Angiogenic growth factors in hypoxic pregnancy. Maternal and fetoplacental hypoxia do not alter circulating angiogenic growth factors: The emperor's got no clothes?

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ABSTRACT
Context: Placental hypoxia alters production of angiogenic growth factors (AGFs), thought to be causally involved in the development of the pregnancy-specific disease preeclampsia (PE). Consistent with this, the incidence of PE is increased at high altitude (HA, >2700m).

Objective. We tested the hypotheses that (1) circulating sFlt-1 is increased and free VEGF and PlGF decreased at HA, (2) circulating AGFs correlate with biomarkers of hypoxia, (3) peripheral circulating cells may contribute to hypoxia-associated alterations in AGFs, and (4) cord blood levels of AGFs are altered at HA or in PE.

Design. A prospective, cross-sectional study of healthy and preeclamptic pregnancies at low (400m) vs. HA (3600m) in Bolivia.

Participants. Subjects (170 normal, 39 PE) conceived, gestated and delivered at their altitude of residence. Healthy women had no conditions predisposing to preeclampsia.

Methods: Blood was collected using standard techniques and those designed to inhibit platelet activation. Maternal and fetal AGFs were measured by ELISA and compared between altitudes, between normal and PE pregnancies and in relation to biomarkers of hypoxia.

Results: AGFs did not differ between altitudes. >90% of circulating free VEGF, >30% of PlGF and >25% of sFlt-1 was secreted into samples as a consequence of hemostasis. Biomarkers of hypoxia did not correlate with the AGFs. PlGF was lower and sFlt-1 higher in PE. PlGF correlated with placental mass, whilst significantly more sFlt-1 derived from circulating peripheral cells in PE than normotensive patients.

Conclusions. Chronic hypoxia does not alter circulating AGFs. Smaller placentas likely account for diminished PlGF in PE. Peripheral cell release of AGFs provoked by hemostasis is highly variable, hence clinical tests based AGFs are unlikely to have predictive value for diagnosis of PE.

INTRODUCTION

Much attention has been focused on circulating angiogenic growth factors and their binding proteins 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 factors 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 abolish growth factor-stimulated transactivation by sequestering VEGF and PlGF or by forming inactive heterodimers with the transmembrane receptors Flk and Flt-1 (6). The causal argument is that binding of free VEGF and PlGF by excess sFlt-1 in preeclampsia inhibits its beneficial actions on the vascular endothelium, thus permitting the damage that precipitates preeclampsia symptoms. Why sFlt-1 is elevated in preeclamptic pregnancy is not completely understood.

However oxygen tension is a major regulator of the expression of VEGF, PlGF and sFlt-1 in the placenta (7-14). This has contributed to the current hypothesis that placental hypoxia due to underperfusion causes excess production of sFlt-1 and thus the decreases in PlGF and free VEGF reported in pregnancies complicated by preeclampsia.

We directly test this hypothesis using the natural experiment afforded by human residence at high altitude (3600 m) in which maternal arterial PO2 is diminished by 40%, fetal PO2 by 10% and there is clear evidence of maternal, placental and fetal hypoxia (13, 15-17). By hypoxia in mother and fetus, we specifically refer to lowered blood oxygen tension (PO2), as opposed to blood oxygen content, which is greater at high altitude (18, 19). Epidemiological studies support the role of lowered oxygen tension: high altitude is associated with a 2-4 fold increased incidence of preeclampsia and of intrauterine growth restriction (20, 21).

We prospectively tested four hypotheses. First, we predicted that we would find increased sFlt-1 and decreased free VEGF and PlGF in high-altitude pregnancy due to placental hypoxia (10, 11) consistent with the elevated risk of preeclampsia at elevations >2700 m. Second, we hypothesized that variation in these circulating angiogenic growth factors would correlate with biomarkers of maternal and/or fetal hypoxia, e.g. oxygen tension or erythropoietin concentrations. These questions together comprise a test of the hypothesis that imbalance in angiogenic growth factors causes preeclampsia. We would predict that high altitude pregnancies would favor the angiogenic profile observed in preeclampsia, albeit not as severe in pregnancies that remain normotensive. If Angiogenic growth factors in human pregnancy so, it can be argued that the increased incidence of preeclampsia at high altitude is due to an increased number of women being pushed over a “threshold” of imbalance in circulating angiogenic growth factors by hypoxia-mediated changes in the placental production of these factors. Third, we tested to what extent altered concentrations in angiogenic growth factors, if present, may be due to excess secretion by other cell types within the circulation (22). Finally, some reports suggest that fetal sFlt-1 and/or free VEGF are elevated under conditions such as IUGR, maternal preeclampsia and other conditions related to fetal distress (23-27). We therefore tested the fetal cord blood for the factors of interest and examined them in relationship to fetal indicators of oxygen status.

METHODS

Research design, subjects and sites: The data presented here stem from a sub-project within a cross-sectional, prospective study design that was used to evaluate the effects of altitude and genetic

ancestry on uterine blood flow, maternal O2 delivery to the feto-placental unit and pregnancy outcome (16, 17). Genetic ancestry had no impact on the variables reported here and hence this report compares low (400 m n=90) with high altitude pregnancies (3600 m n= 80) with no differentiation by genetic ancestry. All participants gave written, informed consent to the protocols, which were approved by the collaborating Bolivian institution (Instituto Boliviano de Biología de Altura, Consejo Tecnico), the Bolivian National Bioethics Committee and the Institutional Review Board of the New Jersey Medical School. Inclusion criteria were good health (absence of chronic conditions that predispose to preeclampsia e.g. hypertension, renal disease, obesity), conception, gestation and delivery at the altitude of study, and delivery by elective cesarean section. The latter criteria was required to measure uterine and fetal blood flows as close to time of delivery as possible, and to avoid the confounding impact of labor on analyses of
placental tissue planned as part of the larger study. Women were excluded for drug, alcohol or tobacco use, for gestational diabetes or a positive oral glucose tolerance test. A small subset of women were studied in the second trimester, term and >3 months post-partum so that gestational age-dependent changes in VEGF in relation to the non-pregnant values could be evaluated.
Preeclampsia was defined according to NIH consensus guidelines (28). After exclusion of cases in which the relevant clinical data were insufficient for diagnosis there were 20 preeclamptic pregnancies at high altitude and 19 at low altitude. This sample size does not permit division into the most informative hierarchy, i. e. early onset preeclampsia with versus without IUGR and late onset preeclampsia with versus without IUGR. The data are therefore divided into cases in which there was early onset of symptoms and delivery at <35.9 weeks gestational age and late onset, cases in which the onset of symptoms and delivery occurred later than >36 weeks.
Blood collection, measures of oxygen tension: 1-10 days prior to elective cesarean delivery, mothers completed a health screen and medical history. An arterialized blood sample (warmed hand vein) was drawn for measurement of blood gases as previously described (16). Prior to elective cesarean delivery, at the time that the anesthesiologist placed the maternal IV, a 15 ml blood sample was collected and samples intended for analyses of angiogenic growth factors were distributed into serum separator tubes and into tubes containing a mixture of sodium citrate, theophylline, adenosine and dipyridamole (hereafter called CTAD), which prevents *in vitro* platelet activation and release of platelet-derived factors into the plasma. Similar samples were obtained from the doubly clamped cord of the umbilical vein, from which umbilical venous and arterial blood gases were also measured (17). Samples were excluded from analyses if supplemental oxygen was used prior to maternal blood sampling or clamping of the umbilical
cord. Serum samples were allowed to clot for 30 minutes at room temperature. The CTAD tubes were pre-chilled, placed on ice after blood collection, then immediately centrifuged for 10 minutes at 4000 g at 4o C. The standard serum sample was similarly centrifuged but at room temperature. Both serum and CTAD samples were aliquotted, flash frozen in liquid nitrogen and stored at -80°C until analysis. The selection of the CTAD vacutainers was based on reports indicating that failure to control for clotting time invalidates the use of VEGF as an indicator of disease states (29-31).

