



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

# The elevation in circulating anti-angiogenic factors is independent of markers of neutrophil activation in preeclampsia

### Citation for published version:

Ramma, W, Buhimschi, IA, Zhao, G, Dulay, AT, Nayeri, UA, Buhimschi, CS & Ahmed, A 2012, 'The elevation in circulating anti-angiogenic factors is independent of markers of neutrophil activation in preeclampsia', *Angiogenesis*. <https://doi.org/10.1007/s10456-012-9261-5>

### Digital Object Identifier (DOI):

[10.1007/s10456-012-9261-5](https://doi.org/10.1007/s10456-012-9261-5)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Publisher's PDF, also known as Version of record

### Published In:

Angiogenesis

### General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



# The elevation in circulating anti-angiogenic factors is independent of markers of neutrophil activation in preeclampsia

Wenda Ramma · Irina A. Buhimschi · Guomao Zhao · Antonette T. Dulay ·  
Unzila Ali Nayeri · Catalin S. Buhimschi · Asif Ahmed

Received: 29 December 2011 / Accepted: 18 February 2012 / Published online: 8 March 2012  
© The Author(s) 2012. This article is published with open access at Springerlink.com

## Abstract

**Background** Severe preeclampsia is associated with increased neutrophil activation and elevated serum soluble endoglin (sEng) and soluble Flt-1 (sFlt-1) in the maternal circulation. To dissect the contribution of systemic inflammation and anti-angiogenic factors in preeclampsia, we investigated the relationships between the circulating markers of neutrophil activation and anti-angiogenic factors in severe preeclampsia or systemic inflammatory state during pregnancy.

**Methods and results** Serum sEng, sFlt-1, placenta growth factor, interleukin-6 (IL-6), calprotectin, and plasma  $\alpha$ -defensins concentrations were measured by ELISA in 88 women of similar gestational age stratified as: severe preeclampsia (sPE,  $n = 45$ ), maternal systemic inflammatory response (SIR,  $n = 16$ ) secondary to chorioamnionitis, pyelonephritis or appendicitis; and normotensive controls (CRL,  $n = 27$ ). Neutrophil activation occurred in sPE and SIR, as  $\alpha$ -defensins and calprotectin concentrations were two-fold higher in both groups compared to CRL ( $P < 0.05$  for each). IL-6 concentrations were highest in

SIR ( $P < 0.001$ ), but were higher in sPE than in CRL ( $P < 0.01$ ). sFlt-1 ( $P < 0.001$ ) and sEng ( $P < 0.001$ ) were  $\approx 20$ -fold higher in sPE compared to CRL, but were not elevated in SIR. In women with sPE, anti-angiogenic factors were not correlated with markers of neutrophil activation ( $\alpha$ -defensins, calprotectin) or inflammation (IL-6).

**Conclusions** Increased systemic inflammation in sPE and SIR does not correlate with increased anti-angiogenic factors, which were specifically elevated in sPE indicating that excessive systemic inflammation is unlikely to be the main contributor to severe preeclampsia.

**Keywords** Soluble endoglin · Soluble Flt-1 · Placenta growth factor · Preeclampsia · Neutrophil · Inflammation

## Introduction

Preeclampsia is a pregnancy-specific hypertensive disorder that affects 3–5% of all pregnant women [1]. The exact etiology is unknown, but angiogenic imbalance and systemic inflammation have emerged as leading causes for the clinical signs of the disorder [2–4].

The angiogenic imbalance hypothesis proposed that preeclampsia arises due to loss of vascular endothelial growth factor (VEGF) activity as a result of rise in soluble Flt-1 (sFlt-1) [5]. The loss of VEGF activity due to increased soluble Flt-1 (sFlt-1) has since been demonstrated in preeclamptic women [6–11]. In addition, preeclamptic women have less TGF- $\beta$  activity due to a rise in soluble endoglin (sEng) [12, 13]. Importantly, these changes precede the maternal disorder. Circulating levels of sFlt-1 and sEng are elevated several weeks prior to the onset of the clinical manifestations of preeclampsia [14, 15], while placenta growth factor (PlGF) is reduced in

W. Ramma · G. Zhao · A. Ahmed (✉)  
University/BHF Centre for Cardiovascular Sciences, Queen's  
Medical Research Institute, College of Medicine and Veterinary  
Medicine, University of Edinburgh, 47 Little France Crescent,  
Edinburgh EH16 4TJ, UK  
e-mail: a.s.ahmed@ed.ac.uk

W. Ramma  
e-mail: wendamma4585@gmail.com

I. A. Buhimschi · G. Zhao · A. T. Dulay ·  
U. A. Nayeri · C. S. Buhimschi  
Department of Obstetrics, Gynecology and Reproductive  
Science, Yale University, New Haven, CT 06520, USA

the first trimester of women who subsequently developed the disorder [16–20]. These observations demonstrate an angiogenic imbalance in preeclampsia.

Many studies have shown that increased systemic inflammation occurs during normal pregnancy and that inflammation is further enhanced in preeclampsia. During inflammatory insults, neutrophils are the first leukocytes to be recruited [21]. Neutrophil counts are increased in normal pregnancy, and further elevated in preeclampsia [22]. Mild neutrophil activation in the maternal circulation occurs in response to circulating syncytiotrophoblastic apoptotic debris that originate from the placenta [23] and was reported to be confined to the maternal circulation in pregnancy-induced hypertension [24]. Activated neutrophils degranulate, releasing numerous products in the circulation. Alpha-defensins are specific to neutrophils, while calprotectin is released by both neutrophils and macrophages. Both markers of neutrophil degranulation are elevated in preeclampsia [22, 25, 26]. Interleukin-6 (IL-6), a pro-inflammatory cytokine secreted by both activated leukocytes and endothelial cells, is also elevated in serum and plasma of women with severe preeclampsia [22, 27, 28].

