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ORIGINAL ARTICLE

Uteroplacental Ischemia Is Associated with Increased PAPP-A2

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Abstract

Residence at high altitude (> 2500 m) has been associated with an increased frequency of preeclampsia. Pappalysin-2 (PAPP-A2) is an insulin-like growth factor binding protein-5 (IGFBP-5) protease that is elevated in preeclampsia, and up-regulated by hypoxia in placental explants. The relationships between PAPP-A2, altitude, and indices of uteroplacental ischemia are unknown. We aimed to evaluate the association of altitude, preeclampsia, and uterine artery flow or vascular resistance with PAPP-A2 levels. PAPP-A2, uterine artery diameter, volumetric blood flow, and pulsatility indices were measured longitudinally in normotensive Andean women residing at low or high altitudes in Bolivia and in a separate Andean high-altitude cohort with or without preeclampsia. PAPP-A2 levels increased with advancing gestation, with the rise tending to be greater at high compared to low altitude, and higher in early-onset preeclamptic compared to normotensive women at high altitude. Uterine artery blood flow was markedly lower and pulsatility index higher in early-onset preeclamptic normotensive women compared to normotensive women. PAPP-A2 was unrelated to uterine artery pulsatility index in normotensive women but positively correlated in the early-onset preeclampsia cases. We concluded that PAPP-A2 is elevated at high altitude and especially in cases of early-onset preeclampsia with Doppler indices of uteroplacental ischemia.

Keywords High altitude · Hypoxia · PAPP-A2 · Pappalysin-2 · Preeclampsia · Uterine artery pulsatility index and blood flow

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Introduction

The hypoxia of residence at high altitude (above 2500 m, 8250 ft) reduces infant birth weight [1], due primarily to slowing of fetal growth [2]. Not all high-altitude residents, however, are equally affected. Multigenerational populations (Tibetans and Andeans) exhibit half the birth weight reduction and maintain higher levels of uterine artery blood flow compared with shorter-resident groups (Han and European) [3–7]. Lower uterine artery blood flow is also associated with preeclampsia [8], a disease that complicates 3–8% of pregnancies, contributes to 15% of preterm births and 10–15% of maternal deaths, and accounts for an even greater proportion of adverse pregnancy outcomes especially in the developing world [9, 10]. Preeclampsia is diagnosed on the basis of new-onset hypertension and end-organ system involvement, namely, proteinuria, thrombocytopenia, impaired liver function, renal insufficiency, pulmonary edema, or cerebral/visual disturbances [11]. Of interest, the incidence of preeclampsia is increased two- to fourfold at high altitudes in Colorado and Bolivia [1, 12, 13] and has also been reported in Tibetans [14], suggesting that the protection afforded long-resident high-altitude populations from fetal growth restriction does not extend to preeclampsia, although existing data are sparse.

Pappalysin-2 (PAPP-A2) is an insulin-like growth factor binding protein-5 (IGFBP-5) protease that is elevated in preeclampsia [15–19]. Given the late appearance of symptoms and minimal treatment options for preeclampsia, concerted efforts have been made to identify biomarkers for earlier detection. Insulin growth factors are thought to play important roles in stimulating extravillous trophoblast invasion and remodeling of uterine spiral arteries [20, 21]. Their bioavailability is regulated by insulin-like binding proteins (IGFBPs) and associated proteases [21–23]. Pregnancy-associated plasma protein A2 (PAPP-A2) is one such protease whose natural substrate is IGFBP-5 and, to a lesser extent, IGFBP-3. In normal pregnancy, placental PAPP-A2 expression is robust in the first trimester, decreases progressively in the second trimester, and then rises again in the third trimester [24]. Assuming that PAPP-A2 cleaves IGFBP5 and releases IGF to potentiate trophoblast invasion and vascular remodeling, then the elevation seen in preeclampsia may reflect a compensatory attempt to stimulate uterine vascular growth/remodeling or reduce resistance in downstream vessels (i.e., spiral or myometrial arteries).

