Influence of protein Z plasma level on thrombin generation assay

Authors :

Bérangère S. Joly

Bénédicte Sudrié-Arnaud

Jeanne-Yvonne Borg

Véronique Le Cam Duchez

Author affiliations:

Bérangère S. Joly
Centre Hospitalier Universitaire de Rouen, Hématologie biologique, Unité Hémostase, Rouen, France; Hôpital Lariboisière, APHP, Université Paris Diderot, Hématologie biologique, Paris, France.
E-mail: berangere.joly@aphp.fr

Bénédicte Sudrié-Arnaud
Centre Hospitalier Universitaire de Rouen, Hématologie biologique, Unité Hémostase, Rouen, France.
E-mail: benedicte.arnaud@gmail.com

Jeanne-Yvonne Borg
Centre Hospitalier Universitaire de Rouen, Hématologie biologique, Unité Hémostase, Rouen, France.
E-mail: jeanne-yvonne.borg@chu-rouen.fr

Corresponding author:
Véronique Le Cam Duchez, Centre Hospitalier Universitaire de Rouen, Hématologie biologique, Unité Hémostase, INSERM U1096, Rouen, France.
E-mail:
veronique.Le-Cam-Duchez@chu-rouen.fr

Abstract

Introduction: Protein Z (PZ) is a vitamin K-dependent factor, involved as a cofactor of PZ-dependent protease inhibitor (ZPI) that inhibits the activated factor X on phospholipid surface. A severe PZ deficiency could be associated with ischemic arterial diseases. The aim of this study was to evaluate the influence of PZ plasma levels on thrombin generation (TG) and to detect a hypercoagulable state in patients with PZ deficiency.

Patients and Methods: Young patients with personal history of arterial thrombosis and PZ deficiency were included. PZ concentrations were assessed using ELISA method. TG assay was performed using platelet-free plasma (PPP) containing 5 pM or 1 pM tissue factor and 4 µM phospholipids with and without thrombomodulin. Then, we focused on the influence of successive overloads with purified PZ in two patients with PZ deficiency (Pat1PZdef and Pat2PZdef) and industrial PZ deficiency (IndPZdef).

Results: First, in 13 patients with PZ deficiency, none influence of PZ level was reported on TG assay parameters. Second, TG profiles performed on Pat1PZdef, Pat2PZdef and IndPZdef were close to the reference TG profile. In presence of TM, there was no difference in TG parameters according to PZ plasma levels. After each overload, real PZ plasma levels were underestimated; differences in standards of purified PZ between the two manufacturers might explain such results.

Conclusion: The absence of influence of PZ concentration on TG assay is reliable to the absence of well-demonstrated clinical consequences of PZ deficiency in arterial thrombosis. Clinical consequences of protein Z deficiency warranted further studies.

Keywords

Protein Z, thrombin generation assay, protein Z deficiency

1. Introduction

Protein Z (PZ), a vitamin K-dependent protein, was identified in bovine plasma in 1977 [1] and in human plasma in 1984 [2]. The amino acid (aa) sequence of bovine PZ (396 aa) was described in 1985 [3] and reported extensive homology to the other vitamin K-dependent coagulation factors: 13 Gla residues within the NH_2 -terminal 40 residues, 2 "EGF-like" domains and 1 Beta-hydroxyaspartic acid at position 64. However, the absence of active serine site and active histidine site in the homologous region with the family of serine proteases enhanced the lack of catalytic triad. This is consistent with the inability to activate PZ into a serine protease by limited hydrolysis [2, 3]. Moreover, PZ only has a cofactor function, and not a proteolytic function [4], like protein S (PS). In 1991, Hogg and Stenflo reported that bovine PZ interacts with thrombin [5], and suggested that bovine PZ mediates binding between thrombin and phospholipid (PL) surfaces. In contrast, human PZ binds thrombin poorly resulting in a minimal impact on thrombin association with PL [6]. This is due to a difference in aa sequence between bovine and human PZ. The complete aa sequence of human PZ was previously described by Sejima and Ichinose [4, 7], who reported the lack of 36 aa C-terminal enhancing thrombin binding [6].

In 1997, Mc Donald *et al* reported that PZ binds PL surface thanks to gamma-carboxyglutamic acid-rich (GLA) domain, such as vitamin K-dependent factors, but PZ presents slower kinetics [8]. The inhibition of activated factor X (FXa) mediated by PZ underlines the need for another plasma protein for inhibition: PZ-dependent protease inhibitor (ZPI) was isolated and characterized by Han *et al* [9, 10]. ZPI belongs to the serpin family, producing rapid inhibition of procoagulant PL and Ca^{2+} and FXa within PZ. Two possible pathways for PZ mediated inhibition of FXa by ZPI were evoked [11] and Tabatai *et al* described a plasma circulating complex between PZ and ZPI [12]. Moreover, Yin *et al* demonstrated that in presence of PZ, thrombin generation (TG) was significantly delayed and peak

thrombin concentration was reduced by more than 50% [13].

