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Vaginal bleeding in early pregnancy and circulating markers of thrombin generation

David N. Hackney, MD, MSCR^a, Richard K Miller, PhD^a, Eva K Pressman, MD^a, Charles W Francis, MD^b, and Hyagriv N Simhan, MD, MSCR^c

^ADepartment of Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester New York

^DDepartment of Medicine, University of Rochester School of Medicine, Rochester, NY

^CDepartment of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, PA

Abstract

Objective—To determine if subjects experiencing acute vaginal bleeding in early pregnancy have increased plasma markers of thrombin generation compared to non-bleeding controls.

Methods—Subjects with clinically apparent acute (within 24 hours of sample collection) vaginal bleeding between 6 and 20 weeks estimated gestational age and without known thrombophilias were enrolled, along with non-bleeding controls, and underwent collection of maternal plasma. Concentrations of thrombin-antithrombin (TAT) and fragment 1 + 2 (F1+2) were determined by enzyme linked immunosorbent assay. Differences between bleeding and non-bleeding subjects were assessed through linear regression with adjustment for gestational age.

Results—20 subjects with vaginal bleeding and 20 controls were included. Bleeding was significantly associated with increased concentrations of TAT ($p=0.007$) and F1+2 ($p=0.044$) when corrected for gestational age. Among bleeding subjects, there was no association between markers of thrombin generation and the subject's description of bleeding quantity, though higher concentrations were associated with a longer self-reported duration of bleeding.

Conclusions—Clinically apparent vaginal bleeding in early pregnancy is associated with increased circulating maternal markers of thrombin generation. Thus, these maternal markers may have a future role in risk stratification.

Keywords

Vaginal bleeding; threatened abortion; threatened miscarriage; thrombin; thrombin-antithrombin complexes; fragment 1 and 2

Corresponding author: David N Hackney MD, MS, Division of Maternal Fetal Medicine, Department of Obstetrics and Gynecology, University of Rochester Medical Center, 601 Elmwood Ave, Rochester, NY 14642, Phone: (585)-275-7480, Fax: 585-256-1416, david_hackney@URMC.rochester.edu.

Declarations of Interest:

The authors report no declarations of interest.

Introduction

Approximately 12% of all pregnant patients experience clinically apparent vaginal bleeding in early pregnancy.[1] Their risk of preterm delivery is significantly increased, making clinical bleeding one of the most common risk factors for preterm birth. The literature on clinical bleeding events, however, is limited by substantial heterogeneity among study results. For example, the incidence of “bleeding” within published cohorts ranges from 1% to 25%, with the resultant Odds Ratios for preterm birth ranging from 1.1 to 3.0[1]. This significant heterogeneity complicates both patient counseling and clinical research efforts.

Much of the heterogeneity presumably arises from the subjective nature of the bleeding events. Clinicians, researchers and patients may differ on what degree of blood constitutes “bleeding”, and the quantification of the bleeding is typically based upon patient report. Optimally, a more quantifiable measures would exist that could be used uniformly by different investigators and clinicians. Maternal plasma markers of thrombin generation should intuitively be increased in subjects with bleeding. However, a past history of first or second trimester bleeding was not associated with increased concentrations of TAT at 24 or 28 weeks in the Preterm Prediction Study [2], nor was the presence of “light” vaginal bleeding associated with elevated TAT in patients experiencing acute preterm labor [3]. In both of these cases, however, the measurements were either performed remotely from the bleeding event or in the context of preterm acute labor[3–4]. The objective of this study was to determine if circulating markers of thrombin generation were increased in patients with clinical vaginal bleeding in early pregnancy when measured at or near the time of the bleeding event itself. Additionally, we sought to determine if these markers were associated with the patient’s subjective assessment of the bleeding’s quantity or duration.

