Serum Proinflammatory Cytokine Responses to Influenza Virus Vaccine among Women during Pregnancy versus Non-Pregnancy

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Objective
This study aimed to comprehensively describe inflammatory responses to trivalent influenza virus vaccine (TIV) among pregnant women and determine whether responses differ compared to non-pregnancy.

Methods
Twenty-eight pregnant and 28 non-pregnant women were vaccinated. Serum cytokines were measured at baseline, and 1, 2, and 3 days post-vaccination. Anti-influenza antibody titers were measured at baseline and 1 month post-vaccination.

Results
Overall, following vaccination, tumor necrosis factor (TNF)-α and interleukin(IL)-6 increased significantly, peaking at 1 day post-vaccination ($P's < 0.001$). Pregnant versus non-pregnant women showed no differences in IL-6, TNF-α, or IL-1β responses. Pregnant women showed no change in IL-8 and increases in migration inhibitory factor (MIF), while non-pregnant showed decreases in both. Pregnancy did not significantly alter antibody responses.

Conclusions
Inflammatory responses to TIV are mild, transient, and generally similar in pregnant and non-pregnant women. Given the variability evidenced, vaccination may provide a useful model for studying individual differences in inflammatory response propensity.

Introduction

During pregnancy, substantial immune adaptation occurs. Pregnancy has been associated with decreased inflammatory responses and maintained/increased anti-inflammatory responses to immune challenges in women as well as in animal models.[1–6] It has been postulated that this immune adaptation may prevent rejection of the fetus by the maternal immune system. Abnormalities in immune adaption to pregnancy may affect pregnancy outcomes such as risk of preeclampsia, poor fetal growth, and preterm birth.[7–10]

For clear ethical reasons, studies of the inflammatory response in humans during pregnancy have

focused almost exclusively on *in vitro* stimulation models.[4,5] Because *in vitro* techniques involve isolation of specific cells, removal of cells from the complex *in vivo* environment, and exposure to higher levels of antigen than normally occurs *in vivo*, the clinical relevance of *in vitro* assessments is often unclear. By providing insight into immune function in the complex, multifaceted, naturally occurring environment, *in vivo* models arguably provide data with clearer clinical relevance.

Influenza virus vaccination provides a unique opportunity to study the *in vivo* inflammatory response during pregnancy. Vaccination is considered safe and beneficial to pregnant women, who are at higher risk than the general population for complications, hospitalization, and death due to influenza.[11–14] Routine influenza vaccination is recommended by the Centers for Disease Control (CDC) and American College of Obstetricians and Gynecologists (ACOG) for all healthy pregnant women in any trimester.[15,16]

In addition to providing a model for studying propensity to inflammatory responding in general, data on inflammatory responses to TIV are of clinical value. In pregnancy, maternal exposure to influenza infection has been linked to increased risk of schizophrenia in offspring[17–19] and inflammatory responses to infection are implicated in this link. Because influenza vaccination induces an inflammatory response, potential effects on fetal development have been cited as a cause for possible concern.[20]

Previous studies have reported that influenza virus vaccine elicits a mild but statistically significant inflammatory response during pregnancy at 1–-2 days post-vaccination.[21] An important limitation of data to date is the cross-sectional design and lack of a non-pregnant comparison group.

Given the limitations of available data, the aims of this study were as follows: (i) to comprehensively describe inflammatory responses to seasonal influenza vaccine among women during pregnancy with longitudinal measurement at baseline, and 1, 2, and 3 days post-vaccination and (ii) to compare inflammatory responses in women during pregnancy versus non-pregnancy. It was hypothesized that inflammatory responses in pregnant women would be mild and transient, with a peak at one to 2 days post-vaccination. It was also hypothesized that inflammatory responses would be attenuated in magnitude among pregnant women as compared to non-pregnant women. This study also included assessment of antibody responses prior to and at 1 month post-vaccination, allowing for examination of equivalence in vaccine immunogenicity in pregnancy versus non-pregnancy.

Methods

Participants

This study included 28 pregnant women and 28 non-pregnant women who were assessed prior to and at 1, 2 and 3 days, and approximately 1 month following seasonal trivalent influenza virus vaccine (TIV) during the 2011–2012 influenza season. Women were recruited primarily from staff and faculty at The Ohio State University Wexner Medical Center through newsletters and online advertisements.

