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MATERNAL SERUM INTERLEUKIN-6, C-REACTIVE PROTEIN, AND MATRIX METALLOPROTEINASE-9 CONCENTRATIONS AS RISK FACTORS FOR PRETERM BIRTH < 32 WEEKS AND ADVERSE NEONATAL OUTCOMES

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Abstract

OBJECTIVE—Elevated concentrations of Interleukin-6 (IL-6), C-reactive protein (CRP), and Matrix Metalloproteinase-9 (MMP-9) in fetal and neonatal compartments have been associated with an increased risk for preterm birth (PTB) and/or neonatal morbidity. The purpose of this study was to determine if the maternal serum concentration of IL-6, CRP, and MMP-9 in women at risk for preterm birth (PTB) who are not in labor, and have intact membranes, are associated with an increased risk for preterm birth < 32 weeks and/or neonatal morbidity.

STUDY DESIGN—Maternal serum samples collected from 475 patients enrolled in a multicenter randomized controlled trial of single versus weekly corticosteroids for women at increased risk for preterm delivery were assayed. Serum was collected at randomization (24-32 weeks gestation). Maternal serum concentrations of IL-6, CRP, and MMP-9 were subsequently determined using enzyme-linked immunoassays. Multivariate logistic regression analysis was performed to explore the relationship between maternal serum concentrations of IL-6, CRP and MMP-9, and preterm birth

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< 32 weeks, Respiratory Distress Syndrome (RDS), Chronic Lung Disease (CLD), Intraventricular Hemorrhage (IVH), Necrotizing Enterocolitis (NEC) and Any Sepsis (S).

RESULTS—Maternal serum concentrations of IL-6 and CRP, but not MMP-9, above the 90th percentile, at the time of randomization, were associated with preterm birth < 32 weeks. In contrast, there was no significant relationship between RDS and NEC and the maternal serum concentration of IL-6, CRP, or MMP-9 (univariate analysis). The development of CLD was associated with a high (above 90th percentile) IL-6 and CRP in maternal serum, even after adjustment for gestational age (GA) at randomization, and treatment group. However, when GA at delivery was added to the model, this finding was non-significant. Neonatal sepsis was more frequent in neonates born to mothers with a high maternal serum concentration of CRP (above >90th percentile). However, there was no significant association after adjustment for GA at randomization, and treatment group. Logistic regression analysis for each analyte indicated that high maternal serum concentrations of IL-6 and CRP, but not MMP-9, were associated with an increased risk of IVH (O.R. 4.60, 95% C.I. 1.86-10.68; O.R. 4.07, 95% C.I. 1.63-9.50), after adjusting for GA at randomization and treatment group. Most babies (25/30) had grade I IVH. When GA at delivery was included, elevated IL-6 remained significantly associated with IVH (O.R. 2.77, 95% C.I. 1.02-7.09).

CONCLUSION—An elevated maternal serum concentration of IL-6 and CRP are risk factors for preterm birth < 32 weeks and subsequent development of neonatal IVH. An elevated maternal serum IL-6 appears to confer additional risk for IVH even after adjusting for gestational age at delivery.

Keywords

Maternal serum; cytokines; preterm birth; neonatal morbidity

INTRODUCTION

Preterm birth complicates 12.7% of all pregnancies in the U.S., and is responsible for 75% of perinatal mortality and more than half the long term morbidity of survivors.[1] Most serious neonatal illness and death is concentrated in the 1 to 2 percent of preterm neonates who deliver prior to 32 weeks of gestation, and a considerable body of evidence suggests that intrauterine infection/ inflammation play a key role in the pathogenesis of at least one third of spontaneous preterm deliveries at less than 32 weeks of gestation.[2, 3] Microorganisms and their products can initiate an inflammatory response mediated by chemokines, cytokines and other inflammatory mediators including matrix degrading enzymes[1-4]. An intra-uterine inflammatory response has been implicated in the mechanisms of preterm parturition associated with infection.[1,3] A fetal systemic inflammatory[2] response has been associated with fetal injury and multi-system organ involvement.[4,5] Most morbidity among preterm infants is attributed to immature organ function. However, since a substantial proportion of preterm infants that delivered prior to 32 weeks are exposed to intrauterine infection[1,3,6] and its associated proinflammatory responses with production of cytokines [7], they are at increased risk for development of serious neonatal complications.[3,4,5] Intrauterine infection is usually chronic and asymptomatic until labor begins or the membranes rupture.[1]

