Participation of protein Z-dependent protease inhibitor and protein Z system in the pathomechanism of thrombotic complications

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Thrombotic complications of unknown etiology remain a serious diagnostic and therapeutic problem. Occurrence of the inherited polymorphisms of genes encoding proteins involved in the coagulation cascade is one of the possible causes of these complications. In recent years, protein Z (PZ) and PZ-dependent protease inhibitor (ZPI) have been

added to the list of prothrombotic factors. PZ is a glycoprotein serving as a cofactor of ZPI, which is responsible for the inhibition of prothrombinase. Expression of the PZ gene is under the control of many transcriptional factors; several polymorphisms alternate the rate of gene expression. The present article describes the significance of the ZPI-PZ system in venous and arterial thrombosis, adverse pregnancy outcomes and antiphospholipid syndrome complications.

Key Words: *Adverse pregnancy outcomes; Arterial thrombosis; Protein Z; Protein Z-dependent protease inhibitor; Venous thrombosis*

The term 'thrombophilia' refers to disorders associated with a persistent hypercoagulable state within the venous system and rarely within the arterial system. Patients with inherited or acquired thrombophilia are often characterized by atypical localization of thrombosis, early age of thrombotic episodes and increased risk of relapses. In recent years, several precipitating factors have been identified such as factor V Leiden, prothrombin gene G20210A polymorphism, antithrombin deficiency, protein C or S, increased plasma factor VIII activity, hyperhomocysteinemia or the presence of antiphospholipid antibodies (1). However, there are still cases in which it is impossible to positively identify the precipitating factor of thrombosis, estimate the risk of recurrence and determine what impedes the optimal planning of secondary thromboprophylaxis (eg, in 30% of thrombosis cases at an unusual site, such as cerebral venous thrombosis, it is impossible to identify the etiological factor) (2). Therefore, it is necessary to discover and characterize new factors that may be responsible for the state of hypercoagulability. In the past 25 years, interests of many investigators focused on the protein Z (PZ) and PZ-dependent protease inhibitor (ZPI) system. In the current article, we present the physiological function of PZ and ZPI as well as their contribution to the pathological thrombosis cascade (3,4).

Structure and function of PZ

PZ is a 360 amino acid glycoprotein with a half-life of approximately 2.5 days. It is synthesized mostly in the liver. The PZ structure presents high homology to plasma factors VII, IX, X and protein C and contains four domains: N-terminal gamma-carboxyglutamic (GLA) domain (containing 13 gamma-carboxylated glutamic acid residues essential for interaction with negatively charged phospholipid surfaces in the presence of calcium ions and interaction among the GLA domain of PZ, factor Xa and ZPI [5]), two epidermal growth factor (EGF)-type

domains (EGF1 and EGF2) and serine protease (SP)-type domain (6). Due to the lack of histidine and serine in the catalytic place, PZ does not possess zymogen activity (7). On the other hand, occurrence of the GLA domain makes the activity of this protein depend on the gamma-carboxylation process (the same as some other factors), which occurs in the presence of vitamin K. As a result, the activity of PZ decreases during therapy with vitamin K antagonists and probably in women using oral contraceptives. The presence of the GLA domain is also necessary for effective secretion of the protein by cells (8).

PZ (as a cofactor of ZPI) circulates in plasma and forms a complex with ZPI due to multiple ionic interactions and hydrophobic packing (9). ZPI inhibits prothrombinase activity (factor Xa complex) in the presence of phospholipids and calcium ions. The presence of PZ enhances this process by 1000 times. In contrast to the mechanism of antithrombin 'suicidal inactivation of substrate', the action of ZPI does not lead to the formation of a stable complex. ZPI individually (without PZ contribution) inhibits factors IXa and XIa. However, the physiological significance of this process remains uncertain (3,10). The order in which the ZPI/PZ complex components bind factor Xa also needs to be determined. It is suggested that antithrombin and the PZ/ZPI system complement each other – factor Xa-antithrombin complex is mainly responsible for inhibiting factor Xa on the endothelium cell surface (containing active glycosaminoglycans), whereas the PZ/ZPI complex is responsible for inactivation of the prothrombinase on platelet phospholipids and other cell membranes (Figure 1) (10-13).

A growing body of evidence suggests that the PZ/ZPI complex does not affect the activity of other coagulation factors such as thrombin, factor VII or IX, activated protein C, tissue plasminogen activator or plasmin (14). Intriguingly, the fact that thrombin binds to phospholipids only in the presence of

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PZ suggests that lowering of its activity may result in bleeding (13). However, in vivo studies (12) did not confirm this suggestion.

