Acta Odontologica Scandinavica, 2013; 71: 683–688

informa
healthcare

ORIGINAL ARTICLE

Plasma cytokines levels in aggressive and chronic periodontitis

LETÍCIA GRANDO MATTUELLA[1], MILENE BORGES CAMPAGNARO[1], ANDRÉIA ESCOSTEGUY VARGAS[2], LÉDER LEAL XAVIER[1], RUI VICENTE OPPERMANN[3], JOSÉ ARTUR BOGO CHIES[2] & LETÍCIA ALGARVES MIRANDA[1]

[1]*Laboratório de Biologia Tecidual, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil,* [2]*Laboratório de Imunogenética, and* [3]*Departamento de Odontologia Conservadora – Periodontia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil*

Abstract

Objective. The present study evaluated the Th1/Th2 cytokine profile in plasma from healthy controls and different types of periodontitis patients. ***Materials and methods.*** The concentration of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ was determined in healthy controls ($n = 18$) and patients with chronic ($n = 19$) and aggressive periodontitis ($n = 19$) using a flow cytometric multiplex immunoassay. Means and standard deviations were calculated and compared using Kruskal-Wallis test. Spearman rho coefficient was used to correlate cytokines in the studied groups. ***Results.*** Although there was no significant difference in the concentration of cytokines between groups, there was a tendency to lower levels of IL-5 and IL-10 in the aggressive periodontitis group. Stronger correlations were observed between IL-2/IL-4 and IL-2/IL-10 in healthy controls (0.938 and 0.669, respectively) compared with chronic (0.746 and 0.532) and aggressive periodontitis groups (0.395 and 0.266). When compared to healthy (0.812) and chronic periodontitis (0.845) groups, the correlation of IL-4/IL-5 was weaker in the aggressive group (0.459). ***Conclusion.*** No difference between systemic levels of Th1/Th2 was observed. In aggressive periodontitis patients, nevertheless, a trend towards low levels of Th2 cytokines could suggest a contribution to the development of such an exacerbated manifestation of this disease.

Key Words: *periodontitis, cytokines, plasma, flow cytometry multiplex assay*

Introduction

Periodontitis is a chronic tissue destructive condition in which the tooth supporting collagen fibers of the periodontal ligament and bone are broken down, mainly due to the development of an exacerbated immune inflammatory response to the dental biofilm [1]. The local ecological conditions of the gingival sulcus and host response can influence the opportunistically overgrow of periodontal bacteria [2] and be able to promote destructive periodontitis resulting in irreversible loss of connective tissue and bony attachment [3]. According to the American Academy of Periodontology [4], chronic periodontitis is classified as a slow progression disease related to an accumulation of bacteria biofilm. This form of the disease usually occurs in people over 30/40 years old. Aggressive periodontitis is characterized by a rapid periodontal destruction and generally occurs in young patients with no concomitant systemic diseases. Familial aggregation can occur and sometimes the severity of the disease is not compatible with the amount of biofilm, suggesting a high susceptibility to tissue destruction. As a result, these susceptible patients are twice as likely to lose their teeth when compared to healthy subjects matched by age and sex [5]. Immunological features, such as cytokine profiles, have been studied in an attempt to better understand the behavior of the immune system in these highly susceptible patients [6,7,9,10,12,13].

Cytokines, small soluble proteins that convey information from one cell to another [14], play essential roles in immune and inflammatory responses and the outcome of infection may be attributable to the

Correspondence: Letícia Algarves Miranda, Av. Ipiranga 6681, Bloco 12A, Sala 144, Porto Alegre, RS, 90619-900, Brazil. Tel: + 55 51 3320 3545. E-mail: leticia.miranda@pucrs.br

(Received 8 February 2012; revised 31 May 2012; accepted 31 May 2012)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2012.715191

relative balance between such molecules. A functional definition of cytokines distinguishes between Th1 and Th2 cytokines. Briefly, Th1 cytokines (with TNF-α and IFN-γ as typical representatives) are involved in the activation of T cytotoxic cells and macrophages, consequently stimulating cellular immunity and inflammation, whereas Th2 cytokines (IL-4, IL-5 and IL-10) promote hummoral immunity, counterbalancing Th1 and, consequently, acting as anti-inflammatory molecules [15].