PAPP-A2 levels are upregulated by hypoxia in placental explants [25, 26] but whether circulating PAPP-A2 levels are increased by hypoxia alone or related to uterine artery blood flow and/or indices of uterine vascular resistance is unknown. We therefore measured PAPP-A2 longitudinally in a cohort of normotensive Andean women residing either at low (400 m) or high (3600 m) altitudes in Bolivia and in a separate cohort of Andeans living at 4100 m who either had been diagnosed with preeclampsia or were normotensive controls. In both cohorts, we measured uterine artery diameters, volumetric blood flow, and pulsatility indices to determine their relationship with PAPP-A2 levels. We considered that these studies would provide further insight into the role of PAPP-A2 in the pathogenesis of preeclampsia and determine if chronic hypoxia altered PAPP-A2 levels and if levels influenced vascular growth/remodeling or vasoreactivity as reflected by their association with uterine artery Doppler indices.

Methods

Subjects

Two cohorts of subjects participated, each with informed consent to procedures approved by the institutional review boards at the University of Colorado and the Colegio Médico in Bolivia. The first was comprised of longitudinally studied women at pregnancy weeks 20 and 36 that were a subgroup of those reported previously [4, 27]. All women were of Andean descent and lived either at low (400 m, $n = 9$) or high altitude (3600 m, $n = 23$). The second cohort was studied during the same time period and consisted of normotensive ($n = 22$) or preeclamptic ($n = 26$) Andean residents of high altitude (4100 m) studied on a single occasion between pregnancy weeks 23 and 42. Preeclamptic women were divided into subgroups based on whether they were diagnosed $<$ or $\geq$ week 34 (early onset, $n = 7$, late onset, $n = 19$, respectively). For all subjects, inclusion criteria were singleton gestation, the absence of known risk factors for pregnancy complications (diabetes, chronic hypertension, obesity, history of preeclampsia in a prior pregnancy, autoimmune diseases), and no known non-Andean ancestors. Self-reported ancestry was confirmed with a panel of 100 ancestry-informative genetic markers as previously described [4, 27].

Studies at high altitude were performed at the Instituto Boliviano de Biología de Altura in La Paz (3600 m) or the Hospital Boliviano Holandés in El Alto, Bolivia (4100 m), and low-altitude studies at the Clinica Siraní in Santa Cruz, Bolivia (400 m). Each woman completed a questionnaire to determine her self-identified ancestry; altitude of birth, childhood, and current residence; reproductive and medical history; household income; and pre-pregnancy body weight. Maternal height was measured and a blood sample withdrawn using standard venipuncture into EDTA tubes (BD Vacutainer®), centrifuged at 2000 g for 15 min at 4 °C, and plasma aliquoted and stored at −80 °C. Uterine artery diameter, time-averaged mean flow velocity, and the pulsatility index as a measure of uterine vascular resistance were averaged from duplicate or triplicate measurements, as previously described [4, 27]. In brief, using an ATL3000 machine and a 4-MHz curved linear array probe, each uterine artery was identified in longitudinal view at the point where it appears to crossover the external iliac. The diameter at peak systole and end-diastole for each vessel was measured with color imaging with care being taken to vary the depth of imaging

so as to obtain clear vessel margins. Since color imaging tends to enlarge the diameter obtained, we later removed the effect of color imaging by using the linear relationship between the with and without color values as measured for the common iliac artery at similar anatomical depth as previously described [28]. After measuring the diameter at a high (~ 90°) angle of insonation in each vessel, the probe was rotated to obtain a < 35° angle of insonation and the sampling frame adjusted to obtain optimal velocity signals. Flow velocities were measured from quality tracings of three to six beats, and the time-averaged mean flow velocity (TAM) as well as the peak systolic velocity (PSV), end-diastolic velocity (EDV), and pulsatility index ((PI) = [PSV–peak EDV]/mean flow velocity) values recorded. Automatic Doppler measurement mode was used to calculate the TAM. Volumetric flow was calculated as (πr^2 x × 60) where r is the vessel radius and expressed in ml/min. Measurements were performed bilaterally, and both sides averaged for a single measurement per subject.

