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Increased infection with key periodontal pathogens during gestational diabetes mellitus

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Abstract

Aim—Gestational diabetes mellitus (GDM), gingivitis, infection with specific periodontal pathogens and systemic inflammation each increase the risk for poor pregnancy outcome. We set out to monitor the interactions of gingivitis and GDM with respect to oral infection and the systemic inflammatory burden.

Materials and Methods—Four case-control groups ($n = 117$) were recruited, (1) No gingivitis, No GDM ($n = 27$); (2) Gingivitis, No GDM ($n = 31$); (3) No gingivitis, GDM ($n = 21$); and (4) Gingivitis, GDM ($n = 38$). Oral infection with three key periodontal pathogens was determined by PCR. Systemic inflammation was determined by quantification of CRP by EIA.

Results—Gingivitis during pregnancy was associated with oral infection with *Porphyromonas gingivalis*, *Filifactor alocis* and *Treponema denticola* and combinations thereof (all $p < 0.01$). GDM was also associated with increased infection with individual and multiple oral pathogens (all $p < 0.05$). Gingivitis during pregnancy led to a 325% increase in systemic CRP (mean, 2495 versus 8116 ng/ml, $p < 0.01$).

Conclusions—Diabetes and gingivitis act in concert to increase risk biomarkers for poor pregnancy outcome.

Keywords

CRP; gestational diabetes; gingivitis; periodontopathogens; pregnancy

Increased gingival inflammation is well documented during pregnancy (Ehlers et al. 2013, Figuero et al. 2013, Niederman 2013) while periodontal diseases increase risk for maternal (hypertension and preeclampsia) and fetal complications (low birth weight and pre-term birth) (Marin et al. 2005, Guimaraes et al. 2012, Pralhad et al. 2013, Takeuchi et al. 2013, Ha et al. 2014, Jacob & Nath 2014, Rao et al. 2014). For example, active periodontal

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Conflict of interest

The authors have no conflict of interest.

inflammation, as determined by bleeding on probing, has been reported to correlate with reductions in fetal femur length, birth weight and birth length (Takeuchi et al. 2013). Indeed, it has even been suggested that periodontal disease severity may predict adverse pregnancy outcome (Al Habashneh et al. 2013).

Gestational diabetes mellitus (GDM) occurs in 2 to more than 10% of pregnancies (Moses & Cheung 2009) and also increases risk for pregnancy complications, such as placental abnormalities, preeclampsia, emergency cesarean delivery and stillbirth, as well as future development of type 2 diabetes mellitus (Retnakaran et al. 2003, Barden et al. 2004, Gabbay-Benziv & Baschat 2014, Ngai et al. 2014, Ovesen et al. 2014).

Diabetes and periodontal inflammation clearly interact, with overt diabetes associated with an increased risk of more severe periodontitis while periodontitis has been associated with worsened glycemic control in subjects with diabetes (Bascones-Martinez et al. 2011, 2014, Lakschevitz et al. 2011, Negrato et al. 2013, Sima & Glogauer 2013). There are only a few studies that address the interrelationships of GDM and gingivitis during pregnancy, specifically, and exacerbation of gingival inflammation by gestational GDM is apparent (Mittas et al. 2006, Xiong et al. 2006, 2009).

Gingivitis is a bacterial-induced disease and specific dental plaque pathogens, including *Porphyromonas gingivalis* and *Treponema denticola*, have been associated with poor pregnancy outcome (Leon et al. 2007, Carrillo-de-Albornoz et al. 2012, Cassini et al. 2013, Ercan et al. 2013, Tellapragada et al. 2014). Furthermore, poor glycemic control has been reported to result in increased subgingival infection with these same two pathogens (Aemaimanan et al. 2013). Increased low grade systemic inflammation has been suggested to be related to pregnancy complications, such as preeclampsia (Best et al. 2013, Wang et al. 2014), with elevated levels of the acute phase protein, C-reactive protein (CRP), suggested to be an effective early biomarker for GDM (Maged et al. 2014).

We hypothesized that both pregnancy-associated gingivitis and gestational diabetes mellitus would each be associated with increased oral infection with key oral pathogens and set out to monitor oral infection with *P. gingivalis*, *T. denticola* and the recently emergent oral pathogen, *Filifactor alocis* (Wang et al. 2013), as well as the systemic inflammatory burden, in 117 pregnant women with and without gingivitis and with and without gestational diabetes.

