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Maternal plasma soluble fms-like tyrosine kinase-1 (sFlt-1) and free vascular endothelial growth factor (VEGF) at 11-13 weeks of gestation in preeclampsia

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ABSTRACT

Objective: To investigate the maternal plasma concentration of soluble fms-like tyrosine kinase-1 (sFlt-1) and free vascular endothelial growth factor (free-VEGF) at 11-13 weeks of gestation in patients destined to develop preeclampsia (PE) and to examine whether any possible differences in maternal plasma levels are related to uterine artery pulsatility index (PI) and maternal serum placental growth factor (PlGF).

Methods: Plasma free-VEGF, plasma sFlt-1, serum PlGF and uterine artery PI were measured at 11-13 weeks in 90 cases that subsequently developed PE and in 180 unaffected controls.

Results: In the majority of cases of PE and controls the levels of free-VEGF were undetectable. In the pregnancies that developed PE, compared to unaffected controls, uterine artery PI was higher, serum PlGF was lower but there was no significant difference in levels of sFlt-1.

Conclusion: Measurement of free-VEGF and sFlt-1 in maternal blood at 11-13 weeks of gestation is not useful in the prediction of pregnancies destined to develop PE.

Introduction

The placenta expresses the vascular endothelial growth factor receptor-1 (VEGFR-1) mRNA which produces VEGFR-1 or through an alternative splicing process produces soluble fms-like tyrosine kinase-1 (sFlt-1) (Clark *et al.,* 1998; Maynard *et al.,* 2008; He *et al.,* 1999). While VEGFR-1 is retained within the cell membrane of trophoblastic cells, sFlt-1 is secreted into the maternal circulation and acts as an antagonist to the angiogenic factors, VEGF and placental growth factor (PlGF) (Kendall and Thomas 1993; Banks *et al.,* 1998; Levine and Karumanchi 2005).

Preeclampsia (PE), which is an important cause of maternal and perinatal mortality and morbidity, is thought to be the consequence impaired placentation due to inadequate trophoblastic invasion of the maternal spiral arteries (World health organization 2005; Lewis 2004; Khong *et al.,* 1986; Pijnenborg *et al.,* 1991). This impaired placentation is manifested in the findings of Doppler ultrasound studies which reported increased impedance to flow in the uterine arteries (Yu *et al.,* 2005; Plasencia *et al.,* 2007). In PE the maternal plasma or serum concentration of free-VEGF and PlGF is decreased whereas the concentration of sFlt-1 is increased (Tables 1 and 2).

There is extensive evidence that the altered concentrations of PlGF and sFlt-1 precede the clinical onset of the disease (Table 2). In the case of PlGF, the decreased maternal levels are evident from the first-trimester of pregnancy and there

is a significant association between the level of PlGF and uterine artery pulsatility index (PI) (Akolekar *et al.,* 2008). In the case of sFlt-1 there is contradictory evidence concerning first-trimester maternal circulating levels in pregnancies that subsequently develop PE with some studies reporting an increase (Baumann *et al.,* 2008) and others a decrease (Erez *et al.,* 2008; Vatten *et al.,* 2007) or no difference (Levine *et al.,* 2004; Rana *et al.,* 2007; Thadani *et al.,* 2004) from normal. The maternal circulating levels of free-VEGF are also decreased prior to the clinical onset of PE (Levine *et al.,* 2004; Polliotti *et al.,* 2003) but there are no reports concerning the levels in the first-trimester of pregnancy.

The aim of our study was to investigate the maternal plasma concentration of sFlt-1 and free-VEGF at 11-13 weeks of gestation in patients destined to develop PE and to examine whether any possible differences in maternal plasma levels are related to uterine artery PI and maternal serum PlGF.

Methods

Study population

This was a case-control study drawn from a large prospective study for hypertensive complications of pregnancy in women attending for their routine first hospital visit in pregnancy at King's College Hospital, London, UK. In this visit, which is held at 11^{+0}-13^{+6} weeks of gestation, all women have an ultrasound scan to firstly, confirm gestational age from the measurement of the fetal crown-rump

length (CRL), secondly, diagnose any major fetal abnormalities and thirdly, measure fetal nuchal translucency (NT) thickness as part of screening for chromosomal abnormalities. In addition, the maternal serum pregnancy associated plasma protein-A and free beta-human chorionic gonadotropin are determined and the results are combined with the fetal NT to calculate the patient-specific risk for trisomy 21 (Kagan *et al.,* 2008; Snijders *et al.,* 1998).