Periodontal disease is a complex condition. The dominance of a hummoral-type response is suggested in chronic lesions [12], but there is room to speculate that, in the early stages of the disease, cell-mediated reactions interfere with, or even direct, the disease progression. Analyses of cytokine profiles (i.e. type and levels of both Th1 and Th2) in periodontal tissues affected by chronic periodontitis have been performed using distinctly different strategies, such as *in situ* hybridization and immunohistochemistry [13], ELISA [7,9,11], RT-PCR [10,12] and more recently with cytometric bead arrays (CBA) [6,8]. Such studies in aggressive periodontitis are more scarce [9,10] and the CBA methodology has not been used so far in these patients. Some controversial results have been found, for example, elevated levels of IL-10 [6,9,13] and decreased levels of IL-2 [6,13] and IFN-γ [13] were observed in periodontitis compared to healthy controls. IL-4 was significantly present in aggressive [13] but not in chronic periodontitis [11]. IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were not statistically significant in chronic periodontitis compared to a control group [8].

In order to further contribute to the characterization of the cytokine profile presented by periodontitis patients, especially those with the aggressive form of the disease, we evaluated the Th1/Th2 cytokine profile, defined by the detection of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ in plasma from patients with aggressive and chronic periodontitis, using a CBA.

Materials and methods

Subjects

Fifty-six patients were selected from the Department of Periodontology at the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil. Their age ranged from 13–64 years. After clinical examination, the patients who fulfilled the clinical inclusion criteria (see below) were invited to participate in the present study, which was submitted to and approved by the UFRGS ethics committee (Protocol Nr.: 47/05). All patients and individuals from the control group were thoroughly informed about the purpose and methods of the study and written consent was obtained from each of them.

Subjects were classified into the following groups: healthy control ($n = 18$), chronic ($n = 19$) and aggressive periodontitis ($n = 19$). Control healthy subjects had at least 20 teeth in the mouth, a gingival bleeding index less than 20%, attachment loss and/or probing depth in proximal sites ≤3 mm [16]. All periodontal examinations were performed by post-graduated periodontologists. Chronic periodontitis subjects were aged between 35–60 years and had at least 20 teeth, attachment loss ≥4 mm in at least 10 teeth, probing depth ≥6 mm in at least five teeth and periodontal bleeding in at least 10 teeth. Aggressive periodontitis subjects were those who had four or more teeth with attachment loss ≥4 mm in individuals between 14–19 years old or ≥5 mm in individuals between 20–29 years old [5]. A questionnaire was applied to all participants regarding their educational level, smoking habits and general health. For the present research, individuals diagnosed with human immunodeficiency virus and/or diabetes, pregnant or lactiferous women and anyone who took immunomodulatory/anti-inflammatory/antibiotics drugs in the 6 months prior to the study were excluded.

Sample collection

Blood samples (15 mL) were obtained by venipuncture from each patient using BD™ Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ, USA) which were immediately centrifuged in order to isolate the plasma. Plasma samples were aliquoted and stored at -20°C until manipulation.

CBA analysis

The concentration of plasma cytokines was determined by means of flow cytometry using the BD™ CBA Human Th1/Th2 Cytokine Kit I (BD Biosciences, San Jose, CA, USA), a multiplex immunoassay which quantitatively measures soluble analytes on the basis of their different fluorescence intensities [6,8,17]. The CBA kit employed allows for the discrimination of the following cytokines: IL-2, IL-4, IL-5 and IL-10, as well as IFN-γ and TNF-α. The detection limits were as follows: IL-2, 2.6 pg/ml; IL-4, 2.6 pg/ml; IL-5, 2.5 pg/ml; IL-10, 2.8 pg/ml; IFN-γ, 7.1 pg/ml and TNF-α, 2.8 pg/ml. Sample processing and data analysis were performed according to the manufacturer's instructions. Sample data were acquired using a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

The unit of analysis was established as the individual and α was set at 0.05. Patient data was compared with

Table I. Characteristics of the studied subjects.

