokines such as IL-1, IL-8, and TNF-α, which in turn promotes the recruitment of macrophages [45]. Concordantly with these studies performed *in vitro*, in periodontitis tissue samples, an increase in mRNA of IL-1β, IL-6, IL8, and TNF-α, regulated upon activation normal T cell expressed and secreted (RANTES) and monocyte chemotactic protein-1 (MCP-1), was observed, compared to healthy gingiva [46]. In the same context, a higher expression of IL-1β was observed in gingival fluid from deeper sites of periodontitis patients [47].

As a consequence of this inflammatory response, ecological changes in the subgingival region occur, which contribute to the ecological successions in the subgingival area that are associated with periodontitis progression. Interestingly, some periodontitis-associated bacteria have been shown to contribute directly to the chronic inflammation by activating specific intracellular pathways.

Because of the polymicrobial nature of periodontitis and considering that interbacterial interactions occurring in the subgingival biofilm contribute to the disease, current models of periodontitis include the study of the effect of multiple species in the stimulation of immune response. Very recently, Herrero et al. [48] showed that the exposure of epithelial and fibroblast cultures to a dysbiotic biofilm increased the expression of IL-6, IL-8, IL-1β, TNF-α, and MMP-8. In the same context, other studies showed that epithelial cells produce higher cytokine levels when they are exposed to either monospecies or multispecies biofilms [49]. Interestingly, an increased expression of IL-8, C-X-C motif chemokine ligand 3 (CXCL-3), CXCL-1, IL-1, IL-6, colony-stimulating factor 2 (CSF2), and TNF-α was observed in cells stimulated with the multispecies biofilms. Similarly, polymicrobial infection (*P. gingivalis*, *T. denticola*, and *T. forsythia*) using a murine calvarial bone model affected the expression of several genes related to cell proliferation, cell cycle, apoptosis, transport, immune response, and inflammatory response. In the proinflammatory context, the cytokines that increased the most were IL-1, IL-6, and TNF-α, which are precisely those related to chronic inflammation and chronic bone damage [50].

Nonetheless, despite the fact that multispecies infection constitutes a more realistic model considering the polymicrobial etiology of the disease, many studies using planktonic monospecies bacteria have permitted to determine the contribution of key species to the inflammatory process. For example, studies using *T. denticola* monoinfections have shown that the bacterium can activate Toll-like receptor 5 (TLR5) through the flagellin, the main component of the bacterial flagellum. This interaction leads to an increase in IL-1β and TNF-α [51]. *T. denticola* can also suppress the action of antimicrobial peptides such as human β-defensin 3, regulating the signaling pathway activated by TLR2 [52]. Additionally, works by Tanabe et al. [53] demonstrated that *T. denticola* peptidoglycan induces the secretion of proinflammatory cytokines such as IL-8, IL-6, and TNF-α, in murine macrophages, stimulating the production of PGE2 and decreasing their viability. However, *T. denticola* can also counteract the increase of these cytokines, as it has been shown in a study conducted in peripheral blood mononuclear cells, where it was determined that *T. denticola* hydrolyzes IL-1β, IL-6, and TNF-α through the PrtP complex (dentilisin or chymotrypsin-like protease (CTLP)) [54].

On the other hand, the infection of mice with *T. forsythia* increased levels of IgG and IgM, both markers of immune response activation. Moreover, an increase in CD4+ T lymphocytes was shown [55]. Intriguingly, this bacterium has a glycosylated S layer [56], which is important for the mechanical stabilization and protection of the bacterium. A study by Settem et al. [57] showed that glycosylation of S layer of *T. denticola* can deregulate the immune response by preventing Th17 production, probably inhibiting the recruitment of neutrophils to the site of infection. This effect produces tissue and bone destruction.

A Gram-positive anaerobic bacterium that has been emerging as a periodontitis-associated species is *F. alocis*. Infection of gingival epithelial cells (GECs) by *F. alocis* stimulates the production of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α [58]. This is important, since these cytokines are related to the stimulation of osteoclasts and bone resorption [58]. Moreover, these cytokines have been shown to increase in an *in vivo* model (mouse subcutaneous chamber model) and to increase the influx of neutrophils to the site of infection [59].

In spite of the growing evidence showing the relevance of a number of species in the progression of periodontitis, one of the most studied species is *P. gingivalis*. Through such studies, nowadays, we have a good understanding of its role in the pathogenesis of periodontitis. This bacterium is internalized by macrophages and is also able to induce its own internalization by GECs. Once the bacterium is inside the GECs, it can use the machinery of the host cell for its survival and persistence. For example, infected GECs activate antiapoptotic pathways, such as the JAK/STAT and phosphatidylinositol 3-kinase (PI3K)/Akt, which inhibit the intrinsic pathway of apoptosis probably to persist for longer periods. Both pathways have also been related to inflammation. Some cytokines such as IL-6, TNF-α, or IFN-γ function through the JAK/STAT pathway [60]; additionally, the JAK/STAT pathway activates NF-κB and stimulates TNF-α production [61]. The PI3K/Akt pathway, on the other hand, is involved in the increase of TLR4 mRNA, in response to bacterial lipopolysaccharide (LPS) [62]. Finally, phosphorylation of Akt and its consequent activation induces NF-κB, which increases the transcription of antiapoptotic genes [63].

Periodontitis-associated species seek to prolong bacterial growth within the infected cell and also evade the immune system. Once *P. gingivalis* is internalized, it is incorporated into early phagosomes, where it prevents fusion to the lysosome and therefore its degradation [64]. *P. gingivalis* secretes the nucleoside diphosphate kinase (NDK) enzyme that removes ATP through the P2X7 receptor. In macrophages, this receptor stimulates the production and secretion of IL-1β, the apoptosis of the host cell, and killing of bacteria [65].

Moreover, infection of human monocytic cell line with *P. gingivalis* activates NLRP3 and AIM2 inflammasome through caspase 1 activation, which produces the processing of pro-IL-1β to its active form IL-1β [66]. During periodontitis progression, tissue damage occurs both by the direct effect of bacterial virulence factors and the deregulation of the immune system response. *P. gingivalis* interacts with the GECs through the TLRs mediated by the recognition of *P. gingivalis* virulence factors such as fimbria and the LPS. It has been shown that this interaction increases the transcription of TLR2 and TLR4 in GECs [67]. Intriguingly, *P. gingivalis* can modify the lipid A region of its LPS by incorporating different units of acyl groups to its structure. A tetra-acylated structure of *P. gingivalis* lipid A is a TLR4 antagonist with anti-inflammatory potential [68]. However, the penta-acylated structure of *P. gingivalis* lipid A is a TLR4 agonist with proinflammatory potential [68] that activates the NF-κB and MAPK-p38 pathways [69]. Nevertheless, *P. gingivalis* has developed strategies to evade or delay the immune response. For example, within its virulence factors, it possesses gingipain proteases that degrade the CD14 protein (a coreceptor of TLR4 and TLR2), interfering with the optimal recognition of bacterial LPS [70].

P. gingivalis can also modify the expression of adhesion receptors—like E-selectin—for leukocyte adhesion and transmigration, preventing its upregulation. In this context, gingipain proteases produced by *P. gingivalis* degrade the intracellular adhesion molecule 1 (ICAM-1) in GECs, disrupting neutrophils-oral epithelial cell interaction [71]. These proteases affect also the integrity of the cytokines IL-6, IL-8, IL-12, and TNF-α, which are produced in response to the infection [72–75].

Interestingly, in addition to the inflammation induced by periodontitis-associated bacteria, some "core species" have also been linked to inflammation. For example, it has been demonstrated that *F. nucleatum* upregulates the production of MMP-13 and IL-8, through the MAPK/p38 pathway in epithelial cells [76]. Moreover, *F. nucleatum* increases IL-8 mRNA levels through the activation of NF-κB in human GECs [77].

Similar to *P. gingivalis*, *F. nucleatum* also activates NLRP3 inflammasome, inducing the releases of damage-associated molecular patterns (DAMPs) like high mobility group box 1 protein (HMGB1) and proteins that recruit and activate caspases (ASC), increasing the inflammation in GECs [78]. After infection, HMGB1 is released into the extracellular space, which is required for the activation of the inflammasome and the caspase 1 activation [79, 80]. On the other hand, ASC functions as an adapter of the NLRP3 inflammasome assembly and is secreted by macrophages during inflammation [81].

Limited data exist regarding the effect of combined subgingival species in carcinogenesis. Coinfection studies using *F. nucleatum* and *P. gingivalis* show that they induce a synergic virulence response in a mouse periodontitis model, with a stronger inflammatory response triggered by elevated levels of TNF-α, NF-κB, and interleukin IL-1β [82], as well as higher levels of attachment and invasion into host cells [83, 84].

3. Systemic Diseases Associated with Chronic Inflammation in Periodontitis

Although the inflammatory processes occur locally in the oral cavity, several studies have determined that the chronic inflammation during periodontal diseases or the dissemination of bacterial components could cause various extraoral diseases. Some of these diseases and a brief description of their associations with periodontal disease are summarized as follows:

(i) *Cardiovascular diseases*: many studies have linked the presence of periodontal diseases with cardiovascular diseases [5, 85, 86]. Among them, Peng et al. [86] determined through a retrospective cohort study that periodontal therapy promoted a decreased risk of cardiovascular disease. Also, different meta-analyses have managed to link the presence of periodontal diseases with an increased risk of cardiovascular disease [85, 87]. Moreover, some periodontitis-associated species have been linked to such diseases. Thus, Damgaard et al. [88] linked the presence of IgG antibodies against *P. gingivalis* with the presence of cardiovascular disease in serum from 576 participants and Bale et al. [89] proposed that *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *T. denticola*, and

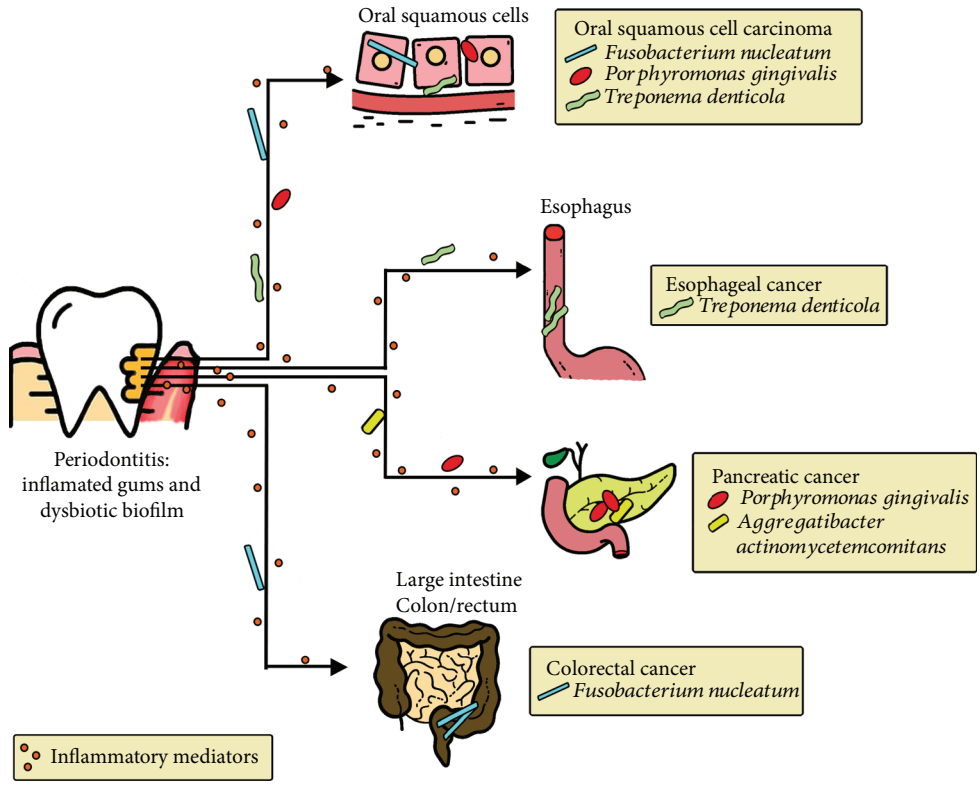

Figure 1: Association of periodontal bacteria with orodigestive cancer. Periodontitis has been associated with orodigestive cancers through the chronic inflammation generated in the oral cavity and the concomitant mobilization of inflammatory mediators to distal sites in the human body, as well as a direct carcinogenic effect mediated by periodontitis-associated bacterial species either directly in oral cells or by migrating from the oral cavity.

F. nucleatum are related to higher risk of atherosclerosis. Interestingly, some cardiovascular diseases are related to chronic inflammation. Two of them, myocarditis and endocarditis, are diseases characterized by a high infiltration of lymphocytes and monocytes. *P. gingivalis* is proposed as an aggravator of autoimmune myocarditis in an *in vivo* model [90].

(ii) *Rheumatoid arthritis (RA)*: RA is an autoimmune disease characterized by the thickening of the synovium, a tissue that exists inside the joints. IL-1 and TNF-α are highly related with the pathogenesis of RA, but other cytokines like IL-4 and IL-17 have also a role in this disease. Many studies confirm a relationship between periodontitis and RA [4, 91], like Mikuls et al. [92] who were able to determine that periodontitis and the presence of *P. gingivalis* is related to the self-activity, characteristic of RA. Additionally, both *F. nucleatum* and *P. gingivalis* are highly prevalent in patients with RA [93].

(iii) *Cancer*: it has been shown that patients affected by periodontal disease have a higher risk of suffering from some type of cancer [34]; specifically, a positive association between periodontal disease and orodigestive cancers (oral, esophageal, gastric, colonic, and pancreatic) has been well established [2, 3], as well as other types of cancers such as breast, prostate, and bladder [48, 94–96]. A deeper explanation of such associations and possible mechanisms involved in these associations will be addressed in following paragraphs.

4. Association between Periodontitis and Orodigestive Cancer

As stated above, multiple epidemiological studies showed a strong association between orodigestive cancers and poor oral health [97–102], periodontal diseases [103–106], tooth loss [98, 99, 101, 102, 106, 107], and periodontal diagnostic parameters such as clinical attachment loss (CAL) and alveolar bone loss [108, 109]. Additionally, patients showing gastric precancerous lesions were more likely to have higher percentages of sites with gingival bleeding [97, 110].

Together with the increasing evidence associating periodontal diseases with several types of extra oral cancer, the question of how these bacteria exert their effect in distal sites in the human body is gaining more and more attention. Thus, some types of cancer have associated carcinogenesis with the chronic inflammation generated in the oral cavity and the concomitant mobilization of inflammatory mediators to distal sites in the human body (Figure 1) [3, 111], while other studies have associated it with a direct carcinogenic effect mediated by periodontitis-associated bacterial species either directly in oral cells or by migrating from the

oral cavity (Figure 1) [112]. Interestingly, despite the natural dissemination of oral bacteria due to swallowing of saliva, which contains a large number of bacteria, explaining therefore its involvement in orodigestive tract [113, 114], there is also evidence showing dissemination through the bloodstream (Figure 1) [115].

Systemic spread of oral bacteria either after routine activities or dental procedures was early reported by Cobe [116]. Particularly, oral anaerobes are released to circulation after some daily activities, such as tooth brushing, flossing, and chewing [117], and also immediately after therapeutic oral procedures such as scaling and root planning [118]. Therefore, dental or oral surgery is considered to be a predisposing factor for anaerobes bacteremia in both adults and children [119, 120]. However, in periodontal disease, migration of bacteria from the oral cavity to other organs in the human body is likely to occur through the blood circulation probably because there is a 3-log increase in the biomass of the subgingival biofilm and the mean surface area where this biofilm is contacting the ulcerated gingiva is approximately $20\,cm^2$ [1, 121], providing a portal of entry for oral bacteria into the vessels and thereby allowing them to spread to distant sites [122]. These bacteremias are usually polymicrobial, with higher numbers of Gram-negative bacilli and species of the genera *Peptostreptococcus*, *Clostridium*, *Fusobacterium*, among others [115].

As stated above, *F. nucleatum* is part of the subgingival microbiota and it is present in most subjects maintaining its proportion from health to disease, probably acting as a metabolic cornerstone for the whole community. Interestingly, extensive evidences associating bacteremia caused by *F. nucleatum* with underlying malignancy have been reported [123]. Moreover, comorbidity between *F. nucleatum* bacteremia and several types of cancer has been found in hospitalized patients [124–126]. Particularly, *F. nucleatum* is considered as a risk factor for colorectal cancer (CRC) (Figure 1) [7, 127, 128], as the bacterium is overrepresented in colorectal tumor tissues versus normal tissues in CRC patients [129–131]; moreover, higher loads of the bacterium have been found in CRC compared to premalignant lesions [127]. It is worth noting that as the bacterium is found together with other oral species in CRC such as *Parvimonas micra*, *Peptostreptococcus stomatis*, *Gemella morbillorum*, *Porphyromonas* spp, *Leptotrichia* spp., and *Campylobacter* spp., it strongly suggests that the source of the microbes is the oral cavity [130, 132–135]. More recently, *F. nucleatum* was also associated with other malignancies as oral cancer (Figure 1) [7], with higher levels of this species found in oral squamous cell carcinoma (OSCC) patients compared to controls [136, 137]. Similar to CRC, other periodontitis-associated taxa, such as *Dialister* spp., *Peptostreptococcus* spp., *Filifactor* spp., *Treponema* spp., and *Parvimonas* spp., were also enriched in these tumors [138]. This is interesting since a combined effect of such species could contribute to cell transformation.

Remarkably, the periodontitis-associated species *P. gingivalis* is the oral bacteria most commonly associated with cancers of the orodigestive tract and it probably has a positive effect in mortality [6, 139]. Among these cancers, *P. gingivalis* shows a strong correlation with OSCC [136], as well as with pancreatic cancer (Figure 1) [6, 140]. This species has been found in tumor tissues from patients with OSCC along with other oral anaerobes as species of the genera *Veillonella*, *Fusobacterium*, *Prevotella*, *Actinomyces*, and *Clostridium* [141], indicating that a combined effect of multiple bacterial species may be involved in carcinogenesis. Similar results have been observed in gingival squamous cell carcinoma where *P. gingivalis* is augmented compared to normal tissues [142], probably due to its invasive ability. In fact, tissue invasion is probably one of the significant ways of oral bacteria dissemination, since both *F. nucleatum* and *P. gingivalis*—the oral species mostly associated with orodigestive cancers—invade gingival tissues and have been found composing 15% to 40% of the total bacteria within the gingival tissue obtained from periodontal lesions [143]. The occurrence of both species in the tissue is likely to happen as a consequence of an intimate interaction between them in the oral cavity and probably also in extra oral sites.

Remarkably, the sole presence of a bacterium in tumorous tissue is not necessarily indicative of its role in the disease. A recent metatranscriptomic analysis showed that although both *P. gingivalis* and *F. nucleatum* were active in OSCC tumor sites compared to healthy control tumor-matched sites, only *F. nucleatum* showed a significant difference in transcriptional activity, as shown by linear discriminant analysis effect size (LefSe) analysis [144]. This indicates that either different species have a role in different stages of the tumorigenesis or that close interactions between microbial species in the tumoral tissues may modify the gene expression of the companions, as it has been shown in an *in vitro* multispecies community model [145, 146]. Interestingly, although it is not a periodontitis-associated species, *F. nucleatum* has been found to be transcriptionally active in different forms of periodontal diseases [147, 148]. Moreover, synergistic interactions between *F. nucleatum* and two periodontitis-associated bacteria, *T. denticola* and *P. gingivalis*, have been reported in chronic periodontitis [149].

This is interesting, since in addition to *P. gingivalis*, other periodontitis-associated taxa have been associated with orodigestive cancers. While carriage of *A. actinomycetemcomitans* correlates with higher risk of pancreatic cancer [150], *T. denticola* has been detected in both tongue squamous cell carcinoma [151] and esophageal cancer tissues (Figure 1) [152]. The question of how these species interact with each other in carcinogenesis has not been fully understood. It has neither been elucidated how migrating oral bacteria affect the local microbiome in distal sites and therefore alter host cell responses. For instance, Arimatsu et al. [153] showed that oral administration of *P. gingivalis* induces changes in the ileal microbiota in a mouse model, increasing systemic inflammation.

5. The Mechanism of Cancer Promotion by Periodontitis-Associated Bacteria

Although the exact mechanisms involved in cancer promotion by periodontal bacteria have not been completely

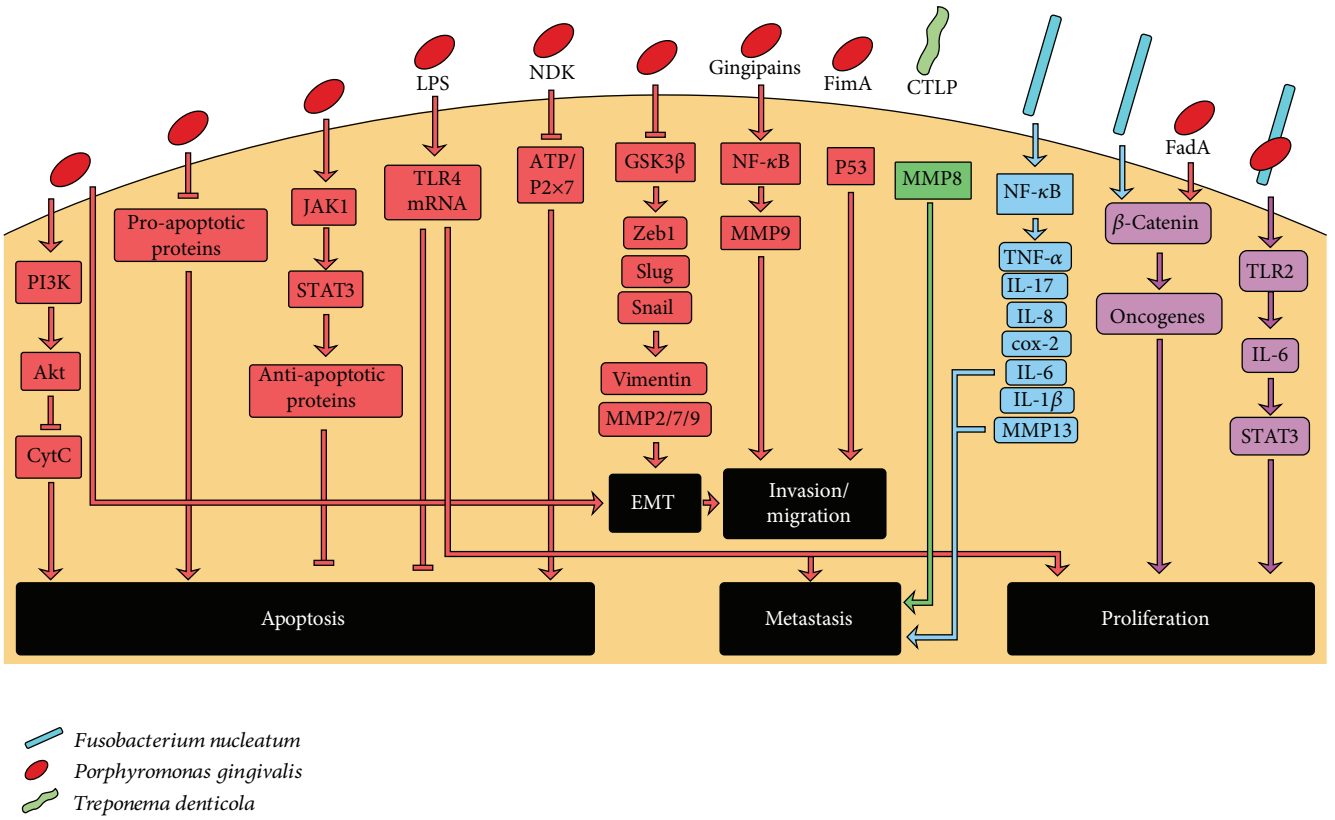

Figure 2: Host response mechanisms of cellular transformation induced by periodontal bacteria. Inhibition of apoptosis, epithelial-mesenchymal transition (EMT), invasion and migration, metastasis, and proliferation are triggered through the activation of prooncogenic pathways by *P. gingivalis* (red arrows), *T. denticola* (purple arrows), *F. nucleatum* (yellow arrow), and *P. gingivalis*+*F. nucleatum* coinfection (orange arrows).

elucidated, local inflammatory effects triggered by bacterial infection have been associated with cellular transformation [6]. Moreover, among all the subgingival species found in tumorous tissue, there is only information regarding carcinogenic mechanisms triggered by a few of them.

P. gingivalis was shown to activate carcinogenesis through several mechanisms (Figure 2). First, the bacterium has been associated directly with activation of oncogenic pathways, such as the promotion of survival in GECs through both the activation of the PI3K/Akt pathway and the inhibition of cytochrome c release [11], as well as with the reduction of the expression of proapoptotic proteins [10]. Additionally, *P. gingivalis* blocks apoptosis through the JAK/STAT pathway in GECs and therefore modulates the intrinsic cell death pathway and regulates the expression of several antiapoptotic proteins [154]. The LPS of *P. gingivalis*, in particular the O-antigen region, contributes to the apoptosis inhibition and induces proliferation in GECs [67]. This effect is associated with increased expression of TLR4 [67].

P. gingivalis was also shown to induce GECs migration in a manner dependent on the overexpression of Zeb1 [155], an activator of the epithelial-mesenchymal transition (EMT). Moreover, *P. gingivalis* increases proliferation and promotes invasion and migration in an *in vitro* model of persistent infection [9]. Likewise, *P. gingivalis* infection inhibits the activity of glycogen synthase kinase 3 (GSK3b), an important EMT regulator, in primary oral epithelial cells [156]. Additionally, other EMT-associated transcription factors, as well as mesenchymal intermediates, such as vimentin, MMP-2, MMP-7, and MMP-9, are increased and associated with higher levels of cell migration.

Several virulence factors are involved in the direct activation of inflammation and cell proliferation mediated by *P. gingivalis* [6]. Among them, nucleoside diphosphate kinase (NDK), FimA, and the LPS of *P. gingivalis* participate in the first stages of carcinogenesis, while gingipains and GroEL are associated with later stages. NDK inhibits proapoptotic mechanisms in oral epithelial cells by inhibiting the ATP/$P2X_7$ cell death signaling [65, 157, 158]. FimA attenuates the host p53-mediated tumor suppression and cell cycle progression in oral epithelial cells [6, 67] and controls the epithelial–mesenchymal transition [155]. Gingipain proteases of *P. gingivalis* activate NF-κB and MMP-9 in oral squamous carcinoma cells, which is important for cancer cell invasion and metastasis [159, 160]. Finally, GroEL produced by *P. gingivalis* increases tumor volume and the mortality of mice implanted with the mouse colon carcinoma cell line (C26) [161]. Recently, Mfa1 fimbria was shown to induce oncogenic signaling, producing myeloid-derived dendritic suppressor cells (MDDSCs) from monocytes activating the pAKT1-pFOXO1 pathway through dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) receptor [162].

Although comparatively less information exists regarding carcinogenic mechanisms triggered by *F. nucleatum*, three virulence factors have been associated with CRC promotion: the adhesin FadA, the LPS, and the autotransporter protein Fap2 (Figure 2) [7]. FadA induces inflammation and activation of procarcinogenic pathways directly in colorectal cells, activating E-cadherin-β-catenin signaling [163]. The LPS of *F. nucleatum* induces the production of inflammatory cytokines both in the gingiva and in the colonic tissue [129, 164]. Consistently, increased expression of proinflammatory cytokine such as IL-6, IL-12, IL-17, and TNF-α has been found in *F. nucleatum*-enriched colorectal adenoma subjects compared to nonadenoma controls [165]. Finally, Fap2 decreases the cytotoxicity of immune cells, favoring cancer progression [166]. *In vivo* studies showed that *F. nucleatum* increases tumor multiplicity and recruitment of tumor-infiltrating immune cells in a mouse model of intestinal tumorigenesis [167]. In this model, *F. nucleatum* generates a proinflammatory microenvironment associated with an NF-κB-mediated response (COX-2, IL-1β, IL-6, IL-8, IL-10, and TNF-α) [167], which provides a critical link between inflammation and cancer [168] and is implicated in potentiating colorectal tumorigenesis in mice [167, 169]. In addition, *F. nucleatum* increases the proliferation and invasion ability of colonic epithelial cells, promoting EMT, activating NF-κB signaling, and increasing the production of IL-6, IL-1β, and MMP-13 [170].

Even less studies evaluated the association of other periodontitis-associated taxa with cancer, among them the contribution of *T. denticola* to carcinogenesis has recently been reported (Figure 2). This species is a highly invasive anaerobic bacteria and possesses a chymotrypsin-like proteinase (CTLP) as a major virulence factor. Recently, CTLP was detected within orodigestive tumor tissues including OSCC, tongue, tonsil, and esophagus [171]. Intriguingly, CTLP converts pro-MMP-8 and pro-MMP-9 to their active forms, which are associated with metastasis in tongue, esophageal, gastric, pancreatic, and CRC [8, 12, 172].

As mentioned above, systemic spread of periodontitis-associated bacteria is usually polymicrobial. In this context, although combined effect of periodontal bacteria is well established in the etiology of periodontitis, its contribution to cancer onset is less understood. Therefore, it is relevant to understand if these bacterial cooccurrences have synergistic or antagonist effect in respect to the activation of inflammatory pathways associated to cancer.

In this context, it has been shown that coinfection of oral epithelial cells with *P. gingivalis* and *F. nucleatum* triggers the TLR2 pathway resulting in IL-6 production and STAT3 activation, which in turn stimulate cell proliferation (Figure 2) [173]. In addition, infection of oral epithelial cells with cocultures of *P. gingivalis* and *F. nucleatum* induces a slight increase in cell migration [156]; however, the pathways that are altered and could explain this effect have not been defined.

6. Conclusion

Periodontitis is a dysbiotic disease, in which chronic inflammation is produced in response to a disease-associated multispecies bacterial community established in the subgingival area. The recruitment of immune cells and the production of several inflammatory mediators contribute to the tissue damage. Additionally, the direct effect of periodontitis-associated bacteria as well as other subgingival microorganisms equally prevalent both in healthy and diseased subjects "core species" contributes to the chronicity of the disease through the activation of specific inflammatory pathways.

Chronic inflammation has also been associated with several systemic diseases, like cancer. The literature demonstrates that either inflammatory mediators produced during periodontitis development could mediate carcinogenesis or periodontal bacteria can exert its effect directly in transforming cells. Interestingly, several oral bacteria, also found in high loads in the periodontal pocket, have been shown to activate inflammatory pathways associated with several stages of cellular transformation (Figure 2). Among them, these bacteria can induce NF-κB-mediated responses, promote cell survival, activate oncogenic pathways, reduce proapoptotic proteins expression, increase cell migration and invasion, increase the expression of EMT-associated proteins, enhance metastasis, etc. In spite of this knowledge, more studies are needed to elucidate the mechanisms triggered by other periodontal bacteria and also understand the tumorigenic effect of combined bacterial infections. Such studies are relevant because, although the combined effect of species such as *P. gingivalis* and *F. nucleatum* has been studied in the etiology of periodontitis, the consequences of its effect in carcinogenesis remain poorly understood. Moreover, since bacterial spreading to distant sites on the human body occurs in coexistence, it is relevant to know the synergistic or antagonistic effects that these interactions may have in oral and extra oral carcinogenesis.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Production of Soluble Receptor Activator of Nuclear Factor Kappa-B Ligand and Osteoprotegerin by Apical Periodontitis Cells in Culture and Their Modulation by Cytokines

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RANKL, a bone-destructive cytokine, and OPG, its osteoprotective counterpart, are expressed in periapical lesions (PLs), which represent hystopatological manifestations of apical periodontitis. However, their regulation in PLs has not been elucidated yet. Therefore, our aim was to study the production of RANKL and OPG and their modulation by pro- and anti-inflammatory cytokines in PL cell cultures. Isolated PL cells were cultured alone or with addition of TNF-α, IFN-γ, IL-17, IL-4, IL-10, and IL-33, respectively. The levels of RANKL and OPG in supernatants were measured by ELISA. The proportion of $CD3^+$ (T cells) and CD19+/CD138+ (B cells/plasma cells) within isolated PLs was determined by immunocytochemistry. The levels of RANKL were higher in cultures of symptomatic PLs compared to asymptomatic PLs and PLs with the dominance of T cells (T-type lesions) over B cells/plasma cells (B-type lesions). A higher proportion of osteodestructive processes (RANKL/OPG ratio > 1.0) were detected in symptomatic PLs. The production of RANKL was upregulated by IFN-γ and IL-17 and higher concentrations of IL-33. IL-10 and lower concentrations of IL-33 augmented the production of OPG. The addition of either RANKL or anti-RANKL antibody to the cultures did not modify significantly the production of OPG. In conclusion, this original PL cell culture model suggests that increased bone destruction through upregulated production of RANKL could be associated with exacerbation of inflammation in PLs with the predominance of Th1 and Th17 responses and increased secretion of IL-33. In contrast, IL-10 and lower levels of IL-33, through upregulation of OPG, may suppress osteolytic processes.

1. Introduction

Apical periodontitis is an opportunistic infection around the apical region, which is a consequence of spreading bacteria from the necrotic pulp [1]. This is a common disease in adults, with roughly one in three individuals affected [2]. The histopathological base of the disease consists of granuloma and radicular cysts, usually named periapical lesions (PLs). They are chronic processes, due to the inability of host defense mechanisms to eradicate the infection [3]. The pathophysiology of PL involves a complex host immune/inflammatory response to the bacteria and their products. The same mechanisms may also cause the destruction of soft and hard tissues surrounding the root apex [4]. PLs are characterized by the infiltration of the periodontal tissue with different inflammatory cells such as neutrophil granulocytes, T and B cells, plasma cells, macrophages, dendritic cells, mast cells, and other cells of the innate immunity [5]. The composition of infiltrating cells and the functional and phenotypic properties of both infiltrating and stromal cells depend on the activation status of PLs which is under control of a series of cytokines [3]. The histopathologic endpoint of PL is bone loss, which may occur to increase vascularization at the apex, thus blocking the infection in the root canal [6, 7].

Bone loss is caused by osteolytic activity of osteoclasts in which the receptor activator of nuclear factor kappa-B ligand (RANKL) plays a crucial role. RANKL was initially identified as a cell membrane-bound ligand responsible for stimulation of osteoclast differentiation and bone resorption [8, 9], by mediating the cell-to-cell interaction between osteoblasts and osteoclast precursors. RANKL is also produced as a secreted ligand by osteoblasts, fibroblasts, and activated T and B cells as well as by the cells of the monocyte-macrophage lineage [10]. The metalloprotease-disintegrin TNF-α-converting enzyme mediates its cleavage into a soluble form. By activating its cognate RANK receptor on the surface of monocytes and macrophages, RANKL triggers the fusion of macrophages and their differentiation into mature osteoclasts with the bone resorption activity [11].

The action of RANKL is antagonized by osteoprotegerin (OPG). OPG is a soluble decoy receptor which blocks RANKL and, by preventing its interaction with RANK, inhibits osteoclast activation and subsequent bone resorption [12]. OPG is largely expressed by some epithelial cells, vascular endothelial cells, and lymphoid cells [13, 14]. The overall efficiency of RANKL on osteoclast formation and bone resorption is tightly coupled to the activity of OPG, as its natural inhibitor. Therefore, it is very important to study concomitantly the expression of these two molecules in sites of hard-tissue resorption, preferably as their relative RANKL/OPG ratio. The RANKL/RANK/OPG system is involved in pathogenesis of osteodestructive processes, including periodontitis and dentoalveolar development. Several papers described the expression of RANKL and OPG in both human and experimental animal PLs, by using immunohistochemistry, mRNA, or biofluid analyses, and the results have been summarized in a recent comprehensive review [15]. Generally, the review provides the evidence that higher RANKL expression and higher RANKL/OPG ratio are associated with periapical bone loss, but it does not give any conclusive information about their role as predictive markers, or their clinical significance. However, recent data showed that RANKL may play an immunoregulatory role since RANKL inhibition resulted in an unremitting proinflammatory response in experimental PLs, persistent high proinflammatory and effector CD4 responses, decreased migration of T regulatory cells (Tregs), and lower levels of IL-10 and TGF β [16]. All these data related to PLs are in contrast to a recent systematic review on biomarkers of alveolar bone resorption in gingival crevicular fluid, which showed that RANKL could be a central biomarker indicating osteoclastic activity and a diagnostic indicator for chronic periodontitis [17].

The expression of RANKL and OPG is under control of numerous factors, including cytokines, which play a crucial role in the regulation of immune/inflammatory reactions within PLs and are critical determinants of lesion outcome [4, 18]. In this context, proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) as well as some chemokines, such as IL-8, orchestrate the recruitment and activation of innate immune cells, presumably neutrophil granulocytes and monocytes in the early inflammatory phase and T and B cells in the later inflammatory phase, respectively. A large number of clinical and experimental results suggest that T-helper 1 (Th1) cells, by producing interferon-γ (IFN-γ), are involved in the progression of PLs and bone destruction, whereas T-helper 2 (Th2) cytokines, such as interleukin 4 (IL-4), IL-5, IL-10, and IL-33, are associated with the humoral immune response and attenuation of tissue damage [3, 4, 6]. It seems that Th9 and Th22 pathways may also contribute to human and experimental periapical lesion stability [19]. IL-17 may play a role in exacerbation of inflammation in PLs [20] and stimulation of osteolysis [21]. On the other hand, Foxp3+CD4 +CD25+ Tregs downregulate immune response, inflammation, and osteolysis in PLs. Their effect is predominantly mediated by transforming growth factor-β (TGF-β) and IL-10 [22–24].