Simple, rapid, noninvasive, and safe tests of markers of asymptomatic intrauterine infection that are associated with adverse neonatal outcomes could be useful in development of strategies for risk stratification and prediction of morbidity among women with or without symptoms of labor. The associations between elevated serum concentrations of IL-6, CRP and MMP-9 in fetal and/or neonatal compartments and preterm delivery and/or neonatal morbidities have been recognized.

There is paucity of data in the current literature concerning the association between maternal serum concentrations of IL-6, CRP, and MMP-9 and preterm delivery (< 32 weeks) and

morbidities in neonates of women at increased risk for spontaneous preterm delivery with intact membranes who are not in labor.

IL-6 is a useful marker for intrauterine infection, PTB, and neonatal morbidities. IL-6, a pleotropic proinflammatory cytokine, is a major mediator of host response to inflammation and infection, and is an early marker of the acute phase response. The presence of increased concentration of IL-6 in cervico-vaginal fluid[8], amniotic fluid [4,5,9,10,11,12], fetal blood[2], umbilical cord blood at delivery[13,14,15,16,17,18], and neonatal blood[19] is an independent risk factor for PTB[7,8], neonatal morbidity[7,9,13,14] bronchopulmonary dysplasia (BPD)[19], IVH[18,16], sepsis[17], periventricular leukomalacia (PVL)[12,15], and cerebral palsy.[4,5]

CRP is a marker for intrauterine infection and inflammation, preterm birth and neonatal morbidities. CRP is a sensitive marker of systemic inflammation that accompanies both acute and chronic inflammatory disorders and is synthesized primarily by hepatocytes in response to various cytokines released from the site of tissue injury or inflammation. CRP is not specific for infection, but is a marker used for diagnosis of many inflammatory, infective, and malignant conditions. Assays for CRP are widely available in most clinical laboratories. Increased concentrations of CRP in amniotic fluid[20], and umbilical cord at delivery[21] are associated with intrauterine infection[20,21] and preterm delivery.[20]

MMP-9 is a marker of intrauterine infection and PTD, and neonatal morbidities. Metalloproteinases (MMPs) are a family of potent zinc-dependent enzymes that belong to the proteases' class of metalloproteinases. They are synthesized by a variety of cell types found in amnion, chorion and decidua. They degrade macromolecules of the extracellular matrix (ECM) components, and have a very important role in maintenance and breakdown of ECM of fetal membranes, leading to membrane rupture.[22] MMP-9 expression increases at parturition, degrades type IV collagen, the major collagen component of the amniotic basement membrane, and may induce apoptosis.

The presence of increased concentrations of MMP-9 in the amniotic fluid [23] fetal blood [24], and neonatal blood [25] were associated with intraamniotic infection[23], preterm birth[23] neonatal Bronchopulmonary Dysplasia (BPD) and IVH.[25] MMP-9 is involved in feto-neonatal development, may have a role in development of lung injury and fibrosis [26], and may contribute to the pathogenesis of BPD and/or IVH in critically ill preterm neonates [25,26] via their action on the remodeling of the ECM, vasomotor regulation, and platelet aggregation.[25]

The purpose of this study was to determine if the maternal serum concentrations of IL-6, CRP, MMP-9, in women at increased risk for preterm birth, who are not in labor, and have intact membranes, are associated with an increased risk for preterm birth < 32 weeks and/or neonatal IVH,CLD,RDS,NEC, and sepsis.

MATERIAL AND METHODS

Study design

This is a secondary analysis utilizing data and maternal serum samples collected during a randomized, double-masked, placebo-controlled, multicenter clinical trial of repeated versus a single course of antenatal corticosteroids (AC). The primary trial was performed at 18 centers of the National Institutes of Child Health and Human Development (NICHD) Maternal-Fetal Medicine Units (MFMU) Network. Recruitment took place from March 2000 to April 2003. The overall study population and methods for this trial have previously been described.[27] The original clinical trial was approved by the institutional review boards at all centers, and informed consent was obtained from all participants. The secondary analysis described here was approved by the institutional review board of Wayne State University School of Medicine.