	Periodontitis group			
	Healthy group	Chronic periodontitis	Aggressive periodontitis	*p*-value
Male/female gender, %	12/6 (66.7)	14/5 (73.7)	16/3 (84.2)	0.462
Age, mean (SD)	28.8 (4.36)[a]	49.4 (9.76)[b]	35.6 (8.48)[b]	0.031*
Ethnic group, %				0.204
Caucasian	18 (100)	16 (84.2)	16 (84.2)	
Non-Caucasian	0 (0.0)	3 (15.8)	3 (15.8)	
Smoking status, %				0.366
Non-smoker	14 (77.8)	11 (57.9)	11 (57.9)	
Smoker/former smoker	4 (22.2)	8 (42.1)	8 (42.1)	

*Statistically different at 0.05, different letters mean statistical difference.

ANOVA test for age and Chi-squared test for gender, ethnic group and smoking status. Concentration of cytokines in pg/ml was presented as median and percentiles 25 and 75 and compared using Kruskal-Wallis test. Spearman rho coefficient was used to correlate between cytokines in healthy, chronic and aggressive periodontitis groups and Pearson's correlation test to ascertain the relationship between age and each cytokine within the three studied groups. A value was considered zero when the level of the cytokine was below the methodological limit of detection and the sample was not excluded from the analysis. SPSS Statistics® 18.0 software was used to analyze the data.

Results

General characteristics of the subjects included in the study are presented in Table I. There was no statistical difference between the cytokines concentrations when the three groups were compared (Table II). However, some trends could be observed. IL-5 and IL-10 levels were lower in patients with aggressive (0.0 and 0.0, respectively) when compared to chronic periodontitis (1.2 and 1.9, respectively) and with healthy controls (0.6 and 0.0, respectively) ($p = 0.060$ and $p = 0.097$, respectively). Besides, IFN-γ levels decreased from healthy to chronic and to aggressive periodontitis groups, respectively (1.8, 1.4, 1.1) ($p = 0.435$). Chronic periodontitis patients presented higher levels of all cytokines when compared to healthy individuals, except IFN-γ (Table II). The pattern of cytokine profiles showed a high individual variability. Pearson's correlation analysis did not detect any relationship between age and cytokine levels when the three studied groups were analyzed (data unshown).

Tables III, IV, V describe the correlations between plasma cytokines of all the studied groups. Spearman rho coefficients between IL-2/IL-4 and IL-2/IL-10 were stronger in healthy individuals (0.938 and 0.669, respectively) compared with chronic (0.746 and 0.532, respectively) and aggressive periodontitis groups (0.395 and 0.266, respectively). A similar pattern was observed when IL-2/IFN-γ cytokines were analyzed, with the weakest correlation observed in the aggressive periodontitis group (0.600). When compared to the healthy (0.812) and chronic (0.845) groups, IL-4/IL-5 correlation was weaker (0.459) in the aggressive periodontitis group.

Table II. Concentration of plasma cytokines in pg/ml (median and percentiles 25 and 75) in chronic and aggressive periodontitis groups compared with healthy individuals.

	Periodontitis			
	Healthy group	Chronic periodontitis	Aggressive periodontitis	*p*-value
IL-2	0.6 (0.0–2.3)	1.6 (0.0–2.2)	1.4 (0.0–1.8)	0.592
IL-4	1.5 (0.0–2.6)	1.9 (0.0–2.5)	1.5 (0.0–2.0)	0.645
IL-5	0.6 (0.0–1.5)	1.2 (0.0–1.4)	0.0 (0.0–0.0)	0.060
IL-10	0.0 (0.0–2.4)	1.9 (0.0–3.0)	0.0 (0.0–1.5)	0.097
TNF-α	0.7 (0.0–2.9)	2.1 (0.0–3.1)	1.6 (0.0–2.4)	0.400
IFN-γ	1.8 (0.0–3.2)	1.4 (0.0–3.2)	1.1 (0.0–2.1)	0.435

p-value corresponds to an all-groups comparison by Kruskall Wallis test.