The development of PLs is a dynamic process in which osteolytic and osteoprotective mechanisms are tightly balanced. However, up to now no one study investigated how a particular cytokine modulates these processes in PLs by acting on RANKL and OPG production, and this was a primary interest in our study.

To address these questions, we used an original approach by studying the production of RANKL and OPG in PL cell cultures. We described this cell-culture model in our previous paper and found it as very suitable to study the pathophysiological mechanisms involved in the progression and restriction of PLs [25]. Therefore, the concrete aim of this work was (1) to examine the production of RANKL and OPG by PL cells in culture, to determine their ratio, and to show the relationship between these parameters and clinical presentation of PLs, their size, and their lymphocyte composition within isolated PL cells; (2) to study the modulatory effect of proinflammatory cytokines (TNF-α, IFN-γ, and IL-17), anti-inflammatory cytokines (IL-4 and IL-10), and IL-33, a cytokine showing anti- and proinflammatory activity [26] on RANKL and OPG production by PL cells; and (3) to check whether the production of OPG by PL cells is modulated by RANKL.

2. Materials and Methods

2.1. Periapical Lesion Samples. Human PLs ($n = 43$) were extracted at the Department for Oral Surgery, Clinic for Stomatology, Military Medical Academy (MMA), Belgrade, Serbia, at the time of teeth extraction or apicotomy. The study was approved by the Ethical Committee of MMA in compliance with the Helsinki Declaration, followed by an informed consent from patients. The average age of the patients was 35 years (range: 21–65 years). The patients with malignant and autoimmune diseases, as well as patients on the immunosuppressive/immunomodulatory therapy, or those on the therapy of systemic modifiers of bone metabolism, were excluded. All the patients included had not been treated with antibiotics for one month before PL excision. PLs were radiographically diagnosed using the standard equipment for intraoral radiography (Carestream CS 2200 Roentgen apparatus; Carestream Dental, Atlanta, GA, USA) and extraoral radiography of the maxillofacial region (orthopantomography and dental cone beam computed tomography (CBCT); LargeV Instrument Corp. Ltd, Beijing, China). The size of

radiolucent PLs on radiographs and tomographs was analyzed by adequate softwares, and smallest and largest diameters were measured. Three patients had two lesions on two different teeth. According to the presence or absence of clinical symptoms, PLs were classified as symptomatic ($n = 22$) or asymptomatic ($n = 21$).

The lesions were divided according to their size into small and large PLs. Small lesions ($n = 18$) were PLs whose mean diameter was less than 4.0 mm. The lesions whose mean diameter was higher than 5.0 mm were classified as large lesions ($n = 25$). No further division between specimens, regarding sex, age, etiology, or tooth type, was done. After extraction, PLs were immediately placed in a medium consisting of RPMI-1640 (Sigma-Aldrich, Taufkirchen, Germany) and antibiotics/antimycotic solution (Sigma-Aldrich) containing penicillin (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml) and then transported to the laboratory.

Some PL cell cultures ($n = 12$) were used to study the modulatory effect of pro- and anti-inflammatory cytokines on RANKL and OPG production.

2.2. Isolation of Cells from PLs. The cells from PLs were isolated by a procedure which has been previously introduced by our research group [5, 25] with some modifications. Briefly, periapical tissue was placed in a Petri dish containing 1 ml RPMI-1640 medium and cut into 2-3 mm diameter pieces using a scalpel. The tissue was then digested for 15 min with 0.05% collagenase type IV (Sigma-Aldrich) and 0.02% DNAse (Sigma-Aldrich) in 5 ml RPMI-1640 medium in a cell incubator at 37°C. After that, the tissue was pressed through a stainless-steel mesh using a syringe plunger, filtered, and resuspended in RPMI-1640 medium containing 1 mM EDTA (Sigma-Aldrich). The remaining of the tissue was subjected to another round of digestion by using 0.05% collagenase/0.02% DNAse and 0.1% trypsin (Sigma-Aldrich) for 20 min. The released cells were pooled, washed twice by centrifugation in the RPMI medium containing 0.5 mM EDTA at room temperature (400 g for 10 min), and counted. The viability of cells, determined by Trypan Blue dye, was higher than 90%. After that, cytospins were prepared from each sample of cells using a cytocentrifuge (Schandon 4, Thermo Fisher Scientific, Waltham, MS, USA) on poly-L-lysine- (Sigma-Aldrich) coated glass slides. The cytospins were stained with May–Grünwald–Giemsa (Sigma-Aldrich) or used for immunocytochemistry.

2.3. Immunocytochemistry. For immunostaining, anti-CD3 monoclonal antibody (mAb) (Abcam, Cambridge, UK), anti-CD19, and anti-CD138 (mAbs) (both from Serotec, Oxford, UK) were used. Rabbit anti-mouse peroxidase-conjugated Ig was purchased from DAKO (Glostrup, Denmark). Cytospins were fixed with 2% pararosaniline (Sigma-Aldrich) for 2 min at room temperature, washed with phosphate-buffered saline (PBS) for 10 min, blocked with rabbit serum for 20 min, and washed with PBS. After that, cytospins were incubated for 60 min at room temperature with either anti-CD3 mAb, as a pan T cell marker, or the combination of anti-CD19/anti-CD138 mAbs, as markers of B cells and plasma cells, respectively, followed by washing with PBS. After that, the slides were blocked with 0.3% H_2O_2 in PBS for 20 min and then incubated with 1 : 50 dilution of polyclonal rabbit anti-mouse peroxidase-conjugated antibody. The immunoperoxidase reaction was developed with diaminobenzidine (Sigma-Aldrich). Controls were samples incubated with an irrelevant mAb, mouse anti-rat CD4 (OX-38) (Serotec), nonreactive with human cells. Cytospins were analyzed by light microscopy (Olympus, Hamburg, Germany). On each cytospin, at least 500 cells were counted. The percentages of positive cells were determined on the basis of total counted cells. Based on the predominance of T cells or B cells/plasma cells, PLs were divided into T-type and B-type lesions, respectively [3].

2.4. Cell Cultures. The cells isolated from PLs were cultivated in 96 wells, with round-bottomed plates (ICN, Costa Mesa, CA) (1×10^5 cells/well, 200 μl) in the complete culture medium consisting of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich) and standard culture solutions of antibiotics [25]. Phorbol myristate acetate (PMA) (20 ng/ml) (Sigma-Aldrich) and Ca^{2+} ionophore (A 23187, 1 M) (Sigma-Aldrich) were used for cell stimulation [27]. After 24 h, the cell supernatants were collected, centrifuged, and frozen at −70°C until the levels of cytokines were determined. Certain cultures were used to study the modulatory effects of cytokines on RANKL and OPG production. The following cytokines were used: IFN-γ; (20 ng/ml), TNF-α (10 ng/ml), IL-17 (25 ng/ml), IL-4 (20 ng/ml), IL-10 (10 ng/ml), RANKL (10 ng/ml and 30 ng/ml), and IL-33 (1 ng/ml and 30 ng/ml). The concentrations of these cytokines were optimized in our previous experiments on peripheral blood mononuclear cells (PBMNC) and several PL cell cultures. All these cytokines were purchased from R&D (Lorton, VA, USA), except IL-33, which was obtained from BioLegend (San Diego, CA, USA). A neutralizing antibody to RANKL was obtained from R&D and used at the concentration of 2 μg/ml.

2.5. Cytokine Assay. The concentrations of RANKL and OPG in culture supernatants were detected by using specific ELISA kits (Abcam) following the instructions of the manufacturer. The levels of cytokines were determined on the basis of the standard curve, constructed by known concentrations of these cytokines. The cut of values for RANKL and OPG was 10 pg/ml and 1 pg/ml, respectively.

2.6. Statistical Analysis. To assess whether the differences between the groups/samples were significant, either Wilcoxon signed-rank tests or Friedman test with Dunn's multiple comparison posttest was used, since the data did not follow the Gaussian distribution according to the Kolmogorov-Smirnov normality test. To assess whether RANKL and OPG levels correlate significantly, the Spearman correlation test was used and the values of $p < 0.05$ were considered to be statistically significant. The statistical analysis was carried out using GraphPad Prism (GraphPad Software, CA, USA).

3. Results

3.1. Production of RANKL in PL Cell Cultures in Relation to Clinical Characteristics of the Lesions and T/B Cell Predominance. In the first part of this work, the levels of soluble RANKL and OPG were determined in 43 different PL cell cultures. The results varied between samples such that the mean level of RANKL was 142.7 ± 116.2, the mean level of OPG was 91.8 ± 58.2, and the mean RANKL/OPG ratio was 2.5 ± 2.2. No correlation between RANKL and OPG levels was found ($r = 0.30$; $p = 0.08$). The proportion of PLs (62.8%) with active bone resorption processes (RANKL/OPG ratio > 1.0) was higher than the proportion of PLs (37.2%) in which bone resorption processes were suppressed (RANKL/OPG ratio < 1.0).

The difference in the level of RANKL between symptomatic lesions (207.9 ± 119.8) and asymptomatic lesions (88.3 ± 67.7) was statistically significant ($p < 0.001$). No significant differences between large (122.4 ± 116.4) versus small (189.0 ± 112.3) lesions were found. The mean proportion of T cells was 18.6 ± 13.8, and the mean percentage of B cells/plasma cells was 16.3 ± 14.3. The number of PLs with the dominance of T cells over B cells/plasma cells (T-type lesions) was 27, whereas the number of PLs with the dominance of B cells/plasma cells over T cells (B-type lesions) was 16. An example of immunocytochemical images of the lesions is given in Figure 1. The difference in the mean levels of RANKL between T-type (189.6 ± 96.3) versus B-type lesions (116.2 ± 55.2) was statistically significant ($p < 0.05$) (Figure 2(a)). The differences in the levels of OPG between any of the examined groups were not statistically significant (Figure 2(b)). The difference in the RANKL/OPG ratio was significantly higher only between symptomatic (3.10 ± 2.91) compared to asymptomatic PLs (1.18 ± 0.73) ($p < 0.05$) (Figure 2(c)). The proportion of PLs with the RANKL/OPG ratio > 1.0 in symptomatic lesions (77.3%) was significantly higher (χ^2 $F = 4.044$; $p = 0.044$) compared to the proportion of PLs with the same ratio in asymptomatic lesions (47.6%). No differences were found when the ratio was compared between the large versus small PLs, as well as T-type versus B-type PLs (Table 1).

3.2. Modulatory Effect of Pro- and Anti-Inflammatory Cytokines on RANKL and OPG Production in Culture of PL Cells. The second part of this work was related to the modulatory effect of pro- and anti-inflammatory cytokines on RANKL and OPG production, which was studied in 12 separate PL cell cultures. The proportion of PLs reflected generally their distribution in the whole group (the proportion of symptomatic and asymptomatic PLs was equal: $n = 6$; the proportion of small size PLs versus large PLs was 5/7; and the proportion of T-type versus B-type PLs was 7/5).

IFN-Υ and IL-17A augmented the production of soluble RANKL ($p < 0.05$ and $p < 0.01$, respectively) (Figure 3(a)). The level of OPG was only increased in the presence of IL-10 ($p < 0.05$) (Figure 3(b)), whereas IL-4 and TNF-α did not modulate the production of both RANKL and OPG (Figure 3(a) and Figure 3(b)). IL-17A increased the RANKL/OPG ratio, whereas IL-10 had the opposite effect (Figure 3(c)).

3.3. Modulatory Effect of IL-33 on RANKL and OPG Production in Culture of PL Cells. The same 12 cultures were used to study the effect of IL-33 on RANKL and OPG production by PL cells. Based on preliminary results on PBMNC and PL cells, showing a dose-dependent effect of IL-33 on RANKL/OPG production and their ratio, we selected two doses (1 ng/ml and 30 ng/ml) of IL-33 for final experiments. As presented in Figure 4, lower concentrations of IL-33 upregulated OPG production and decreased the RANKL/OPG ratio ($p < 0.05$), whereas higher concentrations augmented RANKL production compared to the control ($p < 0.01$) and increased the RANKL/OPG ratio compared to the low dose of IL-33 ($p < 0.01$).

3.4. RANKL Does Not Modulate the Levels of OPG in Culture of PL Cells. Finally, we tested whether the addition of exogenous RANKL modulates the production of OPG. According to mean levels of OPG ($n = 12$), it can be concluded that RANKL, at both of the two concentrations used (10 ng/ml and 30 ng/ml), did not modulate significantly the production OPG. A similar result was obtained by addition of a neutralizing anti-RANKL antibody (Figure 5).

4. Discussion

In this study, we showed that RANKL and OPG are produced in culture of cells extracted from the periapical tissue. This procedure has been introduced previously by our group with the aim to study the pathogenesis of human apical periodontitis [25]. Its advantage over other used approaches so far in humans, related to the expression of biomolecules associated with bone resorption/reparation processes, such as immunohistochemistry, mRNA expression, whole tissue homogenization, or biofluid collection, is the possibility to study the mechanisms involved, based on the manipulation with experimental conditions *in vitro*. In this context, the modulation of RANKL and OPG production by pro- and anti-inflammatory cytokines has been studied for the first time in this work.

The first *in vivo* demonstration of the involvement of RANKL and OPG in PLs came by immunohistochemical studies by using experimental pulpal exposure animal models. These and subsequent studies revealed a locally enhanced RANKL/OPG ratio in PLs and confirmed the implication of this molecular system in pathological periapical bone resorption, similarly as in other bone-destructive pathoses [15]. Several publications were related on RANKL and/or OPG in human PLs. Among them, Tay et al. showed the presence of RANKL in radicular cysts, by using an immunohistochemistry method [28]. Immunolocalization of RAN KL was overlapped with staining for TRAP, a marker of osteoclasts. Sabeti et al. confirmed the presence of RANKL at the gene expression level in inflammatory PLs of undisclosed nature [29]. RANKL mRNA was also detected and semiquantified in periapical granulomas, whilst its expression was below detection limit in healthy periodontal ligament [30]. Expression of RANKL on inflammatory cells isolated from PLs was further investigated using flow cytometry. The results showed that monocytes (CD14+) and dendritic

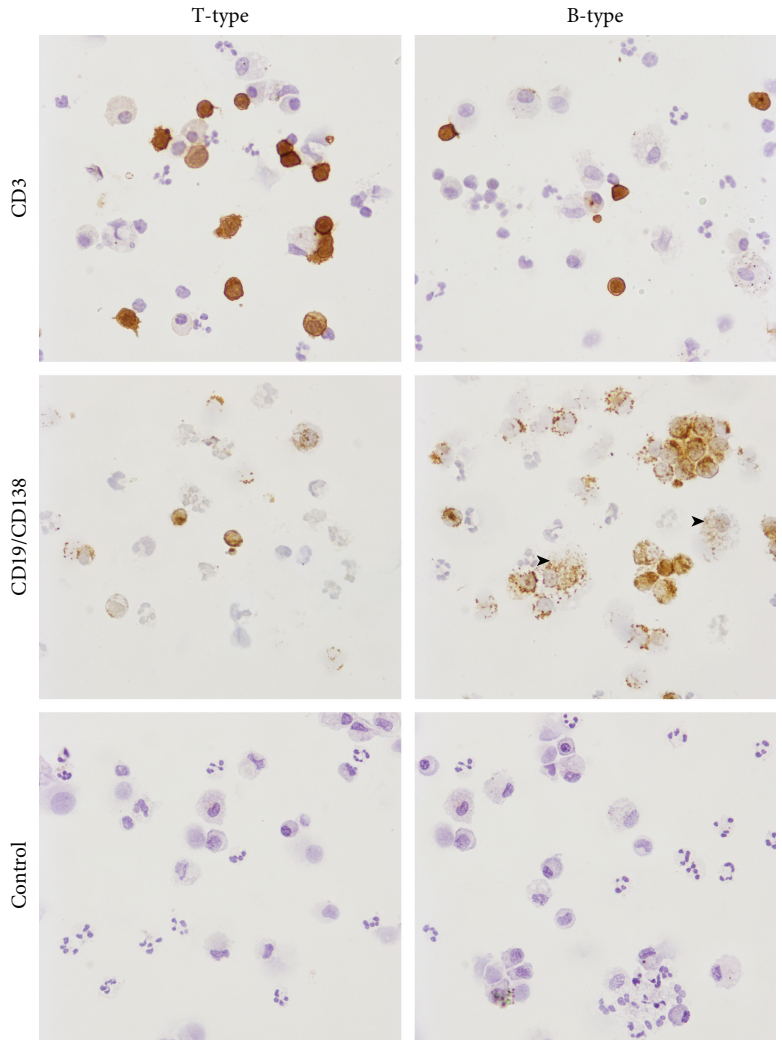

Figure 1: Immunocytochemical presentation of T-type and B-type periapical lesions. Cytospins of periapical lesion (PL) cells were stained with an anti-CD3 (marker of T cells) or a combination of anti-CD19 (B cell marker) and anti-CD138 (marker of plasma cells), as described in "Materials and Methods." A representative sample of T-type PL (predominance of T cells over B cells/plasma cells) or a sample of B-type PL (predominance of B cells/plasma cells) over T cells is presented. Macrophages, which are positively stained with CD138 (intracytoplasmic granular staining), are excluded from the analysis, based on morphological criteria. Some of them are marked by arrows. Controls are stained by an irrelevant (anti-rat CD4) monoclonal antibody, nonreactive with human tissues. Magnifications: ×200.

cells (CD83+) were the main producers of RANKL in granuloma [30]. By using an immunohistochemical study, Menezes and coworkers compared RANKL and OPG levels between apical granulomas and radicular cysts [31]. They found that the ratio of OPG^+/total cells and $RANKL^+$/total cells was higher in granulomas than in cysts, but the RANKL/OPG ratio did not differ between these two types of PLs. They also showed that various cell types expressed both RANKL and OPG, and the staining of macrophage-like cells ($CD68^+$) was of the highest intensity. Another study demonstrated a significantly higher expression of RANKL mRNA levels in granulomas in comparison with cysts [32]. These results are in contrast to those published by Fan et al., who did not identify any difference between total RANKL or OPG protein levels or their ratio between granulomas and radicular cysts [33]. We also did not find the differences in these parameters between large- and small-size PLs. Although we did not classify our PL samples by histological criteria, radiological appearance was very suggestive that the majority of large size PLs resembled cysts, in contrast to small-size PLs. Considering RANKL and OPG expression and radiographic size of periapical granulomas (smaller or greater than 5 mm in diameter), Menezes et al. demonstrate a trend towards higher RANKL and lower OPG expression in smaller lesions, but similarly with our results, the differences were not statistically significant [34]. However, in their study the frequency of RANKL>OPG samples was higher compared to the group of larger lesions. No association of RANKL and OPG protein expression, detected by immunochemistry, with lesion size was also observed by Santos et al. [35]. Their study demonstrated the possible involvement of RANKL, TNF-α, IL-33, cathepsin K, and OPG in the development of radicular cysts and periapical granulomas, with emphasis on the highest immunoreactivity of

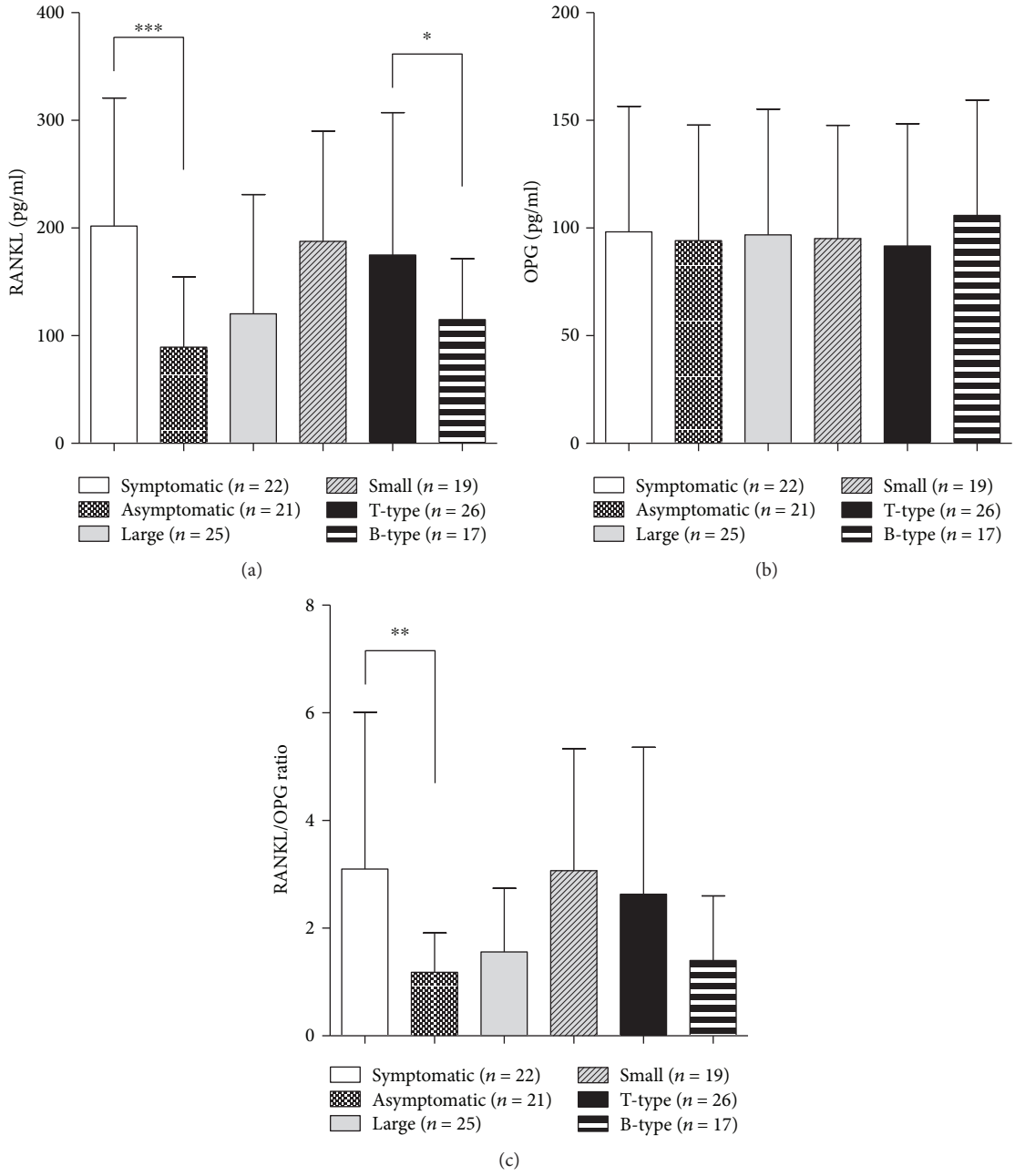

Figure 2: The levels of RANKL (a), OPG (b), and values of RANKL/OPG ratio (c) in PL cell cultures in relation to clinical characteristics of the lesions and T/B cell predominance. Periapical lesion (PL) cells were isolated from 43 PLs and cultivated for 24 hours as described in "Materials and Methods." The levels of soluble RANKL and OPG in supernatants were measured by ELISA, and after that, the RANKL/OPG ratio was calculated. The differences between groups were tested by using the pair Student t-test. The statistically significant difference was marked on the graph. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, as indicated.

cathepsin in cysts and TNF-α and OPG in granulomas. Based on these results, they supposed that OPG could determine the slower growth of granulomas compared to cysts due to its blocking activity against RANKL.

We showed that symptomatic lesions were characterized by higher production of RANKL and higher RANKL/OPG ratio, a phenomenon which has not been explored enough in previous studies. Our results are partly comparable with those published by Fan et al. who observed significantly more RANKL-positive cells in severely inflamed lesions compared to lightly inflamed counterparts. However, the RANKL/OPG ratio was statistically similar between inflammations graded as light, moderate, or intense [36]. The explanation why the production of RANKL is significantly higher in symptomatic PLs could be found by evaluating the association between high production of proinflammatory cytokines in symptomatic PLs [3] and RANKL expression. Among them, IL-1 and TNF-α have been shown to predominate both in the early phase of apical periodontitis and during the exacerbation, and both phases are characterized by the presence of clinical

Table 1: RANKL/OPG ratio in PL cell cultures depending on the clinical presentation of PLs and T/B cell predominance.

RANKL/OPG ratio	Sy n (%)	As n (%)	$\sum n$ (%)	χ^2 F	p
<1.00	5 (22.7)	11 (52.4)	16 (37.2)	4.044	*0.0443*
>1.00	17 (77.3)	10 (47.6)	27 (62.8)		
	Large n (%)	Small n (%)	$\sum n$ (%)		
<1.00	10 (40.0)	6 (33.3)	16 (37.2)	0.1991	0.6555
>1.00	15 (60.0)	12 (66.7)	27 (62.8)		
	T-type n (%)	B-type n (%)	$\sum n$ (%)		
<1.00	7 (26.9)	9 (52.9)	16 (37.2)	2.978	0.0844
>1.00	19 (73.1)	8 (47.1)	27 (62.8)		

Sy: symptomatic lesions; As: asymptomatic lesions.

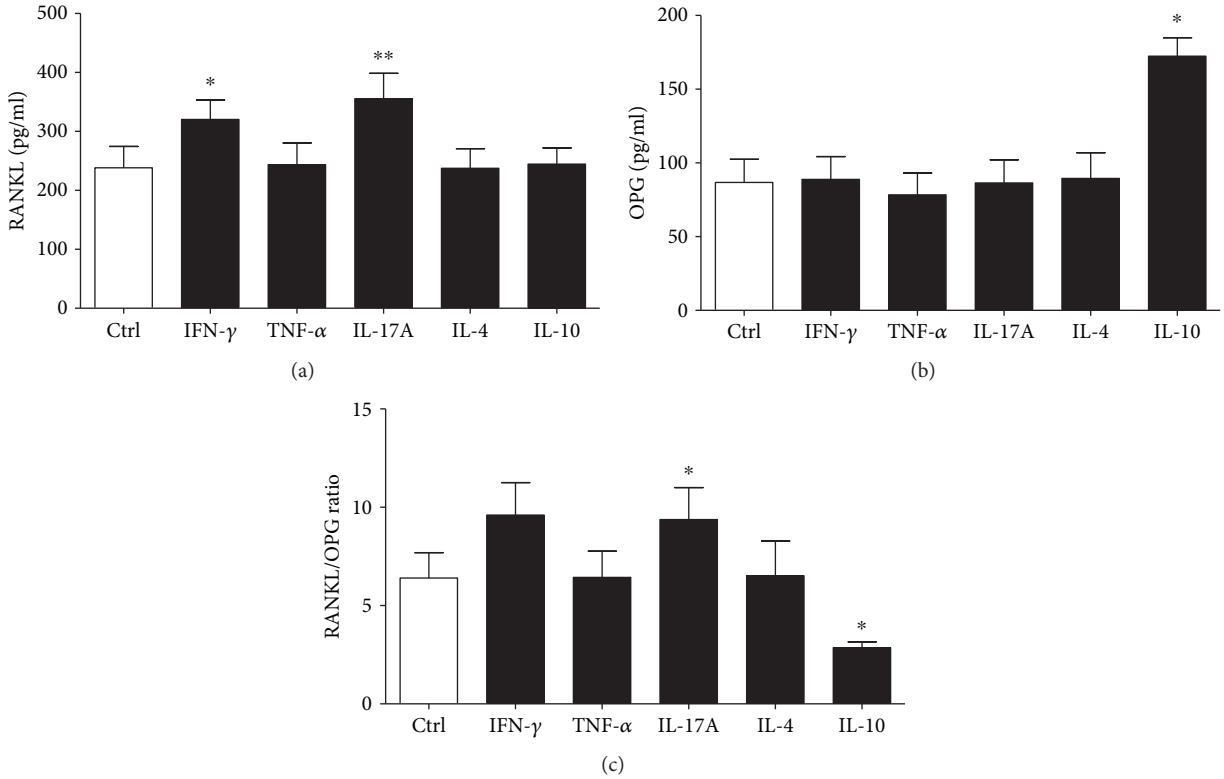

Figure 3: The levels of RANKL (a), OPG (b), and RANKL/OPG ratio (c) in PL cell cultures upon stimulation with pro- and anti-inflammatory cytokines. Periapical lesion (PL) cells were isolated from 12 PLs and cultivated for 24 hours with TNF-α, IFN-γ, IL-17A, IL-4, and IL-10 as described in "Materials and Methods." The levels of soluble RANKL and OPG in supernatants were measured by ELISA, and after that, the RANKL/OPG ratio was calculated. (a) Friedman test ($n = 12$, Friedman statistics 22.1, $p = 0.0005$) and Dunn's multiple comparison test, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs Ctrl. (b) Friedman test ($n = 12$, Friedman statistics 15.94, $p = 0.007$) and Dunn's multiple comparison test, $^{*}p < 0.05$ vs Ctrl. (c) Friedman test ($n = 12$, Friedman statistics 21.14, $p = 0.0008$) and Wilcoxon signed-rank test, $^{*}p < 0.05$ compared to Ctrl.

symptoms [3]. According to Kitaura et al., TNF-α acts directly to promote osteoclastogenesis, by increasing the expression of RANKL in macrophages and stromal cells [37]. Other studies suggest a direct effect of this cytokine on the bone resorption or through RANKL without modification of its expression. The resorption activity of osteoclasts generated by TNF-α in the absence of RANKL was critically dependent upon IL-1, which was expressed by the influence of TNF-α [38]. Zhang et al. found that TNF-α potently stimulated RANKL-induced osteoclastogenesis via coupling the RANK signaling pathway [39]. Further results indicated that IL-1 and LPS stimulate the production of osteoclasts through two parallel processes such as direct enhancement of RANKL and suppression of OPG expression, which is mediated by PGE2 production [40]. Kubota et al. demonstrated that TNF-α promotes the expression of OPG in synovial fibroblasts, predominantly through TNF-RI which may contribute to self-protection from the bone destruction [41]. We did not find any modulation of both RANKL and OPG production by TNF-α, suggesting that TNF-α has not a

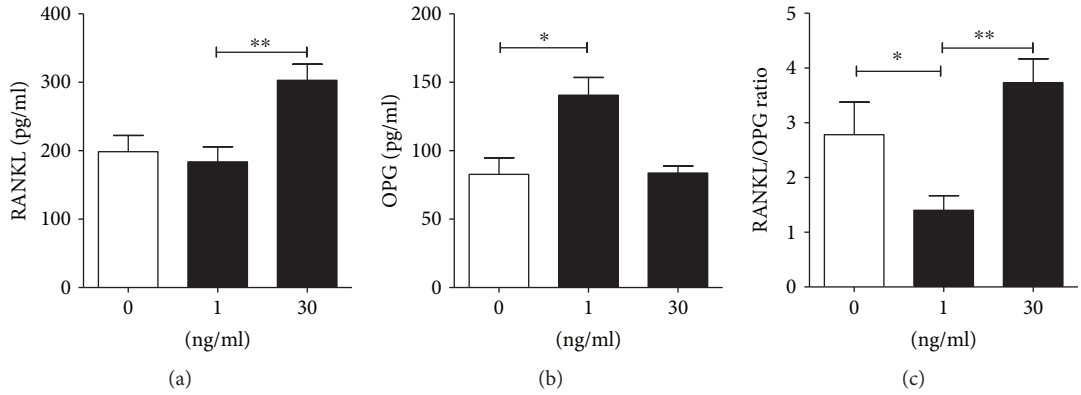

Figure 4: The levels of RANKL (a), OPG (b), and RANKL/OPG ratio (c) in PL cell cultures upon stimulation with IL-33. Periapical lesion (PL) cells were isolated from 12 PLs and cultivated for 24 hours with 1 ng/ml or 30 ng/ml of IL-33 as described in "Materials and Methods." The levels of soluble RANKL and OPG in supernatants were measured by ELISA, and after that, the RANKL/OPG ratio was calculated. (a) Friedman test ($n = 12$, Friedman statistics 10.33, $p = 0.0017$) Dunn's multiple comparison test, $^{**}p < 0.01$ as indicated. (b) Friedman test ($n = 12$, Friedman statistics 9.33, $p = 0.0055$) Dunn's multiple comparison test, $^{*}p < 0.05$ as indicated. (c) Friedman test ($n = 12$, Friedman statistics 12.00, $p = 0.0001$) and Dunn's multiple comparison test, $^{*}p < 0.05$ and $^{**}p < 0.01$ as indicated.

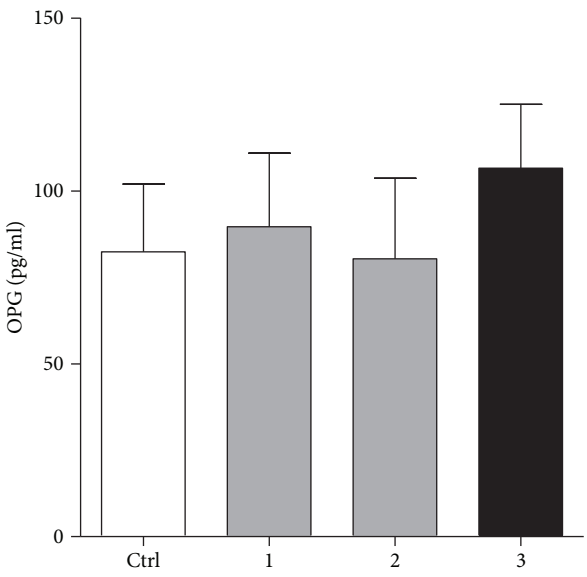

Figure 5: Modulation of OPG production in PL cell cultures by RANKL or anti-RANKL neutralizing antibody. Periapical lesion (PL) cells were isolated from 12 PLs and cultivated for 24 hours with 10 ng/ml (labeled as 1 on x axis), 30 ng/ml (labeled as 2) of RANKL, or anti-RANKL neutralizing antibody (2 μg/ml) (labeled as 3) as described in "Materials and Methods." The levels of OPG in supernatants were measured by ELISA, and after that, the RANKL/OPG ratio was calculated. All differences in relation to control (Ctrl) were not statistically significant ($p > 0.05$).

dominant effect on the expression of these bone-remodeling mediators in the mixture of the PL cell population, composed predominantly of inflammatory cells. It is interesting that we did not observe any significant modulation of OPG production by either addition of exogenous RANKL or its neutralization by anti-RANKL antibody, suggesting that, at least in this culture model, the expression of OPG is not under direct influence of RANKL. It is interesting that this phenomenon has not been examined in other cell systems. Another cytokine which is upregulated in symptomatic lesions is IL-17 [20]. IL-17$^+$ T cells are important inducers of RANKL expression and can cause alterations in the RANKL/OPG balance [42]. In addition, RANKL is expressed by IL-17$^+$ cells. However, a study showed that Th17 cells do not induce osteoclastogenesis in the absence of osteoblasts, which strongly suggests that RANKL expressed on Th17 cells alone is not sufficient to induce the differentiation of osteoclasts. This is partly dependent on a small amount of IFN-γ produced by Th17 cells, which could counterbalance the RANKL action [42]. It has been shown that IL-17 significantly enhances the expression of RANKL but also inhibits the expression of OPG in human periodontal ligament cells through mitogen-activated protein kinases and nuclear factor-κB (NF-κB) signals [43]. We showed that IL-17A augments RANKL, but not OPG secretion by PL cells, which is in accordance with the osteodestructive role of Th17$^+$ cells. The increased number of activated T helper (Th) cells in PLs could be the reason why we detected a higher proportion of bone-destructive lesions (RANKL/OPG ratio > 1.0) in T-type lesions, compared with those with the predominance of B cells and plasma cells and in which humoral immune response prevails [3]. Among them are Th17$^+$ cells. This is also in accordance with our findings that PL cells produce higher levels of RANKL in the presence of IL-17A.

Our previous results also showed that Th1 cells, which produce IFN-γ, are also numerous in PLs [25]. The role of IFN-γ in the pathogenesis of PLs and bone destruction is still controversial since IFN-γ can function as a pro- or antiresorptive cytokine [4, 32]. IFN-γ blunts osteoclast formation through direct targeting of osteoclast precursors but indirectly stimulates osteoclast formation and promotes bone resorption by stimulating antigen-dependent T cell secretion of RANKL and TNF-α [44]. The opposite results were published by Takayanagi et al. who showed that IFN-γ strongly suppresses osteoclastogenesis by interfering with the RANKL-RANK signaling pathway [45]. The analysis of the *in vivo* effects of IFN-γ in mouse models revealed that IFN-γ has both direct antiosteoclastogenic and indirect pro-osteoclastogenic properties *in vivo*. Our findings, which

showed that IFN-γ stimulates the production of RANKL by PL cells in culture, are in accordance with those showing that Th1 cells, through induction of RANKL, play a direct role in bone resorption in Th1-dominant diseases [46].