Women were excluded from participation if they reported chronic health conditions with implications for immune or neuroendocrine function including HIV, lupus, arthritis, hypertension, asthma, and diabetes. Women were also excluded if they were taking medications that may alter immune or inflammatory parameters including daily antivirals (e.g., valacyclovir HCl) or statins. Pregnant women were excluded if they reported fetal anomaly or preeclampsia. All pregnant women were <33 weeks of gestation to allow for adequate time for the 1 month post-vaccination visit prior to delivery. Per phone call the day before the first study visit, women who reported an acute illness with cold or flu-like symptoms within the past 7 days were rescheduled. Women who were eligible and chose to participate completed a written informed consent. Participants received compensation for their participation. The study was approved by The OSU Biomedical Institutional Review Board.

Demographic and Psychosocial Measures

Demographic and descriptive information regarding height, current weight, pre-pregnancy weight, age, race, education level, marital status, and income was collected. The following health behaviors were assessed at the initial study visit: smoking, participation in regular physical activity (i.e. at least 1 hour per week of vigorous activity), and frequency of prenatal vitamin use (for pregnant women only).

Measurement of Serum Inflammatory Markers

Inflammatory markers were assessed at baseline, 1, 2 and 3 days post-vaccination. At each study visit, whole blood was collected into vacutainer tubes

while subjects were in a seated position. On follow-up days, blood samples for the same woman were collected within a 2-hr window of collection of the baseline sample for that particular woman to ensure that sample time points were approximately 24 hr apart. Samples were immediately centrifuged, aliquoted, and placed in −80°C freezer storage until analysis. Serum levels of interleukin(IL)-6, tumor necrosis factor (TNF)-α, IL-8, and IL-1β were assayed in duplicate with ultrasensitive multiplex kits from Meso Scale Discovery (MSD) and chemiluminescence methodology using the Immulite 1000 (Siemens Healthcare Diagnostics, Inc., Deerfield, IL, USA). Serum levels of macrophage migration inhibitory factor (MIF) were assayed in duplicate using ultrasensitive multiplex kits from R&D Systems (Minneapolis, MN, USA) per kit instructions.

Measurement of Antibody Responses to Vaccination

Serum from baseline and 1 month post-vaccination was assayed using the haemagglutination inhibition (HAI) test. HAI antibody titers reported as <1:10 were valued at 1:5 for statistical purposes. Consistent with prior studies, for example,[22] seroconversion was defined as a pre-vaccination antibody titer $\leq$1:10 and a post-vaccination titer of $\geq$1:40 or among women with a pre-vaccination titer >1:10, a fourfold increase in the titer. Seroprotection was defined as an antibody titer $\geq$ 1:40.

Physical Measurements

For pregnant women, body mass index (BMI) was calculated (kg/m^2) using height as measured by a nurse at the study visit and self-reported weight prior to pregnancy. For non-pregnant women, BMI was calculated based on current height and weight as measured by a nurse at the study visit.

Influenza Virus Vaccination

Each woman received Fluarix (GlaxoSmithKline, Research Triangle Park, NC, USA) seasonal trivalent influenza virus vaccination. During the 2011–2012 influenza season, each 0.5 mL dose contained 45 μg haemagglutinin (HA), with 15 μg HA of each of the following three virus strains: A/California/7/09 (H1N1), A/Victoria/361/2011 (H3N2), and B/Wisconsin/1/2010.

Statistical Analyses

First, pregnant and non-pregnant women were compared in terms of demographic and behavioral characteristics to assess the comparability of groups. T-tests and chi-square tests were used to evaluate group differences.

Next, inflammatory responses were analyzed using a separate linear mixed model for each outcome. Each model contained fixed effects for pregnancy status and time (baseline, 1, 2, or 3 days post-vaccination) and a time by pregnancy status interaction term. A random subject effect was included to account for correlation among measures from the same subject. Contrasts for change from baseline for each cytokine were tested at each post-vaccination time point for pregnant versus non-pregnant women by linear combinations of the mixed model parameter estimates.

