Correlation between periodontal disease, inflammatory alterations and pre-eclampsia

G. T. Politano[1], R. Passini[2], M. L. Nomura[2], L. Velloso[3], J. Morari[3], E. Couto[2]
[1]Department of Pediatric Dentistry, Dental School São Leopoldo Mandic, Campinas, São Paulo, Brazil, [2]Department of Obstetrics and Gynecology and [3]Department of Internal Medicine, School of Medical Sciences, University of Campinas, Campinas, São Paulo, Brazil

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Background and Objective: Several studies have hypothesized that periodontal disease may increase the risk of pre-eclampsia. The correlation between the two diseases would probably be based on hypertension-related cytokine release in the local periodontal environment. The aim of this study was to evaluate the association between periodontal disease and pre-eclampsia, and the correlation of the two conditions with interleukin-6 *(IL-6)* and tumor necrosis factor-α *(TNFα)* mRNA expression.

Material and Methods: A case–control analysis of 116 pregnant women, 58 with pre-eclampsia (cases) and 58 normotensive pregnant women (controls) was performed. In addition to collection of socio-demographic data and periodontal evaluation, peripheral blood samples were collected for laboratory analysis of *IL-6* and *TNFα* mRNA expression by real-time PCR.

Results: There was an association between periodontitis and pre-eclampsia (adjusted odds ratio 3.73; 95% confidence interval 1.32–10.58). Increased *TNFα* mRNA expression was observed in pre-eclamptic women; however, there was no correlation between periodontitis and systemic cytokine expression. In the case group, systemic cytokine mRNA levels were similar in pregnant women with and without periodontitis (means ± SD): 0.73 ± 0.24 vs. 0.82 ± 0.38 for *TNFα* and 1.31 ± 1.49 vs. 1.09 ± 0.74 for *IL-6*, respectively.

Conclusion: Periodontitis was clinically related to pre-eclampsia; however, the supposed mechanism that correlates the two diseases, i.e. a systemic inflammatory process involving cytokines *TNFα* and *IL-6* in the presence of periodontal disease, could not be confirmed in this study.

Gabriel Tilli Politano, DDS, MSc, PhD, Antônio Lapa, 1032, Cambuí, Campinas – São Paulo, CEP 13025-242, Brazil
Tel: +55 19 32522903, +55 19 96350820
Fax: +55 19 32522903
e-mail: gabrielp@mpcnet.com.br;
gabriel@clinicapolitano.com.br

Pre-eclampsia, a pregnancy-specific disorder, is characterized by an increase in systolic arterial pressure (≥ 140 mmHg) and/or diastolic pressure (≥ 90 mmHg) and proteinuria (≥ 300 mg/24 h), after 20 wk of gestation. This condition is potentially dangerous for both the mother and the fetus, and control measures must be implemented with the aim of reducing the risk of progression to eclampsia, which is characterized by convulsions (1).

Although the etiology of pre-eclampsia remains obscure, several risk factors could be associated, such as maternal infection. A systematic review (and meta-analysis) was conducted to verify whether maternal infection was associated with the risk of pre-eclampsia. Among all infectious processes (urinary tract infection, periodontal disease, *Chlamydia pneumoniae* infection, HIV infection,

malaria, periodontal infection and cytomegalovirosis), periodontal disease showed the highest odds ratio [OR 1.76; 95% confidence interval (CI) 1.43–2.18]. Nevertheless, the lack of studies concerning the etiology of pre-eclampsia and the difficulty in establishing a cause–effect relationship between this disorder and periodontal disease were emphasized (2).

Although there is some controversy in the literature concerning pre-eclampsia, some studies have concluded that this disorder is of a pronounced systemic inflammatory nature, since higher levels of tumor necrosis factor-α (*TNF*α) and interleukin-6 (*IL-6*) were found in pre-eclamptic pregnant women, and these cytokines are especially known to cause endothelial damage (3).

Several studies have found a relationship between pre-eclampsia and periodontal disease (4–7). However, the majority of them presented methodological deficiencies and difficulties in proving clinical findings.