Both the relationship between anti-angiogenic factors and neutrophil activation and the magnitude of their increase remains unknown. To dissect the contribution of neutrophil activation and anti-angiogenic factors in severe preeclampsia we investigated the relationships between the circulating markers of neutrophil activation/inflammation status ( $\alpha$ -defensins, calprotectin and IL-6) and anti-angiogenic factors (sEng and sFlt-1) in severe preeclampsia or systemic inflammatory state during pregnancy.

## Methods and materials

### Patients and biological specimens

We analysed blood samples from 88 women with singleton pregnancies recruited in the Low- and High-Risk Clinics and Labour and Delivery Unit at Yale-New Haven Hospital between December 2004 and March 2010. All women were followed prospectively from enrollment until delivery. Women with severe preeclampsia (**sPE**) ( $n = 45$ , gestational age (GA) median [interquartile range]: 30 [27–32] weeks) were enrolled at the time of clinical diagnosis. Controls (**CRL**) were healthy normotensive women who had an uncomplicated pregnancy and delivered at term (CRL,  $n = 27$ , GA: 29 [26–32] weeks). In addition, samples were collected from a group of pregnant women presenting with symptoms of systemic inflammatory response (**SIR**) syndrome (SIR, GA: 30 [27–32] weeks), who were admitted for clinical work-up targeted to identify the underlying cause. The Yale University Human Investigation Committee

approved the research protocol, and written informed consent was obtained from all participants.

Gestational age was established based on last menstrual period and/or early ultrasound evaluation ( $<20$  weeks of gestation) in all cases [29]. Severe preeclampsia was defined based on the American College of Obstetricians and Gynecologists criteria: gestational age  $>20$  weeks, blood pressure of 160 mm Hg systolic or higher or 110 mm Hg diastolic or higher on 2 occasions at least 6 h apart, and/or proteinuria of at least 5 g in a 24-h urine specimen or 3+ or greater on 2 random urine samples collected at least 4 h apart [30]. Other elements of the diagnosis included: IUGR ( $<10$ th percentile), persistent neurologic symptoms (headache, visual changes), epigastric or right upper-quadrant pain, pulmonary edema or cyanosis, oliguria (urinary output  $<500$  mL/24 h), serum creatinine  $>1.2$  mg/dL, elevated liver enzymes (greater than two times the normal) and thrombocytopenia ( $<100,000$  cells/ $\mu$ L) [30].

All patients with SIR presented with fever of  $>38^{\circ}\text{C}$  ( $100.4^{\circ}\text{F}$ ) and at least one of the following conditions: maternal leukocytosis ( $>15,000$  cells/ $\text{mm}^3$ ), maternal tachycardia ( $>100$  beats/min) or fetal tachycardia ( $>160$  beats/min). All SIR patients had a transabdominal amniocentesis to confirm or exclude intra-amniotic infection. In the context of a negative amniotic fluid analysis, a thorough imaging and microbiological workup was conducted which included ultrasonography, MRI, blood, urine and sputum cultures as deemed necessary in the context of additional clinical manifestations. Final diagnoses were as follows: intra-amniotic infection ( $n = 7$ ), pyelonephritis ( $n = 3$ ), bloodstream infection ( $n = 3$ ), gastroenteritis ( $n = 1$ ), pneumonia ( $n = 1$ ), appendicitis ( $n = 1$ ). Serum and plasma were collected during clinical assessment or admission, as previously described [31]. Blood (serum and citrated plasma) were collected by venipuncture, prior to intravenous fluid administration. Serum tubes were allowed to clot at room temperature for 30 min. All samples were spun at 800 g at  $4^{\circ}\text{C}$  for 15 min, the supernatant aliquoted and immediately stored at  $-80^{\circ}\text{C}$  until analyzed.

### Immunoassays and other biochemical measurements

Serum sEng, sFlt-1 and PlGF were measured using ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). ELISA assays for serum IL-6 (eBioscience, San Diego, CA) and calprotectin (Hycult Biotechnology, Uden, Netherlands), and plasma  $\alpha$ -defensins [32] (HNP1-3, Hycult Biotechnology, Uden, Netherlands) were performed according to the manufacturer's protocol.

### Statistical analysis

Normality testing was performed using the Shapiro–Wilk test. Comparisons between two groups were performed

using Mann–Whitney tests or between 3 groups using Kruskal–Wallis on ranks followed by Dunn’s tests as appropriate. For immunoassay results, logarithmic transformations were applied before statistical comparisons were performed. Relationships between variables were explored using Spearman’s Rank order correlations. Comparison between strength of correlations was achieved based on a z statistic. A probability level of <0.05 was considered statistically significant. Exact P values for non-parametric pairwise comparisons were obtained with SPSS (PASW Statistics v.18, IBM Armonk, NY). Multiple stepwise regression analysis was used to explore concurrent relationships between inflammatory and anti-angiogenic markers as dependent variables and demographic or clinical characteristics as independent variables. Variables were entered in the model based on  $P < 0.05$  and removed if

$P > 0.1$ . Statistical analyses were performed with Graph-Pad Prism (v 4.0; GraphPad Software Inc, La Jolla, CA), SigmaPlot 11.0 (Systat Software Inc, San Jose, CA) or MedCalc (Broekstraat, Belgium) softwares.

