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THE RELATIONSHIP OF LONGITUDINAL LEVELS OF COMPLEMENT Bb DURING PREGNANCY WITH PREECLAMPSIA

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Abstract

PROBLEM—To determine the understudied relationship between Bb during pregnancy in
subjects with preeclampsia compared with normotensive controls.

METHOD OF STUDY—Nested case-control study.

RESULTS—Average Bb levels significantly decreased over time in pregnancy (weekly slope
(SE): -0.0094 (0.0005), $p < 0.01$). Cross-sectionally, at less than 10 weeks, Bb levels decreased
with increasing gestational age in women who remained normotensive (weekly slope (SE): -0.007
(0.02) and for women who developed preeclampsia (weekly slope (SE): -0.059 (0.03) $p = 0.12$).
Among women who developed preeclampsia, Bb levels were greatest when samples were drawn
in the gestational window of 15–20 weeks [(weekly slope (SE): 0.06 (0.02)] while levels among
normotensive women were inversely related with gestational age [weekly slope (SE): -0.02
(0.01)]. The differences in slopes between cases and controls between 10 and 21 weeks' gestation
was statistically significant ($p = 0.003$).

CONCLUSIONS—We suggest dysregulation of Bb activation between 10 and 20 weeks'
gestation in women who develop preeclampsia.

Keywords

Complement Bb; gestational age; preeclampsia

Introduction

Preeclampsia is a hypertensive disease of pregnancy that complicates approximately 5% of
all pregnancies and is associated with maternal morbidity and mortality, intrauterine growth
restriction, preterm birth and adverse neonatal outcomes.^{1–3} Preeclampsia is characterized
by the clinical manifestations of *de novo* hypertension and proteinuria, occurring in the

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second half of pregnancy. Investigators have shown a role for shallow trophoblast invasion of the spiral arteries⁴, circulating anti-angiogenic factors⁵, dysregulation of the immune system⁶ and an exaggerated inflammatory response⁷ in the etiology of this syndrome.

Our group^{8–10} and others^{11–20} have demonstrated a role for the complement system in preeclampsia. The complement system is a complex series of over 30 proteins, found in the circulation, body fluids and on cell surfaces and is an essential component of the innate immune system.^{21, 22} The main effector function of the complement system is to defend the host against microbial infection and to remove immune complexes as well as ischemic, necrotic and apoptotic cells.²³ The classical, lectin and alternative complement pathways (described elsewhere^{24–26}) are activated by pathway-specific triggering mechanisms to generate anaphylatoxins which are proinflammatory activation fragments, opsonins, which coat target surfaces to facilitate removal of damaged tissue and, the terminal membrane attack complex that directly lyses damaged cells.²⁷ Importantly, the complement system is tightly controlled by a number of fluid-phase and cell surface proteins to avoid injury to bystander tissues. Dysregulation of control of the complement system can lead to a severe inflammatory response and host tissue damage^{23, 28, 29}

We reported from our previous research a link between the complement activation fragment Bb (a marker of alternative complement pathway activation²⁸) measured in a single blood draw in early pregnancy (<20 weeks' gestation) with the subsequent development of preeclampsia.³ We also demonstrated that these associations varied with gestational age at blood draw. The purpose of this current study was to build on these results using a newer assay and a research infrastructure developed as part of the University of Colorado, Department of Obstetrics and Gynecology, Colorado Baby Blanket program. Our specific aims were to determine: 1) the natural history of Bb throughout pregnancy, and 2) the relationship between complement Bb measured at distinct time points during pregnancy with preeclampsia.

Materials and Methods

The Colorado Baby Blanket Research program was started and approved by the Colorado Multiple Institutional Review Board in 2011. The purpose of the program was to link biological samples collected at 3 time points during pregnancy with clinical data collected during pregnancy and at delivery. We enrolled women into the program if they were less than 23 weeks' gestation at the first prenatal visit (Visit 1) and planned to have their babies at the University of Colorado Hospital (UCH). In brief, the women were referred to research personnel by obstetric providers. Following consent, the women had additional research-related blood samples drawn along with the routine prenatal labs. The women were followed prospectively during pregnancy with the collection of additional research samples with the oral glucose tolerance test in the second trimester (Visit 2) and during the last trimester (Visit 3) if the woman was still pregnant. At delivery, outcome data were collected and entered into the Baby Blanket dataset housed in the Division of Biostatistics and Bioinformatics at National Jewish Health, Denver. At this assessment the gestational age at blood draw was verified using results from ultrasound examinations.