Therefore, reduced PZ blood concentrations might be expected to reduce inhibition of blood coagulation predisposing to thrombosis. There have been several clinical studies on PZ deficiency in many different clinical contexts but all with inconclusive and controversial results. Indeed, PZ deficiency has been described as a risk factor for bleeding [14] but these data were not confirmed in other studies [15, 16]. Other studies assessed PZ plasma level in several thrombotic events including stroke, myocardial infarction, cerebral or deep venous thrombosis and pregnancy complications. However, many discrepancies were observed in the results of these numerous studies reported in several reviews [17, 18].

The aim of this present study was to evaluate the influence of PZ plasma levels on coagulation activation and to detect a hypercoagulable state in patients with PZ deficiency using TG assay.

2. Patients and Methods

2.1. Patients

In a first part, fifteen young patients (age <56 years old) with personal history of arterial thrombosis (stroke or myocardial infarction) and PZ deficiency but no other coagulation abnormalities or anticoagulant therapy, were included in this study conducted at Rouen University Hospital, France. Blood samples were collected from tubes containing 0.109 M of trisodium citrate (1:10) (Venosafe Plastic tubes, Terumo, Japan) and double centrifuged (2250g-15min-20°C). Platelet-free plasmas were kept frozen at -80°C until the assays.

In a second part, we focused on two other patients with PZ deficiency and without anticoagulant therapy (Pat1PZdef and Pat2PZdef). Pat1PZdef was a 52-year-old woman admitted for stroke to the Neurology Department of Rouen University Hospital, France, with a PZ level of 0.43 mg/L (normal range from 0.9 to 2.7 mg/L) and no other coagulation abnormalities. Pat2PZdef was a 33-year-old man, admitted for myocardial

infarction to the Cardiology Department of Rouen University Hospital, France, with a PZ level of 0.31 mg/L controlled to 0.48 mg/L. In this latter patient we evidenced isolated and persistent antibodies IgG antiphospholipid (Phopho-LISA IgG/IgM, Theradiag, Marne la Vallée, France) but no anticardiolipin, no anti-beta2 GPI antibodies and no lupus anticoagulant.

2.2. Methods

PZ assays were performed on plasma samples in our homeostasis unit at Rouen University Hospital, France using ELISA method (Asserachrom PZ, Diagnostica Stago, Asnières, France).

For both selected deficient PZ plasmas, progressive PZ overloads were performed using purified PZ (Hyphen Biomed, Neuville/Oise, France).

Moreover, we used an industrial PZ deficient plasma (Protein Z deficient plasma, Hyphen Biomed, Neuville sur Oise France), (IndPZdef), restored with distilled water and containing less than 1% of PZ and normal range for other coagulation factors. This industrial deficient PZ plasma was overloaded too.

For each spiked plasma, real PZ concentration was assessed.

TG was measured according to the method previously described by Hemker et al [19]. TG assay was performed in a Fluoroscan Ascent® fluorometer (Thermoscientific Labsystems, Helsinki, Finland) and Thrombinoscope™ software (Thrombinoscope BV, Maastricht, The Netherlands) [20-22], in three different conditions (all reagents from Diagnostica Stago, Asnières, France):

*condition 1: using PPP normal reagent containing final concentrations of 5 pM tissue factor (TF) and 4 μM PL,

*condition 2: using PPP low reagent, containing final concentrations of 1 pM tissue factor (TF) and 4 μM PL, [23].

For both these conditions, endogenous thrombin potential (ETP), lagtime, peak concentration and time to peak [21], were analyzed by Thrombinoscope™ software, and velocity was calculated for each sample.

*the third condition used recombinant human thrombomodulin (TM) (Diagnostica Stago, Asnières, France,) obtained to boost protein C pathway. Using our standardized procedure, TG was measured with and without TM, and ratio of ETP was calculated with and without TM as ETP(TM+)/ETP(TM-) [19, 24].

In each set of TG experiment, we performed an assay on our in-house internal quality control (IQC) (normal pooled plasmas) in order to validate the set of experiment.

Parameters of TG assay were normalized against the IQC of the same set of experiment or against the mean of IQC assays. Finally, we standardized our set of experiments using a protocol described by Dargaud *et al* [25-27].

2.3. Statistical analysis

Data are expressed as mean ± standard deviation (SD). The intra-assay coefficient of variation (CV) for TG assay was calculated from consecutive measurements of IQC. Statistical analysis was performed on NCSS 2007 statistical software (Statistical Solutions, Cork, Ireland). We studied correlation between PZ final concentration and respectively lag time, peak, ETP and time to peak.

