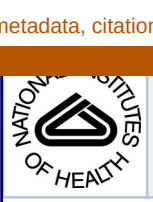

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Haptoglobin Phenotype and Abnormal Uterine Artery Doppler in a Racially Diverse Cohort

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Abstract

Objective—The anti-oxidant and proangiogenic protein haptoglobin (Hp) is believed to be important for implantation and pregnancy, although its specific role is not known. The three

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Declaration of Interests

The authors report no conflicts of interest.

phenotypes (1-1, 2-1 and 2-2) differ in structure and function. Hp 2-2 is associated with increased vascular stiffness in other populations. We examined whether Hp phenotype is associated with abnormal uterine artery Doppler (UAD) in pregnancy.

Methods—We conducted a secondary analysis of a preeclampsia prediction cohort nested within a larger placebo-controlled randomized clinical trial of antioxidants for prevention of preeclampsia. We determined Hp phenotype in 2,184 women who completed UAD assessments at 17 weeks gestation. Women with notching were re-evaluated for persistent notching at 24 weeks gestation. Logistic regression was used to assess differences in UAD indices between phenotype groups.

Results—Hp phenotype did not significantly influence the odds of having any notch (p=0.32), bilateral notches (p=0.72), or a resistance index (p = 0.28) or pulsatility index (p = 0.67) above the 90th percentile at 17 weeks gestation. Hp phenotype also did not influence the odds of persistent notching at 24 weeks (p=0.25).

Conclusions—Hp phenotype is not associated with abnormal UAD at 17 weeks gestation or with persistent notching at 24 weeks.

Keywords

haptoglobin; pregnancy; vascular resistance; women; race; ethnicity

Introduction

Uterine artery Doppler velocimetry (UAD) is a vascular screening test that identifies pregnant women with an increased risk for maternal-placental syndromes, including preeclampsia, intrauterine growth restriction and preterm birth [1]. Abnormal UAD is typically defined as a notch in the uterine artery velocity waveform at the beginning of diastole, or by a resistance or pulsatility index that exceeds the 90th percentile for gestational age [2–4]. These abnormalities suggest high resistance and reduced elasticity and compliance in the uterine vasculature [5]. Failed systemic vascular adaptation to pregnancy [5], maternal cardiac defects [4] and failed dilatory remodeling of the spiral arteries that supply the placenta [6–8] may contribute to abnormal UAD.

Haptoglobin (Hp) is a powerful anti-oxidant [9] and pro-angiogenic [10] protein that is believed to be important for implantation and pregnancy, although its specific role is not known [11, 12]. Hp sequesters iron by binding free hemoglobin following hemolysis [9]. Certain phenotypes may also effect vascular stiffness [13, 14] and modulate natural killer cell invasiveness [12, 15]. The three Hp genotypes give rise to three distinct phenotypes (Hp 1-1, 2-1 and 2-2; Figure 1S), which differ in size, structure, and function [9]. Hp 2-2 is the weakest anti-oxidant [9]. Hp 2-2 is also associated with increased systemic vascular resistance [14], reduced large and small artery elasticity [14], and increased pulse wave velocity in individuals with Type 2 diabetes [13]. This may contribute to the two-fold increase in cardiovascular event risk in Hp 2-2 diabetics, compared to Hp 1-1 and 2-1 diabetics [16].

Small case-control studies suggest that Hp phenotype may predict adverse pregnancy outcomes, including preeclampsia [17, 18], gestational diabetes [19], and premature rupture of membranes [20]. However, we were unable to confirm a relationship between Hp phenotype and preeclampsia risk in large cohorts of low-risk nulliparous women [21] or women with Type 1 diabetes [22] participating in randomized controlled trials of antioxidants for preeclampsia prevention. This highlights the importance of using large cohort studies when examining Hp phenotype and pregnancy outcome to reduce the likelihood of spurious findings [23].

The potential effects of Hp phenotype on abnormal UAD have not been investigated. Hp 2-2 is associated with increased vascular stiffness in Type 2 diabetes [13, 14], however little is known about Hp phenotype and vascular stiffness in other populations. We conducted a pre-planned secondary analysis to determine whether Hp phenotype was associated with abnormal UAD in the trial of low risk nulliparous women [21]. We hypothesized that Hp 2-2 women would be more likely to have abnormal UAD at 17 weeks gestation and persistent notching at 24 weeks gestation. We tested this hypothesis in a preeclampsia prediction cohort nested within a randomized clinical trial of antioxidants for preeclampsia prevention.