Patient population and data collection

Women between 23 weeks and 0 days and 31 weeks and 6/7 days, with intact membranes, who were at increased risk for spontaneous preterm delivery, and had already received one course of AC 7 to 10 days earlier, were randomized to receive additional weekly courses of betamethasone or identical-appearing placebo. Exclusion criteria were insulin dependent diabetes mellitus, systemic corticosteroid use during pregnancy, chorioamnionitis, non-reassuring fetal status, or a major fetal anomaly. The first 67 enrolled patients received repeat courses of betamethasone until 33 6/7 weeks. Subsequently, due to concerns of possible fetal risk, the number of repeat courses was limited to four. Maternal, clinical and demographic data were collected at the time of randomization, delivery and discharge.

At the second interim analysis of the primary trial the external data safety and monitoring committee (DSMC) found a tendency toward decreased birthweight in the repeat steroid group without reduction in the primary outcome. The latter combined with difficulties encountered with recruitment resulted in stopping the trial in April 2003.

There were 495 patients randomized, 252 in the repeat AC group and 243 in the placebo group with no significant differences between the two groups in demographic parameters.[27]

Maternal serum

A sample of maternal blood was collected at randomization prior to study drug administration. After clot formation at room temperature the blood sample was centrifuged for 10 minutes at 3400 RPM; the serum was divided to aliquots, frozen, and stored for future analysis.

Neonatal data and outcomes

Neonates were followed until either discharge from the hospital, or up to 120 days, at which time relevant data from the nursery records was obtained. Cranial ultrasounds were performed by 14 days of age on all infants to assess the occurrence of IVH and PVL. Protocol guidelines for scanning, including cranial ultrasound specifications and quality measures, were given to all sites. The films were forwarded to the Biostatistic Coordinating Center (BCC). RDS was defined as requiring oxygen from 6 to 24 hours of age, clinical features of RDS within 24 hours of age, respiratory support from 6 to 24 hours of age, and an abnormal x-ray within 24 hours of age. Presence of IVH or PVL (periventricular lucency in the white matter) were determined by central cranial ultrasound readings, conducted by a panel of expert radiologists blinded to the clinical data. Classification of IVH was according to the Papile classification system.[28] CLD was defined as the need for supplemental oxygen at 36 weeks' corrected age in infants born before 34 weeks. Clinical NEC diagnosis was made regardless of stage. The diagnosis of sepsis required the presence of a clinically ill infant in whom systemic infection is suspected with a positive blood, CSF, or catheterized/suprapubic urine culture; or in the absence of positive cultures, clinical evidence of cardiovascular collapse or an unequivocal X-ray confirming infection.

IL-6, CRP, and MMP-9 Immunoassays

Enzyme-linked immunoassays were used to determine concentrations of IL-6, CRP, and MMP-9 in maternal serum samples. High sensitivity IL-6 assays were purchased from R&D Systems (Minneapolis, MN), high sensitivity CRP assays were obtained from ALPCO Diagnostics (Salem, NH), and MMP-9 assays were purchased from Amersham Biosciences (GE Healthcare, Piscataway, NJ). Maternal serum samples were incubated in duplicate wells of the micro titer plates, which had been pre-coated with antigen specific (IL-6, CRP, or MMP-9) monoclonal antibodies. During this incubation IL-6, CRP or MMP-9 present in the standards or maternal serum samples was immobilized by their specific pre-coated antibodies