An increased plasma level of *IL-6* was found in patients with periodontitis after scaling. The authors also emphasized the possible systemic implications of this induction, since *IL-6* has many biological functions (8). In another study, the clinical and immunological correlations between periodontal disease and pre-eclampsia were evaluated, and it was concluded that severe oral infection seems to increase the risk for the occurrence of pre-eclampsia. The authors, using ELISA, also showed that systemic *TNF*α levels were correlated with bleeding on probing, but not with probing depth or clinical attachment level (9).

The aim of the present study was to perform a clinical and immunological evaluation of the association between periodontal disease and pre-eclampsia, as well as the correlation of the two diseases with *IL-6* and *TNF*α mRNA cytokine expression, by real-time PCR.

Material and methods

Study sample

A case–control methodology was used in this study, previously approved by the Institutional Review Boards of the Faculty of Medical Sciences University of Campinas (UNICAMP), Brazil (protocol number 055/2007). The research was conducted in accordance with the World Medical Association Declaration of Helsinki (10). A total of 58 women with pre-eclampsia (cases) attending two reference hospitals in Campinas, São Paulo, Brazil, and 58 normotensive women receiving prenatal care at two basic health units in the same region were enrolled in this study between July 2007 and July 2009. Normotensive and pre-eclamptic women volunteered to participate during a prenatal visit or if they were hospitalized for treatment of hypertension. After the evaluation, all pregnant women were allowed to have their scheduled dental care visits free of charge at the reference institutions in Campinas. Treatment for pre-eclampsia and prenatal care were performed normally.

Women were diagnosed with pre-eclampsia if they had blood pressure ≥140/90 mmHg after 20 wk of gestation and proteinuria, which was considered to be present when one 24 h urine collection showed a total protein excretion ≥300 mg (1). Women were diagnosed as normotensive if they had blood pressure < 140/90 mmHg, and if this had been the case during the pre-gestational period as well.

Exclusion criteria were as follows: evidence of maternal infection (except for periodontal infection); pregnant and < 20 wk gestation; multiple gestations; previous diagnosis of pre-eclampsia; gestational or pregestational diabetes; nefropathy; patients with cardiac abnormalities requiring antibiotic prophylaxis before dental interventions; currently undergoing periodontal treatment (during the gestational period); < 15 teeth.

Once the medical authorization form was obtained, all study participants signed a written informed consent. In addition, the women included in the study were interviewed using a structured questionnaire comprising information on the participant's age at delivery, gestational age, number of previous pregnancies, use of prenatal care, use of alcohol and tobacco, race and Brazilian socio-economic status (11). The economic classification used in this research took into consideration various characteristics of the pregnant woman's family, such as housing, assets in the home (such as electrical household appliances, television sets, DVDs and automobiles) and the residents' educational status. These data were used to stratify the classification into good, regular or poor economic condition.

Blood sampling and laboratory analysis for *IL-6* and *TNF*α mRNA detection

Blood samples (3.0 mL) from each pregnant patient were collected in tubes containing ethylenediaminetetraacetic acid. The material was then processed within 24 h at the UNICAMP cellular signalization laboratory.

Leukocytes were isolated from whole blood samples by centrifugation at 1330 *g* and 4°C. The supernatant (plasma) was discarded, and 1.0 mL of ammonium chloride (2 M) and 140 μL of ammonium bicarbonate (1 M) were added. Leukocytes were resuspended and isolated by vortexing for 10 s and centrifugation.

Isolation of RNA from leukocytes was performed using TRIzol® (Invitrogen, Carlsbad, CA, USA), strictly in accordance with the manufacturer's instructions. The integrity of the RNA was confirmed by 1% agarose gel electrophoresis. Samples (3 μg) of RNA were reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). As a result, complementary DNA (cDNA) was obtained.

Real-time PCR

The primers and probes for *IL-6* and *TNF*α were obtained from Applied Biosystems (TaqMan Gene Expression Assay ID Hs00174131_m1 and Hs00174128_m1, respectively), using the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as an endogenous control.

System validation was performed before the relative quantification experiments in order to ensure similarity of

the amplification efficiency (close to 100%) of both genes (relevant genes × endogenous control).

Relative real-time PCR was performed in triplicate for the quantification of relevant gene expression. Reactions were set up using 6.75 μL Taqman Universal PCR Master Mix 2×, 0.625 μL primer and probe mix, 1.125 μL water and 4.0 μL cDNA. Thermal cycling began with denaturing for 2 min at 50°C, 10 min at 95°C followed by 40 cycles at 95.0°C for 15 s and at 60°C for 1 min. Relative gene expression values were obtained using an ABI Prism1 7500 Sequence Detector System (SDS; Applied Biosystems®).

