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ORIGINAL ARTICLE

Protein Z, protein S levels are lower in patients with thrombophilia and subsequent pregnancy complications

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Summary. *Objective*: We posit that low levels of protein S (PS) and protein Z (PZ) contribute to adverse pregnancy outcome (APO). *Patients*: We evaluated 103 women with subsequent normal pregnancy outcome (NPO), 106 women with APO, and 20 women with thrombophilia (TP). *Methods*: We compared first trimester (1st TRI) PZ levels in 103 women with NPO, 106 women with APO, and in 20 women with TP. We compared plasma levels of PZ and free PS antigen during the second (2nd TRI) and third trimesters (3rd TRI) of pregnancy in 51 women with APO and 51 matched women with NPO. *Results*: The mean 1st TRI PZ level was significantly lower among patients with APO, compared to pregnant controls (1.81 ± 0.7 vs. 2.21 ± 0.8 μg mL^{-1}, respectively, $P < 0.001$). Of patients with known TP, those with APO had a tendency for lower mean PZ levels compared to those TP women with NPO (1.5 ± 0.6 vs. 2.3 ± 0.9 μg mL^{-1}, respectively, $P < 0.0631$). There was a significant decrease in the PZ levels in patients with APO compared to NPO (2nd TRI 1.5 ± 0.4 vs. 2.0 ± 0.5 μg mL^{-1}, $P < 0.0001$; and 3rd TRI 1.6 ± 0.5 vs. 1.9 ± 0.5 μg mL^{-1}, $P < 0.0002$). Protein S levels were significantly lower in the 2nd and 3rd TRIs among patients with APO compared to patients with NPO (2nd TRI 34.4 ± 11.8% vs. 38.9 ± 10.3%, $P < 0.05$, respectively; and 3rd TRI 27.5 ± 8.4 vs. 31.2 ± 7.4, $P < 0.025$, respectively). *Conclusions*: We posit that decreased PZ and PS levels are additional risk factors for APO.

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Introduction

Several investigators have demonstrated that uteroplacental vascular abnormalities are associated with pregnancy complications defined as pre-eclampsia, fetal loss, intrauterine growth restriction (< 10th percentile), abruptio placentae and preterm delivery (< 37 weeks) [1–3]. Common thrombophilic (TP) conditions have been demonstrated to occur with increased frequency among women who have experienced one or more pregnancy complications by some [4,5], but not all investigators [6,7]. There are minimal prospective data on the risk of pregnancy complications when carrier states of these TP conditions are evaluated [8]. Recurrence risks for pregnancy complications in women with TP have been reported to range from 30 to 77% [9].

In addition, when a TP is identified, multiple TP conditions are present in 10% of cases [10]. This raises the speculation that additional and or as yet unmeasured abnormalities may increase the prothrombotic milieu that is present in pregnancy.

Normal pregnancy is associated with decreased levels of protein S (PS) activity and free PS antigen in the vast majority of patients [11]. In addition, most normal pregnancies acquire some degree of resistance to activated protein C (APC), when measured by the first generation global assays and tests that measure endogenous thrombin potential [12,13]. Factor X (FX), its activation to FXa and participation in the activation of prothrombin, is a central element in the generation of thrombin [14]. Therefore, it is possible that impairment in, or decrease in, the control of FXa in the addition to other prothrombotic changes may play an important role in the control of the hemostatic system with possible adverse effects in pregnancy.

Protein S functions as part of the natural protein C anticoagulant system as a cofactor for APC in the degradation of FVa and FVIIIA [15]. Protein S has an APC independent anticoagulant activity through its binding to cell surface negatively charged phospholipids. This inhibits the activity of the complexes (FVIIIa–FIXa–cell surface phospholipid) and FXa–FVa–cell surface phospholipid) thereby decreasing the generation of thrombin [16,17].

