ORIGINAL ARTICLE

Longitudinal Profiling of Inflammatory Cytokines and C-reactive Protein during Uncomplicated and Preterm Pregnancy

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Problem
Previous studies have investigated the utility of inflammation markers as predictors of preterm birth, but none have compared trends in levels between uncomplicated and preterm pregnancy.

Method of study
We explored longitudinal changes in plasma cytokines, including IL-1β, IL-6, IL-10, and TNF-α, as well as C-reactive protein in pregnant women from a nested case–control study.

Results
IL-6 was associated with increased odds of spontaneous preterm birth, defined by presentation of spontaneous preterm labor and/or preterm premature rupture of the membranes. Associations were strongest later in pregnancy. IL-10 was associated with increased odds of placentally mediated preterm birth, defined by presentation with preeclampsia or intrauterine growth restriction, and odds ratios were also highest near the end of pregnancy.

Conclusion
Maternal inflammation markers were associated with increased risk of preterm birth, and relationships differed by etiology of preterm delivery and gestational age at sample collection.

Introduction

Preterm birth is a significant public health problem, occurring in over 10% of births worldwide and contributing largely to neonatal mortality and various morbidities in childhood and later in life.[1] Despite the severity of this problem, most cases result from unknown causes and mechanisms of preterm birth remain poorly understood. This limits the ability to predict and prevent preterm birth. Current interventions are estimated to decrease the burden of preterm birth rates only minimally over the next 5 years.[2]

Intrauterine inflammation at the maternal–fetal interface is a well-established contributor to preterm birth.[3] The initial stages of this inflammation generally occur in an asymptomatic setting which has led to intense investigation of biomarkers that may identify those with an increased inflammatory burden and thereby aid in the prediction of prematurity risk. The most strongly predictive markers are from matrices close to the maternal–fetal interface, such as amniotic and cervicovaginal fluid.[4] Plasma or serum markers measured at various time points during pregnancy have so far proven less useful. Despite their lack of predictive value, a description of profiles of systemic maternal inflammation markers over gestation may be important for a better understanding of normal pregnancy and for

characterizing mechanisms leading to preterm birth. These profiles have not been well characterized to date.

Previous studies characterizing longitudinal changes in circulating maternal biomarkers have been limited in sample size, number of time points and repeated measures available, and have focused on uncomplicated pregnancy. In the present study, we analyzed the changes in levels of inflammatory biomarkers, including cytokines IL-1β, IL-6, IL-10, and TNF-α, and C-reactive protein (CRP) over the course of normal pregnancy, and compare these normal profiles to those from pregnant women who delivered preterm.

Materials and methods

Study Population

This nested case–control study draws from a cohort of over 1600 women who delivered at the Brigham and Women's Hospital in Boston between 2006 and 2008.[5] Subjects were recruited early in pregnancy (<15 weeks gestation) and provided demographic and anthropometric information as well as urine and blood samples at an initial visit (visit 1: median gestational age [GA] = 9.71 weeks, range = 4.71–16.1 weeks). Additional samples were collected, processed, and stored for future use at up to three subsequent visits: visit 2 (median GA = 17.9 weeks, range = 14.9–21.9 weeks); visit 3 (median GA = 26.0 weeks, range = 22.9–29.3 weeks); and visit 4 (median GA = 35.1 weeks, range = 33.1–38.3 weeks). For the present study, women who delivered live singleton infants were selected and their samples extracted from storage for measurement of biomarkers of inflammation. All women who delivered preterm, at <37 weeks completed gestation, were included (n = 130), as were 350 random controls. Cases were examined overall as well as in subsets by type, or etiology, of preterm birth.[6] These groups included (i) spontaneous preterm births, or those preceded by preterm premature rupture of the membranes (PPROM) and/or spontaneous preterm labor (n = 56) and (ii) placental preterm births, those resulting from preeclampsia or intrauterine growth restriction (IUGR; <10th percentile in weight for gestational age) during pregnancy (n = 35). Other cases (n = 39) were medically indicated preterm births, which resulted from other maternal or fetal complications. This group was not examined in this analysis, as there is no hypothesis regarding a unifying biological mechanism for this set of cases.

Biomarker Analysis

A total of 1585 plasma samples (n = 391 for cases, n = 1194 for controls) were analyzed for biomarkers of inflammation at the University of Michigan Cancer Center Immunological Monitoring Core (Ann Arbor, MI, USA). CRP was measured using a DuoSet enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) with sensitivity to 10 pg/mL. Only one sample measured was below the limit of detection (LOD) and was replaced with the LOD/$\sqrt{2}$.[7] One sample was above the upper limit of detection (reported as >100 μg/mL) and was replaced with 100 μg/mL. The distribution of CRP levels was lognormal and all values were ln-transformed for analysis.