The PZ level can be determined using immunoassays with anti-PZ antibodies. Commercially available immunoassays are Asserachrom Protein Z (Diagnostica Stago Inc, USA) and Zymutest Protein Z (HYPHEN BioMed, France). It was reported that PZ level is distributed in a wide range of concentrations among healthy people and may depend on ethnic factors (4). As a result, it is difficult to quote strict normal ranges of PZ level. According to the manufacturer's instruction, the expected mean ($\pm$ SD) values of PZ level among healthy subjects are 1.56 ± 0.06 $\mu\text{g/mL}$ (range 0.26 $\mu\text{g/mL}$ to 3.78 $\mu\text{g/mL}$; Asserachrom Protein Z) and 2.8 $\mu\text{g/mL}$ (range 1 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$, Zymutest Protein Z). However, both assays are registered only for research purposes and should not be used as a diagnostic tool. Values of PZ level among healthy controls are listed in Table 1 (15-18).

During physiological pregnancy, a gradual increase in PZ level was observed and the concentration returned to initial levels six to 12 weeks after delivery. An increased PZ level among obese pregnant women was also reported (4). However, one study (19) reported a decreased PZ level in pregnant women. Such contradictory data may result from the presence of gene polymorphisms in particular populations (3). Known nonvascular conditions influencing PZ level are listed in Table 2 (3,4,17,20-23).

Expression of the PZ gene (*PROZ*), 14 kbp in size, located in locus 13q34 (in the proximity of coagulation factor genes VII and X) (24), takes place under control of various transcription factors. The promoter A region and two enhancer regions (B and C) are located within *PROZ*. Hepatocyte nuclear factor 4a may bind region A, Sp1 (ubiquitous transcriptional factor) binds regions A and C, whereas region B is bound by an unidentified factor (24). In the *PROZ* gene, many polymorphic variants were found that may significantly change the level and function of PZ. For instance, polymorphisms of G103A in intron A and G79A in intron F is associated with lower PZ levels among carriers of allele A, whereas a polymorphism of C24T in intron G results in a higher PZ level among the carriers of allele TT (25).

PZ and venous thromboembolic risk

Although after in vitro research, significant reduction of thrombin generation in the presence of PZ was observed, in studies (14) with transgenic mice (with *PROZ* knockout), increased risk of thrombosis was not observed. However, in mice with simultaneous PZ gene knockout and the presence of factor V Leiden coagulopathy associated with high mortality, intravascular thrombosis was observed. The mortality rate was particularly high among homozygotes (3).

An association between venous thromboembolism (VTE) episodes and PZ level is still being studied; present studies show conflicting results. Lichy et al (26) concluded that gene G79A polymorphism did not influence the risk of cerebral venous thrombosis, whereas Le Cam-Duchez et al (27) suggested that patients with cerebral venous thrombosis more often had polymorphic variants of G79A nucleotide compared with the control group (occurrence of at least one allele A – OR 2.57; 95% CI 1.23 to 5.34). In the same study, no significant association of A13G polymorphism within the PZ gene promoter was

Figure 1 A complex consisting of activated factors X (Xa) and V (Va), membrane phospholipids and calcium ions (prothrombinase) forms the last step of the coagulation cascade preceding the conversion of prothrombin into thrombin. Prothrombinase can be activated directly by factor VIIa and tissue factor (TF; extrinsic pathway) or through activated factors XIIa, XIa, IXa and VIIIa (intrinsic pathway). Two serine protease systems (serpins) are mainly responsible for the inactivation of prothrombinase – antithrombin (AT; physiologically activated by heparan sulfate) and protein Z (PZ)-dependent protease inhibitor (ZPI), which forms a complex with PZ. G Glycosaminoglycan (eg, heparan sulfate)

found. In the study by Souri et al (28), a low level of PZ (less than 15%) in one case was associated with increased risk of thrombosis and was caused by the substitution of glutamic acid with glutamine (polymorphism of G79A in intron F). It caused a significant synthesized protein defect, ie, an absence of one of 13 post-translational gamma-carboxylation sites.