Recently, a role for protein Z (PZ) in controlling FXa activity has been elucidated [18]. Protein Z is a 62 kDa vitamin K-dependent plasma protein that serves as a cofactor for a PZ-dependent protease inhibitor of FXa [19]. In addition to antithrombin and the tissue factor pathway inhibitor, PZ has a role in the control of FXa [20]. Protein Z increases rapidly during the first months of life followed by slow increases during childhood, with adult levels reached during puberty [21,22]. Recent studies have shown that PZ deficiency influences the prothrombotic phenotype in patients with the FV Leiden mutation [23]. Low plasma PZ levels have been reported in patients with antiphospholipid antibodies [24]. There are contradictory reports of an association between ischemic stroke and PZ deficiency ($< 1.0\ \mu g\ mL^{-1}$) [25,26], and there is a high prevalence of PZ deficiency in patients with unexplained early fetal loss (10th–19th weeks) [27]. The role of PZ in regulation of the hemostatic system is still under investigation, as there is evidence that PZ deficiency promotes bleeding [28].

We therefore hypothesized that decreased PZ concentrations, in addition to decreased PS levels, would contribute to the prothrombotic state that would increase the risk for pregnancy complications associated with uteroplacental vascular abnormalities.

Materials and methods

Study design

As part of an ongoing study (March of Dimes Study) on the causes of prematurity, we performed a cross-sectional study comparing first-trimester (1st TRI) (0–14 weeks) PZ levels among 103 healthy women with eventual normal pregnancy outcomes and 106 women who experienced pregnancy complications in the index pregnancy. We also assessed PZ levels among 20 patients with documented acquired or inherited thrombophilias, six of whom (30%) experienced one or more of the pregnancy complications. Demographic data and antepartum events on the patients with normal pregnancy outcome and pregnancy complications were collected prospectively as a part of the March of Dimes *P*reterm *D*elivery *P*rediction *S*tudy (PDPS) to minimize recall bias and incomplete information.

In a subgroup of patients, we measured maternal plasma PZ and plasma free PS antigen levels in the second trimester [(2nd TRI) (14–< 27 weeks)] and the third trimester [(3rd TRI) (≥ 27 weeks)] from 51 patients who experienced pregnancy complications in the index pregnancy, and 51 healthy women with normal pregnancy outcomes who were matched for race, maternal age, gestational age at blood draw, parity and gravidity.

Patient groups

Healthy patients with eventual normal pregnancy outcomes (NPO) Patients with normal pregnancies served as the controls and were defined retrospectively following delivery of an uncomplicated singleton gestation of a full term, appropriately grown fetus. Exclusion criteria for the control group include complications: namely, intrauterine fetal death (IUFD), intrauterine growth restriction (IUGR) defined as < 10th percentile for birth weight [29], pre-eclampsia defined by established criteria [30], abruptio placentae, gestational hypertension, preterm labor, preterm rupture of membranes (PROM), bleeding in any trimester and medical disorders such as diabetes, stroke and thromboembolic disease.

Patients with pregnancy complications (APO) Subjects were identified as patients with pregnancy complications if any one or more of the following were present:

1. Unexplained fetal demise ($N = 0$) occurring after 14 weeks, unrelated to major malformations, karyotype abnormalities, maternal or fetal infections, maternal uterine or cervical malformations.
2. IUGR ($N = 6$), defined as birth weight < 10th percentile for gestational age [29].
3. Pre-eclampsia ($N = 26$): defined as normotension prior to 20 weeks' gestation, hypertension (single diastolic blood pressure of 110 mmHg or greater, or consecutive reading of 90 mmHg or greater on more than one occasion at least 4 h apart, and proteinuria developing after 20 weeks [30].
4. Bleeding ($N = 47$): defined as unexplained vaginal bleeding during more than one trimester.
5. Preterm delivery ($N = 21$): defined as a delivery occurring prior to 37 completed weeks of gestation, associated with intact membranes [31].
6. Preterm delivery associated with preterm premature rupture of membranes ($N = 6$): defined as a delivery occurring prior to 37 completed weeks of gestation, associated with ruptured membranes, considered to be present if either fern, pooling, nitrazine are positive, oligohydramnios is documented on sonogram [32].