Plasma samples were also analyzed for inflammatory biomarkers IL-1β, IL-6, IL-10, and TNF-α using the Milliplex MAP High Sensitivity Human Cytokine Magnetic Bead Panel which allows for measurement of multiple cytokines at once using a small sample volume (EMD Millipore Corp., St. Charles, MO, USA). We selected these cytokines because previous studies of these cytokines in maternal blood were most frequently associated with preterm birth, and/or they have been strongly suggested as important in the parturition process.[8–10] Of the 1585 samples analyzed, 176 were measured once and the remaining were measured in duplicate. Correlation between duplicates was high (Spearman R = 0.96–0.99). For individual measures below the LOD (0.128 pg/mL for all cytokines), values reported numerically were kept as is, and values reported as <0.128 were replaced with LOD/$\sqrt{2}$. Following these substitutions, an arithmetic average of duplicate measures was created for statistical analysis. As with CRP, all cytokine levels were lognormally distributed and ln-transformed.

Statistical Analysis

Analyses were performed using R version 2.15.2 2 (R Foundation for Statistical Computing, Vienna, Austria) and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). We examined the distributions of CRP and cytokine levels in all samples using geometric means and standard deviations as well as selected percentiles. Spearman correlation coefficients were

used to assess relationships between different biomarkers in all samples measured in cases and controls separately. To examine differences in biomarker levels by demographic characteristics, we created geometric means of biomarkers for each subject from visits 1–3 to represent average exposure across pregnancy for each individual. Measures from visit 4 were excluded from this measure as some cases had already delivered at that time point.[5] Geometric means of individual averages were calculated for categorical covariates, and differences for each level were compared to the reference using a pairwise *T*-test of ln-transformed values. We examined biomarker differences by the following categories: race/ethnicity (White, African American, Other); education level (high school, technical school, junior college or some college, college graduate); health insurance provider [private insurance/health maintenance organization (HMO)/self-pay, Medicaid/supplemental security income (SSI)/MassHealth]; body mass index (BMI) at visit 1 (<25 kg/m^2, 25 to <30 kg/m^2, >30 kg/m^2); tobacco use (no smoking during pregnancy, smoking during pregnancy); alcohol use (alcohol use during pregnancy, no alcohol use during pregnancy); and parity (nulliparous, parous).

To create estimates of differences in biomarkers between cases and controls, we first used logistic regression models to calculate odds of overall preterm birth as well as preterm birth subsets in association with average exposure across pregnancy. Initially, each biomarker was examined in relation to preterm birth in its own model; however, we also examined models with more than one biomarker and with interaction terms in a secondary analysis. Crude models were created without adjustment for any covariates, and full models were built using a forward stepwise procedure, in which covariates examined in bivariate analysis were added one at a time and included in final models if they changed effect estimates by >10%. We also examined odds of preterm birth in association with biomarker levels from individual visits.

In addition to estimating associations, another aim of our analysis was to describe variability and patterns in inflammatory biomarker levels over pregnancy. First, we calculated intraclass correlation coefficients (ICCs) from linear mixed effect models with random intercepts only to estimate reproducibility of biomarker levels across pregnancy. ICC is a ratio of between-individual to between-plus within-individual variability and ranges from zero to one, with a value of one representing perfect reproducibility (i.e., zero within-individual variability).[11] Second, we examined biomarker trajectories in association with GA using a generalized additive mixed model framework (GAMM) with the *mgcv* package in R.[12] GAMM allows adjustment for correlation between biomarker levels within subject using random intercepts as well as random slopes. Covariates included in the GAMM were the same as those used in the logistic regression models. The association between GA and biomarker levels was modeled by a penalized regression spline term with maximum allowable degrees of freedom = 10. Predicted values of biomarker levels were extracted from GAMM models using the *predict* function for reference levels of covariates [maternal age = median (32.2), race/ethnicity = white, education = High school level, health insurance = Private/HMO/self-pay, BMI <25 kg/m^2] and were plotted with confidence intervals. Models were created for: (i) controls alone; (ii) controls and cases of spontaneous preterm birth, with an interaction term between case status and the smoothing term for GA; and (iii) controls and cases of placental preterm birth, also with an interaction term between case status and the smoothing term for GA. Significant differences in levels between controls and cases were evaluated by interaction terms.

Results

Most biomarkers were highly detectable in our population, with IL-6 detected in 97.9% of samples and CRP, IL-10, and TNF-α detected in 99.9% of samples. IL-1β was slightly less detected, with only 78.0% of samples above the LOD. Geometric means, standard deviations, and selected percentiles of biomarkers in all samples are presented in Table I. Spearman correlations between biomarkers are presented in Table II. While all correlations were statistically significant, they were only weak to moderate in strength. Correlations were similar between cases and controls.

