

## Theme Issue Article

# Protein Z, a protein seeking a pathology

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### Summary

Protein Z (PZ) is a vitamin K-dependent factor identified in human plasma in 1984 characterized by an homology with other vitamin K-dependent factors (factor VII, IX, X, protein C). In contrast to these factors, PZ does not possess any enzymatic activity but is involved as a cofactor in the down-regulation of coagulation by forming a complex with the protein Z-dependent protease inhibitor (ZPI). ZPI inhibits the activated factor X (FXa) on phospholipid surface. In mice, the disruption of PZ gene is asymptomatic, but the association with the factor V Leiden mutation leads to a quasi complete mortality during the neonatal period with microvascular thrombosis. In humans, PZ is characterized by an unusual wide distribution in plasma, and a major decrease induced by warfarin. Isolated PZ deficiency does not

seem to constitute a risk for venous thrombosis, but a severe PZ deficiency could increase the risk of well recognized venous thrombotic risk factors such as factor V Leiden, G20210A mutation or hyperhomocysteinemia. Unexpectedly, a relationship between PZ deficiency and ischemic arterial diseases such as stroke, acute coronary syndromes or peripheral arterial disease was described but not confirmed by all studies. PZ deficiency could be also a risk factor for early fetal losses, and increases the arterial risk in antiphospholipid syndrome. This review analyzes the different studies so far published and discusses the various results obtained in order to understand whether or not protein Z deficiency could be considered as an arterial ischemic risk factor.

### Keywords

Vitamin K-dependent factors, arterial thrombosis, coagulation inhibitors, pregnancy

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## Introduction

The regulation of blood coagulation depends on a complex interplay between procoagulant, anticoagulant and fibrinolytic proteins. Different natural anticoagulant systems were identified during the last 40 years (1). The last system identified is the protein Z (PZ) /protein Z-dependent protease inhibitor (ZPI) complex. If ZPI was only recently isolated and characterized (2–4), PZ was first identified in bovine plasma in 1977 (5) and isolated from human plasma in 1984 (6). PZ is a single chain glycoprotein which contains 13  $\gamma$ -carboxyglutamic acid (Gla) residues (7, 8). So far, this content in Gla-residues is the highest of the different vitamin-K dependent proteins to be described. As it is the last of the vitamin K-dependent proteins to elute during anion exchange chromatography, it was named Z corresponding to the last character of the alphabet (9).

## PZ structure

Bovine PZ structure was extensively analysed: it contains 396 amino acid residues and has a molecular mass of 50 kDa (7, 8). Amino acid sequence analysis of bovine PZ performed on fragments obtained by chemical and enzymatic degradation showed an extensive homology with the other vitamin K-dependent plasma proteins, and particularly with the light chain of factor X (10). A modular organization of bovine PZ was deduced: a Gla-domain, followed by an  $\alpha$ -helical region containing a cluster of aromatic residues, two epidermal growth factor (EGF)-like modules, and a serine protease-like module (11). However, despite these structural similarities with the other vitamin K-dependent serine proteases, it was evidenced that PZ could not have any proteolytic activity, since among the active site residues, only Asp is present, whereas His and Ser are replaced by Thr and Ala residues, respectively (10). In addition, a  $\beta$ -hydroxyaspartic acid residue was detected in PZ (12, 10) and an O-glycosylation of a

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serine residue was found in the first EGF-like domain (13). Four oligosaccharide groups are attached to the Asn 59, Asn 191, Asn 289 and Thr 288 residues. In contrast to factors IX, X or protein C (PC), which most often have additional Gla-independent calcium-binding sites in their first EGF-like module (14), the Gla-domainless PZ has no calcium-binding sites (15). The Gla-domain of PZ presents a higher affinity for calcium when linked to the first EGF-like module than when Gla-domain is free. This indicates that the N-terminal EGF-like module is a prerequisite for native conformation and affinity of the PZ Gla-domain (11).

Compared to other vitamin K-dependent proteins, PZ shows 100-fold slower membrane binding and dissociation kinetics. This property seems to correlate with an extra Gla residue at position 11 in PZ (16).

Alpha-thrombin induces a limited proteolysis of bovine PZ between Arg 365 and Gly 366, releasing a 45.6 kDa protein and the COOH-terminal PZ glycopeptide containing 31 amino acid residues (15). This cleaved PZ was commonly detected during purification of vitamin K-dependent factors (7).

