Plasma Levels of Inflammatory Markers Neopterin, Sialic Acid, and C-Reactive Protein in Pregnancy and Preeclampsia

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BACKGROUND

To determine whether the cellular inflammatory marker of activated macrophages and monocytes, neopterin (NEO), and the acute-phase inflammatory markers sialic acid (SA) and C-reactive protein (CRP) are elevated in pregnancy and further elevated in the pregnancy syndrome preeclampsia.

METHODS

Maternal plasma concentrations of NEO, SA, and CRP were measured by high-sensitivity enzyme-linked immunosorbent assay (ELISA) or high-performance liquid chromatography in 20 nonpregnant women, 40 women with uncomplicated pregnancies, 50 women with transient hypertension of pregnancy alone, 49 women with small for gestational age (SGA) infants without preeclampsia, and 47 women with preeclampsia.

RESULTS

The mean concentration of plasma NEO, SA, and CRP were all significantly elevated in all groups of pregnant women compared to nonpregnant women ($P < 0.001$ for all). In addition, maternal plasma NEO concentrations were further elevated in women with preeclampsia compared to the other groups of pregnant women ($P < 0.01$). As expected, the acute-phase inflammatory markers CRP and SA correlated positively with each other. However, CRP was also correlated with the activated macrophage and monocyte marker NEO in women with transient hypertension of pregnancy and with preeclampsia ($P < 0.05$).

CONCLUSIONS

The inflammatory markers NEO, SA, and CRP are all elevated during pregnancy. However, only NEO, a marker of macrophage and monocyte activation, was further elevated in women with preeclampsia. These data suggest that there is a striking increase in inflammation during pregnancy, and cellular immune activation is further elevated during preeclampsia.

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The pregnancy syndrome preeclampsia complicates 3–7% of all pregnancies and continues to be a major contributor to maternal and neonatal morbidity and mortality.[1,2] Inflammation is thought to contribute significantly to the pathophysiology of preeclampsia, including endothelial and placental dysfunction.[3] The focus of this study was to measure maternal plasma concentrations of three inflammatory markers (C-reactive protein (CRP), sialic acid (SA), and neopterin (NEO)) in pregnant compared to nonpregnant women, in subjects with the pregnancy complication preeclampsia compared to uncomplicated pregnant subjects, as well as subjects with transient hypertension of pregnancy alone and small for gestational age (SGA) infants alone without preeclampsia.

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CRP is an important component of the innate immune system and is primarily produced by the liver as an acute-phase reactive protein in response to inflammatory stimuli.[4] Elevated serum CRP provides a sensitive biomarker of chronic systemic inflammation, an independent predictor of future cardiovascular events,[5] and elevations in CRP are associated with and also precede preeclampsia; however this has not been a consistent finding.[6–8]

SA plays a central role in the biomedical functioning of humans.[9] Similar to CRP, SA concentrations are elevated during inflammatory processes, likely resulting from elevated levels of richly sialylated acute-phase glycoproteins. Elevated levels of SA have recently been associated with an elevated risk of stroke and cardiovascular mortality.[10]

NEO is a specific and unique marker of innate inflammation because it is synthesized and released solely by activated macrophages and monocytes.[11,12] Measurements of plasma NEO concentrations have been used to evaluate progression of viral infections, renal transplant rejection, severe systemic inflammatory diseases, nephritic syndrome, and several autoimmune diseases.[11]

Earlier data have shown that inflammatory cytokines and cell surface markers are higher in women with preeclampsia compared to women with uncomplicated pregnancies. We hypothesize that plasma concentrations of NEO, SA, and CRP will be elevated in pregnant women and further elevated in women with preeclampsia. Moreover, plasma concentrations of NEO, SA, and CRP will be positively associated with each other, particularly in women with preeclampsia.

METHODS

Study population. This was a nest case–control study of 206 women enrolled between 1997 and 2002 in an ongoing investigation of preeclampsia at Magee-Women's Hospital (Pittsburgh, PA). The study was approved by the Institutional Review Board and informed consent was obtained from all subjects. All pregnant subjects were nulliparous healthy women without known preexisting medical complications. Exclusion criteria included multiple gestation, prior preeclampsia, illicit drug use, and preexisting medical conditions such as diabetes, chronic hypertension, and renal disease.