Infant birth weight, sex, head circumference, and gestational age at delivery were obtained from medical records completed by hospital personnel or, in the case of home births, by study investigators using a portable weighing scale and measuring tape. Gestational age was computed by weeks from last menstrual period unless values differed by more than 2 weeks from fetal biometry obtained at week 20, in which case ultrasound dating was used.

PAPP-A2 Immunoblot Analysis

Immunoblots were performed using 12.5 μl of plasma combined with 6x reducing loading buffer (Boston BioProducts) per lane. For each cohort, the same pregnancy control sample was run on each blot for normalizing densitometry across blots. Samples were separated by electrophoresis on a 4–20% gradient Criterion Tris-HCL gel (BioRad) and transferred to a polyvinylidene difluoride membrane (PVDF, BioRad). Blocking was performed for 1 h using Start Block buffer (Thermo Scientific PI27538) with 0.05% Tween 20 (Sigma Aldrich). Membranes were then incubated with anti-pappalysin-2 antibody (R&D systems AF1668 polyclonal goat) in a 1:2000 dilution of blocking buffer overnight at 4 °C, washed, and incubated with donkey anti-goat horseradish peroxidase-conjugated antibody diluted in blocking buffer (1:100,000, Sigma Aldrich). Chemiluminescence (Western Lightning Plus-ECL, Perkin & Elmer) was used to visualize the immunoreactive bands following exposure to XR film (LightLabs). Densitometry was performed for PAPP-A2 (~ 220 kDa) using Quantity One software (BioRad). Membranes were then stained for total protein with REVERT Total Protein (Li-Cor) and a uniform nonspecific band around ~ 130–150 kDa scanned and quantified using an Odyssey Fc (Li-Cor) to serve as a loading control for the longitudinal cohort, and a uniform nonspecific band on the pre-eclampsia cohort radiographs at a similar molecular weight was used for normalization for protein loading. PAPP-A2 expression in relative units was normalized both for protein loading and for exposure across all blots for each cohort. Of note, the relative units cannot be directly compared between the cohorts. A representative immunoblot for PAPP-A2 and the normalization 130–150kDA band is shown in Fig. 1. Densitometry results from multiple immunoblots normalized to a standard sample loaded on all blots are presented in the graphs of Fig. 2.

Calculations

Body mass index (BMI) was calculated from the mother's pre-pregnant weight and height at the time of study. Babies were classified as small for gestational age (SGA) if gestational age- and sex-specific birth weights were less than the 10th percentile [29]. Deliveries occurring before week 37 were considered preterm.

Statistics

Continuous variables passing the D'Agostino and Pearson test for normality were compared across time or between altitudes in the longitudinally studied Andean groups using two-way analysis of variance with Tukey's or Sidak's multiple comparisons as appropriate. Comparisons among the cross-sectionally studied normotensive, early- and late-onset pre-eclampsia groups were made with one-way analysis of variance with Dunnett's multiple comparisons. Comparisons of categorical or continuous variables that were not normally distributed were compared using chi-squared tests. Spearman's rho was used to calculate the relationship between PAPP-A2 levels and uterine artery diameter or pulsatility index. GraphPad Prism v 6.0 was used for performing all statistics. Comparisons among groups were considered significant when the two-tailed p was less than 0.05 unless the direction of comparison was specified in advance, in which case a one-tailed test was used. Trends are reported when $0.05 < p < 0.10$. Tabular data are presented as the mean plus or minus standard error of the mean (SEM) or 95% confidence intervals.

Results

Maternal and Infant Characteristics

The high- and low-altitude Andean groups did not differ in maternal age, BMI, or primiparity although the high-altitude women were shorter than their low-altitude counterparts (Table 1). Infant birth weight, gestational age at delivery, sex, and percent SGA were similar in the low- and high-altitude groups.