The human PZ was isolated by a four-step procedure which included barium citrate absorption of the vitamin K-dependent proteins (6). Human PZ has a molecular weight of 62 kDa and can be cleaved by thrombin in a 56 kDa protein. The thrombin cleavage sites are quite different from those described above for  $\alpha$ -thrombin on bovine PZ, since thrombin cleaves human PZ in its NH-2 terminal, and thrombin-cleaved human PZ loses its capacity to be absorbed to barium citrate (17). Amino acid sequence of human PZ was published in 1990 by cDNA cloning and partial amino sequence analysis (18, 19). A homology of 59% was evidenced between the amino acid sequences of bovine and human PZ. Thirteen Gla-residues were detected at the positions 7, 8, 11, 15, 17, 20, 21, 26, 27, 30, 33, 35, 40. Nine potential carbohydrate attachment sites could be detected: five Asn- and three O-linked, as well as an unique Ser-linked (20). Similarly to bovine PZ, His and Ser residues present in the active site of the serine proteases are replaced by Lys and Asp residues, respectively, whereas Asp is conserved. The region around the typical activation cleavage site of coagulation factors is also absent in the human PZ.

Human PZ is 36 residues shorter than bovine PZ and therefore contains only 360 amino acids. This structural difference is important, since it has been shown that bovine PZ promotes the association of bovine thrombin with phospholipid vesicles whereas bovine thrombin alone could not. It was speculated that *in vivo*, PZ could constitute an important factor for localizing thrombin to phospholipid surface. As a result, PZ deficiency could induce a haemorrhagic diathesis (21). However, an additional study demonstrated that bovine PZ binds to thrombin with the residues 366 to 396. Since these residues are missing in human PZ, no physiologically relevant interaction between human PZ, thrombin and phospholipids can occur (22).

Recently, using homology modeling and molecular dynamics, a structural model of human PZ was proposed (23). This approach emphasized the importance of residue Gla at position 30 (Gla-30) for the stabilization of the Gla-domain. It is has to be noted that this Gla residue is well conserved among the different vitamin K-dependent proteins (24). In this model, it was also

shown that the common polymorphism R255H (25) which is more frequent in patients with factor V Leiden (FVL) mutation and thromboembolic complications (26), could modify the local electrostatic charge of PZ leading to an impaired interaction with the ZPI.

## PZ synthesis

The liver is the major source of plasma PZ (27), but a possible synthesis by endothelial cells is also described (28). The PZ gene is localized in chromosome 13q34, spans about 14 kb and consists of 9 exons including one alternative exon (29). The expression of the PZ gene was shown to depend on the liver-enriched transcriptional factor hepatocyte nuclear factor-4  $\alpha$ , and the ubiquitous factor Sp1 plays the role of an enhancer (30). Several polymorphisms in the PZ gene were identified (25). Two of them, A-13G and G79A, which have a high degree of linkage disequilibrium, were shown to influence plasma levels of PZ. The lowest levels of plasma PZ were associated with the GG and AA genotypes for the A-13G and the G79A polymorphisms, respectively (31 – 34). A polymorphism in the intron C (G-42A) seems also to be linked with different plasma levels of PZ, with the lowest PZ levels for the genotype AA (32). The importance of Gla-30 for the secretion of PZ was described. An E30Q mutation was reported for a patient who had a low level of plasma PZ (24). Expression studies of this mutated PZ in baby hamster kidney cells showed that this mutation blocked the secretion of the PZ variant, and could also inhibit the secretion of normal PZ. Similarly, the substitution of Gla-30 by a Lys residue (E30K) is also associated with a defective secretion of PZ (35).

## PZ function

Although PZ was described more than 30 years ago, its physiological function remained unknown for a long time. The evidence of the role of PZ in the control of the coagulation was demonstrated in the last 1990s by the isolation of the ZPI (2) and the description of a marked prothrombotic phenotype in PZ deficient mice homozygous for FVL (36). *In vitro*, it was shown that PZ increases more than 1,000-fold the inhibition of factor Xa by ZPI (3). ZPI inhibits also factor XIa but in a PZ-independent mechanism. Thrombin generation is reduced by the complex PZ/ZPI in a mixture containing prothrombin, factor V, phospholipids and calcium, but not if factor Va is present, indicating that the main role of the PZ/ZPI complex is to regulate the coagulation cascade prior to the formation of the prothrombinase complex (4). In plasma, which contains excess ZPI relative to PZ, all the PZ circulates in a complex form with ZPI (37).