Twenty women were not pregnant, 40 women had an uncomplicated pregnancy, 47 women had preeclampsia, 50 women had transient hypertension of pregnancy without preeclampsia, and 49 women had SGA infants without preeclampsia. Preeclampsia was diagnosed by the presence of gestational hypertension, proteinuria, and hyperuricemia beginning after the 20th week of pregnancy with resolution of gestational hypertension and proteinuria postpartum. We included hyperuricemia in our classification of preeclampsia as it identifies a more homogeneous group of gestational hypertensive women with a greater frequency of adverse fetal outcomes, including preterm birth and SGA infants.[13] Gestational hypertension was defined as a new onset increase in blood pressure, including an absolute blood pressure ≥140 mm Hg systolic and/or ≥90 mm Hg diastolic after 20 weeks of gestation. Proteinuria was defined as ≥300 mg per 24-h urine collection, ≥2+ protein on voided urine sample, ≥1+ protein on catheterized urine specimen, or a protein–creatinine ratio of ≥0.3. Hyperuricemia was defined as plasma uric acid concentration ≥1 s.d. above reference values at the gestational age the sample was obtained (e.g., term, >5.5 mg/dl).[14] Diagnosis of preeclampsia was determined retrospectively based on medical chart review by a jury of research and clinical investigators.

Transient hypertension of pregnancy was defined as the presence of gestational hypertension without proteinuria or hyperuricemia. SGA infants were defined by infant birth weight ≤10th centile for gestational age, after adjusting for race and gender, in an otherwise uncomplicated pregnancy. SGA infants with clinical or pathological evidence of chronic intrauterine infection or chromosomal abnormalities were excluded from the study.

Blood samples. Maternal venous EDTA plasma samples were collected predelivery upon admission to labor and delivery, and EDTA plasma samples from nonpregnant women were collected during the luteal phase of the menstrual cycle. Plasma samples were aliquoted and stored at −70 °C for later analysis. Gestational age was determined by best obstetric estimate (first trimester ultrasound when available).

Quantification of NEO. Plasma NEO was measured in duplicate by high-performance liquid chromatography with fluorescent detection (Waters 717 plus, Milford, MA). The mobile phase was ammonium bicarbonate (10 mmol/l, pH 6.8) at a flow rate of 1 ml/min. 100 µl of plasma was mixed with 100 µl of 0.5 mol/l perchloric acid, vortexed, and centrifuged at 14,000*g* for 5 min. 100 µl of collected supernatant was mixed with 40 µl of 100 mmol/l ammonium bicarbonate buffer at 0.45 mmol/l. 20 µl of the prepared samples were injected into the equilibrated high-performance liquid chromatography system and fluorometric detection performed at an excitation wavelength of 353 nm and emission at 438 nm. The standard curve was linear from 1.25 to 40 nmol/l. A quality control plasma sample was included in each analysis, and the interassay variability was 6.5%.

Quantification of CRP. CRP was measured in duplicate by a high-sensitivity enzyme-linked immunosorbent assay (ELISA), as described previously.[15] The sensitivity of the assay was 0.2 ng/ml, and spike and recovery tests indicated 91–103% recovery. The intra-assay variability was 3.9% and interassay variability was 7.4%.

Quantification of SA. SA was determined in duplicate using a diagnostic kit (Roche, Indianapolis, IN). The standard curve was linear from 10 to 125 mg/dl, and the sensitivity of the assay was 5 mg/dl. The interassay variability was 9%.

Quantification of plasma creatinine. Creatinine was quantified as an indicator of renal function in the subject groups. Plasma creatinine was measured in duplicate using a modified high-performance liquid chromatography method based on that of Kock *et al.*[16] The interassay variability was <4%.

Cellular fibronectin, triglycerides, and uric acid. Cellular fibronectin (cFN) and triglycerides were quantified as indicators of endothelial dysfunction and dyslipidemia. Plasma cFN was measured in duplicate for each sample by an ELISA specific for the ED-A domain of human cFN. This ELISA technique is as described by Powers *et al.*[17] The sensitivity of the ELISA was 12.5 ng/ml and the interassay variability was 7%.

Plasma triglycerides were measured in duplicate using a diagnostic kit (Pointe Scientific, Lincoln Park, MI). The coefficient of variation between runs was 7%.