Methods

The study was reviewed and approved by the University of Rochester Human Subjects Review Board. Subjects with vaginal bleeding were recruited prospectively from the ultrasound unit at the time of an unscheduled ultrasound. The minimum criteria for “bleeding” was a quantity sufficient for the subject’s provider to order an “emergent” or otherwise unscheduled ultrasound, and the subject verbally endorsing the presence of bright red vaginal bleeding within 24 hours of both study enrollment and plasma collection. Other inclusion criteria were: 1) estimated gestational age between 6 0/7 and 20 0/7 weeks, 2) singleton gestation, 3) fetal cardiac activity, 4) intent to delivery at the University of Rochester Medical Center or affiliated hospital. Exclusion criteria were: 1) current use of anticoagulation 2) history of thrombosis or embolism 3) known thrombophilia, liver disease, or other chronic medical condition with the potential to alter the coagulation system 4) drugs of abuse, including cocaine 5) identification of a non-uterine source of vaginal bleeding, as determined by the subsequent review of the outpatient medical record. This determination was based on the referring provider’s evaluation. Laboratory testing for drugs of abuse was not specifically performed as part of the study and was based upon prenatal record review. Control subjects without vaginal bleeding were recruited from women in the hospital-affiliated private practice and resident clinics and who otherwise met the inclusion and exclusion criteria. Before enrollment, control subjects were verbally asked about the

presence of vaginal bleeding during the pregnancy, and were excluded if there was any self-reported prior bleeding or “spotting”. Control subjects were enrolled in three groups in order to coordinate plasma collection with the timing of routine blood draws: 6–11 weeks (prenatal battery), 11–14 weeks (first trimester screen) and 15–20 weeks (second trimester screen or maternal serum alpha-fetoprotein testing). Because markers of thrombin generation are known to increase with gestational age[5], an equivalent number of control and bleeding subjects were frequency matched within these three broad ranges, but not otherwise matched to specific individual gestational ages.

Prenatal records and ultrasound reports were obtained and reviewed for all subjects after enrollment. Subchorionic hematomas (SCH) were defined as echolucencies between the chorion and the uterine wall. One of the study authors (DNH) reviewed all images to confirm the presence or absence of a SCH. At the time of enrollment, subjects with bleeding were also queried regarding the presence or absence of prior invasive obstetric procedures (amniocentesis or chorionic villous sampling) and for the duration of bleeding. Additionally they were asked to rate the quantity of their current bleeding as: 1) “Light”, defined as not sufficient for a menstrual pad or other sanitation, 2) “Medium”, defined as necessitating a menstrual pad, though it was never completely soaked and did not need to be changed frequently or 3) “Heavy”, defined as repeatedly having to change menstrual pads.

All enrolled subjects underwent venipuncture for the collection of 3–4 mL of blood in a sodium citrate vacutainer, which was thereafter centrifuged for the collection of plasma, frozen and stored at -20°C . Samples were aliquoted for the minimization of freeze-thaw cycles. Concentrations of thrombin-antithrombin (TAT) and prothrombin fragment 1 + 2 (F1+2) were measured using commercially available enzyme-linked immunosorbance assays (ELISA) (Affinity Biologicals Inc., Ancaster, ON, Canada) according to the manufacturer’s recommendations and a previously described protocol[6]. All samples were run in duplicate, with an intra-assay correlation coefficient of 98.1% for TAT and 98% for F1+2. All results that were outside of the standard curve underwent additional dilutions. The individual performing the ELISA was blinded to the case allocation.

Clinical characteristics between subjects with and without bleeding were analyzed with the t-test, Fisher’s Exact test or Mann-Whitney U where appropriate. All tests were two-sided with a p value of 0.05 designating significance. The relationship between TAT and F1+2 concentrations and gestational age in the non-bleeding control group was evaluated through linear regression. Correlations between TAT and F1+2 were evaluated through Spearman’s rho. Differences in TAT and F1+2 concentrations in bleeding versus non-bleeding subjects were evaluated through linear regression with adjustment for gestational age, as well as an unadjusted Mann-Whitney U. The association between self-reported duration of bleeding and TAT and F1+2 was evaluated through linear regression, and the association with the reported quantity of bleeding was evaluated through Mann-Whitney U. Post-regression diagnostics were evaluated through standardized Pearson’s residuals, Cook’s D and DFBETA, as well as visual inspection of residual versus fitted values plots. All analyses were performed with Stata 11 (College Station, TX, USA)

