

Association between periodontal condition and subgingival microbiota in women during pregnancy: a longitudinal study

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ABSTRACT

Objective: In this study, the gingival conditions and the quantitative detection for *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia* in pregnant women were determined. Material and Methods: Quantitative determinations of periodontal bacteria by using a SyBr green system in women during pregnancy were performed. Women at the 2nd and 3rd trimesters of pregnancy and non-pregnant women were included in this study. *A. actinomycetemcomitans* was observed in high numbers in women at the 2nd and 3rd trimesters of pregnancy with a significant difference ($p < 0.05$). *F. nucleatum* and *P. intermedia* were also observed in high levels. Results and Conclusion: Our results show that pregnant women are more susceptible to gingivitis, and the presence of *A. actinomycetemcomitans* in subgingival biofilm might be taken into account for the treatment of periodontal disease.

Keywords: Gingivitis. Periodontal disease. Pregnancy.

INTRODUCTION

Periodontal diseases are chronic polymicrobial infections determined by the interaction between microorganisms and immune, environmental, behavioral and/or hereditary factors, causing an inflammatory response of the periodontal tissues^{14,18}. This disease affects individuals of different ages and it is characterized by a chronic tissue-destructive inflammation and dental attachment loss.

The pathogenesis of periodontal disease is a result of the accumulation of bacterial species in subgingival biofilm, particularly by gram-negative anaerobic and microaerophilic bacteria, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, and *Campylobacter rectus*⁷.

Studies have shown a relationship between the periodontal disease and systemic changes in pregnant women, such as diabetes, cardiovascular

and lung diseases, and pregnancy complications, as well as hormonal alterations during pregnancy causing gingival inflammation⁹. The periodontal diseases have been implicated as risk factors for adverse pregnancy outcomes, such as prematurity and low birth weight^{11,12}, as well as the presence of different periodontal bacteria, such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens*, and *C. rectus* in oral microbiota from pregnant women^{10,20}.

Gingival alterations associated to the biofilm formation during pregnancy are often observed, and this may increase the severity of gingivitis. In addition, variations in the oral microbiota and cellular metabolism have been observed, as well as an increase in estrogens levels, such as progesterone, affecting the vascular permeability, producing edema, and increasing the gingival fluid^{21,23}.

The identification of microbial agents producing severe periodontal diseases and the efficacy of suitable therapeutic method might help to the

maintenance of the periodontal health and it might be important to the development of better programs aimed to pregnant women and newborn health.

In this study, we hypothesized that there is a possible association between the periodontal condition and periodontal bacteria in women at the 2nd and 3rd trimesters of pregnancy, and it was evaluated.

MATERIAL AND METHODS

Participants

Twenty three pregnant women from 18 to 35 years old were evaluated, and nine of them were selected in accordance to the inclusion criteria. The participants were recruited at the National Health System of Vila Nova de Colares (Serra, ES, Brazil), and all of them had medical prenatal and dental care. Nine non-pregnant women aged from 18 to 35 years old belonging to an evangelical community and that did not use any hormonal contraceptive method were also included as controls. Pregnant women were at the 2nd (between 15th and 26th weeks) and 3rd trimester (between 30th and 36th weeks) of gestation, and gestational age was confirmed by ultrasound. All participants displaying at least 15 teeth, excluding third molars, were included in this study. Women with chronic diseases, smokers, alcohol dependency, use of systemic antibiotics in the last three months, use of prophylactic antibiotics for periodontal examination in the last six months, or extensive prosthetic rehabilitation, were excluded. In the course of the sample collections, 11 pregnant women were treated with antibiotics (10 with urinary infection and one with sinusitis), and three women did not attend the scheduled appointments, and they were excluded. All participants signed an informed consent form, approved by the Ethics Committee of the Federal University of Espírito Santo, Vitória, ES, Brazil (Protocol No. 259/10).

The demographic and socio-economic data and the medical and dental history were also collected. The demographic data were: time of pregnancy (first collection at 21st weeks and second collection at 33rd weeks), no use of anti-conceptive, ethnic in pregnant women (2 white, 3 black, 4 mulatto), and non-pregnant (5 white, 1 black, 3 mulatto). Socio economic level in pregnant belonged to 1 class B, 6 class C, 2 class D, and in non-pregnant belonged to 6 class B, 3 class C.

The clinical examination was conducted by a single trained dentist. Clinical evaluation was performed on all teeth (except third molars) and at six sites *per* tooth by using North Carolina periodontal probes (PC-PUNC 15, Hu-Friedy manufacturing Inc., Chicago, IL, USA). In all the pregnant women, the clinical monitoring was

not affected by any discomfort or adverse effect commonly observed during pregnancy, such as nausea, vomiting, weakness, or blood pressure alterations.