*In vivo*, PZ-deficient mice (PZ<sup>-/-</sup>) have an apparently normal phenotype, but PZ gene disruption in association with homozygous FVL results in intrauterine and perinatal thrombosis and a near complete mortality. In addition, the severity of vascular thrombosis and hepatic fibrin deposition in homozygous FVL-mice was related to PZ genotype [severity in PZ<sup>-/-</sup> > PZ<sup>(+/-)</sup> > wild type] (36). In humans, the consequences of PZ deficiency are less evident, as described below.

**Table 1: Protein Z (PZ) and arterial thrombosis.** P, patients; C, controls; ACS, acute coronary syndrome; PAD, peripheral arterial disease; CRVO, central retinal vein occlusion; Aφ, acute phase; FU, follow-up; N.I., not indicated; N.D., not determined; n.s., non significant.

	Pathology	Population characteristics			Protein Z (µg/ml)		p	Cut-off (µg/ml)	Deficiencies (%)		Polymorphisms			
		Ethnicity	N (P/C)	Mean age (years)	P	C			P	C	A-13G G allele % (P/C)	p	G79A A allele % (P/C)	p
Vasse et al. [68]	Stroke	French	169/88	33	1.56	2.29	Yes	1	19.5	5	N.D		N.D	
Kobelt et al. [73]	Stroke	Swiss	157/192	41	1.45	1.5	n.s	N.D			N.D		N.D	
Heeb et al. [40]	Stroke	Hispanic (54%) Others (46%)	154/206	58	2.04	2.41	< 0.001	1.46	N.D	N.D	N.D		N.D	
Lopaciuk et al [72]	Stroke	Polish	99/100	38	1.56	1.64	n.s	0.5	N.D	N.D	N.D		N.D	
McQuillan et al [38]	Stroke	Australian	79/186	66	1.14	1.16	n.s	N.I						
Lichy et al. [33]	Stroke	German	200/199	40	N.D	N.D					4.2/7	n.s	15.7/24.4	0.007
Staton et al. [34]	Stroke	Australian	Aφ: 151/164 FU: 79/164	67	Aφ: 1.51 FU: 1.15	1.13	Aφ: < 0.001 FU: n.s				26.5/20.7	n.s	35.8/40.9	n.s
Obach et al. [66]	Stroke	Spanish	390/147	67	N.D	N.D					N.D		20/22	n.s
Refaai et al. [41]	Stroke	American (54%) African-American (46%)	110/399	N.I	N.I	N.I	n.s	N.I			N.D		N.D	
Fedi et al [44]	ACS	Italian	223/265	62	1.51	1.73	< 0.001	0.56	15.7	4.9	N.D		N.D	
Sofi et al [75]	ACS	Italian	193/N.I	66	A?: 1.69 FU: 1.6	N.I	< 0.001 A? vs FU	0.61	A?: 10.4 FU: 19.2		N.D		N.D	N.D
Cesari et al. [76]	ACS	Italian	244/352	64	1.43	1.74	< 0.001	N.I			12.3/15.3	n.s	36.9/33.2	n.s
Morange et al. [77]	ACS	French (58%) Irish (42%)	297/593	N.I	1.58	1.64	n.s	0.93	10	11	N.D		N.D	
Refaai et al. [41]	ACS	American (78%) African-American (22%)	382/375	N.I	N.I	N.I	Low PZ 0.02 women 0.02 smokers	N.I			N.D		N.D	
Sofi et al [79]	PAD	Italian	120/360	75	1.59	1.73	< 0.05	0.60	24.2	5.3	N.D		N.D	
Pardos-Gea et al. [80]	PAD	Spanish	15/82	N.I	1.02	2.44	0.001	N.I			N.D		N.D	
Ayoub et al [71]	Sneddon's syndrome	French	28/78		1.47	1.93	0.02	1	31	3.8	N.D		N.D	
Koren-Michowitz et al [81]	CRVO	Israeli	6 without other risk factors/42	60	1.38	2.01	0.022	0.8	20	6	N.D		N.D	
Oztürk et al. [82]	Behcet's disease	Turkish	24/24	35	1.08	1.41	< 0.05	N.I						
Koutroubakis et al. [83]	Ischemic colitis	Greek	33/33	64	1.38	1.86	0.001	1	18.2	3	N.D		N.D	

