Original Article

Ultra-high sensitive C-reactive protein during normal pregnancy and in preeclampsia: a pilot study

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Introduction: Angiogenic and inflammatory factors have been shown to play an important role in the pathogenesis of preeclampsia. However, there is little information on their interaction. The aims of this study were to investigate the longitudinal pattern of inflammatory markers, such as interleukin-6 (IL-6) and C-reactive protein (CRP) using a novel ultra-high sensitive assay method (uhsCRP), and to explore their relationship with angiogenic factors such as placental growth factor (PLGF), soluble fms-like tyrosine kinase-1 (sFlt-1), and vascular endothelial growth factor (VEGF) in normal pregnancies and pregnancies complicated by preeclampsia.

Materials and methods: Serum levels of uhsCRP, IL-6, PLGF, VEGF and s-Flt-1 were longitudinally determined in 16 women with normal, singleton healthy pregnancies at 7–13, 17–22, 27–31 and 37–41 weeks of gestation by ELISA. uhsCRP was measured using a ultra-high sensitivity ELISA test. Serum of women with preeclampsia ($n = 15$) was available only once, usually in the third trimester of pregnancy. Women with premature rupture of membranes (PROM) or infection such as chorioamnionitis were excluded. Spearman rank correlation, logistic regression, ROC analysis, ANOVA and Mann–Whitney *U*-test were used for statistical purposes.

Results: In normal pregnancies, serum uhsCRP showed a gestational age-dependent increase ($r = 0.40$; $P < 0.001$). In women suffering from preeclampsia, uhsCRP levels were higher than in gestational age-matched controls (18010 ± 4763 versus 3026 ± 587 ng/ml; $P < 0.001$). Similarly, serum IL-6 levels increased throughout pregnancy and correlated with uhsCRP in normal pregnancies and in preeclampsia ($n = 64$, $r = 0.37$; $P < 0.01$ and $n = 15$, $r = 1.00$, $P < 0.0001$). uhsCRP levels were positively correlated with sFlt-1 levels ($n = 64$, $r = 0.34$; $P < 0.01$).

Conclusion: The increases in uhsCRP (and IL-6) serum levels with advancing gestation indicate a shift towards an inflammatory state during normal pregnancy. The excessive rise in uhsCRP and sFlt-1 in preeclampsia indicate that both may be involved in its pathogenesis. uhsCRP may be useful as an early marker for preeclampsia and studies defining the pattern of its rise throughout pregnancies at risk are urgently needed.

Keywords: angiogenic factors, inflammation, preeclampsia, pregnancy, ultra-high-sensitivity , C-reactive protein

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; CRP, C-reactive protein; CVD, cardiovascular diseases; hsCRP, high-sensitivity CRP; IL-6, interleukin-6; IL-8, interleukin-8; NS, not significant; PLGF, placental growth factor; PROM, premature rupture of membranes; ROC analysis, receiver-operating characteristic curve analysis; SD, standard deviation; sFlt-1, soluble fms-like tyrosine kinase-1; SGA, small for gestational age; TMB, 3,3′,5,5′-Tetramethylbenzidine; TNF-α, tumour necrosis factor-alpha; uhsCRP, ultra-high sensitive C-reactive protein; VEGF, vascular endothelial growth factor

INTRODUCTION

Preeclampsia, a pregnancy-specific disease characterized by hypertension and other signs of systemic endothelial dysfunction, contributes substantially to perinatal maternal and neonatal morbidity. Moreover, following preeclampsia, both the mother and her child have a higher risk to suffer from cardiovascular diseases (CVD) later in life [1–5]. Normal pregnancy shows features of a mild chronic inflammation [6]. Women with cardiovascular risk factors are predisposed to pregnancy-associated hypertensive disorders including pre-eclampsia [7,8]. Risk factors for atherosclerosis (such as hypertension, dyslipidaemia, overweight, familiar disposition and diabetes) are also strong predictors for preeclampsia attesting to a similar pathogenesis [9] and oxidative stress [10]. Higher soluble fms-like tyrosine kinase-1 (sFlt-1) levels and sFlt-1/placental growth factor (PLGF) ratios characteristically found in women with preeclampsia were shown to be also positively associated with signs of arterial aging during pregnancy and 1-year postpartum [11]. Moreover, endothelial cells play a pivotal role in the pathogenesis of these two disorders [12,13]. Indeed, ischemia and oxidative stress has been

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proposed to cause the endothelial injury observed in preeclampsia [14,15] as well as in atherosclerosis [16,17]. Like other components of the immune system, C-reactive protein (CRP) might have – in addition to protective – also potentially harmful effects. A growing body of evidence suggests that CRP might be implicated in the pathogenesis of atherosclerosis [13,16,17] and abdominal aortic aneurysms [18] as well as in the tissue damage occurring in acute myocardial infarction [19–23]. Increased CRP serum levels have been identified as an independent risk indicator for cardiovascular events [24].