Clinical periodontal examination

All participants underwent a clinical periodontal examination. This was performed by one dental clinician with the patient lying on a specific litter or hospital bed. Intra-examiner variability was tested by performing repeated measurements in 12 pregnant women, on two consecutive days. The percentage agreement was 95.2% for probing depth and 95.9% for clinical attachment level.

Each tooth was measured and examined for plaque index (scored as being present or absent), probing depth (in millimeters), bleeding on probing (deemed positive if it occurred within 15 s after probing) and clinical attachment level (in millimeters) at six sites per tooth (mesiobuccal, buccal, distobuccal, lingual, distolingual and mesiolingual) with a Williams probe (Hu-Friedy, Chicago, IL, USA). Probing depth was recorded in millimeters from the free gingival margin to the base of the gingival sulcus or periodontal pocket. Clinical attachment level measurements were determined using the cemento-enamel junction as a reference point.

Patients were classified as having periodontitis when two or more sites showed pocket formation (≥4 mm), clinical attachment level (≥4 mm) and bleeding on probing (4).

Statistical analysis

Calculation of an appropriate sample size was based on studies evaluating the incidence of periodontal disease parameters (probing depth, bleeding on probing, prevalence of periodontitis and clinical attachment level) in pre-eclamptic/normotensive women (12), resulting in 58 cases and 58 controls. Furthermore, studies assessing the presence of cytokines (*IL-6* and *TNFα*) in pregnant women with or without pre-eclampsia were reviewed (13–16), resulting in 26 cases and 26 controls for *IL-6*, and 50 cases and 50 controls for *TNFα*. A sample size of 116 (58 in each group) was chosen, because it was the highest sample size value.

A statistical program and software (SAS version 9.2; SAS Institute, Cary, NC, USA) were used for data processing and data analysis. The difference between case and control groups of normally distributed continuous variables was assessed using Student's unpaired *t*-test. The Mann–Whitney *U*-test was used to evaluate the differences between the groups for the other variables (non-normal distribution). Fisher's exact and chi-squared tests were used to analyze the nominal data.

Bivariate association between periodontitis and pre-eclampsia was analyzed using the chi-squared test. A multivariable logistic regression model was used to assess the relationship between the two diseases, adjusting for age and gestational age. Crude and adjusted odds ratios and their 95% confidence intervals were calculated. A value of $p \leq 0.05$ was considered to be significant. The Mann–Whitney *U*-test was used to compare periodontal variables between the groups.

The Mann–Whitney *U*-test was also used to compare cytokine *IL-6* and *TNFα* mRNA expression between groups and to analyze the relationship between periodontitis and cytokine mRNA expression. Correlations between periodontal data (plaque index, probing depth, bleeding on probing and clinical attachment level) and cytokine expression were determined by Spearman's rank test. Population attributable risk was estimated using Levin's Formula.

Results

The patient characteristics of both groups are given in Table 1. Mean age and mean gestational age were significantly higher in the pre-eclamptic group when compared with those in the normotensive group ($p \leq 0.05$). No significant association was found between the other variables studied (use of prenatal care, use of alcohol and tobacco, color/race, parity and socio-economic status) and pre-eclampsia.

Table 1. Personal characteristics of pre-eclamptic cases and normotensive controls

Variable	Pre-eclamptic cases ($n = 58$)	Normotensive controls ($n = 58$)	*p*-Value
Age (years; mean ± SD)	28.62 ± 6.93	24.69 ± 5.37	0.0009[a]
Gestational age (weeks; mean ± SD)	32.24 ± 4.24	28.92 ± 6.01	0.0035[b]
Economic status (*n* (%))			
Good economic situation	4 (6.9)	11 (19.0)	0.1716[c]
Regular economic situation	44 (75.9)	42 (72.4)	
Poor economic situation	10 (17.2)	5 (8.6)	
Race (*n* (%))			
Caucasian	23 (39.7)	33 (56.9)	0.0632[c]
Noncaucasian	35 (60.3)	25 (43.3)	
Primiparous (*n* (%))	21 (36.2)	28 (48.3)	0.1882[d]
Use of alcohol (*n* (%))	3 (5.2)	3 (5.2)	1.0000[c]
Proper utilization of prenatal care (*n* (%))	58 (100)	58 (100)	—
Tobacco use (*n* (%))	6 (10.3)	8 (13.8)	0.7767[c]

[a]Student's *t*-test.
[b]Mann–Whitney *U*-test.
[c]Fisher's exact test.
[d]Chi-squared test.
$p \leq 0.05$ was considered to be significant.

