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ORIGINAL ARTICLE

Evaluation of periodontal pathogens in amniotic fluid and the role of periodontal disease in pre-term birth and low birth weight

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Abstract

Background. Pre-term birth and/or low birth weight (PTLBW) is a serious problem in developing countries. The absence of known risk factors in ~ 50% of PTLBW cases has resulted in a continued search for other causes. The aim of this study was to examine the effect of periodontitis on pregnancy outcomes. ***Methods.*** Samples were taken from 50 pregnant women who underwent amniocentesis. Polymerase chain reaction was performed on amniotic fluid samples obtained during amniocentesis and on subgingival plaque samples to determine the presence of *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Treponema denticola, Tannerella forsythia, Fusobacterium nucleatum, Prevotella intermedia, Campylobacter rectus* and *Eikenella corrodens*. Plaque index, gingival index, bleeding on probing, probing depth and clinical attachment level were evaluated. Medical records were obtained after birth. ***Results.*** Social and demographic variables were similar among the Gingivitis (G), Localized Periodontitis (LP) and Generalized Periodontitis (GP) groups. Four subjects gave birth to PTLBW neonates. *Campylobacter rectus, T. forsythia, P. gingivalis* and *F. nucleatum* were detected in the amniotic fluid and subgingival plaque samples of three patients who gave birth to PTLBW neonates. The amniotic fluid sample from the fourth patient was not positive for any of the tested pathogens. ***Conclusion.*** These findings suggest that the transmission of some periodontal pathogens from the oral cavity of the mother may cause adverse pregnancy outcomes. The results contribute to an understanding of the association between periodontal disease and PTLBW, but further studies are required to better clarify the possible relationship.

Key Words: *periodontitis, microbiology, pregnancy complications*

Introduction

Periodontitis can present a systemic inflammatory challenge because of the large epithelial surfaces that can ulcerate in the periodontal pockets. Ulceration can allow bacteria and their products to reach other parts of the body [1]. Cross-sectional, case-control and cohort studies have indicated that periodontitis may confer a 2-fold risk for cardiovascular disease and a 7-fold risk for premature birth [2].

Pre-term and/or low birth weight (PTLBW) is a major medical, social and economic problem and accounts for a large proportion of maternal and neonatal mortality and long-term sequelae [3]. Factors such as smoking, alcohol or drug use during pregnancy, inadequate pre-natal care, race, low socioeconomic status, hypertension, high or low maternal age, diabetes and genitourinary tract infections increase the risk for PTLBW [2]. The role of infection has gained importance in efforts to determine the etiology of PTLBW. The strongest data for infection-induced pre-term labor indicate an association between bacterial vaginosis and premature birth [4]. Although the etiology of ~ 50% of pre-term deliveries remains unknown, increasing evidence supports an effect of non-genital maternal infections [5].

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Cross-sectional and prospective data, as well as the results of animal experiments, continue to bolster the hypothesis that periodontal infection is an independent risk factor for prematurity and growth restriction [2,6–8]. Periodontal infections may mediate PTLBW through contamination of the fetoplacental unit by periodontal pathogens or through effects of inflammatory mediators on the fetoplacental unit. A third potential mechanism involves the effects of lipopolysaccharides of periodontal pathogens [9]. Infection of mice with *Fusobacterium nucleatum*, *Campylobacter rectus* and *Porphyromonas gingivalis* has induced preterm delivery [8,10,11]. *Porphyromonas gingivalis* has also been isolated from amniotic fluid and subgingival samples from pregnant women diagnosed with threatened or premature labour [12]. However, in a study by Katz et al. [13], *P. gingivalis* was detected in the placenta in a case of normal birth. In another study, Dörtbudak et al. [14] examined the amniotic fluid of 36 women at risk for pregnancy complications. Bacteria were never found in the amniotic fluid samples studied, but bacteria from subgingival plaque samples, including orange and red complex bacteria, were found in 18% of full-term and 100% of pre-term neonates. In a study by Leon et al. [12], 26 women with threatened premature labor were examined and amniotic fluid samples were collected. Microbial invasion of the amniotic cavity was indicated in 30.8% of the samples based on *P. gingivalis* polymerase chain reaction (PCR), although bacterial cultures were negative.

