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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

when measured at 450 nm alone, and the r^2 for the correlation between the two measures was 0.99 ($n = 64$ samples). For sFlt-1 subtracting values obtained at 570 nm from those made at 450 nm resulted in concentrations that were 5 ± 6% lower when measured at 450 nm alone ($n = 42$ samples), with an r^2 for correlation between the two measures of 0.91. A review of the literature suggested that (where reported) most laboratories have used only the 450 nm wavelength. We therefore completed studies using the 450 nm wavelength only and these are the values reported.

3.2. Assay variability

Our intra-assay coefficients of variation (CV) are reported as mean ± SD. The CV for duplicate samples where VEGF concentrations were detectable at >5 pg/ml had a mean of 3.3 ± 32.8% ($n = 42$ samples), and 16.2 ± 117.6% where values were lower than 5 pg/ml (limit of detection) but above 0 ($n = 29$ samples). The remaining samples ($n = 25$) either had one value above and one value below 0 (these were considered non-detectable, as invariably the positive value was <5 pg/ml). Again, the high CV is due to the very low VEGF concentrations present. For PlGF the intra-assay CV was 3.6 ± 8.9% and for sFlt-1 the CV was 3.2 ± 6.1%. The inter-assay coefficient of variation was 12 ± 21% for VEGF, 9 ± 8% for PlGF and 15 ± 10% for sFlt-1.

We compared our measurements of variability in the assay with those of R&D systems, the manufacturer of these assay kits. They report their assay coefficients of variation (CVs) based on 20 and 40 replicates for intra- and inter-assay variation, respectively. We re-calculated R&D's potential CV at their low, moderate and high standard concentrations, assuming that only duplicates rather than 20–40 replicates were tested. Following this calculation, the potential intra-assay CV is 14–20% for free VEGF, 17–26% for PlGF and 8–12% for sFlt-1 under standard laboratory protocols using duplicate samples. This is an underestimate of potential variability as R&D reports CVs from non-pregnant subjects, whose concentrations for VEGF are 10–100 fold higher and for sFlt-1 as much as 10-fold lower and much less variable than values observed in pregnancy. Nonetheless, the results above show that our estimates of variability compare well with those of the manufacturer under the same conditions.

3.3. Subjects

Table 1 shows the characteristics of the subjects in this study, healthy women and their neonates.

3.4. Free VEGF

The detection limit of the assay is reported as ≥5.0 pg/ml. For the free VEGF assays, no dilution was necessary. Pregnancy substantially reduces the circulating levels of free VEGF (Fig. 1A, $n = 30$ serially studied pregnancies), which do not differ between mid- and late pregnancy. All women had greater levels of free VEGF >3 months postpartum, in most cases at least an order of magnitude higher than their pregnancy values. Fifty percent of the maternal samples had values less than 5 pg/ml at 20 weeks and 40% had values less than 5 pg/ml when measured at term.

We examined the extent to which clotting in serum separator tubes may provoke platelets or other circulating cells to release VEGF. We analyzed paired samples collected into serum separator versus those collected into CTAD tubes in a subset of normotensive, near-term mothers (Fig. 1B) and in the umbilical venous cord blood of their neonates (Fig. 1C). CTAD treatment diminished the circulating levels of free VEGF in the mothers at term from a median 4.1 pg/mL (range 0–61.2) to a median of 0 pg/mL (range 0–20.6, $p < 0.0001$, $n = 40$). The mean difference between serum and CTAD values was 83% (range 60–100%). Nearly twice as many CTAD than serum samples had VEGF concentrations that were undetectable (63%) and/or below the detection limit of 5 pg/ml (83%, Fig. 1B). While fetal cord blood serum concentrations of VEGF were >100 fold greater than in their mothers (Fig. 1C), collection of fetal blood into CTAD abolished free VEGF in 26% of the samples and lowered values to < 5 pg/ml in an additional 24%. The remaining positive values were decreased by more than 40-fold from a median of 265 pg/mL (range 1–921) to a median of 5 pg/mL (range 0–31) in CTAD samples ($p < 0.0001$, $n = 34$). The mean percentage change between serum and CTAD VEGF levels was 98% (range 85–100%). As the figure shows, the serum/CTAD differences are highly variable between individuals, precluding the development of a correction calculation between the two methods. Excluding values below detection limits, serum and CTAD derived values of VEGF did not correlate. Maternal and fetal VEGF concentrations did not correlate with gestational age, placental or birth weight. Fetal VEGF concentrations did not correlate with those of their mothers. Thus the presence of free VEGF in both maternal and fetal cord blood is largely an artifact of the method used for blood collection; most pregnancy samples do not show detectable levels of VEGF and those that do are extremely low.