Normotensive and preeclamptic women were of similar height and parity, but the women with preeclampsia tended to be slightly older and had a higher BMI. Early-onset preeclamptic

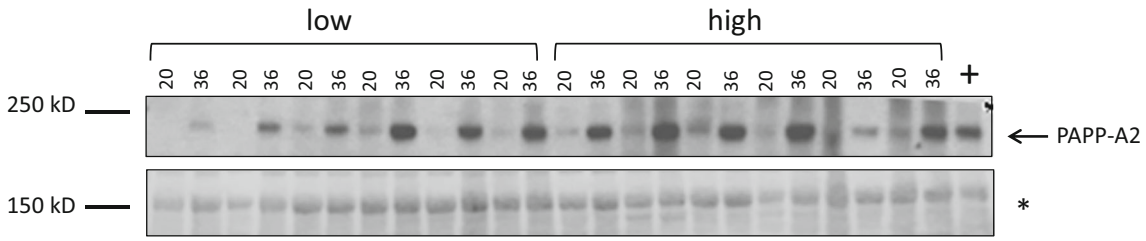

Fig. 1 PAPP-A2 levels increase with gestational age both at low and high altitude. Representative immunoblot of maternal PAPP-A2 plasma levels. Paired samples from 20 and 36 weeks gestation from Andean women residing at low altitude ($n = 6$) or high altitude (n = 6). The band at ~ 220 kDa in top panel represents PAPP-A2 immunoreactivity and is noted by the arrow. The lower panel showing a nonspecific band at ~150 kDa (*) was used for normalization. Positive control sample for PAPP-A2 is denoted by +

women gave birth to infants of an earlier gestational age and weighed less than those born to the normotensive or late-onset groups (Table 1).

PAPP-A2 Levels

PAPP-A2 levels rose with advancing gestation in the longitudinally studied low- and high-altitude Andean women (Figs. 1 & 2). There was a trend for an interaction between the effects of altitude and time, suggesting a greater gestational age related rise in PAPP-A2 in the high- compared to the low-altitude Andeans (Table 1).

Comparing the cross-sectional groups, PAPP-A2 levels were higher in all the preeclamptic cases than normotensive women and in the early onset than normotensive women (Fig. 2). PAPP-A2 levels rose with gestational age in the normotensive women but were unrelated to gestational age in the early- or late-onset preeclamptic women (Fig. 3) or the two preeclamptic groups combined (data not shown).

Uterine Artery Diameter and Pulsatility Index

Uterine artery diameter and blood flow were greater in the longitudinally studied high- versus low-altitude groups, and the uterine artery pulsatility index decreased with advancing gestation at both altitudes (Table 1). Uterine artery diameters were similar in the cross-sectionally studied normotensive and preeclamptic groups, but the pulsatility index was higher, and blood flows lower especially in the early-onset cases.

PAPP-A2 levels were not related to the uterine artery pulsatility index in the cross-sectionally studied normotensive women or in any of the longitudinally studied groups but were positively correlated with the pulsatility index in the early-onset women or in all preeclamptic cases combined and also tended to be so related in the late-onset women (Fig. 4). There were no relationships between PAPP-A2 levels and uterine artery diameter or blood flow in any longitudinal or cross-sectional group (data not shown).