However, our explanations about the dominance of osteodestructive processes in T-type lesions are simplified because other Th subsets (Th2, Th9, Th22, and Tregs) as well as different subpopulations of $CD8^+$ T cells are also present in PLs. Th2 cells, by producing IL-4, IL-5, and IL-13, are believed to be associated with the humoral immune response in which anti-inflammatory processes dominate [3]. However, we found that only half of B-type lesions belong to the group of bone antiresorptive PLs (RANKL/OPG ratio < 1.0). It has been shown that IL-4 directly prevents osteoclast precursors from differentiating into osteoclasts in a signal transducer and activator of transcription (STAT) 6-dependent manner [47]. In addition, IL-4 suppresses RANK expression but enhances OPG expression in osteoblastic cells [47]. We did not show any modulatory effect of IL-4 on RANKL and OPG production in PL cell cultures, suggesting that most inflammatory cells from the PLs respond differently to IL-4 than osteoclasts do.

The only subpopulation of T cells which strongly inhibits inflammation and bone destruction is the Treg subset. Tregs produce IL-10 and TGF-β, which downregulate the production of RANKL and increase the production of OPG [48]. IL-10 directly inhibits osteoclast precursors by suppressing RANKL-induced NFATc1, c-Fos, and c-Jun expression [49]. Our results, showing an increased production of OPG and decreased RANKL/OPG ratio in PL cell cultures, support previous findings. However, we did not detect any significant change in the production of RANKL.

An interesting finding in our study was related to the dual effect of IL-33 on RANKL and OPG production. We showed for the first time that lower doses of this cytokine augment OPG production in PL cells, in contrast to higher doses which augment RANKL. It is known that IL-33 belongs to the IL-1 family and is closely related in structure to IL-18 and IL-1β. The cytokine is synthesized as a biologically inactive precursor and then cleaved by the enzyme caspase-1 to be secreted as active mature forms. IL-33 stimulates target cells by binding to its ST2 receptor which is followed by activation of NF-κB and MAPK pathways via identical signaling events to those observed for IL-1β. IL-33 is also a nuclear factor abundantly expressed in high endothelial venules. The major targets of IL-33 *in vivo* are tissue-resident immune cells such as mast cells, group 2 innate lymphoid cells (ILC2s), and Tregs. However, it also acts on many other cells including Th1 cells and CD8 cells. Initially, IL-33 was proven as a potent stimulator of Th2 immune response (allergy diseases), but we know now that IL-33 is a crucial immune modulator with pleiotropic activities in type 2, type 1, and regulatory immune responses, playing important roles in allergic, fibrotic, infectious, and chronic inflammatory diseases [26]. The knowledge of the role of IL-33 in periodontal diseases is relatively scarce, and the results are contradictory. In this context, Malcolm et al. demonstrated that the expression of IL-33 and ST2 was elevated in gingival tissues from patients with chronic periodontitis as compared with healthy tissues. Similarly, IL-33 expression was observed to be higher in periodontal tissues of *Porphyromonas gingivalis*–infected mice. IL-33 upregulated the expression of RANKL in such mice. In contrast, administration of OPG, by targeting RANKL, abrogated periodontal destruction [50]. Therefore, IL-33 could act as a proinflammatory cytokine with osteodestructive consequences. Similarly, Velickovic et al. showed higher expression of IL-33 and ST2 in periapical granulomas and radicular cysts when compared to healthy periapical tissues, suggesting that IL-33/ST2 signaling may be involved in periapical inflammation and tissue fibrosis [51]. Araujo-Pires et al. did not find any significant difference in the expression of IL-33 between active (RANKL>OPG) and inactive (RANKL<OPG) human periapical granulomas [52]. However, the opposite results were found in ST2 knockout mice in which deletion of ST2 signaling augmented the RANKL/OPG ratio in PLs. The number of $CD3^+$ RANKL+ cells along with TRAP+ osteoclasts was higher in PLs of $ST2^{-/-}$ mice compared with wild-type (WT) mice, whereas the percentages of $CD3^+$ OPG+ cells were higher in WT mice. In addition, ST2 deletion increases inflammatory bone loss, which was associated with enhanced Th1/Th17 cell-mediated periapical immune responses and increased osteoclastogenesis [53]. Based on all these results, our findings suggest that during exacerbation of inflammation in PLs, IL-33 could potentiate bone resorption through increased production of RANKL. However, during resolution of inflammation, low secretion of IL-33, through augmented production of OPG, could act as a bone-protective cytokine.

In conclusion, PL cells in culture produce a significant amount of both RANKL and OPG, so we propose this model as a useful alternative to study mechanisms involved in the pathogenesis of apical periodontitis. Some of them, which were examined in this work, suggest that both bone resorptive and bone-protective processes are present in all stages of PL development and are finely balanced by cytokines involved in PL pathogenesis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

Authors' Contributions

The following are contributions of the authors in this study: Miloš Duka—design of the study, participation in the clinical part of experiments, and drafting of the manuscript; Mile Eraković—participation in the clinical part of experiments and analysis of clinical data; Zana Dolićanin—participation in the immunological part of experiments and immunocytochemistry and analysis of data: Dara Stefanović—analysis of radiological parameters; Miodrag Čolić—design of the study, participation in the immunological part of experiments,

analysis of data, and manuscript final revision. All authors approved the final version of the manuscript for submission.

Acknowledgments

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Research Article

Apoptosis Transcriptional Profile Induced by *Porphyromonas gingivalis* HmuY

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This study aimed at evaluating the transcriptional profile of apoptosis-related genes after *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) derived from individuals with periodontitis (P) and healthy nonperiodontitis (NP) control subjects with *P. gingivalis* HmuY protein. PBMCs from the P and NP groups were stimulated with HmuY *P. gingivalis* protein, and the expression of genes related to apoptosis was assessed by custom real-time polymerase chain reaction array (Custom RT^2 PCR Array). Compared with the NP group, the P group showed low relative levels of apoptosis-related gene expression, downregulated for FAS, FAS ligand, TNFSF10 (TRAIL), BAK1, CASP9, and APAF1 after *P. gingivalis* HmuY protein stimulation. Furthermore, the P group exhibited low levels of relative gene expression, downregulated for CASP7 when the cells were not stimulated. Our data suggest that *P. gingivalis* HmuY protein might participate differently in the modulation of the intrinsic and extrinsic apoptosis pathways.

1. Introduction

Periodontitis is a multifactorial disease, with significant participation of the host, environmental factors, and bacterial components. It is known that keystone pathogens, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, in the subgingival biofilm elicit a host inflammatory response, which can lead to periodontal breakdown. [1]. The microbial diversity of the oral cavity is immense, and the host response during periodontitis is complex, with components of the innate and adaptive immune system that lead to chronic inflammation and bone resorption [2].

Recent metagenomic and mechanistic studies are consistent with a new periodontal pathogenesis model that proposes that periodontal diseases are caused by a synergistic and dysbiotic microbial community, not by a selected group of bacteria known as "periodontopathogens". Bacteria found in low abundance in the microbiota have an effect throughout the community and are critical components for the development of dysbiosis. They are known as "keystone" pathogens [3]. However, an increased abundance in these known pathogens is observed, related to the presence as well as the severity of the disease, indicating a microbial variation in the dysbiotic process [4].

Virulence factors of *P. gingivalis*, the main keystone pathogen in periodontitis, can determine a great immunogenicity to stimulate innate and adaptive immune host responses. Among them are capsule components, lipopolysaccharide (LPS), fimbriae, and outer membrane proteins,

such as associated and secreted gingipains. Gingipains specific for arginine (HRgpA and RgpB) and lysine (Kgp) play an important role in the infection, supporting the onset and progression of periodontitis [5].

P. gingivalis can invade the periodontal tissues and use a panel of virulence factors to evade the innate immune and inflammatory responses [6]. It has been shown that this pathogen has the ability to invade epithelial cells [7], which can be an escape mechanism from host defenses, favoring the penetration of the microorganism into the bloodstream and thus acting systemically in the human body [8].

P. gingivalis ability to obtain nutrients and evade the host immune response in the microenvironment is directly related to its survival, proliferation, and infection. One of the essential nutrients for its development is iron. This component is most abundant in the host in the form of heme [9]. The heme capture can be performed by a *P. gingivalis* HmuY, which sequesters and delivers heme to the outer membrane receptor HmuR [10–12].

There is a syntrophy among the species of the oral biofilm, revealing the interspecies cooperation in the acquisition of nutrients [13]. This mechanism reveals how HmuY works together with proteases produced also by other bacterial species to acquire heme from hemoglobin and may represent mutualism between *P. gingivalis* and *Prevotella intermedia* cohabiting the periodontal pocket [14]. However, HmuY is recognized by highly specific antibodies, suggesting that this protein can serve as a specific antigen of this bacterium [15].

P. gingivalis HmuY also seems to act in the programmed cell death process. Human peripheral blood mononuclear cells (PBMCs) cultured with the protein appear to be unable to complete the process of apoptosis, resulting in death characterized by the release of inflammatory cell content in the microenvironment, such as late apoptosis and necrosis, which can prolong the tissue destruction process [16]. This protein induces high levels of Bcl-2 in the mononuclear cells of individuals with periodontitis, resulting in the inhibition of apoptosis and thereafter enabling increased survival of $CD3^+$ T cells, which prolong the chronic inflammatory condition in periodontitis [17].

The modulation of cell death molecules and mechanisms evolved by periodontopathogens during the establishment of periodontitis has been demonstrated [18–21]. However, the apoptosis of mononuclear cells under bacterial challenge is unclear. The present study identifies specific *P. gingivalis* components that are involved in these events, which is the novelty of the study. In addition, the role of HmuY in triggering the extrinsic and intrinsic apoptosis pathways is not well understood. Thus, this study was carried out to evaluate the transcriptional profile of genes involved in apoptosis mechanisms in PBMC under *P. gingivalis* HmuY protein stimulation.

2. Materials and Methods

2.1. Participants. The present study was approved by the Feira de Santana State University Institutional Review Board (No. 0203.0.059.000-11). All volunteers in this study signed the free and informed consent form. A total of 8 individuals with severe periodontitis (P group) and 8 nonperiodontitis controls (NP group) were recruited between 2013 and 2014 from the College of Dentistry at the Feira de Santana State University, Bahia, Brazil. Volunteers with diabetes, pregnancy, smoking habit, any autoimmune disease, cardiovascular disease, prior periodontal treatment, and anti-inflammatories and antibiotics used within two and six months before inclusion in this study, respectively, were the exclusion criteria.

2.2. Disease Classification. A dentist (P.C.C.F.) was trained in the calibration process for periodontal examination (kappa interexaminer agreement value = 0.932) using a Williams periodontal probe (Hu-Friedy, Chicago, IL, USA). The following clinical parameters were evaluated: probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP).

Participants were diagnosed as having periodontitis (P group) if they had at least four teeth with at least one site with a probing depth greater than or equal to 4 mm, clinical attachment loss of 3 mm or more, and bleeding on probing at the same site [22]. All individuals with periodontitis had a diagnosis of severe form of the disease, since they had at least 2 interproximal sites with clinical attachment loss greater than or equal to 6 mm (not affecting the same tooth) and at least 1 interproximal site with probing depth greater than or equal to 5 mm [23].

Those participants who did not meet these criteria were considered not to have periodontitis (NP group).

2.3. Antigen. The *P. gingivalis* HmuY protein (NCBI ID number: CAM 31898) was overexpressed, purified, and lyophilized as described previously [24] and subsequently dissolved in PBS to a final concentration of 2.5 μg/mL.

2.4. Blood Collection and Cell Culture. Peripheral venous blood (20 mL) was collected from the volunteers in heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation in accordance with the manufacturer's guidelines (SepCell, StemCell Technologies Inc., USA) and then cultured in flat-bottom 24-well plates (10^6 cells/well) in RPMI (Roswell Park Memorial Institute) medium (LGCBio, São Paulo, SP, Brazil), containing 10% fetal calf serum (inactivated by heat) and 1% antibiotic/antimycotic drug (R&D Systems, Minneapolis, MN, USA). Cells (10^6 per well) were incubated with 5 μg/mL of pokeweed mitogen (PWM), 2.5 μg/mL of *P. gingivalis* HmuY protein, or in the absence of antigens (nonstimulated cells), under 5% CO_2 in humid conditions for 48 h at 37°C.

2.5. Gene Expression Analysis. RNA was extracted from the cells using a miRNeasy Mini Kit (SABiosciences Corp.) in accordance with the manufacturer's instructions. cDNA was synthesized using 500 ng of the total RNA and a RT^2 First Strand Kit (SABiosciences Corp.).

The Custom Human RT^2 Profiler PCR Array: CAPH12794 (SABiosciences Corp.), designed for this study, was used for the sample analysis. Altogether, 45 different genes were simultaneously amplified in the sample using

384-well plates. To verify the presence of a single amplicon, a melting curve was performed.

PCR arrays were carried out using Applied Biosystems QuantStudio 12K Flex Real-Time PCR System 384-well block (Applied Biosystems). 10 μL of a mixture composed of 2× SABiosciences RT^2 qPCR Master Mix and cDNA was applied into each well. The Ct values were calculated for each gene using the Applied Biosystems software. Baseline and threshold measurements were determined according to the manufacturer's guidelines (SABiosciences, Qiagen).

2.6. Statistical Analysis. The software supplied by Qiagen (https://www.qiagen.com/br/shop/genes-and-pathways/data-analysis-center-overview-page) identified glucuronidase beta (GUSB) as the most stably expressed housekeeping gene for data normalization. The fold change in gene expression (compared to positive control – PBMC – P group) was calculated using the ΔΔCt method. A more than twofold change in gene expression (compared to positive control – PBMC – P group) was considered as the up- or downregulation of a specific gene expression.

The RT^2 Profiler PCR Array Data Analysis software does not perform any statistical analysis beyond the calculation of p values using Student's t-test based on 2^(-ΔCT) values for each gene P group compared to the NP group. The Microarray Quality Control (MAQC) published results indicating that a ranked list of genes based on fold change and such a p value calculation was sufficient to demonstrate reproducible results across multiple microarray and PCR Arrays including the RT^2 Profiler PCR Arrays [25].

3. Results

The comparison between the periodontitis (P) and nonperiodontitis (NP) groups demonstrated that there were no statistically significant differences in the sex ($p = 0.96$) or age ($p = 0.57$) (Table 1). On the other hand, periodontal status was worse in the periodontitis individuals, who showed a higher percentage of teeth with bleeding on probing ($p = 0.03$), probing depth ≥ 4 mm ($p = 0.001$), and clinical attachment loss ≥ 3 mm ($p = 0.001$).

3.1. Expression Profile of Apoptosis Gene Targets: Heat Map. The analysis of the mRNA expression pattern indicated differences between the P and NP groups both in cells without stimulation (nonstimulated cells) and under *P. gingivalis* HmuY protein stimulus (HmuY). The variability in the pattern of global gene expression between the P and NP samples indicated heterogeneity, particularly in those stimulated with HmuY protein. Under both conditions of culturing, stimulation with HmuY (HmuY) and without antigenic stimulation (nonstimulated cells), PBMC of individuals of the NP group showed a higher number of genes upregulated, and on the other hand, individuals with periodontitis showed a downregulated gene expression profile. Furthermore, differences were more evident in the NP and P groups when stimulated with *P. gingivalis* HmuY protein (HmuY), as can be observed in Figure 1.

In nonstimulated cells, the expression of CASP7 and FASLG was upregulated in a higher number of individuals of the NP group (3 and 4 individuals, respectively). Patient NP1, without the diagnosis of periodontitis, showed upregulation in most of the genes examined. In the P group, the upregulation of CASP7, FAS, BAK, and CASP3 occurred in one case for each gene and in two cases for BCL2L1. Most of the genes evaluated were downregulated in the cells cultured without stimulation in this group.

BCL2L1 was upregulated in four out of eight individuals without periodontitis after HmuY stimulation, while only one individual showed upregulation when the cells were cultured without stimulus. Similarly, three individuals showed upregulation of TNFSF10, FAS, FASLG, CASP9, and APAF1 under HmuY challenge. In the P group, after stimulation with HmuY protein, the individual who showed upregulation in BID, BAX, CASP3, and BCL2L1 also showed indetermination in TNF, TNFSF10, FAS, FASLG, CASP9, and APAF1, differently from the cells grown without stimulus, which showed downregulation in the case of all these genes. The other individual who showed upregulation in BCL2L1 also demonstrated upregulation in TNF. The pattern of regulation changed from downregulation to indetermination in most of the cases.

3.2. Apoptosis-Related Gene Expression in the Extrinsic Pathway. PBMC from the P group showed downregulation and lower levels for FAS ligand compared to those from the NP group ($p = 0.01$), as observed in Figure 2, under stimulation with *P. gingivalis* HmuY protein. Under the same conditions, the downregulation of FAS and TNFSF10 in the P group, but not statistically significant ($p = 0.09$; $p = 0.06$, respectively) (Figures 2(d) and 2(f)), was observed. When PBMCs were cultured without stimulus, no statistically significant difference was found for FAS ($p = 0.31$), FAS ligand ($p = 0.43$), or TNFSF10 (TRAIL) ($p = 0.22$) expression between the P and NP groups (Figures 2(a)–2(c)).

3.3. Apoptosis-Related Gene Expression in the Intrinsic Pathway. The downregulation and lower levels of CASP9 ($p = 0.003$) and APAF1 ($p = 0.048$) in PBMC in the P group compared to PBMC from the NP group were observed, when the cells were cultured under stimulation of *P. gingivalis* HmuY protein (Figures 3(e) and 3(f)). Downregulation was found in the P group for the expression of BAK1, compared to the NP group, but this difference is not statistically significant ($p = 0.085$) (Figure 3(a)). There was no statistically significant difference between the P and NP groups in the BAK1 ($p = 0.35$), CASP9 ($p = 0.25$), and APAF1 ($p = 0.6$) expression for unstimulated PBMC (nonstimulated cells) (Figures 3(d)–3(f)).

3.4. Caspase 7 Gene Expression. PBMC from the P and NP groups stimulated with *P. gingivalis* HmuY protein (HmuY) did not demonstrate a statistically significant difference for caspase 7 ($p = 0.40$) (Figure 4(a)). However, there was downregulation and lower levels for caspase 7 in unstimulated cells from the P group in comparison to the NP group ($p = 0.05$) (Figure 4(b)).

Table 1: Clinical findings of patients with periodontitis (P) and nonperiodontitis (NP) subjects.

	NP	P	p value
Number of men/women	9/17	7/13	0.958
Age (years) (mean ± SD)	37.92 ± 10.8	41.95 ± 9.7	0.565
% Bleeding on probing (mean ± SD)	8.45 ± 10.7	37.97 ± 16.9	0.028
% Probing depth ≥ 4 (mean ± SD)	1.23 ± 1.84	14.93 ± 8.91	0.001
% Clinical attachment loss ≥ 3 (mean ± SD)	19.60 ± 15.53	57.04 ± 17.25	0.001

SD = standard deviation, p = significance level ($p \leq 0.05$).

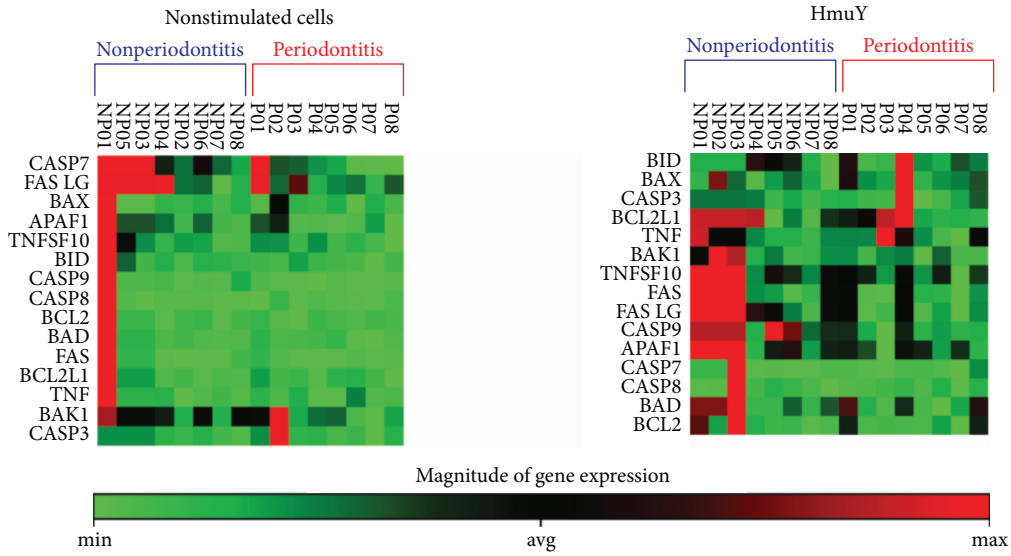

Figure 1: Gene cluster identification involved in apoptosis mechanisms by PBMC from individuals with periodontitis (P) and nonperiodontitis (NP) under *P. gingivalis* HmuY protein (HmuY) stimulus and in the absence of stimulus (nonstimulated cells). The green-black-red color gradient represents relative levels of gene expression, indicating under-even-over regulation, respectively.

4. Discussion

The most common outcome in inflammatory/infectious conditions is cell death by necrosis [26]. However, previous studies have demonstrated both induction and inhibition of apoptosis mechanisms in different cells types through the challenge of oral microorganisms and/or their components [21, 27–31]. It was demonstrated that the upregulation of apoptosis-related proteins occurred in gingival epithelial cells challenged with *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* [32]. In addition, *P. gingivalis* products seem to be able to induce the expression of genes related to apoptosis in osteogenic bone marrow stromal cells [33] and THP-1 cells [34]. The potential of *P. gingivalis* HmuY protein to interfere with cell death mechanisms in PBMC has been shown previously [16, 17].

In this study, the involvement of genes encoding apoptosis-related proteins that participate in the intrinsic and extrinsic pathways of cell death that were expressed differently in the PBMC of the P and NP groups was demonstrated. In the extrinsic pathway, HmuY was able to downregulate the expression of FASL in individuals with periodontitis. In the intrinsic pathway, *P. gingivalis* HmuY protein downregulated the caspase 9 and APAF1 expression in cells from diseased individuals. Thus, it seems that *P. gingivalis* uses this protein to suppress apoptosis of the defense host cells through both pathways, corroborating previous studies that proposed a role of delaying apoptosis [16] and increasing the production of BCL-2 [17] by HmuY.

Several studies have demonstrated many factors to suppress or inhibit apoptosis, showing the dependence of genes which encode pro- and antiapoptotic proteins in the balance of this process [35, 36]. In the presence of a bacterial challenge, it is possible to observe DNA damage associated with apoptosis and expression of p53, BCL-2 [37], FAS, FASL, and active caspase 3 in gingival tissues [38]. *P. gingivalis* can induce apoptosis in human gingival epithelial cells through the increase of FASL expression and the increase of the gene transcription mediated by NFκB [39]. The oligomerization of APAF1, induced by its binding to cytochrome c, forms an apoptosome, a known structure that recruits and activates a caspase initiator, caspase 9, which, in turn, cleaves and activates caspase effector caspase 3 and caspase 7, leading to apoptosis [40].

Regarding FAS, TNFSF10 (extrinsic pathway), and BAK1 (intrinsic pathway), consistently with the results shown above, the downregulation of mRNA expression was observed in cells from individuals with periodontitis

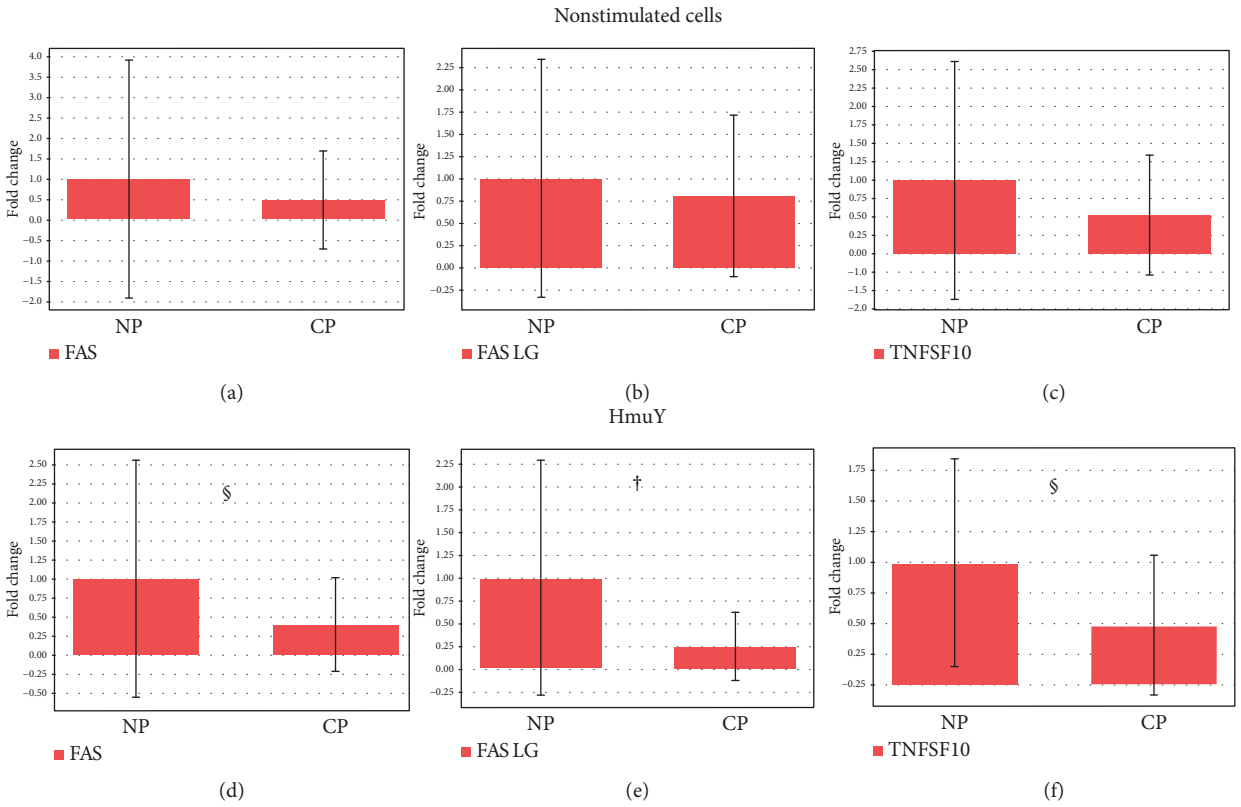

Figure 2: Apoptosis-related extrinsic pathway gene expression in the PBMC of the (a-c) P and NP groups without stimuli and under (d-f) *P. gingivalis* HmuY protein stimulation. (a, d) FAS (TNF receptor superfamily, member 6); (b, e) FAS LG (TNF superfamily, member 6 ligand); (c, f) TNFSF10 (tumor necrosis factor ligand superfamily-10). $^{\dagger}p = 0.01$ and $^{\S}p < 0.1$.

under HmuY stimulus. However, the differences found between this group and individuals without the disease showed borderline p values. Therefore, these results must be confirmed in studies using a larger sample size to improve the statistical power. These findings showed that *P. gingivalis* HmuY protein may impair or retard the process of apoptosis by downregulation of genes that initiate the extrinsic and intrinsic signaling pathways in periodontitis. It is known that in the extrinsic pathway, apoptosis can be induced by cell surface receptors, such as FAS, TNFR1, and TNFSF10 [41–43], while intrinsic apoptotic stimuli can activate BAX and BAK and, consequently, cause mitochondrial outer membrane permeabilization [40].

In a previous study, using *P. gingivalis* lipopolysaccharide, the upregulation of mRNA for IL-1β and FAS ligand was identified in a mouse periodontitis model [44]. Furthermore, the expression of mRNA for FAS and FAS-L in human gingival epithelial cells (CEGH) was upregulated by heat-killed *P. gingivalis*, and programmed cell death was induced [39].

In the assessment of caspase 7, downregulation in the mRNA expression in cells from periodontitis individuals cultured without stimulus in comparison to cells from control individuals has been demonstrated. This difference was not observed when the cells were cultured in the presence of *P. gingivalis* HmuY protein. Caspase 7 has been found in inflammatory conditions induced by bacterial infections to be involved as an effector molecule in apoptosis responsible for inducing cell detachment and ROS production in a redundant way with caspase 3 [45, 46].

Thereby, the negative modulation through the inhibition of the apoptotic process can enable *P. gingivalis* to infect host cells and provide an escape mechanism from the host immune system. These findings suggest that this keystone pathogen can reduce or inhibit PBMC apoptosis through downregulation of mRNA genes which are involved in the intrinsic and extrinsic pathways of early proapoptotic signaling in programmed cell death under *P. gingivalis* HmuY protein stimulation in individuals with periodontitis. This process could lead to continuous production of proinflammatory cytokines and chemokines in the microenvironment of the inflamed periodontal tissue, improving survival and chemotaxis of cells involved in immune defense mechanisms. It can result in a chronic inflammatory state of destruction of periodontal tissues and possibly in the establishment of a dysbiotic process in affected individuals.

We are aware that a limitation of the present study is the relatively small sample size, which suggests the need for further studies with larger numbers of participants to establish the role of the *P. gingivalis* HmuY protein in inflammatory and molecular mechanisms of cell death, providing new insights into the immunopathogenesis of periodontitis. In addition, the experiments were carried out using PBMCs and cannot be extrapolated to the microenvironment of

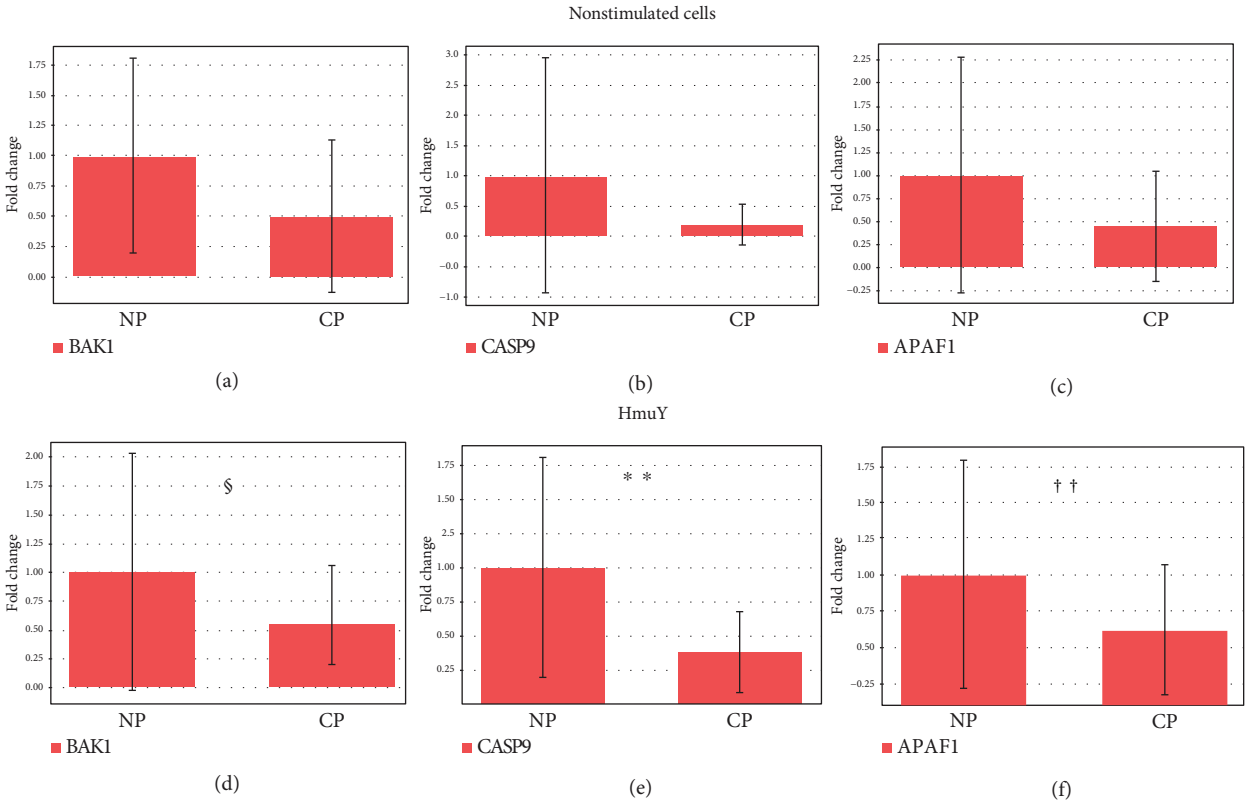

Figure 3: Apoptosis-related intrinsic pathway gene expression in the PBMC of the (a-c) P and NP groups without stimuli and under (d-f) *P. gingivalis* HmuY protein stimulation. (a, d) BAK1 (BCL2-antagonist/killer 1); (b, e) CASP9 (caspase 9); (c, f) APAF1 (apoptotic peptidase activating factor-1). $^{**}p = 0.01$, $^{\dagger\dagger}p = 0.05$, and $^{\S}p < 0.1$.

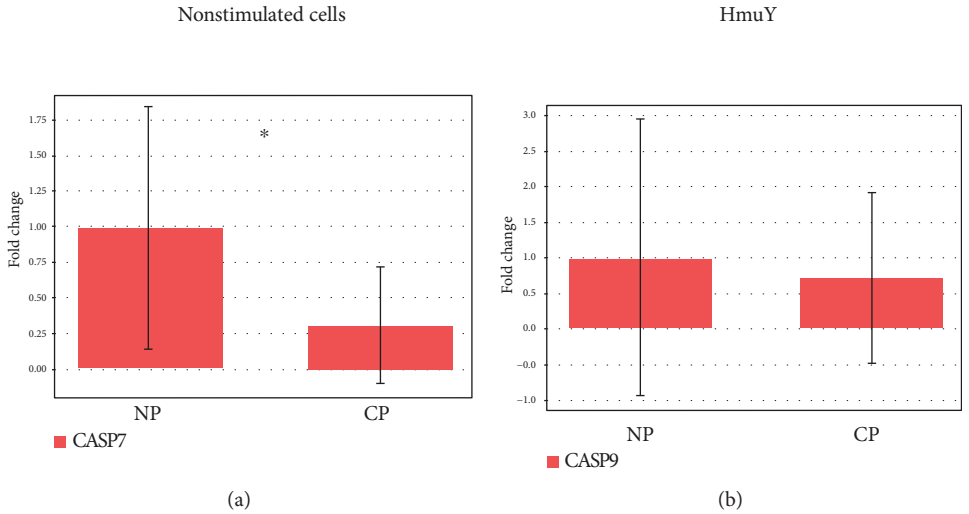

Figure 4: Caspase 7 expression in the PBMC of the (a) P and NP groups without stimulation (nonstimulated cells) and under (b) *P. gingivalis* HmuY protein stimulation. Caspase 7 (apoptosis-related cysteine peptidase). $^{*}p = 0.05$.

the periodontal lesion, in which resident cells play a major role in the pathogenesis of the disease *in vivo*.

In conclusion, *P. gingivalis* HmuY protein might contribute to the survival of PBMC in periodontitis by regulating the transcriptional profile of genes involved in apoptosis, such as FASL, caspase 9, APAF1, FAS, TNFSF10 (TRAIL), and BAK1. It can lead to the perpetuation and aggravation of the inflammatory condition of the periodontal structures through the release of more inflammatory mediators which contribute to the breakdown of tissues.

Data Availability

The data underlying the findings of this study are available in the online repository: https://repositorio.ufba.br/ri/bitstream/ri/21723/1/TESE%20PAULO%20CIRINO.pdf [47].

Conflicts of Interest

The authors have declared no competing interests.

Acknowledgments

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Review Article

Contribution of Statins towards Periodontal Treatment: A Review

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The pleiotropic effects of statins have been evaluated to assess their potential benefit in the treatment of various inflammatory and immune-mediated diseases including periodontitis. Herein, the adjunctive use of statins in periodontal therapy *in vitro*, *in vivo*, and in clinical trials was reviewed. Statins act through several pathways to modulate inflammation, immune response, bone metabolism, and bacterial clearance. They control periodontal inflammation through inhibition of proinflammatory cytokines and promotion of anti-inflammatory and/or proresolution molecule release, mainly, through the ERK, MAPK, PI3-Akt, and NF-κB pathways. Moreover, they are able to modulate the host response activated by bacterial challenge, to prevent inflammation-mediated bone resorption and to promote bone formation. Furthermore, they reduce bacterial growth, disrupt bacterial membrane stability, and increase bacterial clearance, thus averting the exacerbation of infection. Local statin delivery as adjunct to both nonsurgical and surgical periodontal therapies results in better periodontal treatment outcomes compared to systemic delivery. Moreover, combination of statin therapy with other regenerative agents improves periodontal healing response. Therefore, statins could be proposed as a potential adjuvant to periodontal therapy. However, optimization of the combination of their dose, type, and carrier could be instrumental in achieving the best treatment response.