**Results**

Demographic, clinical and outcome characteristics

Gestational age at sample collection was not different among the 3 groups (Table 1). Compared to CRL, women with SIR were more likely to be non-Caucasian. Women with sPE had higher blood pressures and proteinuria compared to both non-preeclamptic groups. Twelve of the patients with sPE (29%) had IUGR (<10 percentile). There

**Table 1** Demographic, clinical and outcome characteristics of the study groups

Variable	sPE n = 45	CRL n = 27	SIR n = 16	P value
<b>Demographic and clinical characteristics</b>				
Age (years) <sup>†</sup>	27 [20–36]	29 [23–32]	31 [21–38]	0.745
Non-Caucasian race, n [%] <sup>§</sup>	28 [62]	10 [37]	13 [81]	0.013
Weight (kg) <sup>†</sup>	82 [69–100]	74 [63–84]	83 [64–97]	0.377
Nulliparity, n [%] <sup>§</sup>	28 [62]	11 [41]	10 [63]	0.172
Gestational age (weeks) <sup>†</sup>	30 [27–32]	29 [26–32]	30 [28–33]	0.443
Systolic blood pressure (mmHg) <sup>†</sup>	170 [160–180] <sup>a,b</sup>	114 [101–127]	108 [101–124]	<0.001
Diastolic blood pressure (mmHg) <sup>†</sup>	101 [92–110] <sup>a,b</sup>	68 [60–76]	60 [51–72]	<0.001
Proteinuria—urinary dipstick <sup>†</sup>	3 [2–3] <sup>a,b</sup>	0 [0–0]	0 [0–0]	<0.001
24 h-protein excretion (g/24 h) <sup>†</sup>	2.6 [1.3–5.3]	NA	NA	NA
Neurological manifestations, n [%] <sup>§</sup>	22 [49] <sup>a,b</sup>	0 [0]	0 [0]	<0.001
IUGR, n [%] <sup>§</sup>	12 [27] <sup>a,b</sup>	0 [0]	0 [0]	0.001
HELLP syndrome, n [%] <sup>§</sup>	13 [29] <sup>a,b</sup>	0 [0]	0 [0]	<0.001
<b>Laboratory characteristics</b>				
WBC ( $\times 1,000/\text{mm}^3$ ) <sup>†</sup>	11.7 [8.6–14.7]	10.5 [8.3–12.5] <sup>b</sup>	13.6 [11.8–20.8] <sup>a</sup>	0.006
Hemoglobin (g/dL) <sup>†</sup>	12.3 [11.2–13.2] <sup>b</sup>	12.2 [11.5–13.0] <sup>b</sup>	10.8 [10.2–12.2] <sup>a</sup>	0.013
Hematocrit (%) <sup>†</sup>	36.1 [33.4–38.8] <sup>b</sup>	33.9 [33.6–37.2] <sup>b</sup>	32.7 [29.6–35.3] <sup>a</sup>	0.004
<b>Differential count</b>				
Neutrophils (%) <sup>†</sup>	75 [65–84] <sup>b</sup>	76 [67–79] <sup>b</sup>	82 [80–87] <sup>a</sup>	0.009
Lymphocytes (%) <sup>†</sup>	17 [11–27] <sup>b</sup>	18 [15–23] <sup>b</sup>	9 [7.3–12] <sup>a</sup>	<0.001
Monocytes (%) <sup>†</sup>	6 [4–8]	5 [4–7]	6 [4–8]	0.840
ANC ( $\times 1,000/\text{mm}^3$ ) <sup>†</sup>	8.9 [6.2–11.9] <sup>b</sup>	7.6 [5.8–10.1] <sup>b</sup>	11.6 [10.0–16.2] <sup>a</sup>	0.002
<b>Outcome characteristics</b>				
Delivery <34 weeks, n [%] <sup>§</sup>	42 [93] <sup>a,b</sup>	0 [0] <sup>b</sup>	7 [44] <sup>a</sup>	<0.001
Gestational age at delivery (weeks) <sup>†</sup>	29 [28–32] <sup>a,b</sup>	39 [38–40] <sup>b</sup>	36 [29–39] <sup>a</sup>	<0.001

Gestational age equates to the gestational time at which blood was taken and the time of delivery is given as the “gestational age at delivery” IUGR intrauterine growth restriction; HELLP Hemolysis, ELevated liver enzymes and Low Platelets; WBC white blood cell count; ANC absolute neutrophil count

<sup>†</sup> Data presented as median [interquartile range] and analyzed by Kruskal–Wallis ANOVA

<sup>§</sup> Data presented as n [%] and analyzed by Chi square test

<sup>a</sup>  $P < 0.05$  versus CRL; <sup>b</sup>  $P < 0.05$  versus SIR

were significant differences in hematological parameters among groups. Compared to CRL, women with SIR had higher WBC and neutrophil counts and were significantly lymphogenic. Among sPE women, there were wide variations in absolute neutrophil counts (ANC) ranging from 3,000 to 20,000 cells/mm<sup>3</sup>. There was a direct correlation between ANC and 24 h-proteinuria ( $R = 0.508$ ,  $P = 0.003$ ). There was no relationship between ANC and blood pressure levels or presence of IUGR or HELLP syndrome. As expected, sPE women delivered at earlier gestational ages compared to CRL and SIR.

#### Neutrophil activation and inflammation

Neutrophil activation, assessed by  $\alpha$ -defensins and calprotectin concentrations in the maternal circulation, showed that the median plasma level of  $\alpha$ -defensins in sPE was only increased by 1.5-fold compared to CRL (Table 2, Fig. 1). Women with SIR exhibited a further significant increase in plasma  $\alpha$ -defensins compared to both sPE and CRL groups (Fig. 1). The median calprotectin concentration was increased by approximately twofold in sPE compared to CRL, reaching a similar level to that observed in SIR (Fig. 1). Inflammation in sPE group was significantly higher than CRL, as demonstrated by the twofold increase serum IL-6 levels in sPE compared to the CRL group. The SIR group exhibited significantly higher IL-6 compared to both sPE and CRL groups (Fig. 1 and Table 2).