3. Results

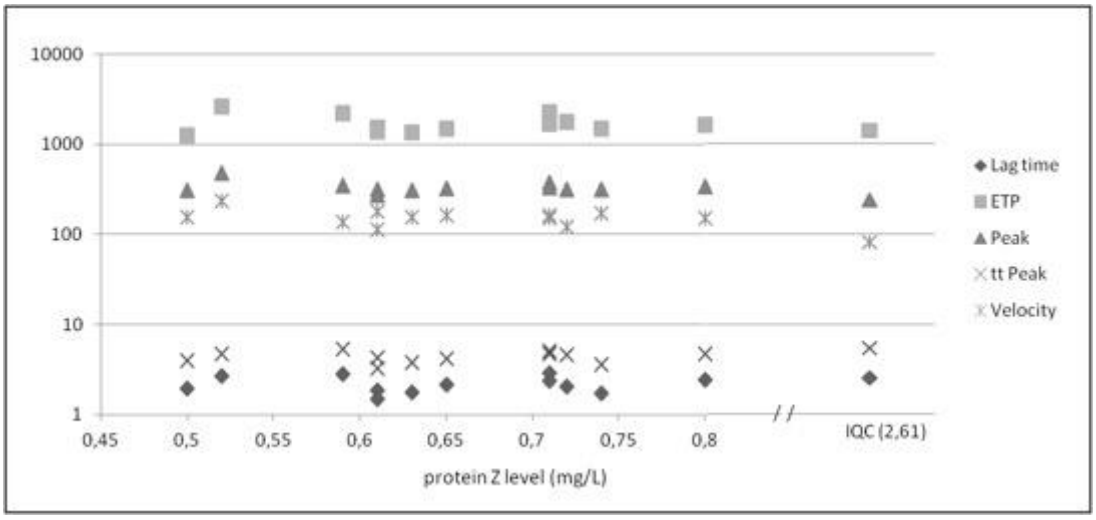
3.1. TG assay performances

The intra-assay CV was calculated from 8 measurements of IQC in the same set of experiments. The CV for each parameter were respectively 2% for lag time, 4% for ETP and 2% for both the peak and time to peak. Forty-four assay runs were performed for IQC; inter-assay reproducibility was correct for both TG parameters (mean CV of 11% for lag time, 9% for ETP, 11.9% for peak and 9.3% for time to peak).

3.2. Patients with PZ deficiency

First, we performed PZ assays on plasma collected from 13 patients with PZ deficiency. PZ level did not influence the parameters of TG assay (Fig. 1). Nevertheless, this comparison was performed in patients with likely differences in coagulation state.

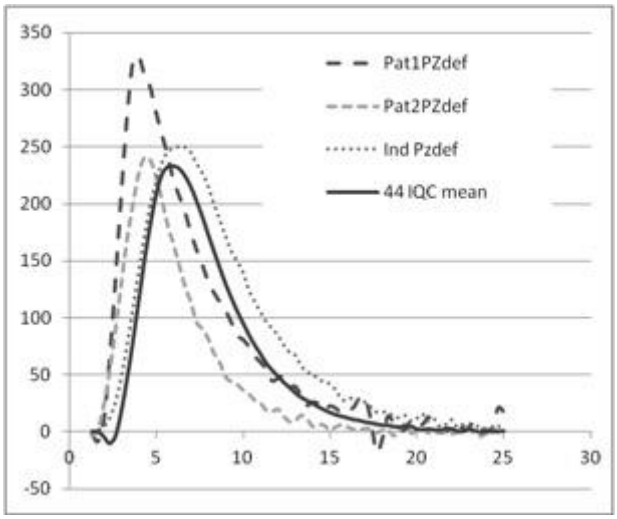
Figure1: Parameters of thrombin generation assay for 13 patients with protein Z deficiency and the internal quality control (protein Z plasma concentration: 2.61 mg/L).