Studies including the coexistence of PZ deficiency and other etiological factors of thrombophilia seem to lead to more unequivocal conclusions. In a study by Kemkes-Matthes et al (13) comprising 46 patients with factor V Leiden, increased risk of VTE at a lower age in patients with coexistence of PZ deficiency was found. In another study by Martinelli et al (17) including 443 patients with first venous thromboembolic episodes, the authors did not confirm the correlation between PZ level and the risk of thrombosis; nevertheless, patients with a low PZ level and mutation of factor V Leiden had an 18-fold higher risk of developing venous thrombosis – with gene G20210A polymorphism, patients had a fourfold higher risk and with hyperhomocysteinemia, patients had a sixfold to 10-fold higher risk. Another polymorphism, which may worsen the course of VTE among factor V Leiden carriers, is the substitution of arginine by histidine in locus 255 within the pseudocatalytic domain. A similar role may be played by the coexistence of Lys25Arg and Ser40Gly polymorphisms although in vitro studies did not confirm any difference between these substitutions and wild variants (29).

TABLE 1
Mean values of the protein Z level in healthy subjects

Number of subjects	Mean $\pm$ SD (range), $\mu\text{g/mL}$	Assay	Reference
88	2.29 $\pm$ 0.64 (1.01–3.57)	Asserachrom Protein Z, Diagnostica Stago, USA	Vasse et al (15)
125	1.77 $\pm$ 0.61 (ND)	Asserachrom Protein Z	van Goor et al (16)
443	1.79 $\pm$ ND (0.25–7.82)	Asserachrom Protein Z	Martinelli et al (17)
197	1.44 $\pm$ 0.64 (0.21–3.44)	Asserachrom Protein Z	Santacroce et al (18)

ND No data

TABLE 2
Known factors influencing protein Z level

Decreased	Increased
Liver disease	Oral contraceptives
Vitamin K antagonists (warfarin)	Hypertriglyceridemia
Ischemic colitis	
Nephrotic syndrome	
Renal insufficiency and hemodialysis	
Respiratory distress syndrome	
Thalassemia	
Pre-eclampsia	

Adapted from references 3,4,17,20-23

From a theoretical point of view, the impact of PZ on thrombosis may be indirect and may depend on ZPI function. Thus, the latter protein was also studied with the purpose of analyzing its activity on thrombotic complications. In studies (30) on mice models, an increased risk of thrombosis in cases of ZPI deficiency was observed. Van de Water et al (31) analyzed nonsense mutations of the ZPI gene in position R69 and W30, and the results showed a more frequent presence of these mutations among patients with thrombosis than controls (4.4% versus 0.8%; OR 5.7; 95% CI 1.25 to 26.0). Similar observations were reported by Corral et al (3,32) with respect to haplotype H5 including the nonsense mutation Arg67Stop. Carriers of haplotype H5 had a more than threefold risk of venous thrombosis and more often had a familial history of thrombosis, implying that this polymorphism may be of the same importance as factor V Leiden. However, a prospective study (33) and meta-analysis (34) comprising 5000 patients did not confirm any association between the R67X and W303X polymorphisms and VTE. Contradictory conclusions may arise because of influence from additional factors associated with analyzed populations (eg, from southern or northern Europe) (35).

PZ and ischemic stroke risk

Vasse et al (15) demonstrated that PZ deficiency in patients with ischemic stroke (mean age 33 years) is more frequent than in healthy persons (20% versus 5%), which indicates that there is a moderate stroke risk in patients with PZ deficiency (estimated RR = 4). On the other hand, no statistically significant correlation was found in the same analysis in persons with venous thrombosis (mean age 37 years). It should be emphasized that in some individuals with PZ deficiency, other thrombosis factors were also detected – anticardiolipin antibodies were detected in two individuals and protein S deficiency was detected in one individual. Heeb et al (36) demonstrated that there is a correlation between reduced PZ concentration and ischemic stroke; for the whole analyzed group (mean age 57 years), the OR was 2.6 (95% CI 1.5 to 4.3), for the male population, the OR was 3.6 (95% CI 2.0 to 6.4) and in men

without diabetes, the OR was 3.8 (95% CI 2.2 to 6.8). Similar data were obtained in another study with 100 cerebral stroke or transient ischemic stroke patients; no correlation was found between the G79A gene polymorphism and increased cerebral stroke risk, although the AA allele was associated with a lower PZ concentration. Low PZ concentration was an independent factor that increased the risk for cerebral stroke. In the acute phase of ischemic stroke, PZ concentration in the lowest quartile significantly increased the stroke risk, especially if age, sex, smoking, arterial hypertension, diabetes or hormonal contraceptives were taken into account – OR 3.0 (95% CI 1.1 to 8.7). It is surprising that in patients with type 2 diabetes, the PZ concentration was higher than in patients without carbohydrate metabolism disorder (16).