Materials and Methods

Materials

Chelex 100 was purchased from Bio-Rad Laboratories (Hercules, CA, USA), PCR primers came from Bio-Synthesis Inc. (Lewisville, TX, USA), while PCR SuperMix and UltraPure distilled water were from (Invitrogen, Carlsbad, CA, USA). Gifu anaerobic medium (GAM) was purchased from Nissui Pharmaceutical (Tokyo, Japan) while brain heart infusion (BHI) medium came from Becton Dickinson (Sparks, MD, USA). Fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA, USA). All other media components came from Sigma Aldrich (St. Louis, MO, USA). High sensitivity cotinine immunoassays were

obtained from Salimetrics (State College, PA, USA). Finally, CRP EIA kits were ordered from Cayman Chemical Company (Ann Arbor, MI, USA).

Study population

All individuals were recruited between September 2012 and March 2013 at the Endocrinology and Metabolism outpatient clinic, Aydın State Hospital, Aydın, Turkey. The study was conducted in full accordance with ethical principles, including the World Medical Association's Declaration of Helsinki, as revised in 2008. The protocol was approved by the Ethics Committee of Medical Faculty of Ege University (protocol number 13-2/9). The study protocol was explained and written informed consent was received from each individual before enrollment in the study. Medical and dental histories were obtained from each individual and then saliva and serum samples were obtained before clinical periodontal examination. The inclusion criteria were being in the third trimester, not having any other diagnosed systemic diseases requiring usage of medications such as antibiotics, non-steroidal anti-inflammatory drugs, corticosteroids or drugs that cause gingival overgrowth. Smoking status was determined according to the self-reports of the pregnant women and then verified biochemically by salivary cotinine analysis. The exclusion criterion was having a body mass index (BMI) value ≥ 30 (kg/m²).

Gestational diabetes mellitus was diagnosed according to the current American Diabetes Association criteria (American Diabetes 2013), i.e. a 75 g oral glucose tolerance test was administered, with fasting plasma glucose measurement at 1 and 2 h, at 24–28 weeks of gestation in women not previously diagnosed with overt diabetes. GDM was diagnosed if the following threshold values were met: fasting, ≥ 92 mg/dl (5.1 mmol/l); 1 h, ≥ 180 mg/dl (10.0 mmol/l); and 2 h, ≥ 153 mg/dl (8.5 mmol/l).

Gingivitis was diagnosed as previously described (Armitage 2003, Ozcaka et al. 2012, 2013), i.e., when bleeding on probing (BOP) was present at $\geq 50\%$ of all sites and probing depth (PD) was ≥ 3 mm at $\geq 90\%$ of the measured sites, no more than one site with a PD ≥ 4 mm and clinical attachment level (CAL) ≥ 1 mm. All participants had ≥ 20 teeth present. Periodontally healthy women were required to present with $\leq 30\%$ BOP, and no clinical sign of alveolar bone loss. Plaque index (PI) was recorded as present or absent following visual examination (O'Leary et al. 1972).

Saliva and serum sampling

Whole, unstimulated saliva samples were obtained by expectoration into polypropylene tubes before any clinical measurement or periodontal intervention in the morning, following an overnight fast, during which participants were requested not to drink (except water) or chew gum, essentially as previously reported (Zeller et al. 2014). The saliva samples were clarified by centrifugation (800 g) for 10 min. at room temperature, and 500 μ l amounts were placed in polypropylene tubes and immediately lyophilized. Venous blood (5 ml) was taken from the antecubital vein by standard venipuncture. Serum was separated by centrifugation at 1500 g for 10 min. and immediately frozen at -40°C then lyophilized. Samples were shipped to the University of Louisville for biochemical and microbiological analysis. Ethical approval to analyse biomedical samples originating in Turkey was obtained

from the Institutional Research Board of the University of Louisville (protocol number 13.0670).

Determination of systemic CRP concentrations

Circulating CRP was measured in serum using EIA kits, according to the manufacturer's instructions (Cayman Chemical Co.), using an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The reported sensitivity of the assay is 50 pg/ml.

Determination of salivary cotinine concentrations

Salivary cotinine was measured using a high sensitivity cotinine immunoassay (Salimetrics) and an Emax Precision Microplate Reader, as previously reported (Zeller et al. 2014). The reported sensitivity of the assay is 0.15 ng/ml.

Growth of periodontopathogens

P. gingivalis ATCC 33277; *F. alocis* ATCC 38596; and *T. denticola* ATCC 35405 were grown to mid-to late log phase at 37°C (80% N₂, 10% H₂, 10% CO₂) in GAM; BHI supplemented with FBS (5%), l-cysteine (0.1%) and arginine (20%); and new oral spirochete broth (with veal heart infusion replaced with BHI and KH₂PO₄ omitted) (Ohta et al. 1986), respectively, in an anaerobic chamber (Coy Laboratories, Grass Lake, MI, USA).