(form antigen antibody complexes). Repeated washing and aspiration was conducted to remove all other unbound materials from the assay plate prior to incubation with specific antibody-enzyme reagents. The assay plates were washed again and incubated with a substrate solution and color developed in proportion to the amount of antigen bound in the initial step of the individual assays. The color development was stopped with the addition of an acid solution and the intensity of color was read using a programmable micro titer plate based spectrophotometer (SpectraMax M2 micro plate workstation, Molecular Devices Corporation, Sunnyvale, CA). The concentrations of IL-6, CRP or MMP-9 in maternal serum samples were determined by interpolation from individual standard curves composed of human IL-6, CRP or MMP-9. The calculated inter-assay coefficients of variation (CV) for IL-6, CRP and MMP-9 immunoassays in our laboratory were 5.78%, 6.28% and 4.95%. Calculated intra-assay CVs for IL-6, CRP and MMP-9 were 3.24%, 2.63% and 2.16%. The calculated detection limits (sensitivity) for IL-6, CRP and MMP-9 immunoassays were 0.14 pg/ml, 1 ng/ml, and 1.2 ng/ml respectively.

Statistical analysis

Treatment (repeat AC) and placebo groups were compared with respect to concentration of IL-6, CRP and MMP-9 at randomization. The association between maternal serum concentration of IL-6, CRP, and MMP-9 at the time of randomization with rates of neonatal morbidities (IVH, RDS, CLD, NEC, and sepsis) was examined. Chi-square or Fisher's Exact test was used to compare categorical variables and the Wilcoxon Rank Sum test was used to compare continuous variables. Odds ratios and 95% confidence limits were calculated to examine the association between high concentrations of these analytes (> 90^{th} percentile) and rates of neonatal morbidities. Multiple logistic regression analysis was used to explore these associations while adjusting for possible confounders. Initially, the regression models included gestational age at randomization, treatment group, and analyte. Gestational age at delivery was included in final regression models. A nominal two-sided P value less than 0.05 was considered to indicate statistical significance. No adjustments were made for multiple comparisons.

RESULTS

Serum samples were collected at baseline (gestational age of 28.1 +/− 2.4 weeks), from 475 of 495 mothers that participated in this study. Maternal CRP and MMP-9 serum concentrations were not significantly different between repeat AC (treatment group) and placebo groups at randomization, but there were slight differences (P=0.042) for IL-6 (Table I).

Maternal serum concentrations of IL-6 and CRP, but not MMP-9, above the 90th percentile at the time of randomization were associated with preterm birth < 32 weeks of gestation (Table II). In contrast, univariate analysis showed no significant relationship between RDS and NEC, and maternal serum concentrations of IL-6, CRP, or MMP-9. Neonatal sepsis was more frequent in neonates born to mothers with high maternal serum CRP (above 90^{th} percentile). However, this association was not significant after adjustment for GA at randomization and treatment group. (Table III). The development of CLD was associated with high IL-6 and CRP (above 90^{th} percentile) in maternal serum both in the univariate analysis and after adjusting for gestational age (GA) at randomization and treatment group (Table III). However, when gestational age at delivery was added to the model, there was no longer a significant association between either high IL-6 (O.R. 0.89, 95% C.I. 0.24 - 3.07) or CRP (O.R. 1.54, 95% C.I. 0.46 - 4.85) and rates of CLD.

Table IV shows the percent of neonates with IVH grade 1-4 by level of each of the analytes ≤ 90^{th} and > 90^{th} percentiles. There were 30 cases of IVH, 25 grade I, 3 grade II, 1 grade III and 1 grade IV. In univariate analysis patients with higher levels of IL-6 and CRP had significantly higher rates of IVH.

Regression analysis for each analyte indicates that high maternal serum concentrations of IL-6 and CRP, but not MMP-9, were associated with increased risk of IVH (O.R. 4.60, 95% C.I. 1.86-10.68; O.R. 4.07, C.I. 1.63-9.50) after adjusting for gestational age at randomization and treatment group (Table V). When gestational age at delivery was included elevated IL-6 remained significantly associated with IVH (O.R. 2.77, 95% C.I. 1.02-7.09).

DISCUSSION

The principal finding of this study was that elevated maternal serum concentrations of IL-6 and CRP, but not MMP-9, were associated with preterm birth < 32 weeks of gestation and subsequent development of IVH in neonates of women at increased risk for preterm birth who are not in labor and had intact membranes. An elevated maternal serum IL-6 appears to confer additional risk for IVH even after adjusting for gestational age at delivery and treatment group. The analyte maternal serum concentrations were not significantly different between repeat AC and placebo groups at baseline.

