

## Assessment of Protein C and Protein S of Pregnancy Loss Victims

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### ABSTRACT

**Background:** The haemostatic changes that result in thrombophilia during the pregnant state have been linked to pregnancy loss. **Objective:** Assessment of Protein S, and Protein C assays in pregnancy loss victims in Abia State, South East, Nigeria. **Materials and Methods:** This was a cross-sectional study involving women in their reproductive years. Study population was stratified into 3 groups and the Protein C and Protein S concentrations measured and compared among the three groups. **Results:** A total of 130 apparently healthy Nigerian women of child-bearing age were enrolled in the study. The study groups consisted of 70 women who had just lost a pregnancy, 30 women with normally progressing pregnancy and 30 nonpregnant women. The protein C concentration for the pregnancy-loss subjects was significantly lower than that of the normal pregnancy at  $p \leq 0.01$  while that of Protein S showed non-significance ( $p > 0.05$ ). **Conclusion:** Protein C deficiency is associated with increase in pregnancy loss.

**Key words:** Pregnancy loss, Protein C, Protein S.

## INTRODUCTION

Human reproduction is accompanied by physiological changes that occur primarily to nurture pregnancy as they ensure the proper development, growth and ultimate survival of the embryo/foetus. These changes include (but are not limited to) cardiovascular, respiratory, metabolic/endocrinal, renal, haematologic and immunologic changes (Priya *et al.*, 2016). Some of these changes influence normal biochemical values while others may mimic symptoms of medical disease (Priya *et al.*, 2016). The pregnant state is not failure-proof. Pregnancy loss (also termed miscarriage or abortion) is the spontaneous loss of an intra-uterine pregnancy without outside intervention, and occurring before 20 weeks of gestation (Abdullah and Mahdi, 2013). Pregnancy is associated with significant changes in the haemostatic profile (Chandra *et al.*, 2012) creating a hypercoagulable state, and which has been attributed to alterations of the proteins that govern blood clotting (Gersh *et al.*, 2009), thus enforcing a thrombophilic state (Walker, 2000). There are varied reports regarding the levels of Protein C and Protein S during pregnancy. Oruc *et al.*, (2000), reported reduced levels of both Protein C and Protein S in pregnancy (most probably dilutional). Said *et al.*, (2010) and Petrozza *et al.*, (2014) reported of reduced levels of Protein S during pregnancy. While Petrozza *et al.*, (2014) noted no change in Protein C levels in pregnancy when compared with the nonpregnancy, Said *et al.*, (2010) observed a significant increase in Protein C activity during pregnancy.

Women with a history of recurrent pregnancy loss (RPL) are in a procoagulant state even when they are not pregnant (Petrozza and Berin, 2014). This thrombophilic state has been linked to pregnancy loss (Mekaj *et al.*, 2015). In this study we investigated the relationship between

pregnancy loss and deficiencies of both Protein C and Protein S amongst women in Abia State, Nigeria.

## **MATERIALS AND METHODS**

A total of 130 apparently healthy women of childbearing age (18-45 years) were enrolled in this study. It was designed as a cross-sectional study. The study population was divided into three groups as follows: pregnancy-loss (70 subjects), normal pregnancy (30 subjects) and non-pregnant control (30 subjects). The Study lasted for the period of four months (between December 2017 and March 2018).

### **Study Setting**

The study took place at the following hospitals: Federal Medical Centre, Umuahia, Nazareth Specialist Hospital Aba, (a specialist gynaecological clinic), General Hospital, Aba and General Hospital, Ohafia, all in Abia state, Nigeria.

### **Blood collection**

3mls of the blood were collected via venepuncture and put into plain vacuum containers. For the pregnancy loss subjects, this collection was done within 48 hours of pregnancy loss. The serum was retracted after 30 minutes of clotting. The serum was used for the estimation of Protein C and Protein S levels.

### **Laboratory Analysis**

The microtitre plate provided in this kit has been pre-coated with antibody. Standard, samples and HRP conjugated antibody were added to wells according to the manufacturer, Melsin

Laboratories, China. After incubation and washing to remove the uncombined enzyme, chromogen Solution A and B was added. The colour of the liquid changed to blue. At the effect of acid, the colour finally became yellow. The colour change is measured spectrophotometrically at a wavelength of 450nm. The concentration of Protein C and Protein S in the sample was then determined by comparing the O.D of the samples to the standard curve.