A secondary review of each completed maternal record was conducted without knowledge of the biomarker level by the Principal Investigator of the study (A.M.L.) and by a maternal fetal medicine specialist (R.S.G) in the event of uncertainty regarding pregnancy outcome data. A small number of women delivered outside UCH (n=17). Pregnancy outcome data were collected on these women from a detailed questionnaire administered to the study participant (n=7) by our research personnel or by review of the medical record from the outside hospital with patient consent (n=10).

Women included in the study delivered before December 25, 2013 (n=874). We excluded women who: withdrew from the study (n=10), were lost to follow-up (n=60), had a deviation in study protocol at enrollment (missed, inadequate or lost sample, recruitment over 23 weeks' gestation n=6), had a multiple birth (n=17), delivered before 20 weeks' gestation (n=51) or had any of the following: a repeat delivery during the study period (n=4), a congenital anomaly (n=34), preexisting diabetes (n=14), a clotting disorder (n=12), autoimmune disease (n=13), chronic hypertension or cardiac disease (n=56). In addition, women who developed gestational hypertension during the index pregnancy (n=45) were also removed from the study. In the final analytic dataset (n=552 records), 28 (5%) women developed preeclampsia. For each case of preeclampsia, we randomly selected 4 normotensive controls (n=112) from the cohort. At the time of the repository pull, 4 controls were discovered to have inadequate or missing samples leaving a total of 136 in the analytic dataset. Of the 136 women included in the nested-case control study 136 had 1 sample, 127 had two samples and, 103 had 3 samples.

Clinical variables

Preeclampsia was classically defined as gestational hypertension and proteinuria.¹ Gestational hypertension was defined as a systolic blood pressure greater than 140 mm Hg or a diastolic blood pressure greater than 90 mm Hg on at least 2 occasions at least 6 hours apart after 20 weeks' gestation in women known to be normotensive before pregnancy and before 20 weeks' gestation. Preeclampsia was defined as: (1) gestational hypertension with proteinuria (300 mg or greater per 24 hour period) or at least 1 or greater on dipstick or (2) in the absence of proteinuria gestational hypertension with cerebral symptoms, epigastric or right upper quadrant pain with nausea or vomiting, or thrombocytopenia and abnormal liver function tests.¹ Collected maternal risk factors for preeclampsia included: maternal race/ethnicity, age, parity (nulliparous vs multiparous), cigarette smoking at conception (yes/no) and a history of hypertensive disease in a previous pregnancy (yes/no). Prepregnancy body mass index (BMI) was calculated as the maternal prepregnancy weight divided by the square of the height (kg/m²) and was categorized as follows: underweight (BMI 18.5 kg/m²), normal weight (BMI 18.5–24.9 kg/m²), overweight (BMI 25–29.9 kg/m²), and obese (BMI >30 kg/m²).³⁰ The maternal prepregnancy weight was self-reported by the woman. The maternal height was obtained from the prenatal medical record.

Sample preparation and assay for complement activation fragment Bb

Each EDTA-plasma sample was centrifuged after phlebotomy, and the supernatant was removed, aliquoted, and placed in a freezer at –80°C. The complement activation fragment

Bb ($\mu\text{g/ml}$) was measured at the Complement Laboratory at National Jewish Health, which holds clinical laboratory certification from CAP/CLIA as well as ISO 15189. The Microvue complement Bb Plus immunoassay (Quidel, San Diego) was used to measure Bb. This assay was introduced in 2008 to create a more sensitive, broader range and robust immunoassay. Differences between the Bb plus assay and the older Bb immunoassay include: a) substitution of a monoclonal anti-Bb for the polyclonal conjugate, b) qualification of new standards and controls values assigned against “gold standard” purified Bb and, c) implementation of a new tetramethylbenzidine substrate system. Validation of the assay was done to CLIA standards. The interassay coefficient of variability (CV) is 11.3%, and the 2 standard deviation reference range is 0.41 to 1.49 $\mu\text{g/mL}$ as established by the laboratory. The technicians conducting the assay were blinded to the case status of the samples.