We recorded maternal characteristics and medical history, stored serum and plasma at -80°C for subsequent biochemical analysis and performed transabdominal color Doppler for measurement of the left and right uterine artery PI and recorded the lowest value (L-PI) (Poon *et al.,* 2009a and 2009b). Written informed consent was obtained from the women agreeing to participate in the study, which was approved by King's College Hospital Ethics Committee.

The base cohort study population, wherein the present case-control study was nested, was examined between March 2006 and March 2007 and constituted 8,234 singleton pregnancies. In 147 (1.8%) cases there was subsequent development of PE, 135 (1.6%) cases developed gestational hypertension (GH) and 7922 cases were unaffected by PE or GH. In addition, there were 30 (0.4%) pregnancies, in which there was at least one hypertensive blood pressure but on the basis of the available data it was not possible to determine if the diagnosis was PE, were also excluded from further analysis. There was available stored maternal

blood from 90 of the 147 cases that developed PE and maternal plasma sFlt-1 and free-VEGF were measured in all these 90 cases that developed PE, which included 30 who required delivery before 34 weeks (early-PE) and 60 cases of late-PE, and 180 unaffected controls. Each case of PE was matched with two controls for storage time because blood was collected on the same date. Each control delivered a phenotypically normal neonate at term with weight appropriate for gestational age and did not develop any hypertensive disorder of pregnancy. None of the samples in the case-control study were previously thawed and refrozen. The maternal characteristics in the 90 cases of PE with available blood were not significantly different from the 57 cases of PE without blood (maternal age, p=0.917; body mass index, p=0.390; crown rump length, p=0.989, nulliparity, p=0.735; women of Black racial origin p=0.498, and smoking status, p=1.00).

This study is part of a research program on the early prediction of pregnancy complications and some of the data from these patients on serum PlGF and uterine artery L-PI were included in previous publications (Akolekar et al., 2008; Poon et al., 2009b). The values on serum PlGF were available in 87 patients in the PE group and 177 patients in the control group of the present study.

Maternal history

Patients were asked to complete a questionnaire on maternal age, racial origin, cigarette smoking during pregnancy, method of conception, medical history,

medication, parity, obstetric history and family history of PE in the mother. The questionnaire was then reviewed by a doctor together with the patient. The maternal weight and height were measured and the body mass index (BMI) was calculated in Kg/m^2.

Outcome measures

The definition of PE was that of the International Society for the Study of Hypertension in Pregnancy (Brown *et al.,* 2001). The diastolic blood pressure should be 90 mmHg or more on at least two occasions four hours apart developing after 20 weeks of gestation together with significant proteinuria in previously normotensive women. Significant proteinuria is defined by 300 mg or more in 24 hours or two readings of at least ++ on dipstick analysis of midstream or catheter urine specimens if no 24-hour collection is available. In PE superimposed on chronic hypertension significant proteinuria (as defined above) should develop after 20 weeks of gestation in women with known chronic hypertension (history of hypertension before conception or the presence of hypertension at the booking visit before 20 weeks of gestation in the absence of trophoblastic disease).

The newborn was considered to be small for gestational age (SGA) if the birth weight was less than the 10^{th} percentile after correction for gestation at delivery and sex, maternal racial origin, weight, height and parity (Gardosi and Francis, 2007).

Sample analysis

Plasma sFlt-1 and free-VEGF and serum PlGF were measured by enzyme linked immunoassay (ELISA) technique using DuoSet® human sFlt-1 and free VEGF immunoassays and Quantikine® free PlGF immunoassay (R&D Systems Europe Ltd., Abington,UK). The lower limit of detection of the assays were 15 pg/mL for sFlt-1, 5 pg/mL for VEGF and 7 pg/mL for PlGF. Samples whose duplicate values differed by more than 15% were analyzed.

