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# Evidence for extraplacental sources of circulating angiogenic growth effectors in human pregnancy

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ABSTRACT

Pregnancy complications such as preeclampsia (PE) and intrauterine growth restriction (IUGR) are associated with reduced blood flow, contributing to placental and fetal hypoxia. Placental hypoxia is thought to cause altered production of angiogenic growth effectors (AGEs), reflected in the circulation of mother and fetus. Vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and their soluble binding protein (sFlt-1) are, in turn, postulated as being causally involved in PE via induction of systemic endothelial cell dysfunction. To dissect the role of AGEs, accurate measurement is of great importance. However, the values of AGEs are highly variable, contributing to heterogeneity in their association (or lack thereof) with preeclampsia. To test the hypothesis that variability may be due to peripheral cell release of AGEs we obtained blood samples from normal healthy pregnant women ($n = 90$) and the cord blood of a subset of their neonates using standard serum separation and compared results obtained in parallel samples collected into reagents designed to inhibit peripheral cell activation (sodium citrate, theophylline, adenosine and dipyridamole-CTAD). AGEs were measured by ELISA. CTAD collection reduced maternal and fetal free VEGF by 83%, and 98%, respectively. Free PlGF was decreased by 29%, maternal sFlt-1 by >20% and fetal sFlt-1 by 59% in the CTAD-treated vs. serum sample ($p < 0.0001$). In summary blood collection techniques can profoundly alter measured concentrations of AGEs in mother and fetus. This process is highly variable, contributes to variation reported in the literature, and renders questionable the true impact of alteration in AGEs on pregnancy pathologies.



## 1. Introduction

Much attention has been focused on circulating angiogenic growth effectors (AGEs) as being causally related to the human-specific pregnancy disease preeclampsia [1–4]. Such observations contribute to a long and continuing history of positing single or even a combination of circulating factors as causal agents in preeclampsia [5]. Among the circulating angiogenic growth effectors implicated in preeclampsia are free vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and the soluble fms-like tyrosine kinase 1 (sFlt-1), a binding protein for VEGF and PlGF. The soluble form of Flt-1 can limit growth factor-stimulated transactivation by sequestering VEGF and PlGF or by forming inactive heterodimers with the transmembrane receptors Flk and Flt-1 [6]. In preeclampsia, the causal argument is that binding of free VEGF and PlGF by excess sFlt-1 inhibits their beneficial actions on the vascular endothelium, enabling the systemic endothelial cell damage postulated as precipitating the symptoms of preeclampsia [7].

Placental hypoxia due to hypobaria, ischemia or chronically lowered blood flow (e.g. preeclampsia, high altitude residence) is reflected at the molecular level by an increase in Hypoxia-Inducible Factor (HIF), a transcription factor which acts as a key regulator of gene expression [8–11]. HIF target genes include the angiogenic growth effectors (VEGF, PlGF, sFlt-1), which are thus differentially regulated by oxygen tension in the placenta [12–15] and are

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consequently altered in the circulation of mother and fetus in preeclampsia [3,16–18]. In addition to placental production, Rajakumar and colleagues have shown that peripheral blood mononuclear cells may also be a source of sFlt-1, a factor that could contribute to preeclamptic pathology [19]. In fact VEGF and PlGF are produced by a wide variety of cell types in addition to trophoblast and endothelium, most notably macrophages, neuronal and tumor cells [20–23].

In dissecting the role of AGEs in pathologies such as preeclampsia, accurate measurement of the *in vivo* levels of these effectors is of great importance. Accuracy, however, is suspect given that the values of AGEs reported in the literature are highly variable. This has contributed to the heterogeneity in their association (or lack thereof) with preeclampsia [1,3,24,25]. The effects of blood collection techniques and preservative or anticoagulant reagents on the measurement of AGEs have not been systematically evaluated. Several studies have reported that variability in the measurement of AGEs is reduced by the use of plasma rather than serum [26–28]. The variability in AGE measurements and the potential for contributions from peripheral blood cells have led several researchers to conclude that blood sampling for the purpose of AGE measurement is best performed using reagents designed to inhibit platelet activation and the peripheral cell release of growth factors. They recommend use of a combination of sodium citrate, theophylline, adenosine and dipyridamole, "CTAD" [29–31]. We chose the CTAD method based on these reports and others indicating that failure to control for clotting time invalidates the use of VEGF as an indicator of disease states [26,27,30]. However there are no reports using this methodology for the other angiogenic growth effectors. In this study we measured maternal and fetal AGEs in pregnancy, comparing the values obtained for identical samples collected into CTAD with those obtained from samples collected using the standard serum separation method, the method most widely used in large-scale clinical studies.