Subjects with acquired or inherited thrombophilia (s) Patients were considered to have a thrombophilic condition if one or more of the following were identified: (i) presence of FV Leiden mutation; (ii) presence of prothrombin gene mutation 20210 A; (iii) deficiency of protein C; (iv) deficiency of protein S; (v) deficiency of antithrombin; (vi) presence of anticardiolipin antibodies; (vii) presence of lupus inhibitor (anticoagulant); and (viii) hyperhomocysteinemia.

Data collection

Basic data regarding past medical, surgical, gynecologic and obstetric histories, sociodemographic characteristics, antepartum, intrapartum and postpartum course or complications and neonatal outcome were extracted from the Bellevue Hospital, New York University Medical Center Institutional Review Board (IRB)-approved Peri-grant perinatal database [32], or through a prospective IRB-approved longitudinal study of pre-eclampsia. These data, together with all pertinent laboratory data, were entered into a relational database (4th Dimension, ACI US, Inc., Cupertino, CA, USA) through a series of existing entry screens custom designed for this purpose. The screens contain a host of validity and range checks to minimize the possibility for data entry error. In addition, journal entry reports list the details of each record entered or updated, and these reports are compared with the source documents.

Biochemical assays

Protein Z was measured by a commercially available immunoassay (Diagnostica Stago, Parsippany, NJ, USA) [33]. The inter- and intracoefficient variances are 8 and 5%, respectively.

We measured free PS antigen levels by Liatest free PS kit on STA Compact from Diagnostica Stago (Parsippany, NJ, USA). This is an immunoturbidimetric, antigenic assay for free protein S [34]. The inter- and intracoefficient variances are less than 10%. Data were analyzed by the *t*-test, ANOVA and χ^2 tests.

Results

Table 1 displays the maternal age, gestational age at blood draw and obstetric data for the 103 women with NPO, 106 women with APO and 20 women known to be thrombophilic (TP), with six of these women having had APO. Mean gestational age at delivery and birth weight were significantly lower in APO and TP groups compared to the normal pregnancy group (*t*-test, $P < 0.0001$ and 0.0002, respectively). Tables 2 and 3 show demographic and clinical data for the subgroups of APO ($N = 51$) and NPO ($N = 51$) patients. There are no statistically significant differences of patients' demographic data between these two subgroups, except the maternal age (27.8 ± 5.1 vs. 29.3 ± 5.3, $P = 0.025$). In these two subgroups of 51 NPO and 51 APO, there was a significant difference in gestational age at delivery ($P = 0.0025$) but only a trend in birth weight ($P = 0.0510$,Welch *t*-test for disparate SDs).

The mean 1st TRI maternal plasma PZ level for the entire group was significantly lower among APO ($N = 106$) patients (1.81 ± 0.7 μg mL^{-1}) compared to pregnant controls with NPO ($N = 103$) (2.21 ± 0.8 μg mL^{-1}; ANOVA, $P < 0.001$, with Bonferonni correction), whereas mean 1st TRI maternal plasma PZ level of TP patients fell between the mean of the NPO and APO patients (2.02 ± 0.9 μg mL^{-1}, $P =$ NS). The subset of pregnant patients with TP who had APO (6/20, 30%) had lower plasma PZ levels compared to pregnant patients with TP with NPO, but the difference did not reach statistical significance (14/20) (*t*-test, 1.5 ± 0.6 vs. 2.3 ± 0.9 μg mL^{-1}; $P = 0.0631$).

The IUGR group had the lowest mean PZ levels and the PPROM had the highest mean PZ levels (1.38 ± 0.66 μg mL^{-1}L and 2.03 ± 0.7 μg mL^{-1}, respectively). However, due to the small number of samples in each group, the difference between different APO groups is not statistically significant (ANOVA, $P < 0.59$). Linear regression analyses of PZ levels against birth weight and gestational age at delivery were calculated. The data showed no correlation between PZ levels and birth weight, or PZ levels and gestational age at delivery at any of the trimesters of PZ sampling ($r^2 \geq 0.01$).

