Expression and diagnostic value of plasma protein Z and protein Z-dependent protease inhibitor in fetal growth restriction

Ci Pan[1], Yulian Huang[2], Xiaopei Guo[2], Cunhua Gu[2], Jing Guo[2], Xiaohua Luo[2, 3]

[1]Prenatal Diagnosis Center, 3rd Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[2]Department of Obstetrics and Gynecology, 3rd Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[3]Henan Provincial Clinical Research Center for Perinatal Medicine, 3rd Affiliated Hospital of Zhengzhou University, Zhengzhou, China

ABSTRACT

Objectives: Investigating the expression levels of plasma protein Z (PZ) and protein Z-dependent protease inhibitor (ZPI) in fetal growth restriction (FGR) and to explore their diagnostic value in FGR.

Material and methods: In this study, the number of pregnant women with FGR, healthy pregnant women (Healthy Control, HC), and childbearing-age women without pregnancy (Blank Control, BC) is 79, 79, and 60, respectively; their plasma PZ and ZPI levels in each group are determined by ELISAs. Then, the correlations between these indices and FGR were assessed using Spearman analysis. Moreover, these indices' diagnostic values for FGR are evaluated using the receiver operating characteristics (ROC) curves.

Results: The plasma levels of PZ and ZPI are significantly decreased in the HC and FGR groups compared against the BC group (P < 0.001), whilst the levels of PZ and ZPI in the FGR groups are lower than those in the HC group (P < 0.01) notably. PZ plasma concentration has positive relationship with ZPI concentrations in the HC and FGR groups. The combination of PZ and ZPI, with the Area under the Curve (AUC) 0.92 (95% CI = 0.88–0.96), the sensitivity 0.82, and the specificity 0.88, outperforms everyone.

Conclusions: Plasma PZ and ZPI are significantly decreased in pregnant women with FGR, which can be used for pregnant women's FGR screening.

Key words: fetal growth restriction; protein Z; protein Z-dependent protease inhibitor; coagulation dysfunction; diagnosis

Ginekologia Polska 2022; 93, 12: 987–992

INTRODUCTION

Fetal growth restriction (FGR) is also known as intrauterine growth restriction (IUGR). It is defined as fetuses with an estimated fetal weight or abdominal circumference that is less than the 10th percentile for gestational age [1]. FGR is one of the common complications of pregnancy probably caused by maternal, fetal, placental and external factors, and it has a significant impact on fetal morbidity and mortality as well as long-term neurological dysfunction [2]. Previous studies have shown that FGR is an important cause of perinatal morbidity and mortality, with 52% of stillbirths and 10% of fetal perinatal deaths related to FGR [2]. Fetal growth is a complex process controlled by maternal, placental or fetal factors. Maternal age, nutritional status, living environment and some pregnancy complications have a close relationship with the growth and development of the fetus [3]. The abnormal structure or function of the placenta is also an important factor affecting the growth and development of the fetus in utero. Although the primary pathophysiological mechanisms of these causes differ from one another, they usually share the same final common pathway: decreasing the supply of nutrients to the fetus by affecting the blood perfusion between the uterus and placenta [4].

During pregnancy the body's coagulation and anticoagulation systems are in dynamic balance helpful to maintain the normal function of the placenta. Abnormal blood

coagulation in pregnant women can promote forming thrombus between the uterus and placental villus, as well as reduce exchanging placental nutrients, thus leading to the occurrence of FGR [5–6]. In addition, previous studies have shown that placental fibrin deposition and vascular thrombosis significantly reveals increase in patients with FGR, including villous arterial thrombosis, perivillous fibrin, and utero-placental and intervillous thrombosis [5].