All analyses utilized log-transformed cytokine data to normalize the data distributions. For one subject, IL-6 and IL-1β measures were $\geq$3 SD above the mean and were considered outliers and excluded from analyses. Fifty-four women had complete data at all four study time points. Two subjects had one missing data point each, one at day 1 and one at day 3. These subjects remained in the analyses; the mixed models utilize the non-missing data to inform the missing data points during parameter estimation. Eighty-three of the IL-1β data points (38.6%) were below the detection limit of 0.6 pg/mL. These data points were set to one-half the lower detection limit, 0.3 pg/mL.

Finally, we examined antibody responses among pregnant versus non-pregnant women by chi-square analyses or Fisher's exact test when necessary, with the antibody response for seroconversion and seroprotection defined as described above.

Results

Demographic and Behavioral Characteristics

Demographic and behavioral characteristics of the study sample are presented in Table I. In the sample overall, women were predominately White (75%). The average age was 29.1 (SD = 5.7) years. Pregnant women were predominately in the 2nd trimester (n = 16; 57%) at the time of vaccination [average weeks of gestation = 28.4 (SD = 17.9)]. Twenty-seven women (48%) indicated that they had received the influenza virus vaccine in the previous

Table I Demographic Characteristics

	Pregnant (n = 28)	Non-pregnant (n = 28)	P-value
Age, mean (SD)	28.6 (5.5)	29.6 (5.9)	0.49
Race			
African – American	8 (29%)	6 (21%)	0.53
White	20 (71%)	22 (79%)	
Ethnicity, Hispanic/Latino	1 (4%)	3 (11%)	0.30
Nulliparity	11 (39.3%)	11 (39.3%)	0.96
BMI, mean (SD)	24.9 (5.2)	24.1 (7.0)	0.64
Marital Status			
Married	18 (64%)	13 (46%)	0.40
Unmarried, but in a relationship	5 (18%)	7 (25%)	
Single	5 (18%)	8 (29%)	
Income			
Less than $30,000	10 (36%)	10 (36%)	0.95
$30,000–$74,999	8 (29%)	9 (32%)	
$75,000 or more	10 (36%)	9 (32%)	
Education			
High school or less	8 (29%)	7 (25%)	0.46
Some college	4 (14%)	9 (32%)	
Bachelor's degree	5 (18%)	4 (14%)	
Some graduate school or higher	11 (39%)	8 (29%)	
Smoking			
Current	2 (7%)	5 (18%)	0.15
Past	5 (19%)	9 (32%)	
Never	21 (75%)	14 (50%)	
Vigorous activity, ≥1 hr per week	11 (39%)	18 (64%)	0.06
Hours of sleep night prior to vaccination, mean (SD)	7.4 (1.7)	6.8 (1.9)	0.18
Vaccinated previous year	12 (43%)	15 (54%)	0.42

year. Income was distributed similarly across ranges of less than $30,000 (36%), $30,000–$74,999 (30%), and $75,000 or more (34%). Women with bachelor's degrees or higher made up 50% of the sample. Thirty-one women (55%) were married, with another 21% unmarried but in a relationship.

Pregnant and non-pregnant women did not differ significantly in age [$t(54) = 0.70$, $P = 0.49$], body mass index [$t(54) = 0.47$, $P = 0.64$], or race [$\chi^2(1) = 0.38$, $P = 0.54$]. In terms of prior births, 11 of 28 (39.3%) in each group (pregnant and non-pregnant) were nulliparous. Pregnant versus non-pregnant women did not differ significantly in rates of smoking ($\chi^2(2) = 3.83$, $P = 0.15$), hours of sleep in the night prior to vaccination ($t(54) = 1.37$, $P = 0.18$), or rates of vaccination in the previous year ($\chi^2(1) = 0.64$, $P = 0.42$). Non-pregnant women reported marginally higher rates of vigorous activity ($\chi^2(1) = 3.50$, $P = 0.06$), with 64% reporting one or more hour per week of vigorous activity compared to 39% of pregnant women.

Inflammatory Responses following Influenza Virus Vaccine

In terms of baseline serum inflammatory markers, pregnant and non-pregnant women did not differ in IL-6, TNF-α, or IL-1β. Baseline IL-8 and MIF were significantly higher in non-pregnant than in pregnant women (P's < 0.001). Inflammatory responses to vaccination are presented in Fig. 1. Mixed model analyses demonstrated that in the sample overall, significant increases in IL-6 were seen at 1 and 2 days post-vaccination, with a peak at 1 day post-vaccination (day 1, $P < 0.001$; day 2, $P = 0.01$). Similarly, in the overall sample, an increase in TNF-α was observed at 1 day post-vaccination ($P < 0.001$). In contrast, there was a significant decrease in IL-8 at 1 day post-vaccination ($P < 0.001$). In the overall sample, there were no significant changes in either MIF or IL-1β in response to vaccination.