No significant association was detected between elevated maternal serum concentrations of IL-6, CRP and MMP-9 and RDS and NEC. The significant association found between maternal serum concentrations of IL-6 and CRP and CLD, even after adjustment for GA at treatment group and randomization, disappeared after GA at delivery was added to the model. The significant association between maternal serum concentrations of CRP and sepsis disappeared after adjusting for GA at randomization and treatment group.

Several prior studies have explored the association between maternal serum concentrations of analytes and preterm delivery and/or neonatal morbidities. Most studies that explored the relationship between IL-6 [29-35] or CRP [10,11,36-38] and preterm delivery [29-31,34-36], or infectious and non-infectious neonatal outcomes [10,11,30,32,34,37] focused on pregnancies with either preterm labor (PTL) [10,29,34,35,37], or preterm rupture of membranes (PPROM). [11,29,30,32]

Since infection is a major cause of spontaneous preterm delivery in the late second and early third trimester higher maternal serum concentrations of some analytes in women at increased risk for spontaneous early preterm delivery would be expected. There are consistent associations in the fetal and/or neonatal compartments[5-26] between IL-6, CRP and MMP-9 and preterm delivery and neonatal morbidities, in pregnancies complicated by preterm labor or PPROM. However, such consistency is lacking regarding associations between maternal serum concentrations of analytes[29-38] and outcomes. There is doubt on the potential usefulness of maternal serum measurement of analytes for the detection of risk for preterm delivery in women with preterm labor or PPROM. IL-6 holds more promise than most other analytes tested. While some have found a statistically significant association between high maternal serum concentrations of IL-6 and preterm labor,[29] others[34] have found no association. A systematic review of maternal serum CRP as a predictor of chorioamnionitis in pregnancies with PPROM included eight primary studies that met the inclusion criteria. [38] Three studies in the review concluded that CRP was useful, but 5 studies concluded the opposite. These differences may be related to study design, timing of blood collection, the underlying characteristics of study populations and incomplete control for confounding. These data suggest that the maternal compartment differs from the fetal compartment and that the fetal inflammatory compartment is not necessarily reflected in maternal serum.

Very few studies have explored the association between maternal serum concentrations of IL-6, CRP or MMP-9 and early preterm delivery and/or neonatal morbidities in women that are at increased risk for preterm delivery (or at low risk for preterm delivery) but who are not in labor and have intact membranes. The MFMU Network Preterm Prediction Study, a nested case-

control study of low risk pregnant women at 24 weeks of gestation, found that maternal serum concentration of IL-6 was not a significant marker for preterm birth before 35 weeks.[33] Recently Vogel et al[39] evaluated concentrations of 17 inflammatory markers (including IL-6) in both maternal serum and cervicovaginal secretions during the second trimester (12-25 weeks) in 69 asymptomatic women with singleton pregnancies without labor or PPROM. However, all participants were at significantly increased risk for preterm delivery having had at least one prior spontaneous preterm birth. High maternal serum concentrations of IL-6 were associated with an increased risk of spontaneous preterm birth before 35 weeks[39].

Our study was a large, multicenter, prospective trial with clear definitions for all clinical and outcome variables, and high data quality. However, it also has some limitations, including a small sample size. Although originally designed for a sample size of 2400, after the trial was halted early, only 495 women had been randomized. As a result, there were fewer babies with neonatal morbidities, e.g. very few with grade III/IV IVH. Yet this study is still one of the largest prospective studies that has explored the relationship between maternal serum analytes and preterm delivery and neonatal morbidities. It is possible, theoretically, that a single course of AC, that all patients received prior to randomization, affected the results.

Development of strategies for risk stratification and prediction of morbidity in preterm birth < 32 weeks of gestation include identification of simple, rapid, and safe markers of intrauterine infection in women that are at increased risk for early preterm birth, not in labor, with intact membranes. This study provided new information, suggesting that elevated maternal serum concentrations of IL-6 and CRP are risk factors for early preterm delivery (< 32 weeks) and neonatal IVH. Even after adjustments for gestational age at delivery elevated IL-6 confers additional risk for IVH.

