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Increased inflammatory biomarkers in early pregnancy is associated with the development of pre-eclampsia in patients with periodontitis: a case control study

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Aim(s): To explore the relationship between biomarkers of systemic inflammation in plasma and gingival crevicular fluid in early pregnancy and the subsequent development of pre-eclampsia in patients with periodontitis.

Materials and Methods: A case-control study was performed. From a cohort composed of 126 pregnant women, 43 normotensive healthy pregnant women were randomly selected, and 11 cases of preeclampsia were identified. Plasmatic and gingival crevicular fluid (GCF) samples were collected in early pregnancy (11–14 wk gestation). The levels of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were measured in the plasma and GCF samples, whereas the level of C-reactive protein (CRP) was measured in plasma samples. Biomarkers were determined by ELISA assays. The data were analysed using descriptive statistics, and the association between variables was estimated through logistic regression models.

Results: There was observed an association between pre-eclampsia and plasmatic levels of CRP (OR: 1.07; $p = 0.003$). Additionally, pre-eclampsia also was associated with IL-6 levels in GCF samples in early pregnancy (OR: 1.05; $p = 0.039$). A multiple logistic regression model suggests that increased levels of IL-6 in GCF (OR = 1.06; $p = 0.02$; CI 95% 1.007–1.117) in early pregnancy increase the risk of developing pre-eclampsia.

Conclusion(s): Pregnant women with periodontitis who later development pre-eclampsia, shows increased levels of IL-6 in GCF and CRP in plasma during early pregnancy. Periodontal disease could contribute to systemic inflammation in early pregnancy via a local increase of IL-6 and the systemic elevation of CRP. Therefore, both inflammatory markers could be involved in the relationship between periodontal disease and pre-eclampsia.

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Pre-eclampsia (PE) is a maternal multi-organ disease that clinically manifests in the second half of pregnancy. Its complications have become one of the primary causes of maternal and foetal morbidity and mortality in the world, as they cause nearly 40% of premature births before 35 wk of gestation. Moreover, PE has been strongly associated with an increased risk of later-life death resulting from cardiovascular disease, independent of other risk factors, and it occurs in approximately 5–10% of all pregnant women worldwide. Despite the amount of resources invested in the study and treatment of PE, it is still difficult to predict and is thus difficult to treat. The pathogenesis of PE is not completely understood, but it is generally accepted that endothelial dysfunction of the maternal vascular system and infection play a key role in the clinical manifestations of the disease. PE is most likely the result of a generalised inflammatory response, including activation of inflammatory and endothelial cells (1–6).

Several studies have suggested a link between PE and periodontal disease (7–9). In the literature, an association between maternal periodontal disease and the risk of adverse pregnancy outcomes, i.e., preterm delivery and intrauterine growth restriction, has been described (10–13). In the case of hypertensive disorders in pregnancy, women with evidence of periodontal disease had an increased risk of PE compared with those without periodontal disease. Moreover, the association has been also related to the severity of periodontal disease: the risk of PE is higher when the periodontitis is severe (7). However, the biological mechanism involved in the association between periodontitis and PE has not been resolved (14).

Periodontal disease is a chronic destructive inflammatory pathology that affects the tooth-supporting tissues, and it is one of the most prevalent chronic infections in humans. The disease is caused by dental plaque, which is a biofilm in which Gram-negative anaerobic microorganisms predominate. Oral microorganisms initiate periodontal disease, but the periodontal breakdown is primarily mediated by the inflammatory response of the host (12,15).

It has been proposed that daily episodes of bacteraemia or the dissemination of bacterial endotoxins that originate from the periodontal lesions may induce systemic activation of the inflammatory response (16,17) and trigger the synthesis of pro-inflammatory cytokines (18). These cytokines further activate the inflammatory response to generate a chronic low-grade systemic effect that includes the up-regulation of interleukin-6 (IL-6), tumour necrosis factor (TNF-α) and C-reactive protein (CRP) (17,19–21). This response also includes the activation of inflammatory cells and endothelial cells, and it may result in endothelial dysfunction (22–25). Several pathologies that produce a chronic low-grade inflammation, such as diabetes mellitus and obesity, are risk factors for the development of hypertension during pregnancy (26–28), and it has been hypothesised that the exacerbation of the inflammatory response during pregnancy by other pathologies may be a predisposing factor for the occurrence of PE (29). Within this context, periodontal disease in pregnant patients may be another condition that could affect the development of PE as the result of a local and systemic increase of pro-inflammatory molecules.