The aim of this study was to examine the microbes present in amniotic fluid and subgingival plaque samples from pregnant women, using PCR.

Materials and methods

This study was approved by the Ethics Committee of Hacettepe University, Ankara, Turkey. The study population was drawn from women who underwent amniocentesis due to double or triple screening test positivity. In total, 50 women undergoing amniocentesis at the Department of Obstetrics and Gynecology, Hacettepe University between January and December 2009 consented to participate in the study. All subjects were informed about the aim of the study and all provided written consent approved by the Hacettepe University Ethics Committee. Amniocentesis was performed by two gynaecologists (OD, OO) between 16–18 weeks of pregnancy. Women with known congenital uterine or vaginal malformations, fetal malformation, multiple gestation, a chronic disease (diabetes, hypertension, epilepsy, cardiac disease, lung disease, renal disease or a positive test for human immunodeficiency virus), a history of antibiotic use or periodontal treatment during pregnancy or fewer than 20 teeth were excluded. Gestational age assessment was based on menstrual cycle history and defined by ultrasonographic fetal biometry between weeks 16–18. Full-term birth was defined as giving birth in week 37 or later to a child weighing more than 2500 g.

The demographic characteristics of the participants, such as age, marital status and educational status were obtained using a detailed questionnaire. Gestational week and birth weight data were obtained from medical records.

During amniocentesis, 2-mL samples of amniotic fluid were collected from each participant for microbiological examination. The samples were stored at 80°C until analysis.

Clinical periodontal measurements

After the amniocentesis, all participants were invited to the Department of Periodontology, Hacettepe University for periodontal examination and subgingival plaque sampling to be performed on the same day as the amniocentesis procedure. The following clinical periodontal parameters were measured by a skilled clinician (EE) for all teeth except third molars: probing depth (PD), clinical attachment level (CAL), plaque index (PI) [15], gingival index (GI) [16] and bleeding on probing (BOP) [17]. Six sites were examined on each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual. PD and CAL measurements were recorded to the nearest millimeter using a manual periodontal probe (Hu-Friedy, Chicago, IL). Radiographic exposure was avoided because the women were pregnant. Periodontitis was defined according to the extent of the disease [18]. Localized periodontitis (LP) was defined as PD ≥4 mm and CAL ≥3 mm at the same site on two or three teeth; and generalized periodontitis (GP), as PD ≥4 mm and CAL ≥3 mm at the same site on four or more teeth [18]. All teeth were examined with the exceptions of third molars, incompletely erupted teeth, teeth in areas with extensive caries lesions, teeth with fractures or iatrogenically damaged restorations and teeth with surfaces where the cement–enamel margins could not be distinguished. Periodontal treatment was performed during the post-partum period.

Microbiological sampling and processing

Subgingival bacterial samples were taken after the clinical measurements. A total of four periodontal sites with the deepest PDs, representing one site on each of four different teeth in different quadrants, were chosen for microbial sampling. Supragingival plaque was removed, the sample site was isolated from saliva and a sterile endodontic paper point was inserted in the periodontal pocket until resistance was felt. The point was left in place for 20 s and then placed in a vial containing 0.1 mL of Tris-EDTA buffer (1 M TE) for storage at 80°C until analysis [19].

The patients were informed of their periodontal status and treatment requirements. Professional oral prophylaxis, including supragingival scaling, was applied, but other treatments were postponed until after the patients gave birth.

The presence of eight periodontitis-related species (*P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, *Tannerella forsythia*, *F. nucleatum*, *Prevotella intermedia*, *C. rectus*, *Eikenella corrodens*) was determined by 16S ribosomal DNA (rDNA) amplification using the PCR method described by Slots et al. [20]. The samples were analyzed in the microbiology laboratory of Hacettepe University Children's Hospital.