Protein Z (PZ) is a vitamin K-dependent plasma protein with N-terminal Gla domain. On one hand, it promotes binding thrombin and phospholipid surface and helps blood coagulation. On the other hand, these proteins can also combine with protein Z-dependent protease inhibitor (ZPI) forming a complex that jointly inhibits the activity of activated coagulation factor X (Xa) in the coagulation pathway, so the indirect anticoagulation will be developed during this process [6–9]. The ZPI-PZ complex inhibits the coagulation response which inhibits the FXa produced by the external or internal FXase complex prior to its incorporation into prothrombin. The ZPI-PZ minimally regulates FXa during prothrombin activation [10]. PZ with reducing levels lead to procoagulant states and are associated with thrombotic diseases, such as ischemic stroke, venous thromboembolic disease, cardiovascular disease, and pregnancy complications [11]. At present, studies have proved that PZ is bound up with placental abruptions in preeclampsia and recurrent abortion, but there are still few studies on the correlation between changes in PZ and ZPI levels and FGR [12–15].

Aiming at improving the maternal and infant outcomes of patients, it is very significant to clarify the etiology and pathogenesis of FGR and to strengthen the prevention and treatment of FGR Therefore, in this study, the plasma levels of PZ and ZPI in pregnant women with FGR during pregnancy, normal pregnant women as well as non-pregnant women of childbearing age, were detected to analyze the correlation between the two and FGR group, and to explore the diagnostic value of the two for FGR.

MATERIAL AND METHODS
Subjects

The FGR group was made up of 79 FGR patients who were diagnosed and hospitalized for delivery in the Third Affiliated Hospital of Zhengzhou University from September 2018 to March 2020. There are 39 patients in the second trimester and 40 in the third trimester. According to FGR guidelines approved by the American College of Obstetricians and Gynecologists (ACOG) in 2019, each patient was diagnosed [1]. In addition, 79 healthy pregnant women were selected as healthy control (HC) including 42 cases in the second trimester and 37 cases in the third trimester. They received routine perinatal care in outpatient department during the same period. Moreover, 60 women at child-

bearing-age were undergoing pre-pregnancy examination and they were selected as the blank control group (BC group).

Exclusion criteria

Chronic pregnancy complications, such as hypertension, diabetes, hypothyroidism; pregnancy with autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis; pregnancy-complicating diseases of major organs; fetal developmental abnormalities or chromosomal abnormalities; placental abnormalities; acute and chronic infectious diseases; history of smoking, alcohol abuse or mental illness.

This study was authorized by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University (No.05). All subjects had signed the informed consent form before venous blood collection.

Sample collection and preservation

We collected 2 mL of fasting peripheral venous blood from all subjects using an EDTA vacuum anticoagulation tube and centrifuged at 4°C, 3500 rpm for 5 min. Then, the supernatants were collected separately and stored at –80°C for the following examinations.

Detection of PZ and ZPI levels

Levels of PZ and ZPI in plasma were determined by double-antibody Enzyme-linked immunosorbent Assays (ELISAs). The performance of ELISAs is based on the manufacturer's instructions of ELISA kits (ab137989 and ab181132, Abcam, UK). Also, 50 μL of standards and patients' plasma samples were added onto the microtiter plate each well. Then cover wells with sealing tape and incubate for two hours at room temperature. After incubation, remove liquid from each well and wash five times. Add 50 μL of PZ antibody or ZPI antibody to each well and incubate for one hour. Then wash microplate and add 50 μL of SP conjugate to each well. Incubate for 30 minutes and wash microplate again. Add 50 μL of chromogen substrate to each well and incubate for about 20 minutes or till the optimal blue color density develops. Then add 50 μL of stop solution to each well to terminate the reaction. The absorbance values of each well in the microtiter plate at 450 nm were detected immediately.