With regard to periodontal parameters, the percentage of sites with probing depth ≥4 mm, clinical attachment level ≥4 mm or probing depth and CAL ≥4 mm were individually associated with pre-eclampsia (Table 2), thus prevalence of periodontitis was significantly higher in the case group (adjusted odds ratio 3.73; 95% CI 1.32–10.58; Table 3). Adjusted population-attributable risks estimated for the sample of this study showed that if periodontitis were eliminated it would be 29.3% before and 45.9% after adjusting for the effect of those variables which differed between the groups.

As regards cytokine mRNA expression from leucocytes, the transcription amounts of *TNFα* gene expression were significantly higher in the pre-eclamptic group (mean ± SD, 0.77 ± 0.32) in comparison with those in the normotensive group (mean ± SD, 0.63 ± 0.34; $p < 0.05$). However, there were no statistically significant differences as regards *IL-6* levels (mean ± SD in the pre-eclamptic group 1.2 ± 1.17; and mean ± SD in the normotensive group 1.2 ± 1.5; $p > 0.05$). In the case group, systemic cytokine mRNA levels were similar in pregnant women with and without periodontitis (means ± SD): 0.73 ± 0.24 vs. 0.82 ± 0.38 for *TNFα* and 1.31 ± 1.49 vs. 1.09 ± 0.74 for *IL-6*, respectively.

Spearman's rank correlation index showed that there was no association between any of the periodontal parameters (plaque index, probing depth, bleeding on probing and clinical attachment level) and systemic *IL-6* and *TNFα* expression.

Discussion

There is growing evidence to suggest an association between maternal periodontal disease and the risk for adverse pregnancy outcomes, such as preterm birth (17), low birth weight (18) and pre-eclampsia (4,9,19–21).

The present study also found an association between periodontal disease parameters (percentage of sites with probing depth ≥4 mm, clinical attachment level ≥4 mm or probing depth and clinical attachment level ≥4 mm and prevalence of periodontitis) and pre-eclampsia. As a result, it is appropriate to inform women in the fertile period or during early pregnancy about the importance of good oral health. The findings of this study provide further evidence that there is an association between maternal periodontitis and pre-eclampsia (adjusted odd ratio of 3.73). This odds ratio value was considered very meaningful, since pre-eclampsia (as well as other multifactorial diseases) is related to several risk factors, showing an odds ratio similar to that for periodontitis. Taken together, these factors are responsible for the increased risk of pre-eclampsia. Thus, it would be presumptuous to offer periodontal treatment to pregnant women with the aim of reducing the risk of general health problems.

Adjusted population-attributable risks estimated for the sample of this study showed that if periodontitis were eliminated, the risk for pre-eclampsia would be excluded in 49.5% of the cases. Although these data emphasize the importance of periodontal disease

in pregnancy, additional work is needed to evaluate the influence of periodontal treatment in pregnant women, especially within the scope of conducting larger, prospective investigations. The results of the present study show that there is sound scientific justification for recommending regular follow-up dental examinations prior to or during pregnancy as a means of eliminating periodontal infection, right from the first day of gestation.

In the present study, clinical periodontal examination was performed in all the women by one calibrated examiner, thus eliminating inter-examiner variability and improving data quality. Intra-examiner variability was tested by performing repeated measurements in 12 pregnant women, on two consecutive days. Thus, the percentage agreement was 95.2% for probing depth and 95.9% for clinical attachment level.