1. Introduction

Periodontitis is an inflammatory disease of infectious origin characterized by progressive destruction of periodontal soft and hard tissues leading to tooth loss. The main symptoms comprise gingival inflammation, formation of periodontal pocket, alveolar bone loss, abscess, or tooth mobility [1]. The pathogenesis of periodontitis involves a complex interaction of immune and inflammatory cascades initiated by bacteria of the oral biofilm [2]. Persistent inflammation and dysbiosis worsen periodontal tissue damage, and the host response plays a vital role in this phenomenon contributing to tissue destruction [3].

The conventional treatment comprising scaling and root planing (SRP) presents limitations in certain cases involving deep periodontal pockets, inaccessible areas, or severe periodontitis [4]. Therefore, several adjunctive pharmacological therapeutics have been tested to improve its outcomes. In this context, systemic and local deliveries of drugs such as antibiotics, bisphosphonates, anti-inflammatory drugs, anticytokines, probiotics, and prebiotics have been tested so far to reduce bacterial load and to control inflammation [5–9]. Likewise, the use of statins in periodontal treatment has been explored recently [10]. Statins, or inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), are a group of drugs, used primarily to treat hyperlipidemia and to prevent cardiovascular diseases [11]. After their discovery in the 70s, they have been widely prescribed worldwide [12]. They differ mainly in their ring structure, and these structural differences modify their pharmacological properties including hydrophilicity and lipophilicity. The lactone ring is present in an active form (already hydrolyzed)

Table 1: Physical properties of different types of statins.

Drug	Source	Solubility	Molecular mass (Da)
Atorvastatin	Synthetic	Lipophilic	1209.42
Simvastatin	Natural	Lipophilic	418.6
Lovastatin	Natural	Lipophilic	404.5
Mevastatin	Natural	Lipophilic	390.52
Pravastatin	Natural	Hydrophilic	446.52
Fluvastatin	Synthetic	Lipophilic	411.47
Cerivastatin	Synthetic	Lipophilic	459.56
Pitavastatin	Synthetic	Lipophilic	421.46
Rosuvastatin	Synthetic	Hydrophilic	481.54

in all statins except for simvastatin, lovastatin, and mevastatin, in which the lactone ring is activated (hydrolyzed) in the liver. The lactone form of the statins enables their transport, metabolism, and clearance [13] (Table 1).

Apart from their lipid-lowering properties, statins possess pleiotropic effects due to their anti-inflammatory, antioxidative, antibacterial, and immunomodulatory properties [14–17]. Statins have also been reported to have anabolic effects on the bone by augmenting bone morphogenetic protein-2 (BMP-2) expression, thus contributing towards the differentiation and activity of osteoblasts (OBs) [18]. In view of their beneficial properties, statins have been presented as new potential candidates for improving periodontal therapy outcomes [19, 20].

In several preclinical and clinical studies, statins have exhibited contradictory results [21–23] depending on the mode of delivery (local vs systemic), anatomy and severity of the lesions, type of disease, and treatment approach (nonsurgical vs surgical). Therefore, the aim of this literature review was to establish a better understanding of the prophylactic and therapeutic effects of all statin types administered locally or systemically as adjuvant to nonsurgical/surgical periodontal treatment in existing preclinical models and clinical settings and to explore the biological mechanisms underlying these healing and proregenerative effects in the management of periodontitis.

2. Methods

2.1. Literature Search. Studies published in English language only were included, and the last search was carried out in September 2018. Regarding studies performed on animal models and clinical trials, a systematic literature search was performed in the PubMed/MEDLINE and ScienceDirect databases. A hand search has also been performed after checking references of the identified articles. Concerning *in vivo* studies, the following keywords were used for the search: periodontitis OR periodontal disease OR alveolar bone loss OR periodontal attachment loss OR periodontal pocket AND simvastatin OR statin OR rosuvastatin OR atorvastatin OR cerivastatin OR mevastatin OR lovastatin OR pravastatin OR Fluvastatin OR pitavastatin OR Hydroxymethylglutaryl-CoA Reductase Inhibitors AND mouse OR dog OR pig OR rat OR rodent OR rabbit OR monkey OR in vivo. A study was considered eligible if it met the following criteria: (1) experimentally induced periodontitis (EIP) and/or acute/chronic periodontal defects (ACP), (2) treatment of EIP and/or ACP with statins (local or systemic or combination) with or without SRP or other periodontal treatment modalities, and (3) at least one periodontal parameter assessed as outcome. Exclusion criteria for *in vivo* studies were the following: (1) periapical lesions, (2) tooth extraction models, (3) orthodontic movements, (4) calvarial models, (5) long bone defects, and (6) drug-induced gingival enlargement.

Concerning clinical studies, the following keywords were used for the search: periodontitis OR periodontal disease OR alveolar bone loss OR periodontal attachment loss OR periodontal pocket AND simvastatin OR statin OR rosuvastatin OR atorvastatin OR cerivastatin OR mevastatin OR lovastatin OR pravastatin OR Fluvastatin OR pitavastatin OR Hydroxymethylglutaryl-CoA Reductase Inhibitors. A study was considered eligible if it met the following criteria: (1) randomized and controlled clinical trials, (2) cohort clinical studies, (3) longitudinal studies, (4) patients with diagnosis of chronic or aggressive periodontitis, (5) systemic or local administration of statins with nonsurgical or surgical periodontal treatment, and (6) at least one periodontal parameter: pocket depth (PD), clinical attachment level (CAL), bone loss (BL), or tooth loss (TL) assessed as outcome. Exclusion criteria for clinical studies were the following: (1) no follow-up, (2) no periodontal treatment, and (3) reviews, letters, and case reports.

2.2. Study Selection. Titles and abstracts of the studies were screened independently by two reviewers (CP and FB) and categorized as suitable or not for inclusion. Full reports were reviewed independently for studies appearing to meet the inclusion criteria or for which there was insufficient information in the title and abstract to allow a clear decision. Disagreements between the authors were resolved after discussion with a third reviewer (OH).

2.3. Risk of Bias Assessment. Risk of bias was assessed using the Cochrane Collaboration's tool for assessing risk of bias which provided guidelines for the following parameters: sequence generation, allocation concealment method, blinding of the examiner, address of incomplete outcome data, and free of selective outcome reporting. The degree of bias was categorized as follows: low risk if all the criteria were met, moderate risk when only one criterion was missing, and high risk if two or more criteria were missing. Two reviewers (FB and CP) independently performed the quality assessment, and any disagreement was resolved by a third investigator (OH) (Supplemental Table 1).

3. Results

3.1. Effect of Statins on the Inflammatory-Immune Crosstalk. Localization of *periodontium* at the interface between the teeth and jaws exposes periodontal tissues to continuous bacterial challenge which could contribute to exacerbation of the immune response during periodontal

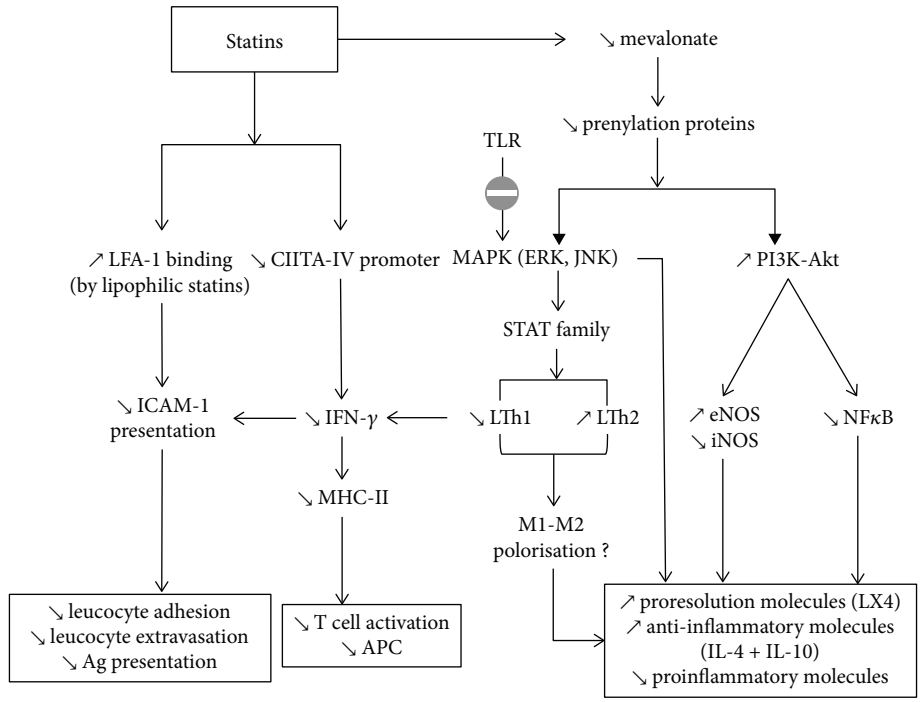

Figure 1: Effect of statins on the inflammatory-immune crosstalk. Direct LFA1 site binding by lipophilic statins decreases ICAM-1 presentation leading to reduced leukocyte chemotaxis and antigen presentation. Statins inhibit MHC-II induction by IFN-γ leading to decreased T-cell activation. Statins lower mevalonate release, leading to resolution of inflammation via the ERK, MAPK, and PI3K-Akt pathways.

wound healing. Recruitment of inflammatory cells at the periodontal site, including polymorphonuclear (PMN) leukocytes, macrophages, and lymphocytes, is associated to the release of a complex nexus of cytokines. When the inflammatory front migrates toward the alveolar bone, it stimulates osteoclastogenesis and subsequent alveolar bone destruction [24]. Therefore, the importance of inflammation control at the soft tissue level cannot be undermined.

The effects of statins on the inflammatory-immune crosstalk involved in the periodontal wound healing have been evaluated. Statins decrease the levels of proinflammatory cytokines (interleukin-1 beta (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α)) and increase the release of anti-inflammatory mediators (IL-10) and chemokines [25, 26]. There are several pathways implicated in the action of statins, notably suppression of HMG-CoA reductase, thereby inhibiting Rac and p21Ras phosphorylation. As Rac and p21Ras are coupled to the transcription of proinflammatory molecules via MAP kinase (MAPK) pathways, therefore, statins also suppress nuclear factor kappa B (NF-κB) activation, thus reducing the expression of proinflammatory molecules [27] (Figure 1).

3.1.1. Effect of Statins on Inflammatory Molecules. In vitro, the effect of statins on inflammatory mediators' secretion was demonstrated to be cell specific. For instance, in human oral epithelial cells [15] and OBs [28], statins reduced IL-6, IL-8 release, whereas, in T-cells [29, 30], statins increased the expression of IL-4, IL-5, IL-10 and IL-13. *In vivo*, statins confirmed the reduction of cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), IL-1β, IL-6, IL-8, TNF-α, interferon-gamma (IFN-γ), C-reactive protein (CRP), colony-stimulating factors (CSF2, CSF3), recruitment of mononuclear inflammatory cells, and several Toll-like receptors (TLRs) in various EIP or ACP models [26, 31–35]. Clinical trials also corroborated the downregulation of inflammation by the use of statins, as demonstrated by increased IL-10 level in gingival crevicular fluid (GCF) from hyperlipidemic patients treated with statins [19].

3.1.2. Effect of Statins on Proresolution Molecules. Periodontal wound healing and regeneration involve a constant "tug-of-war" between the proinflammatory and anti-inflammatory/proresolution mediators [36, 37]. Anti-inflammatory effects of statins enhancing resolution of periodontal inflammation, that is, initiated by several endogenous chemical and lipid mediators, such as the lipoxins (LXs), resolvins (RVs), protectins, and maresins, could possibly explain the positive treatment outcomes [38, 39]. However, further studies need to explore the exact effect of statins on the proresolution mediators.

3.1.3. Effect of Statins on Host Modulation. Literature reports contradictory results regarding the effect of statins on different types of immune cells. For instance, in an ACP model, simvastatin did not change circulating white blood cell (WBC) counts in a study [33], whereas leukocyte infiltration was decreased by atorvastatin gavage in an EIP model [40]. Similarly, regulatory T (Treg) cells that control adaptive immunity against pathogens and activate other effector

immune cells were reported to be regulated by statins. In this regard, atorvastatin and simvastatin demonstrated an increase in the number of human Treg cells and differentiation of CD4 into Treg *in vitro* [41, 42].

Furthermore, TLRs have an important role in the immune-inflammatory crosstalk with a consequent impact on periodontal wound healing response. In the context of periodontal treatment, targeting TLRs has been proposed as it could enhance antimicrobial properties, suppress adverse inflammation, or activate tissue repair [43]. Interestingly, simvastatin inhibited the stimulation of several TLRs (1, 2, 3, 4, 6, 7, and 9) by *Aggregatibacter actinomycetemcomitans* (*A.a*) LPS *in vivo*, reducing its capability to escape innate immune response [33]. Hence, statins play an instrumental role in the modulation of inflammatory and immune responses.

3.1.4. Inhibition of Major Histocompatibility Complex Class II (MHC-II) by Statins. In case of nonresolving periodontal lesions, bacterial antigens are processed and presented by antigen-presenting cells and macrophages. Such process is associated to massive immune cell recruitment implicated in tissular destruction [2]. In this regard, statins are able to inhibit MHC-II expression due to inhibition of the inducible promoter IV of the class II transactivator (CIITA) as observed in several cell types, including monocytes and macrophages [44]. This effect renders statins to have a potential host-modulating impact on periodontal treatment.

3.1.5. Lymphocyte Function-Associated Antigen-1 LFA1 Site Binding by Statins. Lymphocyte function-associated antigen-1 (LFA-1), an integrin with its main ligand intercellular adhesion molecule-1 (ICAM-1), is activated on the surface of fibroblasts (FBs) by IFN-γ and represents a critical phase in the early stage of inflammation. ICAM-1 regulates LFA-1-dependent neutrophil transmigration and recruitment to the inflammation site [45]. Several studies have demonstrated the inhibition of LFA-1 by statins in many inflammatory and immune diseases other than periodontitis. Statins inhibit ICAM-1 upregulation and chemotaxis of monocytes [46]. Lovastatin, simvastatin, and mevastatin, but not pravastatin, were able to inhibit the LFA-1/ICAM-1 interaction *in vitro* by binding to the L-site of LFA-1 [47]. In this way, statins limit the exacerbation of immune-mediated inflammatory response at the lesion site. However, the impact of statins on LFA-1 binding in the context of periodontal wound healing remains unexplored.

3.1.6. Effect of Statins on Nitric Oxide Synthase (NOS). NOS plays an important role in host defence and homeostasis and has been implicated in the pathogenesis of periodontitis, where it is expressed in FBs, epithelial cells, rests of Malassez, macrophages, osteoclasts (OC), and vascular endothelial cells [48, 49]. In chronic periodontitis, bacterial challenge induces proinflammatory cytokine release and a higher expression of inducible NOS (iNOS) and NOS derived from FBs and WBCs that migrate to the periodontal lesion [50–52] leading to inflammation-mediated bone resorption [53]. Various studies demonstrated a NOS-inhibiting effect by the use of statins. For instance, *in vivo*, rosuvastatin significantly reduced inflammation-mediated tissue destruction and gingival iNOS expression [54].

Concerning the underlying mechanism of action, statins attenuate the production of reactive oxygen species (ROS) induced by NADPH oxidase by suppressing Rac's geranylation. Phosphatidylinositol-3 active kinase (PI3-Akt) is a kinase that phosphorylates and stimulates eNOS. Mevalonate is able to inhibit PI3-Akt; therefore, by reducing the concentration of mevalonate, statins upregulate eNOS-derived NO production resulting in vasorelaxation that leads to improved angiogenesis and wound healing response [27].

3.1.7. Effect of Statins on Matrix Metalloproteinases (MMPs). MMPs degrade extracellular matrix proteins, especially collagen, contributing to the degradation of periodontal tissue including alveolar bone [55]. Most statins have been reported to potently inhibit the expression of MMP-1, MMP-8, and MMP-9 upregulated by LPS as demonstrated for simvastatin in mononuclear cells *in vitro* [56]. Moreover, *in vivo*, a decrease of MMP-1, MMP-2, MMP-8, and MMP-9 was observed by the use of statins [31, 57–59]. Thus, statins prevent periodontal tissue and alveolar bone destruction by inhibiting the release of MMPs.

3.2. Effect of Statins on Bone Metabolism. Statins have an impact on bone metabolism through increase of osteogenesis, decrease of OB apoptosis, and osteoclastogenesis [60]. Statins allow periodontal regeneration via the Ras/Smad/extracellular signal-regulated kinase (Erk)/BMP-2 pathway that enhances bone formation [61] and by antagonizing TNF-α through Ras/Rho/mitogen-activated protein kinase (MAPK) that causes osteoclastic differentiation [62]. Moreover, they significantly increase OB differentiation factors such as alkaline phosphatase (ALP), osteocalcin (OCN), bone sialoprotein (BSP), BMP-2 [63], osteopontin (OPN), and vascular endothelial growth factor (VEGF) [64] (Figure 2).

3.2.1. Role of Statins in the Promotion of Osteogenesis. Inhibition of HMG-CoA by statins decreases prenylation of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GPP) leading to increased levels of BMP-2 and VEGF through the PI3-Akt pathway. Interestingly, both VEGF and BMP-2 regulate OB differentiation and bone formation during bone repair and regeneration [65, 66]. Concerning BMP, simvastatin and lovastatin increased the levels of BMP-2, consequently, increasing OB activity *in vitro* [58, 63]. Statins present a cost-effective option when compared with growth factors such as BMP-2 [67, 68].

Hydrophobic statins (simvastatin, atorvastatin, and cerivastatin) also increased mRNA expression of VEGF in OBs [69]. Likewise, simvastatin increased osteoprotegerin (OPG) expression in periodontal tissue [58] and enhanced matrix calcification in human bone marrow stem cells by diminishing the mean size of the fibroblastic colony-forming units (CFU-Fs) [70]. *In vivo*, statins stimulated bone growth and repair by increasing angiogenesis [71]. In particular, the lactone-form statins (lovastatin and simvastatin) stimulated OB differentiation of mouse periodontal ligament cells

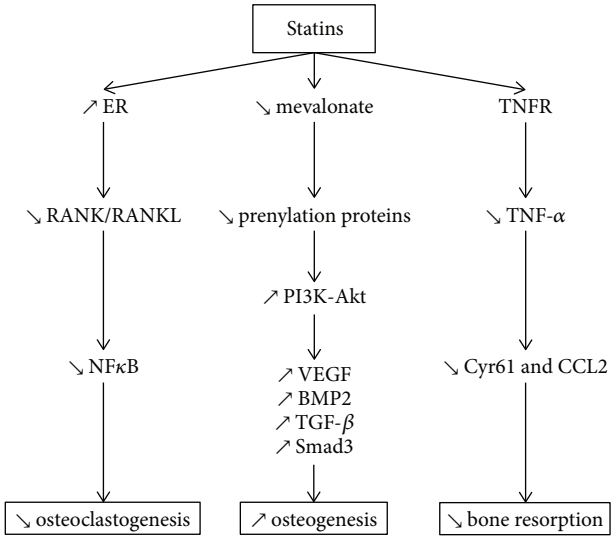

Figure 2: Effects of statins on several pathways involved in bone metabolism. Statins decrease osteoclastogenesis via RANK/RANKL and NF-κB signaling. Statins promote osteogenesis by increasing VEGF, BMP2, and TGF-β expression through the PI3-Akt pathway. Statins prevent inflammation-mediated bone resorption by decreasing TNF-α, via TNFR.

(PDLs) via the ERK1/2 pathway (phosphorylation) and enhanced intercellular matrix mineralization [63].

3.2.2. Role of Statins in the Inhibition of Bone Destruction. Statins act through certain pathways that avert bone degradation. Several clinical trials confirm the reduction of alveolar bone loss by statins, as an adjunct to SRP [72]. Many studies reported significantly decreased bone resorption by the use of simvastatin, rosuvastatin, and atorvastatin [26, 28, 32, 73]. Interestingly, simvastatin reduced TNF-α-induced synthesis of Cysteine-rich 61 (Cyr61) and chemokine ligand 2 (CCL2) [74] that are potential osteolytic mediators in inflammatory bone diseases, in human OB, thereby decreasing bone loss. Besides, statins increase bone formation by inhibiting OB apoptosis, augmenting TGF-β against the Smad3 signaling pathway. As an evidence, pitavastatin, mevastatin, and simvastatin induced the expression of Smad3 in nontransformed OBs (MC3T3-E1) [75]. Consequently, statins prevent bone destruction and also promote bone healing and regeneration.

3.2.3. Role of Statins in the Inhibition of Osteoclastogenesis. Statins suppress osteoclastogenesis through the OPG/receptor activator of the nuclear factor kappa-B ligand (RANKL)/-RANK signaling pathway. Statins (simvastatin, atorvastatin, and fluvastatin) inhibited, *in vitro* and *in vivo*, the expression of the receptor activator of RANK which along with RANKL is required for the differentiation of OC precursors [26, 31, 33, 58, 76]. Nevertheless, IL-10 is also implicated in inhibiting bone resorption by preventing the RANK/RANKL pathway ([77]); hence, statins could potentially reduce the inflammation-mediated bone resorption [25]. Another mechanism for osteoclastogenesis involving unprenylated Rap GTP-binding protein 1A (Rap-1A), a RAS super family of small GTP-binding protein member, has been studied in the context of statins. Rosuvastatin, pravastatin, cerivastatin, and simvastatin caused accumulation of unprenylated Rap-1A in rabbit osteoclast-like cells and macrophages, inhibiting osteoclast-mediated resorption. Interestingly, hydrophilic statin (cerivastatin) was more effective than hydrophobic statin (rosuvastatin) to inhibit OC prenylation [78]. Additionally, the mRNA expression of cathepsin K, a key marker of OC differentiation, is reduced by simvastatin through inhibition of Src signaling and modulation of MAPK including ERK1/ERK2. Moreover, upregulation of AKT leads to a decrease of OC activity via RANKL and BMP-2 [79].

3.3. Antibacterial Effect of Statins. Periodontitis is a polymicrobial disease involving keystone pathogen such as *Porphyromonas gingivalis* (*P.g*) that is able to hijack the adaptive immune response. Therefore, elimination of the periodontal pathogens is the cornerstone of periodontal treatment. Uncontrolled infection hinders periodontal wound healing and may worsen the therapeutic outcome by reducing the clinical attachment gain. Statins exhibit antimicrobial effects attributed to an increased bacterial clearance from the infection site as demonstrated in a model of sepsis (Figure 3) ([80]). Hence, statins could provide an additional benefit during periodontal wound healing (Table 2).

Cholesterol is an integral component needed by bacteria for maintaining their membrane integrity. Statins can counter bacteria by inhibiting the intermediate in the isoprenoid biosynthesis pathway necessary for membrane stability, which is substituted by cholesterol and protects bacteria from the toxic effect of statins. Statins, therefore, kill bacteria directly and by lowering accessible host cholesterol content for bacterial growth and protection. Such effects may be due to the disruption of teichoic acid structures reducing biofilm formation ([81]). Statins display antibacterial activity towards anaerobic bacteria, including periodontal pathogens such as *A.a* and *P.g*. For instance, low concentration of simvastatin was proven to be effective against *A.a* and *P.g* even if *A.a* was more sensitive (MIC $< 1\,\mu$g/mL) than *P.g* (MIC until $2\,\mu$g/mL dilution) [82]. The hydrophobic nature of simvastatin may explain its antibacterial activity against periodontal pathogens where it disrupts the bacterial membrane in a "soap-like" manner causing its death [83]. Nevertheless, not all statins exhibit antibacterial activity. The degree of HMG-CoA reductase inhibition corresponds directly to the cholesterol-lowering capabilities of statins [84] but it does not seem commensurate with their antibacterial potency [85].

Some other mechanisms are modulated by the action of statins on lipoxin A4 (LXA4) production, a proresolving lipid mediator that enhances bacterial clearance, consequently reducing the severity of periodontal disease [86, 87]. Furthermore, the mechanistic target of rapamycin (mTOR) signaling, regulated principally by TLRs via two major pathways (NF-κB-dependent pathway and a PI3-Akt-dependent pathway), is also involved in bacterial clearance [88]. It is known that statins inhibit isoprenoid synthesis, impeding intracellular signaling molecules like Rho or Rac [89].

Therefore, it is plausible that statins possess certain antibacterial properties that could facilitate periodontal

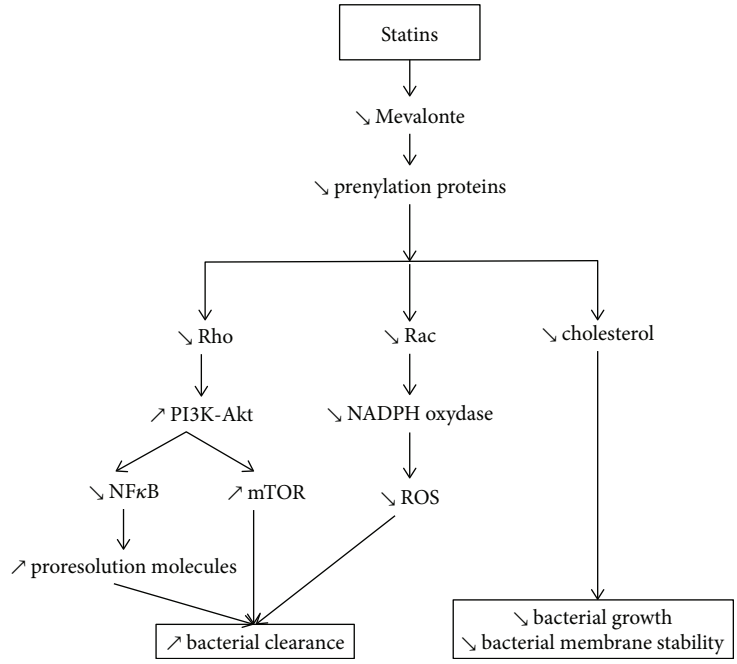

Figure 3: Antibacterial effect of statins. Statins arrest bacterial growth and disrupt their membrane stability by decreasing cholesterol. Statins increase bacterial clearance by decreasing NF-κB and ROS signaling (via the PI3K-Akt and NADPH oxidase pathways, respectively) and by enhancing proresolution molecule release.

Table 2: Representative *in vitro* studies evaluating the impact of statins on periodontal pathogens.

Reference	Experimental design	Local drug delivery Type of statin dose	Results	Periodontal consideration
[82]	MIC was determined against *P.g* (ATCC 33277) and *A.a* (ATCC 25586) using serial dilution method	Simvastatin, 1 μg/mL to 500 μg/mL	↘ *P.g* ↘ *A.a*	Simvastatin had an antibacterial effect against the keystone pathogens involved in periodontal disease
[138]	*A.a* (ATCC 43719), *P. nigrescens* (ATCC 33563), or *P.g* (ATCC 33277) were cultured on a trilayer functional CS membrane with EGCG and lovastatin	Lovastatin 0.1, 0.5, 1, and 2 mg	↘ *P.g* ↘ *A.a*	Lovastatin had an antibacterial effect against periodontopathogenic bacteria

treatment. However, since periodontitis is a polymicrobial disease, the susceptibility of various other periodontal pathogens to statins must also be evaluated.

3.4. Effects of Statins in Induced Periodontitis Models. Statins have been tested in several induced periodontitis models to evaluate improvement in periodontal parameters and their underlying biological mechanisms. *In vivo*, 35 studies were identified based on the inclusion criteria (Figure 4), out of which 16 involved local statin delivery (Table 3), 17 used systemic route (Table 4), and 2 employed a combination of both modes (Table 5). In the studies evaluating local statin application, 8 studies involved the treatment of EIPs while the remaining 8 investigated the treatment of ACP models, one of which was induced by LPS injection of *Escherichia coli* (*E. coli*) [90]. Concerning the systemic administration of statins (Table 4), 14 out of the total 17 studies treated EIPs, whereas the 3 remaining studies involved ACP models by LPS injections of *A.a* [32, 33] and *P.g* into the gingiva [76].

Regarding the mode of periodontitis induction, in total, 24 out of 35 studies had EIP with ligatures (cotton, nylon, or silk), whereas 11 used ACP including the 4 studies where periodontitis was induced by bacterial LPS. Studies were mostly performed in rodents (Tables 3, 4, and 5). In ACP models, the surgically created lesions were mainly intrabony defects, fenestration defects, dehiscence defects, furcation class II defects, and 3-walled intrabony defects.

In 6 studies, animals with systemic diseases (i.e, osteoporosis [26, 91, 92], metabolic syndrome [32], cyclosporine A-associated alveolar bone loss [35], hyperlipidemia [54], or hypertension [93] were used to evaluate the effect of statins treatment. Overall, 22 studies involved treatment with simvastatin, 7 with atorvastatin, 3 with rosuvastatin, 2 with lovastatin, and only one with fluvastatin. Some studies investigated more than one type of statin. *In vivo*, the systemic

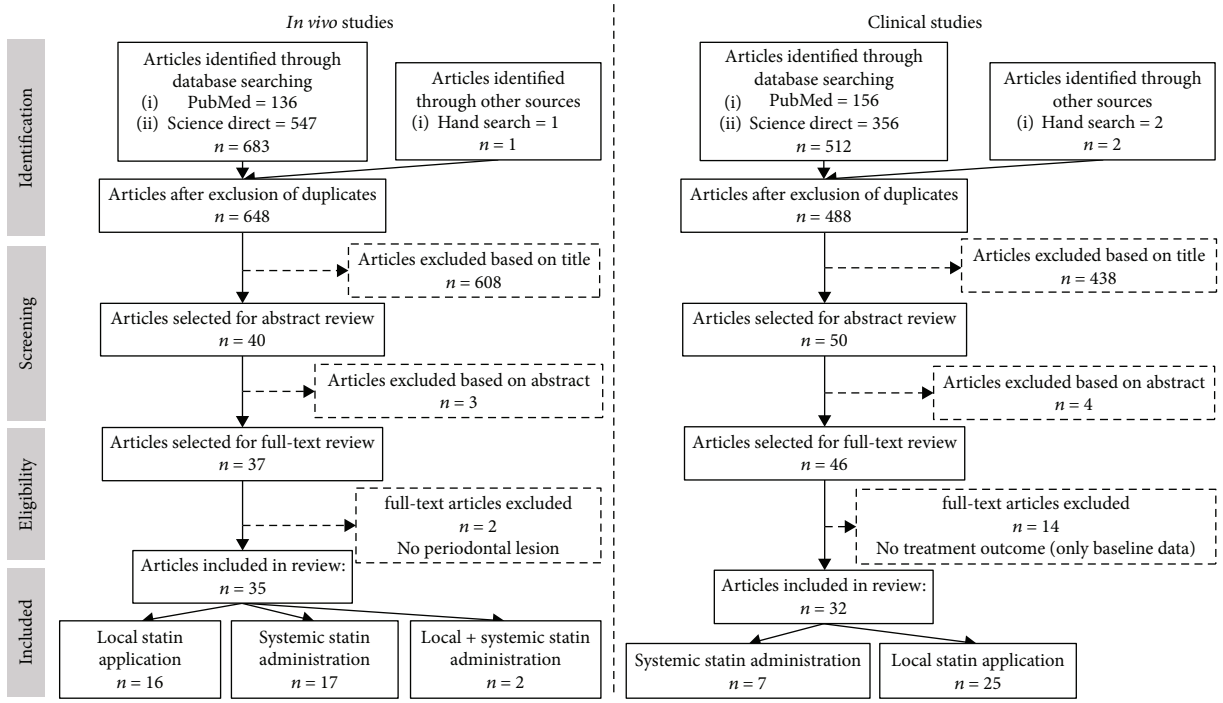

Figure 4: Selection of the studies.

dosage used ranged from 0.3 to 30 mg/kg with 20 mg/kg as the most commonly tested dose. The dose of locally delivered statins varied with the type of carrier/scaffold used (Table 3). Five studies demonstrated insignificant improvements [94–98]. Interestingly, 3 of them involved surgical treatment of ACP models by local statin application [94, 96, 98] and one study employed nonsurgical local statin therapy [95], whereas only one EIP was treated with systemic statin delivery [97]. One study even demonstrated a negative impact of statin use [99].

3.5. Clinical Outcomes. The selected studies evaluating the effect of statins in the context of periodontal treatment included 23 controlled and randomized clinical trials, 8 cohort studies, and 1 longitudinal study (Figure 4). Primary outcomes varied between improvement of clinical attachment level (CAL), reduction of pocket depth (PD), tooth loss, radiographic bone defect depth, periodontal inflamed surface area (PISA), and serum and/or GCF proinflammatory cytokines level. Most of the studies focused on the local administration ($n = 25$) of statins (Table 6), while 7 investigated the impact of systemic route (Table 7). Essentially, effects of statins have been evaluated as an adjunct to both nonsurgical and surgical treatments, mainly in the context of chronic periodontitis in healthy patients.

3.6. Statins as a Local Adjunct to Nonsurgical Periodontal Treatment. The effect of local delivery of statins as an adjunct to nonsurgical periodontal therapy (SRP) was studied in 20 clinical trials (Table 6). Atorvastatin and simvastatin have been the most commonly studied statins. Amongst the identified studies, 13 demonstrated a significant PD reduction, CAL gain, and IBD fill in healthy patients, 2 in well-controlled type II diabetes patients, and 3 in smokers. At contrary, in 2 studies, the test groups using atorvastatin or simvastatin did not show any significant differences when compared with the control [21, 100]. For instance, with simvastatin, the mean PD gain was 1.23 ± 0.57 mm for the control group versus 1.83 ± 0.07 mm for the test group ($p = 0{,}112$) and the mean CAL gain was 2.09 ± 0.08 mm for the control group versus 2.43 ± 0.01 mm for the test group ($p = 0.889$) after 45 days. Nevertheless, authors found a statistically significant reduction of PI, BOP, IL-6, and IL-8 levels [21].

Only 4 studies compared the outcomes obtained with more than one statin; however, contradictory results were observed. For instance, one study did not show any significant difference between atorvastatin and simvastatin [100], whereas better results were obtained with atorvastatin in another study [101]. Nevertheless, two studies highlighted greater efficacy with rosuvastatin in comparison with atorvastatin [20, 102].

Interestingly, studies that have investigated the effects of statin treatment on the biological markers from GCF showed that simvastatin administration reduced significantly IL-6, IL-8 and increased the anti-inflammatory IL-10 [21, 100, 103].