Statistical analyses

Statistical analyses were performed using SPSS 23.0 software (Chicago, USA). The quantitative data conforming to normal distribution were expressed as mean ± standard deviation (X ± SD) and abnormal distribution data were presented as median (percentiles), M (P25, P75). Two independent samples t-test and nonparametric Mann-Whitney U were used to compare the differences between two groups. One-way ANOVA and Kruskal-Wallis test were used

Table 1. Comparisons of general information of subjects among the BC, HC, and FGR groups

Terms	BC	HC	FGR	χ^2/Z	p
Age (years)	24 (27.5, 30)	28 (26, 30)	29 (27.75, 31)	2.91	0.23
Gravidity	1 (1.2)	2 (1.3)	2 (1.3)	5.37	0.068
Parity	1 (0.1)	0 (0.1)	0.5 (0.1)	3.48	0.18
Gestational age*	—	28.49 ± 3.98	29.23 ± 4.43	0.83	0.41
BMI#	—	23.48 ± 1.97	23.61 ± 2.32	0.30	0.77

* — The gestational age was calculated based on the last menstruation and verified through early-stage ultrasonography; FGR — fetal growth restriction; BC — Blank Control; HC — Healthy Control; BMI — body mass index

to compare the differences among multiple groups. Pearson analysis was used to analyze the correlation between PZ and ZPI concentrations. The Spearman test was performed for simple correlations. Besides, the diagnostic value of each indicator or indicator combination was assessed using the receiver operating characteristics (ROC) curves. In each analysis, the difference was statistically significant at P < 0.05.

RESULTS

The baseline characteristics of the study participants, including age, gravidity, parity, gestational age, body mass index (BMI), are presented in Table 1. The results show that there was no significant difference in the age (p = 0.23), gravidity (p = 0.068), parity (p = 0.18) among the BC, HC, and FGR groups. In addition, no significant difference was identified in the gestational age (p = 0.41) and BMI (p = 0.77) between the HC and FGR groups.

To explore the variation of PZ and ZPI during pregnancy, the expression of PZ and ZPI were determined and compared among the BC, HC, and FGR groups. The results indicate that the expression of PZ and ZPI were significantly lower in BC and FGR groups comparing to HC group (Tab. 2). For further investigation, the expression of PZ and ZPI between the second and third trimester were also compared in the HC and FGR groups. However, significant differences were only identified in PZ between the second and third trimester of the FGR group (P = 0.023, Tab. 3). The data suggested that PZ and ZPI were significantly increased during the second and third trimester and abnormally decreased expression of PZ and ZPI in plasma might be potential indicators for FGR.

Pearson linear correlation analysis showed that the plasma PZ concentration was positively correlated with ZPI concentration both in the HC group (R^2 = 0.42, P < 0.0001, Fig. 1A) and FGR group (R^2 = 0.25, P < 0.0001, Fig. 1B). Considering these findings, the diagnostic values of PZ and ZPI were estimated using ROC curve. The results showed that all indices presented significant diagnostic values and the diagnostic values in turn were: ZPI (AUC = 0.88, P < 0.001) < PZ (AUC = 0.90, P < 0.001) < PZ + ZPI (AUC = 0.92, P < 0.001) (Tab. 4 and Fig. 1C).

Table 2. Comparisons of plasma PZ and ZPI among BC, HC, and FGR groups (±s)

Terms	n	PZ (ng/mL)	ZPI (ng/mL)
BC	60	16.80 ± 3.41	6.54 ± 1.11
HC	79	34.67 ± 6.25[a]	13.54 ± 2.44[a]
FGR	79	24.26 ± 4.70[b]	9.62 ± 1.92[b]
p		< 0.001	< 0.001

[a] — Compared with BC group, p < 0.05; [b] — Compared with HC group, p < 0.05; BC — Blank Control; HC — Healthy Control; FGR — fetal growth restriction

Table 3. Comparisons of plasma PZ and ZPI between the second and third trimesters (±s)

Terms	Trimester	n	PZ (ng/mL)	ZPI (ng/mL)
HC	Second	42	33.64 ± 6.14	13.22 ± 2.55
	Third	37	35.75 ± 6.56	13.90 ± 2.29
p			0.14	0.22
FGR	Second	39	23.05 ± 5.17	9.38 ± 2.01
	Third	40	25.44 ± 3.89	9.85 ± 1.83
p			0.023	0.28

FGR — fetal growth restriction ; HC — Healthy Control,; PZ — protein Z; ZPI — Z-dependent protease inhibitor

DISCUSSION

Placental insufficiency is widely accepted to be the primary cause of FGR, but the reasons of placental insufficiency are various and complex. Constricted spiral arteries and increased coagulation result in fetal hypoxia and inappropriate nutrition exchange [16]. Hence, it is important to explore the pathogenesis of coagulation dysfunction to improve the clinical diagnosis and therapy of FGR.