Detection of pathogens

The presence or absence of bacterial DNA was determined by four PCR amplifications per Chelex 100 (25% mass/volume)-treated saliva sample, using primers documented in Table 1. Cycling parameters were as previously reported (Slots et al. 1995, Siqueira & Rocas 2003, Zeller et al. 2014). DNA extracted from cultured periodontopathogens and water served as positive and negative controls, respectively. *P. gingivalis*, *T. denticola*, *F. alocis* and universal 16s amplicons were visualized using the Lonza Flashgel system (Rockland, ME, USA).

Data analyses

The sample size was based on precedent literature examining the relationships between diabetes, pregnancy, CRP concentrations and infection with oral pathogens (Dasanayake et al. 2008, Molnar et al. 2008, Aemaimanan et al. 2013, Ercan et al. 2013, Maged et al. 2014). Statistical significance was determined using INSTAT v3.06 (GraphPad, San Diego, CA, USA). Parametric or non-parametric ANOVA with Tukey or Dunn post-testing, respectively, and Fisher's Exact Test were employed, as appropriate. Correlations between measurements of interest were determined by Spearman rank analysis. Significance was set at $p = 0.05$.

Results

The characteristics of all recruited women are shown in Table 2. The periodontally healthy group with GDM was older than the periodontally healthy group without GDM by an average of 3.4 years. As expected, all clinical parameters were higher in those with

gingivitis compared to the periodontally healthy women while those with GDM had a higher BMI than those who did not. By chance, the groups were matched for smoking (smoking yes *versus* no). Furthermore, there was no correlation between cotinine concentration and BoP ($p = 0.636$), perhaps due to the small number of smokers and low cotinine concentrations.

Women with gingivitis during pregnancy are more likely to harbor periodontopathogens

As expected, because gingivitis is a bacteria-induced disease, pregnant subjects with gingivitis were more likely to harbor periodontopathogens than those who were periodontally healthy, as shown in Table 3.

Women with gestational diabetes mellitus are more likely to harbor periodontopathogens

Gestational diabetes mellitus itself was associated with increased infection with *P. gingivalis*, *F. alocis*, *T. denticola*, or a multiplicity of infection in individuals both with and without gingivitis, as shown in Table 3.

Gingivitis dramatically elevates systemic CRP in pregnant women

The systemic inflammatory burden, as determined by CRP measurement, was higher in those with gingivitis only compared to those without gingivitis (with or without GDM, both $p < 0.01$) but not those with gingivitis and GDM, as shown in Fig. 1. The mean (SD) CRP concentrations for those without gingivitis or GDM, with gingivitis only, with GDM only and those with both gingivitis and GDM was 2495 (3007); 8386 (10006); 1838 (1389); and 8698 (4938) ng/ml, respectively. There was no correlation between cotinine concentration and CRP ($p = 0.835$), again perhaps due to the small number of smokers and low cotinine concentrations. Similarly, there was no correlation between BMI and CRP ($p = 0.265$) for the total population, likely reflecting the importance of gingival inflammation.

Discussion

A recent systematic review of the available epidemiological evidence determined that maternal periodontitis is independently associated with adverse pregnancy outcome but that interpretation of the data is impacted by disease definition (Ide & Papapanou 2013), a conclusion supported by Macedo et al. (2014). Increased bacterial infection and systemic inflammation related to moderate and severe periodontitis are each risk factors for poor pregnancy outcome (Borgnakke et al. 2013, Chapple et al. 2013, Figuero et al. 2013). Specific microbial signatures of the subgingival biofilm were able to distinguish between microbiomes of periodontal health, gingivitis and periodontitis and these profiles may be helpful in establishing risk of disease (Lourenco et al. 2014). Herein, we specifically address the interactions of a different type of periodontal disease, gingivitis, and GDM.

Infection with periodontal bacteria has been reported to be an important indicator of poor pregnancy outcome, particularly pre-term and/or low birth weight infants (Chan et al. 2010, Hirano et al. 2012, Ercan et al. 2013, Santa Cruz et al. 2013, Ye et al. 2013).