In a study of 91 children with ischemic stroke and 70 individuals of a control group (in homozygotes with respect to polymorphism of G791A), the stroke risk was significantly increased even in the absence of factor V Leiden or G20210A polymorphism of the prothrombin gene (OR 3.88; 95% CI 0.41 to 36.5). In heterozygotes, the risk was not increased (37). However, the study is limited by the small number of G79A homozygotes in the control group, and the fact that differences in the PZ concentration were not found.

In the Kobelt et al (38) study, the OR was 4.3 (95% CI 1.7 to 11) for individuals with ischemic stroke and a PZ concentration of 160% or greater, even if traditional risk factors were considered. Similarly, Staton et al (39) reported a higher PZ concentration in the seventh day of stroke compared with the control group. An increased PZ concentration was observed in patients with an allelic AA variant, associated with the promoter A13G polymorphism and with the GG variant of the G79A polymorphism. However, no differences in the frequency of the abovementioned genotypes between the patient and control groups were observed, although for the GG (G79A) variant, the stroke risk was higher than in individuals with only one A allele. The results shown are similar to the study by Lichy et al (40) – the incidence of the A allele (G79A polymorphism) in the F intron in individuals of the control group was higher than in individuals with cerebral stroke (24.4% versus 15.7%), which suggests that it ‘protects’ against strokes in young age. The differences in the results between the above studies may be due to the differences between the groups studied, the coexistence of other diseases (eg, diabetes or hyperlipidemia), which increases the stroke risk, and the period during which material was collected for tests.

The study by Nowak-Göttl et al (41) is also noteworthy. It analyzed children with cerebral stroke or thromboembolism disease along with their families and found a higher incidence of haplotype 1 (ATG) in patients with stroke that was not associated with cerebral vascular disease (OR 1.46; 95% CI 1.02 to 2.1),

thromboembolism (OR 1.54; 95% CI 1.01 to 2.35) or a combination of diseases (OR 1.4; 95% CI 1.08 to 1.93).

Antiphospholipid syndrome

An increased thrombosis risk can also be caused by acquired thrombophilia, in particular by antiphospholipid syndrome (APS). To date, the details of the pathogenesis mechanism, in which the presence of a lupus anticoagulant leads to thrombosis, have not been elucidated; one hypothesis emphasizes the effect of autoimmune processes on the coagulation cascade and anticoagulation factors.

Ayoub et al (42) showed that in Sneddon's syndrome, PZ concentration is lower compared with the control group; furthermore, in this population, the incidence of PZ deficiency, defined as a concentration lower than 1 mg/L, was higher (31% versus 3.8%). McColl et al (43) also showed that in APS, PZ concentrations are lower and independent of disease symptoms.

Sailer et al (44) showed an association between the presence of anti-PZ antibodies in patients with a lupus anticoagulant and thrombosis or obstetric failures. In patients in whom lupus anticoagulant was present, the level of the immunoglobulin M (IgM) anti-PZ antibodies was significantly higher. Furthermore, the highest IgM and IgG levels were found in patients with a history of obstetric failures, although this difference did not reach statistical significance. Forastiero et al (45) found that factor Xa inactivation by PZ/ZPI complex was slower in the presence of β_2 -glycoprotein I and was further slowed in the presence of IgG anticardiolipin antibodies. Although the incidence of PZ deficiency in patients with APS (less than the fifth percentile) was higher, the condition was not associated with ZPI concentration. PZ deficiency was also associated with a sevenfold increase in the risk for arterial thrombosis in patients with APS. Koutroubakis et al (20) suggested that a decreased PZ level, coexisting with factor V Leiden and antiphospholipid antibodies, may play a role in the pathogenesis of ischemic colitis.

On the other hand, Pardos-Gea et al (46) failed to prove an association between PZ deficiency and the presence of autoantibodies, although the mean titre of IgG anti-PZ antibodies was higher in the arterial than venous thrombosis group or in the control group. These reports certainly suggest a need for further studies and observation.

PZ and arterial complications

In a report by Sofi et al (47), a lower concentration of PZ and ZPI, and lower activity of both proteins in chronic ischemia of the lower limbs were noted. Furthermore, a negative correlation between PZ and ZPI, and the presence of cardiovascular risk factors or even advanced clinical peripheral arterial occlusive disease were observed. It is puzzling that in patients with peripheral arterial thrombosis, the concentration of PZ is lower than in patients with cerebral stroke or myocardial infarction (46).