PZ is a kind of vitamin K-dependent plasma protein and commonly synthesized by vascular endothelial cells [17]. Its structure is similar to other K-dependent factors, such as VII, IX, and protein C [18]. ZPI is a member of serpin superfamily, which can rapidly inhibit activation of factor Xa when having PZ, calcium and procoagulant membranes. It also can suppress the activation of factor XI lack of the aforementioned cofactors [19]. Previous studies demonstrated that
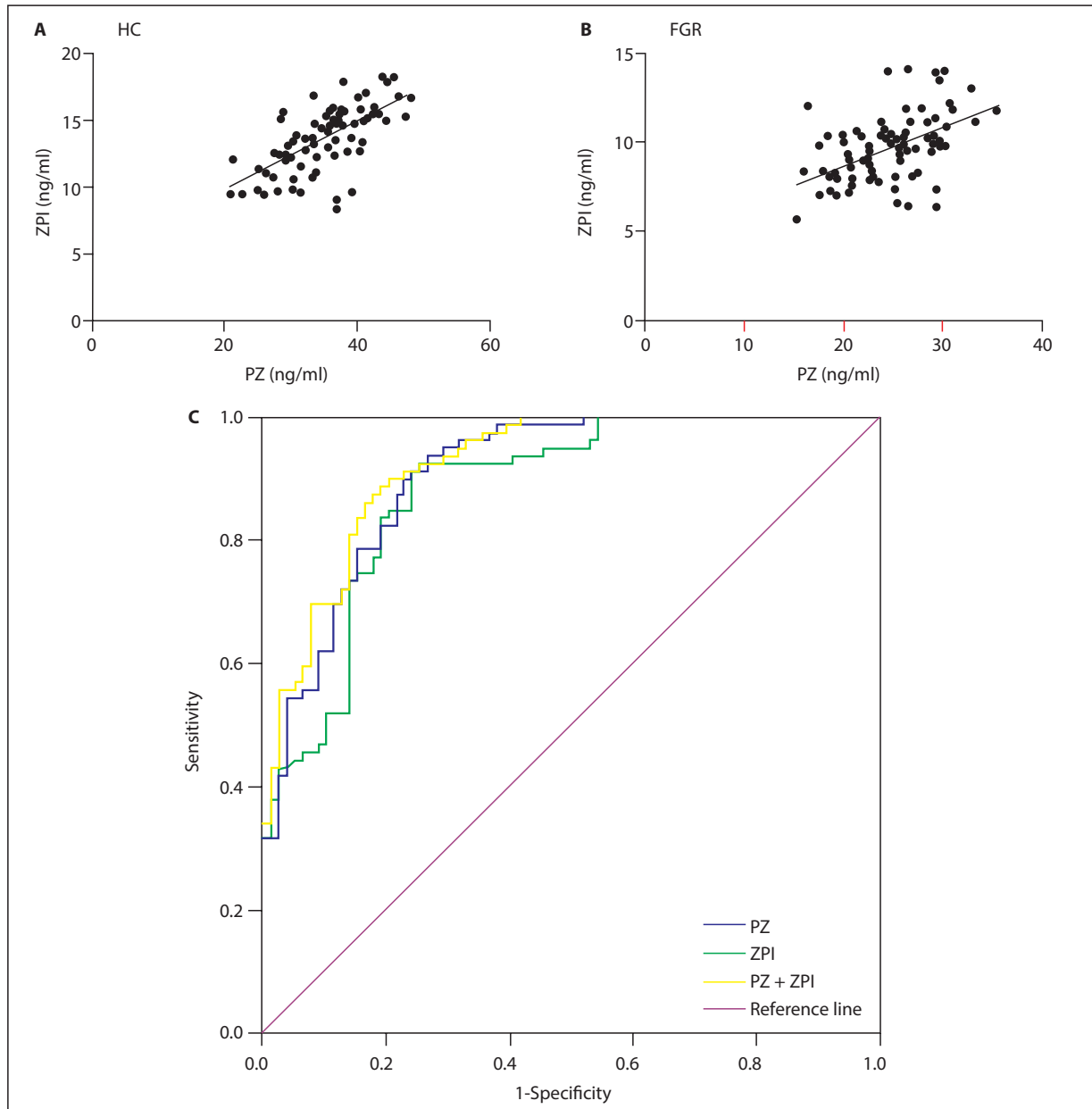
Figure 1. Diagnostic value of PZ and ZPI for FGR. **A.** Correlations between PZ and ZPI in normal pregnant women; **B.** correlations between PZ and ZPI in FGR; **C.** Diagnostic value of PZ, ZPI assessed using ROC curve. The value of specificity is plotted as 1-specificity on the x axis