3.7. Statins as a Local Adjunct to Surgical Periodontal Treatment. Statins have also been inspected for their role in the surgical treatment outcomes. In all identified studies where statins (simvastatin, atorvastatin, and rosuvastatin) were locally administered concomitant to surgical approach (including the use of biomaterials or PRF), a significant reduction of PD, improvement of CAL, and bone defect fill was achieved in the test group in comparison to the control

Table 3: *In vivo* studies evaluating the impact of local statin administration on periodontal wound healing.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Local drug delivery Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
[139]	Rats (retired female breeder) EIP by ligatures Maxillary right M2	Nonsurgical treatment (therapeutic) Simvastatin prodrug 0.5 mg, 1.0 mg, and 1.5 mg Local injections of the drug/SIM/SIM-mPEG carrier 10 μL into the palatal gingiva between maxillary M1 and M2 Three weekly injections until euthanasia	↗ amount of uninflamed connective tissue in the M1-M2 interproximal area ↘ bone loss, especially with 1.5 mg SIM/SIM-mPEG ↘ percentage of neutrophils	Simvastatin limited periodontal breakdown by reducing bone loss and the extent of gingival inflammation
[73]	Rats (male) ACP (maxillary bone defect) Maxillary M1 extraction followed by socket healing, preparation of a critical-sized periodontal defect (2.0 mm diameter and 1.0 mm depth) on the mesial aspect of the M2, and manual removal of the residual bone and cementum on mesial aspect of M2	Surgical treatment (therapeutic) Simvastatin 1 mg Encapsulated in double-walled PDLLA-PLGA microspheres Combinations: simvastatin-BSA, simvastatin-PDGF, simvastatin	↗ neo-osteogenesis ↗ bone mineral density ↗ bone volume fraction ↗ number and thickness of trabeculae ↘ trabecular separation ↗ cementogenesis of the periodontal apparatus ↘ inflammatory cell infiltration	Simvastatin promoted osteogenic differentiation, reduced inflammation, and facilitated osteogenesis. Sequential PDGF-simvastatin delivery was able to accelerate osteogenesis, bone maturation, fiber realignment, and cementogenesis of the periodontal apparatus, thus accelerating periodontal regeneration
[94]	Rats (male) ACP (tooth-associated alveolar bone defect model) extraction of M1 followed by 4 weeks of socket healing, preparation of a critical-sized intrabony periodontal defect in the M1 edentulous ridge next to the mesial aspect of the M2 finished by a 2.6 mm diameter and 1.0 mm deep osteotomy (completely removing the mesial wall of the osteotomy), and cementum removal (to expose the mesial aspect of M2)	Surgical treatment (therapeutic) Simvastatin 1 mg PDLLA-PLGA hybrid microspheres encapsulating simvastatin/PDGF/BSA to fill the defects	↗ neo-osteogenesis (histologically) PDL fibers not inserted on the root surface (mainly parallel) ↗ bone volume fraction % (not significant)	Simvastatin histologically improved bone healing but better healing response was observed in the group receiving PDGF
[95]	Rats (female) ACP (fenestration defects) Defects 2 mm high, 4 mm wide, and 1.5 mm deep over mandibular molar roots	Nonsurgical treatment (therapeutic) Simvastatin 0.5 mg Local injection of 0.5 mg SIM per site dissolved in 70% ethanol or as SIM-ALN-CD Three weekly injections	↗ insignificant improvement of bone fill compared to other groups New cementum formation (not significant) But better bone healing	Simvastatin had a local bone healing effect which can be augmented by addition of certain other regenerative molecules like ALN

Table 3: Continued.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Local drug delivery Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
		Treatment started 15 days after the defect preparation	response after systemic ALN administration followed by simvastatin injections	
[138]	Dogs (male) ACP (maxillary bone defect) Extraction of all maxillary PM2 followed by healing and preparation of one-walled intrabony defects (4 × 5 × 4 mm: buccolingual, mesiodistal, and depth, respectively) on the mesial and distal sides of maxillary bilateral PM1 Removal of residual cementum by SRP	Surgical treatment (therapeutic) Lovastatin 0.1, 0.5, 1, or 2 mg per trilayer functional CS with the EGCG membrane area (cm^2)	↗ new bone formation in the EGCG14-CS-lovastatin 1 group (62.03%) > BioMend® group (46.07%) > control group (42.32%) Evidence of new cementum deposition observed on the root surface No inflammatory cell infiltrate was noted in the $EGCG_{14}$-CS-lovastatin 1 group Fibrous connective tissue approximated to the surgical defect	The trilayer functional CS membrane with EGCG and lovastatin enhanced periodontal regeneration and bone formation rate
[140]	Dogs (male) ACP (maxillary bone defect) Extraction of maxillary 2^{nd} and 3^{rd} incisors followed by 8 weeks of socket healing and, later, preparation of three-walled intrabony defects (4 × 4 × 5 mm: buccolingual, mesiodistal, and depth, respectively) on the mesial side of maxillary bilateral canines Removal of residual cementum by SRP	Nonsurgical treatment (therapeutic) Lovastatin 4 mg dissolved in chloroform to form a 3 wt % PLGA solution Local injections of PLGA-lovastatin-CS-tetracycline 0.3% nanoparticles prepared as a hydrogel by mixing with gelatin (10 mg/100 mm^3) to fill the defects	↗ new deposits of cementum on the root surface ↗ active plasmacytoid osteoblastic rimming along the trabecular surface of the bone adjacent to the defect ↗ percentage of new bone formation (41.32%) No evident inflammation	PLGA-lovastatin-chitosan-tetracycline nanoparticles showed a good osteogenic potential. They promoted new bone and cementum formation
[96]	Rats (male) ACP (mandibular bone defect) Preparation of surgical defects 0.8 mm in diameter through the alveolar bone over the mesiobuccal root of the mandibular M1 bilaterally	Surgical treatment (therapeutic) Simvastatin 2.5% gel Defect was filled with 2.5% simvastatin gel Single topical application	↘ marrow spaces in simvastatin-treated defects ↗ collagen fibril organization ↗ OPN in bone matrix ↗ alveolar bone regeneration	Simvastatin gel improved the quality of the new bone and decreased bone resorption

Table 3: Continued.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
		Local drug delivery		
[99]	Dogs (males and females) ACP (mandibular bone defect) Preparation of bilateral 3-walled intrabony defects (4 × 4 × 4 mm) distal of the mandibular PM2 and mesial of the PM4 and class II furcation defects at the buccal furcation of the mandibular M1 measuring 4 mm occlusal apically and 4 mm buccolingually followed by healing and SRP of defect sites	Nonsurgical treatment (therapeutic) Simvastatin 0.5 mg or 2.0 mg in 30 μL methylcellulose gel Three weekly injections	↗ edentulous ridge thickness (29% greater with simvastatin) ↗ bone loss in class II furcation defects ↗ length of new cementum in the interproximal intrabony defect ↗ bone height with simvastatin (2 mg) No new cementum was observed in furcations	Simvastatin was not appropriate for the treatment of class II furcation defects. However, it improved bone healing in intrabony defects and edentulous ridges significantly
[22]	Rats (male) EIP by ligatures Maxillary M2 bilaterally	Nonsurgical treatment (therapeutic) Atorvastatin 2% *w*/*v* containing CS gel Local 100 μL volume application every other day until euthanasia	↘ IL-1β, IL-6, and IL-8 ↗ IL-10 (time dependent) ↘ alveolar bone resorption (significantly with ATV + CS application and insignificantly with ATV alone) ↘ attachment loss Improvement of inflammatory and osteoclastic activity score over time	Atorvastatin with chitosan downregulated inflammation-mediated bone resorption
[90]	Rats (female) EIP by injection of *E. coli* LPS 10 μL of endotoxin injection (1 mg/mL of LPS in PBS) between M1 and M2	Nonsurgical treatment (preventative) Simvastatin 0.5 mg of simvastatin and 3.75 mg of SIM-ALN-CD in H2O Three weekly 12 μL injection bilaterally into the palatal/interproximal gingiva of M1 and M2 Treatment started one week before induction	↗ bone preservation during experimental periodontitis by prophylactic SIM-ALN-CD injection ↘ subsulcular inflammation ↘ alveolar bone loss ↘ OC number	Simvastatin protected against alveolar bone loss and soft tissue inflammation
[98]	Dogs (female) ACP (mandibular bone defect) Preparation of dehiscence defects (5 × 3 mm) bilaterally on the lateral aspect of the mandibular PM2 mesial roots and removal of	Surgical treatment (therapeutic) Simvastatin Graft surgery with HA grafts bilaterally covered with resorbable bilayer collagen membranes hydrated with 10 mg simvastatin	↗ width of new bone in edentulous ridge Distance between CEJ and the alveolar crest was more coronal in dehiscence defects treated	Simvastatin improved new bone formation where periosteum existed and did not induce severe side effects except for moderate swelling that, eventually, subsided

Table 3: Continued.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Local drug delivery Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
	root cementum Split-mouth design	(graft surgery performed at the time of defect preparation) Local injection 10 mg SIM (0.5 mg/kg) in ethanol (100 μL) Three weekly injections (one week after the graft surgery and defect preparation)	with simvastatin (insignificant) Three weeks post-op after simvastatin injection (firm swelling about 1 × 1 cm to 3.5 × 3.5 cm in size), disappeared in 2 months	
[59]	Rats (male) EIP by ligatures Left mandibular M1	Nonsurgical treatment (therapeutic) Simvastatin 1 mg/mL (Natrosol + simvastatin gel solution) into the periodontal pocket SRP and irrigation with simvastatin Single injection	↘ MMP-8 expression ↘ bone loss	Simvastatin reduced periodontal bone loss
[141]	Rats (male) EIP by ligatures Maxillary M2	Nonsurgical treatment (therapeutic) Simvastatin 0.2 mg in 50 μL PBS topically injected into the buccal gingivae Twice a week for 70 days	↗ ALP activity ↗ bone nodule formation No inflammatory cells around the new bone ↘ bone loss Simvastatin recovered the ligature-induced alveolar bone resorption (46% reversal of bone height)	Simvastatin increased bone regeneration and reduced inflammation
[142]	Rats (male) EIP by ligatures Mandibular left M1	Nonsurgical treatment (preventative) Simvastatin 0.5 mg/kg body weight orally Followed by laser therapy Treatment started 1 day before induction and daily until euthanasia	↘ bone loss ↘ carbonylated proteins in gingiva	Simvastatin reduced bone loss
[91]	Rats (female ovarectomized) EIP by ligatures Mandibular right M1	Nonsurgical treatment (protective) Simvastatin 10^{-6} M, 3×10^{-7} M, 10^{-7} M subperiosteal injections (0.05 mL)	↘ periodontal breakdown ↘ bone loss in alveolar bone crest zone in a dose-dependent manner ($10^{-7} > 10^{-6} > 3 \times 10^{-7}$)	Simvastatin reduced bone loss in a dose-dependent manner

Table 3: Continued.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Local drug delivery Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
		Twice a week since the first day of ligature insertion to the 25th day		
[143]	Rat (female) EIP (ligature) Maxillary M2 bilaterally	Nonsurgical treatment (therapeutic) Simvastatin SIM-PPi conjugate Different treatments including SIM-PPi (dissolved in 25%, 2.56 mg, equivalent to 1.5 mg SIM) and SIM acid (dissolved in PBS, 1.56 mg, equivalent to 1.5 mg of SIM) locally injected (10 μL) into the palatal gingiva between the maxillary M1 and M2 On the first day of weeks 1, 2 and 3 after ligature placement	↗ alveolar bone crest preservation with SIM-PPi ↗ bone volume ↗ trabecular thickness ↗ trabecular number ↘ trabecular separation ↘ neutrophil and lymphocyte score ↘ OC score	Simvastatin improved periodontal bone regeneration and decreased periodontal inflammation

Table 4: *In vivo* studies evaluating the impact of systemic statin administration on periodontal wound healing.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Systemic drug delivery Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
[31]	Rats (male) EIP by ligatures Maxillary left M2	Nonsurgical treatment (protective) Atorvastatin 1 mg/kg, 5 mg/kg, and 10 mg/kg 1 hour before induction and thereafter once daily	↘ MMP-2, MMP-9 ↘ RANK-L, RANK ↗ OPG ↗ GSH levels ↘ IL-1β, TNF-α, and MPO (dose dependent) ↘ COX-2 level ↘ MDA activity ↘ alveolar bone loss is dose dependent	Atorvastatin protected against alveolar bone loss in a dose-dependent manner
[58]	Rats (female) EIP by ligatures Maxillary left M2	Nonsurgical treatment (protective) Simvastatin 3, 10, and 30 mg/kg/day 1 hour before induction and thereafter once daily	↗ BMP-2 and OPG levels ↗ TRAP activity ↘ MPO activity (dose dependent) ↘ IL-1β and TNF-α ↗ IL-10 ↘ gingival GSH ↗ gingival MDA and NOX ↘ iNOS, MMP-1, MMP-8, RANK, and RANKL expression No differences in AST and ALT levels Inhibition of alveolar bone loss	Simvastatin prevented inflammatory bone resorption and possessed antioxidant properties
[144]	Rats (male) EIP by ligatures Maxillary left M2	Nonsurgical treatment (protective) Atorvastatin 1, 3, and 9 mg/kg Atorvastatin mixed in sterile saline by gavage 30 min before ligature placement and then daily until euthanasia	↘ alveolar bone loss in the furcation area as well as in proximal faces of upper M2 (47% reduction with 9 mg dose compared to that with the control) Insignificant bone loss protection with 1 and 3 mg doses	Atorvastatin had protective effect against alveolar bone loss
[40]	Rats (male) EIP by ligatures Maxillary left M2	Nonsurgical treatment (protective + therapeutic) Atorvastatin 0.3 mg/kg or 27 mg/kg by gavage In combination with ALN 30 min before ligature placement and thereafter once daily until euthanasia or 5 days after the start of periodontitis induction and then daily until euthanasia	↘ TRAP and MPO activity ↘ cementum resorption ↘ neutrophilia and lymphomonocytosis ↘ alveolar bone loss both prophylactically (39%) and therapeutically (53.4%) with lower dose of ALN + ATV (0.01 mg/kg+0.3 mg/kg, respectively) Prevented BALP reduction with lower dose of ALN + ATV No effect on serum transaminases	Atorvastatin reduced alveolar bone loss, cemental resorption, and inflammatory cell infiltration both prophylactically and therapeutically

Table 4: Continued.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
		Systemic drug delivery		
[145]	Rats (male) EIP by ligatures Maxillary left M2	Nonsurgical treatment (protective) Atorvastatin 0.3, 3, and 27 mg/kg by gavage 30 min before ligature placement and thereafter once daily until euthanasia	↘ alveolar bone in a dose-dependent manner (39% for 3 mg/kg and 56% for 27 mg/kg doses) Prevented the reduction of BALP serum levels (27 mg/kg) Prevented leukocytosis (27 mg/kg)	Atorvastatin prevented alveolar bone loss with both prophylactic and therapeutic doses
[32]	Rats (female with metabolic syndrome) ACP (injection of 20 μg of *A.a* LPS in PBS) into the palatal gingiva between the maxillary M1 and M2, thrice per week for 4 weeks	Nonsurgical treatment (protective) Simvastatin 20 mg/kg/day Daily via gavage for 4 weeks Treatment started on the same day as injection of LPS	↘ LPS induced alveolar bone loss in both lean and fat rats (significantly) ↘ infiltration of mononuclear cells ↘ inflammatory score ↘ LPS stimulated RANKL and CSF2 expression in both lean and fat rats ↘ bone resorption	Simvastatin downregulated inflammation-mediated bone resorption
[33]	Rats (female) *ACP injection of* 20 μg/rat of *A.a* LPS through the palatal gingiva between the maxillary M1 and M2 thrice per week for 8 weeks	Nonsurgical treatment (protective) Simvastatin (20 mg/kg/day) daily via oral gavage for 8 weeks	↘ LPS induced alveolar bone loss (31%) ↘ LPS induced osteoclastogenesis ↘ TNF-α, IL-1α, IL-1β, IL-6, CSF-2, CSF-3, MCP-1, and MMP-9 ↘ LPS induced TLR family members' expression	Simvastatin downregulated inflammation-mediated bone resorption
[25]	Rats (male) EIP by ligatures Maxillary M2	Nonsurgical treatment (protective) Rosuvastatin 20 mg/kg in water by gavage 1 h before ligation and then once daily until euthanasia	↗ IL-10 ↘ IL-1β ↗ MDA ↗ GSH ↘ inflammatory infiltrate ↘ OC number ↗ OB number ↘ alveolar bone loss (significantly)	Rosuvastatin protected against alveolar bone loss
[54]	Rats (male) EIP by ligatures Hyperlipidemia induction through diet Maxillary M2	Nonsurgical treatment (protective) Rosuvastatin 20 mg/kg in water by gavage 1 h before ligation and then once daily until euthanasia	↘ gingival iNOS (significantly) ↘ inflammation and hyperemia ↘ alveolar bone loss	Rosuvastatin protected against inflammation-induced bone degradation
[34]	Rats (male) EIP by ligatures Mandibular M1 and maxillary M2 bilaterally	Nonsurgical treatment (therapeutic) Simvastatin 10 mg/kg in water once daily orally until euthanasia Treatment started 8 days after periodontitis induction	↘ alveolar bone loss ↘ IL-6 ↘ CRP	Simvastatin decreased inflammation and alveolar bone loss

Table 4: Continued.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Systemic drug delivery Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
[93]	Rats (male hypertensive) EIP by ligatures Mandibular M1 bilaterally	Nonsurgical treatment (protective) Rosuvastatin 2 mg/kg oral gavage Treatment started since the day of induction daily until euthanasia	↘ bone loss in furcation area ↘ attachment loss ↘ TRAP-positive multinucleated cells	Rosuvastatin reduced alveolar bone loss and osteoclastogenesis
[97]	Rats EIP by ligatures Mandibular M1	Nonsurgical treatment (protective + therapeutic) Simvastatin Different treatments: simvastatin-simvastatin: aqueous suspension of simvastatin by gavage (35 mg/kg/day) administration before and after periodontitis induction; simvastatin-water: simvastatin administration before and filtered water after periodontitis induction; and water-simvastatin: water administration before and simvastatin after periodontitis induction	No significant differences between groups receiving simvastatin before the induction of periodontitis and those that received water No protective effect of simvastatin against the development of periodontitis	Simvastatin did not possess protective or therapeutic effects against periodontitis development
[146]	Rats (male) EIP by ligatures Mandibular left M1	Nonsurgical treatment (therapeutic) Simvastatin 25 mg/kg Dissolved in saline Treatment started 14 days after the initiation of periodontitis induction	↗ TG levels ↘ MDA level ↗ IL-10 ↘ MMP-9 ↘ bone loss No difference on TNF-α levels	Simvastatin promoted the anti-inflammatory mediators to counter alveolar bone loss
[35]	Rats (male, cyclosporine A-induced alveolar bone loss) EIP by ligatures Mandibular right M1	Nonsurgical treatment (protective) Simvastatin 20 mg/kg orally daily for 30 days The treatment and induction started on the same day	↗ Ca2+ concentrations (significantly) No effect of simvastatin treatment in the presence of periodontal disease on serum ALP levels but it blocked the cyclosporine A-mediated decrease of ALP No significant effect on alveolar bone turnover but with concomitant cyclosporine A and simvastatin delivery Simvastatin completely inhibited cyclosporine A-induced bone loss	Simvastatin did not prevent alveolar bone loss in periodontitis but it completely countered the cyclosporine A-induced bone loss
[147]	Rats (male) EIP by ligatures Mandibular right M1	Nonsurgical treatment (protective) Simvastatin 20 mg/kg The treatment and induction started on the same day	↗ ALP activity in periodontal inflammation ↘ alveolar bone loss	Simvastatin protected against alveolar bone loss

Table 4: Continued.

Reference	Experimental periodontitis induction model (i) Animal (ii) Method (iii) Site	Periodontitis treatment (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
		Systemic drug delivery		
[76]	Mice (male) ACP (*P.g* LPS injection) 1 mg/kg *P.g* LPS injection at the gingiva of left mandibular M2 on days 4 and 7	Nonsurgical treatment (protective) Fluvastatin 3 mg/kg IP injections on days 1, 4, and 7	↘ LPS induced OC (by >50%) ↘ LPS-induced bone erosion ↘ RANKL	Fluvastatin prevented inflammation-induced bone erosion
[26]	Rats (male, GIOP) EIP by ligatures Maxillary left M2	Nonsurgical treatment (protective) Atorvastatin 27 mg/kg ATV orally 30 min before induction and once daily afterwards	↘ bone loss ↘ MPO, TNF-α, IL-1β, IL-6, and IL-8 ↗ IL-10, GSH, SOD, and CAT levels ↘ RANKL and DKK-1 ↗ OPG, WNT10 β, and β-catenin expressions and BALP activity	Atorvastatin prevented alveolar bone loss in periodontitis and reduced inflammation

Table 5: *In vivo* studies evaluating the impact of a combination of local and systemic statin administration on periodontal wound healing.

	Local + systemic drug delivery			
Reference	*Experimental periodontitis induction model* (i) Animal (ii) Method (iii) Site	*Periodontitis treatment* (i) Type of treatment (ii) Type and dose of statin (iii) Mode and time of statin delivery	Results	Periodontal considerations
[57]	Rats (male) EIP by ligature mandibular M1	Nonsurgical treatment (therapeutic) Atorvastatin Systemically (5 mg/kg in a volume of 0.5 mL) and locally (0.1 mg/kg in a volume of 0.05 mL) at a dose of 0.1 mg/kg in a volume of 0.05 mL	↗ alveolar bone area % ↗ VEGF ↘ MMP-9 ↘ alveolar bone and attachment loss Local application showed better results on periodontium healing	Atorvastatin increased the alveolar bone regeneration while decreasing the periodontal inflammation and attachment loss
[92]	Rats (female ovarectomized) EIP by ligatures Maxillary M1 and M2 bilaterally	Nonsurgical treatment (therapeutic) Simvastatin Local injection (0.8 mg/0.05 mL) Oral (25 mg/kg) For two months until euthanasia	↗ alveolar crest height (28% with local & oral and 27% with local) ↗ BV/TV ↗ trabecular thickness ↘ trabecular separation	Simvastatin reduced bone degradation when administered locally, systemically, or both locally and systemically together

The animals included in the studies are healthy unless stated otherwise. Treatment was considered (1) "preventative" when it started at least one day before the start of EIP/ACP induction, (ii) "protective" when it started the same day as that of EIP/ACP induction, and (iii) "therapeutic" when it started at least one day after the start of EIP/ACP induction.

group [104–108] (Table 6). Amongst these studies, the mean difference of PD between the test and control groups ranged from 1.3 ± 0.21 mm to 2.51 ± 0.22 mm ($p < 0.001$). Thus, the mean difference of CAL between the test and control groups ranged from 1.16 ± 0.09 mm to 2.35 ± 0.08 ($p < 0.001$). Moreover, the mean difference of bone defect fill between the test and control groups ranged from 1.336 ± 0.714 to 3.08 ± 0.07 ($p < 0.001$).

3.8. Impact of Systemic Administration of Statins on Nonsurgical Periodontal Treatment Outcomes. The impact of systemic administration of statins on nonsurgical periodontal treatment outcomes was evaluated in a few studies (Table 7). From the 7 studies identified, 4 demonstrated significant improvements regarding reduction of PD, CAL gain, and/or tooth loss in comparison to the control group [56, 109–111]. At contrary, 3 other studies did not show any significant differences in periodontal outcomes between the statin-treated and control groups [112–114]. These discrepancies could be due to the very short follow-up of the abovementioned 3 studies (3 months) compared to the other ones (from 3 months to 7 years follow-up). Moreover, one of the studies did not compare the treatment group with a control group [110].

4. Discussion

Statins exhibit multiple effects, including modulation of inflammatory-immune crosstalk, bone regeneration, and antibacterial activity, to promote periodontal wound healing and regeneration (Figure 5). They act through several closely interrelated pathways highlighting potential therapeutic targets. The hydrophobic or hydrophilic nature of statins determines their efficacy, action on periodontal pathogens, and treatment response and appears to be largely cell and tissue dependent [69, 78]. Further insight into this may help selecting the best statin.

Moreover, the mode of statin delivery also affects the treatment outcomes. Oral systemic administration of statins reduces periodontal inflammation and consequent tooth loss [111] but the low resultant dose available to the tissues after hepatic bypass renders them relatively less efficacious [60]. On the other hand, a higher dose to enhance efficacy can manifest systemic side effects such as statin-induced myopathy, hepatotoxicity, nephrotoxicity, pulmonary manifestations, ophthalmological manifestations, gastrointestinal hemorrhage risk, and oral manifestations (dryness, itch, bitterness, and cough) [115, 116]. Therefore, to avoid these side effects, various local application strategies have been tested that allow site-specific delivery reducing the required dose, frequency of application, and bioavailability in the blood [60, 117, 118], concomitantly improving patient compliance [119].

The development and selection of an optimal statin delivery carrier are crucial as it enhances the statin retention on the lesion and acts as a scaffold for cell growth and differentiation [120]; therefore, it should be capable to withstand the oral environment, continuous fluid exchange inside the pocket, and salivary influx.

Several studies demonstrate that anti-inflammatory properties of statins vary according to the type and dose of statin used [121]. On a cellular level, modulation of macrophage polarization from a proinflammatory M1 to a proresolution M2 phenotype by systemic delivery of immune modulatory drugs resolved persistent inflammation associated with chronic periodontitis [122]. In this context, statins' ability to switch M1 to M2 to promote periodontal wound healing and regeneration needs to be explored. Furthermore,

Table 6: Clinical studies evaluating the impact of local statin administration on periodontal wound healing.

Reference Study area Type of study	Drug Mode of delivery Dose	Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
			Local drug delivery		
[130] (India) RCT with split-mouth design	Simvastatin in methylcellulose gel 1.2 g of SIM	30 Periodontitis (Armitage 1999) Healthy patients (nonsmokers) Sites with periodontal pocket measuring ≥ 5 mm and vertical bone loss ≥ 2 mm in different quadrants of the mouth	Nonsurgical treatment Group I: SRP + placebo gel Group II: SRP + SIM gel 6 months follow-up	All subjects tolerated the drug ↗ periodontal parameters with or without SIM ↗ CAL ($p = 0.02$) ↗ INFRA 2 ($p < 0.01$) ↘ PD significantly ($p = 0.04$) ↘ INFRA 1 ($p < 0.01$)	Simvastatin increased periodontal regeneration and CAL gain
[23] (India) RCT	Rosuvastatin 1.2% rosuvastatin (RSV) gel	90 Chronic periodontitis Healthy patients (nonsmokers)	Nonsurgical treatment Groups I: SRP + placebo gel Group II: SRP + 1.2% RSV gel Group III: SRP + 1% MF gel 12 months follow-up	↗ CAL ↘ PD significant ↗ bone fill ↘ PI ↘ mSBI ↘ DDR	Rosuvastatin increased periodontal regeneration and CAL gain
[102] (India) RCT	Atorvastatin and rosuvastatin 1.2% atorvastatin or 1.2% rosuvastatin gel local drug delivery (1.2 mg/0.1 mL)	90 No data Healthy patients (nonsmokers) Mandibular class II furcation defects with PD ≥ 5 mm and horizontal PD ≥ 3 mm	Nonsurgical treatment Group I: SRP + placebo Group II: SRP + 1.2% RSV gel Group III: SRP + 1.2% ATV gel 9 month follow-up	↘ PI and mSBI in all groups The 2 statins lead to the following: ↘ PD ↗ mean gain in CAL ↗ mean percentage of DDR Statistically greater results for RSV than for ATV	Statins increased periodontal regeneration and CAL gain
[103] (India) Cohort study	Simvastatin SIM gel (1.2 mg/0.1 mL)	50 Chronic periodontitis Healthy patients (nonsmokers)	Nonsurgical treatment Group I: SRP alone Group II: SRP + SIM gel 3 months follow-up	↘ IL-6 and IL-8 ↗ IL-10 significantly ↘ PI, mSBI, and PD No effect on CAL	Simvastatin gel decreased periodontal inflammation and promote periodontal regeneration
[21] (India) RCT	Simvastatin 1.2% simvastatin gel	46 Chronic periodontitis Healthy patients (nonsmokers)	Nonsurgical treatment Group I: SRP Group II: SRP + SIM gel 45 days follow-up	↘ PI, GI, and SBI No significant difference for PD and CAL ↘ mean IL-6 levels No significant difference for IL-8 levels	Simvastatin gel decreased periodontal inflammation

Table 6: Continued.

Reference Study area Type of study	Drug Mode of delivery Dose	Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
			Local drug delivery		
[104] (India) Cohort study with split-mouth design	Simvastatin Combination of DFDBA and a 10^{-8} M solution of the drug simvastatin	15 No data Healthy patients (nonsmokers) Identical bilateral infrabony defect	Surgical treatment (Kirkland flap) Group A: DFDBA alone Group B: DFDBA + SIM 24 weeks follow-up	↘ PD ↗ mean gain in CAL (better with DFDBA + SIM) ↘ infrabony defect depth (greater reduction with DFDBA + SIM) ↗ linear defect fill (better with DFDBA + SIM)	Simvastatin increased periodontal regeneration and CAL gain
[148] (India) RCT	Atorvastatin 1.2% atorvastatin gel (ATV gel (1.2 mg/0.1 mL)	75 Well-controlled type 2 diabetic patients (nonsmokers) Chronic periodontitis	Nonsurgical treatment Group 1: SRP + ATV Group 2: SRP + placebo 9 months follow-up	↗ mSBI ↘ PD ↗ CAL gain ↘ IBD depth and DDR No significant difference for PI at all time intervals evaluated	Atorvastatin increased periodontal regeneration
[125] (India) RCT	Atorvastatin 1.2% atorvastatin gel (ATV gel (1.2 mg/0.1 mL))	71 Smokers Chronic periodontitis	Nonsurgical treatment Group 1: SRP + ATV Group 2: SRP + placebo 9 months follow-up	↘ PD ↗ mean CAL gain ↘ mean percentage of DDR ↘ mSBI ↘ IBD depth No statistically significant difference in the site-specific PI score and full-mouth PI score between the groups at any visit	Atorvastatin increased periodontal regeneration and CAL gain
[105] (India) Cohort	Atorvastatin 1.2% ATV gel	96 Healthy patients (nonsmokers) Chronic periodontitis	Surgical treatment Group I: OFD + PRF Group II: OFD + PRF + 1.2% ATV Group III: OFD alone 9 months follow-up	ATV gel and PRF alone showed significantly the following: ↘ PD ↗ mean CAL gain ↘ IBD depth	Atorvastatin increased periodontal regeneration and CAL gain

Table 6: Continued.

Reference Study area Type of study	Drug Mode of delivery Dose	Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
			Local drug delivery		
				No statistically significant difference in PI and mSBI scores between the groups at 9 months	
[101] (India) RCT	Atorvastatin and simvastatin 10 mL of 1.2% ATV gel (1.2 mg/0.1 mL) and 10 mL of 1.2% SIM gel (1.2 mg/0.1 mL)	96 Healthy patients (nonsmokers) Chronic periodontitis	Nonsurgical treatment Group I: SRP + 1.2% ATV Group II: SRP + 1.2% SIM Group III: SRP + placebo 9 months follow-up	The 2 statins lead to the following: ↘ PD ↘ mSBI ↘ IBD depth ↗ mean CAL gain Statistically greater results for ATV than for SIM for PD reduction, CAL gain and percentage of IBD reduction	Atorvastatin increased periodontal regeneration and CAL gain
[149] (India) RCT	Simvastatin Single topical transmucosal injection 1.2 mg SIM	60 Chronic periodontitis Healthy patients (nonsmokers)	Nonsurgical treatment Group I: SRP + placebo Group II: SRP + SIM 6 months follow-up	↘ mSBI ↘ mean PD ↗ mean CAL ↗ IBD fill ↘ GI	Simvastatin increased periodontal regeneration and CAL gain
[126] (India) RCT	Simvastatin SIM 1.2 μg/inj. (0.12 μg/mm3) Methylcellulose gel	72 Chronic periodontitis Healthy patients (nonsmokers) Mandibular buccal class II furcation defects	Nonsurgical treatment Group I: SRP + placebo Group II: SRP + 1.2 mg SIM 6 months follow-up	↘ SBI and PB ↗ CAL ↗ IBD fill	Simvastatin increased periodontal regeneration and CAL gain
[150] (India) RCT	Atorvastatin 1.2% ATV methyl cellulose gel	60 patients Chronic periodontitis Healthy patients (nonsmokers)	Nonsurgical treatment Group I: SRP + 1.2% ATV Groups II: SRP + placebo gel 9 months follow-up	↘ PD ↘ mSBI ↗ mean CAL gain ↗ IBD fill	Simvastatin increased periodontal regeneration and CAL gain
[151] (India) RCT	Simvastatin 1.2% SIM gel	38 Chronic periodontitis Well-controlled type II diabetes Nonsmokers	Nonsurgical treatment Group I: SRP + SIM Group II: SRP + placebo 9 months follow-up	↘ PD ↗ mean CAL gain ↗ mean radiographic bone fill ↘ mSBI	Simvastatin increased periodontal regeneration and CAL gain

Table 6: Continued.

Reference Study area Type of study	Drug Mode of delivery Dose	Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
			Local drug delivery		
[152] (India) RCT	Rosuvastatin 1.2% rosuvastatin (RSV) gel	65 Chronic periodontitis Healthy (nonsmokers)	Nonsurgical treatment Group I: SRP + RSV Group II: SRP + placebo 6 months follow-up	↘ mSBI ↘ PD ↗ mean CAL gain ↗ IBD fill	Rosuvastatin increased periodontal regeneration and CAL gain
[20] (India) RCT	Atorvastatin + rosuvastatin 1.2% RSV and 1.2% ATV gel	90 Chronic periodontitis Healthy (nonsmokers)	Nonsurgical treatment Group I: SRP + placebo Group II: SRP + 1.2% RSV gel Group III: SRP + 1.2% ATV gel 9 months follow-up	The 2 statins lead to the following: ↘ mSBI ↘ PD ↗ mean CAL gain ↗ IBD fill Statistically greater results for RSV than for ATV for PD reduction, CAL gain, IBD reduction, and msSBI reduction	Atorvastatin and rosuvastatin increased periodontal regeneration and CAL gain
[106] (India) RCT	Rosuvastatin 1.2% RSV gel	90 Chronic periodontitis Healthy (nonsmokers)	Surgical treatment 2/3-walled intrabony defects Group I: OFD alone Group II: OFD + PRF Group III: OFD + PRF + 1.2% RSV gel 9 months follow-up	↘ PD ↗ mean CAL gain ↗ IBD fill	Rosuvastatin increased periodontal regeneration and CAL gain
[107] (India) RCT	Rosuvastatin 1.2% RSV gel	110 Chronic periodontitis Healthy (nonsmokers) Mandibular degree II furcation defects	Surgical treatment Group 1: OFD + placebo gel Group II: OFD + PRF + HA Group III: OFD + RSV 1.2 mg gel + PRF + HA 9 months follow-up	↘ PD ↗ mean CAL gain ↗ IBD fill ↘ PI and mSBI	Rosuvastatin increased periodontal regeneration and CAL gain
[153] (India) RCT	Atorvastatin 1.2% atorvastatin gel	90 Chronic periodontitis Healthy patients (nonsmokers) Intrabony defect	Nonsurgical treatment Group I: SRP + ALN Group II: SRP + 1.2% ATV Group III: SRP + placebo group 9 months follow-up	↘ PD ↗ mean CAL gain ↗ IBD fill ↘ mSBI	Local delivery of atorvastatin increased periodontal regeneration

Table 6: Continued.

Reference Study area Type of study	Drug Mode of delivery Dose	Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
			Local drug delivery		
[154] (India) RCT	Simvastatin 0.1 mL SIM gel (1.2 mg/0.1 mL)	24 Aggressive periodontitis Healthy patients (nonsmokers) Intrabony defect	Nonsurgical treatment Group I: SRP + placebo gel Group II: SRP + SIM gel 6 months follow-up	↘ PD ↗ mean CAL gain ↗ IBD fill ↘ mSBI All patients tolerated the drug with no postapplication complications No statistically significant difference between groups I and II regarding PI	Simvastatin increased periodontal regeneration
[108] (India) RCT	Simvastatin 1.2 mg Simvastatin gel	20 Chronic periodontitis Healthy patients (nonsmokers)	Surgical treatment PD ≥ 5 mm in the mandibular molar region bilaterally Group I: OFD + SIM Group II: OFD + placebo gel 9 months follow-up	↗ IBD fill for group I Significant results at 9 months in both groups: ↘ GI, PD ↗ mean CAL gain	Simvastatin increased periodontal regeneration
[155] (India) RCT	Simvastatin 10 μL prepared SIM gel (1.2 mg/0.1 mL)	40 Chronic periodontitis Healthy patients Smokers only	Nonsurgical treatment Group I: SRP + SIM 1.2% Group II: SRP + placebo 9 months follow-up	↘ mSBI ↘ PD ↗ mean CAL gain ↗ IBD fill	Simvastatin increased periodontal regeneration and CAL gain
[156] (India) RCT	Simvastatin 1.2% simvastatin gel	60 Chronic periodontitis Healthy (nonsmokers)	Nonsurgical treatment Group A: SRP + placebo Group B: SRP + SIM gel 6 months follow-up	↘ mSBI and PD ↗ mean CAL gain ↗ IBD fill ↘ IL-6 levels	This study showed the efficacy of SIM as a local drug delivery system in the treatment of chronic periodontitis not only in clinical but also in molecular levels
[137] (Chile) RCT	Atorvastatin 2% atorvastatin dentifrice	36 Chronic periodontitis Controlled diabetic only All types of smoking status	Nonsurgical treatment Group I: SRP + ATV dentifrice Group II: SRP + placebo dentifrice 1 month follow-up	↘ PISA ↘ mean PD ↘ % of sites with PD ≥ 5 mm	Simvastatin increased periodontal regeneration and CAL gain

Table 6: Continued.

Reference Study area Type of study	Drug Mode of delivery Dose	Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
			Local drug delivery		
				↗ mean CAL gain ↘ % of sites with CAL ≥ 5 mm ↘ BOP ↘ GI	
[100] (India) Cohort study	Atorvastatin + simvastatin Drug in sodium alginate suspension administered with calcium chloride solution, subgingival delivery 1.2% simvastatin, or 1.2% atorvastatin	45 Moderate to severe chronic periodontitis Healthy (nonsmokers)	Nonsurgical treatment Group I: SRP alone Group II: SRP + 1.2% SIM Group III: SRP + 1.2% ATV 6 months follow-up	The test groups did not show any statistically significant difference when compared with the control group	No significant benefit for periodontal regeneration with the use of statin

Table 7: Clinical studies evaluating impact of systemic statin administration on periodontal wound healing.