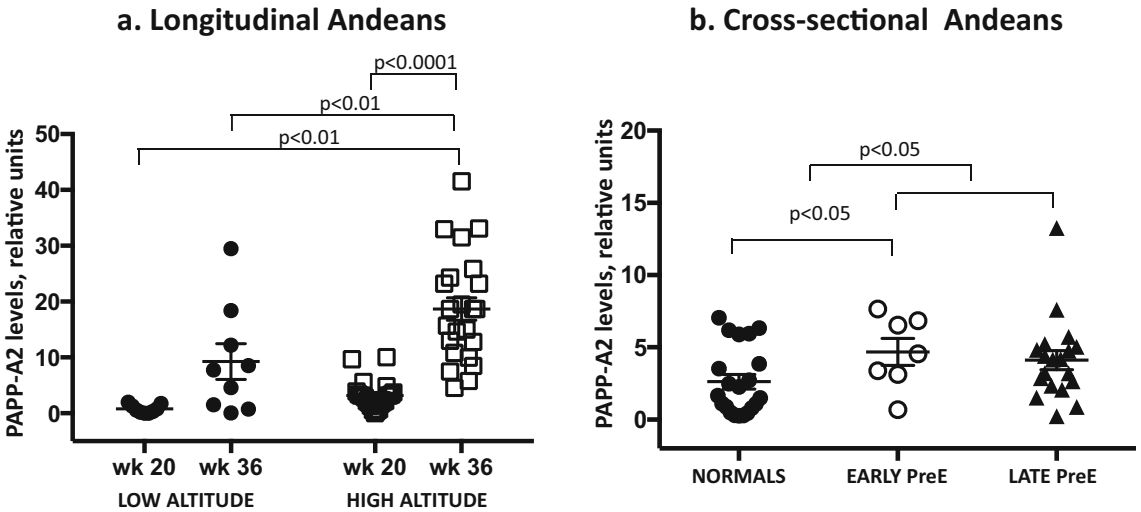

Fig. 2 Panel A: PAPP-A2 level's rose with advancing gestation in the longitudinally studied Andean women residing at low or high altitude. Panel B: Using one-tailed tests, PAPP-A2 levels were higher in all preeclamptic than in normotensive women and also higher in the early-onset preeclamptic vs. normotensive cases. PAPP-A2 levels were determined by immunoblot and expressed as relative units

Table 1 Maternal and infant characteristics

	Longitudinal Andeans					Cross-sectional Andeans			
	Low alt	High alt	*P*-alt	*P*-time	*P*-int	Normals	Early PreE	Late PreE	P
Maternal (n)	9	23				22	7	19	
Age (yr)	25 ± 2	25 ± 2	NS	–	–	27 ± 2	35 ± 2	28 ± 2	NS (.07)
Height (cm)	157 ± 2	151 ± 1	< 0.01	–	–	151 ± 1	151 ± 3	151 ± 2	NS
BMI (kg/m^2)	24 ± 1	26 ± 1	NS	–	–	29 ± 1	32 ± 2	33 ± 1*	< 0.01
Primiparous (%)	44 (4,85)	30 (10,51)	NS	–	–	41 (19,63)	14 (0,49)	53 (28,77)	NS
PAPP-A2, wk 20 Wk 36	0.78 ± 0.28 9.26 ± 3.22	3.19 ± 0.64 18.67 ± 2.33	< 0.01	< 0.01	NS(.08)	2.62 ± 0.51	4.68 ± 0.94*	4.11 ± 0.65	NS (.07)
Uterine a diam, wk 20 Wk 36	0.46 ± 0.02 0.50 ± 0.02	0.63 ± 0.01 0.63 ± 0.01	< 0.01	NS	NS	0.63 ± 0.02	0.59 ± 0.02	0.66 ± 0.03	NS
Uterine a PI, wk 20 Wk 36	1.21 ± 0.28 1.01 ± 0.17	1.05 ± 0.09 0.82 ± 0.07	NS	< 0.01	NS	0.72 ± 0.03	1.56 ± 0.29*	0.93 ± 0.08	< 0.0001
Uterine a flow, wk 20 Wk 36	230 ± 41 519 ± 65	327 ± 53 670 ± 111	< 0.01	NS (.06)	NS	474 ± 37	186 ± 28*	456 ± 52	< 0.01
Infant									
Birth wt (g)	3428 ± 118	3228 ± 68	NS			3088 ± 120	1908 ± 362*	2768 ± 127	< 0.001
Gestational age (wk)	39.7 ± 0.7	39.6 ± 0.4	NS			39.3 ± 0.4	33.7 ± 1.8*	38.3 ± 0.3	< 0.001
Male (%)	56(26,62)	47(41,65)	NS			35 (10,61)	67 (13,100)	59 (33,85)	NS
SGA (%)	0(0,0)	5(0,16)	NS			18 (1,36)	43 (0,92)	47 (23,72)	NS

Mean ± sem or 95% confidence intervals (in parentheses)

Abbreviations: alt = altitude, diam = average of right and left uterine artery diameters (cm) at week 20 or 36, flow = volumetric flow (ml/min), int = interaction, PAPP-A2 = pregnancy-associated plasma protein, PI = pulsatility index, PreE = preeclampsia, SGA = small for gestational age and sex, UtA = uterine artery, wt = weight