Results

Twenty subjects with, and 20 subjects without, vaginal bleeding were enrolled, for a total sample size of 40. No subjects were excluded secondary to the later identification of substance abuse or a non-uterine source of bleeding. Baseline clinical characteristics between the two groups are presented in Table I. Two subjects in the control group and three in the bleeding group had had a prior spontaneous preterm birth, though for one of the subjects in the bleeding group it had occurred in the context of a spontaneous triplet gestation. One of the subjects in the bleeding group had experienced four prior spontaneous abortions, though had had a negative workup (including negative anticardiolipin antibodies) and also had had uncomplicated term deliveries and was thus not excluded. No other subjects in either group had otherwise had more than two spontaneous abortions, and the percentage of subjects with prior abortions did not differ between the two groups. The two groups differed significantly in maternal age (which was higher in the bleeding group) and there was a trend towards increased parity in the bleeding group.

TAT and F1+2 were correlated significantly (Spearman's rho 0.86, $p < 0.001$). Within the control group, there was a significant positive association in linear regression between gestational age and the concentration of TAT ($p = 0.002$, $R^2 = 40\%$) and F1+2 ($p = 0.012$, $R^2 = 26\%$). Linear regression was then performed with TAT or F1+2 concentration as the outcome and gestational age and bleeding as the predictors. When corrected for gestational age, bleeding was associated with increased concentrations of TAT ($p = 0.007$) and F1+2 ($p = 0.044$). Because of the potential differences in maternal age and parity, stepwise backwards logistic regression was then performed with maternal age and parity added to bleeding and gestational age as predictors and $p > 0.05$ the cut-off for inclusion in the final model. Maternal age and parity were both eliminated from the model. Concentrations of both analytes are presented graphically in Figures 1 and 2, respectively. In an unadjusted analysis (Mann Whitney U), the association between bleeding and markers of thrombin generation was significant for F1+2 ($p = 0.013$), though was a trend for TAT ($p = 0.07$).

Among the subjects with vaginal bleeding, the median self-reported duration of bleeding was 2 days, with a range of 1 to 98. In linear regression, duration of vaginal bleeding was significantly associated with the concentration of TAT ($p = 0.023$, $R^2 = 21.6\%$) and F1+2 ($p = 0.03$, $R^2 = 35.2\%$). Ten subjects with bleeding identified it as “light”, 7 as “medium” and 3 as “heavy”. Because only 3 subjects reported “heavy” bleeding, they were not analyzed separately. There were no significant differences in the concentrations of TAT ($p = 0.49$) or F1+2 ($p = 0.88$) in subjects with “light” versus “moderate” bleeding. Seven subjects with bleeding were identified as having a subchorionic hematoma on ultrasound. The presence of a subchorionic hematoma was not associated with an increased concentration of TAT ($p = 0.72$) or F1+2 ($p = 0.4$). None of the subjects with bleeding reported a prior invasive procedure. The median concentrations and range for both analytes within different subgroups of subjects with vaginal bleeding are presented in Table II.

Two subjects with particularly high concentrations of TAT were identified as potential outliers on the basis of standardized Pearson's residuals (2.93 and 3.11). Of note, in both

cases, the values fell within the range of TAT concentrations observed in pregnant subjects in several other cohorts[2, 7–8]. Additionally, both subjects were not only in the bleeding groups, but had reported two of the longer durations of bleeding (98 and 15 days). Thus the concentrations were felt to be biologically plausible. As a measure of their influence and leverage on the regression results, Cook's distance and DFBETA were measured on both subjects for both TAT and F1+2, and in both cases was less than 1 (maximum Cook's distance 0.6 for F1+2 and maximum DFBETA 0.94, both in the latter subject). The potential outliers did not generate a violation of the normality assumption of linear regression as determined by visual inspection and normality testing of the residual histogram. Visual inspection of the residual versus fitted value plot demonstrated a potential violation of homoskedasticity. Thus the linear regression was repeated with robust estimators, in which the association between bleeding and TAT remained significant ($p=0.009$), though was a trend for F1+2 ($p=0.053$).