Reference Study area Type of study	Drug Mode of delivery Dose	Systemic drug delivery Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
[109] (USA) Retrospective cohort study	Not reported	1021 Chronic periodontal disease All types of patients (diabetic, smokers, antibiotic users, anti-inflammatory users…)	Nonsurgical treatment Hyperlipidemic vs healthy Mean follow-up = 7.1 years	Any statin use during the first 3 years after the initial periodontal exam was associated with a 48% decreased tooth loss rate in year 4 and subsequent years	Statins reduced tooth loss in chronic periodontitis
[112] (Mexico) RCT	Atorvastatin 20 mg/day	38 Chronic periodontitis Healthy (all types of smoking status)	Nonsurgical treatment Group I: SRP + ATV Group II: SRP + placebo 3 months follow-up	↘ dental mobility ↘ distance from the crestal alveolar bone to the cementoenamel junction	Atorvastatin reduced tooth mobility and bone loss
[110] (Turkey) No control group Longitudinal	Atorvastatin 10 or 20 mg	20 Chronic periodontitis Hyperlipidemic patients (nonsmokers)	Nonsurgical treatment SRP 6 months follow-up	↘ median values for the PI, GI, PD, and BOP (%) ↗ median value of CAL gain All lipid parameters decreased after the periodontal treatment No comparison with the control group	Atorvastatin reduced periodontal breakdown Improved periodontal health may influence metabolic control of hyperlipidemia
[113] (Turkey) Cohort study	Atorvastatin 10 or 20 mg	80 Chronic periodontitis Healthy or hyperlipidemic patients (nonsmokers)	Nonsurgical treatment Group I: healthy patient + SRP Group II: hyperlipidemic patients + prescribed diet (HD) Group III: hyperlipidemic patients + atorvastatin (HS) 3 months follow-up	↗ BOP ↘ IL-6 (serum and GCF) ↘ TNF-α (GCF) levels	Systemic atorvastatin had beneficial effects on periodontal inflammation
[111] (Germany) Cohort study	Simvastatin ($n = 87$), lovastatin ($n = 27$), pravastatin ($n = 53$), fluvastatin ($n = 37$), atorvastatin ($n = 34$), and cerivastatin ($n = 42$)	2689 All types of periodontal disease Hyperlipidemic vs normolipidemic All types of smoking status	All types of periodontal treatment Group I: participants undergoing statin treatment Group II: patients without statins 5.3 years mean follow-up	No effect on PD and CAL ↘ tooth loss	Statins had the beneficial effect of protecting against tooth loss
[56] (USA) Cohort study	Simvastatin Not reported	117 Chronic periodontitis Diabetic vs healthy All types of smoking status	Nonsurgical treatment Group I: nondiabetic patients not taking statin Group II: nondiabetic patients taking statin Group III: diabetic patients not taking statin	↘ PD in diabetic patients ↗ CAL in nondiabetic patients ↘ MMP-1 level in GCF of nondiabetic and diabetic patients No difference was found for	Statin intake was associated with reduced PD in diabetic patients and MMP-1 level in GCF in either nondiabetic or diabetic patients

Table 7: Continued.

Reference Study area Type of study	Drug Mode of delivery Dose	Number of patients Periodontal status Type of patients	Type of treatment Study design (groups) Follow-up	Results	Periodontal considerations
		Systemic drug delivery			
			Group IV: diabetic patients taking statin 6 weeks follow-up	MMP-8 and MMP-9 levels in GCF	
[114] (India) Cohort study	Atorvastatin 20 mg/day	107 Chronic periodontitis Hyperlipidemic vs normolipidemic Nonsmokers	Nonsurgical periodontal treatment Group 1: hyperlipidemic + SIM Group 2: hyperlipidemic + diet Group 3: normolipidemic patients 3 months follow-up	↘ GI Mean change in PD is negatively associated with LDL-C Mean change in GI is positively associated with HDL-C	Patients with hyperlipidemia were more prone to periodontal disease Statin intake had beneficial effects on periodontal inflammation

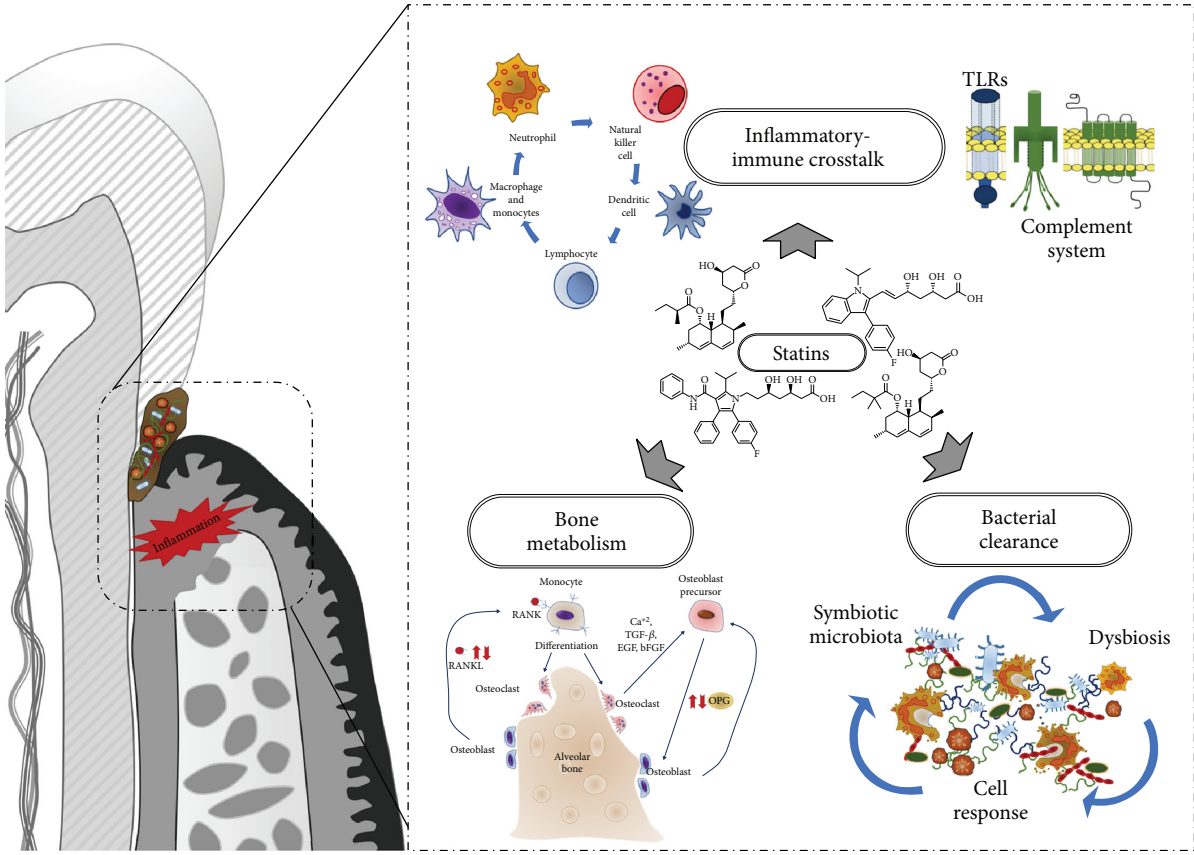

FIGURE 5: Pleiotropic effects of statins in the context of periodontitis management. Statin biological properties might be of interest for the management of periodontitis as they act on each tissular compartment and mechanisms including inflammatory-immune crosstalk, bone metabolism and bacterial clearance.

it is yet to be established if statin-induced reduction in plasma total cholesterol and LDL cholesterol levels in the periodontal space could decrease macrophage recruitment to improve the treatment outcome.

Despite the documented anti-inflammatory properties of statins, a local high-dose statin application causes considerable soft tissue inflammation [123]. Accordingly, studies determined that reducing the simvastatin dose from 2.2 mg to 0.5 mg reduced inflammation without compromising its bone growth potential [67]. A 10 mg/kg/day dose in rats is equivalent to 70 mg/day for humans, so it is a high systemic dose compared to that commonly used in clinical practice (20-40 mg/day) [124].

Concerning locally applied statins, most clinical studies investigated the 1.2% dose (mainly atorvastatin, simvastatin, and rosuvastatin) [20, 23, 125, 126]. Therefore, other doses should be tested to compare efficacy.

Most of the review articles have focused on the use of statins as adjunct to the nonsurgical SRP in clinical settings [127–129]. Here, this review encompasses the use of statins (local, systemic, or combination), alone or in addition to other drugs or scaffolds, in nonsurgical or surgical periodontal treatment *in vitro*, *in vivo*, and in clinical trials. However, the potential of statins in surgical periodontal therapy remains relatively less explored except for a few studies where treatment outcomes were improved, primarily, with the combination of some other regenerative agents such as allograft or PRF [105, 106]. Cognizant of the numerous studies involving statins, not all statin types have been studied so far; thus, exploring all natural and synthetic statins to compare their efficacy and safety could be instrumental.

Notably, 17 out of 32 clinical studies were carried out by the same group of researchers on similar population; therefore, generalizations should be drawn with caution. Additionally, in most studies involving statins, the follow-up period was no longer than 9 months [103, 130]. Hence, it is imperative to follow clinical studies for periods longer than those commonly investigated so as to achieve a deeper and more genuine insight into their long-term benefits. Discrepancies amongst outcomes between time points are of importance to clearly conclude. For instance, the meta-analysis performed by Sinjab et al. [131] declared the outcomes of the control group of a study [20] to be better by considering the data up to 6 months follow-up, whereas the meta-analysis performed by Ambrósio et al. regarded the treatment group of the same study to have better outcomes as the follow-up data until 9 months was taken into account [132].

Moreover, the studies carried out so far mainly involved hyperlipidemic patients, diabetic patients, or smokers. Systemic diseases, such as obesity or metabolic syndrome, have been linked with periodontitis [133]. It has been demonstrated that such conditions modify significantly the host

response to periodontal pathogens [134] but also could impaired treatment response. For instance, in a rat model of metabolic syndrome, the effects induced by statins in rats with metabolic syndrome were different in comparison with rats without [32] highlighting the potential modulation of pharmacologic effect due to the systemic condition. Even if clinical trials performed in diabetes patients or exhibiting hyperlipidemia showed promising results when statins were administered concomitantly to nonsurgical periodontal treatment [56, 110, 113, 114], more studies are required to better understand the differential biological mechanisms modulated by statin's administration. It would also be of importance to assess statins' tolerance and efficacy in subjects with different systemic conditions where periodontal treatment response is impaired (e.g., liver diseases, kidney dysfunction, and immunocompromised states).

In clinical trials, the local application of statins with surgical periodontal treatment always showed significant improvements in periodontal parameters [105, 106]. However, *in vivo*, statin application in ACP models showed contradictory results [99] which could be explained by the limitations of animal models to simulate conditions identical to human periodontal disease. Nevertheless, as a direct optimization of treatment protocols in humans is not ethically permissible, the utility of preclinical models to get directions and overall assessment of the expected treatment outcomes in clinical scenarios cannot be undermined.

Concerning the systemic administration of statins, a study reported that using a combination of two pharmacokinetically different statins (20 mg/day of atorvastatin plus 40 mg/day of pravastatin) in hyperlipidemic patients for one year improved their lipid profiles compared to those on monotherapies [135]. Besides, a case of a hyperlipidemic patient experiencing certain side effects with a high dose of systemic simvastatin who could well tolerate a combination of reduced doses of simvastatin and rosuvastatin instead has also been reported [136]. To the best of our knowledge, no two statins have been combined for periodontal treatment so far; nonetheless, combination of two statins could be tested for its impact on periodontal treatment response.

Likewise, the impact of incorporating statins with antimicrobial agents, growth factors, or other proregenerative molecules within a local application system could be studied as adjunct to SRP. Statin integration into gels [21] or dentifrice [137] could enhance ease of application and patient's compliance and could be potentially beneficial in the maintenance phase to counter periodontal breakdown that persists after conventional periodontal treatment. The literature does not report the impact of statins on patients with extremely poor oral hygiene; nonetheless, it could be interesting to explore the impact of statins on oral hygiene indicators.

5. Conclusion

Statins have been studied in depth in the context of bone regeneration, but soft tissue healing remains relatively less explored. Further research into it could present statins as a potential adjunctive therapeutic strategy with a positive impact on both hard and soft periodontal tissue healing. Furthermore, the impact of statins on proresolution molecules has not been investigated in the context of periodontal wound healing and regeneration. This could unveil new vistas for statins as regenerative therapeutics. Since all available statins have not been tested yet, new studies need to evaluate the impact of other statins on antibacterial, inflammatory, immune, and osteoprogenitor responses. To conclude, choosing an optimum dose of statins, based on the mode of drug delivery and the carrier employed, may enhance the positive impact of statins on the periodontal treatment outcomes. Moreover, combining statins with growth factors or other drugs in an efficient carrier system may be beneficial to promote periodontal regeneration.

Abbreviations

M1:	First molar
M2:	Second molar
M3:	Third molar
mPEG:	Polyethylene glycol monomethyl ether
PDLLA-PLGA:	Poly-(d,l-lactide) and poly-(d,l-lactide-co-glycolide
BSA:	Bovine serum albumin
PDGF:	Platelet-derived growth factor
PM:	Premolar
PDL:	Periodontal ligament cells
EGCG:	Epigallocatechin-3-gallate
CS:	Chitosan
BALP:	Bone alkaline phosphatase
LPS:	Lipopolysaccharide
PBS:	Phosphate buffered saline
ALN-CD:	Alendronate-β-cyclodextrin
SIM:	Simvastatin
CEJ:	Cementoenamel junction
HA:	Hydroxyapatite
TGF-β:	Transforming growth factor beta
E. coli:	*Escherichia coli*
PPi:	Isopropyl alcohol
TRAP:	Tartrate-resistant acid phosphatase
GSH:	Glutathione
MDA:	Malondialdehyde
MPO:	Myeloperoxidase
GIOP:	Glucocorticoid-induced osteoporosis
DKK1:	Dickkopf-related protein
CAT:	Enzyme catalase
SOD:	Enzyme superoxide dismutase
MMPs:	Matrix metalloproteinases
MCP:	Monocyte chemotactic protein
CSF:	Colony-stimulating factor
A.a:	*Aggregator actinomycetemcomitans*
P.g:	*Porphyromonas gingivalis*
COX:	Cyclooxygenase
ALP:	Alkaline phosphatase
AST:	Aspartate aminotransferase
ALT:	Alanine aminotransferase
IP:	Intraperitoneal
TG:	Triglyceride
ATV:	Atorvastatin
PD:	Pocket depth

RANKL: Receptor activator of the NF-κB ligand
RANK: Receptor activator of NF-κB
OPG: Osteoprotegerin
OPN: Osteopontin
BV/TV: Bone volume/tissue volume
CAL: Clinical attachment level
SRP: Scaling and root planing
INFRA: Radiographic infrabony defect fill
MF: Metformin
DDR: Defect depth reduction
DFDBA: Demineralized freeze-dried bone allograft
OFD: Open flap debridement
BOP: Bleeding on probing
GI: Gingival index
PI: Plaque index
mSBI: Modified sulcus bleeding index
IBD: Intrabony defect
PRF: Platelet-rich fibrin
PISA: Periodontal inflamed surface area
LDL-C: Low-density lipoprotein cholesterol
HDL-C: High-density lipoprotein cholesterol
OB: Osteoblasts
OC: Osteoclasts
EIP: Experimentally induced periodontitis
ACP: Acute/chronic periodontal defect
NOX: Nitrate/nitrite levels
VEGF: Vascular endothelial growth factor.

Conflicts of Interest

The authors declare no conflicts of interest related to this study.

Authors' Contributions

CP and FB performed the electronic search and drafted the manuscript. OH drafted and critically revised the manuscript. IB, PS, and NB-J critically revised the manuscript. All authors reviewed the final version of the manuscript. Catherine Petit and Fareeha Batool contributed equally to this work.

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Supplementary Materials

Risk of bias assessment of included clinical studies. *(Supplementary Materials)*

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Research Article

CEMP-1 Levels in Periodontal Wound Fluid during the Early Phase of Healing: Prospective Clinical Trial

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Objectives. Cementogenesis seems to be significantly compromised during tissue inflammation. In dental practice, surgical procedures are performed with the aim to regenerate periodontium including cementum. However, inflammation that occurs during the initial healing phases after surgery may impair regeneration of this tissues. The aim of the present study was to assess if surgical procedures designed to regenerate periodontium might affect levels of cementum protein-1 (CEMP-1) in periodontal wound fluid during early phase of healing. *Materials and Methods*. In 36 patients, 18 intrabony periodontal defects were treated with regenerative therapy (REG group) and 18 suprabony periodontal defects were treated with open flap debridement (OFD group). In the experimental sites, gingival crevicular fluid was collected immediately before surgery, and periodontal wound fluid was collected 4, 7, 14, and 21 days after surgery. CEMP-1 levels were detected by indirect enzyme-linked immunosorbent assay technique. *Results*. At the analysis, it resulted that there was a significant average difference in CEMP-1 values between the REG and OFD groups at baseline ($p = 0.041$), the CEMP-1-modeled average in the OFD group was lower by 0.45 ng/ml. There was a significant trend in CEMP-1 over time, and this trend was different among the 2 groups: the REG group showed a statistically significant rising CEMP-1 trend (0.18 ng/ml a week $p = 0.012$), while the OFD had a trend that was significantly lower (-0.22 ng/ml a week compared to the REG group trend $p = 0.023$), the OFD group lost on average 0.05 ng/ml a week. In REG sites, GCF protein levels resulted also related to clinical parameters. *Conclusions*. During the initial inflammatory phase of periodontal healing, CEMP-1 levels decrease regardless of the surgical protocol applied. The surgical procedures used to regenerate periodontal tissue are able to reverse this trend and to induce significant increase of CEMP-1 in periodontal wound fluid after the first week postop.

1. Introduction

Gingival crevicular fluid (GCF) is a physiological serum transudate that flows through the junctional epithelium to gingival sulcus and that can be collected at the gingival margin or within the gingival crevice. This fluid is called periodontal wound fluid (PWF) when it is derived from postsurgical healing sites. After periodontal surgical trauma, cell-signaling protein molecules (e.g., growth factors, chemokines, or cytokines) and products of cellular activity (enzymes and adhesion molecules) are released in the wound-healing area. Levels of cytokines, chemokines, and angiogenic biomarkers within the gingival crevicular fluid and in periodontal wound fluid have been studied in clinical trials to assess the ongoing angiogenesis, connective tissue, and bone formation activities during wound-healing phases (inflammation, granulation tissue formation, and tissue neoformation/remodeling) [1, 2]. Morelli et al. [3] evaluated changes of angiogenic markers in wound fluid after placement of a soft tissue autograft or of a living cellular construct for treatment of mucogingival defects. The authors observed that these procedures heal in two different ways and that levels of the analyzed biomarkers

also resulted different. Eren et al. [4] characterized the wound-healing activity and inflammation of localized gingival recession defects treated with coronally advanced flap plus platelet-rich fibrin compared with coronally advanced flap plus connective tissue graft, and they observed that platelet-rich fibrin may promote early wound healing by elevating the levels of tissue inhibitor of matrix metalloproteinases-1 and suppressing the levels of proinflammatory and remodeling molecules (matrix metalloproteinase-8 and IL-1b) in gingival crevicular fluid at 10 days after surgery. Pellegrini et al. [5] assessed the levels of proteins related to epithelium, connective tissue, and bone healing as possible biological indicators of clinical outcome at 6 months after surgery. Comprehension of physiology of cementum and related molecules is important for the development of potential new therapies in periodontal regeneration. However, clinical studies evaluating specific markers for cementum activity are lacking. Cementum protein-1 (CEMP-1) is a tissue-specific protein for cementum. This protein is only expressed by cementoblasts, their progenitors, and by periodontal ligament-derived cells [6, 7]. CEMP-1 has been detected lining the cementum surface, in the perivascular area and within the periodontal ligament throughout the root surface [6]. One in vitro study observed that CEMP-1 stimulates migration and proliferation of periodontal ligament cells and promotes cell differentiation, maturation, and deposition of mineralized extracellular matrix resembling cementum [7]. It also reduces the level of osteoblastic markers and increases the amount of cementoblastic markers. Furthermore, overexpression of CEMP-1 was found to slightly increase cementogenesis and differentiation of cementoblasts [7]. The levels of CEMP-1 reduce significantly after stimulation of cementoblasts with IL-1β, and cementogenesis may be significantly compromised during tissue inflammation [8]. In dental practice, surgical procedures are performed with the aim to regenerate periodontium including cementum, ligament, and alveolar bone. However, inflammation that occurs during the initial healing phases after surgery may impair this tissue regenerative activity.

The aim of the present study was to assess if surgical procedures designed to regenerate periodontium might affect levels of CEMP-1 in periodontal wound fluid during initial healing.

2. Materials and Methods

This is a prospective clinical trial. The study has been approved by the ethical committee of Università degli Studi di Milano (Italy) (18.10.11, number 30/11). All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

2.1. Study Population. A total of 36 volunteers were enrolled: 18 patients that had an intrabony periodontal defect requiring regenerative therapy (REG group) and 18 patients that had a horizontal periodontal defect (without intrabony components) requiring open flap debridement (OFD group). Enrolled patients presented the following inclusion criteria:

(i) age range: 25-80 years old

(ii) nonsmoking (former smokers were included if they had not smoked within 6 months of the study initiation)

(iii) suffering from periodontitis at stage 3 grade B [9] and completed the initial periodontal therapy from at least 3 months

(iv) defect anatomy OFD group: presence of at least one tooth with probing pocket depth (>5 mm) and clinical attachment level with an intrabony defect ≤ 3 mm

(v) defect anatomy REG group: presence of at least one tooth with probing pocket depth (>5 mm) and clinical attachment level of ≥5 mm associated with an intrabony defect of >3 mm

(vi) good oral hygiene: full mouth plaque and bleeding scores ≤ 20% at the beginning of the study (baseline)

(vii) experimental teeth had to be vital or properly treated with root canal therapy

(viii) subgingival margins, open margins, overhanging margins, and inadequate restorations had to be absent in the experimental site

The following exclusion criteria were applied:

(i) patients with clinically significant or unstable organic diseases, immune-compromised, taking steroid medications, chronically treated (i.e., two weeks or more) with any medication known to affect periodontal status (i.e., antibiotics or nonsteroidal anti-inflammatory drugs), and taking antibiotics within 90 days of baseline

(ii) patients displaying compromised healing potential such as those affected by connective tissue disorders or bone metabolic diseases; patients with conditions requiring antibiotic prophylaxis

(iii) pregnant or lactating women, or women who were of childbearing potential and not using birth control or abstinence

(iv) current smokers or former smokers who had smoked in the previous 6 months

(v) patients affected by active infectious diseases

2.2. Study Procedures. The study timeline is reported in Figure 1. After verification of the inclusion/exclusion criteria and sign of the informed consent, each patient was enrolled into the study. Gingival crevicular fluid was harvested and clinical measurements and radiographs were taken (baseline). Before the surgical appointment, all patients

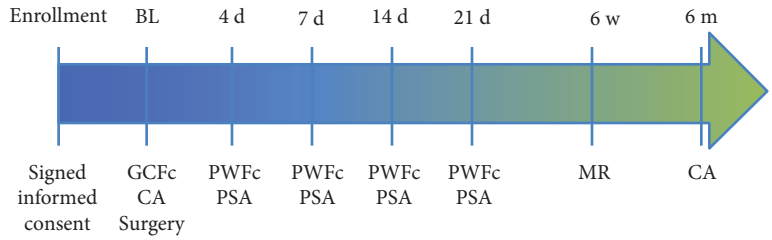


Figure 1: Timeline of the study. GCFc: gingival crevicular fluid collection; CA: clinical assessments (probing pocket depth, clinical attachment level, full mouth plaque and bleeding, photographs, X-ray); PWFc: periodontal wound fluid collection; PSA: postsurgical clinical assessments of healing; BL: baseline; d: days; MR: membrane removal.

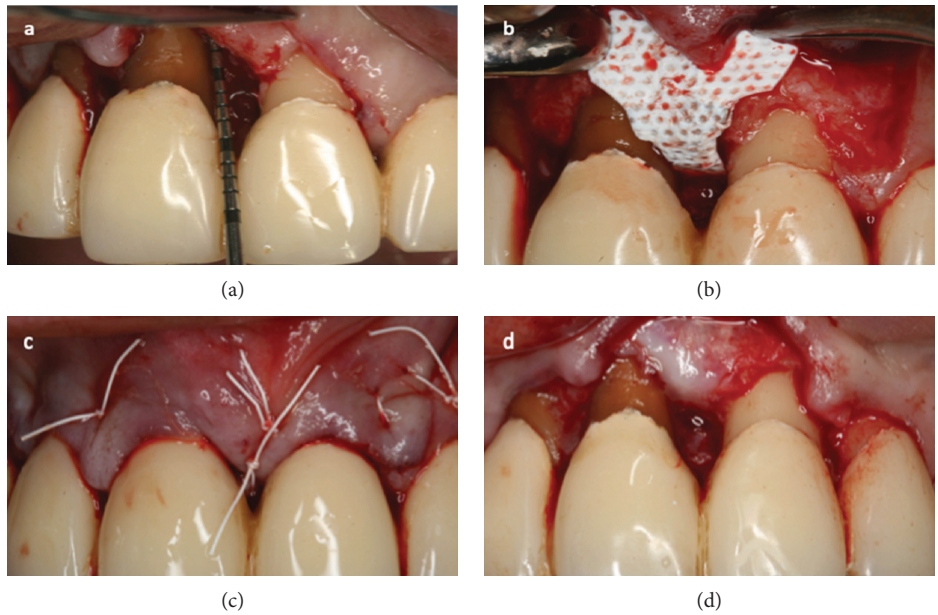


Figure 2: In the sites with intrabony periodontal component, after flap elevation, the granulation tissue was removed and roots were planned (a). The defect was covered with nonresorbable titanium-reinforced completely inert membrane (dense polytetrafluoroethylene) alone with no bone substitutes (b) and closed with a single-modified internal mattress suture (c). The membrane was removed 6 weeks after surgery (d).

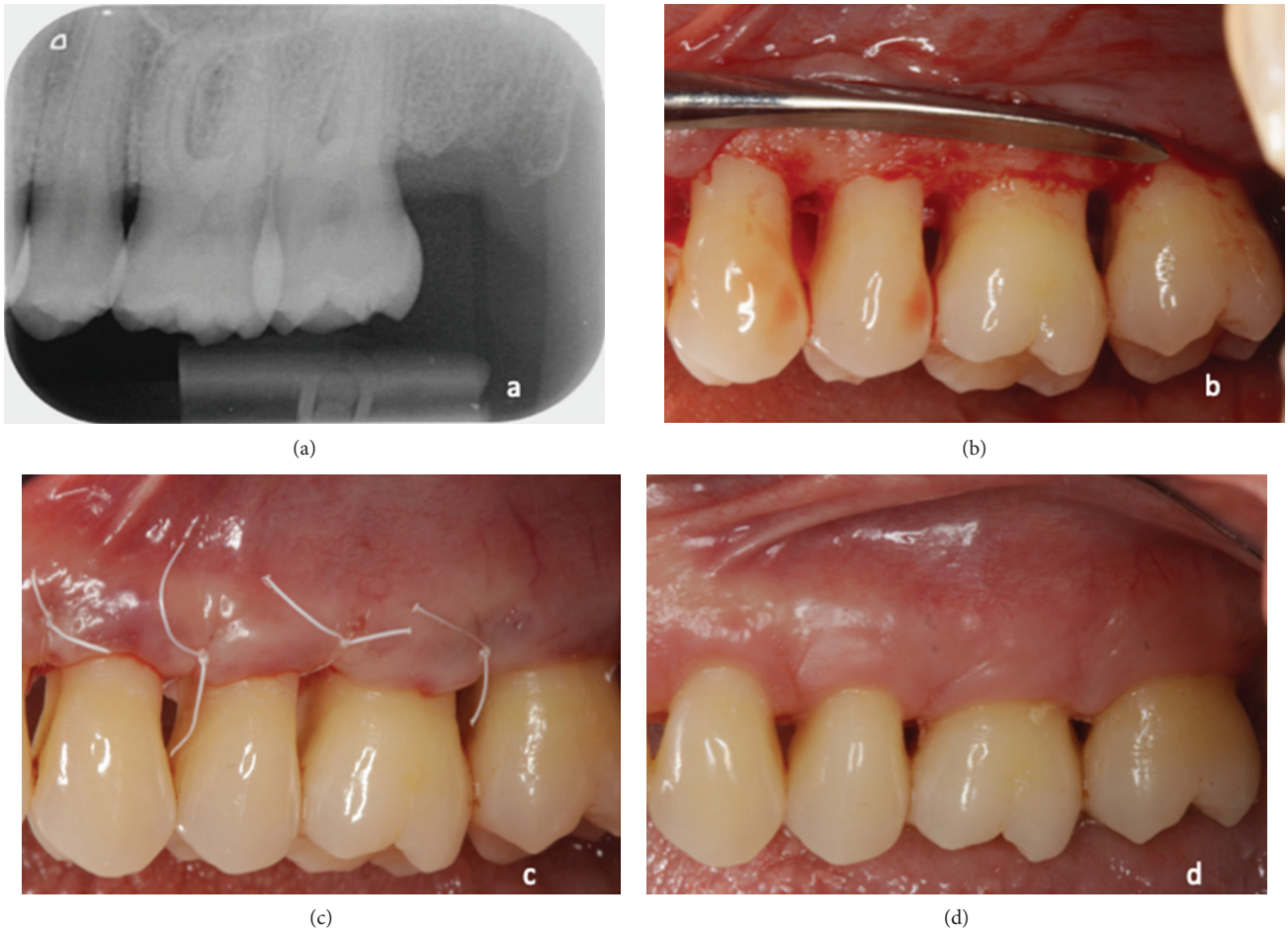
underwent professional oral hygiene procedure and instructions were given to eliminate any infective complications. Each patient's compliance to the experimental protocol was also confirmed.

In both groups (open flap debridement and regenerative therapy), local anesthesia with mepivacaine 2% 1 : 100.000 epinephrine was administered. In all the sites (OFD and REG), full-thickness flap was incised and elevated.

In the 18 REG sites, the simplified papilla preservation technique was adopted whenever the width of the interdental space was 2 mm or narrower, while the modified papilla preservation technique was applied when the interdental sites were wider than 2 mm [10, 11]. The intrasulcular interdental incision (SPPF or MPPT) was extended to the buccal and lingual aspects of the mesial and distal teeth adjacent to the defect. After flap elevation, the granulation tissue was removed, and the intrabony defects were cleaned by hand instrumentation, ultrasonic scalers, and the root planning was done. Vertical releasing incisions were performed when flap reflection caused tension at the extremities of the flap(s). REG defects were covered with a nonresorbable titanium-reinforced completely inert membrane (dense polytetrafluoroethylene) (Cytoplast®, Osteogenics Biomedical, Lubbock, Texas, USA) alone with no bone substitutes and closed with a single modified internal mattress suture (polytetrafluoroethylene 6/0); thus, a tension-free primary closure of the papilla was obtained (Figure 2).

In the OFD sites, modified Widman Flap was performed [12]. After flap elevation, the granulation tissue was removed, and the horizontal defects were cleaned by hand instrumentation, ultrasonic scalers, and the roots were planned. The OFD sites were closed with a single external mattress suture (polytetrafluoroethylene 5/0) (Figure 3).

Postoperative pain and edema were controlled with ibuprofen. Patients received 600 mg at the beginning of the surgical procedure and were instructed to take another tablet



(a) (b) (c) (d)

Figure 3: Modified Widman Flap was performed in sites with suprabony periodontal defects (a). After flap elevation, the granulation tissue was removed and roots were planned (b). Sites were closed with single external mattress suture (c). Sutures were removed 1 week after surgery (d).

6 hours later. Subsequent doses were taken only if necessary to control pain. Patients with ulcers, gastritis, and other contraindications to nonsteroidal anti-inflammatory drugs received 500 mg acetaminophen. All patients were instructed to intermittently apply an ice bag on the operated area (20 minutes per hour for 24 hours). All patients were instructed to discontinue tooth brushing and avoid trauma at the surgical site for a period of time ranging between 3 and 4 weeks. A 60-second rinse with 0.12% chlorhexidine digluconate was prescribed 3 times/day for the first 3 to 4 weeks.

At 4, 7, 14, and 21 days after surgery, periodontal wound fluid was collected by a blind operator (EC). Clinical measurements were taken 6 months after surgery.

2.3. Gingival Crevicular Fluid or Periodontal Wound Fluid Harvesting and Analysis. In all the patients, gingival crevicular fluid or periodontal wound fluid was collected from the study site at baseline, and 4, 7, 14, and 21 days after surgery (Figure 1) as previously reported [13]. Prior to crevicular fluid collection, supragingival plaque biofilm in the area around each sample was removed and the site was air-dried. Two methylcellulose paper strips (Periopaper®, ProFlow Inc., Amityville, NY) were gently inserted in the gingival sulcus or periodontal pocket for 1-2 mm. The fluid sample was then collected for 30 seconds, and the strips were placed into the Eppendorf tubes. The samples of gingival crevicular fluid and periodontal wound fluid were subsequently kept on dry ice and stored at -20°C until analysis.

Prior to biomarker analysis, gingival crevicular fluid and periodontal wound fluid were thawed at room temperature, and proteins were eluted with 100 μL of 1× phosphate buffered saline solution and Protease Inhibitor PMSF (Thermo Fisher Scientific, Italy). To retrieve the crevicular fluid sample, the paper strips were centrifuged at 15 000×g for 5 min for 4 times, and the total volume of 100 μL was obtained. The indirect enzyme-linked immunosorbent assay technique was used to detect and quantify levels of CEMP-1 protein (ng/ml) in the crevicular/wound fluid using the MBS702364 kit (MyBioSource) according to manufacturer's instruction. The indirect enzyme-linked immunosorbent assay test was performed measuring the absorbance at 450 nm by means of a spectrophotometer plate reader (Wallac Victor II plate reader).

Table 1: Data on study population.

Parameter	Mean ± sd in the REG group	Mean ± sd in the OFD group	P value between the groups
Age (years)	55.9 ± 9.2	58.3 ± 11.6	ns
Full mouth plaque score (%)	5.7 ± 2.8	6.5 ± 2.9	ns
Full mouth bleeding score (%)	3.9 ± 1.8	4.6 ± 2.9	ns
Probing pocket depth at experimental sites (mm)	8 ± 1.8 (BL) 4.1 ± 1 (6 m)	5.3 ± 0.6 (BL) 2.9 ± 0.7 (6 m)	ns
Clinical attachment level at experimental sites (mm)	9.7 ± 2.9 (BL) 5.4 ± 2.1 (6 m)	6.2 ± 1.5 (BL) 4.3 ± 1.1 (6 m)	ns
Experimental sites that bleed on probing at baseline (%)	61.1% (BL) 5.5% (6 m)	44.4% (BL) 0 (6 m)	ns

REG: sites that underwent to regenerative therapy; OFD: sites treated with open flap debridement; NS: not statistically significant; BL: baseline; 6 m: 6 months.

2.4. Clinical and Radiographical Analysis. The following clinical measurements were taken at baseline and 6 months after surgery:

(i) full mouth plaque and bleeding score at the four sites for all teeth

(ii) probing pocket depth, recession, and clinical attachment level (calculated as the sum of probing pocket depth and recession) were assessed at six sites of each tooth treated

Reduction of probing pocket depth and gain of clinical attachment level were calculated, respectively, as the difference between probing pocket depth or clinical attachment level at baseline and probing pocket depth or clinical attachment level at 6 months.

All measurements were taken with a UNC periodontal probe (Hu-Friedy Manufacturing Company Inc., Chicago, IL, USA).

Intraoral radiographs of the defect were taken using Rinn's attachment and a long cone parallel technique at baseline and 6 months after periodontal surgery.

Intraoral photographs of the experimental sites were taken before surgery, after defect debridement, after flap closure, at weeks 1, 2, and 3, and at 6 months.

2.5. Statistical Analysis. Each patient represented a statistical unit, and only one defect was treated for each volunteer. In both groups, mean and standard deviation were calculated for probing pocket depth (mm) and clinical attachment level (mm) at baseline and at 6 months postop and for levels of CEMP-1 (ng/ml) at baseline and 4, 7, 14, and 21 days after surgery.

Preliminary analysis was carried out at baseline measuring the difference in mean CEMP-1 (ng/ml) levels between the REG and OFD groups with a t-test. As a first evaluation, t-tests of average differences between CEMP-1 at baseline and treatment day 21st were carried out between the groups (i.e., REG and OFD); furthermore, t-tests for paired data were applied to both the REG and OFD groups between CEMP-1 levels at baseline and day 21st. These evaluations were only carried out between measurements at baseline and at day 21st to avoid pseudoreplication and multiple testing issues.

To properly characterize the data, a linear mixed model was used, in order to account for repeated measures on patients over time [14]. The study treatment group (REG vs OFD) and time (observation week) and their interaction to capture nonparallel growth trends were included as fixed effects; patients were considered as random effects in particular with respect to time (days). Finally, since the same patients were measured over time, a first order autocorrelation structure was used. The nlme library from the R-project statistical suit was used to fit the linear mixed model. A level of significance of 5% ($p < 0.05$) was considered.

3. Results

A total of 36 nonsmoker patients, 18 for each group, were enrolled. One patient in the OFD group discontinued early, and analysis was performed on 18 patients of the REG group (12 females and 6 males; mean age, 55.9 ± 9.2) and on 17 patients of the OFD group (10 females and 7 males; mean age, 58.3 ± 11.6). Demographic and clinical data of patients are reported in Table 1, and no significant differences were found between the groups. Uneventful wound healing occurred in all the operated sites. In the sites treated with regenerative procedure, no membrane exposure occurred, and all membranes were removed at 5-6 weeks after surgery.