Distributions of demographic characteristics from our study population are presented in Table III. Women were predominantly white (59%), well-educated (68% junior college, some college, or college graduate), and non-smokers (92%). Biomarker levels showed some significant differences within demographic groups, most clearly for CRP (Table III). CRP levels were higher in African American

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Table I Distribution of Inflammation Biomarkers in Samples (n = 1585) from Cases (n = 130) and Controls (n = 350) Combined and Intraclass Correlation Coefficients (ICC) with 95% Confidence Intervals (CI)

	Percentiles							
	Geometric Mean (S.D.)*	25th	50th	75th	90th	95th	Max.	ICC (95% CI)
CRP (μg/mL)	5.80 (2.89)	2.82	5.36	12.2	24.8	34.9	104	0.66 (0.62, 0.70)
IL-1β (pg/mL)	0.30 (3.47)	0.14	0.26	0.50	1.17	2.80	67.8	0.80 (0.78, 0.83)
IL-6 (pg/mL)	1.54 (3.24)	0.83	1.35	2.45	6.12	12.6	555	0.80 (0.77, 0.82)
IL-10 (pg/mL)	13.9 (2.58)	9.08	13.4	19.8	33.9	59.4	2095	0.81 (0.78, 0.83)
TNF-α (pg/mL)	3.05 (1.87)	2.19	3.02	4.30	6.07	7.44	111	0.80 (0.77, 0.82)

*Indicates geometric mean and geometric standard deviation.

compared to White mothers, in mothers with a high school education only compared to mothers with a college degree, in mothers with public compared to private health insurance, in mothers who were overweight (BMI 25 to <30 kg/m^2) or obese (BMI >30 kg/m^2) compared to normal weight prepregnancy, and in mothers who were parous compared to mothers who were nulliparous. Fewer differences were observed by cytokine levels. No significant differences in biomarker levels were observed by tobacco or alcohol use during pregnancy, potentially due to the small number of alcohol (n = 19) and tobacco (n = 31) users in our study population. By case status, the only significant difference observed in the crude comparisons was that IL-6 concentrations were significantly higher in cases compared to controls.

Some biomarkers levels were associated with increased odds of preterm birth in the initial analysis using geometric mean levels measured from visits 1–3 for each individual (Table IV). In unadjusted models, IL-6 was associated with significantly increased odds of overall preterm birth (odds ratio [OR] = 1.29, 95% confidence interval [CI] = 1.09, 1.54). The association became slightly stronger when spontaneous preterm births were examined separately (OR = 1.33, 95% CI = 1.07, 1.66) and remained in full models adjusting for maternal age, race/ethnicity, education level, health insurance provider, and BMI. An ln-unit increase in IL-10 was also associated with increased odds of overall preterm birth in adjusted models (adjusted OR [AOR] = 1.28, 95% CI = 1.01, 1.62), but this relationship appeared to be primarily driven by the association with placental preterm births (AOR = 1.67, 95% CI = 1.12, 2.49). Increased odds of placental preterm birth were observed in association with CRP and TNF-α in crude models, but in adjusted models, the effect estimates were not statistically significant. No significant associations were observed for IL-1β.

We additionally created logistic models with multiple biomarkers. For overall preterm birth, the most elevated OR was in association with IL-6 and IL-10 levels. A model with both biomarkers indicated that IL-6 was more strongly associated with overall preterm birth (AOR = 1.25, 95% CI = 0.98, 1.59) compared to IL-10 (AOR = 1.07, 95% CI = 0.79, 1.45). Similarly, in models of spontaneous preterm birth when both IL-6 and IL-10 were included at once the association between IL-10 and preterm birth was diminished (AOR = 1.05, 95% CI = 0.70, 1.57) while

Table II Spearman Correlations between Biomarkers from All Samples Measured in Cases (n = 379 Samples, 130 Subjects) and Controls (n = 1143 Samples, 130 Subjects)

	IL-1β	IL-6	IL-10	TNF-α
Cases				
CRP	0.06*	0.23*	0.12*	0.17*
IL-1β		0.40*	0.27*	0.21*
IL-6			0.61*	0.37*
IL-10				0.46*
Controls				
CRP	0.09*	0.26*	0.05	0.18*
IL-1β		0.24*	0.10*	0.16*
IL-6			0.37*	0.25*
IL-10				0.31*

*Spearman correlation coefficient statistically significant ($P < 0.05$).

the association with IL-6 remained stronger (AOR = 1.30, 95% CI = 0.95, 1.78). Finally, for placental preterm birth, IL-6, IL-10, and TNF-α were all strong predictors and we tested the effect of including all three in one model. The results showed that the strongest predictor was IL-10 (AOR = 1.64, 95% CI = 0.93, 2.91) while the effect estimates for IL-6 (AOR = 0.92, 95% CI = 0.58, 1.47) and TNF-α (AOR = 1.38, 95% CI = 0.66, 2.88) were reduced. Interactions between biomarkers were also examined but none were statistically significant (data not shown).