The aim of the present study was to explore the relationship between biomarkers of systemic inflammation such as CRP, IL-6 and TNF-α in the plasma and gingival crevicular fluid (GCF) in early pregnancy in patients with periodontitis who subsequently development pre-eclampsia.

Material and methods

Study design

A case-control study was conducted. The selection criteria for the subjects were that they be pregnant women with a diagnosis of preeclampsia. In total, 11 cases were recruited from the prenatal cohort of 126 women enrolled in the obstetrics and foetal medicine unit of the University of the Andes Health Care Centre, Santiago, Chile, between the years 2009 and 2010. The controls were randomly selected from the same cohort to generate a sample of 43 control subjects. Both groups consisted of patients with singleton gestation of equal socioeconomic level. Clinical enrolment and anthropometric data were collected at 11–14 wk gestation, and a dental evaluation and full-mouth periodontal exams were then performed by one periodontics, with a high intra-examiner reliability (0.806–0.812 Kappa test). All patients received oral hygiene products, a soft toothbrush and a dentifrice (Colgate, Piscataway, NJ, USA), and they were instructed not to rinse or brush with any antimicrobial compound, such as chorhexidine or triclosan. After delivery, all women with periodontal inflammation received case-specific treatment. All clinically relevant data for the study were stored in a computer database. Women were excluded if they smoked, had fewer than 18 teeth, had used systemic or topical anti-microbial/anti-inflammatory therapy for the previous 3 mo or had a history of previous periodontal treatment. Women who had teeth with urgent-care needs were treated during pregnancy, and these women were also excluded from the study. Written informed consent was obtained from the women who agreed to participate in the study, which was approved by the University of the Andes Ethics Committee.

The variables studied were pre-eclampsia, age, initial weight, height, body mass index (BMI), number of teeth, plasma CRP, plasma IL-6, plasma TNF- α, Gingival crevicular fluid (GCF) IL-6, GCF TNF- α and periodontal status.

Definition of study groups— Pre-eclampsia group was defined as women who were diagnosed with PE if, during the second and third trimester of pregnancy, they developed a blood pressure over 140/90 and proteinuria, which was considered to be present when one 24-h urine collection showed a total protein excretion ± 300 mg. Control group was defined if women have a normal pregnancy,

described as a pregnancy that progressed without the development of any serious obstetric disease and resulted in the delivery of a healthy infant after 37 completed weeks with a normotensive blood pressure status.

Women were diagnosed with periodontitis if they had four or more teeth showing one or more sites with a probing pocket depth (PPD) of 4 mm or higher as well as a clinical attachment loss (CAL) of 3 mm or higher at the same site, inflammation and bleeding on probing (BOP). Women who showed BOP at more than 25% of the sites and gingival redness but did not have clinical attachment loss were diagnosed as having gingivitis. Pregnant women who did not exhibit PPD greater or equal to 4 mm and an attachment loss $\geq$ 3 mm and in whom fewer than 25% of the sites showed BOP were classified as normal.

Sample collection and assessment

Blood samples— Blood samples were collected at enrolment by standard venipuncture using EDTA-containing tubes between 8:00 and 10:00 AM. The plasma was separated and immediately frozen and stored at −80°C until required for analysis.

GCF samples— Gingival crevicular fluid samples were collected between 11–14 wk. After the tooth was isolated with a cotton roll, supragingival plaque was removed with curettes (Hu Friedy, Gracey, IL, USA), without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. GCF was collected with paper strips (Pro-Flow, Amityville, NY, USA). The strips were placed into the sulci/pocket until mild resistance was sensed and left in place for 30 s. Strips contaminated by saliva or bloods were excluded from the sampled group. After GCF collection, the strips were placed in Eppendorf vials containing 100 μL of phosphate-buffered saline (PBS) with 0.05% Tween-20 (PBS-T). GCF was extracted by centrifugation at 10,000 g for 5 min (Hermle Labortech Nik. Z-233 MK-2, Wehingen, Germany). The elution procedure was repeated twice, and samples were stored at −80°C until further analysis. GCF samples were obtained from four periodontal pockets (1 × quadrant) in the periodontally affected sites (probing depth of 5 mm, attachment loss of 3 mm in the same teeth).