The possibility that periodontal disease could be associated with gestational health problems is justified by the fact that oral infection can stimulate the inflammatory process. Some studies have shown bacterial colonization of the amniotic fluid in patients with preterm labor (22), as well as periopathogenic micro-organisms in the placentae of women with pre-eclampsia (23), suggesting systemic dissemination of these periodontopathogens. Furthermore, systemic dissemination of periodontopathogens to the vascular area involved in cytotrophoblast invasion and placentation could play a role in the development of pre-eclampsia. With the use of the first trimester extravillous cytotrophoblast

Table 2. Periodontal parameters in pre-eclamptic and normotensive groups

Variable	Pre-eclamptic cases ($n = 58$)	Normotensive controls ($n = 58$)	*p*-Value	Odds ratio (95% confidence interval)
Bleeding on probing (% of sites)	12 (0.16)	9 (0.13)	0.0794[a]	—
Plaque index (% of sites)	53 (0.27)	44 (0.23)	0.0540[a]	—
Probing depth ≥ 4 mm (mean ± SD; % of sites)	5.4 (12.3)	2.8 (5.8)	0.0481[a]	—
CAL ≥ 4 mm (mean ± SD; % of sites)	6.3 (12.9)	3.4 (5.9)	0.0334[a]	—
PD and CAL ≥ 4 mm (mean ± SD; % of sites)	5.3 (12.3)	2.6 (5.0)	0.0471[a]	—
Presence of periodontitis (%)	41 (70.3)	29 (50)	0.0227[b]	2.41 (1.12–5.18)

CAL, clinical attachment level.
[a]Mann–Whitney *U*-test.
[b]Chi-squared test.
$p \leq 0.05$ was considered to be significant.

Table 3. Relationship between periodontitis and risk of pre-eclampsia after adjusting for the effect of those variables which differ between groups (age and gestational age)

Variable	Pre-eclamptic cases ($n = 58$), %	Normotensive controls ($n = 58$), %	Odds ratio[a] (95% confidence interval)
Periodontitis			
Absent	29 (50)	41 (70.7)	1.00
Present	29 (50)	17 (29.3)	3.73 (1.32–10.58)

[a]Chi-squared test.

cell line, the authors showed that human cytomegalovirus impaired placentation by its inhibitory effect on cytotrophoblast invasiveness (24). These data emphasize the effects of infection on placentation; however, inevitably there are some differences between viruses and bacteria (25,26).

It is known that the micro-organisms present in periodontal infection promote local increase in the levels of various cytokines, such as interleukin-1β, prostaglandin E_2 (9), *IL-6* and *TNFα* (27) in the gingival crevicular fluid. As it has also been proved that although the origins of some cytokines, such as *IL-6* and *TNFα*, are partly unknown, they are associated with pre-eclampsia (13–16), and therefore the question of whether their production in the compromised periodontal site could increase the risk of hypertension should be discussed.

Canakci *et al.* (9) showed evidence that the levels of cytokines interleukin-1β, *TNFα* and prostaglandin E_2 were increased in the gingival crevicular fluid of pregnant women with pre-eclampsia when compared with those of normotensive pregnant women. The extensive vascularization of the periodontal ligament allows one to suppose that chronic local infections could be sources of cytokines that disseminate and act systemically in the vascular endothelium, promoting endothelial lesions (13–16,26,28–31) and therefore increasing the risk of pre-eclampsia.

In contrast, if pregnant women with pre-eclampsia, irrespective of periodontal infection, have increased levels of inflammatory cytokines in peripheral blood, one cannot discard the possibility that these mediators could increase the risk of periodontitis. That is to say, since periodontitis and pre-eclampsia are multifactorial diseases and have risk factors in common, when one thinks of the effect of the cytokines, it is difficult to establish which of the two diseases is the cause and which is the consequence of the other.

To analyze the correlation between the systemic inflammatory cytokines and the measurements found in the oral cavity (plaque index, probing depth, bleeding on probing and clinical attachment level), Spearman's rank correlation index was used. In other words, an evaluation was made to verify whether the increase in these measurements in isolation would signify an increase in mRNA of the inflammatory cytokines in the peripheral blood. The results of the present study showed that there was no positive or negative correlation between the oral indexes and the two cytokines studied.

In contrast, a previous study found a significant correlation between the plasma levels of *TNFα* and bleeding on probing in women with pre-eclampsia (9). The cytokine detection methodology may be responsible for these differences. Instead of evaluating plasma cytokine levels, as was done by Canakci *et al.* (9), the present study was designed to perform mRNA isolation from leukocytes by real-time PCR. In the case of cytokine detection, there are some disadvantages, since most cytokines have short circulating half-lives and are rapidly trapped by receptors. Thus, the results may not truly represent accurate cytokine quantification (32). Real-time PCRs were developed for mRNA analysis, thus enabling the gene activation for specific protein production to be identified at extremely low levels (33).