#### Angiogenic factors

Compared to CRL, the sPE group had an ~20-fold increase in the median serum sEng and sFlt-1 concentrations (Fig. 2) accompanied by decreased PlGF (Table 2). Importantly, despite the elevated inflammatory status in

SIR (Fig. 1), sFlt-1 and sEng concentrations in the SIR group were similar to CRL (Fig. 2).

#### Inter and intra-process relationships

To further dissect the relationships between the two biological processes (inflammation and anti-angiogenic status) in preeclamptic women, we performed correlation analyses of biomarkers representative for each process (intra-process correlations) using the whole cohort (3 groups). We then analysed intra and inter-process relationships within the sPE group. Whole cohort correlations showed that inflammatory markers  $\alpha$ -defensins, calprotectin and IL-6 correlated strongly with each other. This indicated that they were indeed representing measurements of the same biological process (Table 3A). Similarly, the placental angiogenic factors (sEng, sFlt-1 and PlGF) also correlated with each other, suggesting that their release in the circulation likely occurs via related pathways (Table 3B). Significant positive intra-process correlations between the angiogenic markers, as well as the markers of inflammation, were also observed within the sPE group (Table 4). However, inter-process correlations in the sPE group revealed lack of correlation between the markers of inflammation and an anti-angiogenic state. The only inter-process correlation that reached statistical significance was between calprotectin and PlGF (inverse association,  $P = 0.011$ ). A graphical representation of these observations is shown in Fig. 3. Each case in our cohort is plotted in the 3D space relative to its  $x$ ,  $y$  and  $z$  coordinates represented by its sFlt-1, sEng and IL-6 concentration, respectively. As shown, the only variation along the  $y$  axis (depth: IL-6) is observed among patients in the SIR group (green squares). In contrast, sPE cases (red circles) scatter in the front plane of the graph delineated by their variation in the  $x$ - (length: sFlt-1) and  $z$ - (height: sEng)

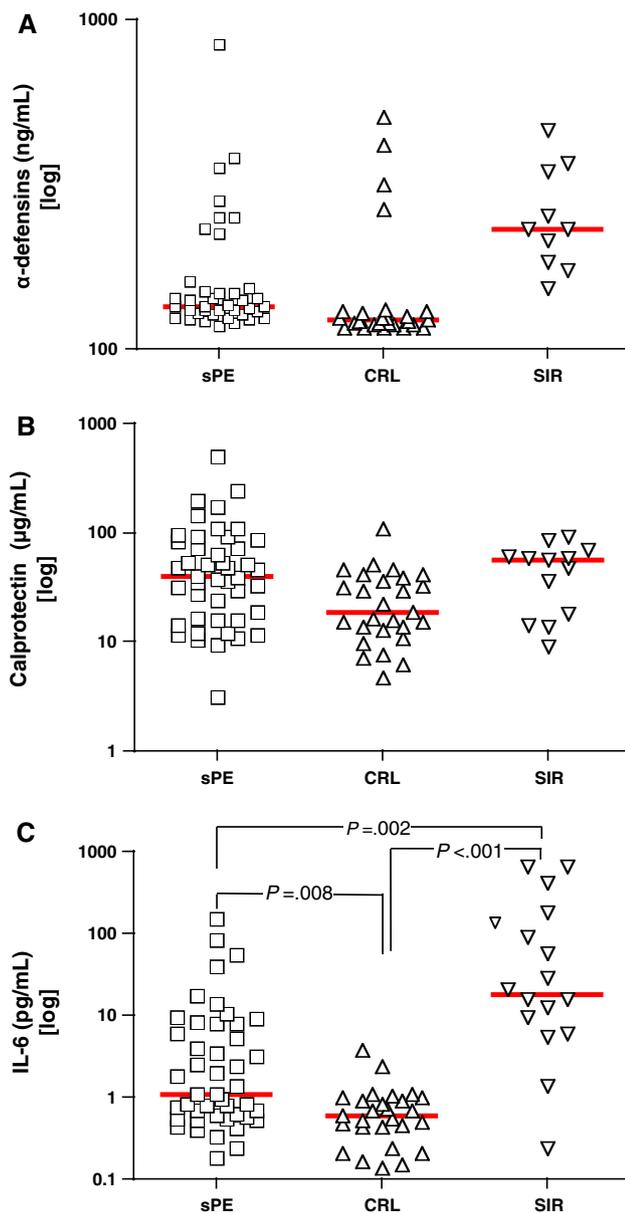
**Table 2** Levels of inflammatory and angiogenic markers in maternal circulation

Variable	sPE n = 45	CRL n = 27	SIR n = 16	<i>P</i> value
<b>Inflammatory markers</b>				
$\alpha$ -Defensins (pg/mL) <sup>†</sup>	135.7 [127.8–153.7] <sup>a,b</sup>	122.9 [119.3–130.2] <sup>b</sup>	233.0 [182.3–351.3] <sup>a</sup>	<0.001
Calprotectin ( $\mu$ g/mL)	40.7 [16.2–86.4] <sup>a</sup>	18.8 [13.0–39.4]	56.8 [16.5–65.3] <sup>a</sup>	0.010
Interleukin-6 (pg/mL)	1.1 [0.6–7.9] <sup>a,b</sup>	0.6 [0.4–1.0] <sup>b</sup>	18.6 [7.1–162.1] <sup>a</sup>	<0.001
<b>Pro and anti-angiogenic markers</b>				
sEndoglin (ng/mL)	70.1 [41.3–109.4] <sup>a,b</sup>	4.3 [3.5–6.1]	4.9 [7.3–7.0]	<0.001
sFlt-1 (ng/mL)	21.5 [15.2–30.5] <sup>a,b</sup>	1.5 [1.1–1.8]	2.5 [1.5–4.1]	<0.001
PlGF (pg/mL)	65.5 [33.0–101.2] <sup>a,b</sup>	432.9 [359.6–815.2]	203.0 [142.0–536.8]	<0.001
sFlt-1/PlGF ratio	384.6 [180.3–641.9] <sup>a,b</sup>	3.4 [1.9–6.1]	9.2 [4.4–13.6]	<0.001