Assays: All ELISA kits were purchased from or donated by 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.

Validation studies: For each protein of interest we tested linearity by serial dilution of a pooled sample comprised of serum from healthy women who were a minimum of three months postpartum 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 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 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 the 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 samples). 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 undetectable levels of free VEGF, and the remainder had very low values, yielding a small denominator when calculating percentages. For PlGF, 64 samples tested using 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. 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 those are the values reported in the results.

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=36 samples) and 16.2 ± 117.6% where values were lower than 5 pg/ml but above 0 (n= 29 samples). The remaining samples either had one value above and one value below 0 (these were considered non-detectable, as invariably the positive value was < 5 pg/ml). The high CV is due to the very low VEGF concentrations present. For PlGF the intra-assay CV was 3.6 ± 8.9 %. 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 sFlt1.

Free VEGF: The detection limit of the assay is reported as < 5.0 pg/ml. No dilution was necessary. Samples were analyzed from 71 mothers and umbilical venous cord blood from 40 neonates (20 from each altitude). We report here the means of positive values (>0) observed, the number and percent of samples that fell below the 5 pg/ml detection limit, and the number and percent that were non-detectable.

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. We measured a total of 151 maternal serum samples and 40 matched CTAD plasma samples. All 39 preeclamptic mothers had paired serum and CTAD samples. All samples had detectable levels of PlGF and none were below 7 pg/ml. PlGF was not tested in the fetal cord blood due to limited volumes of serum and CTAD plasma.

sFlt-1: Two different assays were used, the R&D Systems Quantikine human sVEGF R1 ELISA kit (DVR100, formulation prior to 2006) and the revised product (as of January 2006). The revised product yielded values ~ 6-fold higher than the older product; correlation between the values of

samples tested with the two kits was acceptable (r2 = 0.88); divergence was at the upper end of the range. The reported detection limit of the assay ranged from 1.5-13.3 pg/ml, with a mean of 3.5 pg/ml. We measured serum and CTAD plasma samples from the 170 normal mothers and umbilical venous cord blood from 135 of their neonates and all 39 preeclamptic mothers and babies. With the old kit a 5-fold dilution was required; 80 maternal samples were assayed using the old kit, 40 from each altitude. With the new kit, a 20-fold dilution was required in the maternal samples, and a 5-fold dilution in the fetal samples. Values reported in table and figures 170 unless otherwise indicated are from the newer R&D formulation (the old kit was discontinued prior to assays completion). All serum samples had detectable levels of sFlt-1 and none were below 13.3 pg/ml. In the preeclamptic samples 20-100 fold dilutions were required to obtain values within the range of the standards, and even then, the outliers indicated in the figures and tables fell outside of this range.

Statistical analysis: For the matched serum versus CTAD plasma samples, a paired t-test was used to determine whether the conditions of blood collection influenced the results. Values for the angiogenic growth factors did not have a Gaussian distribution and were thus analyzed by the Mann Whitney U test to compare between altitudes. The data are presented as box and whiskers plots with the median indicated by the bar, the 25th and 75th centiles by the box, and the maximum and minimum by whiskers. Maternal and fetal demographic and clinical data are reported as the mean ± standard error of the mean. Serial data on free serum VEGF were log-transformed and analyzed using a repeated measures ANOVA followed by the Student Neuman-Keuls test for pair-wise differences (between trimesters and post-partum). Comparison between the normal and preeclamptic subject groups was by ANOVA. Categorical data (e.g. infant sex) was analyzed by chi square. Regression analyses were used to compare the relationship between angiogenic growth factor concentrations, birth or placental weight and maternal or fetal measures of oxygenation. Values were considered significant where p was less than 0.01, utilizing a Bonferroni correction based on the fact that the circulating factors of interest in normal women were tested three times, by altitude, compared with preeclampsia and by serum vs. CTAD blood collection procedures.

RESULTS:

Normotensive participants: Table 1 shows the characteristics of the healthy women and their neonates. Mothers were generally similar in their demographic characteristics, but birth weight was lower in the high-altitude pregnancies despite similarity in gestational age. Adjustment for variation in maternal height, body mass index, weight gain with pregnancy, parity and for neonatal gestational age and sex did not appreciably change the birth weights (Table 1). In short, demographic factors do not explain the altitude-associated decrement in birth weight, which is accompanied by reduced body length and abdominal circumference but preserved head circumference. Mothers at 3600 m were hypoxemic ($p<0.0001$), with an arterial PO_2 of 54±1 mmHg (range 41-72) whilst at 400 m PaO_2 was 93±1 (range 79-115). The fetuses also had lower umbilical venous PO_2 (27±1 at 3600 m, range 15-41 vs 31±1 400 m, range 9-46, $p<0.01$) although the difference was much less than in the mothers (-10% fetal vs. -40% maternal).

Free VEGF: Pregnancy substantially reduces the circulating levels of free VEGF (Figure 1A) at both low and high altitude and does not differ between mid- and late pregnancy. All women had greater levels of free VEGF postpartum, in most cases an order of magnitude higher than their pregnancy values. Serum VEGF concentrations were similar at high versus low altitude at all time points (Figure 1A). Amongst all pregnancy samples 38% of the mothers had non-detectable levels of free VEGF; values less than 5 pg/ml were detected in an additional 24%. We next 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 in a subset of normal, term mothers at each altitude (Figure 1B, n=20 at each altitude) and in the umbilical venous cord blood of their neonates
(Figure1C). CTAD-treatment diminished circulating levels of free VEGF in the mothers by more than 90% at both altitudes. Nearly twice as many CTAD than serum samples had VEGF 215 concentrations that were undetectable (68%) or below the detection limit of 5 pg/ml (90% Figure 1B). While fetal cord blood serum concentrations of VEGF were >100 fold greater than in their mothers (Figure 1C), collection of fetal blood into CTAD vacutainers abolished free VEGF in 60% of the samples and lowered values to < 5 pg/ml in an additional 20%. The remaining positive values were decreased by more than 100-fold (Figure 1C). Maternal and fetal VEGF concentrations did not correlate with placental or birth weight. Fetal VEGF concentrations did not correlate with that of their mothers.
Thus the presence of free VEGF in both maternal and fetal blood is largely an artifact of the method used for blood collection; the majority of samples do not show detectable levels of VEGF and those few that do are extremely low. PlGF: PlGF was similar at 400 and 3600 m, not decreased at high altitude as predicted (Figure 2A). The values obtained from CTAD samples also did not differ by altitude (Figure 2B). However CTAD treatment decreased PlGF by more than one-third relative to the paired serum samples (Figure 2B). At both altitudes, maternal PlGF concentrations were positively correlated with placental weight (Figure 2C).
sFlt-1: An initial series of maternal serum samples (n=40 per altitude), using the original assay kit from R&D (pre January 2006) showed that altitude elevated maternal circulating sFlt-1 in serum samples (Figure 3A left panel $p<0.01$) replicating our results from a prior study with fewer mothers at a lower altitude (14). However when this assay was repeated using the new sFlt-1 assay on the same serum samples, the altitude-associated difference did not attain significance (Figure 3A right panel). The newer R&D formulation, designed to increase detection sensitivity, raised absolute sFlt-1 values by ~6-fold, but associated with this was an increase in variability that eliminated the prior, statistically significant result at high altitude. Because the old kits were discontinued, comparison of serum with CTAD samples was completed with the new formulation only. Collection of blood into CTAD tubes reduced sFlt-1 concentrations in healthy pregnant women by 22±3% and 25±4% at 400 and 3600 m, respectively (Figure 3B).
In fetal serum samples sFlt-1 values were lower than in the mothers, but did not differ between attitudes in either the serum or CTAD samples (Figure 3C). CTAD treatment decreased cord blood sFlt-1 concentrations. The CTAD-associated decrease in sFlt-1 in cord blood tended to be greater at 400 than 3600 m (-72±9 % vs. -44 ± 10%, $p = 0.06$, Figure 3C). sFlt-1 concentrations were not related to birth or placental weight, nor were they correlated between mother and fetus.
In summary, the results for these three angiogenic growth factors show that none differ by altitude in healthy, normal pregnancies. Moreover, essentially all circulating free VEGF, a third of circulating PlGF and a quarter of sFlt-1 in serum samples collected under standard clinical conditions is not derived from the placenta, but from other cells in the maternal and fetal circulation. The degree to which hemostasis alters growth factor concentrations is highly variable from individual to individual precluding any form of standard correction for hemostatic effects.Finally, PlGF shows a strong relationship with placental weight, suggesting that placental size is a primary determinant of maternal circulating concentrations of this growth factor.
Participants with Preeclampsia: The characteristics of and clinical data pertinent to the mothers and infants in the preeclampsia samples are given in Table 3. As would be expected, gestational age at