Statistical analysis

In the majority of cases of PE (54 of 90) and controls (118 of 180) the levels of free-VEGF were undetactable and no statistical analysis was performed. In contrast, sFlt-1 and PlGF were measured in all samples. The following steps were taken for the statistical analysis. First, the distribution of plasma sFlt-1 was transformed using the equation: $Y = \log_{10}(\text{sFlt-1} - k)$ to approximate a Gaussian distribution. The distributions of uterine artery L-PI and PlGF were made Gaussian after logarithmic transformation. Second, multiple regression analysis was used to determine which of the factors amongst the maternal characteristics and gestation were significant predictors of log sFlt-1 in the unaffected group. The measured uterine artery L-PI was converted into MoM after adjustment for gestation, maternal age, BMI and racial origin, as previously described (Poon *et al.,* 2009b). Similarly, the measured PlGF was converted into MoM after adjustment for fetal CRL, maternal weight, racial origin

and cigarette smoking status as previously described (Akolekar *et al.,* 2008). Third, Mann-Whitney test with *post hoc* Bonferroni correction was used to compare median values of sFlt-1, uterine artery L-PI and PlGF between the outcome groups. Fourth, regression analysis was used to determine the significance of association between sFlt-1, uterine artery L-PI and PlGF in the outcome groups.

The statistical software package SPSS 16.0 (SPSS Inc.,Chicago, IL) was used for data analyses.

Results

The maternal and pregnancy characteristics of each of the outcome groups are compared in Table 3. In the early-PE group compared to the unaffected group, there were more Black women, more women had PE in their previous pregnancy, more were chronic hypertensive on antihypertensive medication, they delivered at an earlier gestation and had a lower birth weight centile. In the late-PE group compared to the unaffected group, women had a significantly higher BMI, there were more Black women, more women had PE in their previous pregnancy, more were chronic hypertensive on antihypertensive medication, they delivered at an earlier gestation and had a lower birth weight centile.

Multiple regression analysis in the unaffected group demonstrated that log sFlt-1 did not change with fetal CRL (p=0.227), maternal age (p=0.874), racial origin (p=0.963),

parity (p=0.524), maternal weight (p=0.987), method of conception (p=0.531) or smoking status (p=0.672).

The measured uterine artery L-PI was converted into MoM after adjustment for gestation, maternal age, BMI and racial origin, as previously described (Poon *et al.,* 2009b). Similarly, the measured PlGF was converted into MoM after adjustment for fetal CRL, maternal weight, racial origin and cigarette smoking status as previously described (Akolekar *et al.,* 2008).

In the pregnancies that subsequently developed early-PE and late-PE, the median plasma sFlt-1 concentration was not significantly different from that in the unaffected group, whereas in both early-PE and late-PE the uterine artery L-PI was significantly increased and serum PlGF was significantly decreased (Table 4).

In the group that developed PE there was no significant association between plasma sFlt-1 and uterine artery L-PI (p=0.736), serum PlGF (p=0.676), gestation at delivery (p=0.102) or birth weight centile (p=0.153). In the unaffected group there was no significant association between plasma sFlt-1 and serum PlGF (p=0.299), gestation at delivery (p=0.264) or birth weight centile (p=0.729) but there was a significant association between plasma sFlt-1 and uterine artery L-PI (r=-0.166, p=0.026).

Discussion

The results of this study demonstrate that at 11-13 weeks of gestation, the median maternal plasma concentration of sFlt-1 in women who subsequently develop PE is not significantly different from the median in unaffected controls. In contrast, serum PlGF is decreased and uterine artery PI is increased and the differences from normal are greater in the early-PE group than in the late-PE group.

The levels of free-VEGF were undetectable in the majority of PE cases and controls. Previous studies in patients with PE reported consistently lower levels of free-VEGF than in normotensive controls (Table 1) (Lyall *et al.,* 1997; Reuvekamp A *et al.,* 1999; Livingston *et al.,* 2000; Maynard *et al.,* 2003; Levine *et al.,* 2004; Muy-Rivera *et al.,* 2005; Buhimschi *et al.,* 2006; Lee *et al.,* 2007). There is also some evidence that decreased levels of free-VEGF precede the clinical onset of PE. Pilliotti *et al.,* (2003) reported that the mean free-VEGF at 14-21 weeks was significantly lower in 20 patients that subsequently developed early-PE compared to 60 unaffected controls. Levine *et al.,* (2004) performed a longitudinal study in 120 patients that subsequently developed PE and 120 unaffected controls and reported lower levels of free-VEGF in the PE group during and for up to five weeks before the clinical onset of PE.