3.1. CEMP-1 Levels. Levels of CEMP-1 in gingival crevicular fluid at baseline and periodontal wound fluid at 4 days and 1, 2, and 3 weeks postop in the sites treated with regenerative approach and open flap debridement are reported in Figure 4.

At baseline, CEMP-1 showed significantly different levels between the REG and OFD groups (OFD mean 0.80, REG mean 1.39; t-test, $p = 0.035$); variability was also different between the two groups (OFD variance 0.46, REG variance 0.78).

Due to these differences at baseline, direct comparisons of CEMP-1 at later time points would have been inappropriate, to make up for this bias a t-test was performed on the difference from baseline CEMP-1 values between the groups

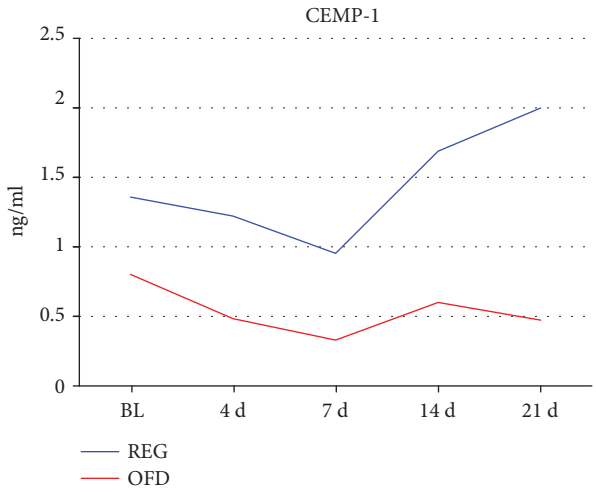


Figure 4: Levels of CEMP-1 in gingival crevicular fluid (at baseline, 0) and periodontal wound fluid at 4, 7, 14, and 21 days (d), postop in sites treated with regenerative approach (REG) and open flap debridement (OFD).

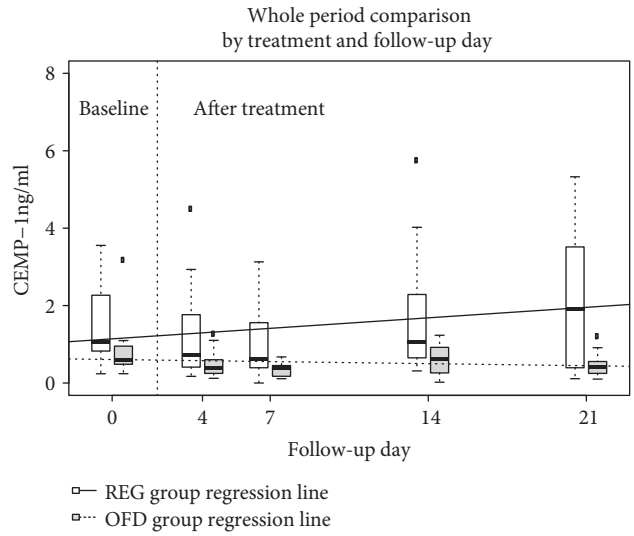


Figure 5: Plot representing distribution of CEMP-1 in ng/ml by treatment and week (baseline is day 0, while days 4 to 21 are after treatment) using box plots. The REG group is white, OFD is grey, the thick black line is the median, the box contains the 1st to the 3rd quartile, and the whiskers are the lowest and highest values within 1.5 times the interquartile range from the box, other values are represented as outliers. The regression lines from the linear mixed model are also plotted (REG, line; OFD, dashed line).

at day 21st. This test was also significant ($p = 0.031$), with the REG group recording a 0.64 ng/ml rise in CEMP-1, and the OFD a 0.32 ng/ml fall between baseline and day 21st. Finally, to determine whether the change from baseline within each group was significant, pairwise t-tests were performed on the REG and OFD groups between baseline and day 21st individually. These tests indicated that the change in the REG group, even though larger (+0.64 ng/ml) was not statistically significant ($p = 0.12$), while the smaller change in the OFD group (-0.32 ng/ml) was barely significant ($p = 0.049$), this result underscores the difference in variance between the 2 groups.

Figure 5 represents the data by timepoint (baseline and 21 days after intervention) and by the treatment group using boxplots. On inspection, strong differences in CEMP-1 levels emerged, both in value and in variability. The REG group CEMP-1 average and median values are higher than the OFD group ones at all time points, but also noticeably more dispersed as shown in the descriptive and preliminary analysis above. The model confirmed what can be seen in the data from inspection i.e., (1) that there was a significant average difference in CEMP-1 values between the REG and OFD groups at baseline ($p = 0.041$), the CEMP-1 modeled average in the OFD group was lower by 0.45 ng/ml, (2) there was a significant trend in CEMP-1 over time and this trend was different among the 2 groups: the REG group showed a statistically significant rising CEMP-1 trend (0.18 ng/ml a week $p = 0.012$), while the OFD had a trend that was significantly lower (-0.22 ng/ml a week compared to the REG group trend $p = 0.023$), the OFD group lost on average 0.05 ng/ml a week.

3.2. Analysis of Correlation. A significant correlation (Spearman correlation) was found in the REG sites among

(i) CEMP-1 levels at 3 weeks and probing pocket depth reduction (($p = 0.041$, $r = 0.486$), ($p = 0.801$ in the OFD sites))

(ii) differential CEMP-1 levels between 3 w and baseline and probing pocket depth reduction (($p = 0.041$, $r = 0.485$), ($p = 0.128$ in the OFD sites))

(iii) differential CEMP-1 levels between 3 w and baseline and probing pocket depth baseline (($p = 0.027$, $r = 0.519$), ($p = 0.102$ in the OFD sites))

4. Discussion

During the early (inflammatory) phase of healing that occurs after periodontal surgery, the levels of inflammatory markers peak, thus negatively affecting periodontal as well as cementum neoformation and shifting healing toward a more reparative than regenerative process [8, 15]. In periodontal regenerative surgery, accuracies are put in place to achieve regeneration of periodontium (alveolar bone and cementum with inserted perpendicularly oriented periodontal ligament fibers) and the trend of regenerative markers may be affected accordingly. In the present study, we evaluated if the levels of CEMP-1 in periodontal wound fluid change during initial healing after surgical procedure designed to regenerate periodontium. From data, it resulted a different trend of PWF CEMP-1 amount after periodontal regenerative surgery compared to periodontal surgery alone. In the REG sites, after initial decrease, the protein level increased significantly, while in the OFD sites remained at levels significantly lower than baseline. The decreased amount of this protein observed in all the treated sites after surgery may have resulted from the activation of inflammatory cells, in the initial healing phase [8]. Initial defect degranulation and subsequent removal of cells that synthesize CEMP-1 may also have

delayed the production of proregenerative proteins as well as CEMP-1. In the OFD sites, the sustention of low levels of the analyzed protein may indicate the ongoing periodontal tissue repair with limited formation of new cementum/mineralized tissue. After OFD, the recruitment and activation of cells may mainly be devoted to epithelium and connective tissue healing as supported by increased amounts of fibroblast growth factor-2 and transforming growth factor-β [1]. On the contrary, in the REG sites, the regenerative procedure seems to invert this tendency increasing the CEMP-1 secretion by cementoblasts; the mineralization activity seems to resume after the initial flexion, thus suggesting the beginning of the cementum regeneration with insertion of periodontal ligament fibers. These data on different amounts of protein for tissue neoformation confirm those of previous study. A different trend of local levels of matrix metalloproteinase-1 and bone morphogenetic protein-7 during early wound healing was found between periodontal surgery associated with regenerative procedure and periodontal surgery alone. Furthermore, this data was found to be related with the clinical outcome of periodontal regenerative surgery, while sites that underwent open flap debridement showed no such association, thus suggesting that the connective tissue neoformation and remodelling was ongoing at the site treated with regenerative therapy [5].

CEMP-1 has been analyzed in the present study as a tissue-specific protein for cementum. This protein is expressed by cementoblastoma-derived cells and periodontal ligament cells and is localized throughout the cementum [6]. CEMP-1 seems to play a role in the early phases of mineralization by promoting the formation of apatite crystals [6]. Within the periodontal ligament, this protein stimulates the recruitment, proliferation, and maturation of mesenchymal stem cells promoting their mineralization activity [16]. Data on class II furcation defects of monkeys treated with Matrigel® matrix alone or associated with human transforming growth factor-β3 demonstrated that this growth factor induces cementogenesis by upregulation of CEMP-1 and that CEMP-1 is an indicator of cementogenesis [17].

The correlation analysis has shown that the increase of CEMP-1 from baseline in the REG sites is moderately but significantly related to PD reduction at 6 months. It can be speculated that the short-term clinical outcome of periodontal regenerative therapy may be predicted by early molecular analysis of wound fluid. The sites with intrabony defects treated with the regenerative approach showed significantly higher levels of CEMP-1 than OFD sites both at baseline and at all postop appointments, and the probing pocket depth at baseline was related to the increase of CEMP-1 from baseline to 3 weeks. Periodontal defects with a vertical bone component have higher physiological remodeling activity and innate regenerative potential than those with suprabony component, and this may induce higher basal levels of CEMP-1. For ethical reason, it was not possible to apply the same surgical protocol to infrabony and suprabony defects. The two interventions selected for this trial are different in their nature, surgical protocol, and expected periodontal healing events (regeneration vs. repair). In the sites where the primary goal of intervention was tissue regeneration, it may be expected that the blood clot stabilized under the barrier membrane is progressively replaced by a new bone, periodontal ligament, and cementum, as demonstrated histologically in humans [18]. Otherwise, in the sites treated with OFD where the primary goal was the pocket reduction without tissue regeneration, no relevant cementogenesis or osteogenesis is expected, and human histological studies reported the formation of long junctional epithelium with parallel-oriented collagen fibers [19, 20]. On the base of these different healing profiles, the postop higher levels of CEMP-1 found in the REG sites than those in the OFD sites may reflect the stronger cementogenesis and tissue mineralization that occur in infrabony defects after regenerative procedure.

Ethical limits of harvesting periodontal tissue after regeneration in patients make the research of alternative noninvasive methods to assess biological events that occur during healing necessary. In clinical practice, evaluation of cementogenesis by analysis of wound fluid may be useful to predict the success of periodontal regeneration as well as to support the development of further regenerative approaches. To our knowledge, the present study is the first to define the possible use of CEMP-1 as an early predictor of clinical outcome and as a marker for cementogenesis, by confirming its presence in gingival crevicular fluid/periodontal wound fluid of patients and finding increased levels of this protein mostly in the sites where regeneration of periodontal complex was expected. Suprabony defects were chosen for comparison since their different healing models supposedly induce a limited CEMP-1 production thus highlighting the role of this protein during healing. As the last point, it is necessary to identify the reference level over which periodontal regenerative therapy can be defined as successful. This reference level may be hardly deductible, due to the high variability in CEMP-1 levels found especially in the REG group. This data that may reflect the interindividual variability in regenerative potential that normally exists between patients, as well as the intraoperator variability for surgical performances. For these reasons, further studies with increased number of samples and histological confirmation in humans are needed.

The application of this protein as an agent promoting cementum regeneration would also be interesting. The effects of bioresorbable scaffold loaded with rhCEMP-1 on the attachment, proliferation, and osteogenic and cementogenic differentiations of human periodontal ligament cells have been investigated histologically in a critical size defect on rodent, and the potential of generating cementum-like tissue in vitro and in vivo has been demonstrated [21].

5. Conclusions

Data from this study supports that, after surgery, during the initial inflammatory phase of periodontal healing, CEMP-1 levels decrease regardless of the surgical protocol applied. The surgical procedures used to regenerate periodontal tissue are able to reverse this trend and to induce significant increase of CEMP-1 in periodontal wound fluid after the first week postop.

Limited to gingival crevicular fluid/periodontal wound fluid, CEMP-1 could be a marker for possible ongoing

cementogenesis during the first 21 days of healing. It would be interesting to design further clinical and histological studies evaluating levels of this protein in tissue and in crevicular fluid of healthy sites as well as after different nonsurgical/surgical procedures with the purpose to define a reference value that may discriminate between healing patterns.

Data Availability

The molecular and clinical data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Research Article

Are There Any Common Genetic Risk Markers for Rheumatoid Arthritis and Periodontal Diseases? A Case-Control Study

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Background. Several studies suggest that there is a biologically plausible connection between rheumatoid arthritis (RA) and periodontal diseases (PD). Both disorders are characterized as multifactorial diseases potentially sharing common risk factors. Based on the inflammatory nature of RA and PD, the impact of genetic variations of genes of the immune system on both diseases was studied in this study. *Materials and Methods*. We conducted a case-control study ($n = 201$) comparing 101 RA patients suffering from periodontal disease of different severities (no/mild PD vs. severe PD) with 100 systemically healthy controls without RA and severe PD. The genotype, allele, and haplotype distributions of 22 SNPs of 13 pro- and anti-inflammatory cytokines were assessed applying sequence-specific PCR. *Results*. Evaluating the impact of cytokine SNPs in RA, we identified the G allele of rs1801275 in IL4Rα ($p = 0.043$) and the G allele of rs361525 in TNFα ($p = 0.005$) as disease-associated risk factors in bivariate analyses. In multivariate analyses, these significant associations could not be proven. The A allele of rs2430561 in IFNγ was indicative for severe periodontitis among the patients with rheumatoid arthritis ($p = 0.039$). Investigating the impact of rs2430561 in IFNγ on comorbidity using binary logistic regression analyses, the A allele was confirmed as an independent risk factor for severe periodontal disease and RA ($p = 0.024$). *Conclusions*. These results emphasize the association of genetic variations in proinflammatory cytokines (TNFα and IFNγ) and cytokine receptor (IL4Rα) and RA and periodontal diseases. In multivariate analyses, the A allele of IFNγ was proven to be a significant marker of RA and PD comorbidities. The study broadens the knowledge about disease-specific differences in genetic composition and provides an improved understanding of a possible association of both diseases.

1. Background

A relationship between periodontal disease (PD) and rheumatoid arthritis (RA) has been emphasized in several clinical studies [1–4]. Both diseases are described as chronic destructive inflammatory diseases sharing remarkable pathological and clinical similarities at cellular and molecular levels [5–7]. Patients suffering from rheumatoid arthritis are more likely to exhibit severe periodontitis or missing teeth than healthy controls [8–10]. On the other hand, patients with periodontal disease were shown to be more susceptible to RA compared with healthy persons [11]. There is a dose-dependent association pattern between severity of periodontitis and RA disease activity [3]. Moreover, the nonsurgical treatment of periodontal disease was shown to have a positive effect on rheumatic complaints [12, 13], and vice versa, the therapy of RA was proven to have a beneficial impact on periodontitis [14]. However, the possible underlying link between both diseases is not completely understood.

An important early clinical sign specific for RA is the occurrence of anti-citrullinated protein antibodies (ACPAs) [15]. It was demonstrated that the periodontopathogen *Porphyromonas gingivalis* (*P.g.*) has the unique capacity of expression of peptidylarginine deiminases, responsible for protein citrullination [16]. This fact corroborated the assumption of the involvement of periodontal infection in the aetiology of RA [17]. Genetic constellation (HLA-DRB*04) was supposed to be associated with a higher odds ratio for the occurrence for borderline significance for ACPA (anti-CCP) [S. Reichert, personal communication]. However, no conclusive scientific evidence regarding the role of periodontal pathogens in RA was provided so far.

Furthermore, it is recognised that increased inflammatory burden accompanied by PD and RA can mutually influence each other and affect further inflammatory diseases [1]. Of particular importance in this context are common features of regulation and dysregulation of inflammatory response [9, 18]. Periodontal disease and rheumatoid arthritis are characterized by excessive chronic inflammatory reactions leading to infiltration of T and B lymphocytes, neutrophils, and monocytes [19]. Both diseases are characterised by an imbalance between proinflammatory and anti-inflammatory cytokines [7, 20–22]. This complex interplay is a determining fact for PD and RA, respectively, and leads to the maintenance of inflammation and induction of bone resorption, joint destruction, and erosion [7, 23].

In recent years, a lot of clinical association studies were conducted in order to confirm the impact of genetic variants on RA [24, 25] and PD [26, 27]. Genetic studies reveal that both diseases are characterized by shared genetic risk factors such as a MHC class II HLA-DRB1 allele [28] or cytokine SNPs, including the KCNQ1 gene [29].

These considerations lead to the hypothesis of a shared genetic profile associated with a higher susceptibility to RA and periodontal disease. In order to support this hypothesis, we evaluated a panel of pro- and anti-inflammatory genes (IL1α, IL1β, IL1R, IL2, IL4, IL1RA, IL-4Rα, IL6, IL10, IL12, IFNγ, TGFβ, and TNFα) possibly involved in the aetiology of both inflammatory diseases. We assessed allele, genotype, and haplotype frequencies of these genes in RA patients suffering from PD of different severities and systemically healthy controls without RA and severe PD.

2. Materials and Methods

2.1. Study Population. In the case-control study, 201 unrelated subjects of Caucasian origin were included. In general, patients and controls were only included if they had a minimum age of 18 years (patients) or 30 years (controls), were not pregnant, and had not taken antibiotics in the past 3 months or undergone subgingival scaling and root planning procedures 6 months prior to the examination. The patients and controls had no known medical or general health conditions that might profoundly contribute to development of periodontitis (except RA in RA patients). In accordance with this, subjects were not included if they were suffering from diabetes mellitus type I or II, Morbus Crohn, coronary heart disease, lupus erythematosus, Behçet disease, or oral pemphigus or pemphigoid or if they developed gingival overgrowth due to specific drugs such as antiepileptics, calcium-channel blockers, or cyclosporine. The controls were excluded if they took anti-inflammatory drugs regularly. The medication of RA patients with nonsteroidal or anti-inflammatory drugs was recorded. During the course of anamnesis age, current or past diseases as well as medication and smoking status were assessed. The medication included nonsteroidal anti-inflammatory drugs (NSAIDs: 50%), disease-modifying antirheumatic drugs (DMARDs: 70%), and biologicals (42%). The periodontal examination comprised the assessment of approximal plaque index (API) [30], the percentage of sites with bleeding upon probing (BOP), pocket depth (PD: distance between the gingival margin and apical stop of the pocket), and the number of missing teeth. To determine the mean clinical attachment loss (CAL: distance between the cement-enamel junction and apical stop of the probe) in cases and controls, six sites around each tooth were measured, and the maximum values were recorded.

The control group is comprised of 100 subjects not suffering from rheumatoid arthritis. This group was recruited consecutively at the Department of Operative Dentistry and Periodontology of the Martin Luther University Halle-Wittenberg from 2005 until 2009. The periodontal examination was carried out by an experienced periodontist using a non-pressure-sensitive periodontal probe (PCPUNC156, Hu-Friedy, Rotterdam, Netherlands). All controls exhibit no or mild periodontitis according to the consensus report for "definition of a periodontitis case and disease progression in risk factor research" [31]. A "mild periodontitis" was defined as the presence of clinical attachment loss of $\geq$3 mm in $\geq$2 nonadjacent teeth. Controls with vestibular values of clinical attachment loss $>$ 3.5 mm caused by traumatic tooth brushing or orthodontic therapy, CAL according overhanging subgingival restorations, or primary endodontic lesions were not considered as cases of periodontitis. Furthermore, pseudo pockets on the last molars with a depth of >3.5 mm were not considered as a periodontitis case [32].

The patient group includes 101 subjects with diagnosed rheumatoid arthritis according to current criteria for classifying rheumatoid arthritis [33]. RA was diagnosed and treated at the Clinic of Internal Medicine II, Department of Rheumatology, Martin Luther University Halle-Wittenberg (Prof. G. Keyßer and Dr. C. Schäfer), at the Department of Rheumatology "Rheumahaus Potsdam" (Dr. M. Bohl-Bühler and Dr. S. Reckert) and at three private practices in Magdeburg and Halle (Saale). From 2012 until 2016, the RA patients were included consecutively without consideration of periodontal status. For periodontal assessment, pressure-sensitive periodontal probes (TPS-probe Vivicare, Vivadent, Schaan, Liechtenstein, or DB764R Aesculap AG & Co. KG, Tuttlingen, Germany) were used. The dental examiners were instructed and trained in the implementation of both periodontal probes. The examiners were educated on a phantom model A-PB (frasaco GmbH, Tettnang, Germany) and under clinical conditions. According to the consensus report, a severe periodontitis case was defined as proximal attachment loss of $\geq$5 mm in >30% of the teeth [31]. Mild periodontitis was defined as mentioned above.

2.2. Genetic Investigations. For genetic investigations, fresh venous blood was obtained from the subjects in ethylenediaminetetraacetic acid- (EDTA-) treated tubes. Preparation of genomic DNA was carried out using a QIAamp blood extraction kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's manual.

The analysis of SNPs was carried out using the Cytokine Genotyping array CTS-PCR-SSP Tray Kit (Collaborative Transplant Study, Department of Transplantation Immunology of the University Clinic of Heidelberg, Heidelberg,

Germany). PCRs were performed with sequence-specific primers for the detection of genotypes and haplotypes. A fragment of 440 bp of the human C-reactive protein and 90 bp fragment of the human β-globin gene was coamplified as positive controls, respectively.

For every PCR, 10 ml of a Mastermix containing 1UTaq-Polymerase (Thermo Scientific, Waltham, USA), 100 ng genomic DNA, and PCR reaction buffer were added. PCR was performed in an Eppendorf Mastercycler Gradient (Eppendorf, Wesseling-Berzdorf, Germany) (2 min. 94°C; 10 cycles: 15 s 94°C, 1 min. 64°C, 30 s 72°C; 20 cycles: 15 s 94°C, 50 s 61°C, 30 s 72°C). After agarose gel electrophoresis, the resulting pattern was evaluated visually. In Table 1, the detected SNPs are displayed.

2.3. Molecular Biological Assessment of Porphyromonas gingivalis. Subgingival plaque samples were taken before subgingival scaling was carried out. The microbial samples were collected from the deepest pocket of each quadrant (insertion of a sterile paper point for 20 s) and pooled in one tube. Bacterial DNA was isolated applying the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. The subgingival occurrence of *P.g.* was detected using the micro-Ident® test of HAIN-Diagnostik (Nehren, Germany) according to the manufacturer's protocol. The method was described in detail in [34].

2.4. Statistical Analyses. Statistical analyses were carried out using commercially available software (SPSS v.25.0 package, IBM, Chicago, IL). Values of $p \leq 0.05$ were considered significant. Continuous data were assessed for normal distribution using the Kolmogorov-Smirnov test. These data were reported as means ± standard deviation (normal distributed values) or median, 25th/75th interquartiles (values not normally distributed). For the statistical evaluation of continuous variables, Student's t-test (normal distributed values), Mann–Whitney U test, or Kruskal-Wallis test (values not normally distributed) was used. Categorical variables were plotted in contingency tables and evaluated using the chi-square test and Yates continuity correction. If the expected cell frequency was <5, Fisher's exact test was applied.

Binary logistic regression analysis was used for investigating the impact of polymorphic variants on the occurrence of PD or RA considering established confounders.

3. Results

3.1. Clinical Evaluation. We performed a case-control study in order to evaluate the impact of genetic variants in selected pro- and anti-inflammatory genes (Table 1) in association with RA. We involved a control group of systemically healthy controls without RA and severe PD ($n = 100$) and a group of patients with RA ($n = 101$) suffering from periodontitis of different severities (severe periodontitis: $n = 25$; no/mild periodontitis: $n = 76$) in our study. The demographical and periodontal characteristics are displayed in Table 2. In general, patients suffering from RA were significantly older, were more often of female gender, and were more often smokers than probands without RA. Corresponding to the inclusion criteria, RA patients exhibited the more severe dental parameters including probing depth and clinical attachment loss. Subdividing the group of RA patients according to their periodontal status, it was obvious that patients suffering from severe periodontitis were more often males. In terms of age and smoking status, no significant differences were shown, although RA patients with severe periodontitis were older ($p = 0.129$) and more frequently smokers ($p = 0.102$) than RA patients with no or mild periodontitis. As expected, all RA patients suffering from severe periodontitis showed the more severe periodontal characteristics.

3.2. Genetic Variants in Rheumatoid Arthritis. Furthermore, we evaluated the impact of genetic variants in selected pro- and anti-inflammatory genes in association with occurrence of RA. Significantly, more G allele carriers of rs1801275 in IL4Rα and of rs361525 in TNFα were in the group of patients suffering from RA compared to the group of probands without RA (Table 3).

In binary logistic regression analyses, including age, gender, smoking status, and the occurrence of *P.g.* as confounding factors, the G allele of IL4Rα ($p = 248$) and the G allele of TNFα SNPs ($p = 0.422$) could not be proven as independently associated with RA. Increasing age, female gender, smoking, and the occurrence of *P.g.*, however, could be shown as significant risk factors for RA in these complex risk models (Table 4).

All other investigated genotypes, alleles, and haplotypes of pro- and anti-inflammatory cytokines were not found to be in association with rheumatoid arthritis in our study.

3.3. Cytokine Allele, Genotype, and Haplotype Frequencies in Association with Periodontitis. In a second evaluation, we tested possible associations between genetic variants and the severity of periodontal disease in the group of RA patients. No significant associations could be proven for all allele, genotype, and haplotype distributions investigated, except for IFNγ SNP rs240561. Patients suffering from more severe periodontitis were more frequently A allele carriers of this genetic variant than patients with no or mild periodontitis ($p = 0.039$, Table 5). In a multivariate risk model, higher age, male gender, smoking, and the higher incidence of *P.g.* but not the A allele of IFNγ SNP rs240561 had predictive value for severe periodontal disease. However, comparing patients with comorbidity of RA and PD with probands without RA, the A allele was significantly associated with both diseases in bivariate (Table 5, $p = 0.039$) and multivariate analyses (Table 6, $p = 0.024$).

4. Discussion

Over the last years, the involvement of genetic variants of cytokines as potential markers for disease susceptibility, progression, therapeutic success, and prognosis of PD and RA received particular attention [35, 36]. Based on the multifactorial pathogenesis of both diseases, it can be expected that genetic variants have rather a modulating than a

Table 1: Genetic specificities of each gene investigated using the Cytokine CTS-PCR-SSP Tray Kit.

Gene	dbSNP-ID	Genotype/haplotype
IL1α	rs18000587	T/C
IL1β	rs16944	C/T
	rs1143634	T/C
IL1R1	rs2234650	C/T
IL1RN	rs31592	C/T
IL4Rα	rs1801275	G/A
IL12B	rs3212227	C/A
IFNγ	rs2430561	A/T
TGFβ1	rs1800470/rs1800471	CG, CC, TG, TC
TNFα	rs1800629/rs361525	GG, AG, GA, AA
IL2	rs2069762/rs2069763	TG, GG, GT, TT
IL4	rs2243248/rs2243250/rs2070874	TTT, TTC, TCT, TCC, GTT, GTC, GCT, GCC
IL6	rs1800795/rs1800797	GG, CG, GA, CA
IL10	rs1800896/rs1800871/rs1800872	GCC, GCA, GAC, ACC, ATC, ATA, ACA, ATA

Table 2: Demographical characteristics and periodontal conditions in relation to rheumatoid arthritis (RA) and periodontal disease (PD).

Variable	Probands without RA ($n = 100$)	All ($n = 101$)	RA patients No/mild PD ($n = 76$)	Severe PD* ($n = 25$)	p value	
	I	II	III	IV	I vs. II	III vs. IV
Demographical and anamnestic parameters						
Age (years) (mean + SD)	45.8 ± 11.1	54.8 ± 13.1	53.6 ± 13.8	58.2 ± 10.3	**<0.001**	0.129
Female gender (%)	50	71.3	77.6	52	**0.002**	**0.014**
Current smoking (%)	20	24.8	21.1	36	**0.005**	0.102
Periodontal conditions (median (25th-75th IQR))						
Plaque index (%)	38 (28.7-59.3)	38.1 (16.1-68)	27.8 (9.5-57.8)	66 (41.5-83.2)	0.372	**<0.001**
Bleeding on probing/tooth (%)	42.4 (23.6-62.9)	38.5 (19.1-68.8)	32.7 (17.8-62.5)	60 (35.6-89.9)	0.934	**0.001**
Bleeding on probing/tooth surface (%)	8.7 (4.8-19.8)	9.5 (3.9-22.7)	8.6 (3.5-16.8)	19 (8.9-39.9)	0.664	**0.001**
Probing depth (mm)	2.5 (2.3-2.8)	4 (3-5.5)	3.5 (2.9-5.5)	5.5 (4.3-7.5)	**<0.001**	**<0.001**
Clinical attachment loss (mm)	2.8 (2.6-3.2)	4.1 (3.2-5.9)	3.5 (3-5)	5.9 (4.7-8.8)	**<0.001**	**<0.001**
Missing teeth (except 8th)	2 (0-3.75)	5 (2-10)	4 (1-9.75)	9 (5.5-15.5)	**<0.001**	**0.001**

*Proximal attachment loss of ≥5 mm in ≥30% of teeth present. Statistical comparisons were made by the chi-square test including Yates correction for categorical variables. Continuous variables were analyzed by the Mann–Whitney U test and presented as median (25th/75th interquartiles (IQR); values not normally distributed) or Student's t-test and mean (standard deviation (SD); normal distribution).

determinant influence in this context. However, epidemiological studies showed that the genetic contribution to rheumatoid arthritis and periodontitis is substantial and may account for 50% of the RA [37] and PD risk profile, respectively [38, 39].

Therefore, we investigated possible associations between a panel of genetic variants of pro- and anti-inflammatory cytokine genes, including allele, genotype, and haplotype distributions, and RA or PD in bivariate and multivariate models considering further established risk markers for both diseases.

4.1. Clinical Evaluation. In the present association study, we included a group of controls without RA and severe periodontitis and a group of RA patients suffering from periodontitis of different severities (no/mild or sever periodontitis). As shown in Table 2, RA patients were significantly older than controls ($p < 0.001$). It is well established, that the incidence of rheumatoid arthritis increases with age [19]. Especially, women in their fourth and fifth decades are more affected by RA [40]. Also in our study, women were shown to be more susceptible to RA than men (Table 2, $p = 0.002$). Another major risk factor of RA is cigarette smoking. Smoking increases the RA risk especially in patients with genetic predisposition [41]. This fact was confirmed by our study, since RA patients were significantly more often smokers than controls without RA

Table 3: Genotype and allele distributions of SNPs in IL4Rα (rs1801275) and TNFα (rs361525) among RA patients and controls.

	Probands without RA and severe PD $n = 100$	RA patients with varying degrees of severity of PD $n = 101$	p value
IL4Rα rs1801275			
AA (%)	72	60	
AG (%)	25	33	
GG (%)	3	7	0.150
A (%)	84.5	76.5	
G (%)	15.5	23.5	**0.043**
TNFα rs361525			
AA (%)	1	1	
AG (%)	15	6.9	
GG (%)	84	92.1	0.186
A (%)	10	1	
G (%)	90	99	**0.005**

Statistical comparisons were made by the chi-square test including Yates correction. RA patients: patients with rheumatoid arthritis.

(Table 2, $p = 0.005$). As it was demonstrated by different studies, patients suffering from rheumatoid arthritis exhibit the more severe periodontal symptoms including probing depth and clinical attachment loss [6, 8].

Within the RA group, the patients were subdivided according their periodontal status. Patients suffering from severe periodontitis were older (n.s.), more often smokers (n.s.), and males ($p = 0.014$) compared to patients without or with mild periodontitis (Table 2). Since age, smoking, and the male gender are major risk factors of severe periodontitis, these tendencies were in accordance with established risk profile of periodontal disease [42].

4.2. Genetic Variants in Rheumatoid Arthritis. A lot of genetic variants have been reported to be implicated in the pathogenesis of rheumatoid arthritis [43, 44]. In this study, the G allele of SNP rs1801275 in the IL4Rα gene and the G allele of SNP rs361525 in the TNFα gene were shown to be associated with RA out of 22 polymorphic variants in 13 cytokine genes (Table 3). However, considering further risk markers of RA, these associations of SNPs in IL4Rα as well as in the TNFα gene and RA did not remain significant. This might indicate that factors like increasing age, the female gender, smoking, and the occurrence of *P.g.* were more strongly associated with RA implying a minor role of these genetic variants in our study.

Studies investigating the impact of rs1801275 in the IL4Rα gene in RA revealed contradictory results [45–49]. In accordance with our results, Moreno et al. could confirm the G allele of rs1801275 as a risk factor for rheumatoid arthritis in RF positive patients [45]. On the other hand, other studies showed an impact of AG and AA genotypes [48] and A allele [49], respectively, on the development of RA. Furthermore, in a meta-analysis, no association of this genetic variant was demonstrated for rheumatoid arthritis [46] and for patients with juvenile idiopathic arthritis [47]. Since the inclusion criteria varied considerably, no consistent risk pattern regarding rs1801275 could be generated. It could be conceivable that genetic markers of IL4Rα could influence its gene expression. This could be of great importance since IL4Rα mediates the intracellular signalling cascades elicited by the anti-inflammatory cytokine IL4, a major regulator of the TH1/TH2 balance. However, functional studies could not prove a genetic influence of this polymorphism on gene expression in patients suffering from allergic asthma [50] or systemic sclerosis [51]. Further studies are needed in order to investigate the potential functional role of this polymorphism in rheumatoid arthritis.

Besides rs1801275 in the IL4Rα gene, a genetic variant in the TNFα (G allele of rs361525) gene was shown to be associated with RA in our study. In a meta-analysis by Lee and Bae, the impact of A allele of rs361525 on RA was demonstrated evaluating 10 case-control studies including patients of different ethnicities [52]. However, taking only 6 studies conducted in Europe into consideration, the G allele was more frequent among RA patients ($p = 0.047$) [43]. These results obviously imply that the disease-related genetic characteristic of rs361525 is dependent on ethnicity. A possible genetic influence of rs361525 on TNFα expression was studied intensively leading to controversial outcomes [53–55]. However, in patients with rheumatoid arthritis [56] or osteoarthritis [57], the GG genotype and G allele of rs361525 were associated with increased TNFα expression, respectively. Therefore, in our RA group, the higher frequency of G allele carriers of rs361525 could be accompanied with higher TNFα expression indicative for RA [58]. Of particular importance for the RA therapy is the treatment with TNFα antagonists [59]. Indeed, it could be demonstrated that genetic variants in TNFα, including rs361525, were associated with responsiveness to TNFα treatment [60, 61].

Our results support the thesis of an association of genetic variants in cytokine genes (IL4Rα and TNFα) with RA. However, in multivariate analysis, a corresponding genetic influence could not be proven (Table 4). This might imply that other risk factors are stronger disease-determining markers and that these genetic variants play a minor role in the aetiology of RA. And indeed, higher age, the female gender, smoking, and the subgingival occurrence of *Porphyromonas gingivalis* were significant predictors in binary logistic regression analysis. These results are in accordance with the established risk model of RA [19, 40, 41].

4.3. Cytokine Allele, Genotype, and Haplotype Frequencies in Association with Periodontitis within the Test Group. A lot of case-control studies, meta-analyses, and GWAS were conducted in order to evaluate the impact of genetic variants on the aetiology of aggressive and chronic periodontites with variable results [62–64].

A shared genetic background was assumed to be the basis among others for the biological plausible link between periodontitis and further inflammatory diseases, including RA. Therefore, studies were performed in order to identify

Table 4: Binary logistic regression analyses investigating the impact of G allele of rs1801275 (IL4Rα) and G allele of rs361525 (TNFα) on the incidence of rheumatoid arthritis.

Variables	Regression coefficient	Odds ratio	95% confidence interval		p value
			Lower	Upper	
IL4Rα (rs1801275)					
Age	0.056	1.06	1.04	1.08	<0.001
Female gender	0.897	2.45	1.55	3.87	<0.001
Current smoker	0.841	2.32	1.48	3.64	<0.001
P.g. positive	0.655	1.93	1.18	3.13	0.008
G allele	0.336	1.40	0.79	2.47	0.248
TNFα (rs361525)					
Age	0.056	1.06	1.04	1.08	<0.001
Female gender	0.887	2.43	1.54	3.84	<0.001
Current smoker	0.863	2.37	1.51	3.71	<0.001
P.g. positive	0.676	1.97	1.21	3.19	0.006
G allele	0.370	1.45	0.59	3.57	0.422

Age, gender, smoking status, and the occurrence of *Porphyromonas gingivalis* (*P.g.*) were considered as confounding factors.

Table 5: Genotype and allele distributions of IFNγ SNP rs240561 in relation to severity of PD.

	Probands without RA and severe PD ($n = 100$)	RA and no/mild PD ($n = 76$)	RA and severe PD ($n = 25$)	p value		
	I	II	III	II vs. III	I vs. II	I vs. III
AA (%)	28.3	26.3	48			
AT (%)	44.4	50	40			
TT (%)	27.3	23.7	12	0.151	0.756	0.110
A (%)	50.5	51.3	68			
T (%)	49.5	48.7	32	**0.039**	0.966	**0.039**

Statistical comparisons were made by the chi-square test including Yates correction. PD: periodontal disease.