Table 4. ROC curves of diagnostic value of combined plasma PZ, ZPI, and PZ + ZPI for FGR

Terms	Specificity	Sensitivity	AUC (95% CI)	Cut-off	p
PZ	0.90	0.77	0.90 (0.86–0.95)	29.62 (ng/mL)	0.000
ZPI	0.76	0.91	0.88 (0.83–0.93)	11.70 (ng/mL)	0.000
PZ + ZPI	0.88	0.82	0.92 (0.88–0.96)	—	0.000

PZ — protein Z; ZPI — Z-dependent protease inhibitor

that abnormal expression of plasma PZ is related to adverse pregnancy outcomes, such as intrauterine fetal death, hypertensive disorders of pregnancy, recurrent spontaneous abortion, etc. [14, 20–21]. Souri et al., had documented that plasma PZ and ZPI levels in healthy pregnant women are significantly higher than those of the non-pregnant

women [22]. In this study, plasma PZ and ZPI levels in the healthy pregnant women are also higher than those of the childbearing age without pregnant women. Further explorations show that plasma PZ expression has a positive relationship with the expression of ZPI in both HC and FGR groups. According to these findings, we speculated that the hypercoagulable nature of pregnancy might be the reason which leads to compensatory increased synthesis of PZ and ZPI in the liver and inhibits the activation of FXa, as well as reducing the risk of thrombosis of the pregnant women.

Currently, the correlations between the expression of PZ and ZPI and FGR remain unclear. Quack et al., found that plasma PZ level in the normal pregnant women has positive relationship with the gestational age, and it returns to a normal level within three months after delivery [23]. Brettelle et al. [14], had demonstrated that approximately half of pregnant women with plasma PZ deficiency has placental vascular abnormalities, thus, they pointed out that the decrease of plasma PZ level may be related to FGR. However, Gowri et al. [24], found that no statistical significance was identified in the reduction of plasma PZ level in pregnant women with FGR. In this study, compared with the normal pregnancy control, the plasma PZ and ZPI levels are significantly downregulated in the FGR group, but there were no significant changes in the second and third trimesters of pregnancy in the normal pregnancy groups. It indicates that insufficient plasma PZ and ZPI levels may be a risk factor for FGR. In addition, further investigations showed that plasma concentrations of PZ and ZPI has a negative relationship with FGR. Our research provides a method to predict FGR for the women whose fetuses are slightly smaller than the gestational age during the second trimester.

With the lack of criteria for screening and diagnosis of fetal growth restriction, the diagnosis is mainly based on medical history, physical examination and ultrasound examination. Early diagnosis of FGR during pregnancy is helpful for early intervention in pregnant and parturient women, as well as providing a reference for improving the prognosis for the mother and child. In this study, the diagnostic values of PZ and ZPI were determined with ROC curve and found that both PZ and ZPI presented higher diagnostic values. Combining these results, we speculated that deficiencies of PZ and ZPI might contribute to the development of FGR and be utilized for the FGR screening in clinic.