ELISA assays

The plasma levels of CRP were measured using a double-antibody ELISA plate with reagents from R & D systems TM® (Human C-Reactive Immunoassay, No. DCRP00, R & D Systems, Inc., Minneapolis, MN, USA) following instructions provided by the manufacturer. The plasma and GCF levels of IL-6 and TNF-α were measured using a double-antibody ELISA plate using reagents from BioSource® (IL-6 Immunoassay kit; BioSource International, San Diego, CA, USA). TNF-α was measured using reagents from eBioscience® (Human TNF-α ELISA Ready-SET-Go ® Catalogue Number: 88-7346) following the instructions provided by the manufacturer. The ELISA plates were read at a wavelength of 450 nm in an automatic ELISA plate reader (Microplate Reader, ELx808; Bio Tek Instruments, Winooski, VT USA).

Statistical analyses

Periodontal disease (periodontitis/gingivitis) was analysed as dichotomous variable. For plasmatic inflammatory markers (CRP, IL-6 and TNF-α) and GCF inflammatory markers (IL-6 and TNF-α), were studied as continuous variables. The descriptive analysis of continuous and discrete variables (age, weight, height, BMI and number of teeth) was performed based on the mean, standard deviation, median and interquartile range, depending on the variable analysed. The difference between variables in both groups (normotensive/pre-eclamptic patients) was determined using the two samples Wilcoxon rank-sum test. The correlation between the plasma and local levels of mediators was explored using rho-Spearman Test. The association strength was assessed using a multiple logistic regression model. The crude and adjusted odds ratio (OR) with a 95% confidence interval (CI) was determined, and the data management and statistical analysis were performed using STATA software (version 11; StataCorp, Lakeway Drive College Station, TX, USA).

Results

Of the initial pregnant cohort of 126 patients, 11 women developed pre-eclampsia, and 43 pregnant women served as random controls. The demographic and pregnancy-related characteristics of the study groups are shown in Table 1. There were no significant differences in age, weight, height and BMI between the groups ($p > 0.05$). The median number of teeth present was 22 in the PE group and 26 in the healthy patients ($p = 0.057$). The mean probing depth

Table 1. Description of personal characteristics of normotensive and pre-eclamptic patients

	Healthy/normotensive patients (n = 43)		Pre-eclamptic patients (n = 11)		
Variables	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	p value*
Age	27.19 (7.18)	26 (11)	28.91 (6.11)	28 (9)	0.4776
Initial weight	65.82 (14.25)	63 (16)	71.04 (17.11)	72.9 (19.5)	0.2874
Height	156.67 (5.45)	156 (7)	157.73 (4.45)	159 (8)	0.4196
BMI	26.85 (5.88)	25.46 (6.47)	28.7 (7.46)	28.2 (10.13)	0.4083
Number of teeth		26 (3)		22 (5)	0.0573

*Two-sample Wilcoxon rank-sum test.
BMI, Body Mass Index; SD, Standard deviation; IQR, Interquartile range.

Table 2. Periodontal status and CRP, TNF-α and IL-6 systemic/local levels in normotensive and pre-eclamptic patients (Simple logistic regression)*

Variables	Healthy/ normotensive patients (n = 43)	Pre-eclamptic patients (n = 11)	OR	p value	Confidence interval (95%)
Periodontitis	33 (76.74%)	9 (81.82%)	1.36	0.719	0.252–7.372
Plasma CRP	10 [10.78]	29.04 [42.05]	1.07	0.003	1.022–1.116
Plasma IL-6	4.43 [9.56]	8.15 [14.683]	1.03	0.433	0.963–1.089
Plasma TNF- α	14.66 [32.86]	35.94 [49.36]	1.02	0.260	0.989–1.043
GCF IL-6	9 [20.85]	15 [42.6]	1.05	0.039	1.002–1.102
GCF TNF- α	0 [0]	0 [0]	–	–	–