Plasma uric acid was measured in duplicate using a diagnostic kit supplied from Pointe Scientific. The assay procedure followed for uric acid was as described by the manufacturer, and the interassay variability was <6%.

Statistical analysis. Data are presented as mean ± s.d. or median and interquartile ranges, where appropriate. The plasma concentration of CRP was not normally distributed, therefore, values were transformed by square root, which normalized the

data distribution, and were used for the statistical analyses. The statistical package JMP 5.0.1a software (SAS Institute, Cary, NC) was used to analyze the data. Analysis of variance was performed with Fisher's protected least significant differences post hoc testing to adjust for multiple comparisons. Correlations were by standard regression analysis with Pearson's product moment (r) reported. Statistical significance was accepted at $P < 0.05$. Based on earlier published differences in CRP between uncomplicated pregnant women compared to women with preeclampsia, a sample size of 19 subjects per group would have 80% power to detect a twofold difference between these two groups with a α of 0.05.[18] The presence of labor at the time of sample collection (82 in labor and 104 not in labor at sample collection) among the pregnant subjects did not significantly influence any of the markers of inflammation in this study, NEO ($P = 0.33$), CRP ($P = 0.76$), and SA ($P = 0.43$).

RESULTS

The demographic and clinical characteristics of the study subjects are presented in Table 1. Maternal age was modestly but significantly higher in the pregnant women with preeclampsia compared to nonpregnant women, and there was no difference in age between the remaining groups of pregnant women. Maternal prepregnancy body mass index (BMI) was also greater in pregnant women with preeclampsia and those with transient hypertension compared to nonpregnant women. The average blood pressure before 20 weeks gestation was similar between all groups of pregnant women. As is typical, the average gestational age at delivery was significantly earlier in the women with preeclampsia compared to the other study groups, and infant birth weight and birth weight centile were lower in the preeclampsia and SGA groups compared to the infants of uncomplicated pregnant women. As expected, women with preeclampsia had higher plasma uric acid concentrations compared to all other groups. As reported earlier, the median concentrations of cFN and triglycerides, markers of endothelial dysfunction, and dyslipidemia, respectively, were also significantly elevated in all pregnant women compared to nonpregnant women (Table 1), and the concentration of both were further elevated in women with preeclampsia compared to the other pregnancy groups. In addition, plasma creatinine was elevated in nonpregnant women and women with preeclampsia compared to the remaining groups of pregnant women, and this difference is consistent with the known difference in glomerular filtration rate between preeclamptic women compared to other pregnant women.

Inflammatory markers are elevated in pregnancy and preeclampsia

The maternal plasma concentration of the three inflammatory markers, NEO, SA, and CRP, were significantly elevated in all groups of pregnant women compared to nonpregnant women (Table 1). In addition, NEO, the specific cellular inflammatory marker of activated monocytes and macrophages, was

Table 1 | Patient demographics, pregnancy information, and markers of inflammation