One of the limitations of this study is the data collection. Since the diagnosis of FGR is in the second and third trimester, the plasma PZ and ZPI levels of FGR pregnant women in the first trimester were not included. However, our research has demonstrated the diagnostic value of PZ and ZPI for FGR. Further studies, such as prospective studies, postpartum follow-up, and larger cohort studies, can determine the predictive value of PZ and ZPI in the first trimester of FGR.

CONCLUSIONS

The reduction of plasma PZ and ZPI levels might be associated with the coagulation dysfunction in pregnant women with FGR. PZ, ZPI were presented significant diagnostic value for FGR and the best diagnostic value was shown in the second and third trimesters. Also, they may also benefit finding the Etiology of FGR. Consequently, it is important to detect the variations of PZ and ZPI in pregnancy plasma to improve the screening and therapy of FGR.

Conflict of interest

All authors declare no conflict of interest.

REFERENCES

1. American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics and the Society for Maternal-Fetal Medicin. ACOG Practice Bulletin No. 204: Fetal Growth Restriction. Obstet Gynecol. 2019; 133(2): e97–e9e109, doi: 10.1097/AOG.0000000000003070, indexed in Pubmed: 30681542.
2. Børte S, Winsvold BS, Stensland SØ, et al. The effect of foetal growth restriction on the development of migraine and tension-type headache in adulthood. The HUNT Study. PLoS One. 2017; 12(4): e0175908, doi: 10.1371/journal.pone.0175908, indexed in Pubmed: 28410431.
3. Monk D, Moore GE. Intrauterine growth restriction--genetic causes and consequences. Semin Fetal Neonatal Med. 2004; 9(5): 371–378, doi: 10.1016/j.siny.2004.03.002, indexed in Pubmed: 15691772.
4. Zhang S, Regnault TRH, Barker PL, et al. Placental adaptations in growth restriction. Nutrients. 2015; 7(1): 360–389, doi: 10.3390/nu7010360, indexed in Pubmed: 25580812.
5. Kraus FT. Fetal thrombotic vasculopathy: perinatal stroke, growth restriction, and other sequelae. Surg Pathol Clin. 2013; 6(1): 87–100, doi: 10.1016/j.path.2012.10.001, indexed in Pubmed: 26838704.
6. Chui A, Zainuddin N, Rajaraman G, et al. Placental syndecan expression is altered in human idiopathic fetal growth restriction. Am J Pathol. 2012; 180(2): 693–702, doi: 10.1016/j.ajpath.2011.10.023, indexed in Pubmed: 22138583.
7. Tabatabai A, Fiehler R, Broze G. Protein Z circulates in plasma in a complex with protein Z-dependent protease inhibitor. Thrombosis and Haemostasis. 2017; 85(04): 655–660, doi: 10.1055/s-0037-1615649.
8. Han X, Fiehler R, Broze G. Characterization of the protein Z–dependent protease inhibitor. Blood. 2000; 96(9): 3049–3055, indexed in Pubmed: 11049983.
9. Huang X, Swanson R, Broze GJ, et al. Kinetic characterization of the protein Z-dependent protease inhibitor reaction with blood coagulation factor Xa. J Biol Chem. 2008; 283(44): 29770–29783, doi: 10.1074/jbc.M805214200, indexed in Pubmed: 18768472.
10. Huang X, Dementiev A, Olson ST, et al. Basis for the specificity and activation of the serpin protein Z-dependent proteinase inhibitor (ZPI) as an inhibitor of membrane-associated factor Xa. J Biol Chem. 2010; 285(26): 20399–20409, doi: 10.1074/jbc.M110.112748, indexed in Pubmed: 20427285.
11. Sofi F, Cesari F, Abbate R, et al. A meta-analysis of potential risks of low levels of protein Z for diseases related to vascular thrombosis. Thromb Haemost. 2010; 103(4): 749–756, doi: 10.1160/TH09-09-0645, indexed in Pubmed: 20076855.
12. Effraimidou S, Farmakiotis D, Topalidou M, et al. Low protein Z levels, but not the intron F G79A polymorphism, are associated with unexplained pregnancy loss. Thromb Res. 2009; 124(1): 24–27, doi: 10.1016/j.thromres.2008.09.017, indexed in Pubmed: 19026439.
13. Erez O, Romero R, Vaisbuch E, et al. Maternal anti-protein Z antibodies in pregnancies complicated by pre-eclampsia, SGA and fetal