Methods

Study Population

We conducted a secondary analysis of a double-blind, multicenter randomized controlled trial in which nulliparous, low risk women received daily doses of 400 IU of vitamin E and 1000 mg of vitamin C, or placebo, from 9–16 weeks gestation until delivery (NCT 00135707) [24]. The trial was conducted between 2003 and 2008 by 16 centers in the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. A subset of 2,434 women who started treatment/placebo by 9–12 weeks gestation were enrolled in a nested preeclampsia prediction cohort (women not in the prediction cohort could start treatment/placebo as late as 16 weeks gestation). Women in the prediction cohort provided extra blood samples at each time point and underwent UAD assessment at 17 weeks gestation. All subjects provided written, informed consent before participating. Hp phenotyping was performed at the University of Pittsburgh (Institutional Review Board approval PRO10010368).

UAD

UAD was performed in women enrolled in the prediction study at approximately 17 weeks gestation. If a notch was present on any waveform, a second ultrasound was scheduled at 24 weeks gestation to identify women with persistent notches. Sonographers completed a certification exam to ensure that procedures were standardized across centers. Doppler examinations were performed transabdominally. If the sonographer could not obtain an adequate transabdominal image, transvaginal Doppler was performed. Three waveforms from the right and left uterine arteries were recorded. The uterine artery was insonated as it entered the uterus, one cm distal to where it crossed the external iliac artery. The resistance index (RI) was calculated as (systolic velocity – diastolic velocity) / systolic velocity. The

pulsatility index (PI) was calculated as (systolic velocity – diastolic velocity) / [(systolic velocity + diastolic velocity) / 2]. The RI and PI of the right and left uterine arteries were averaged. A notch was defined as a clear increase in velocity at the beginning of diastole. UAD indices were calculated at each study site by a sonographer who had completed the study certification exam. Multiples of the median for the RI and PI were calculated as described previously [8]. Expected medians were calculated based on a subject's gestational age, maternal weight and race. A RI or PI greater than the 90th percentile for gestational age was considered abnormal.

Hp Phenotyping

Phenotyping was performed as described previously [18] by individuals who were blinded to patient data. 5 μl serum samples supplemented with human hemoglobin (Sigma-Aldrich, St. Louis, MO) were run on 6% tris-glycine native gels (Invitrogen, Carlsbad, CA). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA).

Hemolyzed samples and samples with low Hp concentrations were phenotyped by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS PAGE) of 1 to 6 μl of serum. Samples were run on 12% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred to PVDF. PVDF was incubated with blocking solution (tris-buffered saline containing 5% non-fat milk, 0.1% Tween 20), and primary (1:5,000, Polyclonal Rabbit Anti-Human Haptogloin, DakoCytomation, Carpinteria, CA) and secondary (1:25,000, Goat anti-Rabbit IgG horseradish peroxidase, Millipore) antibodies. Incubations were performed at room temperature for one hour each.

PVDF was stained for peroxidase activity (SuperSignal West Pico Chemiluminescent Substrate, Fisher Scientific, Pittsburgh, PA) and imaged (FlouroChem Q System, Cell Biosciences, Santa Clara, CA). Hp phenotypes were identified by banding patterns of the Hp-hemoglobin complexes (Figure 1S, native PAGE) or the Hp α_1 and α_2 alleles (SDS PAGE).

Statistical Analysis

Baseline characteristics across phenotypes were compared using the Chi-square test for categorical variables and the Kruskal-Wallis test for continuous variables. Logistic models were used to assess the odds of abnormal UAD at the first Doppler exam and persistent notching at the second exam. All models were adjusted for age, gestational age at Doppler exam, treatment group, characteristics that differed between Hp phenotype groups (age, race, education, and prenatal/multivitamin use at randomization), and BMI, which was associated with UAD indices.

Results

Subjects

The prediction cohort included 2,434 women. We excluded 250 women because pregnancy outcome data were not available (n = 40), the UAD exam was not completed by 21 weeks gestation (n = 206), blood samples were not available (n = 1), or Hp was undetectable (Hp 0,

n = 3). Of the remaining 2,184 women, 49 had the rare 2-1M phenotype. Hp 2-1M individuals produce large amounts of Hp 1-1 and only small amounts of the Hp 2-1 isoforms due to a modification of the α_2 allele (Figure 1S) [9]. The opposite banding pattern is observed in Hp 2-1 individuals, who produce very little Hp 1-1 and large amounts of the Hp 2-1 isoforms. Hp 2-1M women were excluded from statistical analyses of UAD indices due to small sample size. 1,066 women were randomized to receive daily vitamin C and E supplementation. This included 53% of Hp 1-1 women (n = 238), 48% of Hp 2-1 women (n = 488), and 51% of Hp 2-2 women (n = 340). The remaining 1,069 women were randomized to receive placebo.