3.3. Overloading of PZ deficient plasmas

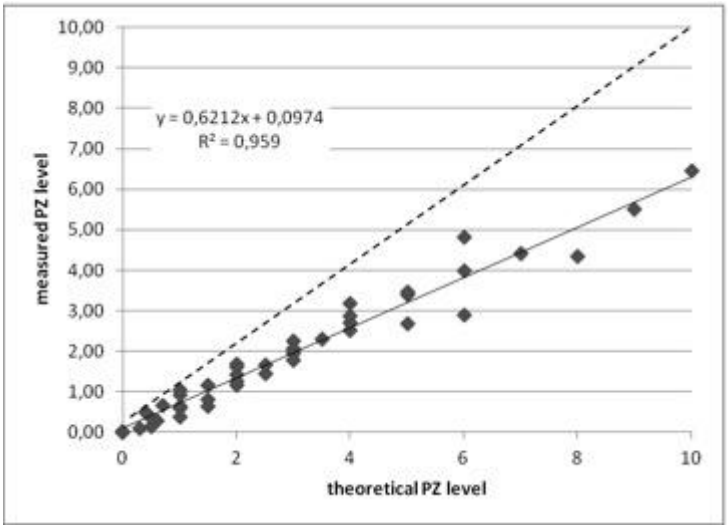
Second, two other patients with PZ deficiency (Pat1PZdef and Pat2PZdef) and industrial PZ deficiency (IndPZdef) were chosen for overload with purified PZ. We compared the TG profile of these 3 PZ deficient plasmas with the mean profile of IQC (44 sets) (Fig. 2), considering the IQC profile as the reference in standardized procedure (condition 1: TF 5 pM, PL 4 µM).

Figure 2: Comparison of thrombin generation assay before overload with purified protein Z for both patient with protein Z deficiency, the industrial protein Z deficient and the internal quality control. Thrombin generation assay was assessed with tissue factor 5 pM, phospholipids 4 µM; 44 measurements of internal quality control were assessed.



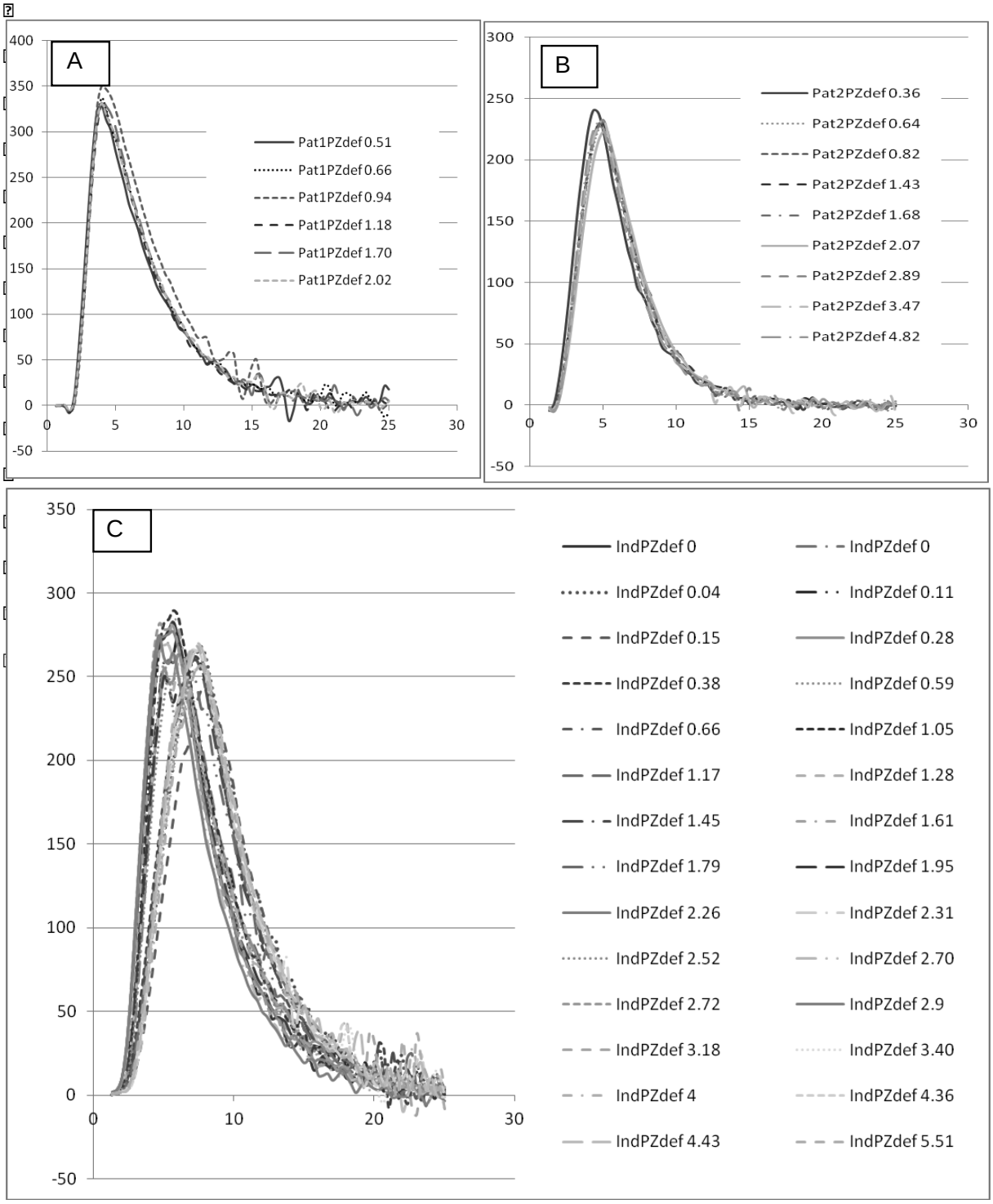
Pat1PZdef plasma had increased hypercoagulability (lagtime: 2 min; peak: 327 nM; ETP: 1816 nM.min; time to peak: 4.17 min) compared to normal plasma (lagtime: 2.58 min; peak: 239 nM; ETP: 1409 nM.min; time to peak: 5.54 min). TG profile of Pat2PZdef and IndPZdef were close to the reference TG profile, while the lagtime of Pat2PZdef was shorter than the reference profile (2.17 min versus 2.58 min). After each overload, real PZ plasma levels were determined. The real concentration was under the theoretical level (Fig. 3).