<sup>†</sup> Data presented as median [interquartile range] and analyzed by Kruskal–Wallis ANOVA followed by multiple post hoc Dunn's tests

<sup>a</sup>  $P < 0.05$  versus CRL

<sup>b</sup>  $P < 0.05$  versus SIR



**Fig. 1** Scatterplots of the markers of neutrophil activation and the inflammatory cytokine interleukin-6. Levels of  $\alpha$ -defensins (a), calprotectin (b) and interleukin-6 (IL-6) (c) in women with severe preeclampsia (sPE, n = 45), pregnant controls (CRL, n = 27) and women with systemic inflammatory response syndrome (SIR, n = 16). The *thick red line* represents the groups’s median. Statistical analysis: Kruskal–Wallis ANOVA followed by multiple post hoc Dunn’s tests

coordinates. As expected, CRL cases (yellow triangles) cluster together around the point of origin indicating minimal variation in the 3 analytes.

**Discussion**

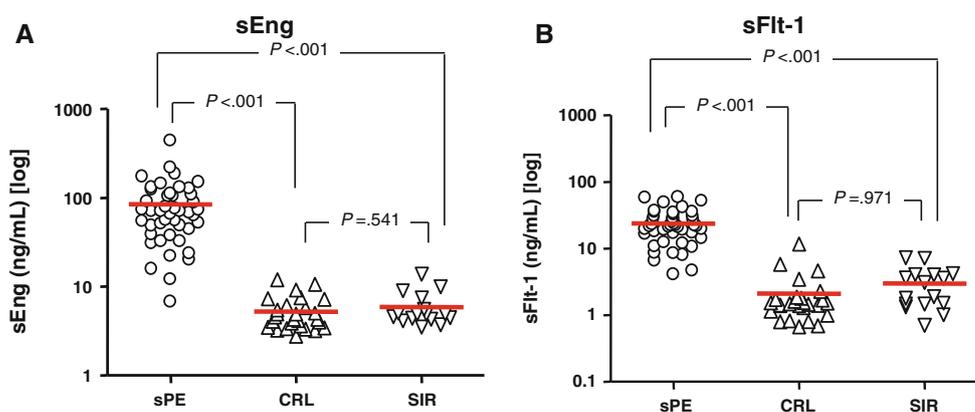
The study examined the contribution of systemic inflammation and anti-angiogenic factors in preeclampsia by

investigating the relationships between circulating markers of neutrophil activation/inflammation and anti-angiogenic factors in severe preeclampsia and systemic inflammatory status during pregnancy. The results reveal two important findings. First, this case–control study demonstrates that increased neutrophil activation and the release of anti-angiogenic factors in preeclampsia occur at different magnitudes in severe preeclampsia. Second, higher levels of anti-angiogenic sFlt-1 and sEng are specific to severe preeclampsia, and did not occur in women with systemic inflammatory state during pregnancy.

The positive correlation between  $\alpha$ -defensins and calprotectin demonstrates that neutrophil degranulation occurs in severe preeclampsia. These markers of neutrophil activation were positively correlated with IL-6, indicating that neutrophil activation is accompanied by inflammation at the time of the clinical manifestation of the disease. The cohort of severe preeclampsia patients exhibited significantly elevated circulating sEng and sFlt-1. However, there was no meaningful relationship between the increase in sEng or sFlt-1 and neutrophil activation, as determined by  $\alpha$ -defensins or calprotectin and IL-6 release, in severe preeclampsia. More importantly, patients with clinically relevant systemic inflammatory state during pregnancy did not exhibit elevated maternal sEng and sFlt-1 despite raised levels of  $\alpha$ -defensins, calprotectin and IL-6. This indicates that the rise in these anti-angiogenic factors is specific to preeclampsia and their release in the maternal circulation is unlikely to be triggered by neutrophil activation.

Activation of neutrophils and their degranulation result in the generation of reactive oxygen species and oxidative stress. Many studies have proposed that oxidative stress could be the main placental problem leading to preeclampsia [33]. Recently, Redman and Sargent suggested that oxidative stress could induce sFlt-1 and sEng release via nuclear factor kappa-B (NF $\kappa$ B) to a similar or greater extent as hypoxia [4]. In our study, women with preeclampsia had a 20-fold increase in serum sEng and sFlt-1 and approximately a twofold increase in markers of neutrophil activation ( $\alpha$ -defensins and calprotectin) and the pro-inflammatory cytokine, IL-6. This is consistent with the results of similar studies on sEng [15], sFlt-1 [14],  $\alpha$ -defensins [34], calprotectin [25, 35] and IL-6 [36]. Our findings extend the results of previous studies by demonstrating that neutrophil activation is not associated with increases in sEng or sFlt-1 in severe preeclampsia. To strengthen these findings, we included a group of pregnant patients with systemic inflammation in the absence of preeclampsia. This group failed to show any elevations in circulating sEng or sFlt-1, despite heightened systemic inflammation, indicating that the rise of anti-angiogenic factors is specific to preeclampsia and independent of systemic inflammation. Although, the present results