delivery, birth and placental weights were lower than in normotensive women in both the early and the late-onset preeclamptic cohorts. The median and interquartile ranges for PlGF and sFlt-1 are also given in Table 3 for the early and late-onset patients, but as there was no significant difference between these groups, the data were consolidated for figures and statistical analyses. Maternal oxygen tension in preeclamptics was 93 ± 3 (range 80-114 mmHg) at 400 m and 59 ± 3 (range 44-75 mmHg at 3600 m); these values are similar to those obtained in the normotensive controls within each altitude. Fetal PO2 was lower in the fetuses of preeclamptic women than controls at both altitudes (400 m, 24 ± 2, range 13-35 mmHg) (3600 m, 23 ± 2, range 10 – 31 mmHg).

VEGF and PE: Our results show that free VEGF in human pregnancy is largely an artifact of blood collection. We therefore did not measure VEGF in the preeclamptic women.

PlGF and PE: There were no altitude-associated differences in PlGF concentrations among the preeclamptic women, but preeclamptic women had lower PlGF concentrations than their normotensive counterparts ($p<0.001$), regardless of altitude (Figure 4A). Early vs. late onset preeclampsia did not differ in PlGF concentrations (Table 2). Collection into CTAD reduced the PlGF values in preeclamptics at each altitude (Figure 4B, $p<0.001$) by 25± 5 % at low altitude and by 32 ± 4% at high altitude ($p=0.72$). The correlation between PlGF and placental weight was significant at both low and high altitude in preeclampsia (Figure 4C). The slopes of the relationship between placental weight and PlGF did not differ between the normotensive and preeclamptic groups ($p=0.24$). Lowered PlGF in preeclamptic pregnancy is possibly due to the smaller placental size associated with this pathology, rather than to differences in oxygenation or placental function.

sFlt-1 and PE: Soluble Flt-1 serum levels did not differ in preeclamptic women at low vs. high altitude (Figure 5A). Collection into CTAD reduced sFlt-1 levels by 62 ± 11% at 400 m and 94 ± 15% at 3600 m (Figure 5B, $p<0.005$). While these values do not differ from each other, the CTAD-associated reduction in sFlt-1 is ~3-5 fold greater among preeclamptics than what was observed in the normotensive women ($p<0.005$). There was no difference in sFlt-1 levels in early versus late-onset preeclamptic women (Table 2), nor did the decrement in sFlt-1 vary by severity of disease, although the variability in early onset cases was much greater than late-onset (Figure 5B).

Associations between variables: We tested the hypothesis that variation in maternal and fetal oxygen tension might be related to variation in the maternal or fetal circulating levels of angiogenic growth factors. We conducted regression analyses of maternal PO2, arterial O2 content and erythropoietin levels and of fetal umbilical venous, arterial PO2, O2 content and 290 erythropoietin (X axis) with PlGF and sFlt-1 concentrations (Y axis). These showed no meaningful associations, with the r2 values ranging from 0.00 – 0.12. This was true whether considered in relation to the entire sample, or within each altitude. The hypothesis that variation in circulating angiogenic growth factors might be related to differences in maternal or fetal oxygenation was thus rejected.

DISCUSSION:

None of the hypotheses tested were supported. Free VEGF, PLGF and their soluble binding protein, sFlt-1, did not differ in low vs. high altitude pregnancies despite maternal, fetal and placental hypoxia at 3600 m. Oxygen tension, content, and biomarkers of hypoxic stimulus like erythropoietin were not related to maternal or fetal circulating angiogenic growth factors. Hypoxemia, across the extended physiological range of 41 - >100 mmHg arterial PO2 in mothers and 9 - 46 mmHg in fetuses does not cause appreciable variation in circulating angiogenic growth factors. For the first time, we report that more than 90% of circulating free VEGF, >30% of PlGF and >25% of sFlt1 was secreted into the test samples as a consequence of hemostasis and is therefore an artifact of blood collection technique.

These growth factors are released by other cell types within the circulation and therefore their excess or insufficiency in pregnancy pathologies does not necessarily reflect changes in placental production. We report here, also for the first time, that there is greater release of sFlt-1 from these non-placental sources in preeclamptic than normotensive pregnant women. We found that PlGF is correlated with placental weight in both normotensive and preeclamptic pregnancy. This suggests that decrement in PlGF in preeclampsia might be a correlate of the smaller placental size characteristiocs of preeclamptic pregnancy rather than a diagnostic feature causally associated with the development of endothelial cell dysfunction. 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, low already, are also very nearly abolished by similar precautions. Studies suggesting deficit in free VEGF in the maternal circulation is a causal factor in preeclampsia should be also rejected; free VEGF in the maternal circulation essentially does not exist, and we predict more recent studies arguing that PlGF and/or sENG are instead the causal culprits in preeclampsia (5, 32) will be equally discounted in future. We conclude from the discussion below and the data presented above that the role of angiogenic growth factors in preeclampsia is similar to that of numerous other circulating factors invoked now and in previous decades, a sequelae of the disease process, an artifact, or a correlate, but not a causal event. Such prior factors include, e.g., worms (33), coagulation factors (34, 35), thromboxane/prostaclyclin imbalance (36, 37), STBMs (38, 39), prooxidants (40, 41), uric acid (42, 43), etc.