Phenotype prevalence differed by race (Table 1), and was similar to published values for white, black and Hispanic North Americans [25]. Age increased progressively across the Hp 2-1M, 1-1, 2-1 and 2-2 groups (Table 1). Hp 2-2 women were older and more educated than women with the other phenotypes, and were more likely to report vitamin use at randomization than women with the Hp 1-1 and 2-1 phenotypes. The phenotype groups did not differ with respect to pre-pregnancy BMI, blood pressure at randomization, smoking, history of a previous pregnancy ending prior to 20 weeks, or family history of preeclampsia. Hp 2-2 women were randomized slightly later than Hp 1-1 women, and completed their first UAD assessment slightly earlier than Hp 1-1 and 2-1 women. Although statistically significant, these 1–3 day differences are unlikely to have had any clinical impact.

Subsequent analyses of UAD indices were adjusted for characteristics that differed between the 1-1, 2-1 and 2-2 groups (race, age, education, and vitamin use). The “White” and “Other” groups were pooled for race adjustments, as the phenotype prevalence in the “Other” group was most similar to whites. There were no interactions between Hp phenotype and race for UAD indices. Treatment group (vitamins vs. placebo) did not significantly affect any of the UAD indices examined; therefore treatment groups were pooled for all analyses. Results were not different in when the placebo group (211 Hp 1-1 women, 537 Hp 2-1 women, and 321 Hp 2-2 women) was analyzed alone.

Relationship Between Hp Phenotype and UAD Indices at 17 Weeks Gestation

334 women (15.5%) had notching at 17 weeks gestation. Hp phenotype did not significantly influence the odds of having any notch or bilateral notching (Table 2) after adjusting for age, gestational age at Doppler exam, race, education, vitamin use, body weight and treatment group. The odds of having a RI and PI above the 90th percentile for gestational age did not differ between women of different Hp phenotypes.

Relationship Between Hp Phenotype and Persistent Notching at 24 Weeks Gestation

268 of the 334 women (80.2%) who had notching at 17 weeks gestation returned for a second UAD assessment at 24 weeks gestation (Table 3). Six women with the 2-1M phenotype were excluded. The percentage of women who did not return was not different between Hp phenotype groups (data not shown). Gestational age at the second UAD exam did not differ between the phenotype groups. 84 women (32.1%) who completed the second UAD exam had persistent notching (any notch or bilateral notch). The odds of persistent notching did not differ significantly between women with the Hp 1-1, 2-1 and 2-2

phenotypes (p=0.25). Only 22 women (8.2%) had persistent bilateral notches. Statistical analyses were not performed due to small sample size.

Discussion

In contrast to our hypothesis, we found that Hp phenotype was not associated with the prevalence of any notch or bilateral notches, or the odds of having a RI or PI above the 90th percentile for gestational age, at 17 weeks gestation. There was also no relationship between Hp phenotype and persistent notching at 24 weeks gestation. This study extends the existing literature regarding Hp phenotype and pregnancy to include UAD. The large sample size, cohort design, and racial and ethnic diversity of the cohort (50% non-Hispanic white, 25% Hispanic, 23% non-Hispanic black) are significant strengths.

Although Hp is believed to be important for implantation and pregnancy, it's functional role is not known [11]. Increases in Hp concentration in the rabbit oviduct and uterus follow the time-course of blastocyst transit, and the blastocyst incorporates maternal Hp into its extra-embryonic matrix [11]. Phenotype effects cannot be examined in the rabbit, as this species only has the Hp 1-1 phenotype [26]. In humans, Hp concentrations in the uterine endometrium increase from the follicular to the luteal phase of the menstrual cycle [27]. The uterine decidua has high Hp concentrations at term [27]. Although Hp concentration depends on phenotype [28], this study did not examine the potential effects of phenotype [27]. Mechanistic studies examining the functional significance of these increases in Hp concentration are needed.