Porphyromonas gingivalis is an anaerobic, Gram-negative bacterium and a causative agent of chronic periodontitis that is associated with several systemic sequelae, including pregnancy complications (Leon et al. 2007). Even at low colonization levels, *P. gingivalis*

employs a variety of strategies to control the commensal microbiota and direct disease progression and is thus regarded as a keystone species (Hajishengallis et al. 2011). *Treponema denticola*, a highly motile, invasive, Gram-negative anaerobe, is also a causative agent of periodontitis that has been associated with pregnancy complications (Cassini et al. 2013). *Filifactor alocis* is an anaerobic, Gram-positive bacterium, that has recently been identified by human microbiome projects as a pathogen associated with chronic periodontitis, aggressive periodontitis and endodontic lesions (Aruni et al. 2011). The potential relationship between *F. alocis* and maternal or fetal health, to the best of our knowledge, has not been examined.

From a mechanistic perspective, *P. gingivalis* has been the most extensively studied oral microbe. For example, injection of anti-*P. gingivalis* antibodies that are cross-reactive with cardiolipin into the tails of pregnant mice has been shown to increase fetal loss, relative to control antibody (Schenkein et al. 2013). Administration of *P. gingivalis*-derived LPS to mice reduces fetal weight and increases fetal resorption (Kunnen et al. 2014). Furthermore, *P. gingivalis* DNA has been found in chorionic tissues of high-risk pregnant women (Hasegawa-Nakamura et al. 2011) and in the amniotic fluid of women bearing pre-term and/or low birth weight infants (Ercan et al. 2013).

Saliva collection is more convenient, and thus likely to facilitate more ready recruitment than the isolation of subgingival dental plaque samples, while there are multiple reports that subgingival bacteria can be readily monitored in saliva and, furthermore, that bacteria detected in saliva correlate with periodontal disease status (Martinez-Pabon et al. 2008, Bagyi et al. 2009, Van Assche et al. 2009). Therefore, saliva, rather than plaque, was employed herein. A high rate of infection with the emerging pathogen, *F. alocis* (48.1–76.3%) and the established but understudied pathogen, *T. denticola* (61.3–86.8%) was observed in all subject groupings. Despite this high background, gingivitis during pregnancy increased infection with periodontal pathogens in women with and without GDM. In those without GDM, gingivitis was associated with an increased frequency of detection of DNA from *P. gingivalis*, *F. alocis* and combinations of the three targeted bacteria. In those with GDM, gingival inflammation resulted in increased infection with *P. gingivalis*, *T. denticola* and combinations of the three targeted bacteria.

GDM itself increased the likelihood of infection with periodontopathogens in those with and without gingivitis during pregnancy. In those without gingivitis, GDM was associated with an increased frequency of detection of DNA from *F. alocis* and combinations of the three targeted bacteria. In those with gingivitis, GDM was associated with increased infection with *P. gingivalis*, *T. denticola* and combinations of the three targeted bacteria. Thus, gingivitis and GDM each increase the incidence of infection with periodontopathogens, an established risk factor for pregnancy complications.

Increased systemic inflammation has been suggested to be related to pregnancy complications, such as preeclampsia (Best et al. 2013, Wang et al. 2014), with elevated levels of the acute phase protein, C-reactive protein (CRP), reported to be an effective early biomarker for GDM (Maged et al. 2014). All women were recruited during the third semester, the stage of pregnancy when CRP levels have been reported to be highest in those

with GDM (Leipold et al. 2005). In women with periodontitis and pre-eclampsia, Herrera et al. (2007) found that CRP levels reflected periodontal disease severity. Here, we establish that CRP, employed as a biomarker of systemic inflammation, is dramatically elevated in women with gingivitis during pregnancy, irrespective of GDM diagnosis. CRP concentration is hypothesized to be related to fat deposition and BMI only in early pregnancy, with impaired glucose metabolism important only later in gestation (Wolf et al. 2003, Leipold et al. 2005, Ozyer et al. 2014). Therefore, it is important to note that enrolled women with GDM had significantly higher mean BMI than those without GDM. In baboons, Ebersole et al. (2010) have reported that immunoglobulin responses to oral bacteria (*Fusobacterium nucleatum*, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Campylobacter rectus*) were associated with systemic inflammation during ligature-induced periodontitis in pregnancy (Ebersole et al. 2010).

It will be important to confirm these results in a larger population to ensure that the sample size (four groups of 21–38 subjects each) has not affected results such as CRP-BMI or smoking-CRP associations. In conclusion, and considering this limitation, these data suggest a bidirectional relationship between GDM and gingivitis, although, it has yet to be established if this concerted influence results in poor pregnancy outcome per se. Nevertheless, GDM and gingivitis are each associated with increased infection with pathogenic oral bacteria, while gingivitis increases systemic inflammation, irrespective of GDM. The present study focuses on microbiological data and CRP level with regard to GDM and gingivitis. Further biochemical analysis on cytokine and collagenase levels are planned in the same study population.