When associations between biomarkers and preterm birth were examined by individual visit, several patterns emerged (Table V). For spontaneous preterm birth, AOR was slightly larger in magnitude in association with IL-6 levels at visits 2–4 (median GA 18–25 weeks), although confidence intervals widened due to smaller sample sizes. For placental preterm birth, AOR in association with IL-6 levels was highest at visit 4 (AOR = 1.90, 95% CI = 1.08, 3.34). IL-10 showed a slightly elevated AOR in association with overall preterm birth at visit 3 (AOR = 1.36, 95% CI = 1.05, 1.78) and similarly AOR of spontaneous preterm birth at visits 2–4, although standard errors were somewhat larger. AOR for placental preterm birth in association with IL-10 levels was all significant and increased steadily for visits 1–4. TNF-α showed no association with overall preterm or spontaneous preterm birth, but, as with IL-10, was associated with steadily increasing AOR of placental preterm birth from visits 1–4. Finally, we did not observe associations between CRP or IL-1β and preterm birth overall or by subtype when examined by individual visit.

All cytokines measured showed excellent reproducibility (ICC >0.75) across the duration of pregnancy (Table I). Temporal reliability for CRP was slightly lower but reproducibility was still good (ICC ≥0.4 and <0.75).[11] We next created GAMM models to examine any patterns in biomarker levels over pregnancy and to test for any differences in these patterns in cases compared to controls. Models included random intercepts and slopes as well as the same set of covariates included in logistic models. In controls alone, we found that CRP levels increased early in pregnancy, peaked at approximately 20 weeks, and then gradually declined until the end of pregnancy (Fig. 1). IL-6 levels had an opposite trajectory, declining until approximately 20 weeks gestation and then increasing toward the end of pregnancy. IL-1β levels declined in a linear fashion [estimated degrees of freedom (EDF) = 1], and TNF-α levels remained somewhat level with slight increases at 20 weeks and toward the end of gestation. Finally, IL-10 levels appeared to be unchanging throughout the entire course of pregnancy (p for smoothing term = 0.23, EDF = 1).

After examining patterns in controls alone, we also examined and tested the difference in the relationship between GA and biomarker level by case status using an interaction term. The first set of models examined cases of spontaneous preterm birth only. IL-6 levels were significantly different in spontaneous cases compared to controls (p for interaction<0.001) and levels appeared to diverge as pregnancy progressed (Fig. 2). IL-10 levels were significantly different in cases of placental preterm birth compared to controls (p for interaction = 0.02), and the difference appeared to be linear across pregnancy (Fig. 3). Interaction terms for other cytokines and CRP and spontaneous or placental preterm birth were not statistically significant (data not shown).

Discussion

In the present study, we examined levels of inflammation biomarkers, including CRP and cytokines, at multiple time points during pregnancy in peripheral blood plasma samples. We found that increases in average levels of IL-6, IL-10, and TNF-α during pregnancy were associated with increased odds of overall preterm birth. IL-6 was the strongest predictor in spontaneous preterm pregnancies, and IL-10 was the

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Table III Distributions of Population Demographic Characteristics and Geometric Means[a] of Inflammation Biomarkers within Groups (n = 480)

Demographic characteristics	n (%)	CRP (μg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	TNF-α (pg/mL)
Race/ethnicity						
White (reference)	281 (58.5)	5.20	0.34	1.40	13.3	2.91
African American	77 (16.0)	8.60*	0.23*	1.77	14.7	3.22
Other	122 (25.4)	6.20	0.28	1.61	15.1	2.99
Education						
High school (reference)	68 (14.2)	7.68	0.24	1.73	15.5	3.26
Technical school	77 (16.0)	6.42	0.37*	1.91	16.6	3.07
Junior college or some college	138 (28.8)	6.43	0.28	1.75	14.2	2.98
College graduate	186 (38.8)	4.78*	0.33	1.19*	12.4	2.85
Missing	11 (2.29)					
Health insurance						
Private insurance/HMO/Self-pay (reference)	84 (17.5)	5.53	0.33	1.47	13.8	2.96
Medicaid/SSI/MassHealth	384 (80.0)	7.89*	0.23*	1.63	14.6	3.14
Missing	12 (2.50)					
BMI at Visit 1						
<25 kg/m^2 (reference)	249 (51.9)	4.08	0.32	1.24	13.8	2.89
25 to <30 kg/m^2	126 (26.3)	6.47*	0.31	1.78*	14.7	2.78
>30 kg/m^2	101 (21.0)	13.1*	0.28	1.90*	13.1	3.47*
Missing	4 (0.83)					
Tobacco use						
Smoked during pregnancy (reference)	31 (6.46)	7.95	0.23	1.54	13.8	3.17
No smoking during pregnancy	443 (92.3)	5.80	0.31	1.52	14.0	2.97
Missing	6 (1.25)					
Alcohol use						
Alcohol use during pregnancy (reference)	19 (3.96)	5.35	0.39	1.07	12.4	2.56
No alcohol use during pregnancy	451 (94.0)	5.94	0.30	1.54	14.0	3.00
Missing	10 (2.08)					
Parity						
Nulliparous (reference)	215 (44.8)	5.36	0.30	1.48	13.6	2.87
Parous	265 (55.2)	6.38*	0.30	1.53	14.3	3.07
Infant gender						
Male (reference)	212 (44.2)	6.41	0.30	1.60	13.5	2.97
Female	268 (55.8)	5.52	0.30	1.44	14.4	2.99
Preterm[b]						
<37 weeks (reference)	130 (27.1)	6.52	0.30	1.94	15.9	3.15
≥37 weeks	350 (72.9)	5.68	0.30	1.37*	13.3	2.92