death. J Matern Fetal Neonatal Med. 2009; 22(8): 662–671, doi: 10.1080/14767050902801751, indexed in Pubmed: 19591071.

14. Bretelle F, Arnoux D, Shojai R, et al. Protein Z in patients with pregnancy complications. Am J Obstet Gynecol. 2005; 193(5): 1698–1702, doi: 10.1016/j.ajog.2005.04.006, indexed in Pubmed: 16260213.

15. Al-Shaikh FS, Sater MS, Finan RR, et al. Protein Z variants associated with protein Z plasma levels and with risk of idiopathic recurrent miscarriage. Reprod Sci. 2013; 20(9): 1062–1068, doi: 10.1177/1933719112473659, indexed in Pubmed: 23420821.

16. Ndour D, Gassama O. Intrauterine growth retardation, fetal growth restriction: impact on brain development. EC Paediatrics. 2019; 8: 810–819.

17. Vasse M, Denoyelle C, Corbière C, et al. Human endothelial cells synthesize protein Z, but not the protein Z dependent inhibitor. Thromb Haemost. 2006; 95(3): 519–523, doi: 10.1160/TH05-11-0723, indexed in Pubmed: 16525581.

18. Szumowska A, Galar M, Bolkun L, et al. Plasma concentrations of protein Z and protein Z-dependent protease inhibitor in patients with essential thrombocythemia. Clin Appl Thromb Hemost. 2016; 22(7): 679–684, doi: 10.1177/1076029615576741, indexed in Pubmed: 25783867.

19. Huang X. Engineering a protein Z-dependent protease inhibitor (ZPI) mutant as a novel antagonist of ZPI anticoagulant function for hemo-

philia treatment. J Thromb Haemost. 2019; 17(10): 1655–1660, doi: 10.1111/jth.14610, indexed in Pubmed: 31423718.

20. Al-Shaikh FS, Sater MS, Finan RR, et al. Protein Z variants associated with protein Z plasma levels and with risk of idiopathic recurrent miscarriage. Reprod Sci. 2013; 20(9): 1062–1068, doi: 10.1177/1933719112473659, indexed in Pubmed: 23420821.

21. Erez O, Hoppensteadt D, Romero R, et al. Preeclampsia is associated with low concentrations of protein Z. J Matern Fetal Neonatal Med. 2007; 20(9): 661–667, doi: 10.1080/14767050701495011, indexed in Pubmed: 17701666.

22. Souri M, Sugiura-Ogasawara M, Saito S, et al. Increase in the plasma levels of protein Z-dependent protease inhibitor in normal pregnancies but not in non-pregnant patients with unexplained recurrent miscarriage. Thromb Haemost. 2012; 107(3): 507–512, doi: 10.1160/TH11-08-0591, indexed in Pubmed: 22274138.

23. Quack Loetscher KC, Stiller R, Roos M, et al. Protein Z in normal pregnancy. Thromb Haemost. 2005; 93(4): 706–709, doi: 10.1160/TH04-08-0532, indexed in Pubmed: 15841316.

24. Gowri V, Mathew M, Gravell D, et al. Protein Z levels in pregnant Omani women: correlation with pregnancy outcome. J Thromb Thrombolysis. 2011; 32(4): 453–458, doi: 10.1007/s11239-011-0629-y, indexed in Pubmed: 21837382.