Protein Z and protein S levels were significantly decreased as gestation progressed in the subgroups of NPO and APO patients (Table 4). For each trimester, mean PZ levels were lower in the APO group compared to NPO (1st TRI $P \leq 0.024$, 2nd TRI $P < 0.0001$ and 3rd TRI $P < 0.0002$). The PS levels were significantly decreased as pregnancy progressed (*t*-test, $P < 0.01$). The differences in PS levels between NPO and APO groups in both 2nd TRI and 3rd TRI were statistically significant ($P < 0.025$). Based upon receiver-operator characteristic (ROC) analysis, selective cutoffs for 2nd TRI PZ levels were determined to predict APO, and odds ratios were

Table 1 First trimester protein Z levels: demographic and clinical data [adverse pregnancy outcome (APO) vs. normal outcome (NPO) vs. thrombophilia (TP)]

Mean	Maternal age (years)	Gravidity	Parity	Gestational age at testing (weeks)	Gestational age delivery (weeks)	Birth weight (g)
NPO (*N*=103)	27.2 ± 5.7	2.5 ± 1.7	1.0 ± 1.2	11.0 ± 2.1	39.3 ± 1.3*	3415 ± 472*
APO (*N*=106)	32.1 ± 6.1	2.9 ± 1.5	0.8 ± 1.0	10.6 ± 2.6	37.1 ± 4.6	3102 ± 684
TP (*N*=20)	32.7 ± 3.0	5.2 ± 2.1**	1.4 ± 1.7	8.9 ± 3.0	37.4 ± 2.1	3142 ± 756

The mean gestational age at delivery and infant birth weight were significantly lower in APO and thrombophilia groups compared to the normal pregnancy group (*t*-test *$P < 0.0001$ and 0.0002, respectively). ** Women with thrombophilia had more pregnancies (*t*-test, $P < 0.05$).

Table 2 Demographic and clinical data for 51 patients with APO

Race	Percentage	Maternal age (years)	Parity (no.)	Gravidity (no.)	Delivery age (weeks)	Birth weight (g)
Asian	14	29.3 ± 5.3	2.6 ± 1.2	0.9 ± 1.1	38.4 ± 2.0	3321.0 ± 637.8
Black	2					
Hispanic	57					
White	27					

Table 3 Demographic and clinical data for 51 patients with normal pregnancy outcome

Race	Percentage	Maternal age (years)	Parity (no.)	Gravidity (no.)	Delivery age (weeks)	Birth weight (g)
Asian	16					
Black	2	27.8 ± 5.1	2.5 ± 1.1	1.0 ± 0.9	39.4 ± 1.1	3535.9 ± 441.2
Hispanic	67					
White	14					

Table 4 Mean protein Z (μg ml^{-1}) and protein S (%) levels in subgroups of APO and NPO throughout the pregnancy

	APO		NPO		
	PZ	PS	PZ	PS	
1st trimester	2.01 ± 0.76		2.34 ± 0.69		APO vs. NPO $P < 0.024$
2nd trimester	1.47 ± 0.45*	34.4 ± 11.8	1.98 ± 0.54**	38.9 ± 10.3	APO vs. NPO $P < 0.0001$ for PZ and $P < 0.025$ for PS
3rd trimester	1.55 ± 0.48*	27.5 ± 8.4†	1.93 ± 0.51**	31.2 ± 7.4‡	APO vs. NPO $P < 0.0002$ for PZ and $P < 0.025$ for PS

*Compared to 1st TRI; $P < 0.001$. †Compared 2nd to 3rd TRI, $P < 0.01$; one way ANOVA. **Compared to 1st TRI; $P < 0.001$. ‡Compared 2nd to 3rd TRI, $P < 0.01$; one way ANOVA.