Table 1
Maternal and neonatal characteristics.

Maternal characteristics	$n = 90$
Age (years)	28 ± 1
Gravidity	2.7 ± 0.2
Parity	1.3 ± 0.2
Proportion primiparous (%)	50%
Height (cm)	159 ± 1
Non-pregnant weight (kg)	60 ± 1
Non-pregnant Body Mass Index (kg/m^2)	24.0 ± 0.4
Weight gain with pregnancy (kg)	11.5 ± 0.5
Infant characteristics	
Birth weight (grams, (unadjusted values)	3480 ± 39
Birth weight (grams, adjusted values)[a]	3485 ± 20
Placental weight (g)	470 ± 10
Birth/placental weight ratio	7.6 ± 0.2
Clinically assessed gestational age at birth (weeks)	38.7 ± 0.1
Birth length (cm)	50.5 ± 0.1
Head circumference (cm)	34.8 ± 0.2
Abdominal circumference (cm)	34.3 ± 0.2
Sex ratio M/F	46/44

[a] Adjusted for differences in gestational age, maternal parity, height and weight gain, and fetal sex.

3.5. PlGF

The detection limit of the test is reported as ≥ 7.0 pg/ml. A 4-fold dilution was determined as optimal and used for all samples. All samples had detectable levels of PlGF and none were below 7 pg/ml. PlGF is secreted by the placenta into the maternal, but not fetal circulation, and was not tested in the fetal cord blood for this reason. Placental growth factor (PlGF) was assayed in serum from term pregnancies (Fig. 2). CTAD treatment decreased PlGF by approximately one-third relative to the paired serum samples, from a median 228 pg/mL (range 97–997) in serum to a median of 144 pg/mL (range 51–643) in CTAD (Fig. 2A $p < 0.001$, $n = 23$). The percentage change in serum vs. CTAD was 29% (range 11–66%), indicative of significant variation in the effects of CTAD between individuals (Fig. 2A). Nonetheless, CTAD and serum values for PlGF were correlated ($r^2 = 0.78$, $p < 0.0001$). Maternal PlGF concentrations were positively correlated with placental weight (Fig. 2B, $r^2 = 0.17$ $p < 0.001$, $n = 77$), but not birth weight or gestational age.