DNA extraction was performed using the QIAGEN DNA mini-kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The oligonucleotide primers used for amplification are shown in Table I. Amplification reactions were performed in a final volume of 50 L, containing 1 × reaction buffer, 2.5 mM magnesium chloride, 0.2 mM each dNTP, 0.5 U of Taq DNA polymerase (MBI Fermentas, Burlington, ON, Canada), 30 pM each of the primers and 5 L of DNA sample. The amplification reactions were performed in an automated thermal cycler (Flexigene; Techne, Inc., Burlington, NJ) programmed for denaturation at 95°C for 1 min, followed by 36 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 2 min.

The PCR products (10 μL of each reaction mixture) were separated by electrophoresis in 1.5% agarose gels with 1 × Tris-acetic acid-EDTA (TAE) running buffer, using x174 as molecular weight standards. After the gels were stained with ethidium bromide, the bands were visualized on an ultraviolet transilluminator (Gel Logic 200; Eastman Kodak, New York, NY) and photographed.

Statistical analyses

The descriptive statistics of continuous variables are expressed as means, standard deviations and median values (interquartile ranges: IQR) and those of categorical variables are expressed as frequencies and percentages. Inter-group differences of categorical variables were analysed with χ^2 tests. Welch's analysis of variance (ANOVA) and Kruskal-Wallis tests were used to examine differences among groups in continuous variables. The groups were compared with independent-sample *t*-tests. Stepwise logistic regression analysis was used to identify factors that may have caused the bacterial presence. The χ^2 test was used to examine the frequency of PTLBW. Box plots were used to show differences among groups. All statistical analyses were performed using the PASW Statistics 18 software (SPSS Inc., Chicago, IL). Values of $p < 0.05$ were deemed to indicate statistical significance.

Results

Participants were grouped according to periodontal status: gingivitis (G; $n = 16$), localized periodontitis (LP; $n = 10$) and generalized periodontitis (GP; $n = 24$). Table II provides the sociodemographic variables and periodontal parameters of the participants. A small proportion of women had experienced previous pre-term births (6.3% in group G, 30% in group LP, 8.3% in group GP). A small proportion (12.5%) of women in group GP were current light smokers (<5 cigarettes/day).

Table I. Primer sequences.

Bacterium	Primer sequence (5′→3′)	Amplicon length
A. actinomycetemcomitans	GCT AAT ACC GCG TAG AGT CGG ATT TCA CAC CTC ACT TAA AGG T	443 bp
T. forsythia	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641 bp
C. rectus	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598 bp
F. nucleatum	GAA GAA ACA AAT GAC GGT AAC AAC GTC ATC CCC ACC TTC CTC CT	705 bp
E. corrodens	CTA ATA CCG CAT ACG TCC TAA G CTA CTA AGC AAT CAA GTT GCC C	688 bp
P. gingivalis	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404 bp
P. intermedia	AAC GGC ATT ATG TGC TTG CAC CTC AAG TCC GCC AGT TCG CG	589 bp
T. denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp

Table II. Sociodemographic variables and periodontal parameters of the participants.

Parameter	*G* (*n* = 16)	*LP* (*n* = 10)	*GP* (*n* = 24)	*p*-value
Age (years) (median–IQR)	35–8	35–11	34–8	
Educational status:				
Elementary	32.2%	40%	16.7%	
High school	18.8%	10%	16.7%	
University	50%	50%	66.6%	
Previous pre-term birth	6.3%	30%	8.3%	
Smoker	0%	0%	12.5%	
PI (mean ± SD)	0.45 ± 0.34	0.79 ± 0.3	0.83 ± 0.4*	< 0.05
GI (median–IQR)	0.35–0.23*	0.68–0.22	0.59–0.49	< 0.05
BOP (%) (median–IQR)	27.5–28**	45.5–39	48–55**	< 0.05
PD (mm) (mean ± SD)	1.76 ± 0.24***	2.03 ± 0.11***	2.27 ± 0.36***	< 0.001
CAL (mm) (mean ± SD)	1.8 ± 0.24*	2.08 ± 0.15	2.29 ± 0.37	< 0.001

IQR, interquartile range; SD, standard deviation.
*significantly different from the other two groups; **significantly different from each other; ***all three groups differ significantly.