Table III. Spearman rho coefficients between cytokines in the healthy group.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
IL-2						
IL-4	0.938					
IL-5	0.813	0.812				
IL-10	0.669	0.554	0.637			
TNF-α	0.597	0.526	0.703	0.703		
IFN-γ	0.733	0.801	0.772	0.772	0.737	

Table IV. Spearman rho coefficients between cytokines in the chronic periodontitis group.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
IL-2						
IL-4	0.746					
IL-5	0.644	0.845				
IL-10	0.532	0.580	0.720			
TNF-α	0.741	0.762	0.738	0.792		
IFN-γ	0.655	0.751	0.744	0.807	0.846	

Table VI shows that the cytokine concentration in plasma samples was above the methodological detection limit in not all cases.

Discussion

In the present study, the expression of cytokines that define Th1 and Th2 immune response types (IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ) was compared in plasma of individuals with different forms of periodontitis. No statistical difference could be observed between groups. We used in aggressive periodontitis patients the CBA technique, a flow cytometry multiplex assay that has clear advantages compared to conventional ELISA, such as obtaining a standard curve for each analyte studied from a single set of diluted standards. Besides, it allows measuring simultaneously six cytokines belonging to the Th1 and Th2 profile, minimizing methodological errors. When the cytokines levels were below the methodological limit we considered as zero and included the sample in the analysis [6]. In this case, zero doesn't mean an absolute number, but rather stands for a value inferior to the detection limit of the test.

Our results are in accordance with a previous work that investigated the systemic levels of some inflammatory mediators, such as IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ, with CBA in patients with chronic periodontitis and healthy controls [8]. Using the same methodology, the authors have not observed statistical difference when all the cytokines above were measured. However, this study has not included an aggressive periodontitis group.

Table V. Spearman rho coefficients between cytokines in the aggressive periodontitis group.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
IL-2						
IL-4	0.395					
IL-5	0.681	0.459				
IL-10	0.266	0.557	0.674			
TNF-α	0.424	0.327	0.521	0.619		
IFN-γ	0.600	0.668	0.521	0.543	0.591	

Table VI. Percentage of detection when the level of each cytokine was above the detection limit in healthy, chronic and aggressive periodontitis groups.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
Healthy	50	56	56	39	56	72
Chronic periodontitis	63	68	21	26	47	53
Aggressive periodontitis	63	63	58	53	68	53

Recently, a new Th cell lineage capable of producing IL-17, the Th17 cells, has been described. This cytokine is an important regulator of neutrophil and macrophage migration and subsequent pathogen elimination. It acts over osteoblast enhancing the receptor activator nuclear factor kappa B ligand (RANKL) expression, inducing directly the differentiation of osteoclast progenitors in mature osteoclast and is able to stimulate C-reactive protein production [18,19]. The Th17 profile has not been included in our analysis since the present manuscript corresponds to an experimental approach designed before the availability of a commercial kit including testing for IL-17 levels. Nevertheless, to our knowledge, gingival crevicular fluid levels of IL-17 were recently assessed comparing aggressive periodontal patients and healthy controls and no differences were observed in the total amount of this cytokine between the groups [20]. Considering that Ay et al. [20] performed their analyses on gingival crevicular fluid, a compartment close to the local of inflammation and showing an absence of correlation among disease and IL-17 total amount, we believe that similar results would be achieved when assessing plasma cytokine levels. However, we cannot rule out the possibility of different plasma IL-17 levels among periodontitis patients and controls and a new set of patients is intended to be collected in order to approach that [18,20].