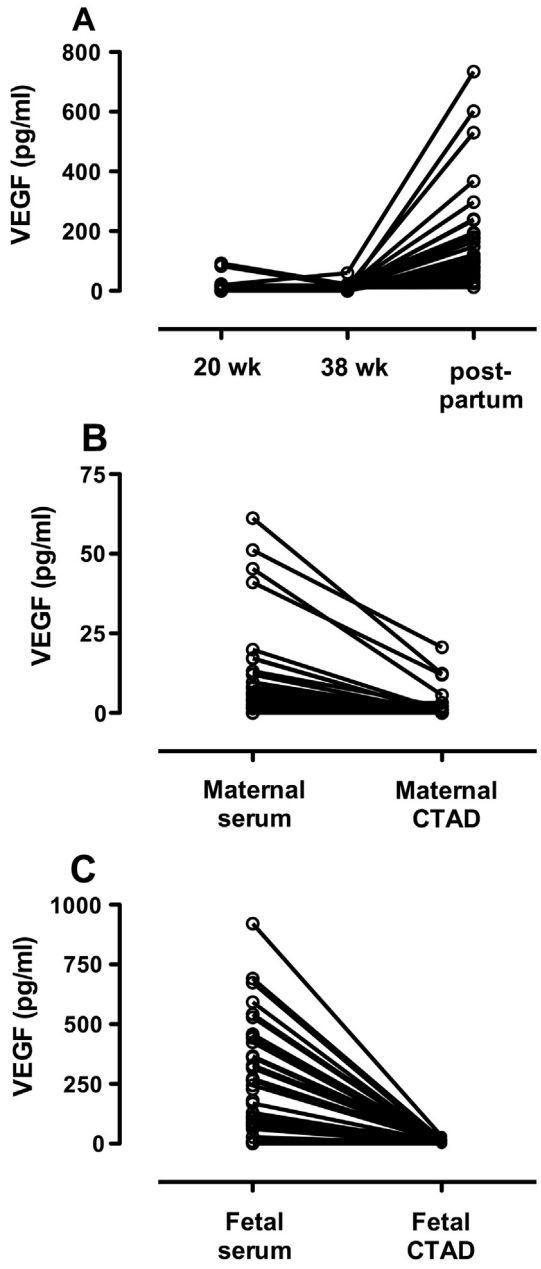

Fig. 1. (A) Thirty women were studied at 20 weeks, 38 weeks of pregnancy and >3 months postpartum. Free VEGF concentrations were reduced during pregnancy ($p < 0.0001$). Values were similar in the second vs. third trimester. In both the pregnant and non-pregnant condition there were >10-fold differences between the highest and lowest values measured. B) Collection of blood into CTAD (see methods) reduced values for maternal free VEGF by 83% ($p < 0.001$; $n = 40$). C) Umbilical venous blood concentrations of VEGF were >10 fold greater in the fetuses than in their mothers. Collection into CTAD reduced the free VEGF concentrations by 98% ($p < 0.01$; $n = 34$). Nil values and those below the detection limit of the kit occurred in 80% of the samples.

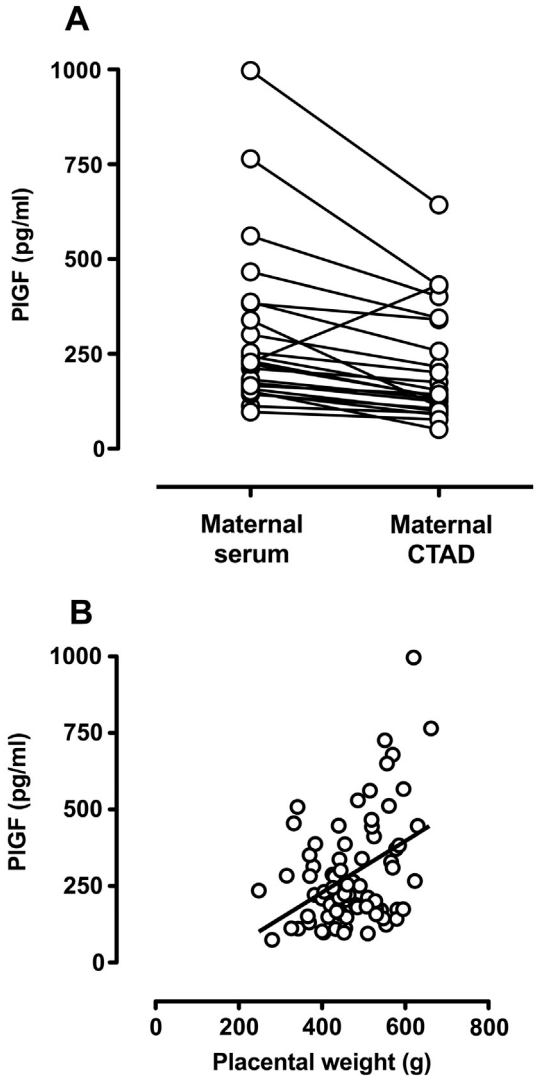

Fig. 2. (A) CTAD treatment decreased maternal PlGF by 29% ($p < 0.0001$; $n = 23$). (B) The serum values for PlGF were positively correlated with placental weight ($y = -109 + 0.84x$, $r^2 = 0.17$, $p < 0.0001$; $n = 77$).

3.6. sFlt-1

The reported detection limit of the assay was 13.3 pg/mL (0.013 ng/mL). A 20-fold dilution was employed in the maternal samples, and a 5-fold dilution in the fetal samples. All serum samples had detectable levels of sFlt-1. Collection of blood into CTAD reduced maternal sFlt-1 concentrations in healthy pregnant women from a median of 10.7 ng/mL (range 3.4–53.1) in serum to 9.3 ng/mL (range 2.3–44.4) in CTAD (Fig. 3A, $p < 0.0001$, $n = 35$), a reduction of 20% (range 1–69%). Correlation between maternal serum and CTAD values was high ($r^2 = 0.89$, $p < 0.0001$). In fetal serum samples sFlt-1 values (Fig. 3B) were >20-fold lower than in the maternal samples. CTAD treatment decreased cord blood sFlt-1 concentrations from a median of 0.4 ng/mL (range 0.2–20.4) in serum to 0.2 (range 0–7.5) in CTAD ($p < 0.001$, $n = 20$), a reduction of 59% (range 32–71%, r^2 for serum vs. CTAD in the 11 paired, detectable values = 0.93, $p < 0.01$). Forty-five percent of the fetal CTAD samples had no detectable levels of sFlt-1. The changes in maternal and fetal sFlt-1 concentrations produced by CTAD were highly variable between individuals as demonstrated by the data in Fig. 3A and B. Neither maternal nor cord blood sFlt-1 concentrations were related to gestational age, birth or placental weight, nor were they correlated between mother and fetus.