Serum CRP levels have been shown to be higher in pregnant than in nonpregnant women [25]. Compared with normal pregnancies, the serum levels of CRP and of factors of the innate immune system were even higher in preeclampsia [26]. However, the information on the usefulness of CRP as a potential marker for preeclampsia is contradictory [25,27–34]. Wolf *et al*. [30] and Tjoa *et al*. [31] have described elevated CRP levels during the first trimester in women who subsequently develop preeclampsia, intrauterine growth restriction or gestational diabetes mellitus, whereas others did not detect such an association [32,33]. Mechanisms involving angiogenic factors and pro-inflammatory cytokines are known to play a key role in the process of placentation and the development of preeclampsia [35]. Interleukin-6 (IL-6) is one of the strongest inducers of hepatic CRP production [35,36]. Altered expression patterns of these factors have been shown to be associated with adverse pregnancy outcome [37,38]. Our group and others have shown that elevated serum levels of sFlt-1 and the soluble form of endoglin herald preeclampsia [34,39,40]. However, the interplay and causal relationship between these factors and CRP has not been examined in detail. CRP is usually measured using a nephelometric method, with a detection limit of approximately 2 mg/l. With the advent of more sensitive assays, such as laser nephelometry, it was possible to measure CRP levels at a lower level, but still above 0.15 mg/l. To distinguish between these two detection methods, the latter was termed 'high sensitive' CRP (hsCRP). In contrast to the 'classical' CRP, which is more a clinical parameter to evaluate the degree of inflammation because of various stimuli, hsCRP has become a promising marker for the evaluation of the cardiovascular risk. Our group has established a novel CRP detection assay based on an immunoanalytical technique [41], which allows a substantial increase in sensitivity, usually bellow 5 pg/ml. To differentiate our ELISA-based test from the nephelometric ones, we have decided to name it ultra-high sensitive CRP (uhsCRP, Table 1).

The aim of this pilot study was to longitudinally investigate the serum levels of uhsCRP, IL-6, PLGF, vascular endothelial growth factor (VEGF) and sFlt-1 in normal pregnancies and, in addition, to compare these data with those obtained from pregnant women affected by preeclampsia.

PATIENTS, MATERIALS AND METHODS

Sixteen healthy, nonsmoking women with singleton pregnancies were prospectively enrolled upon their first presentation in our service at 7–13 weeks of gestation. Blood was drawn longitudinally between 7–13, 17–22, 27–31 and 37–41 weeks of gestation. The serum was stored at −80°C after coagulation and centrifugation. None of these women had premature rupture of membranes or an infectious condition, such as chorioamnionitis. In addition, 15 women who were diagnosed with preeclampsia underwent an antenatal blood collection. Preeclampsia was defined as hypertension (DBP $\geq$90 mmHg and/or a systolic pressure $\geq$140 mmHg) occurring with proteinuria of at least 300 mg per 24 h.

CRP was assayed with a self-established, ultra-highly sensitivity enzyme immunoassay method developed in our laboratory published elsewhere [41]. Briefly, 96-well Nunc Maxisorp microtitre plates were coated overnight with rabbit anti-human CRP [Sigma C3527, 2 mg/ml (Sigma, Dorset, UK) in PBS, 100 μl/well] and the excess sites blocked with BSA (0.5% w/v in PBS, 250 μl/well). Standards (CRP, Sigma C4064, at 25–0.39 ng/ml in 1:2 dilution steps) or samples were added (100 μl/well) in PBS containing 0.5% nonfat milk proteins [Blotto, Pierce Protein Research Products (ThermoFisher, Rockford, Illinois, USA)], followed by an incubation for 2 h at room temperature and with a shaking speed of 500 rpm. The detection antibody was rabbit anti-human CRP conjugated with horseradish peroxidase (HRP, Dako P0227, 1:10 000 in Blotto) in a 1-h incubation (other parameters unchanged). Washing between all incubations was with PBS containing Tween-20 (Sigma, 0.1% v/v, PBST) throughout the procedure. Ready to use 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (100 ml, Zymed Inc., San Francisco, California, USA) was added in a timed sequence. After 10–20 min of incubation at room temperature in the dark, the reaction was stopped by the addition of 100 μl of 2 mol/l hydrochloric acid and the optical density was determined with a dual channel microplate reader at 450 nm against a reference at 590 nm. At midstandard concentration, intra-assay and inter-assay coefficients of variance were 3.1 and 5.5%,