## Physiopathological variations of plasma PZ

The two main characteristics of plasma PZ levels are their wide distribution in normal subjects (32 to 168% of the mean) and the dramatic decrease induced by oral anticoagulants (1 to 16% of normal) (17). The decrease of PZ antigen in the plasma of a normal subject receiving a loading dose of warfarin indicated a plasma half-life of 2–3 days. In a sample of 455 American healthy blood donors, the mean level of plasma PZ collected in EDTA tubes was 2.9 µg/ml (17). However, important differences in mean PZ levels in control groups of different countries have to be noted (Table 1): values vary from 1.16 µg/ml in an Australian study (38) to 2.71 µg/ml in an Uruguayan study (39). These variations are not related to different methods for PZ measurement, since all these studies (except [40]) used the same commercial immunological assay, suggesting that these differences could be related to ethnic variations. In this respect, significant higher levels of PZ were observed in African-Americans than in white Americans (41). Because of the diversity of polymorphisms controlling plasma PZ levels, reference values should be established on a sufficient number of volunteers, to avoid a bias in the selection of the controls. As observed for plasma PZ levels, the frequency of the rare alleles which influence plasma level is different between the various ethnicities tested (from 7 to 20.7 % for the G allele of the A-13G polymorphism; Table 1). If age is usually considered to play a minor role on PZ plasma levels (17), a significant negative correlation was recently reported (42).

Controversial results on the influence of sex on plasma PZ levels were described as well. If most of studies did not identify significant variations, in some studies, the plasma PZ levels were found to be higher in men than in women (31, 43) while the opposite was found in another (44).

The level of PZ is markedly low in newborns (17, 45), particularly in newborns with low gestational age or born from mothers affected by pre-eclampsia (46). Conflicting results concerning newborns with respiratory distress syndrome have also been reported: no significant difference was observed in one study (47), whereas a decrease was mentioned in another (46).

A progressive increase of PZ during normal pregnancy was described, as well as a return to normal levels around 6–12 weeks postpartum (48). This increase during pregnancy is more pronounced in obese (body mass index [BMI] > 28) than in lean women, whereas PZ levels do not significantly differ in the postpartum period (49). In contrast to these two studies, either a decrease of PZ with gestation or no significant variation was reported (50–52). The use of oral contraceptive is associated with higher plasma levels of PZ (53).

The role of inflammation is still debated: a first study reported a significant negative correlation between PZ and interleukin-6 (IL-6) levels in patients with haematological malignancies (54). In the same way, lower PZ levels were reported in 265 controls as well as in 223 patients with acute coronary syndromes (ACS) when fibrinogen level was above 400 mg/dl (44). *In vitro*, purified IL-6, one of the major regulator of fibrinogen biosynthesis, as well as the conditioned medium of lipopolysaccharide activated monocytes did not significantly affect hepatic PZ biosynthesis (55), whereas Oncostatin M, a cytokine of the IL-6 family, increased PZ biosynthesis by microvascular en-

dothelial cells (28), suggesting a possible but weak control of PZ levels by inflammation. In a limited number of patients who underwent primary percutaneous coronary intervention (PCI), a significant increase of PZ was observed within the 72 hours, whereas IL-6 levels did not significantly change during this period, indicating no direct relationship between IL-6 and PZ plasma levels (56). A weak but significant positive correlation was also observed between C-reactive protein (CRP) and PZ levels during pregnancy (49). Comparison of PZ levels in blood samples collected within seven days of an acute ischemic stroke and during the follow-up (3 to 6 months), showed an increase of PZ during the acute phase (38). However, the comparison was performed only in 79 patients during the follow-up, and compared to PZ the levels of 173 patients at diagnosis. The comparison of PZ levels in the subgroup of patients for whom both samples were available would have been more convincing. Nevertheless, even if the mechanism involved is unclear, a possible increase of PZ levels during the acute phase of a thrombotic event must be taken in consideration in studies evaluating a possible link between inherited PZ deficiency and pathology.