Pregnant and non-pregnant women did not differ significantly in IL-6, TNF- α, or IL-1β responses to vaccination. Pregnant women differed from non-pregnant in their IL-8 responses at each follow-up time point (P's < 0.005), with IL-8 decreasing from baseline in non-pregnant women and remaining unchanged in pregnant women. The MIF responses differed at 2 and 3 days post-vaccination (P's < 0.02), with responses decreasing from baseline in non-pregnant women and increasing in pregnant women.

Antibody Responses following Influenza Virus Vaccine

As described, antibody levels were measured by HAI at baseline and approximately 1 month post-vaccination. Data at this time point were missing for two women (one pregnant, one non-pregnant). The majority of women (51/54; 94%) completed this follow-up visit between 27 and 39 days post-vaccination, with the remaining three completing this visit between 43 and 51 days. Seroconversion and seroprotection, as defined above (Measurement of Antibody Responses to Vaccination), were examined for each of the three strains included in

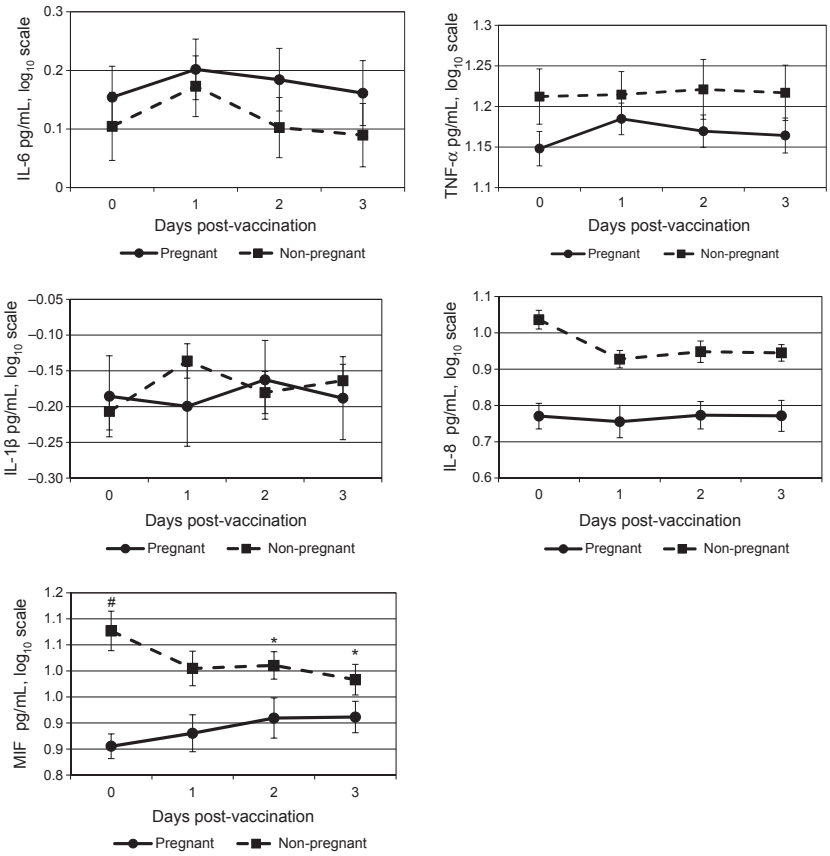

Fig. 1 Serum proinflammatory cytokine responses to trivalent influenza virus vaccine (TIV). Error bars: ±1 SE. Baseline IL-8 and MIF were significantly higher in non-pregnant than pregnant women (*P*s < 0.001). In women overall, TNF-α and IL-6 increased significantly, peaking at one day post-vaccination (*P*s < 0.001). Pregnant versus non-pregnant women showed no differences in IL-6, TNF-α, or IL-1β responses. Pregnant women showed no change in IL-8 and significant increases in migration inhibitory factor (MIF), while non-pregnant showed decreases in both.

the trivalent influenza virus vaccine for the 2011–2012 influenza season (A/California/7/09 (H1N1), A/Victoria/361/2011 (H3N2), and B/Wisconsin/1/2010).