## 2. Methods

### 2.1. Research design and subjects

The data presented here stem from a sub-project within a cross-sectional study design that was used to evaluate the effects of altitude and genetic ancestry on uterine blood flow, maternal $O_2$ delivery to the fetoplacental unit and pregnancy outcome [32,33]. The samples utilized in this study were obtained from pregnant women and the cord blood of their neonates in the sea level arm of the study ($n = 90$). It was not possible to collect matched serum and CTAD samples in all mothers and the umbilical cords of their babies, and some samples were of insufficient volume to permit measurement of all 3 AGEs in duplicate. Therefore the sample sizes are given below with the results for each assay. All participants gave written, informed consent as approved by the Institutional Review Board. Inclusion criteria were singleton pregnancy, good health (absence of chronic conditions that predispose to preeclampsia e.g. hypertension, renal disease, obesity), enrollment in prenatal care and elective/scheduled cesarean delivery. Women were excluded for drug, alcohol or tobacco use, or a positive oral glucose tolerance test. A subset of participants was studied at 24, 36 weeks of pregnancy and >3 months post-partum so that gestational age-dependent changes in VEGF in relation to the non-pregnant values could be evaluated.

### 2.2. Blood collection

Prior to elective cesarean delivery, at the time that the anesthesiologist placed the maternal IV, and without using supplemental oxygen, a 15 ml maternal venous blood sample was collected and samples intended for analyses of AGEs were distributed into serum separator tubes and into pre-chilled tubes containing CTAD (BD, Franklin Lakes, NJ). CTAD specifically prevents platelet activation and release of platelet-derived factors into the plasma, but likely inhibits activation of other peripheral cells as well. Samples obtained from the umbilical cord vein were similarly treated. Serum samples were allowed to clot for 30 min at room temperature. CTAD tubes were centrifuged for 10 min at 2000 $\times g$ at 4 °C immediately after blood collection. The serum sample was similarly centrifuged but at room temperature. Both serum and CTAD samples were aliquoted, flash frozen in liquid nitrogen and stored at −80 °C until analysis.

### 2.3. Assays

All ELISA kits were purchased from R&D systems (Minneapolis, MN). The kits used were the human sVEGF R1/Flt-1 Quantikine ELISA (DVR-100), the free VEGF Immunoassay kit (DVE00) and the human free PlGF ELISA (DPG00). A 4-parameter logistic curve-fit was used for the standard curve and subsequent calculation of the unknown (sample) values, per the manufacturer's recommendations.

### 2.4. Statistical analysis

Maternal and fetal demographic and clinical data are reported as the mean ± standard error of the mean. None of the AGEs passed the D'Agostino and Pearson normality test. Thus for the matched serum versus CTAD samples, a Wilcoxon signed rank test was used to determine whether the conditions of blood collection influenced the results. Spearman's $r$ was used to examine the correlation between serum and CTAD values. Serial data on free serum VEGF were log-transformed and analyzed using repeated-measures ANOVA followed by the Student Neuman–Keuls test for pair-wise differences (between trimesters and post-partum) and are back transformed for figures. Regression analysis was used to compare the relationship between angiogenic growth factor concentrations and gestational age, birth or placental weight. Differences attributable to blood collection techniques are presented in percentages as mean ± SD. Means of the group values are used rather than individual differences, for ease of comprehension and because CTAD reduced so many individual values to zero. Values were considered significant where $p$ was $<0.05$.

## 3. Results

### 3.1. Validation studies

In order to ensure that the CTAD reagent was not interfering with the ELISA assay, the linearity of the standard curves with vs. without the CTAD reagent was evaluated. Briefly, a pooled plasma sample with a known concentration of the AGE of interest was diluted to the same concentration as required by the assay, except that one aliquot was treated with 15 μL PBS and the other with 15 μL CTAD reagent (the estimated maximum quantity of CTAD per sample during collection). This was then added to each of the standards in the standard curve, to yield the same dilution as the experimental samples (e.g. 20-fold dilution for sFlt-1). We then evaluated the linearity of the standard curve and whether or not a value of unity was obtained when comparing the curves for the CTAD vs. control sample. All curves were linear and nearly reached unity, having an $r^2$ of ≥0.98 for each AGE.

For each AGE we also tested linearity by serial dilution of a pooled sample comprised of serum from healthy women who were a minimum of three months post-partum and a pooled sample from mid-pregnancy. For each assay, the serial dilutions were designed to test the dynamic range of the kit as reported by the manufacturer. Initial tests were performed by reading at dual spectrophotometric wavelengths of 570 and 450 nm, with values obtained at 570 nm subtracted from those acquired at 450 nm, as recommended by the manufacturer to correct for optical imperfections in the assay plate. The intra-assay coefficient of variation was calculated for the duplicate samples within each plate and averaged across all plates used. Our inter-assay variation was calculated using the pooled samples mentioned above, loaded in triplicate on every plate. For VEGF, linearity in the serial dilutions correlated with predicted values ($r^2 = 0.83$); divergence was at the upper end of the measurement scale. Linearity tests in the PlGF assay yielded an $r^2$ of 0.96 and for sFlt-1, $r^2$ was 0.98.

Use of the dual wavelength correction for VEGF resulted in values that were 24 ± 8% (mean ± SD) lower where positive values for VEGF were detected ($n = 42$). The $r^2$ for the correlation between samples measured at 450 nm versus 570/450 nm was 0.99. This large variation using dual wavelength correction is due to the fact that most of the pregnancy samples had very low values, yielding a small denominator when calculating percentages. For PlGF, dual wavelength correction resulted in values that were 4 ± 4% greater