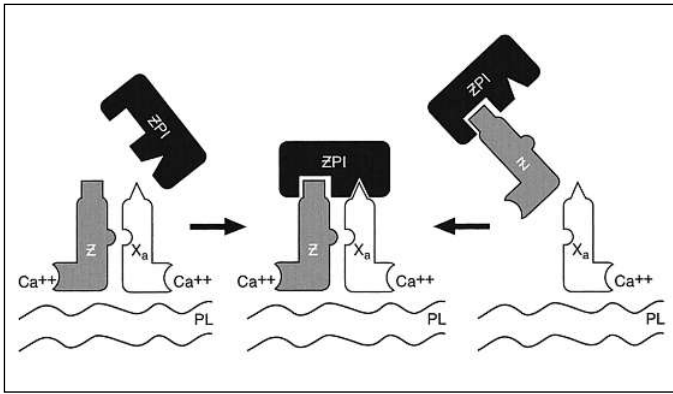
PZ is low in patient with liver disease (27), and in patients with nephrotic syndromes (57, 58). Influence of renal insufficiency and haemodialysis is not clear: in a recent study, a significant decrease ( $p = 0.022$ ) of PZ was observed in 46 patients with chronic renal disease either on haemodialysis or without dialysis (59). However, the difference was not statistically significant when patients are divided in groups on haemodialysis (23 cases) or without haemodialysis (23 cases). No significant variations were previously described in a first limited study on 12 adults with glomerulonephritis without nephrotic syndrome and not on haemodialysis (60). In contrast to the study of Blyth et al. (59), PZ levels were found to be enhanced in patients on haemodialysis (60). The main difference between the two groups of patients on haemodialysis is that in one study (59), CRP was slightly increased, whereas it was in a normal range in the other one (60). Peritoneal dialysis does not seem to modify PZ levels (60, 61).

In addition to the well-known PC deficiency in thalassemia, a clear decrease of PZ is also observed (62). PZ levels are decreased (50.8 %) in patients with malignant tumours, and the deficiency was more pronounced in the most advanced stages of the tumours (63).

PZ levels are also low in patients with disseminated intravascular coagulation and amyloidosis (9). In contrast, PZ levels are increased in subjects with hypertriglyceridemia (40).

## PZ deficiency and bleeding

As PZ was initially thought to act by localising thrombin at the surface of phospholipids, several studies investigated PZ in patients with bleeding tendencies. Kemkes-Matthes and Matthes described a marked decrease of PZ (52% of the control group) in 36 patients with a bleeding tendency of unknown origin and 58.3% of patients had PZ levels below the lowest value of the 36 healthy controls (64). Another study on 48 patients (15 men, 33 women) identified a significant decrease of PZ only in men with a bleeding history, but none of them had PZ level below the values observed in controls (43). A last study (65) failed to detect any PZ decrease in patients with bleeding tendency. In a group of 56



**Figure 1: Protein Z-mediated inhibition of factor Xa by ZPI (reproduced from [9]).** Two mechanisms are proposed to explain the formation of the complex Protein Z(PZ)/ZPI: on the left, PZ and factor Xa form a complex at the phospholipids surface is fixed thereafter. On the right, a pre-formed circulating PZ/ZPI complex binds to the phospholipids surface and interacts with ZPI.

patients with bleeding antecedents, we observed a distribution of PZ levels similar to the control group (unpublished data). More recently, no significant difference in the distribution of the intron G79A PZ polymorphism was reported in hemorrhagic strokes versus a control group (66).

## PZ deficiency and venous thrombosis

As PZ deficiency in mice dramatically increases the thrombotic phenotype of the FVL mutation, the main cause of venous thrombosis (VT) in humans (67), different studies investigated a possible link between PZ deficiency and VT. Four studies carried out on a total of 1,122 patients with venous thromboembolic history reported similar PZ levels in patients and in controls, indicating that PZ deficiency is not an independent risk factor for venous thrombosis (32, 53, 68, 69). However, very low levels of PZ (below the 5<sup>th</sup> or 2.5<sup>th</sup> percentile) could constitute an independent risk factor for VT (32), or the association of low levels of PZ (< 25<sup>th</sup> percentile) with hyperhomocysteinemia increases the risk for VT (69). In agreement with the results observed in PZ-deficient mice, Kemkes-Matthes et al. reported in a series of 46 consecutive patients that thromboembolism in FVL patients occurred earlier in patients with concomitant PZ deficiency than in patient with normal PZ level (70). They also indicated that the R255H substitution in PZ increases the thromboembolic risk of patients with FVL mutation (26). A synergic effect on the risk of VT was also found in patients with FVL (14 patients) or prothrombin G20210A (14 patients) mutation when PZ levels was in the lowest quartile, but surprisingly, this relationship was not statistically significant for patients with PZ levels in the lowest 10<sup>th</sup> percentile (7 patients for FVL and 3 for G20210A ) (69). However, on a larger series (82 patients), the increased risk for the association between FVL mutation and PZ deficiency was not confirmed (53).

In addition, two genetic approaches did not show different frequency of PZ gene polymorphisms in patients with a VT history than in controls (25, 32).

## PZ deficiency and arterial thrombosis

Since our first report showing an increased frequency of PZ deficiency in young patients with a previous history of an ischemic stroke (68), several studies with very conflicting results investigated the role of PZ and arterial thrombosis.