Table 5 Odds for abnormal pregnancy outcome by second trimester PZ levels

PZ Value (%tile)	Odds Ratio for APO (95% CI)	Sensitivity	Specificity
1.30 μg ml^{-1} (20th)	4.25 (1.536–11.759)	93%	32%
1.75 μg ml^{-1} (50th)	2.571 (1.591–4.156)	73%	83%
2.04 μg ml^{-1} (80th)	1.515 (1.233–1.862)	35%	96%

calculated at significant 2nd TRI PZ values, as shown in Table 5.

Discussion

Protein S, in addition to its activity as a cofactor for APC-mediated degradation of FVa and FVIIIa, has anticoagulant activity by its binding to cell surface negatively charged phospholipids and the inhibition of the prothrombinase and tenase complex (FXa/FVa/platelet surface phospholipid/ Ca^{++} in complex) activity. It is possible that a decrease in FXa inhibition (i.e. net increase in FXa activity) may enhance the risk of thrombotic and adverse pregnancy events. In addition to tissue factor pathway inhibitor and antithrombin, PZ, acting as a cofactor for PZ-dependent protease inhibitor, has an important role in the control of FXa.

Gris *et al.* noted a significant number of patients with PZ deficiency in the early fetal loss group with the level of < 1 μg mL^{-1} in 44 of 200 patients, and especially among patients with fetal demise between the beginning of the 10th week and end of the 15th weeks of gestation [27]. These authors found an increased risk of fetal loss associated with PZ deficiency (odds ratio of 6.7, 95% CI 3.1–14.8, $P < 0.001$). They did note that the patients with late fetal loss and recurrent miscarriages had lower PZ levels. In patients with recurrent embryonic loss and PZ deficiency, Gris *et al.* found a inverse correlation between antiprotein Z IgM antibody levels and protein Z concentrations ($P = -0.43$) [35]. However, antiprotein Z IgG antibody and antiprotein Z IgM antibody levels were not correlated with protein Z levels in the entire cohort of 191 patients with pathologic pregnancy and 191 controls. Gris *et al.* have postulated that antiprotein Z antibodies contribute to enhanced immune complex formation, which can result in lower levels of protein Z.

The combination of decreased PZ and PS may act synergistically to enhance the prothrombotic process. In those patients with other measurable and defined thrombophilic disorders or in those with other risk factors or as yet not defined, the decreased PZ and PS may represent an extra hemostatic burden. In our patient groups we found that PZ decreased with gestation, with statistically greater decreases in the APO group. PS was decreased in patients in the 2nd and 3rd trimesters, as has been reported by others [11]. Protein S levels were significantly lower in the APO group compared to the NPO group ($P < 0.025$). Based on our data, the lower limits of normal for PS levels are 29% and 23% for the 2nd TRI and 3rd TRI, respectively. Therefore, we speculate that free PS levels below 29% in the 2nd TRI of pregnancy may indicate a greater risk for APO, if confirmed in a larger study.

Our data suggest that, in addition to other established inherited and acquired prothrombotic changes in pregnancy, the changes in the levels of PZ may have an important role in the regulation of thrombin generation. Low PZ levels, when added to the decreased PS, may represent an additional burden in the hemostatic balance. In addition, the lower PZ and/or PS may compound the prothrombotic milieu that is associated with the other TP disorders. We have reported on the decreased anticoagulant response to soluble thrombomodulin in patients with APO. The mechanism is still to be determined. This would further support the complex prothrombotic changes that have been noted during pregnancy [36]. Further studies with larger groups of patients are indicated to answer these questions. Decreased PZ levels may play an important role in the occurrence of APO. Our data support the finding of PZ deficiency as an additional risk factor for poor pregnancy outcome, as demonstrated in the recent prospective thromboprophylaxis (antenatal daily administration of low dose aspirin 100 mg or subcutaneous enoxaparin (40 mg) trial in women with inherited thrombophilias and prior unexplained fetal loss [37].

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