TABLE 1. Characterization of different CRP assays

	'Classical' CRP	High sensitive CRP (hsCRP)	Ultra-high sensitive CRP (uhsCRP)
Method	Nephelometry	Laser nephelometry	ELISA
Area of application	Clinical (infectious and inflammatory diseases)	Screening (marker for cardiovascular risk evaluation)	Experimental (potential marker for the prediction of preeclampsia)
Commercially available	Yes	Yes	No
Sensitivity	> 2 mg/l	0.15–10 mg/l	<5 pg/ml
Material	Plasma, ascites, pleural effusion, spinal fluid, and so forth	Plasma	Plasma

CRP, C-reactive protein.

TABLE 2. Clinical characteristics

Characteristics	Preeclampsia ($n = 15$)	Controls ($n = 16$)	Significance, *P* value
Age (years)	30.1 ± 0.52	30.5 ± 0.34	<0.05
Gestational age at delivery (weeks)	32 ± 0.68	39.9 ± 0.85	<0.0001
Nulliparous	10 (66.7%)	8 (50%)	NS
Preconceptional BMI (kg/m^2)	26.2 ± 0.81	26 ± 0.78	NS
Cesarean delivery (n, %)	12 (80%)	4 (25%)	<0.01
SBP at inclusion (mmHg)	145.3 ± 12.6	117 ± 0.4	<0.0001
DBP at inclusion (mmHg)	94.5 ± 11.7	74.3 ± 8.7	<0.0001
Birth weight (g)	1780 ± 975	3480 ± 419	<.0001
SGA	9 (60%)	2 (12.5%)	<0.01

Data are presented as mean ± SD or *n* (%). NS, not significant; SGA, small for gestational age (birth weight <10th centile).

respectively. The functional sensitivity of this assay was below 5 pg/ml.

IL-6 was determined with a commercially available ELISA (Quantikine, R&D Systems, Oxford, England). The functional sensitivity was 0.2 pg/ml and the serum was assayed without prior dilution. VEGF was similarly measured with a Quantikine ELISA kit from R&D Systems and following the manufacturers' instructions for serum without modification. sFlt-1 and PLGF was determined with a microplate ELISA set up in our laboratory with antibodies obtained from R&D Systems and as previously described [42].

Data analysis and statistical testing was performed with GraphPad Prism version 5.0 (GraphPad Software, La Jolla, California, USA). All data are shown as mean ± SD. Continuous variables were compared using the student *t*-test or the Mann–Whitney *U*-test, whereas for the longitudinal data, ANOVA was used. D'Agostino test was used to check for normal distribution. Correlations were searched using Spearman rank test. Statistical significance was defined by a *P* value less than 0.05. The study has been approved by the local ethical committee.

RESULTS

The clinical characteristics of the study population are presented in Table 2. Serum uhsCRP levels were found to increase with gestational age in normal, healthy pregnancies ($r = 0.40$; $P < 0.001$, Fig. 1; Spearman rank correlation). When women were stratified in groups ranging from 7 to 13, 17 to 22, 27 to 31 and 37 to 41 weeks of gestation, uhsCRP serum levels increased from 2647 ± 661 to 15.037 ± 4877 ng/ml at term gestation ($P < 0.05$, Student's *t*-test). Mean uhsCRP concentrations of women with preeclampsia ($n = 15$) at the time of delivery were higher when compared with controls ($n = 16$) at term (17960 ± 4591 versus 15037 ± 4877 ng/ml; $P = 0.67$). This finding was further enhanced as gestational age at delivery was significantly lower in women with preeclampsia (32 ± 0.68 weeks, mean ± SD) than in the controls (39.9 ± 0.85 weeks; $P < 0.001$). Gestational age-matched pairs (±1 week of gestation) of women with preeclampsia and normal pregnancy (controls) confirmed the higher uhsCRP levels in women with preeclampsia (preeclampsia versus controls; $n = 7$ for both groups; 16960 ± 5161 versus 3416 ± 1268 ng/ml, respectively; $P = 0.026$, Fig. 2, panel a). Receiver-operating characteristic curves (ROC) analysis was performed to assess the test performance for uhsCRP to diagnose preeclampsia. ROC analysis yielded an AUC of 0. 88 [95% confidence interval (CI) 0.69 to 1; $P = 0.02$; Fig. 2, panel b].