Variable	Nonpregnant ($n = 20$)	Uncomplicated pregnancy ($n = 40$)	Transient hypertension of pregnancy ($n = 50$)	Small for gestational age without preeclampsia ($n = 49$)	Preeclampsia ($n = 47$)
Age (years)[a]	22.2 ± 4.0	23.8 ± 5.8	24.7 ± 7.1	23.2 ± 5.0	27.1 ± 5.5**
Body mass index (kg/m^2)[a]	22.0 ± 2.8	26.1 ± 5.3	27.5 ± 6.4**	24.4 ± 5.4	26.6 ± 6.5**
Race (% African American)[a]	15	35	38	41	13
Gestational age at delivery (weeks)[a]	NA	38.9 ± 2.6	39.3 ± 1.1	39.4 ± 1.3	36.2 ± 2.8*
Average blood pressure before 20 weeks gestation (mm Hg)[a]	NA	112.3 ± 8.6/68.1 ± 5.8	114.8 ± 8.1/69.5 ± 5.9	110.3 ± 8.1/66.4 ± 5.3	115.2 ± 8.8/70.7 ± 7.6
Average blood pressure at delivery (mm Hg)[a]	NA	119.0 ± 8.5/70.8 ± 5.6	150.3 ± 10.8*/89.9 ± 7.5*	117.3 ± 8.6/69.2 ± 5.8	162.5 ± 13.0*/94.3 ± 10.5*
Infant birth weight (g)[a]	NA	3,267 ± 601	3,251 ± 509	2,452 ± 261*	2,446 ± 822*
Birth weight percentile (%)[a]	NA	55.2 ± 26.3	46.6 ± 31.3	4.1 ± 2.9*	31.6 ± 31.9*
Uric acid (mg/dl)[a]	4.7 ± 1.1	5.0 ± 1.1	5.3 ± 0.9	5.5 ± 1.2	7.4 ± 1.4*,**
Creatinine (mg/dl)[a]	0.84 ± 0.1*	0.58 ± 0.1	0.57 ± 0.1	0.56 ± 0.1	0.71 ± 0.2*
Triglycerides (mg/dl)[b]	62.9* (46.1–91.5)	139.8** (190.1–115.7)	164.3** (241.4–127.1)	168.4** (118.3–195.0)	215.1*,** (163.0–304.3)
cFN (μg/ml)[b]	6.1* (3.5–9.1)	17.0** (5.7–25.7)	22.4** (13.5–30.9)	23.9** (13.2–33.3)	35.9*,** (20.1–58.4)
Neopterin (nmol/l)[b]	10.9* (7.6–12.1)	16.7** (11.9–20.1)	17.2** (13.9–20.1)	16.4** (12.7–19.8)	20.2*,** (15.7–23.8)
Sialic acid (mg/dl)[b]	72.0* (62.7–78.8)	104.4** (91.2–106.9)	102.2** (96.7–108.9)	105.3** (91.8–113.6)	103.9** (91.3–111.0)
CRP (mg/dl)[b]	0.02* (0.01–0.25)	0.74** (0.22–1.94)	0.77** (0.29–1.65)	0.57** (0.19–1.49)	0.74** (0.33–1.73)

NA, not applicable.
Data are [a]mean ± s.d., or [b]median (interquartile range).
*$P < 0.05$ vs. uncomplicated pregnant; **$P < 0.05$ vs. nonpregnant.

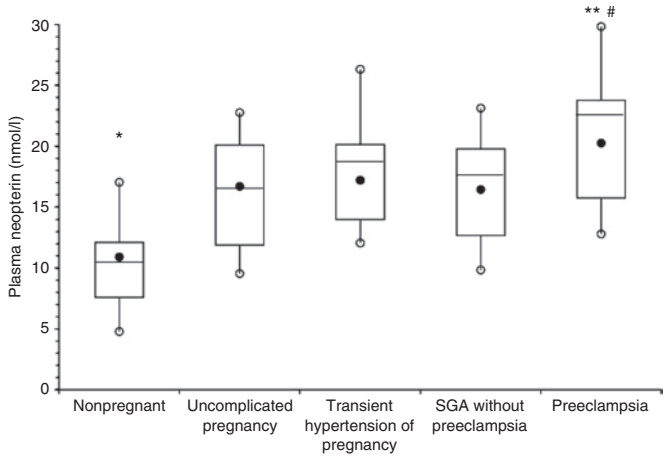

Figure 1 | Maternal plasma neopterin is elevated in pregnancy and further elevated in preeclampsia. Box and whisker plots of maternal plasma neopterin; the filled black circles are the median, the horizontal lines within the box are the mean, the open circles are the 90th and 10th percentiles, and the top and bottom lines of the box are the 75th and 25th percentiles of the data for each group. $^{\#}P < 0.005$ vs. nonpregnant; $^{**}P < 0.005$ vs. uncomplicated pregnancy, and $^{*}P < 0.01$ vs. uncomplicated pregnancy.

further elevated among women with preeclampsia ($P < 0.01$) compared to other three pregnancy groups (Figure 1).

We examined the relationship between the inflammatory markers NEO, SA, and CRP. As expected, there was a significant positive association between the acute-phase inflammatory markers CRP and SA in all groups investigated in this study except the women with transient hypertension of pregnancy (Table 2). There was a significant positive association between the cellular inflammatory marker NEO and the acute-phase inflammatory marker CRP in women with transient hypertension of pregnancy and the women with preeclampsia (Table 2). In contrast, there was no association between the cellular inflammatory marker NEO and SA among any of the individual groups investigated.