[a]Geometric means for each category were created from subject-specific averages (geometric means from levels measured at up to three time points per individual). [b]The large proportion of preterm cases is attributable to the case–control study design and is not indicative of prevalence in the baseline population. *Denotes significant difference in biomarker concentration from reference category based on pairwise T test ($P < 0.05$).

strongest predictor in placental preterm pregnancies. Furthermore, we identified and depicted for the first time longitudinal patterns in these biomarkers in normal and preterm pregnancy and found differences in patterns by these groups.

Inflammation at the maternal–fetal interface is one of most well-established causes of preterm birth.[3,13] Systemic or local infection can initiate a cascade of events leading to premature parturition.[8,13] Pro-inflammatory cytokines are the most well studied in this pathway; however, reduced levels of anti-inflammatory cytokines may be important as well.[14] Because of their well-known involvement in this mechanism, a number of studies have measured cytokines in pregnant women in an attempt to identify predictive clinical markers of premature birth. Most of these studies have only utilized measures from one time point during

Table IV Unadjusted and Adjusted[a] Odds Ratios (95% Confidence Intervals) of Preterm Birth in Association with Ln-Unit Increase in Average[b] Inflammation Marker Concentration

	Unadjusted OR (95%CI)		
	Overall preterm	Spontaneous preterm	Placental preterm
n (cases, controls)	130, 350	56, 350	35, 350
CRP	1.16 (0.94, 1.43)	1.16 (0.86, 1.58)	1.57 (1.08, 2.28)*
IL-1β	1.03 (0.87, 1.23)	1.06 (0.83, 1.34)	1.01 (0.74, 1.38)
IL-6	1.29 (1.09, 1.54)*	1.33 (1.07, 1.66)*	1.30 (0.98, 1.71)
IL-10	1.24 (0.99, 1.54)	1.33 (1.00, 1.78)	1.38 (0.98, 1.92)
TNF-α	1.25 (0.88, 1.78)	1.14 (0.70, 1.86)	2.32 (1.24, 4.33)*
	Adjusted OR (95%CI)		
	Overall preterm	Spontaneous preterm	Placental preterm
n (cases, controls)	127, 332	56, 332	33, 332
CRP	1.07 (0.84, 1.37)	1.23 (0.86, 1.75)	1.03 (0.66, 1.60)
IL-1β	1.00 (0.83, 1.20)	1.02 (0.79, 1.30)	1.04 (0.73, 1.47)
IL-6	1.29 (1.07, 1.56)*	1.33 (1.05, 1.68)*	1.29 (0.90, 1.84)
IL-10	1.28 (1.01, 1.62)*	1.31 (0.97, 1.78)	1.67 (1.12, 2.49)*
TNF-α	1.16 (0.81, 1.66)	1.12 (0.69, 1.82)	1.83 (0.92, 3.61)

*Significantly elevated OR ($P < 0.05$).

[a]Full models adjusted for maternal age, race/ethnicity, education, health insurance provider, and body mass index (BMI) at visit 1.

[b]OR calculated in association with subject-specific averages (geometric means from levels measured at up to three time points per individual).

pregnancy, and previous results have been conflicting for the cytokines examined in the present study.[4,15] Of the pro-inflammatory cytokines we measured, plasma IL-6 concentrations have been associated with increased odds of preterm birth in the largest number of studies, although results from a recent meta-analysis did not show a significant association.[4] In several studies, lower IL-10 levels have been associated with increased risk of preterm birth, which may be expected as IL-10 is anti-inflammatory; however, other studies have reported null associations.[16–19] Our results showed a positive association between IL-10 and odds of preterm birth, which may indicate that IL-10 is also a biomarker of inflammation because of a normal physiologic anti-inflammatory response to an inflammatory state. This hypothesis is supported by the positive correlation between IL-6 and IL-10 observed in this study as well.