*$p < 0.01$ compared with the other two cross-sectional groups

Discussion

Previous in vitro studies have shown that mRNA and protein expression are increased in placental explants exposed to hypoxia [25, 26]. These findings were reproduced in vivo by our finding that maternal PAPP-A2 was elevated in normotensive women living at high compared with low altitude and in the high-altitude preeclamptic vs. normotensive women, suggesting that hypoxia is sufficient to raise PAPP-A2 levels. Further such increases were present in both instances even though the source of the hypoxia likely varied; that is, in the former, the hypoxia was global in nature, whereas it was also likely regional in the latter case due to incomplete spiral arteriolar remodeling as suggested by the preeclamptic women's elevated pulsatility indices. This association between hypoxia and preeclampsia is further supported by prior findings of a hypoxia responsive element (HRE) and two nuclear factor (NF)-κβ binding sites within the PAPP-A2 promoter region [25].

Similar to what has been observed in normotensive women at lower altitudes [24, 30], PAPP-A2 levels rose from 20 to 36 weeks at high altitude, although the rise tended to be greater in the longitudinally studied high- than low-altitude group. There was also a clear relationship between gestational age and PAPP-A2 in the cross-sectionally studied normotensive subjects.

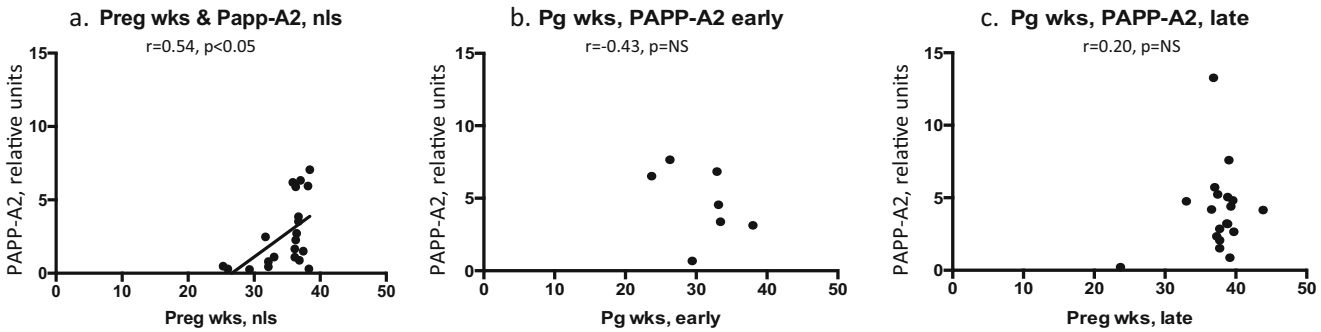

Fig. 3 In Panel A, PAPP-A2 levels in normotensive (nls) Andean women were positively associated with the week of pregnancy (Preg wks) but were unrelated in either early (Panel B)- or late (Panel C)-onset preeclamptic women