Table 6: Binary logistic regression analyses investigating the impact of A allele of rs240561 of IFNγ (a) on the incidence of severe PD in a cohort of RA patients and (b) on the incidence of comorbidity of RA and PD versus controls without both, RA and severe PD.

(a) RA patients with severe PD vs. RA patients who do not have severe PD

Variables	Regression coefficient	p value	Odds ratio	95% confidence interval	
				Lower	Upper
Age	0.031	0.046	1.03	1.01	1.06
Male gender	1.149	0.002	3.15	1.53	6.49
Current smoker	0.893	0.024	2.44	1.13	5.3
P.g. positive	0.944	0.009	2.57	1.26	5.24
A allele	0.684	0.068	1.98	0.95	4.13

(b) Patients with comorbidity of RA and severe PD vs. controls without both, RA and severe PD

Variables	Regression coefficient	p value	Odds ratio	95% confidence interval	
				Lower	Upper
Age	0.131	<0.001	1.12	1.08	1.17
Male gender	0.123	0.767	1.13	0.50	2.55
Current smoker	1.87	<0.001	6.49	2.69	15.6
P.g. positive	1.54	<0.001	4.67	2.07	10.5
A allele	0.977	**0.024**	2.66	1.14	6.20

Age, gender, smoking status, and the occurrence of *Porphyromonas gingivalis* (*P.g.*) were considered as confounding factors.

common genetic risk factors for both diseases [14, 29, 65]. Applying the candidate gene approach, SNPs in IL1β (rs1143634) [13] and KCNQ1 (rs2237892) [29] were shown to be associated with comorbidity of rheumatoid arthritis and periodontal disease.

Also in this study, possible associations of genetic characteristics in cytokine genes and the severity of periodontal disease in RA patients were assessed. Out of the panel of 22 SNPs in 13 cytokine genes, we identified the A allele of SNP rs240561 in IFNγ as a risk indicator for severe periodontitis in RA patients (Table 5). Furthermore, evaluating patients suffering from RA and severe PD, the A allele was a significant predictor for comorbidity also considering further confounders (Table 6).

The scientific knowledge about possible association of rs2430561 to PD [66, 67] and/or RA [68–70] is highly inconsistent. Regarding possible association of this SNP and the susceptibility to PD, a meta-analysis was performed [71]. However, this study failed to prove a genetic association. The IFNγ SNP rs2430561 was demonstrated to be in complete linkage disequilibrium with a polymorphic microsatellite located in the first intron of the IFNγ gene with susceptibility to RA [68; 72]. In contrast, other studies failed to confirm this genetic association to RA susceptibility or severity [69, 70].

In the electrophoretic mobility shift assay, this SNP was demonstrated to be located in a putative nuclear transcription factor NF-κB binding site influencing IFNγ expression [72]. Therefore, studies were conducted in order to assess the influence of this SNP on IFNγ expression resulting in different outcomes [72–75]. However, the inclusion and exclusion criteria of patients as well as the chosen methodological design varied widely. Several studies showed a lower expression associated with the A allele of IFNγ SNP rs2430561 [745]. In contrast, Prabhu Anand et al. could prove a higher IFNγ expression in peripheral blood mononuclear cells accompanied with AA genotype in healthy subjects [74]. Clinical studies investigating the impact of the IFNγ level on periodontitis showed an increased expression in saliva [76] and gingival biopsies in chronic periodontitis as well as in gingival crevicular fluid [77] in periodontal active sites [78]. Also, patients with rheumatoid arthritis were shown to exhibit higher levels of IFNγ in mononuclear cells and tissues from affected organs [79]. IFNγ and its associated signalling pathways were demonstrated to promote the breakdown of soft and hard tissues of the periodontium and induce bone loss [80, 81]. IFNγ is involved in the adaptive immune response due to the activation of macrophages and differentiation of T helper cells [82] including the induction of Th1 cytokines via the JAK/STAT signalling pathway [83]. These IFNγ-associated pathways are important characteristics in the aetiology of inflammatory diseases including PD [84] and RA [79], respectively. And indeed, the T cell-mediated increased IFNγ expression induced by periodontal infections with *P.g.* or *Aggregatibacter actinomycetemcomitans* was shown to be promoting rheumatoid arthritis [74]. Therefore, IFNγ and its functional important genetic variant rs2430561 could provide a biological plausible link between both inflammatory diseases.

4.4. Study Limitations. The present study was performed as a case-control study. It was conducted to establish assumptions of possible associations between genetic variants and periodontitis and rheumatoid arthritis, respectively. However, considering the study design, the verification of these assumptions is not realizable.

Due to one of the strengths of the present study, the homogeneous ethnicity, and due to the strict inclusion and exclusion criteria of the study participants, the sample size was relatively small. This may result in potential bias because of increasing the likelihood of a type II error skewing the outcomes. Therefore, the present investigation could only be considered as a pilot case-control study, and the drawn conclusions should be confirmed and extended in a large-scale cohort. Furthermore, the persons involved in the study are not necessarily representatives of the population as a whole.

In our study, 22 SNPs were tested. In chi^2 tests, significant associations between genetic variants and PD/RA were assessed (Tables 3 and 5). However, if multiple hypotheses are evaluated, the likelihood of incorrectly rejecting the null hypothesis increases, which potentially leads to type I errors. Therefore, a statistical correction for multiple testing should be applied. After Bonferroni correction, the results of Tables 3 and 5 did not remain significant. Therefore, the drawn conclusion should be interpreted with caution.

Because of the integration of subjects from two settings, different methods of the determination of BOP, PD, and CAL were applied and were subject to possible biases. For RA patients, two different pressure-sensitive periodontal probes were used. The overall reproducibility of both probes has been already confirmed in previous studies [85, 86].

Control subjects were dental assessed using a non-pressure-sensitive periodontal probe. It has already been highlighted in earlier studies that both, non-pressure-sensitive periodontal probes and pressure-sensitive probes, are reliable tools for reproducible pocket depth measurements receiving a comparable error rate [87–89].

Finally, the data presented can be considered applicable for Caucasian individuals of central Germany only and must therefore be interpreted with caution. Extrapolation to the general population is not rationally supported.

5. Conclusions

Our results strengthen the thesis that SNP rs2430561 of the proinflammatory cytokine IFNγ may constitute a shared genetic risk factor for PD and RA. This might provide new arguments for the hypothesis of shared inflammatory processes underlying PD and RA. Further studies have to be conducted in order to replicate these findings in larger cohorts.

Abbreviations

API: Approximal plaque index
BOP: Bleeding upon probing
CAL: Clinical attachment loss
DMARD: Disease-modifying antirheumatic drug
GWAS: Genome-wide association study

IFNγ: Interferon gamma
IL4Rα: Interleukin 4 receptor alpha
n.s.: Not significant
NSAID: Nonsteroidal anti-inflammatory drug
PD: Periodontal disease
P.g.: *Porphyromonas gingivalis*
RA: Rheumatoid arthritis
SNP: Single nuclear polymorphism
TNFα: Tumor necrosis factor alpha
vs.: Versus.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

The ethics committee of the Medical School of Martin Luther University Halle-Wittenberg approved the study. The investigations were carried out in accordance with the ethical guidelines of the "Declaration of Helsinki" and its amendment in "Tokyo and Venice."

Consent

All participants signed their written consent to participate in this study. All authors have given their consent for publication.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

SS, HGS, and SR participated in study design and evaluation. EJ and NP examined the patients and controls regarding their periodontal status and were responsible for the collection of subgingival plaque and venous blood samples. SS and NP analyzed subgingival plaque samples. The cytokine genotyping was carried out by SS and NP.

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Research Article

Chemokine Receptor 2 (*CXCR2*) Gene Variants and Their Association with Periodontal Bacteria in Patients with Chronic Periodontitis

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Periodontitis, an inflammatory disease caused by subgingival Gram-negative (G-) bacteria, is linked with loss of the connective tissue and destruction of the alveolar bone. In the regulation of inflammatory response, chemokine receptor 2 (CXCR2), a specific receptor for interleukin-8 and neutrophil chemoattractant, plays an important role. The first aim of this study was to investigate the *CXCR2* gene variability in chronic periodontitis (CP) patients and healthy nonperiodontitis controls in the Czech population. The second aim was to find a relation between *CXCR2* gene variants and the presence of periodontal bacteria. A total of 500 unrelated subjects participated in this case-control study. 329 CP patients and 171 healthy nonperiodontitis controls were analyzed using polymerase chain reaction techniques for three single-nucleotide polymorphisms (SNPs): +785C/T (rs2230054), +1208T/C (rs1126579), and +1440A/G (rs1126580). A DNA microarray detection kit was used for the investigation of the subgingival bacterial colonization, in a subgroup of CP subjects ($N = 162$). No significant differences in allele, genotype, haplotype, or haplogenotype frequencies of *CXCR2* gene variants between patients with CP and healthy controls ($P > 0.05$) were determined. Nevertheless, *Aggregatibacter actinomycetemcomitans* was detected more frequently in men positive for the C allele of the *CXCR2* +785C/T polymorphism (61.8% vs. 41.1%, $P < 0.05$; OR = 2.31, 95% CI = 1.03-5.20) and for the T allele of the *CXCR2* +1208C/T variant (61.8% vs. 38.9%, $P < 0.05$; OR = 2.54, 95% CI = 1.13-5.71). In contrast, no statistically significant associations of *CXCR2* variants with seven selected periodontal bacteria were found in women. Although none of the investigated SNPs in the *CXCR2* gene was associated with CP, the *CXCR2* gene variants can be associated with subgingival colonization of G- bacteria in men with CP in the Czech population.

1. Introduction

Periodontitis is a multifactorial disease that is primarily caused by specific pathogen-associated molecular patterns (PAMPs) and bacterial virulence factors; they trigger an inflammatory host response which results in periodontal tissue destruction and loss of teeth [1, 2]. Chronic periodontitis (CP), the most common form of periodontitis in adults, is either localized or generalized, based on the number of affected sites. The destruction corresponds to the presence of local factors, with a slow-to-moderate rate of progression, but may have periods of rapid progression [3]. CP is strongly associated with "red complex" Gram-negative (G-) bacteria, including *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* [1, 4]. Although *Aggregatibacter actinomycetemcomitans* is supposed to be the main etiological

agent of the aggressive form of periodontitis [5], this bacterium is also connected with CP and some nonoral infections [6].

The host response to anaerobic G- bacteria and their products is an important determinant for progression of periodontal disease. There are a few major risk factors, such as genetic predispositions, systemic diseases, or smoking, which affect the microbial composition in the oral cavity [7, 8]. Cytokines, mediators of host defense and also of periodontal tissue destruction, are considered to be important molecules in the etiopathogenesis of periodontal diseases [9].

Interleukin-8 (IL-8, CXCL8) is known as neutrophil-activating protein-1 (NAP-1) [10, 11]. The effect of IL-8 is mediated by its two receptors—7 transmembrane class A (rhodopsin-like) G protein-coupled receptors (7-TM-GPCRs), so called CXCR1 and CXCR2 [12, 13]. CXCR1 and CXCR2 are expressed on a wide range of leukocytes, including neutrophils, mast cells, and also oral epithelial cells [14, 15]. They are involved in the multiple biological activities, such as initiation and amplification of acute inflammatory reaction, as well as tumor growth, angiogenesis, and metastasis [16–18]. Experimental data suggest that IL-8 and its receptors participate in the elimination of pathogens [19]. A study by Zenobia et al. shows that the recruitment of neutrophils to gingival tissue does not require commensal bacterial colonization but is entirely dependent on *CXCR2* expression [20].

Only a few studies have investigated the variability in *CXCR1* or *CXCR2* genes in relation to CP [21–23], especially in the Brazilian population; however, only the *CXCR2* genotypes and haplotypes have been associated with CP [21]. Based on our previous investigation of *IL-8* gene variability and its association with periodontal bacteria in patients with CP [24], we assumed the role of IL-8 receptor in the etiopathogenesis of periodontal disease.

The first aim of our study was to analyze three SNPs in the *CXCR2* gene +785C/T (rs2230054), +1208T/C (rs1126579), and +1440G/A (rs1126580) in CP patients and healthy nonperiodontitis controls in the Czech population; the second aim was to associate these SNPs with the presence of seven periodontal bacteria in subjects with CP.

2. Materials and Methods

2.1. Subjects. This case-control association study comprised 500 unrelated Caucasian subjects of exclusively Czech ethnicity from the South Moravian Region. Subjects with CP (number of subjects, $N = 329$) were recruited from the Periodontology Department, Clinic of Stomatology, St. Anne's Faculty Hospital, Brno, over the period of 2013–2018. Healthy nonperiodontitis controls ($N = 171$) were selected from patients who had been referred to the Clinic of Stomatology for reasons other than periodontal disease (such as preventive dental check-ups, dental decay, and orthodontic consultations) during the same period as CP patients, and they were of similar age, gender, and smoking status. Similarly, like the patients, all controls were in good systemic health and had minimally 20 remaining teeth. The exclusion criteria included the presence of diabetes mellitus, cardiovascular disorders (such as hypertension or coronary artery diseases), immunodeficiency, current pregnancy or lactation, malignant diseases, immunosuppression due to medication or concurrent illness, the use of anti-inflammatory drugs or antibiotics during a six-week recruitment period, and the inability to consent [25].

Table 1: Demographic data of CP patients and healthy nonperiodontitis controls.

Characteristics	Controls ($N = 171$)	CP ($N = 329$)
Age (mean ± SD, years)	47.56 ± 11.80	54.03 ± 8.99*
Gender (males/females)	82/89	150/179
Smoking (no/yes) (%)	73.68/26.32	72.49/27.18
BMI (mean ± SD, kg m^{-2})	25.45 ± 3.61	26.20 ± 3.77
PD (mean ± SD, mm)	1.21 ± 0.24	3.26 ± 0.81*
AL (mean ± SD, mm)	1.33 ± 0.21	3.94 ± 1.05*
PI (mean ± SD, mm)	0.34 ± 0.14	0.83 ± 0.49*
GI (mean ± SD, mm)	0.38 ± 0.31	0.81 ± 0.36*

AL = attachment loss; CP = chronic periodontitis; GI = gingival index; N = number of subjects; PD = probing depth; PI = plaque index; SD = standard deviation. *$P < 0.05$.

Clinical diagnosis of nonperiodontitis/periodontitis was based on a thorough examination (PI = plaque index, GI = gingival index, etc.), medical/dental history, tooth mobility, and radiographic evaluation. Probing depth (PD) and attachment loss (AL) were collected with a UNC-15 periodontal probe from six sites on every tooth present. The loss of the alveolar bone was determined radiographically, and the decrease in alveolar bone levels was assessed with the Mühlemann index [24]. All participants, no matter whether they agreed or declined to participate or were excluded from the study, were offered periodontitis treatment. The patients were firstly examined by a periodontist, and they had not received scaling and/or root planing minimally six months before measuring periodontal indices [25].

According to their smoking history, the subjects were split into the following groups: nonsmokers (subjects who never smoked) and smokers (former smokers for ≥5 pack-years or current smokers). The pack-years were calculated by multiplying the number of years of smoking by the average number of cigarette packs smoked per day [23]. The demographic data of the studied subjects are shown in Table 1.

2.2. Genetic Analysis. Genomic DNA was isolated from peripheral blood by a standard protocol. It was archived in the DNA bank at the Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic. Three SNPs in the *CXCR2* gene (+785C/T (rs2230054), +1208C/T (rs1126579), and +1440A/G (rs1126580)) were analyzed.

For detection of SNP in the *CXCR2* gene at position +785C/T (rs2230054), the original restriction fragment length polymorphism (RFLP-PCR) method with mismatch primers was introduced. Primers were designed by the

Primer3Output program. PCR was carried out in a volume of 25 μL containing 100 ng of genomic DNA, 0.5 μM of each primer (Fwd: 5′-TCGTCCTCATCTTCCCGCT and Rev: 5′-GGAGTCCATGGCGAAACTTC), 4 U of *Taq* DNA polymerase (Thermo Scientific, Waltham, USA), 2 mM of $MgCl_2$, $MgCl_2$-free reaction buffer with NH_4SO_3 (Thermo Scientific, Waltham, USA), and 0.5 mM deoxyribonucleoside triphosphate mix (Thermo Scientific, Waltham, USA). Denaturation for 5 min at 95°C was followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The last synthesis step was extended to 7 min at 72°C. The restriction of the PCR product (210 bp) was performed in a volume of 25 μL consisting of 15 μL of the PCR product, 10x CutSmart Buffer, and 4 U of *Bsr*BI enzyme (New England Biolabs, Hitchin, United Kingdom), and incubation was done overnight at 37°C. The length of products after restriction digestion was 210 bp for the TT genotype, 210 bp + 193 bp + 17 bp for CT, and 193 bp + 17 bp for CC. The fragments were visualized by 3.0% agarose gel electrophoresis by ethidium bromide. Sizing of the product was performed using a GeneRuler™ 50 bp DNA Ladder (Thermo Scientific, Waltham, USA).

SNP +1208C/T (rs1126579) in the *CXCR2* gene was genotyped using the 5′ nuclease TaqMan® assay C_8841198_10 for allelic discrimination according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). Allele genotyping from fluorescence measurements was then obtained using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3 software was used to analyze real-time and endpoint fluorescence data.

The +1440A/G (rs1126580) SNP was genotyped by allele-specific PCR analysis according to the previously published method [26], with a slight modification. A set of appropriate sequences of allele-specific primers and control primers was used: for the allele-specific DNA fragment (Fwd: 5′-AGGCTGGCCAACGGGG/A and Rev: 5′-TCAT AGCAGCTTATTCACAAGAC) and for the control DNA fragment (Fwd: 5′-TGCCAAGTGGAGCACCCAA and Rev: 5′-GCATCTTGCTCTGTGCAGAT). There is a difference between the sequences of the allele-specific primers used in our study and those in the work of Renzoni et al. [26]. The length of amplified DNA fragments was also different. The presence of an allele-specific band (435 bp) of the expected size in conjunction with a control band (796 bp) was considered to be positive evidence for each particular allele. The absence of an allele-specific band and the presence of a control band were considered to be a negative indication for a particular allele. Briefly, PCR was carried out in a volume of 25 μL containing 100 ng of genomic DNA, 0.5 μM of each allele-specific primer, 0.4 μM of each control primer, 2.5 U of *Taq* DNA polymerase (Thermo Scientific, Waltham, USA), 2 mM of $MgCl_2$, $MgCl_2$-free reaction buffer with NH_4SO_3 (Thermo Scientific, Waltham, USA), and 0.5 mM deoxyribonucleoside triphosphate mix (Thermo Scientific, Waltham, USA). Denaturation for 5 min at 95°C was followed by 35 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min. The last synthesis step was extended to 7 min at 72°C. The fragments were visualized by 2.0% agarose gel electrophoresis by ethidium bromide. Sizing of the product was performed using a GeneRuler™ 50 bp DNA Ladder (Thermo Scientific, Waltham, USA).

2.3. Microbial Analysis. The analyses of seven selected periodontal bacteria based on a DNA microarray detection kit (Protean Ltd., Ceske Budejovice, Czech Republic) have been described previously [24, 27]. The presence of bacterial colonization (*A. actinomycetemcomitans*, *T. forsythia*, *P. gingivalis*, *T. denticola*, *Parvimonas micra*, *Prevotella intermedia*, and *Fusobacterium nucleatum*) in subgingival pockets was examined in a subgroup of 162 CP patients before subgingival scaling. Bacterial load was assessed semiquantitatively: (-) undetected, corresponding to a number of bacteria less than 10^3; (+) slightly positive, which corresponds to a number of bacteria from 10^3 to 10^4; (++) positive, corresponding to a number of bacteria from 10^4 to 10^5; and (+++) strongly positive, corresponding to a number of bacteria exceeding 10^5 [28]. The diagnosis of the specific bacterial infection was considered positive when the number of bacterial cells surpassed 10^3 [23].

2.4. Statistical Analysis. Standard descriptive statistics were applied: mean with standard deviations (SD) or median with quartiles for quantitative variables and absolute and relative frequencies for categorical variables. One-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA was performed to compare continuous variables among the groups. The allele frequencies were calculated from the observed numbers of genotypes. The differences in the allele frequencies were compared by the Fisher exact test, and genotype/haplogenotype frequencies and Hardy-Weinberg equilibrium (HWE) were tested by the χ^2 test. To examine the linkage disequilibrium (LD) between polymorphisms, pairwise LD coefficients (D') and haplotype frequencies were calculated using the SNP Analyzer 2 program (http://snp.istech.info/istech/board/login_form.jsp). Odds ratio (OR), confidence intervals (CI), and P values were calculated. P values less than 0.05 were considered statistically significant. Where appropriate, the Bonferroni correction was used to adjust the level according to the number of independent comparisons to the overall value of 0.05. The adjusted P values are denoted as P_{corr}. Power analysis was performed with respect to the case-control design of the study, taking the incidence rate of markers. Statistical analysis was performed using the statistical package Statistica v. 13 (StatSoft Inc., USA).

3. Results

3.1. Case-Control Study. Our population sample consisted of 232 males and 268 females (CP patients 45.6%/54.4%, controls 48.0%/52.0%). 26.3% of CP patients and similarly 27.2% of healthy nonperiodontitis controls were smokers ($P > 0.05$). No significant differences in means of the body mass index (BMI) between CP patients and controls ($P > 0.05$, mean ± SD: 25.45 ± 3.61 kg m^{-2} vs. 26.20 ± 3.77 kg m^{-2}, respectively) were detected. Groups of cases and controls were different according to PD, AL, PI, and GI ($P < 0.01$); in CP patients, all mean values were higher

Table 2: *CXCR2* genotype and allele frequencies in CP patients and healthy nonperiodontitis controls.

Genotypes Alleles	Controls $N = 171$ (%)	CP $N = 329$ (%)	P value	OR (95% CI)
CXCR2 +785				
CC	41 (24.0)	81 (24.6)	—	1.00
CT	93 (54.4)	166 (50.5)	0.37	0.90 (0.57-1.42)
TT	37 (21.6)	82 (24.9)	0.39	1.12 (0.65-1.93)
C allele	175 (51.2)	328 (49.8)	—	1.00
T allele	167 (48.8)	330 (50.2)	0.37	1.05 (0.81-1.37)
CXCR2 +1208				
CC	45 (26.3)	91 (27.7)	—	1.00
CT	90 (52.6)	174 (52.9)	0.47	0.96 (0.62-1.48)
TT	36 (21.1)	64 (19.5)	0.37	0.88 (0.51-1.51)
C allele	180 (52.6)	356 (54.1)	—	1.00
T allele	162 (47.4)	302 (45.9)	0.35	0.94 (0.73-1.22)
CXCR2 +1440				
AA	35 (20.5)	59 (17.9)	—	1.00
AG	89 (52.0)	171 (52.0)	0.34	1.14 (0.70-1.86)
GG	47 (27.5)	99 (30.1)	0.25	1.25 (0.73-2.15)
A allele	159 (46.5)	289 (43.9)	—	1.00
G allele	183 (53.5)	369 (56.1)	0.24	1.11 (0.85-1.44)

CI = confidential interval; CP = chronic periodontitis; N = number of subjects; OR = odds ratio.

than those in controls. The demographic data of the studied subjects are given in Table 1.

The sample size of the study was optimized to reach relevant detectable effect size keeping the standard level of statistical errors, i.e., type I error 0.05 and type II error 0.20 or power of the test 0.80, respectively. The power calculation was optimized on the basis of the Fisher exact and goodness-of-fit tests as statistical tools used in comparing the principal endpoints in the study. Regarding the background relative frequency of the examined phenomenon as 50%, the reached sample size (171 controls, 329 cases, control : case ratio approx. 0.5) enabled to distinguish the difference in relative distribution of any entity (±13%) as statistically significant. Similarly, regarding the mean relative frequency as 50%, the study is able to detect difference (±13%) with 95% confidence.

3.2. SNPs and Haplotype Analysis. The studied polymorphisms +785C/T (rs2230054), +1208C/T (rs1126579), and +1440A/G (rs1126580) were in HWE in the control group ($P > 0.05$). No significant differences of all allele and genotype frequencies between the CP and control groups were found (see Table 2).

The distribution of genotype frequencies of all studied *CXCR2* gene variants was similar between men and women (data not shown). The SNPs in the *CXCR2* gene (+785C/T (rs2230054), +1208C/T (rs1126579), and +1440A/G (rs1126580)) were in very tight LD with each other to various degrees ($|D'| = 0.72 - 0.94$). Only the haplogenotype TCA/TTG was found more frequently in CP patients than in controls (0.0% vs. 2.4%, $P < 0.05$, $P_{corr} > 0.05$), but the number of subjects in both groups was very low. In our population, no other association between *CXCR2* haplotypes or haplogenotypes and CP was found ($P > 0.05$ for both, see Tables 3 and 4, respectively).

3.3. Microbial Analysis. There were no relationships between variability in the three studied *CXCR2* SNPs and the presence of seven periodontal bacteria in 162 CP patients, and only *A. actinomycetemcomitans* was marginally associated with *CXCR2* +785C/T SNP ($P = 0.06$). *A. actinomycetemcomitans* occurred more frequently in men positive for the C allele of the *CXCR2* +785C/T polymorphism (61.8% vs. 41.1%, $P < 0.05$; OR = 2.31, 95% CI = 1.03-5.20) and for the T allele of the *CXCR2* +1208C/T variant (61.8% vs. 38.9%, $P < 0.05$; OR = 2.54, 95% CI = 1.13-5.71). The presence of *P. micra* was marginally associated with the T allele of +1208 SNP for the group of male CP patients (49.0% vs. 27.3%, $P = 0.05$; OR = 2.56, 95% CI = 0.93-7.08; see Table 5). In contrast, there were no differences between the frequencies of *CXCR2* gene variants and the presence of periodontal bacteria in the group of CP women ($P > 0.05$, data not shown).

4. Discussion

Periodontal disease is characterized by inflammatory processes of tissues surrounding the teeth in response to bacterial stimulation. This inflammatory process is responsible for the progressive loss of the collagen attachment of the tooth to the alveolar bone, leading to bone loss [29]. According to the statistical report by the Ministry of Health of the Czech Republic, 15-20% of the Czech population aged 35-44 years suffered from periodontal disease in 2014 [30].

Table 3: Estimated frequencies (%) of *CXCR2* haplotypes in CP patients and healthy nonperiodontitis controls.

CXCR2 +785C/T	*CXCR2* +1208C/T	*CXCR2* +1440A/G	Controls ($N = 171$)	CP ($N = 329$)	P value	OR (95% CI)
C	T	G	42.3	41.4	0.79	0.96 (0.74-1.26)
T	C	A	39.6	37.9	0.62	0.94 (0.72-1.22)
T	C	G	5.8	8.3	0.14	1.49 (0.87-2.56)
C	C	A	4.7	5.3	0.73	1.11 (0.60-2.09)
T	T	G	2.8	3.7	0.53	1.28 (0.58-2.81)
C	C	G	2.5	2.6	0.92	1.04 (0.46-2.34)
C	T	A	1.6	0.6	0.10	0.31 (0.07-1.30)
T	T	A	0.6	0.3	0.52	0.52 (0.07-3.70)

CI = confidential interval; CP = chronic periodontitis; N = number of subjects; OR = odds ratio.

Table 4: Distribution of *CXCR2* haplotypes (arranged as genotypes) in CP patients and healthy nonperiodontitis controls.

Haplogenotypes +785 +1208 +1440/+785 +1208 +1440	Controls $N = 171$ (%)	CP $N = 329$ (%)	P value	OR (95% CI)
CTG/CTG	27 (15.8)	52 (15.8)	0.55	1.00 (0.60-1.66)
CTG/TCA	67 (39.2)	128 (38.6)	0.51	0.99 (0.68-1.44)
CTG/TCG	6 (3.5)	12 (3.6)	0.58	1.04 (0.38-2.82)
CTG/CCA	5 (2.9)	11 (3.3)	0.52	1.15 (0.39-3.36)
CTG/TTG	6 (3.5)	5 (1.5)	0.13	0.42 (0.13-1.41)
CTG/CCG	6 (3.5)	11 (3.3)	0.55	0.95 (0.35-2.62)
CTG/CTA	2 (1.2)	2 (0.6)	0.42	0.52 (0.07-3.70)
CTG/TTA	0 (0.0)	2 (0.6)	0.43	#
TCA/TCA	21 (12.3)	42 (12.8)	0.50	1.05 (0.60-1.83)
TCA/TCG	12 (7.0)	21 (6.4)	0.46	0.90 (0.43-1.88)
TCA/CCA	9 (5.3)	13 (4.0)	0.32	0.74 (0.31-1.77)
TCA/TTG	0 (0.0)	8 (2.4)	0.03*	#
TCA/CCG	3 (1.8)	5 (1.5)	0.55	0.86 (0.20-3.66)
TCA/CTA	2 (1.2)	0 (0.0)	0.12	#
TCA/TTA	2 (1.2)	0 (0.0)	0.12	#
TCG/TCG	0 (0.0)	5 (1.5)	0.12	#
TCG/TTG	1 (0.6)	3 (0.9)	0.58	1.56 (0.16-15.15)
TCG/CCG	0 (0.0)	1 (0.3)	0.66	#
CCA/CCA	0 (0.0)	3 (0.9)	0.28	#
CCA/CCG	0 (0.0)	1 (0.3)	0.95	#
CCA/CTA	1 (0.6)	1 (0.3)	0.57	0.52 (0.03-8.34)
TTG/TTG	1 (0.6)	3 (0.9)	0.58	1.56 (0.16-15.15)

CI = confidential interval; CP = chronic periodontitis; N = number of subjects; OR = odds ratio. *$P < 0.05$ by the Fisher exact test (without correction for multiple comparisons), but there is a low N in both groups. #OR not calculated because of the presence of zero.

Chemokines and their receptors play important roles in immunological responses, and thus their genetic contribution to various human inflammatory disorders needs investigation [31]. Several reports have suggested that the *CXCR2* variants might influence the susceptibility to chronic inflammatory conditions, especially rheumatoid and respiratory diseases [32–35]. The *CXCR2* gene variability has been associated with several disorders like systemic sclerosis and cryptogenic fibrosing alveolitis, with a strong linkage between the +785C, +1208T, and +1440G alleles [26]. The +785T allele has been found to be protective against chronic obstructive pulmonary disease [32]. In Slovakia, children with the SNP +1208T allele were significantly unrepresented in the recurrent acute pyelonephritis subgroup, and the carriage of the T allele (TT+CT genotypes vs. CC genotype) was linked with a reduced risk of developing this disease [36]. Moreover, analysis of SNP +1208 with serum levels of IL-8, its endogenous ligand, supports an interaction whereby the variant +1208T allele and high serum IL-8 confer synergistic protection against lung cancer [33].

In our research, we focused on 3 polymorphisms in the *CXCR2* receptor +785C/T, +1208T/C, and +1440G/A, which

Table 5: *CXCR2* gene variants and the presence of periodontal bacteria in 62 male CP patients.

Allele frequencies (%)		*CXCR2* +785		*CXCR2* +1208		*CXCR2* +1440	
		C	T	C	T	A	G
A. actinomycetemcomitans	Neg. $N = 90$	41.1	58.9	61.1	38.9	45.6	54.4
	Pos. $N = 34$	61.8*	38.2	38.2	61.8*	29.4	70.6
T. forsythia	Neg. $N = 14$	35.7	64.3	50.0	50.0	35.7	64.3
	Pos. $N = 110$	48.2	51.8	55.5	44.5	41.8	58.2
P. gingivalis	Neg. $N = 38$	47.4	52.6	55.3	44.7	36.8	63.2
	Pos. $N = 86$	46.5	53.5	54.7	45.3	43.0	57.0
T. denticola	Neg. $N = 40$	52.5	47.5	47.5	52.5	40.0	60.0
	Pos. $N = 84$	44.0	56.0	58.3	41.7	41.7	58.3
P. micra	Neg. $N = 22$	31.8	68.2	72.7	27.3	40.9	59.1
	Pos. $N = 102$	50.0	50.0	51.0	49.0*	41.2	58.8
P. intermedia	Neg. $N = 56$	50.0	50.0	53.6	46.4	35.7	64.3
	Pos. $N = 68$	44.1	55.9	55.9	44.1	45.6	54.4
F. nucleatum	Neg. $N = 2$	0.0	100.0	100.0	0.0	0.0	100.0
	Pos. $N = 122$	47.5	52.5	54.1	45.9	41.8	58.2

N = number of alleles; Neg. = negative; Pos. = positive. $^*P \leq 0.05$.

were previously investigated in Brazilian patients with CP [21]. We detected similar allele, genotype, and haplotype frequencies of the all studied *CXCR2* gene polymorphisms between CP patients and controls ($P > 0.05$). In contrast, the +1440GG genotype, originally described by Viana et al. [37], was suggested as a protective factor against CP in Brazilians [21]. The differences between the results in the Czech and Brazilian study [21] could be caused by the interpopulation variability. The *CXCR2* +1440 minor allele frequencies were found to be 46.5% in Czech healthy non-periodontitis controls vs. 57.4% in Brazilian controls [21]. However, our result is in line with the minor allele frequency (43.3%) in the European population [38].

If we arranged haplotypes as genotypes, the carriers of the TCA/TTG (or TTG/TCA) variant seemed to be more susceptible to CP development ($P < 0.05$). On the other hand, the number of carriers of this haplogenotype is too small to demonstrate any significant association with CP after correction for multiple comparisons. Viana et al. found that patients carrying the haplotypes TCA and CCG were more predetermined to CP development, whereas CCA and TCG haplotypes seemed to be protective against CP. In addition, white nonsmoking patients carrying the CTG/TCA variant were more likely to develop periodontal disease, whereas CTG/TCG patients seemed to be protected [21]. Our result matches with a comparable finding elsewhere: TTG/TCA and CTG/TCA haplotypes were associated with CP risk in two different populations, i.e., the Czech and Brazilian [21]. In addition, no CTG/TCG haplotype carrier was present in our population.

A lot of studies have focused on the relationship between the selected gene variants and the presence of periodontal bacteria studied in the recent review and meta-analysis by Nibali et al. [39]. Evidence suggests that genetic factors can influence periodontitis risk, modulating disease elements such as the susceptibility to microbial colonization and the nature of subsequent host-microbe interaction [40, 41]. Our previous research into *IL-8* gene polymorphisms in CP and aggressive periodontitis (AgP) patients shows the association between *IL-8* genotypes and the occurrence of specific periodontal bacteria [24]. In our earlier study, we reported significant differences in the colonization of the oral cavity with *P. gingivalis* (70.5% in CP patients vs. 28% in controls), *T. forsythia* (92.3% in CP patients vs. 56.3% in controls), and *P. micra* (87.2% in CP patients vs. 56.3% in controls) between CP patients and healthy controls [28]. We also determined that *IL-17A* -197A/G (rs2275913) polymorphism was associated with the presence of *T. forsythia* and *T. denticola* in CP patients [27] and that *IL-4* gene polymorphisms in CP patients could predispose to altered cytokine production after bacterial stimulation [42].

Although *A. actinomycetemcomitans* is more often associated with AgP than with CP, Gaetti-Jardim et al. [43] detected the bacteria by the PCR method in 44% of CP patients. In the Brazilian study, the *IL-4* haplotypes, but not the *IL-8* haplotypes, were associated with the presence of *A. actinomycetemcomitans* in CP patients [41]. Nibali et al. reported an association between the variability in the *IL-6* gene and *A. actinomycetemcomitans* in CP patients. The strong association of *IL-6* -174 GG homozygotes with the presence of *A. actinomycetemcomitans* in all subjects and in the subgroup of only white subjects was observed [42]. In our preliminary study, no significant association between the *CXCR2* +1208C/T (rs1126579) SNP and IL-8 plasma levels and the occurrence of the selected periodontal bacteria in 41 CP patients was found [24]. This result was confirmed in the present study on a larger sample size ($N = 162$). After the gender stratification, the presence of *A. actinomycetemcomitans* was significantly associated with *CXCR2* +785C/T and *CXCR2* +1208C/T SNPs, but only in Czech men.

5. Conclusions

This study did not confirm any significant association between the investigated SNPs in the *CXCR2* gene and chronic periodontitis. However, the *CXCR2* gene variants can be associated with subgingival colonization of the selected G- bacteria in men with CP in the Czech population.

Data Availability

The clinical and genetic data used to support the findings of this study are restricted by the Committees for Ethics of the Faculty of Medicine, Masaryk University, Brno (no. 13/2013), in order to protect patient privacy. Data are available from Lydie Izakovicova Holla (holla@med.muni.cz) for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

D.K. and P.B.L. are responsible for the conceptualization, methodology, validation, formal analysis, investigation, data curation, visualization, and writing of the manuscript (original draft preparation), S.L., H.P., and A.F. are responsible for the methodology and writing of the manuscript (review and editing), and L.I.H is responsible for the conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing of the manuscript (review and editing), and supervision. D.K. and P.B.L. contributed equally to this work.

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