### **Normal values**

Normal values of Protein S for Caucasians is 15-25 $\mu$ /ml while that for Protein C is 3.9-5.4 $\mu$ /ml. But the respective levels are lower in the pregnant state as well as in Black Africans (Jerrard-Dune *et al*, 2003). Aside, Momodu and Buseri (2015) attributed inconsistencies in the reference values of these anticoagulants to differences in the sensitivities and specificities of the reagents used as well as to assay techniques. In this study assay was done with ELISA technique and assays below 2.5 $\mu$ /ml for Protein S and 0.7 $\mu$ /ml for Protein C were excluded from the study.

### **Exclusion Criteria**

Those excluded from this study included subjects who were reactive to HIV 1 and II, HbsAg, HCV as well as smokers, patients with co-morbidities like hypertension, pelvic inflammatory disease, known thrombotic illness and patients with abnormal reproductive hormones. Also excluded are subjects with assays below 2.5 $\mu$ /ml for Protein S and 0.7 $\mu$ /ml for Protein C.

## Ethical Approval

Ethical approval was obtained from the Ethics Committees of the Federal Medical Centre, Umuahia, the Hospitals Management Board, Umuahia and the Nazareth Specialist Hospital, Aba.

## Statistical Analysis

Statistical analysis was done using SPSS windows version 20 (IBM Corporation, 2011). Data was grouped into pregnancy-loss, normal pregnancy and non-pregnant control subjects. The Kolmogorov-Smirnov test was used to determine normality. Parametric data were expressed as  $\pm$  SD. Parameters were skewed. Hence, they were expressed as median, 2.5<sup>th</sup> (p2.5) and 97.5<sup>th</sup> (p97.5) percentile, and were log-transformed prior to analysis. ANOVA and Games-Howell post hoc procedures were used to test for group differences. Pearson's correlation was used to determine relationship between parameters. The predictive analysis of parameters on pregnancy loss was explored via binary logistic regression model. Data was considered significant at  $P < .05$ .

## RESULTS

A total of 130 apparently healthy women of childbearing age (18-45 years) were recruited for this study. Subjects consisted of 70 (53.85%) pregnancy loss subjects, 30 (23.08%) normal pregnancy subjects, and 30 (23.08%) non-pregnant control subjects. The mean  $\pm$  standard deviation of age for the test subjects (Pregnancy loss subjects) was  $32.46 \pm 5.41$  years, while those for normal pregnancy and nonpregnant controls were  $31.67 \pm 4.25$  years and  $28.30 \pm$

3.72 years respectively. The majority of this group of subjects (58.57%) were primigravida (gravida I) patients, 31.43% were gravida II, while 10% were gravida III patients (Table 1). Results from Table 1 also show that among the pregnancy-loss group, 41 women (58.57%), were experiencing their miscarriage in their very first pregnancy in life, 22 (31.43%), in their second pregnancy and 7 women (10%) in their third pregnancy. Results show that 65 (92.86%) miscarriages in this group occurred during the first trimester and 5 (7.14%) occurred during the second trimester. Among this group too, 54 (77.14%) women have had one pregnancy loss, 14 (20%) have experienced two pregnancy losses while 2 (2.86%) have experienced three pregnancy losses (in all, the present miscarriage inclusive) (Table 1).

**Table 1: Essential Statistical Details of Pregnancy Loss Subjects**

Parameter	Freq.	%	Mean $\pm$ SD
<b>Pregnancy Loss Subjects</b>			
• Total	70	53.85	NA
<b>Pregnancy Status (Gravidity)</b>			
• Primigravida (Gravida I)	41	58.57	NA
• Gravida II	22	31.43	NA
• Gravida III	7	10.00	NA
<b>Trimester of Pregnancy Loss</b>			
• 1 <sup>st</sup> Trimester	65	92.86	NA
• 2 <sup>nd</sup> Trimester	5	7.14	NA
• 3 <sup>rd</sup> Trimester	NIL	NIL	NA

**Number of Pregnancy Loss(es)  
experienced by subject**

• 1 pregnancy loss	54	77.14	NA
• 2 pregnancy losses	14	20.00	NA
• 3 and above	2	2.86	NA

**Age (y)**

32.46 ± 5.41

Key: Freq. = frequency, y = years, NA = not applicable, NIL = nil, % = percentage, SD = standard deviation, primigravida (gravida I) = pregnant for the first time, gravida II= pregnant for the second time, gravida III = pregnant for the third time