Unfortunately, while intensive oral hygiene can be effective in decreasing gingivitis during pregnancy (Geisinger et al. 2014), women appear to receive less than their normal dental care when pregnant (Steinberg et al. 2013). If confirmed, these results suggest that increased efforts to control gingivitis and glycemic control during pregnancy may help to reduce two important risk factors of poor pregnancy outcome.

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Clinical Relevance

Scientific rationale for the study

Periodontopathogens and elevated inflammation are reported to increase risk of pregnancy complications. Oral hygiene can be effective in decreasing gingivitis during pregnancy yet women appear to receive reduced dental care when pregnant.

Principal findings

Gestational diabetes and gingivitis during pregnancy both increase infection with periodontal pathogens while gingivitis induces a dramatic increase in systemic inflammation, as assessed by CRP, compared to those with diabetes alone.

Practical implications

Diabetes and gingivitis act in concert to increase risk biomarkers for poor pregnancy outcome, suggesting it may be important to increase efforts to control periodontal inflammation during pregnancy.

Fig. 1.

Systemic inflammation (CRP) is elevated in pregnant women with gingivitis. The systemic CRP burden in women with and without gingivitis and with and without GDM are presented. The line and error bars represent mean (SD) values. ** $p < 0.01$.

Table 1

Primers used to detect bacterial DNA in saliva

Primer	Forward primer	Reverse primer
Universal*	AGA GTT TGA TCC TGG CTC AG	ACG GCT ACC TTG TTA CGA CTT
<i>P. gingivalis</i> [†]	AGG CAG CTT GCC ATA CTG CG	ACT GTT AGC AAC TAC CGA TGT
<i>F. alocis</i> *	CAG GTG GTT TAA CAA GTT AGT GG	CTA AGT TGT CCT TAG CTG TCT CG
<i>T. denticola</i> [†]	TAA TAC CGA ATG TGC TCA TTT ACA T	TCA AAG AAG CAT TCC CTC TTC TTA

Primer sequences and amplification conditions were first published by *(Siqueira & Rocas 2003) and [†](Slots et al. 1995).

Table 2

Characteristics of the study population

	Age	Smoking status	Cotinine (ng/ml)	Plaque (%)	Bleeding (%)	Pocket depth (mm)	BMI (kg/m ²)
No gingivitis No GDM (<i>n</i> = 27)	28.7 (4.5)	3/27 (11.1%)	5.5 (14.6)	20.4 (1.9)	20.4 (1.9)	1.0 (0.2)	21.7 (2.3)
Gingivitis No GDM (<i>n</i> = 31)	28.2 (4.5)	3/31 (9.6%)	8.3 (25.7)	72.3 (13.3)***	69.0 (14.0)***	2.4 (0.5)***	21.1 (3.5)
No gingivitis GDM (<i>n</i> = 21)	33.1 (5.0) [#]	4/21 (19.0%)	31.7 (81.7)	20.0 (0)	20.0 (0)	1.0 (0.0)	27.4 (4.1)###
Gingivitis GDM (<i>n</i> = 38)	31.0 (4.9)	5/38 (13.2%)	22.3 (66.9)	72.6 (17.5)***	70.3 (18.4)***	2.6 (0.6)***	28.2 (5.5)***

p 0.05;***### *p* 0.001.* Gingivitis *versus* no gingivitis;# GDM *versus* No GDM.

Table 3

Infection with periodontal pathogens

	<i>P. gingivalis</i>	<i>F. alocis</i>	<i>T. denticola</i>	>1	All 3
No gingivitis No GDM (<i>n</i> = 27)	6 (22.2%)	13 (48.1%)	20 (74.0%)	13 (48.1%)	4 (14.8%)
Gingivitis No GDM (<i>n</i> = 31)	13 (41.9%) **	21 (67.7%) ***	19 (61.3%)	19 (61.3%) **	8 (25.8%)
No gingivitis GDM (<i>n</i> = 21)	7 (33.3%)	16 (76.2%) ###	13 (61.9%)	14 (66.6%) ##	3 (14.3%)
Gingivitis GDM (<i>n</i> = 38)	20 (52.6%) ** [#]	29 (76.3%)	33 (86.8%) ***###	28 (73.6%)	16 (42.1%) [#]

[#] *p* 0.05;**/## *p* 0.01;***/### *p* 0.001.

* Gingivitis versus no gingivitis.

[†] GDM versus No GDM.