The present study and the study by Canakci *et al.* (9) are the only two studies to have analyzed the possible effect of these cytokines on the relationship between periodontitis and pre-eclampsia. Therefore, it is believed that further studies are needed to enable the influence of cytokines on this relationship to be confirmed or excluded. In addition, there are other mechanisms that have so far not been investigated, which might be responsible for the association between these two diseases.

The relationship observed between periodontitis and pre-eclampsia (OR 3.73; 95% CI 1.32–10.58) is consistent with previous articles in the literature (4,19–21). These data emphasize the importance of expectant mothers taking extra care of their oral health. However, the results of the present research and those of other studies should be interpreted with caution, owing to data limitations and varying methodologies, because the prevalence of periodontitis can vary a great deal within the same population depending upon the classification system used (34). In the present study, periodontal disease was categorized according to the classification used in a previous study (4), because this method was consistent with the previous literature.

Nevertheless, there are some limitations to this case–control study that warrant consideration: small sample size, varying periods of time in pregnancy in which the subjects were evaluated, and differences between two groups as regards mean maternal age.

Although some of the pregnant women in this study were of a young age, they were diagnosed with periodontitis. This occurred because the population evaluated in this study consisted of people with low socio-economic status, and in many cases had no access to dental care treatment.

The small number of individuals comprising the sample was due to the difficulty of diagnosing and including pre-eclamptic women during their pregnancy. In many cases, as soon as a patient's pre-eclampsia was diagnosed, the onset of labor occurred. This prevented many women from being included in the present study, because it would have been necessary to analyze their cytokine concentrations during the gestational period.

Among the studies consulted in the literature, almost all performed a periodontal analysis only, which ensured that the women were examined immediately after delivery (12,20,21). Thus, the differences between the groups with regard to age and gestational age were usually not presented.

Recently, large case–control studies have added further evidence to the association between periodontal disease and pre-eclampsia. Nevertheless, even with the relatively small number of pregnant women enrolled in this study and the differences between age and gestational age, there was statistical power to detect cytokine mRNA expression, which is considered the most valuable result found in the present study. Spearman's rank correlation index correlates all periodontal parameters (plaque index, probing depth, bleeding on probing and clinical attachment level) and systemic *IL-6* and *TNFα* expression, which are independent of differences between groups.

Both in pre-eclampsia and in periodontitis, the literature reports the involvement of various other cytokines, in addition to those evaluated in this study. Among them, the proinflammatory interleukins IL-1β, IL-8 and IL-12 (25) and the anti-inflammatory IL-4 and IL-10 (30) may be mentioned. Nevertheless, these studies were conducted in an isolated manner, without associating the periodontal and hypertensive diseases. It is believed that these other cytokines that have been proved to be involved in each of the mentioned diseases may, in a crossed manner, have an influence on the increase in risk of one on the other. However, *TNFα* and *IL-6* were chosen in the present study because they are the cytokines most closely related to pre-eclampsia and also because they are present in compromised periodontal sites (13–16). Therefore, as this research did not study other cytokines involved in local and systemic inflammatory processes, and no other reports of research on them were found in the literature, it is not possible to affirm that they are not involved in the relationship between periodontitis and pre-eclampsia.

Immunological studies about pre-eclampsia and periodontitis have only

recently appeared, and different methodologies have been used. Consequently, in view of these differences, it is not always valid to compare the results obtained. The important aspect is that new lines of research have been opened by the present study, so that further studies with larger populations can be developed during the course of the next few years.

It was concluded that periodontitis is a prevalent oral infection and one that demands care during gestation, because it was associated with pre-eclampsia in the present study. Although other studies have not confirmed that periodontal treatment diminished the risk for the development of pre-eclampsia (35), it is imperative for all pregnant women to undergo a screening examination to provide detailed assessment of their periodontal status, particularly for patients at risk of pre-eclampsia. Nevertheless, the immunological analysis of the studied cytokines was not able to confirm its influence on the process. Thus, future studies are needed to explore the nature of the association between periodontitis and pre-eclampsia further, by studies investigating other cytokines and other mechanisms.

Acknowledgements

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