Figure 3: Comparison of theoretical and measured protein Z concentrations according to theoretical overload. Lines illustrate theoretical protein Z concentrations (black lines), and measured protein Z concentrations (dotted lines).



Nevertheless, the PZ plasma concentrations did not influence the TG profile of Pat1PZdef, Pat2PZdef or IndPZdef (Fig. 4).

Figure 4: Influence of purified human protein Z overloads in thrombin generation profile of protein Z deficient plasmas. Protein Z concentrations measured by ELISA are indicated at the end of each term of legend.



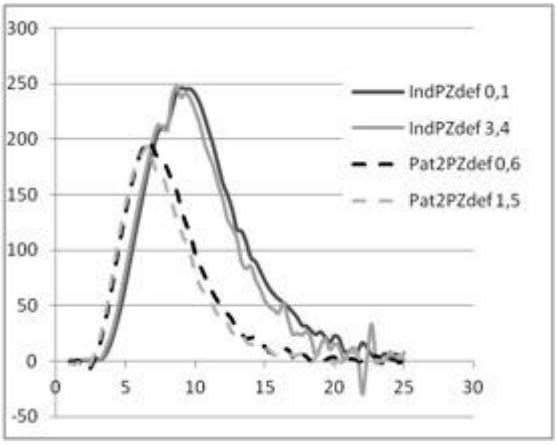
These data were the same after normalization against daily IQC or against the mean of the 44 IQC. Regressions between measured PZ levels and the parameters of TG assay were non-significant (Table 1).

Table 1: Coefficients of regression for thrombin generation parameters, according to the measured protein Z plasma concentrations in plasma protein Z deficient. Each TG assay was normalized against the daily internal quality control or against the mean of 44 internal quality control.

Normalization against daily IQC				
p(regression)	Lagtime	ETP	Peak	TTP
Pat1PZdef	0.264	0.808	0.831	0.264
Pat2PZdef	0.255	0.235	0.659	0.570
IndPZdef	0.513	0.535	0.316	0.630
Normalization against 44 IQC mean				
p(regression)	Lagtime	ETP	Peak	TTP
Pat1PZdef	0.267	0.810	0.829	0.264
Pat2PZdef	0.256	0.249	0.672	0.570
IndPZdef	0.05	0.390	0.902	0.252