Challenges to the interpretation of these results include differences between the collection sites, the combining of two different ethnic groups at each altitude, the variability in the assays themselves, and the recent discovery of additional splice variants of sFlt-1. Ambient temperature and barometric pressure were recorded as part of the blood gas measurement protocol. The 3600m site was cooler and had less annual temperature fluctuation than the 400 m site. If differences in ambient temperature were important this would be more apparent in serum samples as they were allowed to clot and were processed at room temperature whilst the CTAD samples were collected into pre-chilled tubes, transported on ice and centrifuged at 4o C. We tested the serum values against ambient room temperature and found no correlation, nor was any relationship apparent when using cruder measures such as comparing values obtained during summer versus winter. Women of Native American (Andean) ancestry suffer less altitude-associated growth restriction than European migrants, and in theory this might be reflected in their circulating angiogenic growth factor profile. However, we found no evidence to support ancestry-associated differences in the circulating markers of interest. Finally, a close review of the literature suggests that the issue of variability in the assay results deserves closer attention. R&D systems, the manufacturer of the most widely used assay kits, reports their assay coefficients of variation (CVs) based on 20 and 40 replicates for intra- and inter-assay variation, respectively. We recalculated R&D's potential CV at their low, moderate and high standard concentrations, assuming only duplicates rather than 20-40 replicates were tested. (CV was calculated as ± 3 SDs of the mean subtracted from the reported mean and divided by that mean). The potential intra- assay CV is 14-20% for free VEGF, 17-26% for PlGF and 8-12% for sFlt-1. Even this estimate is minimal as R&D tested nonpregnant subjects, whose concentrations for VEGF which are 10-100 fold higher and for sFlt-1 as much as 10-fold lower than values observed in pregnancy. The high CV we report for VEGF is due to the extremely low values measured. For PlGF and sFlt-1 the CVs we report of ~4 ± 9% and 3 ± 6% are acceptable, but the difference in statistical significance for the impact of altitude between the old vs. new formulation for the sFlt-1 assay highlights the issues

discussed above. In summary, variability in the assays themselves, the testing conditions, blood collection protocols, length of storage etc., are all likely to contribute to the heterogeneity in results reported in the relevant literature and may even have obscured our ability to detect hypoxia-associated differences in the present study. Arguing against this is the lack of any correlation between our independent measures of hypoxia and the proteins tested. We are aware that additional splice variants of sFlt-1 have been discovered recently (44), are produced by the hypoxic placental trophoblast (45, 46) and that it is unknown whether current assays measuring sFlt-1 distinguish between these splice variants. Nonetheless we suspect that as the role of endothelial and monocyte secretion of these variants is researched, and further refinement of the mechanisms of interaction between sFlt-1 and activated or otherwise stressed edndothelial cells is accomplished, the argument we raise here against the causal role of agiogenic growth factors in the syndrome of preeclampsia will be supported.

The high-altitude pregnancy model has been useful for testing causal hypotheses in preeclampsia, and for dissociation of normal physiological adaptation to lowered placental oxygen tension from pathological features of preeclampsia. Using the altitude model, we have shown that multiple proteins known to be up-regulated by hypoxia are increased in the high altitude placental tissue, including sFlt-1 (14) and total VEGF (15), observations also supported by *in vitro* studies (8, 12).

We have further shown that global gene expression in the high altitude placenta closely parallels that observed in *in vitro* hypoxia and in preeclamptic placentae (13). VEGF and sFlt-1 are regulated, in part, by HIF-1a. Consistent with this we have shown that placental HIF-1a message and protein are elevated in high-altitude placentae and that protein levels are positively correlated with total VEGF and erythropoietin in the maternal circulation (15). However this similarity between the global profile of placental hypoxia at high altitude and in preeclampsia does not translate into increases in maternal circulating concentrations of angiogenic growth factors in normal high-altitude pregnancy. This contrasts with numerous other physiological parameters where we have shown that altitude values are intermediate between preeclampsia and normalpregnancy (reviewed in (19).

The idea that deficit in free VEGF causes endothelial cell damage in pregnancy is counterintuitive. The normal adult levels of circulating free VEGF are ~100 pg/ml. Maynard and colleagues found that free VEGF was ~13 pg/ml in 11 normotensive women, and ~6 in the 21 preeclamptics studied. However, these values are at or close to the limit of detection and given our and others' results some proportion of the samples must have had non-detectable VEGF. This and other widely cited reports do not indicate what proportion of values for free VEGF were below the detection limit of the assay, nor do they state how values less than the detection limit were analyzed (3, 4). A more interesting question is why is free VEGF abolished in both normal and preeclamptic pregnancy? In theory, lower free VEGF should be beneficial. Hypertension, glomerulosis, altered vascular reactivity and vascular leak are the hallmarks of preeclampsia. But circulating VEGF is elevated, not decreased in these conditions (47-51). It is therefore an excess of free VEGF and not diminution which should contribute to development of preeclampsia. It has been claimed that exogenous administration of free VEGF rescues a preeclampsia-like phenotype

in experimental animals exposed to an excess of sFlt1. However the dose of free VEGF administered was equivalent to what is present in the non-pregnant adult, not what is normal in pregnancy (3, 52). The model does not mimic human preeclampsia as the preeclamptic phenotype was present both in non-pregnant and pregnant animals. This might render the model useful for studying glomerulosis, but this renal lesion is not necessarily pathognomic for preeclampsia (53).

Studies of genetic kidney disease reveal the lesion is due to excess free VEGF-A(165) (54). Since free VEGF is lower in the circulation of preeclamptic mothers it should protect against this lesion. Given that free VEGF is virtually abolished in pregnancy, it appears that the elevation in sFlt-1 and subsequent sequestration of free VEGF must, in general, be beneficial and part of normal pregnancy adaptation, rather than pathological.

PlGF is a homologue of VEGF, differing in that it can bind only to the receptor tyrosine kinase VEGFR-1 (Flt-1) and not the KDR/Flk 1 receptor. Normal adult female levels of PlGF are <50 pg/ml and rise ~10 fold during pregnancy before falling in the third trimester (55). The rise in PlGF in the maternal circulation in the early second trimester is exponential, correlates with Doppler indicators of placental perfusion and likely reflects placental perfusion as well as increase in mass (56). An alternative interpretation is that the opening of the maternal intervillous space to blood flow, which occurs at approximately 10-12 weeks gestation, induces a rise in PlGF due to shear stress or stretch (57, 58). Either way, hypo-perfusion due to impaired development of the spiral arteries and/or failed growth of the placenta, common in severe preeclampsia, may account for the relatively lower levels of PlGF in preeclamptic pregnancy without necessarily having any impact on the maternal endothelium or on the development of symptoms.

An excess of PlGF, like VEGF, is implicated in hypertension and vascular disease (59). Stretch, shear stress, hypoxia and pro-inflmammatory stimuli will induce PlGF expression (57, 58, 60-62). PlGF is an important modifier of VEGF's interaction with endothelial cells, potentiating the mitogenic and permeabilizing effects of the VEGF family of proteins on endothelium (63-65). Human atherosclerotic lesions producing excess PlGF are associated with plaque inflammation and neovascular growth (66, 67). In fact PlGF is known to stimulate monocyte production of proinflammatory cytokines (68), cytokines that are elevated in preeclampsia (69). Hence the evidence favors that preeclampsia should be accompanied by increased, not decreased PlGF. Again the question must be asked, what does a relative paucity of PlGF actually do to endothelial cell health in pregnancy?