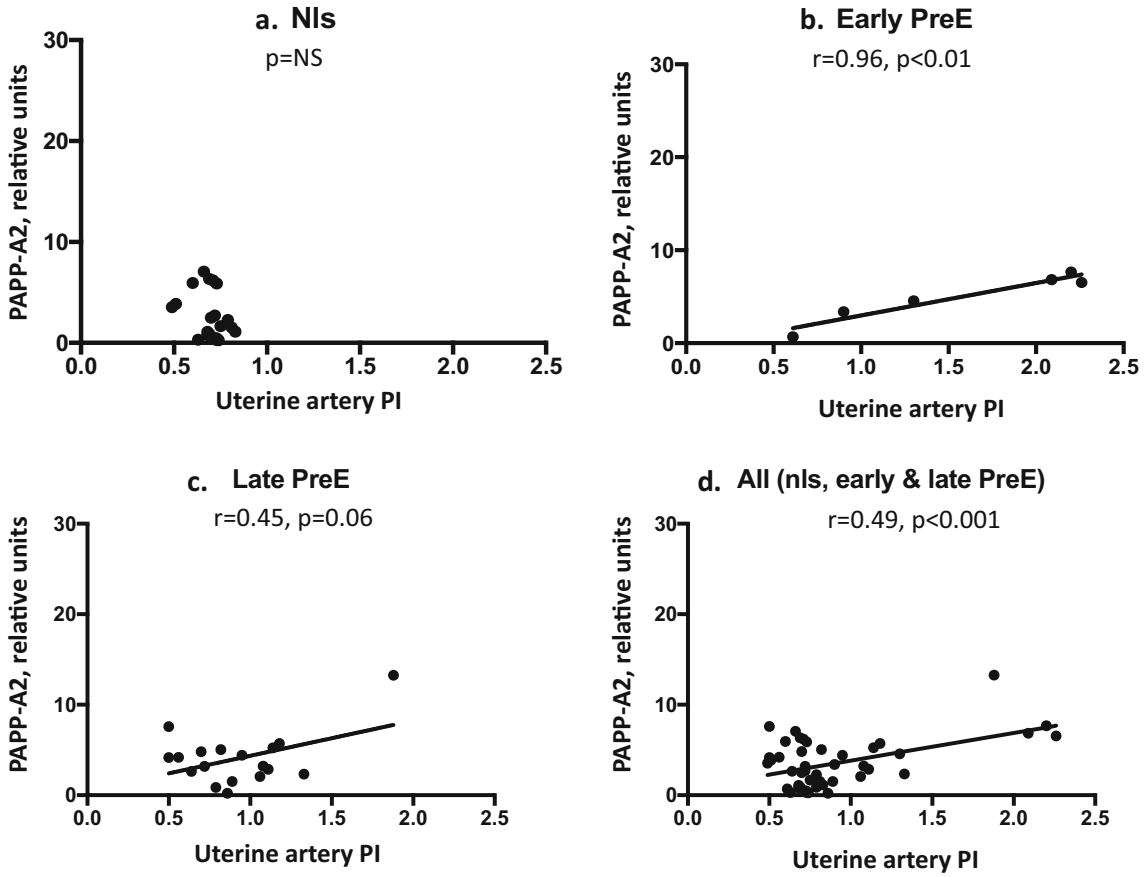

Fig. 4 Panel A: PAPP-A2 levels were unrelated to the uterine artery pulsatility index (PI) in normotensive controls (Nls) residing at high altitude and studied at a single time point. However, PAPP-A2 was strongly associated with the UtA PI in high-altitude residents with early-onset preeclampsia (Panel B) and tended also to be positively correlated in women with late-onset disease (Panel C) or in all subjects combined (Panel D)

However, there was no such relationship in pregnancies affected by preeclampsia (Fig. 3), perhaps due to a lack of appropriate PAPP-A2 downregulation in the second trimester.

PAPP-A2 levels were not strongly correlated with ultrasound Doppler findings, including uterine artery diameter, volumetric blood flow, or pulsatility index in normotensive women regardless of altitude. The lack of a relationship with these flow parameters does not support the concept that PAPP-A2 levels influence the vascular remodeling or vasoreactivity of the maternal uterine vasculature. This is in contrast to prior in vitro studies in which PAPP-A and PAPP-A2 by cleaving IGFBP-4 and IGFBP-5 respectively would enhance extravillous trophoblast (EVT) migration and invasion through the effect on IGF availability [31, 32]. These authors' results showed differential effects of PAPP-A and PAPP-A2 on IGF I and II availability, and impact on trophoblast migration suggests that PAPP-A would have a greater impact on EVT migration than PAPP-A2. Therefore, the low PAPP-A levels seen in early pregnancy associated with preeclampsia would adversely impact EVT invasion, while the high PAPP-A2 seen in later pregnancy associated with preeclampsia may represent a compensatory response [32]. Interestingly, other studies support that PAPP-A2 actually impairs EVT invasion and maternal spiral artery remodeling serving as a mediator of Interleukin-11 suppression of trophoblast cell migration, invasion, and tube formation [33]. Other theories include a physiological effect of PAPP-A2 in maintaining normal pregnancy, but pathological effects after a certain threshold are reached [31].