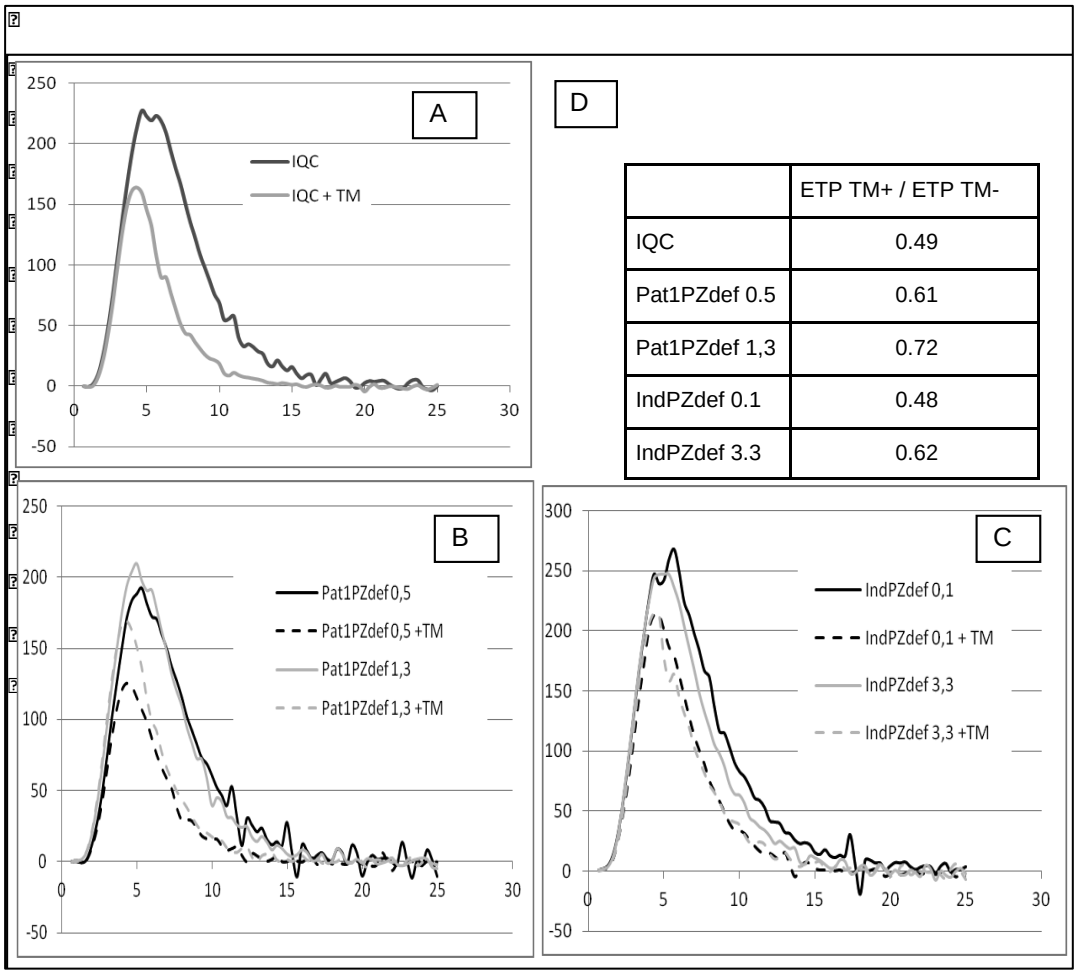
In condition 2, (TF 1 pM), the results were the same when comparing pure Pat1PZdef and IndPZdef with one point of overload for each (Fig. 5).

Figure 5: Comparison of thrombin generation assay for patient with protein Z deficiency and industrial protein Z deficient before and after overload with purified protein Z. Thrombin generation assay was assessed with tissue factor 1 pM, phospholipids 4 µM; protein Z concentrations measured by ELISA are indicated at the end of each term of legend.



Finally, in presence of TM (condition3), there was no difference in inhibition of ETP or peak according to PZ plasma levels (Fig. 6).

Figure 6: Comparison of thrombin generation assay for patient with protein Z deficiency (A), industrial protein Z deficient (B) before and after overload with purified protein Z, and internal quality control (C). Thrombin generation assay was assessed with tissue factor 5 pM, phospholipids 4 µM, with and without thrombomodulin; protein Z concentrations measured by ELISA are indicated at the end of each term of legend. The ratio between ETP with TM and ETP without TM are indicated in the table (D).



	ETP TM+ / ETP TM-
IQC	0.49
Pat1PZdef 0.5	0.61
Pat1PZdef 1,3	0.72
IndPZdef 0.1	0.48
IndPZdef 3.3	0.62

4. Discussion

To our knowledge, this is the first study to observe the effect of plasma PZ level on TG profile. As previously described, PZ, the cofactor of ZPI, contributes to FXa inhibition [11, 12] and thus should influence TG profile. Moreover in 2000, Yin *et al* demonstrated that PZ significantly delayed TG [13]. The authors analyzed the effect of PZ on FXa

activity and TG during *in vitro* studies. Their results suggested that PZ decreased coagulation response in human plasma. First, the comparison between 13 different PZ deficient patients did not reach difference in TG assay profile in our conditions (5 pM FT, 4 μM PL). However, TG assay is a global coagulation test influenced by several coagulation factors [21].

Hence, it could be possible to demonstrate an influence by successive overloads of PZ deficient plasma with a small quantity of purified PZ. In our study, PZ plasma levels did not influence TG profile either for Pat1PZdef or Pat2PZdef.

Second, concerning PZ overloads (Fig. 3), the measurement of PZ concentration was always lower than expected. Differences in standards of purified PZ between the two manufacturers (Hyphen Biomed for IndPZdef and Diagnostica Stago for the purified PZ calibrator Asserachrom PZ) might explain such results. Differences in concentrations are constant and a real correlation exists between observed and expected concentrations. Nevertheless, we obtained concentrations of up to 6 mg/l which is high compared to normal value (0.9 to 2.7 mg/l) but without consequence on TG assay.