**Fig. 2** Scatterplots of the anti-angiogenic factors. Levels of serum soluble endoglin (sEng) (a), soluble fms-tyrosine kinase receptor-1 (sFlt-1) (b) in women with severe preeclampsia (sPE, n = 45), pregnant controls (CRL, n = 27) and women with systemic inflammatory response syndrome (SIR, n = 16). The thick red line represents the groups's median. Statistical analysis: Kruskal–Wallis ANOVA followed by multiple post hoc Dunn's tests



**Table 3** Intra-process relationships among inflammatory (A) versus angiogenic markers (B)

	$\alpha$ -Defensins	Calprotectin	IL-6
<b>A. Inflammatory markers</b>			
$\alpha$ -Defensins	<b><math>r = 1.000</math></b>		
Calprotectin	$r = 0.563$ $P < 0.001$	<b><math>r = 1.000</math></b>	
IL-6	$r = 0.544$ $P < 0.001$	$r = 0.453$ $P < 0.001$	<b><math>r = 1.000</math></b>
	sFlt-1	sEng	PIGF
<b>B. Angiogenic markers</b>			
sFlt-1	<b><math>r = 1.000</math></b>		
sEng	$r = 0.852$ $P < 0.001$	<b><math>r = 1.000</math></b>	
PIGF	$r = -0.692$ $P < 0.001$	$r = -0.778$ $P < 0.001$	<b><math>r = 1.000</math></b>

Data presented as Spearman correlation coefficients and  $P$  values in the entire cohort (n = 88). Italicized values depict non-significant correlations ( $P < 0.05$ ). Perfect correlations are noted by the bold values

conflict with those of an earlier study, which had shown a positive correlation between calprotectin and sEng in preeclampsia [37], the previous results are likely to be the consequence of gestational age differences among groups, as acknowledged by the authors themselves [37]. To eliminate this possible confounder, our patient groups were matched for gestational age at sample collection and the correlation between the two processes was limited to the severe preeclampsia group.

The present study suggests that neutrophil activation is unlikely to be directly involved in the release of maternal anti-angiogenic factors in severe preeclampsia. This conclusion is supported by recent studies demonstrating that complement activation was not associated with the release of angiogenesis-related factors in preeclamptic women [38, 39]. Interestingly, Girardi and co-workers showed that

complement activation induces the release of sFlt-1 from monocytes and causes abnormal placental development and fetal death in mice [40]. Thus, it is possible that inflammatory mediators may have local autocrine or paracrine effects, which could amplify signaling pathways and enhance the autocoid production of anti-angiogenic factors or their actions. However, complement activation did not cause the classical symptoms of preeclampsia (increased blood pressure and proteinuria) in the murine model studied by Girardi and co-workers. This suggests that a threshold concentration of sFlt-1 in the maternal circulation is critical to induce preeclampsia-like symptoms, and that the amount produced by complement-mediated activated monocytes was insufficient to elicit the classical signs of preeclampsia. Indeed, the dose-dependent effect of sFlt-1 is illustrated by the fact that neutralization of sFlt-1 below a critical threshold eliminates the signs of preeclampsia in mice [10].

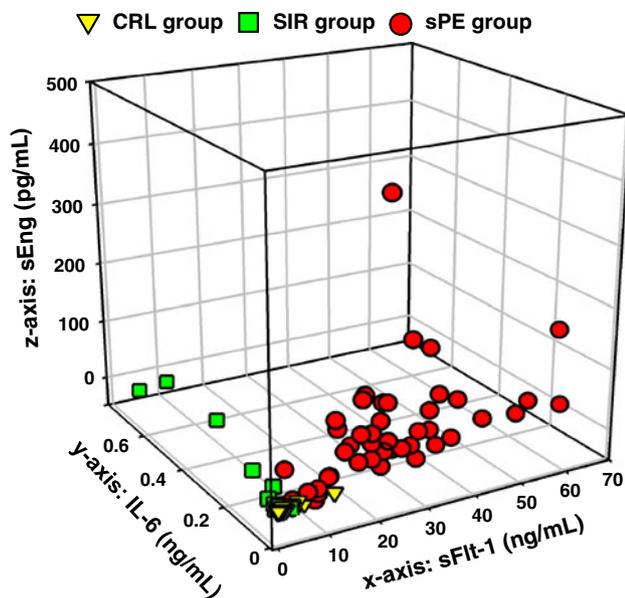
A recent study showed that serial extraction of sFlt-1 from the plasma of severe preeclamptic patients by apheresis reduced circulating sFlt-1 and stabilized maternal blood pressure, prolonging gestation in preeclamptic women [41]. This observation provides strong evidence of the role of anti-angiogenic factors as the likely candidates for severe preeclampsia. In contrast, the hypothesis that systemic inflammatory state per se causes preeclampsia is somewhat at odds with clinical experience of corticosteroids used to accelerate fetal lung maturation. A prospective double-blind randomized clinical trial of betamethasone confirmed the known beneficial effects of this treatment on neonatal outcome, but failed to show any beneficial effect to the preeclamptic mother [42].