In unaffected pregnancies both the serum PlGF and uterine artery L-PI are affected by gestational age and maternal characteristics, including racial origin and

238 weight and these factors need to be adjusted for before comparison between
239 normal and pathological pregnancies (Akolekar *et al.,* 2008; Poon *et al.,* 2009b).
240 The plasma concentration of sFlt-1 was not affected by maternal characteristics or
241 fetal CRL within the narrow gestational range of 11-13 weeks. A longitudinal study
242 of 46 normotensive pregnancies at 8-40 weeks gestation reported that the
243 maternal serum concentration of sFlt-1 increased with gestational age (Romero *et*
244 *al.,* 2008).
245
246 The angiogenic factors VEGF and PlGF are thought to play an important role in
247 placental development and they are also implicated in the vascular adaptation to
248 pregnancy through their action on endothelial cells resulting in vasodilation. In
249 contrast, sFlt-1 antagonises the role of VEGF and PlGF both in placental
250 development and in maintenance of endothelial function (Kaufmann *et al.,* 2004;
251 Lam *et al.,* 2005). In-vitro studies reported that exogenous administration of sFlt-1
252 inhibits VEGF-induced trophoblast invasion and in pregnancies with PE placental
253 expression of sFlt-1 mRNA is increased (Maynard *et al.,* 2003; Zhou *et al.,* 2002).
254 Nagamatsu *et al.,* (2004) reported that in response to reduced oxygen tension
255 cytotrophoblasts produce more sFlt-1 and less free PlGF. Administration of sFlt-1 to
256 pregnant rats produces a PE-like syndrome including hypertension and proteinuria
257 (Maynard *et al.,* 2003). Karumanchi and Bdolah (2004) reviewed the role of sFlt-1 in
258 PE and suggested that excess placental production of sFlt-1 has a causal role in the
259 pathogenesis of clinical manifestations of PE but it is unclear whether it also plays a

role in abnormal placentation. Lockwood *et al.,* (2007) examined the production of sFlt-1 by decidual cells as opposed to trophoblasts and reported that thrombin enhances the production of sFlt-1 by first trimester decidual cells and further hypothesized that sFlt-1 may impair pseudovasculogenesis by altering the local balance of angiogenic factors which in turn would lead to restricted trophoblast invasion and local hypoxia with further release of sFlt-1 by cytotrophoblasts.

The finding that at 11-13 weeks in pregnancies destined to develop PE uterine artery PI is increased and serum PlGF is decreased provides supportive evidence that PE is the consequence of impaired placentation during the first-trimester of pregnancy. Consequently, our finding that at 11-13 weeks plasma sFlt-1 is not altered in pregnancies that subsequently develop PE and the lack of a significant association between plasma sFlt-1 and uterine artery PI or serum PlGF, implies that circulating sFlt-1 during the first-trimester does not reflect its potential role in placentation and the pathogenesis of PE. This is further supported by the findings of the longitudinal study of Levine *et al.,* (2004) who reported that the high levels of sFlt-1 observed in patients with PE are evident for only up to five weeks before the clinical onset of PE and certainly not before 21 weeks of pregnancy. Other studies examining first-trimester maternal circulating levels of sFlt-1 in pregnancies that subsequently developed PE reported that the levels are either increased (Baumann *et al.,* 2008), decreased (Erez *et al.,* 2008; Vatten *et al.,* 2007) or not different from normal (Levine *et al.,* 2004; Rana *et al.,* 2007; Thadani *et al.,* 2004).

Smith *et al.,* (2007) reported that there was no association between maternal serum concentration of sFlt-1 at 10-14 weeks and the risk of developing PE but a higher level of sFlt-1 was associated with a reduced risk of delivery of SGA neonates. In our study there was no association between maternal plasma concentration of sFlt-1 and birth weight centile in either the PE group or the unaffected controls.