While not directly involved in the parturition mechanism, CRP is a strong marker of overall inflammation and systemic maternal levels may indicate changes that would be otherwise difficult to detect.[20] Hence, CRP levels in peripheral blood have been the focus of a similarly large number of studies. However, as with cytokines, a number of positive associations with preterm birth have been observed but predictability is low.[4]

Fewer studies have examined longitudinal changes in peripheral cytokines or CRP during pregnancy, and most of these in normal pregnancies only. A 2007 study with a large number of subjects ($n = 707$) examined differences in cytokine levels during early and mid-pregnancy.[21] IL-6 levels were notably higher later in pregnancy and no change in TNF-α was observed. Another study with a small number of subjects ($n = 20$ with three time points) examined changes in IL-6 and TNF-α over pregnancy and also observed that IL-6 levels increased across gestation but that there was no change in TNF-α levels.[22] Our findings in control subjects are somewhat consistent with these results, as we observed increases in IL-6, but we also observed increases in TNF-α levels later in pregnancy. Additionally, we observed decreases in IL-1β across gestation which has not been previously reported. Two other studies with fewer subjects but a larger number of repeated measurements observed no changes in pro-inflammatory cytokines during pregnancy.[23,24] Another study examined a panel of cytokines measured in maternal peripheral blood three times during pregnancy and twice

Table V Adjusted[a] Odds Ratios (95% Confidence Intervals) of Preterm Birth in Association with Ln-Unit Increase in Inflammation Marker Concentration at Individual Visits

	Overall preterm birth			
	Visit 1	Visit 2	Visit 3	Visit 4
n (cases, controls)	114, 283	107, 282	100, 273	61, 299
CRP	1.04 (0.83, 1.29)	1.10 (0.86, 1.40)	0.94 (0.74, 1.20)	0.95 (0.71, 1.28)
IL-1β	1.03 (0.86, 1.23)	0.97 (0.80, 1.17)	1.05 (0.87, 1.26)	1.04 (0.83, 1.29)
IL-6	1.22 (1.02, 1.45)*	1.29 (1.06, 1.56)*	1.25 (1.01, 1.55)*	1.21 (0.93, 1.56)
IL-10	1.17 (0.94, 1.46)	1.26 (0.98, 1.60)	1.36 (1.05, 1.78)*	1.08 (0.80, 1.46)
TNF-α	1.08 (0.77, 1.53)	1.13 (0.78, 1.64)	1.17 (0.79, 1.73)	1.23 (0.78, 1.95)
	Spontaneous preterm birth			
	Visit 1	Visit 2	Visit 3	Visit 4
n (cases, controls)	50, 283	50, 282	43, 273	21, 299
CRP	1.11 (0.82, 1.50)	1.31 (0.93, 1.83)	0.86 (0.62, 1.19)	1.07 (0.64, 1.77)
IL-1β	1.03 (0.81, 1.31)	1.05 (0.82, 1.34)	1.08 (0.84, 1.39)	0.99 (0.71, 1.39)
IL-6	1.21 (0.97, 1.52)	1.39 (1.10, 1.77)*	1.35 (1.01, 1.79)*	1.41 (0.98, 2.04)
IL-10	1.18 (0.88, 1.58)	1.34 (0.98, 1.83)	1.35 (0.96, 1.92)	1.27 (0.80, 1.99)
TNF-α	1.05 (0.66, 1.68)	1.12 (0.69, 1.84)	0.97 (0.57, 1.64)	1.32 (0.65, 2.68)
	Placental preterm birth			
	Visit 1	Visit 2	Visit 3	Visit 4
n (cases, controls)	32, 283	28, 282	29, 273	13, 299
CRP	1.07 (0.74, 1.55)	0.98 (0.62, 1.53)	0.92 (0.58, 1.48)	1.02 (0.56, 1.86)
IL-1β	1.10 (0.80, 1.52)	0.97 (0.68, 1.39)	1.22 (0.87, 1.73)	1.27 (0.82, 1.98)
IL-6	1.27 (0.92, 1.75)	1.35 (0.94, 1.93)	1.22 (0.81, 1.81)	1.90 (1.08, 3.34)*
IL-10	1.51 (1.04, 2.20)*	1.63 (1.09, 2.45)*	2.03 (1.30, 3.16)*	2.66 (1.41, 5.03)*
TNF-α	1.86 (0.97, 3.53)	1.74 (0.87, 3.48)	2.15 (1.03, 4.50)*	2.41 (0.86, 6.71)

*Significantly elevated OR ($P < 0.05$).