The highest score to the evaluated clinical parameters were registered, as follows: visible plaque index (VPI), gingival bleeding index (GBI); probing depth (PD); clinical attachment level (CAL); and bleeding on probing (BOP). Periodontitis was defined as the presence of four or more teeth with one or more sites of PD \geq 4.0 mm and CAL \geq 3.0 mm, and being localized in two or three teeth and generalized into four or more teeth. Gingivitis was defined when PD \leq 3.0 mm and CAL \leq 2.0 mm in two to five teeth or at least one site with bleeding on probing; and periodontal healthy when PD \leq 2.0 mm and CAL \leq 2.0 mm, and no site with gingival bleeding²⁻⁴.

Sample collection and processing

Supragingival biofilm was removed with sterile gauze and subgingival biofilm samples were collected from the mesial buccal or mesial vestibular of the first or second molar by using two sterilized paper points (No. 30 Tanari, Tanariman Ind Ltd, Manacapuru, AM, Brazil) inserted into gingival crevice for 30 seconds. Twenty-seven subgingival samples from nine women during the 2nd (9 samples) and 3rd (9 samples) semesters of pregnancy and from nine non-pregnant women (9 samples) were collected. The paper points were transferred into a tube containing 300 μ l of TE buffer (Invitrogen, Invitrogen do Brasil Ltd, São Paulo, SP, Brazil), and stored at -80°C.

Bacterial determination by qPCR

Bacterial DNA was obtained by using a phenol-chloroform method⁶ and stored at -80°C until use. DNA from *A. actinomycetemcomitans* ATCC 29523, *F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, and *P. intermedia* ATCC 25611 were used to determine the standard curves, showing a correlation coefficient (R^2) $>$ 0.99 and efficiency from 90% to 110%. Quality, integrity, and quantity of DNA were analyzed by NanoDrop spectrophotometer (NanoDrop 2000 Thermo Scientific, Thermo Fischer Scientific Inc, Wilmington, DE, USA), and 10 μ l of each DNA sample was also checked on 1% agarose gel.

DNA amplifications were performed in a thermal cycler Rotor Gene 6000 with Software 1.7 Series (Corbett, Corbett Life Science, Mortlake, NSW, Australia), by using species-specific primers based on the sequence of the 16S rRNA (Figure 1) and with a Sybr Green system. Amplifications were performed in final volumes of 20 μ l, containing 2 X Sybr Green master mix (Promega, Promega Corporation, Madison, WI, USA), 100 μ M each

primer, and 2 ng DNA. Amplification reactions for all microorganisms were performed in duplicate, as follows: 1 cycle of 95°C (2 min), 40 cycles of 95°C (45 sec) and 60°C (2 min), and suitable annealing temperature (Figure 1). The species-specific primers and quantitative determination conditions are shown in Figure 1. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR, and master mix without DNA was used as negative control.

Statistical analyses

The statistical analyses were performed by using the GraphPad Prism, Version 5.0.

RESULTS

The periodontal characteristics of the women during pregnancy are shown in Table 1. It is possible to note that at 2nd trimester of pregnancy, all the parameters, IPV, ISG, PS, CAL, and SS showed lower values than at the 3rd trimester. In addition, the non-pregnant women showed values (≤ 2 mm) considered as normal. None of the women showed periodontitis. Our results show that, during pregnancy, the gingival inflammation is increased (Table 1).

All the four periodontal bacteria were detected in

both of the analyzed pregnancy periods, as well as in non-pregnant women. In Table 2, it is possible to note the number of copies for each microorganism in pregnant and non-pregnant women. It was observed at the 2nd trimester of pregnancy that the values of copy numbers ranged from 1.49×10^2 to 5.50×10^8 for *A. actinomycetemcomitans*, from 0 to 2.60×10^5 for *F. nucleatum*, from 0 to 7.48×10^2 for *P. intermedia*, and from 0 to 2.81×10^2 for *P. gingivalis*. The bacterial detection at the 3rd trimester of pregnancy was similar to the 2nd trimester, as follows: from 0 to 8.25×10^8 for *A. actinomycetemcomitans*; from 0 to 1.90×10^5 for *F. nucleatum*; from 0 to 2.24×10^2 for *P. intermedia*; and from 0 to 4.43×10^2 for *P. gingivalis*. Interestingly, in non-pregnant women, *F. nucleatum* (from 0 to 3.73×10^5) and *P. gingivalis* (from 0 to 1.46×10^2) were the most prevalent.