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Appendix

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Table I

Analyte Serum Concentrations at Randomization In Repeat Steroid (Treatment) and Placebo Group

	All			Placebo			Treatment			
	N			N			N			p-val
CRP	475			232			243			0.21
		6851.9	(485.0-43920.0)		7420.4	(598.4-47640.0)		6299.4	(444.4-37990.0)	
IL6	470			231			239			0.042
		2.1	(0.6-9.7)		2.2	(0.7-27.5)		2.1	(0.5-8.5)	
MMP9	475			232			243			0.77
		447.8	(174.8-1152.5)		451.8	(174.8-1210.5)		442.0	(177.8-1075.0)	

Data expressed as median with 3rd and 97th percentiles.

IL-6 concentrations expressed in pg/ml; CRP and MMP-9 concentrations expressed in ng/ml.

Table II

Elevated Analytes (IL-6, CRP and MMP-9) at Randomization and Risk of Preterm Birth <32 Weeks of Gestation

Analyte	>90th Percentile (concentration)	Percent Delivery <32 weeks (cases/total)	P-value
IL-6 N=470	No	20.33% (86/423)	0.005
	Yes (>5.15 pg/ml)	38.30% (18/47)	
CRP N=475	No	19.16% (82/428)	< 0.001
	Yes (>25660.0 ng/ml)	46.81% (22/47)	
MMP-9 N=475	No	21.96% (94/428)	0.91
	Yes (> 800.1) ng/ml	21.28% (10/47)	

Table III

Logistic Regression Analysis for Elevated Analytes (IL-6, CRP and MMP-9) and Risk of Neonatal Morbidities

Analyte	N	Odds Ratio	95% CI	P-value
RDS				
IL-6 >90th percentile	470	1.32	0.50-3.07	0.54
CRP >90th percentile	475	1.49	0.62-3.31	0.34
MMP-9 >90th percentile	475	0.88	0.29-2.19	0.79
CLD				
IL-6 >90th percentile	470	3.17	1.15-7.94	0.018
CRP >90th percentile	475	3.74	1.47-8.96	0.004
MMP-9 >90th percentile	475	1.37	0.38-3.86	0.58
Sepsis				
IL-6 >90th percentile	470	2.33	0.79-6.05	0.098
CRP >90th percentile	475	2.21	0.79-5.54	0.11
MMP-9 >90th percentile	475	1.94	0.61-5.18	0.21
NEC				
IL-6 >90th percentile	470	1.43	0.32-4.60	0.59
CRP >90th percentile	475	0.66	0.10-2.49	0.59
MMP-9 >90th percentile	475	0.87	0.13-3.20	0.86

The logistic regression analysis model included gestational age at randomization, treatment group and analytes.

Table IV

Percentage of Babies with IVH Gra de 1-4 by Level of Each Analyte (IL-6, CRP and MMP-9)

Cytokine Level (Randomization)	Cases/Total (%)	Cytokine Level (Randomization)	Cases/Total (%)	P-value
CRP <= 90th percentile N=443	21/397 (5.3%)	CRP> 90th percentile	9/46 (19.6%)	P=0.002
IL6<= 90th percentile N=438	21/394 (5.3%)	IL6> 90th percentile	9/44 (20.5%)	P=0.001
MMP9<= 90th percentile N=443	25/398 (6.3%)	MMP9> 90th percentile	5/45 (11.1%)	P=0.21

Table V

Logistic Regression Analysis for Elevated Analytes (IL-6, CRP and MMP-9) and Risk for IVH

Analyte	N	Odds Ratio	95% CI	P-value
IL-6 >90th percentile	438	*4.60 **2.77	1.86-10.68 1.02-7.09	0.0005 0.038
CRP >90th percentile	443	*4.07 **2.54	1.63-9.50 0.93-6.46	0.002 0.058
MMP-9 >90th percentile	443	*1.84 **1.91	0.60-4.76 0.58-5.39	0.24 0.25

*The logistic regression analysis model included gestational age at randomization, treatment group and analyte.

**Gestational age at delivery added to the above regression model.