In some studies (48,49), an association between reduced PZ level and the occurrence of acute coronary syndrome was shown (2), especially when the concentration in smokers was lower than 565 ng/mL.

In this regard, two prospective studies are noteworthy – the Atherosclerosis Risk in Communities (ARIC) study (50) and the Prospective Epidemiological Study of Myocardial Infarction

(PRIME) study (51). In the ARIC study of the incidence of heart events and cerebral strokes, during a follow-up period of more than nine years, lower PZ concentration was found in women with ischemic heart disease, Caucasian Americans and smokers. Furthermore, smokers with a low PZ concentration (less than the fifth percentile) had a 9.5-fold higher risk of acute coronary syndromes. The A13G and G79A polymorphisms were associated with a low PZ concentration (50). Furthermore, in the PRIME study, a five-year follow-up of two populations of France and Northern Ireland, which differed significantly with respect to the risk for ischemic heart disease, was performed. Although, in the Irish population, the risk for heart events was nearly two times higher (this difference could not be explained by traditional risk factors), no differences in the PZ concentration were found between the ischemic heart disease populations of the two countries; furthermore, in the healthy Irish population, the PZ concentration was higher than in the French population (51).

Pregnancy complications

It is believed that pregnancy complications (miscarriage, pre-eclampsia, placental abruption and intrauterine growth restriction) may, in some cases, result from coagulation system disorder in pregnant women. In addition to long-known causes of thrombophilia – such as factor V Leiden, prothrombin gene G20210A polymorphism, proteins C and S, and antithrombin deficiencies – the effect of the reduced PZ concentration on the course of pregnancy is emphasized (19). Nevertheless, the literature data appear to be contradictory with regard to this matter. In some reports (52), no association between PZ deficiency and obstetric failures was found, while in others, just the opposite was the case – it was shown that this deficiency is an essential risk factor for pregnancy complications. Erez et al (21) observed that in pre-eclampsia, the incidence of PZ deficiency is higher and PZ concentration is lower than in the control group. In the study by Kusanovic et al (53), PZ deficiency was observed more frequently in women who had lost a fetus. Early delivery that was not caused by intrauterine infection of the amniotic fluid was associated with lower PZ concentrations. Furthermore, women with intrauterine hemorrhaging, in whom spontaneous early delivery occurred, had lower PZ concentrations than women who delivered on schedule. In another study (54), significant PZ deficiency was found only in women with spontaneous abortion in early pregnancy (most often between weeks 10 and 15).

Data on PZ deficiency coexisting with autoimmune disorders are interesting because a correlation between the presence of IgM anti-PZ antibodies and PZ deficiency was shown in patients with recurring spontaneous abortions. It appears clinically significant that PZ deficiency or the presence of anti-PZ antibodies can lead to the failure of antithrombotic and antiplatelet prophylaxis in patients with other congenital thrombophilias (55).

On the other hand, Dossenbach-Glaninger et al (56) analyzed the effect of PZ gene polymorphism in intron F (G79A) on recurring obstetric failures in the first trimester. The presence of the A allele which, as mentioned above, is associated with a lower PZ concentration, reduced the risk of spontaneous abortions in women with other coexisting coagulation defects (eg, factor V Leiden, increased factor VIII activity or the presence of

polymorphisms associated with high risk such as Val34Leu in the factor XIII gene and 4G/5G in the PAL-1 gene).

SUMMARY

Currently, studies do not allow unequivocal conclusions with regard to thrombosis risk in PZ deficiency. Nevertheless, in a meta-analysis by Sofi et al (57) performed in January 2010, which included a total of more than 4000 individuals, a correlation was found between low PZ concentration and the incidence of arterial complications (OR 2.67; 95% CI 1.60 to 4.48; $P=0.0002$), obstetric failures (OR 4.17; 95% CI 2.31 to 7.52; $P<0.00001$) and venous thromboembolic disease (OR 2.18; 95% CI 1.19 to 4.00; $P=0.01$). Although in the guidelines "Antithrombotic therapy, and pregnancy venous thromboembolism, thrombophilia" (58) the information concerning PZ deficiency was included only in Table 1 – Question Definition and Eligibility Criteria – taking into account more recent literature, it cannot be concluded that in the future the significance of PZ in the diagnosis and treatment of patients with idiopathic thromboembolic complications will increase.

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