The mean PI values of groups G, LP and GP were 0.45 ± 0.34, 0.79 ± 0.3 and 0.83 ± 0.4, respectively. The PI of group GP was significantly higher than those of groups G and LP ($p < 0.05$). The median GI values of groups G, LP and GP were 0.35–0.23, 0.68–0.22 and 0.59–0.49, respectively. The GI of group G was significantly lower than those of the other two groups ($p < 0.05$). The median BOP percentages of groups G, LP and GP were 27.5–28, 39–45.5 and 48–55, respectively. The BOP percentage differed significantly between groups G and GP ($p < 0.05$). The mean PDs of groups G, LP and GP were 1.76 ± 0.24, 2.03 ± 0.11 and 2.27 ± 0.36 mm, respectively. The PD values differed significantly among all groups. The mean CAL values of groups G, LP and GP were 1.8 ± 0.24, 2.08 ± 0.15 and 2.29 ± 0.3 mm, respectively. The CAL value of group G was significantly lower than those of groups LP and GP.

Microbiological assessment

Table III presents the PCR positive determination rates of the eight periodontal pathogens in the

Table III. Frequencies (%) of bacteria in subgingival plaque (sp) and amniotic fluid (af) samples.

Bacterium	G group	LP group	GP group	*p*-value	Total
A.a (sp)	31.3	50	54.2	> 0.05	46
A.a (af)	0	0	0	> 0.05	0
T. forsythia (sp)	43.8*	80	95.8	< 0.05	76
T. forsythia (af)	0	0	8.3	> 0.05	4
C. rectus (sp)	43.8*	80	83.3	< 0.05	70
C. rectus (af)	0	0	12.5	> 0.05	6
T. denticola (sp)	12.5	30	37.5	> 0.05	28
T. denticola (af)	0	0	0	> 0.05	0
P. intermedia (sp)	31.3	60	62.5	> 0.05	52
P. intermedia (af)	0	0	0	> 0.05	0
P. gingivalis (sp)	18.8*	70	62.5	< 0.05	50
P. gingivalis (af)	0	0	4.2	> 0.05	2
E. corrodens (sp)	31.3	50	54.2	> 0.05	46
E. corrodens (af)	0	0	0	> 0.05	0
F. nucleatum (sp)	50*	90	95.8	< 0.05	80
F. nucleatum (af)	0	0	8.3	> 0.05	4

*differs significantly from the other two groups.

subgingival dental plaque and amniotic fluid samples. *T. forsythia*, *C. rectus*, *P. gingivalis* and *F. nucleatum* were detected less frequently in the subgingival plaque samples of the women in group G. These four pathogens were also detected in the amniotic fluid samples of three women in group GP. None of the evaluated bacteria was found in the amniotic fluid samples of women in groups G or LP.

We found a positive correlation between the detection of *P. gingivalis* and *C. rectus* in subgingival plaque samples and GI in all three groups ($p < 0.05$). We also observed positive correlations between the detection of *F. nucleatum* in subgingival plaque samples and BOP ($p < 0.05$) and between the detection of *A. actinomycetemcomitans* and CAL ($p < 0.05$).

No PTLBW occurred in group G or LP, whereas 16.6% ($n = 4$) of births in group GP were PTLBW. Periodontal bacteria were detected in the amniotic fluid samples of three women whose neonates were PTLBW. The microbiological profiles of the subgingival plaque and amniotic fluid samples from the women who gave birth to PTLBW neonates are given in Table IV. By the end of the study, four subjects had given birth to PTLBW neonates. Periodontal pathogens were found in the amniotic fluid samples from three of these women. The amniotic fluid sample from the fourth woman was not positive for any of the tested bacteria and, to our knowledge, the fourth woman had none of the evaluated risk factors. The women who delivered term and normal weight babies had no bacteria in their amniotic fluid.

Discussion

Several studies [11,12] have demonstrated an association between maternal periodontal disease and PTLBW, but the underlying mechanisms have not been elucidated yet [10,12]. Additional studies are needed to validate this association and to determine whether it is causal. The present study was undertaken to investigate this association in a sample of 50 pregnant women who underwent amniocentesis.