In conclusion, the findings of our study demonstrate that in the first trimester of pregnancy, there is no significant difference in the maternal plasma levels of sFlt-1 between patients destined to develop PE and unaffected controls. These results do not support the hypothesis linking excess production of sFlt-1 with impaired trophoblastic invasion and development of PE. Alternatively, it is possible that sFlt1 increases in particular locations of the placenta itself and interferes with trophoblast migration and pseudovasculogenesis but these changes are not reflected in circulating sFlt-1 during the first-trimester of pregnancy.

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Table 1: Studies reporting the median maternal circulating free vascular endothelial growth factor (VEGF) concentration (pg/mL) in patients during or before preeclampsia compared to controls.

Author	Gestation (wk)	Preeclampsia		Control		P value
		n	VEGF	n	VEGF	
During preeclampsia						
Lyall *et al.,* 1997	26-40	34	2.3	34	12.9	<0.001
Reuvekamp *et al.,* 1999	28-40	30	0.3*	30	18.3*	<0.001
Livingston *et al.,* 2000	27-40	21	6.4*	21	18.7*	<0.001
Maynard *et al.,* 2003	29-40	21	4.1	11	14.0	<0.05
Levine *et al.,* 2004	37-41	21	6.7	26	9.9	<0.05
Muy-Rivera *et al.,* 2005	35-39	131	10.9	175	13.6	NS
Buhimschi *et al.,* 2006	23-40	42	0.1	13	1.6	<0.001
Lee *et al.,* 2007	29-40	20	21.3	20	134.0	<0.001
Before preeclampsia						
Polliotti *et al.,* 2003	14-21	20	2.6	60	6.0	<0.001
Levine *et al.,* 2004	21-32	6	5.1	102	12.8	<0.05

Values in * indicate mean values

491 **Table 2:** Studies reporting the median maternal plasma soluble fms-like tyrosine
492 kinase-1 (sFlt-1) concentration (pg/mL) in patients during or before preeclampsia
493 compared to controls.

494

Author	Gestation (wk)	Preeclampsia		Control		P value
		n	sFlt-1	n	sFlt-1	
During preeclampsia						
Tsatsaris *et al.,* 2003	30-38	19	2690	31	120	<0.001
Levine *et al.,* 2004	29-41	23	4382	23	1643	<0.001
Staff *et al.,* 2005	24-40	32	9932	38	3417	<0.001
Shibata *et al.,* 2005	28-40	22	5221	24	1857	<0.001
Buhimschi *et al.,* 2006	23-40	42	2026	13	434	<0.001
Masuyama *et al.,* 2007	33-40	30	5666*	30	1204*	<0.01
Stepan *et al.,* 2007	20-37	18	8388	15	2602	<0.01
Salahuddin *et al.,* 2007	28-40	19	74700*	20	16600*	<0.01
Lee *et al.,* 2007	29-40	20	1935*	20	298*	<0.001
De Vivo *et al.,* 2008	31-40	52	44870	52	12560	<0.001
Woolcock *et al.,* 2008	25-41	18	3130	18	470	<0.001
Kim *et al.,* 2009	23-40	62	2755*	62	554*	<0.001
Reddy *et al.,* 2009	37-41	10	10100	10	4900	<0.05
Before preeclampsia						
Levine *et al.,* 2004	8-12	21		20		NS
Thadani *et al.,* 2004	7-12	40	1048	80	973	NS
Park *et al.,* 2005	16-23	32	730	128	441	<0.05

Kim *et al.,* 2007	14-23	46	3861	100	2353	<0.001
Rana *et al.,* 2007	11-13	39	3500*	147	3000*	NS
Vatten *et al.,* 2007	4-12	154	135*	392	166*	<0.01
Erez *et al.,* 2008	6-15	56	1405	201	1788	<0.05
Baumann *et al.,* 2008	11-13	46	1764*	92	1537*	<0.05
Lim *et al.,* 2008	14-21	40	4945*	100	2788*	<0.001

Values in * indicate mean values

497 **Table 3.** Maternal and pregnancy characteristics in the three outcome groups