The presence of *A. actinomycetemcomitans* in pregnant women at the 2nd and 3rd trimester compared to the non-pregnant group showed statistically significant values ($p < 0.05$). A randomized longitudinal observation was followed for women during the 2nd and 3rd trimesters of pregnancy. It allowed the evaluation of possible changes in both periodontal status and microbiota during pregnancy, when hormonal variations are observed^{1,8}.

| Microorganism | Oligonucleotides | Amplicon | Melting | Reference |
|--|---|-----------|------------------|---|
| | 5' ----- 3' | size (bp) | temperature (°C) | |
| <i>Aggregatibacter actinomycetemcomitans</i> | CTTACCTACTCTTGACATCCGAA ATGCAGCACCTGTCTCAAAGC | 262 | 60 | Kuboniwa, et al. ¹⁹ (2004) |
| <i>Fusobacterium nucleatum</i> | CTTAGGAATGAGACAGAGATG TGATGGTAACATACGAAAGG | 120 | 56 | Periasamy, et al. ²⁵ (2009) |
| <i>Porphyromonas gingivalis</i> | ACCTTACCCGGGATTGAAATG CAACCATGCAGCACCTACATAGAA | 83 | 57 | Kuboniwa, et al. ¹⁹ (2004) |
| <i>Prevotella intermedia</i> | CGTGGACCAAAGATTCATCGGTGGA CCGCTTTACTCCCCAACAAA | 258 | 55 | Okamoto, et al. ²⁴ (1999) |

Figure 1- Species-specific oligonucleotides used for the quantitative detection

Table 1- Periodontal conditions of women at the 2nd and 3rd trimesters of pregnancy

| Pregnancy | Clinical parameters (values) | | | | |
|---------------------------|------------------------------|---------|------------------------|------------------------|---------|
| | VPI (%) | GBI (%) | PD (mm) | CAL (mm) | BOP (%) |
| | | | Mean±SD | Mean±SD | |
| 2 nd trimester | 60 | 26 | 2.48±0.93 ^a | 2.37±0.90 ^a | 46 |
| 3 rd trimester | 65 | 52 | 2.71±1.0 ^b | 2.50±0.92 ^b | 62 |
| Non-pregnant | 21 | 5 | 1.94±0.80 | 1.93±0.79 | 17 |

^a t-test: $a \neq b$, $p < 0.001$

VPI: visible plaque index; GBI: gingival bleeding index; PD: probing depth; CAL: clinical attachment level; BOP: bleeding on probing

Table 2- Quantitative determination of four periodontal bacteria in subgingival biofilm from women at the 2nd and 3rd trimester of pregnancy and non-pregnant women

| Microorganism | pregnant women (No. of copies) | | non-pregnant women (No. of copies) |
|--|-----------------------------------|-----------------------|---------------------------------------|
| | Second trimester | Third trimester | |
| <i>Aggregatibacter actinomycetemcomitans</i> | | | |
| P1 | 2.58E+10 ² | 2.93E+10 | 0 |
| P2 | 6.05E+10 | 2.45E+10 | 0 |
| P3 | 3.79E+10 ³ | 8.25E+10 ⁸ | 0 |
| P4 | 1.62E+10 | 0 | 0 |
| P5 | 5.37E+10 | 6.65E+10 ⁴ | 0 |
| P6 | 1.49E+10 | 5.05E+10 ³ | 0 |
| P7 | 3.15E+10 ² | 1.73E+10 ⁵ | 0 |
| P8 | 6.17E+10 ⁴ | 3.08E+10 ⁴ | 0 |
| P9 | 5.50E+10 ⁸ | 9.05E+10 ⁴ | 0 |
| <i>Fusobacterium nucleatum</i> | | | |
| P1 | 4.81E+10 | 0 | 0 |
| P2 | 0 | 0 | 0 |
| P3 | 2.98E+10 ² | 3.63E+10 ² | 3.90E+10 ⁴ |
| P4 | 0 | 8.75E+10 ⁴ | 6.99E+10 ² |
| P5 | 0 | 1.90E+10 ⁵ | 1.06E+10 ³ |
| P6 | 3.68E+10 | 6.91E+10 | 2.52E+10 ³ |
| P7 | 0 | 9.26E+10 ² | 3.73E+10 ⁵ |
| P8 | 5.56E+10 ⁴ | 1.08E+10 ⁵ | 4.36E+10 |
| P9 | 2.60E+10 ⁵ | 2.64E+10 ³ | 0 |
| <i>Prevotella intermedia</i> | | | |
| P1 | 1.83E+10 ² | 0 | 5.76E+10 |
| P2 | 0 | 0 | 0 |
| P3 | 0 | 4.62E+10 | 0 |
| P4 | 1.74E+10 | 0 | 1.51E+10 ² |
| P5 | 0 | 2.75E+10 | 0 |
| P6 | 3.22E+10 | 7.75E+10 | 0 |
| P7 | 0 | 0 | 0 |
| P8 | 2.98E+10 | 2.24E+10 ² | 0 |
| P9 | 7.48E+10 ² | 0 | 0 |
| <i>Porphyromonas gingivalis</i> | | | |
| P1 | 0 | 0 | 0 |
| P2 | 0 | 0 | 3.15E+10 |
| P3 | 0 | 0 | 0 |
| P4 | 0 | 0 | 0 |
| P5 | 0 | 1.02E+10 | 0 |
| P6 | 0 | 4.43E+10 | 0 |
| P7 | 0 | 0 | 1.46E+10 ² |
| P8 | 2.81E+10 ² | 2.95E+10 | 0 |
| P9 | 1.18E+10 | 0 | 8.01E+10 |