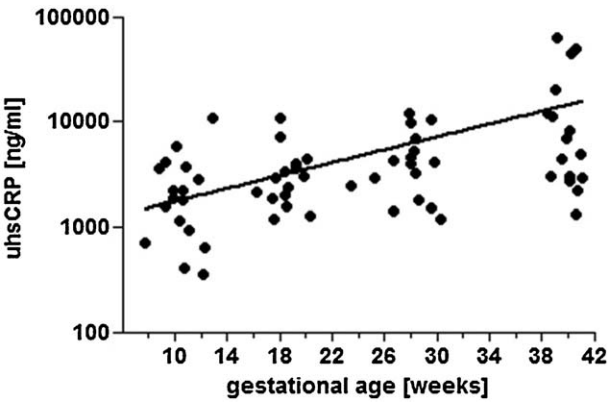

FIGURE 1 Serum levels of ultra-high sensitive assay method correlate with gestational age during normal healthy pregnancies (Spearman rank correlation $r = 0.35$; $P = 0.005$).

Protein expression of IL-6, a known potent stimulus for CRP production in various tissues, was analysed in our samples to investigate a possible impact on uhsCRP protein expression. IL-6 could be detected in the first trimester only in four samples [4/16 (25%)] whereas the detection rate increased to 31.3, 50 and 100% in the second, and third trimester, and at term gestation, respectively, at a threshold of 0.2 pg/ml. A positive correlation was found between the serum concentrations of IL-6 and CRP in healthy pregnancies ($r = 0.37$; $P < 0.01$, Spearman rank correlation). This correlation was even more pronounced in women with preeclampsia ($r = 0.99$, $P < 0.001$, Spearman rank correlation).

The serum concentrations of the soluble Flt-1 increased with advancing gestation in normal pregnancies ($r = 0.56$; $P < 0.001$, Spearman rank correlation) and was positively correlated with uhsCRP ($r = 0.34$; $P < 0.01$, Spearman rank correlation, Fig. 3). ROC analysis were calculated to assess test performance regarding the diagnosis of preeclampsia and yielded an AUC of 0.92 (95% CI 0.87–0.98; $P < 0.0001$), and 0.94 (95% CI 0.87–1; $P < 0.0001$) for sFlt-1, and the sFlt-1/PLGF-ratio, respectively. As uhsCRP correlates with sFlt-1, the combination of uhsCRP and sFlt-1 (AUC: 0.92, 95% CI 0.85–0.98) did not substantially change the test performance when compared with sFlt-1 alone. No correlation was found between uhsCRP and both PLGF as well as VEGF (PLGF: $r = 0.22$; $P = 0.08$; VEGF: $r = -0.09$; $P = 0.46$, respectively).