The oral health status of our study population was determined through structured clinical examinations. No radiographic evaluation was performed because of the patients' pregnancies; nevertheless, accurate periodontal diagnoses were made.

Several factors such as smoking, alcohol or drug use, older or younger maternal age and marital status are related to the risk for PTLBW [21,22]. The patients in our study did not use alcohol or drugs and did not have systemic illnesses that could affect the periodontium or gestational duration or could cause bacterial vaginosis or multiple gestations. All of our participants were 20–44-year-old, married, Caucasian women with a high education level. Although the patients in our study represented a wide age range, those who gave birth to PTLBW neonates were all between 30–35 years old and none were smokers. Several published studies support the role of intra-amniotic infection in the pathogenesis of pre-term labor [8,10–12]. Thus, the presence of bacteria in the amniotic fluid may be one reason for giving birth to a PTLBW neonate.

Previous investigations of periodontal disease and adverse pregnancy outcomes have studied populations that differed from ours [6,7,23,24]. The study populations of Offenbacher et al. [6] and Jeffcoat et al. [7] consisted of younger women and included a higher proportion of subjects from black ethnic groups. The population in a case-control study by Moore et al. [25] differed from those of previous case-control studies with respect to demographic factors and periodontal disease severity, as it included an increased proportion of Caucasian subjects with high socioeconomic status. The characteristics of the study population in Moore et al. may account for the finding of no association between the severity of periodontal disease and pregnancy outcome due to those factors. Similarly, all of the participants in our study were Caucasian and about half were university graduates. These characteristics of our study population may explain the low PTLBW ratio and low periodontal disease severity in the present study.

In a case-control study using a periodontal disease definition similar to ours, Nabet et al. [26] found a significant association between generalized periodontitis and pre-term birth induced due to pre-eclampsia. Their results correlate with ours in that all of the women who gave birth to PTLBW neonates were in the GP group.

Our periodontal findings differ from those of some previously reported studies. In a case control study investigating the possible link between periodontal infection and PTLBW, Buduneli et al. [9] evaluated the periodontal status of 181 women within 3 days post-partum and found no significant difference in periodontal status between the case (PTLBW) and

Table IV. The presence of periodontal pathogens in subgingival plaque (sp) and amniotic fluid (af) samples of patients who gave birth to PTLBW neonates.

	Patient 1		Patient 2		Patient 3		Patient 4	
Bacterium	sp	af	sp	af	sp	af	sp	af
A. a	+	-	-	-	+	-	+	-
T. forsythia	+	+	+	-	+	+	+	-
C.rectus	+	+	+	+	+	+	-	-
T. denticola	-	-	+	-	-	-	-	-
P. intermedia	+	-	+	-	+	-	-	-
P. gingivalis	+	-	+	-	+	+	+	-
E. corrodens	-	-	-	-	+	-	-	-
F. nucleatum	+	+	+	-	+	+	+	-

control (full-term birth) groups, which had mean PDs of 3.45 and 3.20 mm, respectively. These PDs are higher than those in our patients (1.76, 2.03 and 2.27 mm for groups G, LP and GP, respectively). In Offenbacher et al. [6], the group with the healthiest periodontal status had a mean PD of 2.87 mm and the case group had a mean PD of 3.17 mm. Similarly, Davenport et al. [23] reported mean PDs of 3.85 and 3.72 mm for the control and case groups, respectively. Thus, the PD values in the present study are lower than those reported previously.

Periodontal disease severity may be a factor that contributes to adverse pregnancy outcome. Rakato-Alson et al. [5] demonstrated a significant relationship between periodontal disease severity and adverse pregnancy outcomes in a Madagascan population. They found significantly higher PI and papillary bleeding index scores in the pre-term birth group compared with the full-term birth group. Holbrook et al. [27] reported no link between low-grade periodontal disease and pre-term birth in a healthy Caucasian population. In our study population, periodontal disease severity was low among Caucasian women with a high education level and good healthcare.