DISCUSSION

Since during pregnancy, women present nausea, vomiting, weakness, blood pressure alterations, hyperventilation, dyspnea, and edema, clinical monitoring becomes exceedingly difficult, as well as their participation⁸, but in this study, these clinical symptoms did not influence our results.

Molecular methods are currently available for typing and subtyping of periodontopathogens, such as *P. gingivalis*, but they can vary in efficiency and in amount of required labor⁵. Bacterial cultures are used to identify putative anaerobic pathogens from destructive periodontal disease, but can be somewhat cumbersome and expensive. Polymerase chain reaction (PCR) has been used for direct identification of periodontal pathogens from subgingival samples and for elucidating the role of specific bacteria in the periodontal disease due to the ability to accurately detect bacterial species from mixed populations²⁷.

In this study, a real-time PCR method was used to quantify the copies' numbers of four microorganisms in subgingival microbiota from pregnant and non-pregnant women. In addition, studies have shown the presence of periodontal bacteria by using different detecting methods, such as DNA-DNA hybridization or culture of the oral microbiota from pregnant women, but no association between pregnancy and periodontal disease has been demonstrated^{25,29}; however, our results showed a significant relationship with the presence of *A. actinomycetemcomitans* among pregnant women at second and third trimester, and it might be important. The four microorganisms evaluated are considered some of the most important in the development of periodontal diseases, and for this reason they were selected.

Since paper points were used for collection of the subgingival samples in bacterial detection, they have proven to be the most reliable method for bacterial detection from crevicular fluid. Traditional methods of bacterial isolation and identification may limit the bacterial recovery due to the lack or absence of viable cells and growth requirements³⁰.

An increase of the gingival inflammation was observed particularly between the 2nd and 3rd trimesters of pregnancy, showing statistically significant values ($p < 0.05$). A similar increase in VPI, GBI, BOP, PD, and CAL values was also observed when pregnant and non-pregnant women were compared. These observations are similar to other studies^{13,28}. In addition, a severe gingival inflammation (GBI=52%) during the 3rd trimester of pregnancy was observed, in accordance with Tilakaratne, et al.²⁸ (2000).

The exacerbation of gingival inflammation in pregnant women is clinically and histologically

well documented, but its etiology has not been clearly established. Potential mechanisms have been proposed, including alterations in the vascular permeability, immune system, and subgingival biofilm^{17,26}. However, there is little data regarding the subgingival microbiota composition during pregnancy. However, it is known that hormonal variation may promote overgrowth of pathogenic bacteria responsible for gingival inflammation²².

Studies have shown a high prevalence of *P. intermedia* and *P. gingivalis* at the 2nd trimester of pregnancy due to progesterone and/or estradiol being present in high concentrations, which contribute to bacterial growth^{15,16}. Some studies have reported that pregnancy-associated gingivitis is accompanied by an increase in the cell numbers of *P. intermedia*¹, while others have shown no significant difference in *P. intermedia* numbers during the 2nd and 3rd trimesters of pregnancy²³. In this study, *P. intermedia* were observed in higher levels compared to *P. gingivalis*. The inconsistent results in the literature regarding the oral bacteria associated with the onset of periodontal diseases during pregnancy can be explained by the different methodology used in each study^{13,20}.

A high incidence of *F. nucleatum* during the 2nd (77.7%) and 3rd (88.8%) trimester of pregnancy, as well as in non-pregnant women (88.8%), was observed, and it can be explained by the fact that this microorganism is commonly found in subgingival biofilm, and due to its ability of co-aggregation with other bacteria, such as *P. gingivalis*. On the other hand, the presence of *P. gingivalis* in most of the non-pregnant women suggests that its mere presence is not associated with the initiation or progression of periodontal disease strictly enough, and that other factors, such as bacterial virulence and host immune response, may also be important.

Since a small number of participants was evaluated, it is difficult to generalize to other populations; however, no bias concerning the clinical samples collection was observed, and the common symptoms observed during pregnancy had no influence on our results.

Our results show that pregnant women are more susceptible to gingivitis, and that the presence of *A. actinomycetemcomitans* in subgingival biofilm might be taken into account for the treatment of periodontal disease, as well as for the monitoring and prophylactic guidance on the oral health of these patients during pregnancy, particularly in late gestation.

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