Statistical analysis

Descriptive statistics were calculated using means and standard deviations for continuous variables and percentages for categorical variables. Means and proportions were compared using a two-sample t-test and a chi-squared test, respectively. To estimate the change in Bb levels over time, a random coefficient model with an intercept and slope fit for each subject was used. This model included an unstructured covariance matrix for the random coefficients. Change in Bb levels over time were graphically inspected with scatterplot smoothers, specifically loess curves, to aid in the inspection of trends over time³¹ and to determine the appropriate functional form. Inspection of the curve indicated a piece-wise linear regression with a fixed knot (or break point) was needed to compare changes in average Bb levels between cases and controls over gestational age for observations collected during the first visit. Piece-wise linear regression is useful when there are two different linear relationships in the data with a sudden, sharp change. Placement of the knot was determined by fitting a quadratic function, setting the first derivative to zero and solving for gestational age. A sensitivity analysis was performed in which potential clinical confounders (maternal prepregnancy BMI and a history of a hypertensive disease) were included as covariates in the piece-wise linear regression model. Statistical analysis was performed using SAS version 9.3 software (SAS Institute Inc.: Cary, NC, 2011).

Results

Nine (32%) of the 28 cases of women who developed preeclampsia delivered at less than 37 weeks' gestation. The majority of the study participants were non-Hispanic White (62.5%), 15.4% percent were Hispanic, 12.5% were African American and 9.6% were Asian or from other race/ethnicities. The mean $\pm$ SD (years) maternal age was 29.1 $\pm$ 4.5. Fifty-three percent were multiparous, and 15.4% were cigarette smokers at conception. Twenty-six percent of the women were overweight and 24% were obese. The mean $\pm$ SD gestational age at blood draw for visits 1, 2 and 3 (weeks) are 9.3 $\pm$ 3.0 (range 4.3 to 21), 26.9 $\pm$ 1.7 (range 21.6 to 29.9) and 35.6 $\pm$ 1.3 (range 32.4 to 38.3), respectively. The only maternal risk factor associated with preeclampsia was a history of a hypertensive disease in a past pregnancy (Table 1).

We determined if levels of Bb change over the course of pregnancy using our longitudinal data and a standard mixed effects model (or random coefficients model). The data are shown for cases and controls in Figure 1. We demonstrate that circulating Bb levels significantly decreased over time in pregnancy (weekly slope (SE): -0.0094 (0.0005), $p < 0.01$). Estimated mean levels decreased from $0.75 \mu\text{g/ml}$ at 10 weeks to $0.56 \mu\text{g/ml}$ at 30 weeks. The largest amount of variability in Bb levels across subjects is attributed to differences in the starting values (intercepts), whereas all subjects had similar decreasing levels over time (small variability in slopes) (Figure 1). More specifically, a woman who initially had lower Bb levels experienced the same rate of decline compared with women with a higher initial level of Bb.

Differences in Bb levels between women who developed preeclampsia compared with women who remained normotensive during pregnancy were compared for each visit. There were no significant differences in the mean levels of Bb between the groups at any of the 3 visits (Table 2). We plotted the relationship between levels of Bb for women who developed preeclampsia and for women who remained normotensive throughout pregnancy across gestational age (Figure 2). On graphical inspection of Figure 2, a wide spread in gestational age corresponding to collection times for visit 1 is observed (aligned with the results reported above). A u-shaped curve was observed in Bb levels during visit 1 for women who developed preeclampsia. For these women, Bb levels decrease until around 10 weeks' gestation after which an increase in levels is indicated. The two curves are similar across groups for the later time points, in agreement with the results presented in Table 2.

The findings from the plot of the data (Figure 2) focused our attention on the relationship between Bb levels in the two groups over gestational age during visit 1. A quadratic function revealed the minimum of the u-shaped curve to be at 10.2 weeks. To introduce a change in slope to account for the u-shaped curve, a piece-wise linear regression with a fixed knot at 10 weeks was estimated. Ninety-seven women provided samples at less than 10 weeks' gestation for the first visit, 19 of whom developed preeclampsia. The remaining thirty-nine women provided visit 1 samples at 10 weeks' gestation or later and 9 developed preeclampsia. We show in Figure 3 the results from the piece-wise linear regression restricted to visit 1. At less than 10 weeks gestation, Bb levels decreased among cross-sectional samples collected with increasing gestational age in women who remained normotensive throughout pregnancy (weekly slope (SE): -0.007 (0.02) and similarly for those women who developed preeclampsia (weekly slope (SE): -0.059 (0.03) $p = 0.12$). However, among women who developed preeclampsia, average Bb levels were greater when drawn during the first study visit at a later gestational age [(15–20 weeks gestation, weekly slope (SE): 0.06 (0.02)] while levels among normotensive women continued to be inversely related with gestational age (weekly slope (SE): -0.02 (0.01)). The differences in samples collected from increasing gestational ages between cases and controls after 10 weeks was statistically significant ($p = 0.003$). These results were robust after adjusting for maternal prepregnancy BMI and a history of a hypertensive disease in a past pregnancy ($p = 0.004$ after adjustment). In addition to this analysis at visit 1, we also examined the slopes at visits 2 and 3 and found no significant difference between the groups ($p = 0.20$ and 0.15 , respectively).