4. Discussion

We measured AGEs in maternal and umbilical blood samples obtained either by standard serum collection procedures or collection into the CTAD reagent, designed to prevent platelet activation. In maternal samples, 83% of circulating free VEGF, 29% of PlGF and 20% of sFlt-1 present in the serum samples was eliminated in samples collected into CTAD. In fetal cord blood samples, CTAD

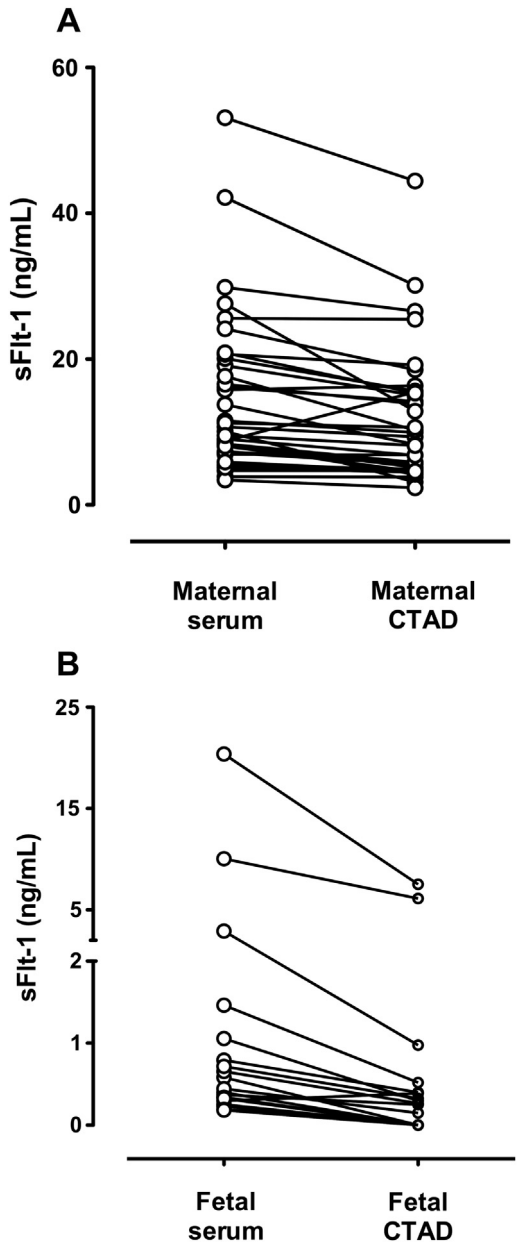

Fig. 3. (A) A Collection of maternal blood into CTAD decreased SFlt-1 concentrations by 20% ($p < 0.0001$; $n = 35$). (B) Neonatal cord blood levels of sFlt1 were lower than in their mothers. Reduction in sFlt-1 attributable to treatment with CTAD was 59% ($p < 0.0001$; $n = 20$).

collection reduced free VEGF by >98% and sFlt-1 by 59%. Thus the serum values for the AGEs are partially an artifact of blood collection technique, as a consequence of hemostasis and/or serum preparation techniques. Moreover, the degree to which hemostasis alters AGE concentrations is highly variable from individual to individual, precluding any form of standard correction for the hemostatic effects. Studies suggesting that fetal compromise is reflected by elevated cord blood values for VEGF or sFlt-1 should be rejected. Free VEGF is not present in the fetal circulation if hemostasis or platelet activation is avoided in blood collection. Fetal sFlt-1 levels, already low, are also very nearly abolished by similar precautions. These growth effectors appear to be released by cells within the circulation and, without appropriate precautions, can be released after sample collection. Thus the excess or insufficiency of AGEs in pregnancy pathologies, when measured in serum samples, will not necessarily reflect changes in placental production. These findings raise significant questions concerning the conclusions reached by many articles in the literature on the association of AGEs with pregnancy pathologies.