## PZ and stroke

In a selected population of young patients (mean age 33 years) with an ischemic stroke, we observed a frequency of approximately 20% of patients with plasma PZ level below 1 µg/ml. Plasmas were collected at least three months after the ischemic event, and the patients did not have other well-recognized arterial risks factors such as hypertension, dyslipidemia, diabetes or cardiac arrhythmia. Such an association between low PZ and ischemic stroke was found by Heeb et al. in a cohort of 154 older patients (mean age 57 years) including patients with well-identified arterial risk factors. However, in smokers, diabetics or women, this association was not detected. The relationship between PZ deficiency and ischemic stroke was observed either in samples taken within the four days of the stroke, or in samples taken two months later (40). A more detailed analysis revealed that low level of PZ is a stroke risk factor in young (below 58 years) but not in older women, suggesting a possible influence of hormonal status on PZ levels (42). An high frequency (31%) of PZ deficiency (< 1 µg/ml) was also reported in a cohort of 36 young patients (mean age 48.9) with antiphospholipid-negative Sneddon's syndrome (71).

In the prospective Atherosclerosis Risk In Communities (ARIC) study, including 16,000 middle-aged individuals for atherosclerotic-related cardiac and stroke events, a trend ( $p = 0.06$ ) with low PZ levels and the development of stroke was only observed in African-Americans (41). The average follow-up was 9.7 years.

In contrast to these observations suggesting a possible link between low PZ levels and ischemic stroke, PZ plasma levels found in the normal range in two studies on a limited number of patients (38, 72) (Table 1). In addition, in the study of McQuillan et al. (38) a significant increase of PZ was observed when PZ was measured within the first seven days after the stroke.

Unexpectedly, in another study where samples were collected at least two months after the stroke, PZ levels above 150% were found in 15.3% of a group of 125 patients (mean age 40 years), whereas such levels were observed only in 7.3 % of healthy controls (73). The authors concluded that high levels of PZ could be an independent risk factor for ischemic stroke

In 51 patients with atrial fibrillation, a common cardiac disorder predisposing to stroke, no significant variation of PZ were observed (74).

To avoid confounding factors such as diabetes, smoking habits, hormonal status, polymorphisms which are associated with PZ plasma levels were also investigated. Lichy et al. observed a significantly lower frequency of the A allele of the G79A polymorphism in a group of 200 young patients (mean age 40.1 years) with a previous history of stroke (56 transient ischemic attacks and 144 complete strokes) than in 199 controls (15.7 % in the cases vs. 24.4% in the controls). They concluded that the low

level of PZ associated with the AA genotype could be protective against stroke (33). In two others studies performed on 151 and 390 patients, the prevalence of A-13G and G79A genotypes was not different between cases and controls (34, 66). A marked difference of frequency of the alleles tested between German and Australian population must be noted (Table 1).

## PZ and coronary syndromes

A similar controversy also concerns acute coronary syndromes (ACS). A first report showed a significant decrease of PZ levels at the admission of 223 patients with an ACS (1.51 µg/ml vs. 1.73 µg/ml in the control group). PZ levels below the 5<sup>th</sup> percentile were observed in 15.7% of the patients (44). The contemporary presence of low PZ levels and smoking habit led to an increase risk of ACS (odds ratio [OR] 9.5, 99% confidence interval [CI] 2.4–37.2). A similar decrease of PZ was found in patients with mono- or multivessel disease. The decrease of PZ was present as well in samples collected at least one year after the ischemic event (75, 76). In addition, in a one-year follow-up study, 19.5% of patients with major adverse cardiac events (MACE) had a PZ level at admission below 0.6 µg/ml, whereas in patients without MACE, the frequency of PZ below 0.6 µg/ml was only 7.9%, suggesting a prognostic value of PZ level at admission (76). The comparison of PZ levels at admission and after one-year follow-up showed that PZ levels were significantly higher during the acute phase of ACS. Interestingly, neither genotype distribution nor allele frequency for A-13G or G79A polymorphisms was significantly different between patients with ACS and controls, whereas plasma PZ levels were significantly lower in patients according to the different genotypes (77).