Soluble fms-like tyrosine kinase-1 (sFlt-1) is a splice variant of VEGF receptor 1. Its existence was hypothesized when the gene for VEGFR-1 was discovered (70), and the protein identified in umbilical vein endothelial cell supernatant in 1993 (71) Production by the placenta in was first reported in 1998 (72) while elevation of sFlt-1 in preeclampsia and by hypoxia was reported as early as 2000 (2, 11). sFlt-1 levels are stable during the early and middle stages of gestation, increase until term (73), and decrease rapidly following delivery (3). In normal pregnancy rise in sFlt-1 correlates with the 3rd trimester rise in blood pressure (74). No such relation is observed in preeclampsia, nonetheless, antihypertensive therapy in preeclamptics reduces sFlt-1 levels (75). There appear to be multiple causes of the elevation in sFlt-1 in preeclampsia apart from hypoxia (14, 76, 77) and feedback relationships appear to exist with inflammation and oxidative stress (78, 79). Consistent with this report, but not quantified as to proportional representation in the maternal circulation, secretion of sFlt-1 by endothelial cells and monocytes was first reported in 2001 (80), and confirmed in preeclampsia more recently (22). Excess concentrations of angiogenic growth factors have been measured in platelet lysates from patients with hypertension (51), thus there is support for the idea that excess secretion of angiogenic growth factors by peripheral cells in preeclamptics may be secondary to hypertension, and not an initiating event in the disease process. Recent reports, consistent with this one, argue against hypoxia as contributing to excess circulating sFlt-1 and instead invoke inflammation (81). This is misleading as hypoxia, oxidative stress and inflammation are all related phenomenon.

As with VEGF and PlGF the damaging role claimed for elevated sFlt-1 in preeclamptic pregnancy is counter-intuitive. sFlt-1 is lower in hypertensive men, and elevated by therapies designed to ameliorate cardiovascular risk. Again, the high altitude model sheds some insight into these hypothetical causal relationships. We have shown an excess of pro-inflammatory cytokines in normal high altitude pregnancy (82) but less oxidative stress in high altitude placentae (83). The former should contribute to a an angiogenic profile favoring preeclampsia while the latter should favor the opposite, neither of which is reflected in the results reported here.

To date no single, nor any combination of factors is definitively present in all cases of preeclampsia, no matter how narrowly the disease is defined (84) (35). Preeclampsia represents a spectrum of disease, with a variable degree of expression in the correlating biochemical markers, that appears to be modified by environment and individual susceptibility (85). Our results do not invalidate the hypothesis that circulating angiogenic growth factors may be involved in the endothelial dysfunction postulated as the proximate cause of the clinical symptoms of preeclampsia (86). However they do support the accumulating evidence in the literature that these proteins play a correlative rather than causal role. Some groups have shown changes in the mean values of maternal circulating angiogenic growth factors several weeks prior to the onset of symptoms (4, 87, 88), while others have not (89). Others have shown that the values obtained have low sensitivity and specificity (55, 90), are unrelated to markers of endothelial cell dysfunction (91) and that changes are due to increase in blood pressure rather than the reverse (74). We found that PlGF in preeclamptics fell within the range of normotensive women in 88% of the subjects at 400 m and 100% at 3600 m. Among preeclamptic subjects, sFlt-1 values were within the range of the normotensive women in 62% at 400 m and 35% at 3600 m. These significant overlaps combined with the variable change in the degree to which activation of peripheral cells contributes to the values measured ex vivo suggests that circulating values are unlikely to attain a sufficient degree of specificity and sensitivity for use as a reliable diagnostic test. Finally, our results argue against hypoxia as causally associated with changes in circulating angiogenic growth factors: lowered maternal or fetal PO2 do not appear to cause an increase in tissue production and release into the circulation of the 3 angiogenic growth factors tested here. Greater attention should be paid in future to alternative explanations for excess or deficit in biomarkers in a variety of disease states. In summary, much of the relationship between maternal circulating angiogenic growth factors and preeclampsia needs to be reconsidered. 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 factors may influence endothelial function to protect against or exacerbate preeclampsia symptoms.

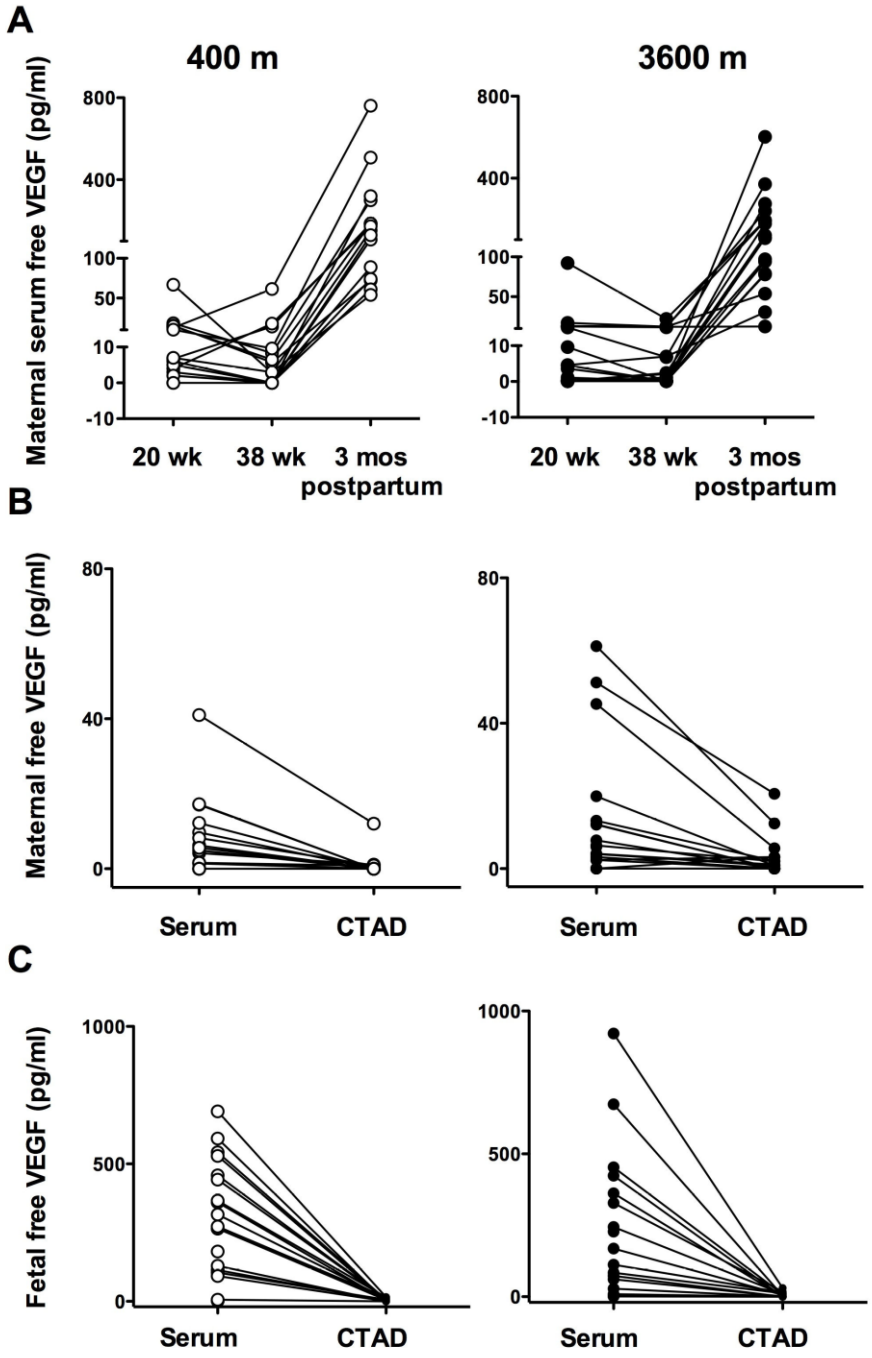
Table 2. Maternal and infant characteristics in preeclamptic pregnancies