498

Characteristics	Control	Early preeclampsia	Late preeclampsia
	(n=180)	(n=30)	(n=60)
Maternal age in years, median (IQR)	32.5 (29.2-36.7)	31.7 (25.4-36.8)	31.5 (26.2-36.7)
Body mass index in Kg/m^2, median (IQR)	24.8 (22.6-28.1)	27.8 (23.7-32.4)	28.2 (24.0-33.5)*
Crown-rump length in mm, median (IQR)	64.7 (60.0-71.1)	68.1 (58.3-73.1)	61.8 (58.3-68.7)
Gestation at sampling in wks, median (IQR)	12.6 (12.3-13.0)	12.6 (12.3-13.4)	12.4 (12.1-13.0)
Gestation at delivery in wks, median (IQR)	39.7 (38.9-40.7)	31.6 (28.7-32.6)*	38.3 (37.2-39.9)*
Birth weight in Kg, median (IQR)	3.5 (3.2-3.7)	1.3 (0.9-1.6)*	2.8 (2.3-3.3)*
Birth weight below the 10th cenile, n (%)	0	24 (80.0)*	25 (41.7)*
Racial origin			
White, n (%)	126 (70.0)	12 (40.0)	26 (43.3)
Black, n (%)	34 (18.9)	13 (43.3)*	27 (45.0)*
Indian or Pakistani, n (%)	10 (5.6)	2 (6.7)	5 (8.3)
Chinese or Japanese, n (%)	4 (2.2)	0	1 (1.7)
Mixed, n (%)	6 (3.3)	3 (10.0)	1 (1.7)
Parity			
Nulliparous, n (%)	88 (48.9)	16 (53.4)	37 (61.7)
Parous – no previous PE, n (%)	85 (47.2)	7 (23.3)*	16 (26.7)*
Parous – previous PE, n (%)	7 (3.9)	7 (23.3)*	7 (11.7)*
Family history of PE – Mother (n, %)	7 (3.9)	4 (13.3)	4 (6.7)
Cigarette smoker, n (%)	7 (3.9)	0	4 (6.7)

Conception			
Spontaneous, n (%)	172 (95.6)	27 (90.0)	58 (96.7)
Ovulation drugs, n (%)	6 (3.3)	2 (6.7)	2 (3.3)
In-vitro fertilization, n (%)	2 (1.1)	1 (3.3)	0
Medical history			
None, n (%)	173 (96.1)	25 (83.3)	57 (95.0)
Chronic hypertension, n (%)	1 (0.6)	4 (13.3)*	3 (5.0)*
Diabetes mellitus, n (%)	3 (1.7)	0	0
Thrombophilia, n (%)	3 (1.7)	1 (3.3)	0
Medication during pregnancy			
None, n (%)	163 (90.6)	25 (83.3)	56 (93.3)
Anti-hypertensives, n (%)	0	2 (6.7)*	1 (1.7)*
Insulin, n (%)	3 (1.7)	0	0
Aspirin, n (%)	2 (1.1)	1 (3.3)	0
Others, n (%)	12 (6.6)	2 (6.7)	3 (5.0)

Comparisons between each outcome group with controls (Chi square test and Fisher exact test for categorical variables and Mann Whitney test with *post hoc* Bonferroni correction for continuous variables): * $p < 0.05$

504 **Table 4.** Median soluble fms-like tyrosine kinase-1 (sFlt-1), uterine artery lowest
505 pulsatility index (L-PI) and placental growth factor (PlGF) in the outcome groups
506

	Unaffected (n=180)	Early preeclampsia (n=30)	Late preeclampsia (n=60)
Plasma sFlt-1 (median, IQR)			
pg/mL	6349 (3697-10153)	7099 (4769-58270)	6840 (4200-11381)
Uterine artery L-PI (median, IQR)			
MoM	0.99 (0.80-1.23)	1.65 (1.31-1.85)*	1.31 (1.13-1.55)*
Unit	1.33 (1.08-1.66)	2.29 (1.91-2.49)	1.88 (1.52-2.19)
Serum PlGF (median, IQR)			
MoM	1.03 (0.83-1.33)	0.61 (0.46-0.84)*	0.82 (0.53-1.03)*
pg/mL	35.5 (27.6-48.6)	20.1 (14.0-33.1)	29.8 (21.5-35.1)

507

508 IQR=interquartile range, MoM= multiple of the unaffected median
509 Comparisons between outcome groups by Mann-Whitney test with *post hoc*
510 Bonferroni correction * p<0.0167.
511