In the present study, a trend to lower levels of IL-5 and IL-10 in aggressive periodontitis (0.0 and 0.0, respectively) was observed when compared to both chronic periodontitis patients (1.2 and 1.9, respectively) and healthy controls (0.6 and 0.0, respectively) ($p = 0.060$ and $p = 0.097$, respectively). Low levels of IL-5 and IL-10 (typical Th2-type response cytokines) observed in the aggressive periodontitis group could lead to a defective control of the immune responses directed against pathogens, consequently resulting in exacerbated inflammation. Through cytokine mRNA analysis, Suárez et al. [10] observed the expression of IL-5 and IL-10 in biopsies from healthy and periodontitis patients, although this expression was absent from the diseased tissue obtained from patients with aggressive periodontitis, which comes in favor of our data. Górska et al. [11] analyzed cytokine concentrations in inflamed gingival

tissues and serum samples from patients with severe chronic periodontitis and compared them to healthy controls. Serum samples showed high individual variability of cytokine profiles, and no association between cytokine concentrations and clinical parameters of periodontitis was found. Nevertheless, the authors point out that, although the IL-10 levels were generally low or even undetectable in serum, the frequency of individuals expressing IL-10 positive cells was much higher in healthy gingival tissues as compared to chronic periodontitis patients, again suggesting that local expression of this molecule can control inflammation. Also, in accordance with our data, Garlet et al. [12] observed, through RT-PCR, lower IL-10 expression in gingival biopsies from patients presenting with aggressive periodontitis.

In addition, we found lower levels of IFN-γ in the periodontitis group compared to healthy controls, in agreement with the findings of Lappin et al. [13]. On the other hand, we observed a higher level of TNF-α in periodontitis patients compared to healthy controls Andrukhov et al. [6].

IL-2/IL-4 Spearman rho coefficient was stronger in healthy controls compared to the periodontitis group. IL-2 plays an essential role in Th2 priming and stabilizes the accessibility of the I*l*4 gene, and STAT5, a key transducer of IL-2 function [21]. We found a weak correlation between IL-4/IL-5 (0.459) in the aggressive periodontitis group. The chemoattraction potential of the cytokines produced by Th1 could favor bone resorption and disease progression. As pointed out by Garlet et al. [12], the chemoattracting characteristics of IL-4 and IL-10 producing cells could control the potentially destructive Th1 response. As IL-10 is associated with suppression of bone resorption [22,23], its low expression in patients with aggressive periodontitis could account for the more severe form of disease.

The present study shows a high variability of cytokines levels in plasma. Recently, it has been suggested that, in periodontitis, this variability and the lower frequency of its detection may contribute to determining the presence and/or severity of the disease [8]. Although Górska et al. [11] affirm that cytokine serum levels are not good disease indicators in periodontitis and that such results may lead to controversial conclusions, the occurrence of an increase in T-cell numbers in peripheral blood and the homing of these cells to the gingiva are well established during the disease process [13]. Furthermore, the level of cytokines in plasma can represent a possible spill-over to the circulation of cytokines produced locally in the inflamed tissues [9]. There is evidence that periodontitis can be associated through this mechanism to an increased risk of a series of systemic conditions such as cardiovascular disease [24] and pregnancy adverse events [25].

Further studies with an increased number of subjects may show more precise results for the relationship between cytokines and periodontitis. However, aggressive periodontitis is a rare condition and few specific publications are found in the literature. In a representative sample of young individuals (14–29 years old) from the metropolitan Porto Alegre/Brazil region, 5.5% of the subjects presented aggressive periodontitis [5].

Our results indicate no difference between systemic levels of Th1 and Th2 cytokines between all groups. Since Th2 cytokines are involved in the regulation of inflammatory responses, the observed trend towards low levels of Th2 cytokines in aggressive periodontitis patients could suggest a contribution to the development of such an exacerbated manifestation of this disease.

Acknowledgments

This study was supported by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (Fapergs, Brazil), PPSUS Fapergs 0615232.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Miranda LA, Fischer RG, Sztajnbok FR, Figueiredo CMS, Gustafsson A. Periodontal conditions in patients with juvenile idiopathic arthritis. J Clin Periodontol 2003;30:969–74.

[2] Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol 1992; 63:322–31.

[3] Stein JM, Machulla HKG, Smeets R, Lampert F, Reichert S. Human leukocyte antigen polymorphism in chronic and aggressive periodontitis among Caucasians: a meta-analysis. J Clin Periodontol 2008;35:183–92.

[4] American Academy of Periodontology. Consensus report on the classification of periodontal diseases. Ann Periodontol 1999;4:38–53.