There are several possible explanations for the absence of a relationship between Hp phenotype and UAD indices. One possibility is that the phenotype-dependent differences in Hp's functions do not have clinically significant effects on the processes that affect UAD during pregnancy. Alternatively, the strengths and weaknesses of the phenotypes may offset one another (i.e. the superior antioxidant capacity of Hp 1-1 may be counterbalanced by the vascular and proangiogenic properties of Hp 2-2), such that the overall effect of Hp phenotype is not clinically significant. A third possibility is that the multi-factorial nature of abnormal UAD may obscure any potential effect of Hp phenotype on the individual pathological mechanisms that contribute to abnormal UAD (failed systemic vascular adaptation, deficient spiral artery remodeling and placentation, and/or cardiac defects). This might occur if Hp phenotype only alters the risk of one type of pathology, or if different Hp phenotypes are associated with different pathologies. In this case, tests specific to each individual mechanism (peripheral vascular function testing, placental bed biopsies and echocardiography) would be necessary to detect a potential effect of Hp phenotype. These expensive and time consuming measurements were not feasible in this large cohort.

Most research examining the relationship between Hp phenotype and adverse pregnancy outcomes focuses on preeclampsia [17, 18, 29, 30]. The largest case-control studies suggested that Hp 1-1 was associated with a lower preeclampsia risk compared to Hp 2-1 [18], or Hp 2-1 and 2-2 [17]. However, we found no relationship between Hp phenotype and preeclampsia risk in 4,500 women from the present trial, suggesting that the results of small case-control studies may have been spurious findings [21]. This highlights the need for

large, prospective studies [21, 23]. Two small case-control studies examined the potential impact of Hp phenotype on other adverse pregnancy outcomes [19, 20]. Austrian women with gestational diabetes were more likely to have the Hp α_2 allele than women with uncomplicated pregnancies [19]. In contrast, Korean women with premature rupture of membranes were more likely to have the Hp 1-1 phenotype (23% vs. 8%) than women with uncomplicated pregnancies [20]. The preeclampsia data suggest that these findings should be verified in large cohorts. The prediction cohort included too few women with gestational diabetes (n = 83) to examine this outcome. Premature rupture of membranes (n = 287) will be examined in a future study.

Limitations

This study has two important limitations. A notch was defined as a clear increase in velocity at the beginning of diastole, rather than by measured criteria. However, all sonographers completed a certification exam to ensure that waveforms were read consistently across sites. Second, UAD was assessed at approximately 17 weeks gestation, and results may differ at other gestational ages. This gestational age was selected for the original study because several large prospective studies reported that abnormal UAD in the second trimester or late first trimester was useful in predicting preeclampsia [1]. Early reports also suggested that Hp phenotype was associated with preeclampsia risk [17, 18]; hence we sought to examine the potential effects of Hp phenotype on UAD both as a vascular test, and as a potential mechanism leading to increased preeclampsia risk. However, subsequent analyses demonstrated that abnormal UAD had poor sensitivity for predicting preeclampsia in this cohort [8], and we were also unable to confirm a relationship between Hp phenotype and preeclampsia risk [21].

In conclusion, Hp phenotype did not identify women at increased risk of abnormal UAD in a racially diverse pregnancy cohort of low risk, nulliparous women. Large cohorts are needed to confirm the results of small case-control studies suggesting that Hp phenotype significantly affects the risk of gestational diabetes [19] and premature rupture membranes [20]. Mechanistic studies are also needed to identify the specific role of Hp in implantation and pregnancy, and to examine the potential role of Hp phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