Maternal characteristics	400 m early onset PE (n=7)	400 m late onset PE (n=12)	3600 m early onset PE (n=10)	3600 m late onset PE (n=10)	p values, all PE relative to altitude-specific controls
Age (years)	26 ± 2	25 ± 1	29 ± 2	27 ± 2	p<0.005
Primiparous (n)	6/7	10/10	9/10	9/10	P<0.0001

Height (cm)	156 ± 3	160 ± 3	160 ± 2	160 ± 3	P = 0.39
Non-pregnant weight (kg)	62 ± 5	62 ± 3	66 ± 5	65 ± 4	p=0.18
Non-pregnant Body Mass Index (kg/m 2)	25.4 ± 1.8	24.2 ± 1.1	27.0 ± 1.7	25.4 ± 1.6	p=0.15
Weight gain with pregnancy (kg)	13.3 ± 3.9	16.8 ± 1.4	11.6 ± 2.5	8.9 ± 2.5	P=0.71
Systolic BP*	138 ± 7	132 ± 5	135 ± 3	140 ± 3	P<0.0001
Diastolic BP*	98 ± 5	92 ± 5	102 ± 5	96 ± 3	P<0.0001
MAP*	125 ± 6	118 ± 5	124 ± 4	125 ± 3	P<0.0001
Infant characteristics					
Birth weight (grams)	2236 ± 225	2895 ± 112	1680 ± 161	2437 ± 99	p <0.0001
Placental weight (g)	269 ± 35	358 ± 27	316 ± 27	324 ± 35	p <0.0001
Birth/placental weight ratio	8.4 ± 0.5	8.3 ± 0.5	5.4 ± 0.4	8.1 ± 0.6	p < 0.0001
Clinically assessed	34.3 ± 0.7	37.0 ± 0.5	33.8 ± 0.6	36.7 ± 0.3	p <0.0001
gestational age wks)					
Sex ratio M/F	2/5	5/7	6/4	8/2	
Serum values (median, inter-quartile range)					
PlGF (pg/ml)	168 (121, 221)	176 (148, 292)	157 (94, 266)	142 (90, 208)	p<0.05 LA p<0.001 HA
sFlt-1 (ng/ml)	84.4 [33.6, 214.5]	29.4 [8.1, 88.4]	57.8 [25.4, 122.3]	24.6 [6.1, 86.0]	p<0.001 LA p<0.001 HA

** All women were taking anti-hypertensive medication, methyl-dopa, 500 mg/day, 485 clinical standard of care in Bolivia

Figure 1

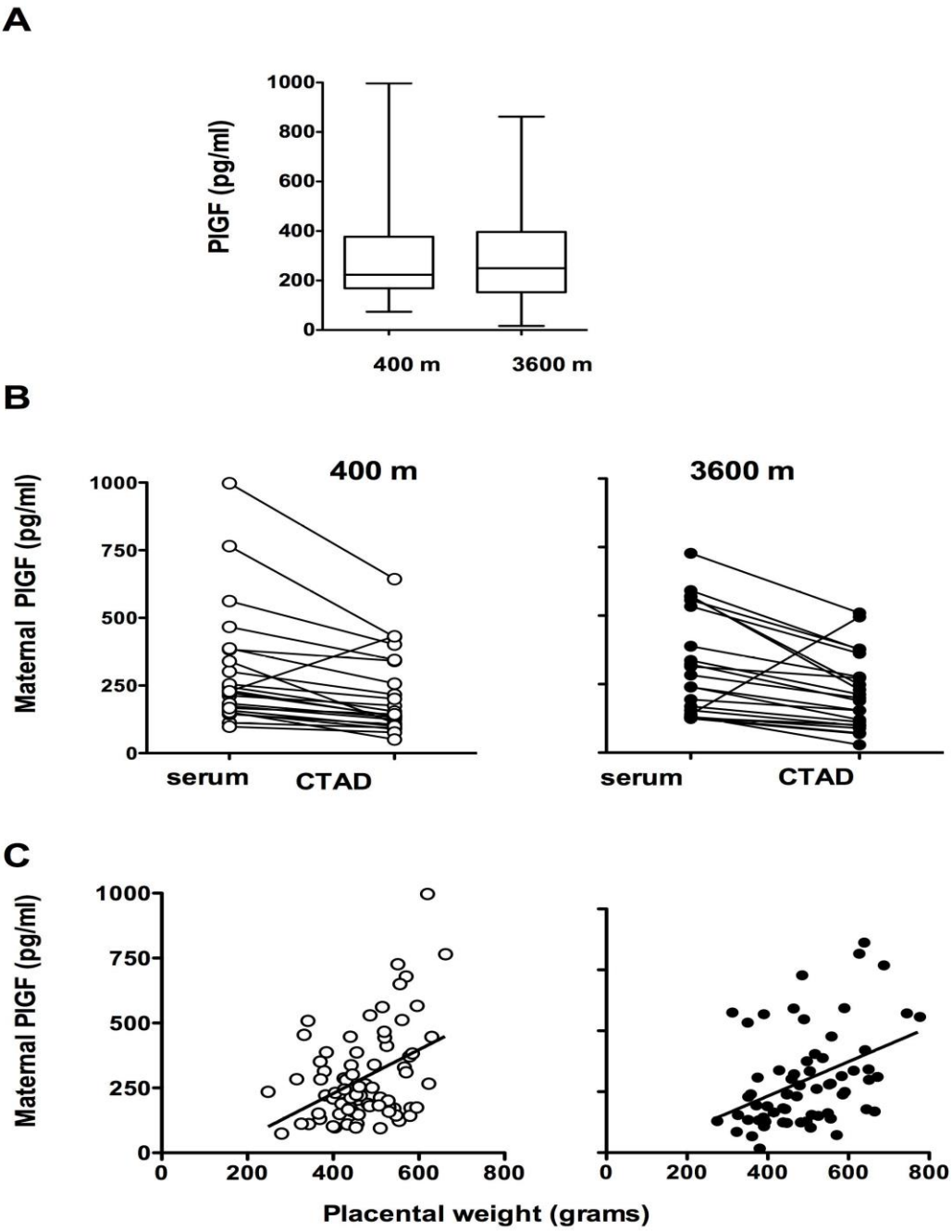


A: In women studied at 20, 38 weeks of pregnancy and >3 months postpartum, free VEGF concentrations were reduced during pregnancy ($p<0.0001$). Values were similar in the second vs. third trimester. Values measured during pregnancy and postpartum did not differ between low (left panel) and high altitude (right panel). In both the pregnant and non-pregnant condition there were >10-fold differences between the highest and lowest values measured (pregnant range = 0 – 82.0, non-pregnant range 30.5 – 754.4 pg/ml).

B: Collection of blood into CTAD (see methods) reduced values for maternal free VEGF by 98 ± 1% at 400 m ($p<0.001$ left panel) and by 87 ± 6% at 3600 m ($p<0.001$ right panel) The magnitude of the decrease was similar at both altitudes ($p=0.11$).