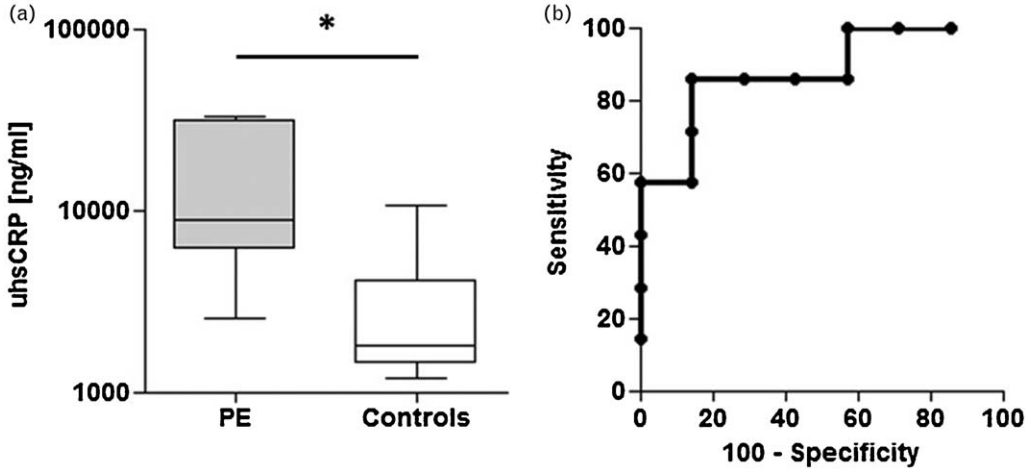

FIGURE 2 (a) Ultra-high sensitive C-reactive protein serum levels were higher in preeclampsia than in gestational age-matched controls ($n = 7$ for both groups; 16960 ± 5161 versus 3416 ± 1268 ng/ml, respectively; $^*P = 0.027$, Student's *t*-test; presented as box and whisker plots. (b) Receiver operating characteristic curve analysis for the diagnosis of preeclampsia yielded an area under the curve of 0.88 (95% confidence interval 0.69–1; $P = 0.02$).

DISCUSSION

Our data showed that in normal pregnancy, the inflammatory marker uhsCRP increased with advancing gestation, which went in parallel with an increase in IL-6 serum levels. Similarly, in our healthy population, sFlt-1 serum levels increased with gestational age. sFlt-1 is a marker of endothelial activation [43] and enables assessing cardiovascular risk [44]. In our healthy pregnant population, both sFlt-1 and uhsCRP rose throughout the whole pregnancy, which underline the inflammatory features of pregnancy *per se* and the concept that pregnancy is a state of low-grade chronic inflammation [6].

CRP is a major acute-phase plasma protein displaying a rapid and pronounced rise in its serum concentration in response to infection or tissue injury. A growing body of experimental and clinical evidence suggests that the production of CRP is not restricted to the liver, but that also cortical tubules as well as glomerular cells of the kidney are able to produce CRP mainly in nephrectomized renal allografts with severe acute rejection [45]. It is of particular interest that Ghezzi *et al.* [46] were able to show that CRP is also present in the amniotic fluid at the time when amniocentesis for genetic analysis is performed, and elevated levels have been associated with adverse pregnancy outcome, such as preterm delivery as well as preeclampsia. Higher amniotic fluid CRP concentrations have also been found in cases with preterm premature rupture of membranes and intra-amniotic infection [47], underscoring its role as an acute phase reactant against micro-organisms also within the intra-amniotic cavity. Our group showed previously that fetal urine contains CRP, which may be an important source for the amniotic fluid CRP [48]. Indeed, because of its molecular mass and also absent placental Fc-receptors, maternal CRP is not able to cross the placental barrier [41,46]. Other epithelial organs, for example, human thymic epithelial cells and epithelial cells of the respiratory tract, are also capable to produce CRP [49,50]. Even neuronal cells seem to synthesize acute phase reactants involved in the pathogenesis of neurodegenerative diseases [51]. It is, therefore, possible that the acute phase response not merely represents a systemic inflammatory pathway, but is probably also part of the local inflammatory response. Our group has demonstrated that the placenta is another important site of CRP production [41]. The majority of the synthesized CRP is released by the placenta into the maternal circulation. This may explain the increasing CRP concentration found in the longitudinal assessment of our population of healthy pregnancies.

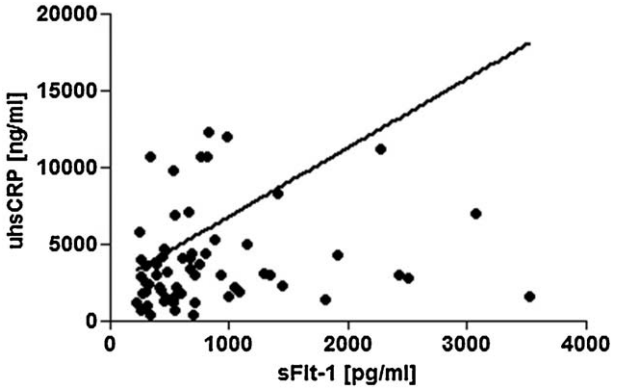

FIGURE 3 In normal pregnancy ultra-high sensitive C-reactive protein serum concentrations correlate with soluble fms-like tyrosine kinase-1 serum levels ($n = 64$, $r = 0.34$; $P < 0.01$, Spearman rank correlation).