Another factor that may affect the reported association between periodontal status and adverse pregnancy outcomes is the definition of periodontitis. In the present study, the definition of periodontitis was based on PD and CAL. However, Lieff et al. [28] determined that the traditionally used definitions were insufficient for clinical periodontal disease assessment. The level of clinical periodontal disease that represents exposure to oral microbes sufficient to impact pregnancy outcome is unknown. Radnai et al. [29] observed that BOP had the strongest association with pre-term birth. They argued that bacteria and/or their products diffused more readily than normal when vascular permeability increased in gingival tissues. Our study results confirmed that patients with periodontal bacteria in the amniotic fluid had generalized periodontitis and higher BOP scores (≥90%; data not shown). The presence of subgingival bacteria in the amniotic fluid may occur in cases of high periodontal disease activity and high BOP scores.

We detected *T. forsythia*, *C. rectus*, *P. gingivalis* and *F. nucleatum* in the amniotic fluid of women with GP. This finding differed from that of Dörtbudak et al. [14], who assessed whether periodontitis predicted premature gestation through the examination of amniotic fluid cytokines and periodontal pathogens. They found no bacteria in the amniotic fluids studied. However, they observed the combined presence of elevated PGE_2, IL-6 and IL-8 levels in the amniotic fluid at 15–20 gestational weeks. The present investigation was limited because it did not examine cytokines or other biochemical markers. However, we did find periodontal pathogens in amniotic fluid samples.

As one of the most prevalent species found in amniotic fluid, *F. nucleatum* has been implicated as the sole infectious agent in pre-term labor. We detected *F. nucleatum* in 8.3% of the amniotic fluid samples from women with GP and PTLBW neonates. *Fusobacterium nucleatum* has been shown to be hematogenously transmitted to the placenta and to cause adverse pregnancy outcomes [8].

In an animal study, Lin et al. [10] subcutaneously inoculated subjects with *P. gingivalis* to induce fetal growth retardation. At the end of the study period, the bacteria were detected in the liver and uterus. Our study detected *P. gingivalis* in a single amniotic fluid sample, which was taken from a woman who gave birth to a PTLBW neonate. This finding is consistent with the results of Lin et al.

In another animal study, Arce et al. [30] showed that *C. rectus* can translocate from a distant site of infection to the placenta and can induce fetal growth restriction. Human placental tissues have not been reported to harbor *C. rectus*, despite evidence of maternal infection and fetal exposure, based on fetal IgG response. Buduneli et al. [9] evaluated the possible link between periodontal infection and PTLBW by means of clinical and microbiological data in post-partum women with low socio-economic level. Their findings indicate that, when subgingival bacteria were evaluated together, *P. micros* and *C. rectus* may have a role in increasing the risk for PTLBW. We did not evaluate the presence of *P. micros*, but, to our knowledge, our detection of *C. rectus* in 12.5% of the amniotic fluid samples from the GP group is the first report of its presence in the human amniotic cavity.

In the present study, bacteria detected in the amniotic fluid were also found in the subgingival plaque. This co-occurrence may explain the source of these bacteria. Microbes could enter the amniotic cavity through four possible mechanisms. The organisms could ascend to the uterus from the vagina and cervix. Based on routine clinical examinations, an obstetrician determined that bacterial vaginosis was absent from our study population. However, as no vaginal samples were obtained from the subjects, we cannot rule out the possibility that there might have been subclinical bacterial vaginosis. This is especially important in the three cases in which infected amniotic fluid demonstrated *C. rectus*. This is a limitation of our study.

The second potential mechanism of microbe entry is the hematogenous transmission of organisms originating elsewhere. Third, retrograde seeding from the peritoneal cavity could occur through the Fallopian tubes. Fourth, inoculation during invasive procedures such as amniocentesis could introduce microbes into the amniotic cavity. We detected periodontal pathogens by PCR in both the subgingival plaque and amniotic fluid samples and we believe that this

explains the source of the bacteria. Our study was limited because we did not use advanced molecular diagnostic techniques to detect sub-species of the bacteria.

Conclusions

In conclusion, periodontal pathogens from the oral cavity may reach the amniotic cavity and cause adverse pregnancy outcomes. This study sheds light on the mechanism underlying the association between periodontal infection and adverse pregnancy outcomes. More studies with larger sample sizes are needed to confirm this association.

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