Because NEO was the only inflammatory marker that was both elevated in pregnancy and further elevated in preeclampsia, we further investigated the relationship between NEO and other biological markers. Despite following a similar pattern of being elevated in pregnancy and further elevated in preeclampsia, there was no relationship between NEO and cFN ($r = 0.04$, $P = 0.77$), triglycerides ($r = 0.2$, $P = 0.18$), or uric acid ($r = 0.0006$, $P = 0.96$) among the women with preeclampsia. Similarly, there was no association between NEO and maternal age ($r = 0.02$, $P = 0.86$), BMI ($r = 0.02$, $P = 0.85$), blood pressure ($r = 0.09$, $P = 0.54$), gestational age ($r = 0.14$, $P = 0.36$), infant birth weight ($r = 0.10$, $P = 0.47$), or birth weight centile ($r = 0.02$, $P = 0.88$) among the women with preeclampsia. Interestingly, there was a significant association between NEO and plasma creatinine ($r = 0.45$, $P = 0.0015$) among the women with preeclampsia, and this relationship was also present among the other groups of pregnant women ($r = 0.17$, $P = 0.05$); however, there was no relationship between NEO and plasma creatinine among the nonpregnant women ($r = 0.09$, $P = 0.69$).

Table 2 | Correlations between inflammatory markers

Group	CRP vs. sialic acid	CRP vs. NEO	Sialic acid vs. NEO
All	$r = 0.47$, $P < 0.0001$	$r = 0.33$, $P < 0.0001$	$r = 0.25$, $P < 0.001$
Nonpregnant women	$r = 0.65$, $P = 0.002$	$r = 0.29$, $P = 0.21$	$r = 0.27$, $P = 0.24$
Uncomplicated pregnancy	$r = 0.47$, $P = 0.02$	$r = 0.22$, $P = 0.18$	$r = 0.07$, $P = 0.75$
Transient hypertension of pregnancy	$r = 0.03$, $P = 0.84$	$r = 0.29$, $P = 0.041$	$r = 0.05$, $P = 0.72$
SGA infants without preeclampsia	$r = 0.50$, $P < 0.001$	$r = 0.16$, $P = 0.29$	$r = 0.28$, $P = 0.053$
Preeclampsia	$r = 0.40$, $P < 0.02$	$r = 0.56$, $P < 0.001$	$r = 0.16$, $P = 0.28$

CRP, C-reactive protein; NEO, neopterin; SGA, small for gestational age.

DISCUSSION

The objective of this study was to investigate differences in three inflammatory markers in pregnant compared to nonpregnant women, and to further investigate differences in these markers in the pregnancy complication including preeclampsia. Our primary results from this study were that the cellular inflammatory marker of macrophage and monocyte activation, NEO, as well as the acute-phase inflammatory markers CRP and SA, are all significantly elevated in pregnant compared to nonpregnant women. In addition, NEO was the only inflammatory marker that was further elevated in pregnant women with preeclampsia compared to the other pregnancy groups.

Healthy human pregnancy requires adaptation of the maternal immune system in order to preserve maternal immune competence while also maintaining tolerance of the semiallogeneic fetus. Shifts in both the adaptive and innate immune systems contribute to this process. Alterations in innate immunity include both cellular and soluble components, reflected by elevated acute-phase proteins, increased numbers of monocytes and granulocytes,[19] and a primary role for uterine natural killer cells in both the local placental environment and in the broader maternal system.[20] Preeclampsia has long been hypothesized to have an immunological basis, with excessive immune activation contributing to the endothelial cell dysfunction evident in preeclampsia.[19]

Numerous studies have reported finding elevated CRP among women with preeclampsia as well as prior to evident clinical symptoms and as much as 30 years postpartum.[6,8,15,21] In many studies, the association between elevated CRP and preeclampsia was significantly attenuated or lost after adjusting for BMI.[18] On the other hand, studies that have matched for BMI have been inconsistent with regard to CRP and the risk of preeclampsia.[6] Importantly, elevated CRP (≥4.9 mg/l) in the first trimester has been associated with a 2.5-fold increased risk of preeclampsia among lean women, and this relationship was absent among overweight and obese women.[22] As evidenced in our own data, there is a profound and significant increase in the circulating concentration of CRP among all pregnant

women compared to nonpregnant women. Furthermore, it is possible that this pregnancy-mediated elevation in CRP may mask potential differences in CRP concentration between different pregnancy outcomes. In addition, we did not observe a relationship between CRP and BMI as has been reported in other studies. However, this lack of an association may be the result of the timing of sample collection (predelivery third trimester) compared to other studies (first or second trimester).