[a]Full models adjusted for maternal age, race/ethnicity, education, health insurance provider, and body mass index (BMI) at visit 1.

postpartum but focused analysis on differences in levels pre- versus post-delivery ($n = 50$).[25]

Several studies have examined IL-10 levels longitudinally during normal pregnancy. Holmes et al.[9] observed that pregnant women had higher levels compared to non-pregnant women at up to three time points during gestation, but there was no difference by timing of measurement. Another study found no changes in IL-10 expression across normal pregnancy in peripheral blood samples.[23] Our results were consistent with these findings, as levels of IL-10 were unchanging in controls throughout the duration of pregnancy. However, our findings contrasted with those from a recent study in which increased LPS-stimulated peripheral blood mononuclear leukocyte IL-10 levels were observed at 25–28 compared to 16–22 weeks gestation in women who delivered preterm (35–36 weeks) compared to term, although results from that study may have been affected by use of a study population in which all women had previously had a preterm birth.[18]

Finally, few studies have examined changes in CRP levels across gestation. In 1990, Nielsen and colleagues examined CRP levels in 60 pregnant women from 4 to 10 time points.[26] They reported that measures were randomly distributed across pregnancy, but no additional statistical techniques were utilized to examine changes. In our analysis, we observed for the first time an increase in CRP levels early in pregnancy up to approximately 20 weeks gestation, followed by a decline in later pregnancy in women who went on to deliver at term. Two other small studies with 2–3 measurements during pregnancy observed no change in serum levels across gestation.[22,27]

We were only able to identify one study that examined changes in inflammation biomarkers across non-normal pregnancy, focusing on preeclamptic

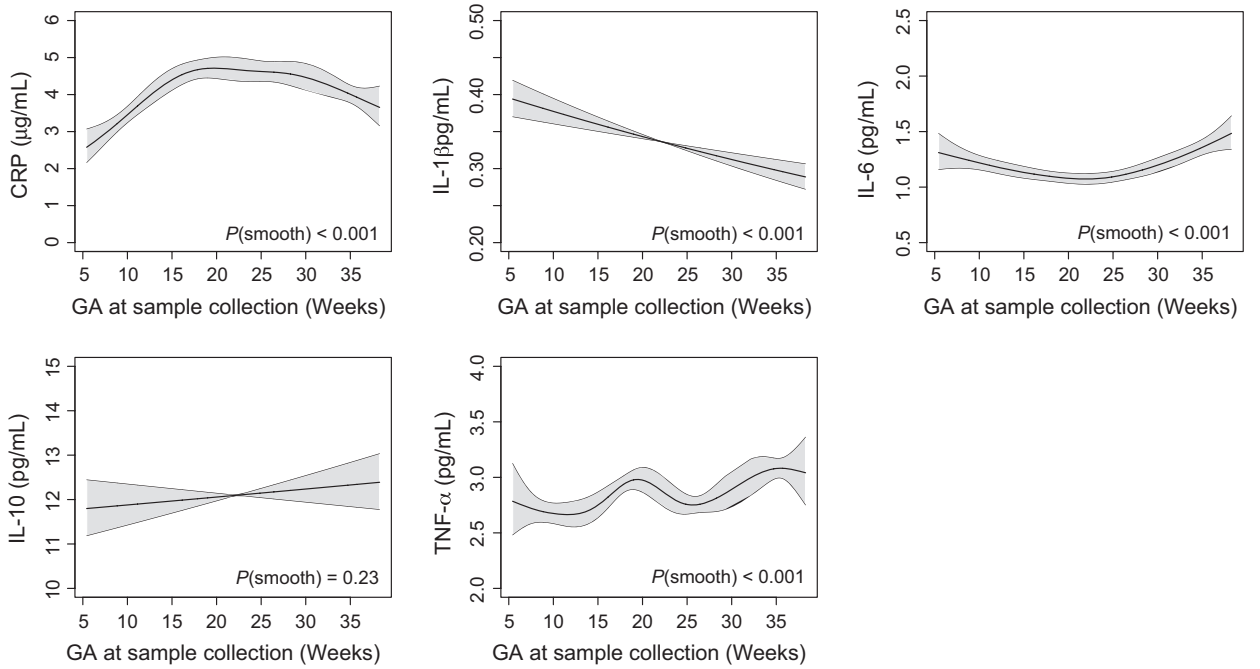

Fig. 1 Plasma Inflammation biomarker levels by gestational age (GA) at sample collection in mothers with term delivery. Levels predicted from smooth term for GA from generalized additive mixed models for subjects with baseline levels for model covariates, including maternal age (32.2), race/ethnicity (white), education level (High School), health insurance provider (Private health insurance/HMO/self-pay), and body mass index (<25 kg/m^2) at visit 1. n = 1135 observations; n = 282 subjects.