Few studies have addressed peripheral cellular content/production/secretion of VEGF, PlGF or sFlt-1. As noted above, VEGF and PlGF are released from several cell types other than trophoblast, including macrophages, neuronal and tumor cells [20–23]. Platelet lysate from both normal and preeclamptic pregnancies contained no sFlt-1 [34], however monocytes have been identified as a source in both pregnancy [19] and kidney disease [35]. PlGF appears to be inducible in epithelial cells [36], and is known to induce VEGF secretion by mononuclear cells [37]. We are aware that additional splice variants of sFlt-1 are produced by the hypoxic placental trophoblast [38,39] and that it is unknown whether current assays measuring sFlt-1 distinguish between these splice variants.

Initial reports analyzing AGEs showed that use of plasma rather than serum reduced the variability in AGE measurement [26–28], however fluctuations have been observed even in plasma preparations, especially when processing procedures take place at room temperature [31]. Several reports have examined the possibility of AGE release by platelets and have suggested use of inhibitors of platelet activation [29–31]. It is apparent from the data reported here that AGEs are potentially produced or bound, stored and secreted/released by other cell types within the circulation and therefore the alteration of AGEs in pregnancy pathologies does not necessarily reflect changes in placental production. Fetal VEGF and sFlt-1 levels are very nearly abolished by CTAD treatment and are therefore unlikely to reflect compromised fetal status as has been reported elsewhere [40–42].

The findings reported here for VEGF, PlGF and sFlt-1 raise significant problems with current methods and the associated results reported in the literature. There are a growing number of papers investigating the role of PlGF and/or SFlt-1 as biomarkers for pathological pregnancies other than preeclampsia and for future disease risk [17,43–48]. In the majority of these reports, no account is taken of the potential release of these AGEs from peripheral blood cells. The primary method of blood collection is the standard serum preparation, despite prior reports suggesting peripheral blood cell release [29–31]. To the extent that we observed a good correlation between PlGF or sFlt-1 levels in CTAD vs. serum, one might expect that the conclusions from serum results are valid. However the absolute values may be incorrect and thus not comparable between studies. Moreover the data reported here is obtained from healthy pregnancies. There are a number of problems in extrapolating this to comparisons with pathological pregnancies in which platelet activation [49], differences in inflammatory cytokines etc. may differentially effect peripheral cell release. Another obvious problem is the potential for differing monocyte populations between control and pathological pregnancies. For example the reduced or absent plasma volume expansion characteristic of PE will alter blood monocyte concentrations. Thrombocytopenia is a frequent occurrence in PE and there is evidence of other potential changes in leucocyte count [50–52]. These effects may invalidate any comparison of AGEs between normal and PE pregnancies in the absence of measures to prevent post-sampling release.

Beyond the major problems cited above, the data reported here highlight other consistent problems in the published literature. First, a number of laboratories do not report their own coefficients of variation for duplicate or triplicate samples (intra-assay) or their plate-to-plate (inter-assay) variability in the AGE ELISAs. Many use the manufacturer's stated CVs instead. Why is this important? Because, as noted above, R&D, the most widely used manufacturer, reports coefficients of variation (CVs) for AGEs that are based on 20

and 40 replicates for intra- and inter-assay variation, respectively. These are not appropriate for assays that use the duplicate or triplicate measures employed by most investigators. Second, although AGEs are not normally distributed, some publications present the data as means and SEM or SD (e.g. [4]). Even if the data are log-transformed prior to analysis, it is still misleading to present means and SD/SEM because it obscures the distribution of values and the degree of separation between cases and controls. Third, very few articles indicate how they handled data such as VEGF where the values are often at or below the level of reliable detection or are simply non-detectable. The assumption that a value below detection limit is at the detection limit is often made, but not reported. For example, in a highly cited article on the subject, the levels of free VEGF in preeclamptics and controls 5 weeks before onset of symptoms was 5.1 pg/mL in PE and 12.8 in controls ($p < 0.002$). No SEM, SD or range was given, nor was it stated how zero values or values below the detection limit of 5 pg/mL were handled [4]. Given the results presented here, and in other papers in which the issue was adequately addressed [53], a significant number of the samples tested in both control and PE groups had to be below the limits of detection. This means that nil values or those below 5 pg/mL must have been assumed to be 5 pg/mL.

In summary, methods for collection, type and storage of diagnostic samples should be standardized, even if it means collecting more than one type of blood sample [28]. More fruitful research should focus on how cells in the peripheral circulation interact with the maternal endothelium and to what extent highly localized release of growth effectors may influence endothelial function.

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