No proteolytic functions are supported by PZ or PS: PS is the cofactor of protein C inhibiting activated factor V and activated factor VIII in presence of PL and calcium, and PZ is the cofactor of ZPI inhibiting FXa in presence of PL and calcium [9, 10]. Several studies have been published on the influence of PS on TG assay. As described by Hezard *et al*, PS deficiency generates hypercoagulability in TG assay, in platelet rich plasma conditions, with and without activated protein C [28]. Duchemin *et al*, in 1994, demonstrated that PS plasma level did not influence TG profile in absence of TM [29]; while inhibition of ETP was correlated with PS levels in presence of TM: lower PS level was correlated with lower ETP inhibition. We did not observe these results with PZ plasma level: in our study, lower PZ level was correlated with higher ETP inhibition for IndPZdef as well as for Pat1PZ def.

Later, Duchemin *et al* reported that PS concentration was positively correlated with lagtime especially with low TF concentration. In contrast, PS concentration did not influence ETP or peak regardless of TF concentration. Dielis *et al* observed an influence of free PS level on lagtime in different conditions for TG assay [30]. In our study, PZ plasma concentration did not influence lagtime, peak or ETP irrespective of TF concentration.

Thus, the mechanisms of action for PS and PZ are similar but the targets of serpin PC and ZPI are different: PC directly inactivates the boosters of TG (activated factors V and VIII) while ZPI inactivates only FXa.

Finally, the absence of influence of PZ concentration on TG assay could explain the absence of well demonstrated clinical consequences of PZ deficiency in venous thrombosis and arterial thrombosis. In 2000 and 2001, two studies demonstrated that PZ deficiency increased thrombotic risk in mice carrying factor V Leiden mutation [11, 13]. Later, the involvement of PZ deficiency in human pathologies was evaluated with opposite results: PZ deficiency and risk factor of venous thrombosis [31, 32], stroke [33, 34], and myocardial infarction [35, 36]. The discrepancies are the same concerning all thrombotic syndromes, as described in literature reviews [17, 18].

5. Conclusion

We were unable to demonstrate the influence of PZ plasma level on thrombin generation assay test according to Hemker's method. Our results likely explain the absence of studies in the literature on the clinical consequences of protein Z deficiency warranting further studies.

Acknowledgements

We thank Agnès Deshayes for her technical help. We are grateful to Nikki Sabourin-Gibbs, Rouen University Hospital, for her help in editing the manuscript.

Conflict of interest.

None declared.

References

1. Prowse CV, Esnouf MP. The isolation of a new warfarin-sensitive protein from bovine plasma. *Biochem Soc Trans* 1977; 5:255-256.
2. Broze GJ, Jr., Miletich JP. Human Protein Z. *J Clin Invest* 1984; 73:933-938.
3. Hojrup P, Jensen MS, Petersen TE. Amino acid sequence of bovine protein Z: a vitamin K-dependent serine protease homolog. *FEBS Lett* 1985; 184:333-338.
4. Ichinose A, Takeya H, Espling E, Iwanafa S, Kisiel W, Davie EW. Amino acid sequence of human protein Z, a vitamin K-dependent plasma glycoprotein. *Biochem Biophys Res Commun* 1990; 172:1139-1144.
5. Hogg PJ, Stenflo J. Interaction of vitamin K-dependent protein Z with thrombin. Consequences for the amidolytic activity of thrombin and the interaction of thrombin with phospholipid vesicles. *J Biol Chem* 1991; 266:10953-10958.
6. Hogg PJ, Stenflo J. Interaction of human protein Z with thrombin: evaluation of the species difference in the interaction between bovine and human protein Z and thrombin. *Biochem Biophys Res Commun* 1991; 178:801-807.
7. Sejima H, Hayashi T, Deyashiki Y, Nishioka J, Suzuki K. Primary structure of vitamin K-dependent human protein Z. *Biochem Biophys Res Commun* 1990; 171:661-668.
8. McDonald JF, Shah AM, Schwalbe RA, Kisiek W, Dahlbäck B, Nelsestuen GL. Comparison of naturally occurring vitamin K-dependent proteins: correlation of amino acid sequences and membrane binding properties suggests a membrane contact site. *Biochemistry* 1997; 36:5120-5127.
9. Han X, Fiehler R, Broze GJ, Jr. Isolation of a protein Z-dependent plasma protease inhibitor. *Proc Natl Acad Sci USA* 1998; 95:9250-9255.
10. Han X, Fiehler R, Broze GJ, Jr. Characterization of the protein Z-dependent protease inhibitor. *Blood* 2000; 96:3049-3055.
11. Broze GJ, Jr. Protein Z-dependent regulation of coagulation. *Thromb Haemost* 2001; 86:8-13.
12. Tabatabai A, Fiehler R, Broze GJ, Jr. Protein Z circulates in plasma in a complex with protein Z-dependent protease inhibitor. *Thromb Haemost* 2001; 85:655-660.
13. Yin ZF, Huang ZF, Cui J, Fiehler R, Lasky N, Ginsburg D et al. Prothrombotic phenotype of protein Z

deficiency. *Proc Natl Acad Sci USA*. 2000; 97:6734-6738.