The higher uterine artery blood flows seen in high- compared to low-altitude Andean women were primarily due to larger uterine artery diameters since flow velocities were similar at the two elevations (Table 1). As previously described, greater uterine artery blood flow in Andean as opposed to European women at high altitude is present by 20 weeks and suggests that multigenerational high-altitude ancestry influences physiological mechanisms that enhance uterine oxygen delivery [4]. Unfortunately, we were unable to collect an adequate number of samples from European women to determine the effect of residence at high altitude on PAPP-A2 levels or their relationship with uterine artery Dopplers in women without such genetic adaptations. Given the association of PAPP-A2 with uteroplacental ischemia, it is possible that women of European ancestry may have higher PAPP-A2 levels compared to Andeans at high altitude.

As expected, PAPP-A2 was higher in pregnancies complicated by preeclampsia. This agrees with other studies detailing the increased RNA and protein in placental tissue and serum of preeclamptic subjects [15, 16, 19, 24, 25]. However, this is the first study to examine the relationship of PAPP-A2 levels with uterine artery Dopplers, diameters, or volumetric flow. PAPP-A2 has previously been shown to be elevated early in pregnancies that subsequently develop preeclampsia [34]. The correlation of PAPP-A2 and uterine artery Dopplers in preeclamptic subjects, particularly those with early-onset preeclampsia, suggests that a factor involved in the pathogenesis of preeclampsia is driving both the elevation of PAPP-A2 and the vascular alterations responsible for raising pulsatility indices. It is not currently known, whether altered PAPP-A2 expression is a rescue response to abnormal placentation or a cause of preeclampsia. Further studies to understand what regulates PAPP-A2 expression could help identify a unifying factor that is involved in the pathogenesis of preeclampsia.

Our study was limited by relatively small sample sizes and the lack of PAPP-A2 measurements in placental tissues. It was not possible to collect placentas given the necessity to recruit women from a large number of hospitals to achieve our study design. It was also not possible to collect infant data when pregnancy ended in fetal demise or in some cases when delivery occurred at home. While we were able to calculate uterine artery volumetric blood flow from measurements of uterine artery diameter and time-averaged mean flow velocity, we did not measure cardiac output or other upstream determinants of blood flow, which can also be affected by preeclampsia. It would have been helpful to have been able to compare a cohort of normotensive high-altitude women of European ancestry to determine if those without genetic adaptations for living at altitude have increased PAPP-A2 associated with the relative uteroplacental ischemia caused by flow reductions from smaller uterine artery diameters. In addition, no Europeans with preeclampsia were studied, and hence, it is unknown whether the effects of preeclampsia on uterine artery blood flow or resistance differ by ancestry. Finally, since at the beginning of this study a robust commercial immunoassay for PAPP-A2 had not been validated, we elected to use western blots for protein quantification due to increased sensitivity in our experience compared to a non-commercial PAPPA-2 ELISA as previously published [18]. The Western blot also needed far less plasma compared to ELISA, conserving the valuable clinical samples. Future studies using currently available ELISAs will be of value for quantifying PAPP-A2 protein levels.

Conclusion

In conclusion, we showed that either global (high altitude) or local (preeclampsia-associated) uteroplacental ischemia is associated with elevated maternal PAPP-A2 levels. We speculate that such a hypoxic upregulation of PAPP-A2 production may contribute to the increased incidence of preeclampsia and fetal growth reduction at high altitude. Future studies are required for identifying the factors linking PAPP-A2 levels with uterine vascular PI in preeclampsia that are not present in normotensive pregnancies, even those at high altitude. PAPP-A2 may serve as a potential biomarker or target for preventive or therapeutic strategies, especially at high altitude [34]. With the advent of ELISAs for measuring PAPP-A2 [30], further testing of PAPP-A2's sensitivity and specificity for predicting preeclampsia is warranted. Such studies may be especially helpful for improving pregnancy outcomes in countries such as Bolivia that have some of the highest maternal and infant mortality rates in the western hemisphere [35].

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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