Comment

There are several key findings from this nested case-control study. First, we found using this new Bb assay that the overall levels of Bb were highest in early pregnancy. Second, the relationship of levels of Bb with preeclampsia varied with the gestational age at blood draw via a u-shaped curve in early pregnancy. On average, Bb levels decrease in women who subsequently developed preeclampsia until around 10 weeks' gestation. In contrast, an increase in Bb levels was observed between 10 and 21 weeks' gestation in women who subsequently developed preeclampsia compared with women who remained normotensive throughout pregnancy. Third, there was no relationship between Bb and preeclampsia in samples taken at two time points in later pregnancy.

The focus of this study was the alternative complement pathway activation fragment Bb. We found the highest levels of Bb in early pregnancy, described by other authors as a pro-inflammatory phase of pregnancy.³² This finding suggests that the alternative pathway is active in early pregnancy in response to the physiological need to protect the developing fetus and placenta by identifying, recognizing, attacking and eliminating microbes or modified tissue cells^{23, 33}, a response totally in keeping with the function of this complement pathway and specifically the potent amplification loop.²⁸

We also found early pregnancy (up to the start of the second half of pregnancy) to be an important time to study the relationship of Bb with preeclampsia. In this gestational period, we demonstrated that the association of developing preeclampsia with Bb levels was dependent on the timing of the blood draw. Increasing Bb levels were observed in women who developed preeclampsia compared with normotensive controls in the period 10 to 21 weeks' gestation. Outside of this time period, Bb levels were similar across cases and controls. It is important to stress the nonlinear relationship of this inflammatory marker over time demonstrated in our statistical analysis. This observation would have been missed (Table 2) if we had not explored and accounted for trends in Bb with gestational age.

The findings up to approximately 21 weeks' gestation are aligned with the results of our previous study.³ In brief, in our previous prospective study we used a different Bb assay, recruited 76% of the cohort (n = 701) between 10 and 20 weeks' gestation, and found that women with an elevated Bb in a blood draw between 10 and 20 weeks' gestation were at a significantly elevated risk of developing preeclampsia.³ In our current nested case-control study, the early pregnancy samples had a larger range in gestational age at blood draw. The average mean Bb levels did not differ between cases and controls when samples collected during the entire range for visit 1 (Table 2) were used. However, after accounting for the non-linear relationship of Bb during pregnancy with preeclampsia, the importance of the time of collection was uncovered. Both studies together provide evidence that consideration of the gestational window (10 to 20 weeks' gestation)³ contributes to whether the association can be identified. We found no relationship between Bb and preeclampsia among the women who had a blood drawn less than 10 weeks' gestation in this or in our previous study. The transition point of 10 weeks' gestation found in both studies is around the time when the maternal circulation to the placenta is established.³⁴ We suggest that there is dysregulation of alternative complement pathway activation during this time of placental

remodeling in women who subsequently develop preeclampsia and suggest that there may be a critical window of gestational age for measuring complement biomarkers. Importantly, we highlight that gestational age may mask the effect of a biomarker if stratification analysis is not conducted over several periods of gestational age.

We hypothesized that levels of Bb would discriminate between women who developed preeclampsia and normotensives throughout pregnancy, but we found separation of levels between the groups only between 10 and 21 weeks' gestation. Moreover, adjusting for other covariates did not impact the results. It is also noteworthy that this lack of discrimination found in our study at visits 2 and 3 is in keeping with a study from Derzsy et al.¹¹ who found no significant difference in levels of Bb (using the same Bb Plus assay) from the third trimester of pregnancy in women with preeclampsia compared with women with uncomplicated pregnancies and healthy non-pregnant women. The longitudinal study of complement activation in pregnancy requires more research and future longitudinal studies will need to study not only the activation but also the regulatory arm of the complement system in a much larger cohort of pregnant women and at more time points during pregnancy.