C: Umbilical venous blood concentrations of VEGF were >10 fold greater in the fetuses than in their mothers, and did not differ at low (left panel) versus high altitude (right panel). Collection into CTAD reduced the free VEGF concentrations by 98±1% at 400 m and by 92±4% at high altitude ($p<0.0001$). Nil values and those below the detection limit of the kit occurred in 80% of the samples. The decrement in VEGF concentrations attributable to CTAD treatment did not differ between altitudes ($p=0.09$).

Figure 2

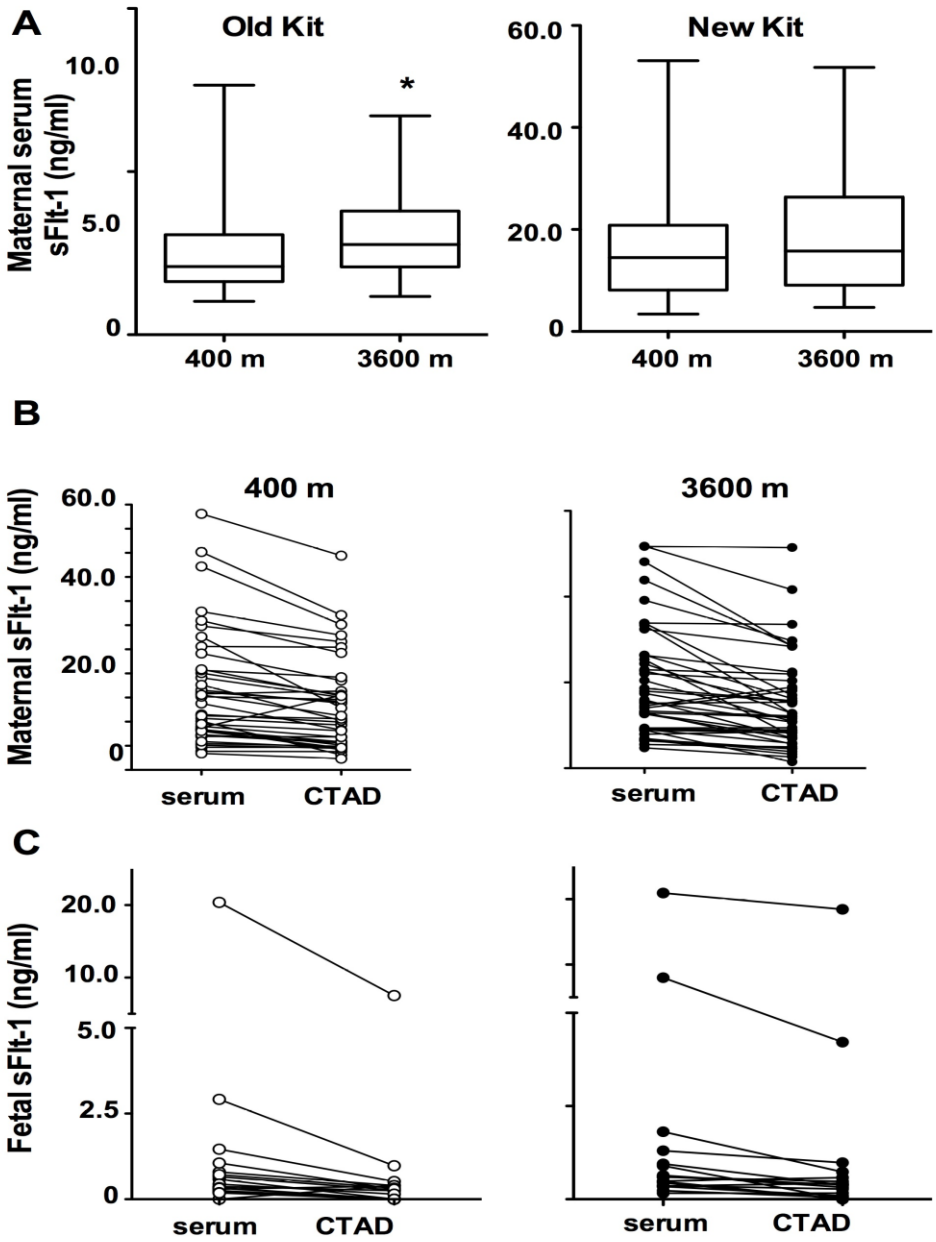


A: PlGF concentrations were similar at low versus high altitude (p=0.93).

B: CTAD treatment decreased PlGF by 32±3% at 400 m and 26± 13% at 3600 m ($p<0.0001$ at each altitude). Elimination of the one sample at each altitude that showed 0 greater PlGF concentration in CTAD than serum samples (which could potentially be due to mislabeled tubes) yielded a decline in PlGF concentrations of 36 ± 2% at 400 m and 38 ± 3% at 3600 m, (range 11-78%). This decrement did not differ between altitudes (p=0.59).

C: The serum values for PlGF were positively correlated with placental weight (400 m $Y= -92.3 + 0.82x$, $r2 = 0.19$, $p<0.0001$; 3600 m $Y = -68.9 + 0.76x$, $r2 = 0.19$, $p<0.0001$). The slopes (p=0.64) and intercepts (p=0.81) did not differ.