In the prospective ARIC study, a relationship between low levels of PZ and coronary heart disease was found only in women and smokers (41), whereas in the Prospective Epidemiological Study of Myocardial Infarction (PRISME) cohort of 10,000 apparently healthy men aged from 50 to 59 years at baseline, with a five-year follow-up, no association was found between low levels of PZ and the risk of ACS (78). However, it must be noted that in patients from North Ireland, where coronary events are two times more frequent than in France, there was a trend for lower PZ levels in patients than in controls (1.61 µg/ml vs. 1.72 µg/ml,  $p = 0.13$ ). We can not exclude that a significant difference could be observed with a longer follow-up

## PZ and other arterial diseases

In a case-control study including 120 old patients (median age 75 years), PZ levels were significantly lower in patients with peripheral arterial disease than in an age and gender-matched group of controls (79). In addition, a significant association between low PZ levels and severity of the disease, evaluated through Fontaine's stages, was evidenced. Another recent study on a limited number of patients reported a greater decrease of PZ levels in patients with peripheral artery thrombosis than in patients with stroke or myocardial infarction (80).

Risk factors associated with atherosclerosis formation predispose to either central retinal vein or artery occlusion. Arterial disease may play a causative role in the development of retinal

venous occlusion. In a series of 36 patients with retinal vessel occlusion, significantly lower PZ levels (1.38 µg/ml) were found in six patients without classical risk factors, compared to 42 controls (2.01 µg/ml,  $p = 0.022$ ) or to the 30 patients with well-recognized risk factors (2.12 µg/ml,  $p = 0.04$ ), suggesting that low levels of PZ could be an independent risk factor for retinal vessel occlusion (81).

Decreased plasma PZ levels were also observed in patients with Behcet's disease, with a more pronounced decrease during the early active phase of the disease, whereas higher levels were observed when disease activity declines with time (82).

A higher frequency of PZ deficiency (< 1 µg/ml) was also described in patients with ischemic colitis than in healthy controls (18.2 % and 3.3 %, respectively) or in patients with diverticulitis (7.7% PZ deficiency) (83).

## PZ deficiency and antiphospholipid antibodies

Low PZ levels were observed in patients with antiphospholipid (aPL) antibodies (39, 84, 85), both in patients with a previous history of thrombosis or without thrombosis (39, 85). PZ deficiency was mainly present in patients having lupus anticoagulant (LA) activity, with or without anticardiolipin antibodies (39). The frequency of PZ deficiency (below the 5<sup>th</sup> percentile of the control group) was higher in patients with definite antiphospholipid syndrome (APS) than in patients who did not fulfil the criteria for APS (24.3% of PZ deficiency versus 10.3%, respectively). The concomitant existence of autoimmune aPL and low levels of PZ was associated with a seven-fold increased risk of arterial thrombosis (85). If it was shown, *in vitro*, that aPL antibodies impair the inhibition of factor Xa by the PZ/ZPI complex, the mechanism of the association of aPL with PZ deficiency remains unknown (85).

LA includes an heterogeneous group of autoantibodies primarily directed against certain plasma proteins such as β<sub>2</sub>-glycoprotein I or prothrombin. As the existence of specific antibodies against PZ was previously demonstrated (86), Sailer et al. evaluated the frequency of anti-PZ antibodies in 102 patients with LA (69 with and 33 without thrombosis) and in 33 controls (87). The prevalence of high anti-PZ antibody levels (above the 75<sup>th</sup> percentile of controls) was higher in LA-patients than in control group, but the difference was statistically significant only for the IgM subtype. In contrast to antibodies against β<sub>2</sub>-glycoprotein I or anticardiolipin, the presence of antibodies against PZ was not associated with the thrombotic risk of LA.

## PZ deficiency and obstetrical pathologies

Gris et al. reported a high frequency (34.8%) of PZ deficiency in women with a first primary episode of early fetal death from the 10<sup>th</sup> to the end of the 15<sup>th</sup> week of gestation (88). It was hypothesized that PZ deficiencies could impair the invasion of the spinal uterine arteries by the cytotrophoblast. The same group described an enhanced frequency and high levels of anti-PZ antibodies (both IgG and IgM) in women with unexplained primary early fetal losses and early fetal death.

Anti-PZ antibodies were independent of classical aPL antibodies (LA, anticardiolipin, and anti-β<sub>2</sub>-glycoprotein I anti-

bodies). Anti-PZ IgG and IgM antibody levels were not correlated with plasma PZ concentrations in both controls and patients (86). In addition PZ deficiency or anti-PZ antibodies were independently associated with a significant decrease of the chance of giving birth to a living child, even in women treated by antiplatelet therapy or low-molecular-weight heparin (89). The association with low PZ levels and different pregnancy complications (growth restriction, intrauterine fetal demise, intra-uterine bleeding) was confirmed in three other studies (50–52, 90). In contrast, in a selected group of 124 women (without inherited or acquired thrombophilia factors) with unexplained fetal losses, PZ levels were similar to those of a control group of 60 women with uneventful pregnancies (91).