In conclusion, our study shows that there was no meaningful relationship between neutrophil activation and angiogenic imbalance in severe preeclampsia. Importantly, the absence of increase anti-angiogenic factors in non-preeclamptic pregnant patients with elevated systemic inflammatory state indicates that it is unlikely that neutrophil activation play a central role in inducing the release

**Table 4** Comparison of inter- vs intra-process relationships in sPE group (n = 45)

Markers	$\alpha$ -Defensins	Calprotectin	IL-6	sFlt-1	sEng	PIGF
$\alpha$ -Defensins	<b>r = 1.000</b>					
Calprotectin	<i>r = 0.603</i> <i>P &lt; 0.001</i>	<b>r = 1.000</b>				
IL-6	<i>r = 0.645</i> <i>P &lt; 0.001</i>	<i>r = 0.372</i> <i>P = 0.012</i>	<b>r = 1.000</b>			
sFlt-1	<i>r = -0.301</i> <i>P = 0.062</i>	<i>r = -0.209</i> <i>P = 0.167</i>	<i>r = -0.122</i> <i>P = 0.423</i>	<b>r = 1.000</b>		
sEng	<i>r = 0.145</i> <i>P = 0.376</i>	<i>r = 0.227</i> <i>P = 0.133</i>	<i>r = 0.080</i> <i>P = 0.597</i>	<i>r = 0.391</i> <i>P = 0.008</i>	<b>r = 1.000</b>	
PIGF	<i>r = -0.211</i> <i>P = 0.196</i>	<i>r = -0.379</i> <i>P = 0.011</i>	<i>r = -0.226</i> <i>P = 0.135</i>	<i>r = -0.023</i> <i>P = 0.882</i>	<i>r = -0.552</i> <i>P &lt; 0.001</i>	<b>r = 1.000</b>

Data presented as Spearman correlation coefficients and *P* values. Italicized values depict non-significant correlations (*P* < 0.05). Other values indicate non-significant correlations (*P* > 0.05). Perfect correlations are noted by the bold values



**Fig. 3** Three-dimensional scatterplot of the study population. Each case is represented by its soluble fms-tyrosine kinase receptor-1 (sFlt-1, *x* axis), interleukin-6 (IL-6, *y* axis) and serum soluble endoglin (sEng, *z* axis). *Red circles* cases with severe preeclampsia (sPE, *n* = 45); *green squares* cases with systemic inflammation (SIR, *n* = 16); *yellow triangles* pregnant controls (CRL, *n* = 27)

of sEng or sFlt-1 during pregnancy. More importantly, since the increase in anti-angiogenic factors, above a critical threshold, is specific to preeclampsia, preeclampsia can be defined as a disease where anti-angiogenic factors disrupt the angiogenic balance, required for healthy pregnancy. In contrast, inflammation is consistently present in a number of pregnancy complications and it is very unlikely that it is the main or even the sole cause of preeclampsia. Our study focused on severe preeclampsia, as these patients have the greatest disease burden for maternal and fetal morbidity and mortality. Additional research is needed to

determine whether these results also apply to mild preeclampsia. Larger longitudinal population-based studies of pregnant women will be needed to definitively ascertain whether angiogenic imbalance or excessive inflammation is the cause preeclampsia, and whether these relationships differ according to disease severity.

**Acknowledgments** This work was supported by grants from the Medical Research Council (G0601295 and G0700288).

**Conflict of interest** The authors declare no competing financial interests.

**Open Access** This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

**References**

- Hogberg U (2005) The World Health Report 2005: “make every mother and child count”—including Africans. *Scand J Public Health* 33:409–411
- Ahmed A, Cudmore MJ (2009) Can the biology of VEGF and haem oxygenases help solve pre-eclampsia? *Biochem Soc Trans* 37:1237–1242
- Redman CW, Sacks GP, Sargent IL (1999) Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* 180:499–506
- Redman CW, Sargent IL (2009) Placental stress and preeclampsia: a revised view. *Placenta* 30(Suppl A):S38–S42
- Ahmed A, Dunk C, Kniss D, Wilkes M (1997) Role of VEGF receptor-1 (Flt-1) in mediating calcium-dependent nitric oxide release and limiting DNA synthesis in human trophoblast cells. *Lab Invest* 76:779–791
- Vuorela P, Helske S, Hornig C, Alitalo K, Weich H, Halmesmaki E (2000) Amniotic fluid-soluble vascular endothelial growth factor receptor-1 in preeclampsia. *Obstet Gynecol* 95:353–357
- Ahmad S, Ahmed A (2001) Regulation of soluble VEGFR-1 by VEGF and oxygen and its elevation in pre-eclampsia and fetal growth restriction. *Placenta* 22:A.7

8. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE et al (2003) Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 111:649–658
9. Ahmad S, Ahmed A (2004) Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. *Circ Res* 95:884–891
10. Bergmann A, Ahmad S, Cudmore M, Gruber AD, Wittschen P, Lindenmaier W, Christofori G, Gross V, Gonzalves A, Grone HJ et al (2010) Reduction of circulating soluble Flt-1 alleviates preeclampsia-like symptoms in a mouse model. *J Cell Mol Med* 14:1857–1867
11. Costantine MM, Tamayo E, Lu F, Bytautiene E, Longo M, Hankins GD, Saade GR (2010) Using pravastatin to improve the vascular reactivity in a mouse model of soluble fms-like tyrosine kinase-1-induced preeclampsia. *Obstet Gynecol* 116:114–120
12. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA et al (2006) Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 12:642–649
13. Walshe TE, Dole VS, Maharaj AS, Patten IS, Wagner DD, D'Amore PA (2009) Inhibition of VEGF or TGF- $\beta$  signaling activates endothelium and increases leukocyte rolling. *Arterioscler Thromb Vasc Biol* 29:1185–1192
14. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH et al (2004) Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 350:672–683
15. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA (2006) Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 355:992–1005
16. Levine RJ, Thadhani R, Qian C, Lam C, Lim KH, Yu KF, Blink AL, Sachs BP, Epstein FH, Sibai BM et al (2005) Urinary placental growth factor and risk of preeclampsia. *JAMA* 293:77–85
17. Buhimschi CS, Norwitz ER, Funai E, Richman S, Guller S, Lockwood CJ, Buhimschi IA (2005) Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia. *Am J Obstet Gynecol* 192:734–741
18. Savvidou MD, Noori M, Anderson JM, Hingorani AD, Nicolaides KH (2008) Maternal endothelial function and serum concentrations of placental growth factor and soluble endoglin in women with abnormal placentation. *Ultrasound Obstet Gynecol* 32:871–876
19. Foidart JM, Munaut C, Chantraine F, Akolekar R, Nicolaides KH (2010) Maternal plasma soluble endoglin at 11–13 weeks' gestation in pre-eclampsia. *Ultrasound Obstet Gynecol* 35:680–687
20. Noori M, Donald AE, Angelakopoulou A, Hingorani AD, Williams DJ (2010) Prospective study of placental angiogenic factors and maternal vascular function before and after pre-eclampsia and gestational hypertension. *Circulation* 122:478–487
21. Savill J (1993) The fate of the neutrophil in vasculitis. *Clin Exp Immunol* 93(Suppl 1):2–5
22. Clark P, Boswell F, Greer IA (1998) The neutrophil and preeclampsia. *Semin Reprod Endocrinol* 16:57–64
23. Sargent IL, Germain SJ, Sacks GP, Kumar S, Redman CW (2003) Trophoblast deportation and the maternal inflammatory response in pre-eclampsia. *J Reprod Immunol* 59:153–160
24. Greer IA, Dawes J, Johnston TA, Calder AA (1991) Neutrophil activation is confined to the maternal circulation in pregnancy-induced hypertension. *Obstet Gynecol* 78:28–32
25. Holthe MR, Staff AC, Berge LN, Fagerhol MK, Lyberg T (2005) Calprotectin plasma level is elevated in preeclampsia. *Acta Obstet Gynecol Scand* 84:151–154
26. Kostakis ID, Cholidou KG, Kallianidis K, Perrea D, Antsaklis A (2010) The role of calprotectin in obstetrics and gynecology. *Eur J Obstet Gynecol Reprod Biol* 151:3–9
27. Tosun M, Celik H, Avci B, Yavuz E, Alper T, Malatyalioglu E (2010) Maternal and umbilical serum levels of interleukin-6, interleukin-8, and tumor necrosis factor- $\alpha$  in normal pregnancies and in pregnancies complicated by preeclampsia. *J Matern Fetal Neonatal Med* 23:880–886
28. Szarka A, Rigo J Jr, Lazar L, Beko G, Molvarec A (2010) Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array. *BMC Immunol* 11:59
29. Hadlock FP, Deter RL, Harrist RB, Park SK (1983) Computer assisted analysis of fetal age in the third trimester using multiple fetal growth parameters. *J Clin Ultrasound* 11:313–316
30. ACOG: ACOG practice bulletin (2002) Diagnosis and management of preeclampsia and eclampsia. No. 33, January 2002. *Obstet Gynecol* 99:159–167
31. Buhimschi CS, Magloire L, Funai E, Norwitz ER, Kuczynski E, Martin R, Richman S, Guller S, Lockwood CJ, Buhimschi IA (2006) Fractional excretion of angiogenic factors in women with severe preeclampsia. *Obstet Gynecol* 107:1103–1113
32. Craddock RM, Huang JT, Jackson E, Harris N, Torrey EF, Herberth M, Bahn S (2008) Increased alpha-defensins as a blood marker for schizophrenia susceptibility. *Mol Cell Proteomics* 7:1204–1213
33. Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK (1989) Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol* 161:1200–1204
34. Prieto JA, Panyutich AV, Heine RP (1997) Neutrophil activation in preeclampsia. Are defensins and lactoferrin elevated in preeclamptic patients? *J Reprod Med* 42:29–32
35. Braekke K, Holthe MR, Harsem NK, Fagerhol MK, Staff AC (2005) Calprotectin, a marker of inflammation, is elevated in the maternal but not in the fetal circulation in preeclampsia. *Am J Obstet Gynecol* 193:227–233
36. Luppi P, Tse H, Lain KY, Markovic N, Piganelli JD, DeLoia JA (2006) Preeclampsia activates circulating immune cells with engagement of the NF- $\kappa$ B pathway. *Am J Reprod Immunol* 56:135–144
37. Staff AC, Braekke K, Johnsen GM, Karumanchi SA, Harsem NK (2007) Circulating concentrations of soluble endoglin (CD105) in fetal and maternal serum and in amniotic fluid in preeclampsia. *Am J Obstet Gynecol* 197(176):e171–e176
38. Derzsy Z, Prohaszka Z, Rigo J Jr, Fust G, Molvarec A (2010) Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol* 47:1500–1506
39. Lynch AM, Murphy JR, Gibbs RS, Levine RJ, Giclas PC, Salmon JE, Holers VM (2010) The interrelationship of complement-activation fragments and angiogenesis-related factors in early pregnancy and their association with pre-eclampsia. *BJOG* 117:456–462
40. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE (2006) Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med* 203:2165–2175
41. Thadhani R, Kisner T, Hagmann H, Bossung V, Noack S, Schaarschmidt W, Jank A, Kribs A, Cornely OA, Kreyssig C et al (2011) Pilot study of extracorporeal removal of soluble fms-like tyrosine kinase I in preeclampsia. *Circulation* 124:940–950
42. Amorim MM, Santos LC, Faundes A (1999) Corticosteroid therapy for prevention of respiratory distress syndrome in severe preeclampsia. *Am J Obstet Gynecol* 180:1283–1288