Discussion

The presence of vaginal bleeding in early pregnancy is associated with increased concentrations of circulating markers of thrombin generation at the time of the bleeding event. Prior studies have not demonstrated this association, albeit within different contexts. No association between early bleeding events and plasma markers in later pregnancy were apparent in the Preterm Prediction Study[2], and Elovitz et al found no association between the presence or absence of "light" vaginal bleeding in subjects with contemporaneous acute preterm labor[3]. In fact, even though our study demonstrated an overall association between clinical bleeding and thrombin generation, many of the bleeding subjects did still have concentrations that would be considered to be within the normal range.

Strengths of our study include the collection of plasma samples acutely at the time of or within 24 hours of the bleeding event itself. Presuming that these markers would decrease as time from the acute event increased, the close proximity of blood draw to bleeding event may have been the primary factor that allowed us to detect a difference. By requiring that the bleeding be sufficient for their provider to order an ultrasound, we ascertained a subject population with more clinically significant vaginal bleeding in order to best detect a difference. Still, despite these efforts, only half of our subjects reported bleeding that was "medium" or "heavy".

Limitations in our study include the fact that, though we attempted to exclude bleeding from a non-uterine source, some of the subjects still may have had bleeding secondary to an infectious etiology. Of note, even if additional cervical cultures or other studies had been collected, it may not have been possible to rule out an infectious or inflammatory event that was either causing or occurring in association with the bleeding, as the pathways of coagulation and inflammation are interrelated and bleeding itself may be a marker for subclinical intrauterine infection. For example, De Felice et al[9] demonstrated a higher incidence of histologic chorioamnionitis in subjects who had previously experienced vaginal bleeding in pregnancy, and Gomez et al[10] had demonstrated a significant incidence of positive amniotic fluid bacterial cultures in subjects with otherwise unexplained vaginal bleeding who underwent amniocentesis. Thus differentiating bleeding from an infectious

versus non-infectious sources is not straightforward, or perhaps even possible prior to delivery.

The evaluation of plasma markers of thrombin generation in early pregnancy is complicated by the fact that they normally rise during the first two trimesters, as has been demonstrated by prior investigators[5] and confirmed here. The source of the increase is within the uterus itself, as opposed to an increase in peripheral thrombin generation, as was demonstrated in the uterine vein studies of Uszynski et al[11]. The exact role of increasing thrombin generation within the placenta is unclear, though it is probably not pathologic at baseline and in fact may be a marker of a healthy, physiologic process. For example, two studies have demonstrated an association between low concentrations of TAT in asymptomatic subjects and the later occurrence of adverse events or placental pathology[2, 8]. Methodologically, one could circumvent the problem of normally rising concentrations by performing measurements on all subjects at an equivalent gestational age, though this is not possible if one is attempting to evaluate an acute and unpredictable event, such as vaginal bleeding. Thus, studies necessitate a correction for gestational age when evaluating TAT and F1+2 concentrations.

Additionally, our study cannot rule out the possibility that subjects with bleeding had intrinsic coagulation abnormalities that resulted in increased baseline rates of thrombin generation. Of note, though thrombophilia screening was not performed systematically as part of this study, no subjects had a known thrombophilia or prior thrombotic event. One subject had four prior spontaneous abortions (in addition to an uncomplicated term delivery) and another had a history of a prior delivery at 27 weeks secondary to severe pre-eclampsia. Both subjects, however, had undergone thrombophilia testing prior to the pregnancy and were negative. None of the other subjects would have had clinical indications for a thrombophilia evaluation, and thus the probability of an unrecognized significant thrombophilia, while not impossible, would be low. With regards to TAT concentrations having reflected baseline elevations instead of being a response to the bleed, a history of prior bleeding was not associated with baseline elevations at 24 or 28 weeks in the Preterm Prediction Study[2]. However, serial measurements of coagulation markers after a bleeding event in pregnancy would be a potential avenue of future investigation.