**Table 2: Comparison of Protein C and Protein S concentrations between Pregnancy-Loss, Normal Pregnancy, and Non-pregnant Subjects.**

Parameter		Pregnancy Loss (n=70)	Normal Pregnancy (n=30)	Non-pregnant (n=30)	<i>F</i>	<i>P</i>
Protein S (µg/ml)	<i>Mdn</i>	9.00	9.30	8.60	0.27	0.77
	P2.5 – P97.5	6.04 – 11.50	4.0 – 9.30	2.60 – 8.60		
Protein C (µg/ml)	<i>Mdn</i>	0.90	1.20	1.20	11.74	0.00
	P2.5 – P97.5	0.70 – 3.89	0.80 – 1.20	0.70 – 1.20		

Key: n = number of subjects, *Mdn* = median, P2.5 = 2.5<sup>th</sup> percentile, P97.5 = 97.5<sup>th</sup> percentile, *F* = F-test statistic, *p* = error probability, \*\* Significant difference observed at *p* < .01, using ANOVA.

**Table 3: Post hoc Testing of Protein C and Protein S concentrations between Pregnancy-Loss, Normal Pregnancy, and Non-pregnant Subjects.**

Immunological Parameter	<i>Post hoc</i> Pair	95% CI	<i>P</i>
Protein S (µg/ml)	PL vs Norm P.	-0.08 – 0.06	0.91
	Norm P. vs Non-P.	-0.08 – 0.12	0.86
	PL vs Non-P	-0.06 – 0.08	0.95

Protein C (µg/ml)	PL vs Norm P.	-0.25 – -0.05	0.00**
	Norm P. vs Non-P.	-0.17 – 0.12	0.92
	PL vs Non-P	-0.30 – -0.05	0.00**

Key: PL = Pregnancy Loss, Norm P. = Normal Pregnancy, Non-P. = Non-pregnant,  $p$  = error probability, 95% CI = 95% Confidence Interval of the Difference, \*\* Significant difference observed at  $p \leq .01$ , \* Significant difference observed at  $p \leq .05$ . All *post hoc* testing were done using Turkey HSD and Games-Howell methods as applicable.

**Table 4: Logistic Regression on the Relationship between Protein S/Protein C and Pregnancy Loss**

	95% CI for exp b			
	<i>B</i> ( <i>SE</i> )	Lower	exp b	Upper
Included				
Constant	-3.57(1.14)**		0.02	
Protein S	0.07(0.06)	0.96	1.07	1.19
Protein C	0.31(0.17)	0.98	1.37	1.91

Key: Key: exp b = change in odds per unit change in predictor,  $B$  = coefficients of predictors,  $SE$  = standard error, 95% CI = 95% Confidence Interval  $R^2 = .09$  (Cox & Snell), .13 (Nagelkerke), Model  $\chi^2(3) = 9.28$ ,  $p = .03$ . \*\*  $p < .01$ .



## DISCUSSION

Heritable thrombotic states have been linked to pregnancy loss, most especially recurrent pregnancy loss (RPL) (Rey *et al.*, 2003; Van Dreden *et al.*, 2012; Yilmaz *et al.*, 2015). RPL is thought to have multiple aetiologies including chromosomal abnormalities, immune dysfunction, thrombophilic disorders and hormonal disturbances (Larsen *et al.*, 2013). Thrombophilia is a predisposition to thrombosis and describes a tendency of increased blood clotting (Walker, 2000). It creates a hypercoagulable state that leads to arterial and/or venous thrombosis at the site of implantation or in the placental blood vessels (Abu-Heija, 2014). It has been reported that defects are noticed in the haemostatic variables just before miscarriages take place (Petrozza and Berin, 2014).

Protein C and Protein S are natural anticoagulants (Parand *et al.*, 2013). When activated, Protein C inhibits clotting by proteolytic cleavage (and thus deactivation) of factors Va and VIIIa, using protein S as a co-factor (Esmon, 2001). Factors Va and VIIIa are important in the coagulation cascade and their inhibition helps to prevent thrombosis, thereby helping to keep blood in a fluid state. Thus deficiencies of Protein C and Protein S results in the development of a procoagulant state (Singla and Jain, 2018), thus worsening the procoagulant state already existing in pregnancy. Protein C equally enhances the viability and growth of trophoblast cells (Iserman *et al.*, 2003), thus ensuring foetal survival. This shift to a procoagulant state is evidenced by a reported shift in the thromboxane/prostacycline ratio in favour of thromboxane which is a known prothrombotic agent, resulting in vasospasm and platelet aggregation in the

trophoblasts, eventually leading to the development of microthrombi and placental necrosis (Singla and Jain, 2018).