*Values are given as median [interquartile range] or n (%).
CRP, C-reactive protein; IL-6, Interleukin-6; TNF-α, Tumour necrosis factor; GCF, Gingival crevicular fluid.

and percentage of sites exhibiting BOP were similar in both groups, and no significant difference in the percentage of sites with plaque was found ($p > 0.05$, data not shown). Both groups were similar in the number of patients with a diagnosis of periodontal disease: periodontitis was found in nine of the 11 (82%) patients who developed PE, and the remaining 2 (18%) patients were diagnosed with gingivitis. In the control group, 77% of the patients were diagnosed with periodontitis, and 23% were diagnosed with gingivitis.

The plasma levels of CRP, IL-6 and TNF-α and the GCF levels of IL-6 and TNF-α from both groups are presented in Table 2. An association between the development of PE and plasma levels of CRP was observed ($p = 0.003$.95% CI: 1.022–1.116), but not plasmatic IL-6 and TNF-α ($p = 0.43$, $p = 0.26$ respectively) in early pregnancy. In GCF samples, there was observed an association between IL-6 levels in early pregnancy and the subsequent development of pre-eclampsia ($p = 0.039$. 95% CI: 1.002–1.102).

The multiple logistic regression analysis suggests that increased levels of IL-6 in GCF (O.R = 1.06; $p = 0.02$; CI 95% 1.007–1.117) in early pregnancy increase the risk of developing pre-eclampsia in patients with periodontitis adjusted by the number of teeth (Tables 3 and 4).

Table 3. Analysis of pre-eclampsia development and initial IL-6 levels in the GCF adjusted by number of teeth and initial body mass index (multiple logistic regression)

Variable	OR	p value	Confidence interval (95%)
GCF IL-6	1.06	0.055	0.999–1.129
Number of teeth	0.79	0.037	0.626–0.986
Body mass index	0.99	0.934	0.862–1.146

GCF, Gingival crevicular fluid; IL-6, Interleukin-6.

Table 4. Analysis of pre-eclampsia development and initial IL-6 levels in the GCF adjusted by number of teeth (multiple logistic regression)

Variable	OR	p value	Confidence interval (95%)
GCF IL-6	1.06	0.026	1.007–1.117
Number of teeth	0.79	0.036	0.628–0.984

GCF, Gingival crevicular fluid; IL-6, Interleukin-6.

Discussion

The present study shows and association between pregnant women diagnosed with periodontitis and who later developed pre-eclampsia with a locally increased IL-6 in the gingival crevicular fluid as well as elevated plasma C-reactive protein in early pregnancy (11–14 wk of gestation).

There is strong evidence to suggest an association between maternal periodontal disease and the risk of adverse pregnancy outcomes (7,10–12), and this association is supported by the finding that oral infection stimulates the immune inflammatory response via a local increase in pro-inflammatory mediators such as interleukin-1β, prostaglandin E2, IL-6 and TNF-α (16–18,30). The extensive vascularisation of the periodontal ligament suggests that chronic periodontal chronic inflammation might be a potential source of inflammatory molecules that could disseminate and act systemically in the vascular endothelium to promote endothelial lesions (31–34) and thereby increase the risk of systemic pathologies. C-reactive protein and IL-6 are associated with periodontal disease, and their increase appears to be correlated with the severity of the disease (20). Moreover, these molecules has been proposed to be related to the elevation of systemic CRP and IL-6 (35), and both mediators might be involved in the association between periodontal disease and other systemic diseases. Several studies have demonstrated an epidemiological link between pre-eclampsia and periodontal disease (7,8). In fact, some studies have found periopathogenic microorganisms in the placenta of women with pre-eclampsia (36), which suggests a systemic dissemination of these periodontopathogens and highlights a potential biological mechanism by which these two pathologies could be related. However, the underlying mechanism is unknown, and the association between both diseases needs to be clarified.