[5] Susin C, Albandar JM. Aggressive periodontitis in an urban population in Southern Brazil. J Periodontol 2005;76: 468–75.

[6] Andrukhov O, Ulm C, Reischl H, Nguyen PQ, Matejka M, Rausch-Fan X. Serum cytokine levels in periodontitis patients in relation to the bacterial load. J Periodontol 2011;82:885–92.

[7] Duarte PM, da Rocha M, Sampaio E, Mestnik MJ, Feres M, Figueiredo LC, et al. Serum levels of cytokines in subjects with generalized chronic and aggressive periodontitis before and after non-surgical periodontal therapy: a pilot study. J Periodontol 2010;81:1056–63.

[8] Queiroz AC, Taba M Jr, O'Connell PA, Nóbrega PB, Costa PP, Kawata VKS, et al. Inflammation markers in healthy and periodontitis patients. A preliminary data screening. Braz Dent J 2008;19:3–8.

[9] Havemose-Poulsen A, Sørensen LK, Stoltze K, Bendtzen K, Holmstrup P. Cytokine profiles in peripheral blood and whole

blood cell cultures associated with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis. J Periodontol 2005;76:2276–85.

[10] Suárez LJ, Ocampo AM, Dueñas RE, Rodríguez A. Relative proportions of T-cell subpopulations and cytokines that mediate and regulate the adaptive immune response in patients with aggressive periodontitis. J Periodontol 2004;75:1209–15.

[11] Górska R, Gregorek H, Kowalski J, Laskus-Perendyk A, Syczewska M, Madaliński K. Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. J Clin Periodontol 2003;30:1046–52.

[12] Garlet GP, Martins W Jr, Ferreira BR, Milanezi CM, Silva JS. Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. J Periodontal Res 2003;38:210–17.

[13] Lappin DF, Macleod CP, Kerr A, Mitchell T, Kinane DF. Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. Clin Exp Immunol 2001; 123:294–300.

[14] Callard R, George AJT, Stark J. Cytokines, chaos, and complexity. Immunity 1999;11:507–13.

[15] Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptative antitumor immunity. Trends Immunol 2002; 23:201–8.

[16] Castro GDC, Oppermann RV, Haas AN, Winter R, Alchieri JC. Association between psychosocial factors and periodontitis: a case-control study. J Clin Periodontol 2006; 33:109–14.

[17] Brietzke E, Stertz L, Fernandes BS, Kauer-Sant'anna M, Mascarenhas M, Escosteguy Vargas A, et al. Comparison of cytokine levels in depressed, manic and euthymic patients with bipolar disorder. J Affect Disord 2009;116:214–17.

[18] Bi Y, Liu G, Yang R. Th17 cell induction and immune regulatory effects. J Cell Physiol 2007;211:273–8.

[19] Kramer JM, Gaffen SL. Interleukin-17: a new paradigm in inflammation, autoimmunity, and therapy. J Periodontol 2007;78:1083–93.

[20] Ay ZY, Yilmaz G, Ozdem M, Kocak H, Sütçü R, Uskun E, et al. The gingival crevicular fluid levels of interleukin-11 and interleukin-17 in patients with aggressive periodontitis. J Periodontol 2012. [Epub ahead of print]

[21] Cote-Sierra J, Foucras G, Guo L, Chiodetti L, Young HA, Hu-Li J, et al. Interleukin 2 plays a central role in Th2 differentiation. PNAS 2004;101:3880–5.

[22] Kawashima N, Stashenko P. Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. Arch Oral Biol 1999;44:55–66.

[23] Sasaki H, Hou L, Belani A, et al. IL-10, but not IL-4, suppresses infection-stimulated bone resorption *in vivo*. J Immunol 2000;165:3626–30.

[24] Beck JD, Slade G, Offenbacher S. Oral disease, cardiovascular disease and systemic inflammation. Periodontology 200023: 110–20.

[25] Guimarães AN, Silva-Mato A, Miranda Cota LO, Siqueira FM, Costa FO. Maternal periodontal disease and preterm or extreme preterm birth: an ordinal logistic regression analysis. J Periodontol 2010;81:350–8.