A significant decrease of PZ was recently described in an important series of 130 women with pre-eclampsia (90), as well as high levels of IgM anti-PZ antibodies (88).

In contrast to these studies suggesting a possible link between PZ deficiencies and obstetrical complications, a recent study analysing the G79A polymorphism, but not plasma PZ levels, observed that the presence of the 79A allele, usually associated with higher levels of PZ, could be protective against fetal loss (92).

## Conclusion

More than 30 years after its isolation from bovine plasma, the physiological role of PZ in human remains unclear. A possible link between PZ and arterial disease is supported by the presence of immunoreactive PZ in atherosclerotic vascular lesions of diabetic and non-diabetic patients, but not in the subendothelial space and microvascular endothelial cells of healthy controls (93). However, the role of PZ in atherosclerosis lesions is unclear: does it promote directly the growth of the plaque or is its presence reactionary? In the first case, it could explain why some studies concluded that high levels of PZ are associated with arterial disease. On the other hand, the PZ presence could constitute a local defence against vascular injury, as it has been shown, for example, for interleukin-10, which is a protective agent against atherosclerosis development (94) and is expressed in the most advanced atherosclerotic lesions (95). Factor Xa induces *in vitro* and *in vivo* the proliferation of vascular smooth muscle cells (96), and specific pharmacological inhibition of factor Xa was shown to reduce restenosis after balloon angioplasty of atherosclerotic femoral arteries (97), suggesting that physiological regulation of factor Xa activity by the complex PZ/ZPI could protect against atherosclerosis development. The analysis of the consequences of a PZ deficiency in diabetic or Apo E-deficient mice could perhaps help to answer this question.

One of the major problems to attempt to correlate any pathological manifestation with PZ deficiency is its wide variation in healthy subjects. However, it is important to rule out an analytical bias, as it was previously observed for Thrombin Activable Fibrinolysis Inhibitor (TAFI). As observed for PZ, a strong gen-

etic control and a wide distribution in normal plasma was described for TAFI. It was clearly established that some immunological assays were sensitive to the frequent 325 Ile isoforms, leading to erroneous plasma TAFI levels (98).

Consequently, an evaluation of the reactivity towards the main genotypes of PZ should be performed for the ELISA assays so far described. Unfortunately, to our knowledge, a plasma functional assay is not currently available. Difficulties to obtain a specific and reliable functional assay arise from the fact that PZ has no direct enzymatic activity and that the PZ/ZPI complex acts only in the early phases of coagulation, before thrombin generation.

In conclusion, at this time, a role for PZ in the pathogenesis of haemostatic disorders in humans remains to be established. From investigations performed so far, no clear link was evidenced between PZ deficiency and bleeding tendency or venous thrombosis and PZ measurement is certainly not useful for these pathologies. However, the consequences of a PZ deficiency on thrombotic arterial disease or obstetrical complications can not be discarded at this time. The confusing results reported, both for plasma PZ levels and polymorphisms, are likely due at least in part to the limited number of individuals enrolled and the choice of the control groups (healthy individuals or sex and age-crossed controls). The different restricted studies so far performed have the interest to highlight the different factors which can influence PZ levels. It is clear, that as for other inherited coagulation factors, plasma PZ measurements should not be performed during the acute phase of the thrombosis.

The fact that the results reported herein from the different studies appear often as being controversial suggests that the ischemic risk associated with PZ deficiency is certainly weak. As observed in animal models, PZ deficiency mainly increases the thrombotic risk associated with other well-identified vascular risk factors such as diabetes, smoking habits or hypertension. For future studies, all these additional risk factors must be imperatively considered.

Interestingly, analysing both PZ plasma levels and PZ polymorphisms Cesari et al. observed that plasma levels were significantly lower in patients with ACS than in controls, for each genotype (77). Consequently, it can not be excluded that plasma PZ deficiencies are acquired, and constitute a marker of vascular disease. Studies evaluating both vascular disease progression and possible variations of plasma PZ levels are needed to confirm this hypothesis. Consequently, to assess the role of PZ in the occurrence of arterial pathology, both PZ levels and polymorphism analysis must be performed only in adequately powered prospective trials with homogeneous groups of patients and controls, and compared with other markers of arterial disease.

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