The sample size of our study was modest, though sufficient for the identification of statistically significant results. However, in some of the secondary analyses, such as the evaluation of subjects with “light” versus “medium” bleeding or with or without a subchorionic hematoma, the possibility exists of a type II error. Thus, caution should be employed in the interpretation of those findings.

Our study was not powered for the evaluation of subsequent obstetric outcomes, as the optimal clinical utility of thrombin generation markers would be in the quantification of future pregnancy risks in subjects with early bleeding events. Such patients account for around 10% of the general obstetrical population, and thus constitute one of the most commonly encountered risk groups for preterm birth. Though thrombin generation markers have been studied with regards to outcome in subjects with preterm labor with and without bleeding[3–4] and in subjects who were asymptomatic at the time of the measurement[2, 6,

12], the relationship with future outcomes in subjects with acute early bleeding events has not been evaluated. The presence of an association potentially could address the significant problem of suboptimal quantification measures and lack of uniform definitions which complicate the literature on bleeding and obstetrical outcomes[1]. In much the same way, sonographic cervical lengths and vaginal fetal-fibronectin have added needed quantization to the clinical entities of cervical “competence” and preterm labor. Determining that thrombin generation markers are, in fact, elevated versus non-bleeding subjects in this population, however, is a necessary first step.

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Figure 1.

Concentrations of thrombin-antithrombin complexes across gestational ages in subjects with or without clinical vaginal bleeding. Shaded areas represent 95% confidence intervals.

Figure 2.

Concentrations of prothrombin fraction 1 and 2 across gestational ages in subjects with or without clinical vaginal bleeding. Shaded areas represent 95% confidence intervals.

Table I
Clinical characteristics in subjects with and without clinical vaginal bleeding

Baseline clinical and demographic data of the included subjects.

	Bleeding	Control	p
	(n=20)	(n=20)	
% African-American (n)	55% (11)	30% (6)	0.2 [*]
% Prior SAB (n)	50% (10)	35% (7)	0.52 [*]
Median gravity (range)	2 (1–6)	2 (1–7)	0.2 [¶]
Median parity (range)	1 (0–2)	0 (0–5)	0.072 [¶]
Median maternal age (range)	27 (18–44)	23 (18–45)	0.042 [¶]
Mean weeks EGA (SD)	12.8 (3.3)	13.3 (3.6)	0.62 [§]

SAB = spontaneous abortion. EGA = Estimated gestational age. SD = standard deviation

^{*} Fisher's Exact Test

[¶] Mann-Whitney U

[§] t-test

Table II
Thrombin-antithrombin and Fragment 1 and 3 concentrations in bleeding subgroups

Concentrations of thrombin antithrombin complexes and fragment 1 and 2 in subjects within different bleeding subgroups. TAT=thrombinantithrombin complexes, F1+2= Prothrombin fragments 1 and 2. SCH=subchorionic hematoma.

	TAT	F1+2
	ng/mL	nM/L
	Mean (range)	Mean (range)
Any vaginal bleeding	31.1 (3.5–112.3)	40.7 (3.4–230.5)
No vaginal bleeding	10.7 (1.4–23.4)	11.9 (1.1–51)
“Light” bleeding	41.1 (5.5–112.3)	57.4 (3.9–230.5)
“Moderate” bleeding	18.1 (3.5–50.9)	23.4(3.4–73.2)
Bleeding w/ SCH	37.1 (3.5–103.5)	54.5(3.7–212.9)
Bleeding w/o SCH	28 (5.5–112.3)	33.2 (3.4–230.5)