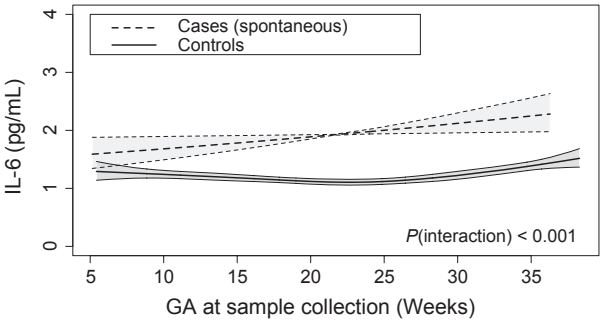

Fig. 2 Plasma IL-6 levels by gestational age (GA) at sample collection in mothers with spontaneous preterm (dashed) compared to term (solid) delivery. Levels predicted from smooth term for GA from generalized additive mixed models for subjects with baseline levels for model covariates, including maternal age (32.2), race/ethnicity (white), education level (High School), health insurance provider (Private health insurance/HMO/self-pay), and body mass index (<25 kg/m^2) at visit 1. n (cases) = 161 observations, 50 subjects; n (controls) = 1087 observations, 282 subjects.

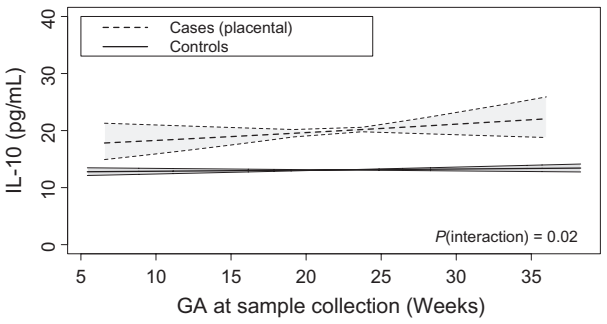

Fig. 3 Plasma IL-10 levels by gestational age (GA) at sample collection in mothers with placental preterm (dashed) compared to term (solid) delivery. Levels predicted from smooth term for GA from generalized additive mixed models for subjects with baseline levels for model covariates, including maternal age (32.2), race/ethnicity (white), education level (High School), health insurance provider (Private health insurance/HMO/self-pay), and body mass index (<25 kg/m^2) at visit 1. n (cases) = 98 observations, 32 subjects; n (controls) = 1087 observations, 282 subjects.

women.[24] As far as we are aware, our study is the first to examine changes in plasma cytokine and CRP levels during pregnancy from women who later deliver preterm. IL-6 levels across pregnancy were significantly different in spontaneous preterm cases compared to controls based on GAMMs, and levels appeared to diverge between the two groups as pregnancy progressed. A similar pattern was observed for IL-10 levels in placental preterm cases compared to controls.

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Our results indicate that the biomarkers measured have moderate to high reproducibility throughout pregnancy. This suggests that (i) measuring levels at multiple time points during pregnancy may not be more useful than a single measurement for predicting preterm birth and (ii) changes observed across gestation are subtle. While it has been reported that peripheral markers may not be useful for predictive purposes, measuring the longitudinal changes over pregnancy may be useful for several reasons. First, these patterns may be helpful for understanding the mechanisms underlying different types of preterm birth. In mothers who went on to have a spontaneous preterm birth, it is notable that while IL-6 increased toward the end of pregnancy, there were no significant differences in cases compared to controls for TNF-α or IL-1β levels. In mothers with placental preterm birth, IL-10 levels were significantly higher across gestation, and levels measured at the end of pregnancy differed most in cases compared to controls. As placental preterm birth is typically characterized by syncytial knot formation and placental infarction occurring at the beginning of pregnancy,[6] these stronger findings in association with levels measured toward the end of pregnancy are different from what we would expect. Future investigation of this relationship could be important for better understanding the mechanism for the relationship between placentation and preterm birth.

These curves may also be used for the identification of internal or external factors contributing to prematurity. We have established that changes in the IL-6 and IL-10 profiles during pregnancy are associated with spontaneous and placental preterm birth, respectively. However, an interesting next step will be to explore through cellular or epidemiology studies what upstream factors could be causing these shifts.

Our study was limited by the availability of plasma samples only; using other matrices for cytokine and CRP measurement, particularly those at the maternal–fetal interface, such as amniotic or cervicovaginal fluid, may be more representative of physiologic changes relevant to pregnancy maintenance.[4] However, collection of a large number of these samples across gestation may not be feasible or ethical for an exploratory study. Despite this limitation, our study has many strengths. It utilized one of the larger populations to date, by number of women and by number of samples per woman, to examine the longitudinal changes in cytokine and CRP levels over pregnancy. We used advanced statistical modeling techniques to explore these nuanced changes and test for differences in those changes in women who delivered preterm compared to term, which has not been done previously. These results provide novel and useful data for future investigation of mechanisms and causes of preterm birth.

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