Notwithstanding the important findings of this study, there were some limitations. The main limitation was sample size, especially low numbers of women with preeclampsia among the cohort recruited after 10 weeks' gestation. However, even with the sample size limitation, we still saw statistically significant results. In addition, only a single sample was collected during the first trimester, making the findings from those comparisons related to increasing gestational age subject to scrutiny. However, these changes are in line with our previous study³ and provide some evidence for our suppositions in the absence of repeated samples during this time period. In view of the findings from our two studies, we strongly advise that future longitudinal studies in pregnancy incorporate the collection of a blood sample around 10 and approximately 20 weeks' gestation. We were also unable to separately examine women who had gestational hypertension alone during pregnancy. These women were excluded from the analysis as the focus of our research question was preeclampsia. This should be considered in future studies as we previously described a relationship between C3a in early pregnancy with gestational hypertension.³⁵ We suggest that this outcome and other pregnancy outcomes related to hypertensive disease of pregnancy such as intra-uterine growth restriction should be included in future research of the role of complement system in pregnancy-related hypertensive disease. Nonetheless, we feel the strength of our study lies in the strong recruitment methodology, study design, meticulous collection of samples, follow-up of study participants and, importantly, the validation of results from our previous study.

We highlight in this study the importance of the timing of the gestational age at blood draw on the relationship of a biomarker with an adverse pregnancy outcome. Interventions for inhibiting complement activation³⁶ or attenuating the effect of inflammation may have a role in modifying adverse pregnancy outcomes associated with dysregulation of inflammation in early pregnancy.

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Figure 1. Bb levels decrease over time in pregnancy

Patient specific Bb levels are displayed for each woman (both cases and controls) over time (grey lines). The overall trend in Bb levels across all women estimated from the random coefficients model is overlaid (thick black line). The slope for the overall trend in Bb levels is $-0.0094/\text{per week}$ ($p < 0.01$).

Figure 2. Scatter plot and fitted curves of Complement Bb in women who developed preeclampsia (black stars and dark curve) and in women who remained normotensive (grey circles and light curve) during pregnancy

The points in the scatter plot are Bb levels in cases (stars) and in women controls (circles) at visit 1 (4.3–21 weeks), visit 2 (21.6–29.9 weeks) and visit 3 (32.4–38.3 weeks) delineated by the vertical lines. The loess procedure was used to fit a curve to the Bb across gestational age for cases (darker line) compared with controls (lighter line). We found: 1) a u-shaped curve with gestational age for samples collected during visit 1 from women who developed preeclampsia, 2) levels in controls decrease consistently over pregnancy and, 3) levels were only slightly higher during visits 2 and 3.

Figure 3. Results from the piece-wise linear regression restricted to visit 1 in women who developed preeclampsia (black stars and dark curve) and in women who remained normotensive (grey circles and light curve) during pregnancy

The lines indicate the resulting slope estimates from the piece-wise linear regression restricted to visit 1 with a fixed knot located at 10 weeks. The model estimates are overlaid on top of the observed Bb levels from women who developed preeclampsia (black stars) and in women who remained normotensive (grey circles). Bb levels decreased in both groups between 4.2 and 10 weeks' gestation. After 10 and up to 21 weeks' gestation Bb levels increased in women who developed preeclampsia. The slope estimates show only a marginal difference between cases and controls up to 10 weeks' gestation ($p = 0.12$) and a statistically significant difference in levels in the time period 10 to 21 weeks' gestation ($p < 0.01$).

Table 1

Demographics in Women with Preeclampsia and Normotensive Controls

	Preeclampsia		
	Yes (n =28)	No (n = 108)	p
Maternal age, Mean $\pm$ SD	29.8 $\pm$ 5.3	29.0 $\pm$ 4.9	0.49
Race/Ethnicity			
Non-Hispanic White	18 (64%)	67 (62%)	0.83
Hispanic	3 (11%)	18 (17%)	0.44
African American	3 (11%)	14 (13%)	0.75
Asian	0 (0%)	3 (2.8%)	0.37
Other	4 (14%)	6 (5.6%)	0.11
Prepregnancy BMI			
< 18.5	1 (4%)	5 (5%)	0.81
18.5 – 25	10 (36%)	53 (49%)	0.21
25 – 30	9 (32%)	26 (24%)	0.38
>30	8 (29%)	24 (22%)	0.48
Nulliparous	16 (57%)	48 (44%)	0.23
Cigarette smoking at conception	6 (21%)	15 (14%)	0.33
Prior hypertensive disease of pregnancy	6 (21%)	4 (3.7%)	<0.01

Table 2

Association of Bb levels at each of the three visits during pregnancy

	Preeclampsia				p
	Yes		No		
	n	Mean $\pm$ SD	n	Mean $\pm$ SD	
Visit 1	28	0.77 $\pm$ 0.26	108	0.77 $\pm$ 0.21	0.98
Visit 2	25	0.58 $\pm$ 0.23	94	0.56 $\pm$ 0.17	0.78
Visit 3	16	0.58 $\pm$ 0.25	95	0.52 $\pm$ 0.16	0.40