Results showed that pregnant and non-pregnant women did not differ significantly in their antibody response to any strain of the vaccine (Table II). Specifically, seroconversion was achieved against A/H1N1 among 70% of pregnant and 74% of non-pregnant [$\chi^2(1) = 0.09$, $P = 0.76$], against A/H3N2 among 63% of pregnant and 59% of non-pregnant [$\chi^2(1) = 0.08$, $P = 0.78$], and against influenza B among 63% of pregnant and 74% of non-pregnant [$\chi^2(1) = 0.77$, $P = 0.38$]. Seroprotection was achieved against A/H1N1 among 89% of pregnant and 85% of non-pregnant [$\chi^2(1) = 0.16$, $P = 0.69$], against A/H3N2 among 81% of pregnant and 93% of non-pregnant [$\chi^2(1) = 1.48$, $P = 0.22$], and against influenza B among 83% of pregnant and 100% of non-pregnant (Fisher's exact test, $P = 0.49$).

Discussion

In this sample of young, generally healthy women, mild and transient inflammatory responses to seasonal trivalent influenza virus vaccine (TIV) were observed in terms of IL-6 and TNF-α, with peak responses at 1 day post-vaccination. Inflammatory responses to vaccination were not significantly different among pregnant versus non-pregnant women for IL-6, TNF-a, or IL-1β. For IL-8, non-pregnant women showed decreases post-vaccination compared to baseline, while pregnant women showed no change. For serum MIF, pregnant women showed increases post-vaccination, while non-pregnant women exhibited decreases.

These data suggest that inflammatory responses to seasonal influenza virus vaccine are not strongly modified during pregnancy. These results contrast prior data showing that inflammatory responses to *in vivo* and *in vitro* immune challenges are attenuated during pregnancy in human and animal models.[1–5]

Table II Antibody Responses to Vaccination among Pregnant and Non-pregnant Women

	Pregnant (n = 27)[a]	Non-Pregnant (n = 27)[a]	P-value (chi-square test)
H1N1 seroconversion	19 (70%)	20 (74%)	0.76
H1N1 seroprotection	24 (89%)	23 (85%)	0.69
H3N2 seroconversion	17 (63%)	16 (59%)	0.78
H3N2 seroprotection	22 (81%)	25 (93%)	0.22
B seroconversion	17 (63%)	20 (74%)	0.38
B seroprotection	25 (83%)	27 (100%)	0.15

[a]One pregnant and one non-pregnant woman had missing data for the 1-month follow-up, for a final sample of 54 for these analyses.

For example, *in vitro* cytokine production of whole blood exposed to lipopolysaccharide (LPS) was significantly lower among 18 women during their third trimester of pregnancy as compared to postpartum, with threefold lower IL-12 production and 40% lower TNF-α production.[4] Lack of such effect in the current study may be related to the mild response among women overall, resulting in a floor effect whereby attenuation was not observable.

In other populations, individual differences in inflammatory responses to vaccination have been used as an *in vivo* model to study propensity toward inflammatory responding to immune triggers in general. For example, in response to TIV, at 24 hr post-vaccination, men with carotid artery disease (CAD) showed an average of 227% increase in C-reactive protein (CRP) as compared to an increase of 40% among men without disease.[23] Similarly, older adults reporting greater depressive symptoms showed elevations in IL-6 at 2 weeks after influenza vaccination, while no IL-6 increase was seen at this time point among those reporting fewer depressive symptoms.[24] Paralleling these findings, we have previously reported that pregnant women reporting greater depressive symptoms showed significantly greater inflammatory responses to TIV.[25]

As perinatal health conditions including gestational hypertension, preeclampsia, and preterm birth have an inflammatory component,[8,9,26–30] a tendency toward exaggerated inflammatory responding to immune triggers may have unique implications in pregnancy. Despite mild changes observed in the group overall, meaningful variability in inflammatory responses was evidenced between individuals. Among pregnant women, for all five inflammatory markers, standard deviations of change scores from baseline to post-vaccination were substantially larger than the mean change score, indicating substantial variability. The coefficients of variation for change scores (100% x standard deviation/mean) ranged from 191 to 8760% (1.9-fold to 87.6-fold greater than the mean). The current sample size did not provide statistical power for examining differential inflammatory responding in relation to risk of adverse perinatal health outcomes. This should be a goal for future studies.