In this study, Protein S and Protein C assays were compared between pregnancy-loss, normal pregnancy, and non-pregnant control subjects as shown in Table 2 and Table 3. Protein C concentration for the pregnancy-loss subjects (*Mdn*: 0.90 µg/ml, *p*<sub>2.5</sub> – *p*<sub>97.5</sub>: 0.70 – 3.89 µg/ml) was significantly lower than that of the normal pregnancy (*Mdn*: 1.20 µg/ml, *p*<sub>2.5</sub> – *p*<sub>97.5</sub>: 0.80 – 1.20 µg/ml) and non-pregnant control subjects (*Mdn*: 1.20 µg/ml, *p*<sub>2.5</sub> – *p*<sub>97.5</sub>: 0.70 – 1.20 µg/ml) (*p* < .01, respectively). However, Protein C assay showed no significant difference between the normal pregnancy and non-pregnant control groups (*p* > .05). However Protein S showed non-significance (*p* > .05) when compared across all groups.

Gene polymorphism occurring in either Protein C or Protein S can lead to abnormalities in either output or functional expressivity of these anticoagulant proteins (Chumpa *et al.*, 2006). This may partly account for any discordant genetic activity expressed by Protein S or Protein C in our study. In fact a subset of subjects in the pregnancy loss group showed increased Protein C activity.

Several studies have reported a positive correlation between RPL and heritable thrombotic states (Van Dreden *et al.*, 2012; Yilmaz *et al.*, 2015). However the existence of conflicting data in literature regarding any association between miscarriage on one hand and Protein C as well as Protein S on the other hand is acknowledged (Mekaj *et al.*, (2015). In their work, Mekaj *et al.*, (2015) found that Protein C and Protein S are causal factors for miscarriage. In contrast with our work, Parand *et al.*, (2013), found a higher frequency of Protein S deficiency in patients with RPL

compared with controls but found that the frequency of Protein C was not significantly different between patients with RPL and healthy women. Protein C and Protein S deficiencies are inherited independently (Chaudhari *et al.*, 2017). Combined deficiencies are rare and come with increased and earlier onset of risk of thrombosis (Chaudhari *et al.*, 2017). In their own work, Ogasarawa *et al.*, (2001) found no differences in the subsequent miscarriage rates between patients with abnormal and normal values of Protein C and Protein S. In the majority of women with inherited thrombophilia, pregnancy is uneventful (Parand *et al.*, 2013). However the risks for miscarriage and some other complications of pregnancy are increased in carriers of thrombophilia. Why certain women with thrombophilia present with gestational vascular complications is still unknown but may be related to a combined effect with another inherited or acquired prothrombotic risk either systemic or localised at the level of the placenta (Altintas *et al.*, 2007) which leads to compromised foetal viability and loss.

Result also showed (from Table 4) that neither Protein C nor Protein S showed significant likelihood of predicting pregnancy loss ( $P > 0.05$ ). However, the overall model was significant with small effect sizes (Model  $X^2(3) = 9.28$ ,  $p = .03$ ,  $R^2 = 0.09$  [Cox & Snell], 0.13 [Nagelkerke]).

## Conclusion

The study assessed serum concentrations of the natural anticoagulants Protein C and Protein S in pregnancy loss, normal pregnancy and nonpregnant controls. Results showed significant association between pregnancy loss and Protein C deficiency. But this association lacks predictive value. However a subset of the pregnancy loss victims showed heightened activity of Protein C activity. In contrast Protein S levels remained stable during the period of pregnancy and showed non-significant variation across all groups.

## **Ethical Approval**

Ethical approval was obtained from the Ethics Committees of the Federal Medical Centre, Umuahia, the Hospitals Management Board, Umuahia and the Nazareth Specialist Hospital, Aba.

## **Limitations of the Study**

The limitations of the study includes, short duration the study was carried out, availability of victims of pregnant loss for the study, some subjects declined to participate in the study.

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