Figure 3

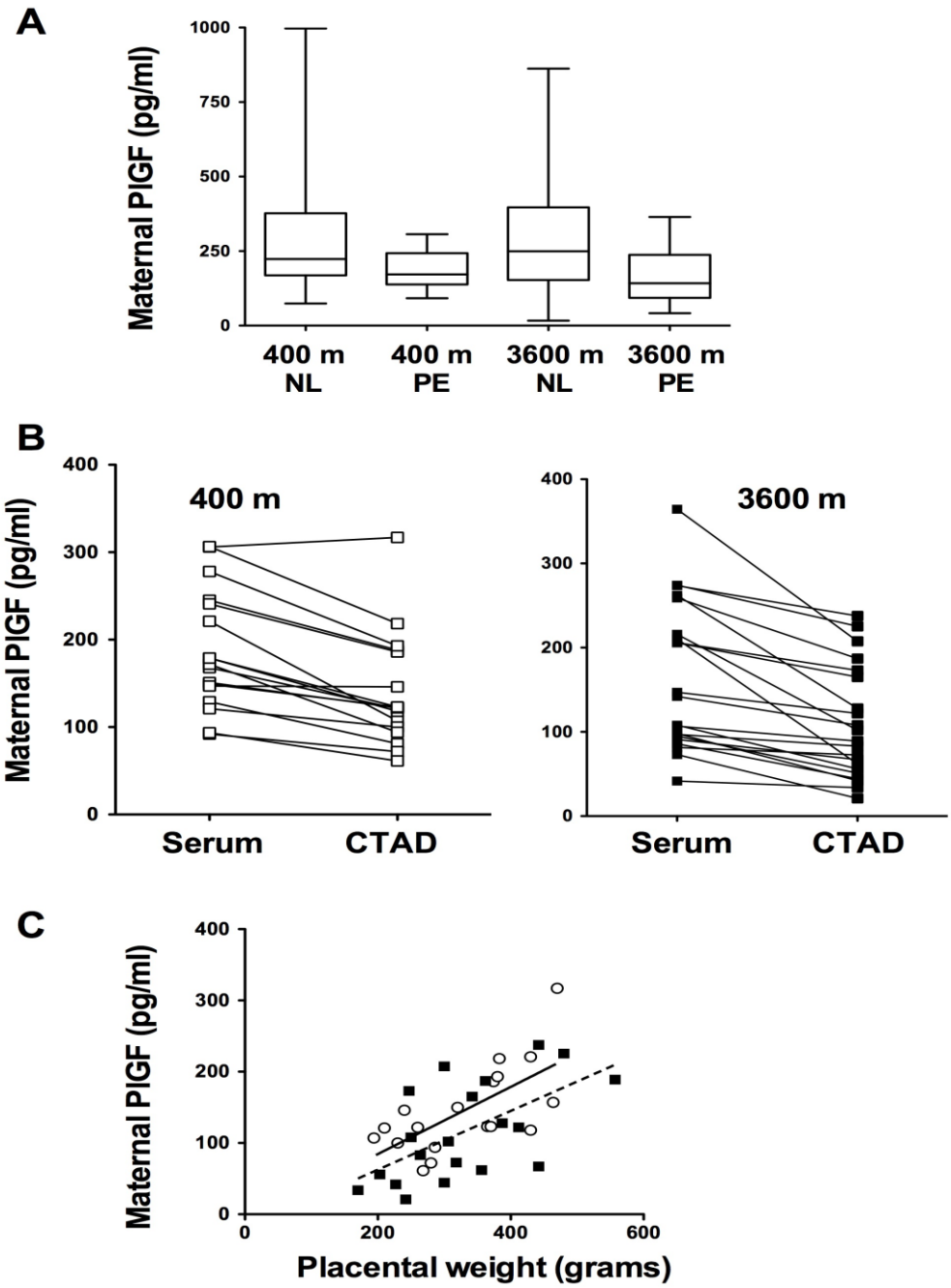


A: When measured using the R&D kit formulated prior to January 2006, sFlt-1 concentrations were greater at high than low altitude (left panel). Using the newer formulation with the same samples increased sFlt1, but this increase was highly variable, ranging from 89% - >1000%. This variability eliminated the statistically significant found using the prior formulation on the same samples (right panel p=0.21).

B: Collection of blood into CTAD decreased PlGF concentrations ($p<0.0001$ each altitude). The decrement was similar at low (left panel) versus high altitude (right panel p=0.47)

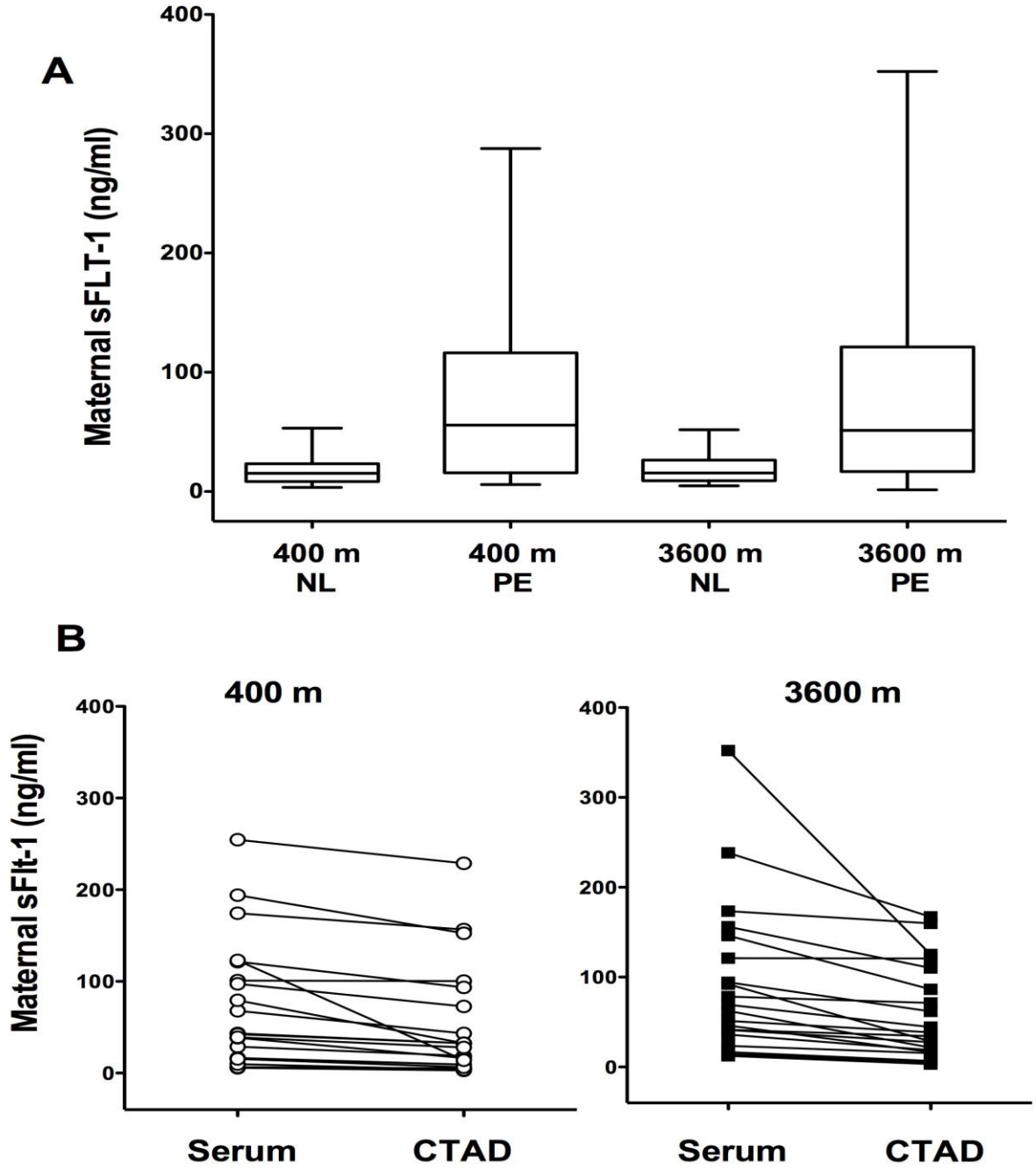
C: The cord blood levels of sFlt1 were lower than in the mothers. Fetal serum sFlt1 did not differ between altitudes in either serum (p=0.47) or CTAD samples (p=0.29). Reduction in sFlt1 attributable to treatment with CTAD tended to be greater at 400 m (72 ± 8%) than 3600 m (52 ± 10%, p=0.02).

FIGURA 4



A: PlGF values were lower in preeclamptic than normotensive women at both low and high altitude, but there was no altitude-associated difference in the values for PlGF in preeclamptics.
B: Collection of samples into CTAD reduced values for PlGF in preeclamptic women at both 400 m (right panel) and 3600 m (left panel).
C: As with normotensive women, PlGF concentrations in preeclamptics correlated with placental weight

FIGURA 5



A: Soluble Flt-1 serum levels were greater than in normotensive women at each altitide, but did not differ in preeclamptic women at low (43, [16, 99] vs. high altitude (66 [38, 133] (p=0.33).
B: Collection into CTAD reduced sFlt-1 levels by 36 ± 5% at 400 m and 42 ± 5% at 3600m (Figure 5B, p<0.005). While these values do not differ from each other, the CTADassociated reduction in sFlt1 is significantly greater among preeclamptics than what was observed in the normotensive women (22 ± 3% at 400 m and 25 ± 4% at 3600 m, p<0.005).

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