Similar to CRP, SA is an acute-phase inflammatory marker, and has been shown to be a strong predictor of coronary heart disease and cardiovascular mortality.[23] Similar to CRP, we have observed that SA is significantly elevated during pregnancy, and that SA is significantly correlated with CRP. However, no differences in SA concentrations were observed between preeclamptic patients compared to women with uncomplicated pregnancies, transient hypertension or women with SGA infants. Reports regarding SA concentrations during pregnancy have been inconsistent. Sydow *et al*. reported that serum SA was not significantly elevated in pregnancy,[24] whereas Alvi *et al*.[25] did report a significant elevation during pregnancy that was in keeping with data from Goni *et al*.[26] Reasons for these discrepancies may be varying populations of women studied and assay differences. In addition, heterogeneity among study populations in developed and developing countries, and differences in levels of chronic subclinical infection may also contribute to differences in inflammatory markers between studies.[6,8,27]

Similar to CRP and SA, NEO is also a marker of inflammation. However, NEO is a unique inflammatory marker because it is synthesized solely by activated macrophages and monocytes.[28] There has been limited investigation of NEO in pregnancy and preeclampsia. Bichler *et al*. were the first to report elevated urinary NEO concentrations in human pregnancy, and Fuith *et al*. were the first to report elevated serum NEO concentrations in pregnancy.[29,30] Similar to our own study, Haeger *et al*. and Kaleli *et al*. reported that plasma NEO concentrations were elevated in women with preeclampsia compared to normal pregnant women.[31,32] In two different studies, Schröcksnadel *et al*. reported that NEO levels are elevated in hypertensive pregnant women.[33,34] More recent data showed that there were no significant differences in NEO concentrations between normal pregnant women and women with preeclampsia between 18 and 38 weeks of pregnancy.[35] However, most of the preeclamptic patients in this study were diagnosed with mild preeclampsia, and smoking, which may increase NEO levels,[36] was not an exclusion criterion in the study. The results of our study confirm the findings of elevated NEO levels during pregnancy and the further elevation in preeclampsia, and that the elevation in NEO in preeclampsia is likely not a consequence of gestational hypertension or SGA, because NEO was not further elevated in these pregnancy groups. Interestingly, NEO was significantly correlated with CRP in patients with transient hypertension of pregnancy and preeclampsia. This finding suggests a possible convergence of soluble and cell-mediated inflammatory factors in these complications near the end of pregnancy. In contrast, despite a similar pattern of being elevated in pregnancy and further elevated in preeclampsia there was no correlation between NEO and uric acid, the endothelial marker cFN or plasma triglycerides, suggesting no direct relationship between NEO and endothelial dysfunction or dyslipidemia observed in preeclampsia. Conversely, NEO was significantly correlated with plasma creatinine concentrations in all pregnant subjects, including patients with preeclampsia. Renal clearance is a significant mechanism by which NEO is eliminated. The mean renal clearance of NEO is 216 ml/min or 1.8 times greater than the glomerular filtration rate of 120 ml/min in nonpregnant subjects.[37] Therefore, it is reasonable that NEO should be correlated with plasma creatinine. However, we did not observe a correlation between NEO and creatinine in nonpregnant subjects. One possible explanation for these findings may be that NEO is elevated in pregnancy and further preeclampsia as a result of innate immune system activation, and that differences in renal function may further accentuate these differences.

Limitations of this study include the analysis of inflammatory and biological markers in late pregnancy at the time of clinically recognizable preeclampsia and the differences in gestational age. However, in addition, there are several strengths of this study that are: the inclusion of a nonpregnant comparison group, other pregnancy complication groups plus transient hypertension of pregnancy, a relatively large sample size compared to previous studies, and the quantification of several inflammatory markers as well as other relevant biomarkers.

In conclusion, we have observed that the inflammatory markers NEO, SA, and CRP are significantly elevated during pregnancy. In addition, NEO, a marker of activated monocytes and macrophages, is further elevated among women with preeclampsia. These data strengthen the hypothesis that pregnancy is marked by a significant activation of the innate immune system, and that this activation is further exaggerated in the pregnancy complication preeclampsia.

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