C-reactive protein is a marker of acute phase response and has proved to be a sensitive biomarker of systemic inflammation (37). This protein is produced in the liver in response to macrophage-derived signals, especially IL-6, as part of the innate immune response to bacterial infection. Levels of CRP are higher among pregnant women (38), and this elevation is further increased in patients with adverse pregnancy outcomes, such as pre-eclampsia, intrauterine growth restriction and preterm delivery (39,40). Moreover, CRP levels are 65% higher in women with periodontitis than in control subjects during pregnancy after adjustment for related factors including age, race/ethnicity,

pre-pregnancy body mass index, alcohol intake, education, income, and gestational age at the time of blood collection (40). Elevated levels of IL-6 are observed in the placenta, amniotic cells, and decidua of pregnancy patients with pre-eclampsia (38). Elevation of IL-6 is also observed in patients with gingivitis and periodontitis (41). In a case-control study, Barak and co-workers found that women with a clinical diagnosis of PE had more severe periodontal disease than healthy controls, and they observed a significant elevation in the GCF levels of PGE2, IL-1β and TNF-α (36).

In a longitudinal study of more than 1000 women, Boggess *et al.* (7) showed that the presence of periodontal disease at delivery was associated with a two-fold increase in the risk for pre-eclampsia compared with women without periodontal infection. Additionally, Canakci *et al.* (9) showed that interleukin-1β, TNF-α and prostaglandin E2 were increased in the GCF of pregnant women with pre-eclampsia when compared with normotensive pregnant women. Another recent study (42) demonstrated an increase in TNF-α mRNA expression in pre-eclamptic women; however, a correlation was not observed between periodontitis and plasmatic cytokine expression.

Our study showed an elevation in plasma levels of CRP and a local increase in IL-6 in the GCF during early pregnancy in patients with periodontitis who later developed PE. Although patients of both groups presented periodontitis, only those which developed pre-eclampsia showed a increase of IL-6 in GCF, and this significant difference was not observed in plasma samples, which would indicates a potential role of local synthesis of IL-6 in periodontal tissue and could contributes with the development of pre-eclampsia.

To date, our study and the studies of Canakci et al. (9) and Politano (42) are the only three studies to have analysed the possible involvement of cytokines in the relationship between periodontal disease and PE. The relevance of systemic maternal inflammation and chronic diseases such as periodontal disease and its relationship with unhealthy pregnancies have yet to be fully explored. Therefore, we propose that CRP and IL-6 could be possible mediators involved in the association between periodontal disease and PE. Moreover, the increase of these molecules during early pregnancy in periodontal patients might be involved in the future development of preeclampsia.

Understanding the pathogenic mechanism of both diseases may help us to identify at-risk pregnant patients to provide maximum benefit and minimised risk during treatment. Furthermore, the prevention of gingival inflammation during pregnancy may be a therapeutic target to prevent PE. Non- surgical periodontal therapy is safe for the mother and foetus, and it is effective in reducing inflammatory signs of maternal periodontal disease (43). Moreover, interventional studies that analysed the effect of periodontal treatment on systemic markers showed a decrease in the plasmatic levels of IL-6 and CRP and an improvement in endothelial function over 6 mo after periodontal treatment (23) Therefore, to reduce adverse birth outcomes, such as PE and premature labour, periodontal treatment could be performed at least 6 mo before conception or at the beginning of pregnancy. Furthermore, it might be appropriate to design preventive strategies for the fertile period of the patients or at least at the beginning of pregnancy that target periodontal health.

Some important limitations should be considered when interpreting the results of the present study: first, the study had a small sample size, and second, the women included in this study belong to a low socioeconomic level. In many cases, they had limited access to dental treatment. Indeed, the women who developed PE in our study had significantly fewer teeth than healthy pregnant women, and this finding, together with the presence of periodontal disease, may indicate that a worsened oral condition affects the development of PE in pregnant patients.

This study provides further evidence of a plausible association between a local increase in IL-6 in the GCF with concomitantly increased plasmatic levels of CRP in early pregnancy with the subsequent development of PE in patients with periodontitis. To date, this study is the first report to suggest that an increase in IL-6 levels in the gingival crevicular fluid in early pregnancy could be used as a local periodontal marker for future development of PE. Therefore, we believe that further prospective longitudinal studies with a larger sample size are needed to assess the impact of these mediators and their predictive value for the development of preeclampsia in women with periodontitis.

Conflict of interest

The authors declare that they have no conflicts of interest in this study.

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