From a clinical standpoint, these results are consistent with prior data showing that inflammatory responses to TIV are mild and transient in pregnant women, supporting the safety of vaccination.[21] In a study of experimental influenza virus infection in 19 healthy adults, fivefold increases in IL-6 were evidenced with significant increases observable from 2–4 days after infection. In comparison, the average response among pregnant women in the current study across the five biomarkers measured was of 1.01- to 1.14-fold magnitude at peak response for the given biomarker. Moreover, elevations in IL-6 were observable at only 1–2 days post-vaccination and were thus of considerably shorter duration than observed in clinical infection.

Given the considerable variability in inflammatory responses to vaccination, it has been suggested that despite mild responses on average, inflammatory responses may be considerably greater among some women, resulting in potential risk to the developing fetus.[20] In this study, among pregnant women, those in the top quartile of responders showed peak increases relative to baseline of 1.3- to 3.1-fold for MIF; 1.3- to 1.9-fold for IL-6; 1.1- to 2.1-fold for IL-1β; 1.2- to 1.8-fold for TNF-α; and 1.1- to 1.4-fold for IL-8. Thus, responses were relatively mild even among the greatest responders. However, these women were generally healthy; responses may differ in women with chronic health conditions.

These data support the notion that despite eliciting an inflammatory response itself, vaccination should provide a protective function by reducing risk of

influenza infection and related exposure to an inflammatory response of considerably greater magnitude. In support of the health benefits of vaccination, pregnant and non-pregnant women did not differ significantly in rates of achieving seroconversion or seroprotection against any of the three influenza virus strains. This is consistent with prior evidence that pregnant women vaccinated in any trimester of pregnancy show similar antibody responses to non-pregnant women.[22,31–34]

In terms of generalizability, this study examined responses during a single influenza season. The composition of the trivalent influenza virus vaccine changes each season. Inflammatory responses may differ based on strains of virus or the novelty of a given strain. In addition, this sample was predominately White and insured. At 47%, rates of vaccination in the prior year were high as compared to the general population; among adults 18–49 years of age, 30.5% were vaccinated in during the 2010–2011 influenza season (the year preceding the current study).[35] However, our analyses showed no significant differences in inflammatory responses based on receipt of vaccination in the prior year. This high prior vaccination rate reflects the fact that many participants were faculty or staff at The Ohio State University Medical Center, among whom seasonal influenza virus vaccination is mandatory.

In this study, 57% of pregnant women were in the 2nd trimester. It would be informative to examine a cohort in which sufficient representation of demographically matched women in the first, second, and third trimester is assessed. However, this is challenging from a research design standpoint; for clear ethical reasons, women cannot be randomized to receive vaccination in a specific trimester (i.e., delay vaccination). Women may be vaccinated in later gestation due to the timing of their pregnancy relative to the influenza season. However, later vaccination may also be related to lack of early prenatal care which, in turn, covaries with poor health behaviors, lack of access to health care, and demographic characteristics. In this study, the majority [19/28 (68%)] had private health insurance, suggesting ready availability to early prenatal care. If future studies examine differences in immune responses to vaccination by trimester, behavioral/demographic characteristics should be carefully considered, particularly in cohorts with greater socioeconomic diversity.

It would be of value to compare inflammatory responses to flu vaccine as an *in vivo* challenge to an *in vitro* inflammatory challenge. As described, the majority of studies in humans to date rely on the latter. It would be informative, from a research standpoint, to understand the extent to which *in vitro* immune challenges correspond to vaccination as an *in vivo* immune challenge, thus providing support for the clinical relevance of *in vitro* challenge studies.

In sum, this study provides novel data on the inflammatory response in the days following influenza virus vaccine in women during pregnancy versus non-pregnancy. These data indicate that among generally healthy women during pregnancy or non-pregnancy, the inflammatory response to TIV is mild and transient. This response was generally similar among pregnant and non-pregnant women. These data lend support for the clinical safety of vaccination during pregnancy. Given the range in magnitude of response evidenced, vaccination may provide a useful model for studying individual differences in inflammatory response propensity, a factor that may have implications for maternal health and pregnancy outcomes.

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Conflict of interests

The authors report no conflict of interests.

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