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Table 1

Subject characteristics

Subject Characteristics		**Hp 1-1 (n = 449)**	**Hp 2-1 (n = 1025)**	**Hp 2-2 (n = 661)**	**Hp 2-1M (n = 49)**	**p**
Age – years		22.7 ± 4.5	23.4 ± 4.8[a]	24.3 ± 4.9[a,b]	20.6 ± 3.4[a,b,c]	<0.01
Gestational age at randomization – week		11.4 ± 1.1	11.4 ± 1.1	11.5 ± 1.0[a]	11.0 ± 1.1[a,b,c]	0.01
Gestational age at Doppler exam – week		17.0 ± 1.1	17.0 ± 1.2	16.8 ± 1.1[a,b]	17.4 ± 1.2[a,b,c]	<0.01
Race or ethnic group - n (% within phenotype)	White	166 (37%)	509 (50%)	410 (62%)	3 (6%)	<0.01
	Black	144 (32%)	221 (22%)	103 (16%)	42 (86%)	
	Hispanic	133 (30%)	276 (27%)	132 (20%)	3 (6%)	
	Other	6 (1%)	19 (2%)	16 (2%)	1 (2%)	
	Significance		*a*	*a,b*	*a,b,c*	
Pre-pregnancy body mass index - kg/m^2		25.8 ± 6.5	25.3 ± 5.8	25.4 ± 6.1	27.1 ± 6.9	0.33
Smoked during pregnancy - n (%)		80 (18%)	162 (16%)	111 (17%)	7 (14%)	0.77
Education - years		12.7 ± 2.6	12.9 ± 2.6	13.4 ± 2.5[a,b]	12.3 ± 1.8[c]	<0.01
Prenatal/multivitamin use prior to randomization - n (%)		332 (74%)	798 (78%)	562 (85%)[a,b]	43 (88%)[a]	<0.01
Previous pregnancy - n (%)		91 (20%)	225 (22%)	157 (24%)	13 (27%)	0.48
Family history of preeclampsia - n (%)		63 (14%)	139 (14%)	82 (12%)	6 (12%)	0.86
Blood pressure at entry (9–12 weeks) - mmHg	Systolic	109 ± 10	110 ± 10	110 ± 10	110 ± 11	0.50
	Diastolic	65 ± 8	66 ± 8	67 ± 8	64 ± 9	0.11

Three subjects with no detectable Hp (Hp 0) were excluded from the analysis. Values are mean ± SD or n (%).

Significant difference ($p<0.05$) from: [a]Hp 1-1, [b]Hp 2-1, [c]Hp 2-2.

Table 2

Hp phenotype and abnormal UAD at the first exam

Outcome	Hp 1-1 (n = 449)	Hp 2-1 (n = 1025)	Hp 2-2 (n = 661)	OR (95% CI)		p[a]
				Hp 1-1	Hp 2-1	
Any Notch[b]	70/433 (16.2%)	158/1004 (15.7%)	95/651 (14.6%)	1.29 (0.91, 1.82)	1.18 (0.89, 1.57)	0.32
Bilateral Notch[c]	27/435 (6.2%)	63/1007 (6.3%)	36/652 (5.5%)	1.19 (0.70, 2.01)	1.18 (0.77, 1.81)	0.72
RI >90th Percentile[a]	38/449 (8.5%)	108/1025 (10.5%)	61/661 (9.2%)	0.87 (0.56, 1.34)	1.19 (0.85, 1.66)	0.28
PI >90th Percentile[d]	41/448 (9.2%)	107/1023 (10.5%)	65/661 (9.8%)	0.89 (0.58, 1.36)	1.06 (0.76, 1.48)	0.67

Values are n/total (%), or OR (95% CI) with Hp 2-2 as the reference group. Abbreviations: OR, odds ratio; CI, confidence interval.

[a]Adjusted for age, gestational age at Doppler exam, race, education, vitamin use, pre-pregnancy BMI and treatment group. 38 women with missing pre-pregnancy BMI data were excluded from all models.

[b]Nine women with missing data for any notch were excluded. Only 323 of the 334 women with notching are included in the table, as 8 women had the 2-1M phenotype and 3 women were missing pre-pregnancy BMI.

[c]Three women with missing bilateral notch data were excluded.

[d]Multiples of the median for the RI and PI were calculated to determine whether values exceeded the 90th percentile for gestational age.

Table 3

Hp phenotype and persistent notchingat 24 weeks gestation

Variable		Hp 1-1 (n =53)	Hp 2-1 (n =134)	Hp 2-2 (n =75)
Gestational age at 1st UAD assessment		16.9 ± 1.0	16.6 ± 1.1	16.6 ± 0.92
Gestational age at 2nd UAD assessment		24.2 ± 1.0	24.3 ± 0.9	24.1 ± 0.9
Persistent notches (any notch or bilateral notches)	n (%)	17 (32.1%)	49 (36.6%)	18 (24.0%)
	OR (95% CI)*	1.16 (0.50, 2.66)	1.68 (0.87, 3.26)	1

Values are mean ± standard deviation unless otherwise indicated. Abbreviations: OR, odds ratio; CI, confidence interval. 268 women with notches at the first assessment returned for a 2nd UAD assessment. Six women with the Hp 2-1M phenotype were excluded.

[a]Adjusted for age, gestational age at Doppler exam, race, education, vitamin use, pre-pregnancy BMI and treatment group.