Endothelial activation is an integral component of the inflammatory response. In relation to local injury, activated endothelium targets inflammatory leukocytes by anchoring them to the vessel wall where they are stimulated to transmigrate into extravascular tissues. The relationship is by two ways. Activated leukocytes, especially granulocytes, can stimulate the endothelium [37]. Redman *et al.* [52] and Sacks *et al.* [53] were able to demonstrate that in response to preeclampsia, a generalized systemic maternal inflammatory response that not only affects circulating leukocytes but also monocytes and lymphocytes, is present. Moreover, some investigators found significantly increased circulating levels of pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6 and IL-8 [35,36]. The observation that this inflammatory state is already present to a milder extent in normal pregnancies

led to the hypothesis that preeclampsia develops when the systemic inflammatory process, common to all women in the second half of gestation as observed in our study, causes maternal regulatory mechanisms to decompensate [52]. The same pro-inflammatory cytokines are strong stimulators of the hepatic (and other) CRP production and, together with the CRP secreted by the placenta, may explain the gestational age-dependent increase of CRP in healthy pregnancies. Similarly, the higher CRP values found in our preeclampsia group may reflect the increased inflammatory burst observed in these women.

There seems to be a complex and still poorly understood action and interaction between inflammatory and angiogenic mechanisms during normal pregnancy. This system is altered in women who develop preeclampsia, and in addition to the angiogenic parameters, it seems that CRP might play an interesting role in this context. However, CRP correlated only with sFlt-1, which is also associated with the maternal inflammatory state during pregnancy [6] and in particular during preeclampsia [26].

It is noteworthy that hsCRP is one of the most frequently used markers for cardiovascular risk assessment. HsCRP is a key laboratory tool in the prognosis and prediction of cardiovascular disease [54,55]. It is of particular interest that women after preeclampsia are at increased risk for cardiovascular diseases later in life [3,56,57] and higher CRP levels are found even several decades following eclamptic pregnancy [58]. This finding might – at least partly – explain the increased risk for CVD in women with a history of hypertensive pregnancy disorders [1–5]. Moreover, hsCRP has also been associated with early pregnancy loss and low-dose aspirin administered to women with increased inflammation defined by higher hsCRP levels, lowered hsCRP concentrations throughout pregnancy and increased live birth rates [59].

Our method presented here is the determination of uhsCRP using a totally different approach (ELISA) when compared with CRP, which is based on the nephelometric detection of agglutinated CRP and hence requiring a minimum concentration of the analyte, which limits the sensitivity of the assay. Laser nephelometry is used for the measurement of hsCRP, which increases the sensitivity by a factor of around 10 but did not change the principle of the assay. Our uhsCRP protocol is based on an antigen–antibody interaction, which, by nature, is much stronger, and thus increases the overall sensitivity of the assay. The limit of detection of hsCRP is commonly within 0.15–10 mg/l. Our ELISA-based immunoassay method is able to detect uhsCRP at a significantly lower concentration than the commercially available hsCRP tests (Table 1). Indeed, we are dealing with CRP values in the range of picogram per millilitres. This, together with the different specificities of the polyclonal antibodies used in our assay, may also explain the discordant reports about the utility of CRP assessment during pregnancy reported in the literature to date [25,27–34].

In conclusion, our small pilot study has shown that the high detection rate of the uhsCRP test allows assessing the degree of the physiologic maternal systemic inflammatory response to pregnancy. Our data demonstrate an association between the innate immune system and markers of inflammation in normal pregnancies and, in particular, in those complicated by preeclampsia. Our findings of substantially increased pro-inflammatory and anti-angiogenic markers in preeclampsia support the hypothesis that CRP and sFlt-1 play a central role in the pathogenesis of preeclampsia. Hopefully these biochemical markers may be used as predictive tools and targets for therapies, which will attenuate endothelial cell dysfunction in preeclampsia and other endothelium-related diseases. Future studies will elucidate whether maternal serum uhsCRP measurements using immunoanalytical methods with functional sensitivities within nanogram to picogram ranges, as the one presented in this study, may be of value in early pregnancy to identify a high-risk population for subsequent hypertensive complications and to detect those women that are at increased risk for cardiovascular complications later in life. Larger prospective studies are eagerly needed to confirm our preliminary results.

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Conflicts of interest

There are no conflicts of interest.

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