14. Kemkes-Matthes B, Matthes KJ. Protein Z deficiency: a new cause of bleeding tendency. *Thromb Res* 1995; 79:49-55.

15. Ravi S, Mauron T, Lammle B, Wuillemin WA. Protein Z in healthy human individuals and in patients with a bleeding tendency. *Br J Haematol* 1998; 102:1219-1223.

16. Gamba G, Bertolino G, Montani N, Spedini P, Balduini CL. Bleeding tendency of unknown origin and protein Z levels. *Thromb Res* 1998; 90:291-295.

17. Corral J, Gonzalez-Conejero R, Hernandez-Espinosa D, Vincete V. Protein Z/Z-dependent protease inhibitor (PZ/ZPI) anticoagulant system and thrombosis. *Br J Haematol*. 2007; 137:99-108.

18. Le Cam-Duchez V, Barbay V, Soria C, Borg JY. [Protein Z, polymorphisms in the protein Z gene and thrombosis]. *Rev Med Interne* 2010; 31:551-557.

19. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb* 2003; 33:4-15.

20. Hemker HC, Al Dieri R. Age-dependency of thrombin generation. *Thromb Haemost* 2006; 95:756-757.

21. Hemker HC, Al Dieri R, De Smedt E, Béguin S. Thrombin generation, a function test of the haemostatic-thrombotic system. *Thromb Haemost* 2006; 96:553-561.

22. Haidl H, Cimenti C, Leschnik B, Zach D, Muntean W. Age-dependency of thrombin generation measured by means of calibrated automated thrombography (CAT). *Thromb Haemost* 2006;95:772-775.

23. Gerotziafas GT, Depasse F, Busson J, Leflem L, Elalamy I, Samama MM. Towards a standardization of thrombin generation assessment: the influence of tissue factor, platelets and phospholipids concentration on the normal values of Thrombogram-Thrombinoscope assay. *Thromb J* 2005;3:16-27.

24. Dargaud Y, Trzeciak MC, Bordet JC, Ninet J, Negrier C. Use of calibrated automated thrombinography +/- thrombomodulin to recognise the prothrombotic phenotype. *Thromb Haemost* 2006; 96:562-567.

25. Dargaud Y, Luddington R, Gray E, Negrier C, Lecompte T, Petros S et al. Effect of standardization and normalization on imprecision of calibrated automated thrombography: an international multicentre study. *Br J Haematol* 2007; 139:303-309.

26. Dargaud Y, Luddington R, Gray E, Lecompte T, Siegemund T, Baglin T et al. Standardisation of thrombin generation test--which reference

plasma for TGT? An international multicentre study. *Thromb Res* 2010; 125:353-356. doi: 10.1016/j.thromres.2009.11.012.

27. Dargaud Y, Wolberg AS, Luddington R, Ragnault V, Spronk H, Baglin T et al. Evaluation of a standardized protocol for thrombin generation measurement using the calibrated automated thrombogram: an international multicentre study. *Thromb Res* 2012; 130:929-934. doi: 10.1016/j.thromres.2012.07.017.
28. Hezard N, Bouaziz-Borgi L, Remy MG, Nguyen P. Utility of thrombin-generation assay in the screening of factor V G1691A (Leiden) and prothrombin G20210A mutations and protein S deficiency. *Clin Chem* 2006 ; 52:665-670.
29. Duchemin J, Pittet JL, Tartary M, Béguin S, Gaussem P, Alhenc-Gelas M et al. A new assay based on thrombin generation inhibition to detect both protein C and protein S deficiencies in plasma. *Thromb Haemost* 1994; 71:331-338.
30. Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyak K, Ten Cate H et al. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost* 2008; 6 :125-131.
31. Santacroce R, Sarno M, Cappucci F, Sessa F, Colaizzo D, Brancaccio V et al. Low protein Z levels and risk of occurrence of deep vein thrombosis. *J Thromb Haemost* 2006; 4:2417-2422.
32. Martinelli I, Razzari C, Biguzzi E, Bucciarelli P, Mannucci PM. Low levels of protein Z and the risk of venous thromboembolism. *J Thromb Haemost* 2005; 3:2817-2819.
33. Vasse M, Guegan-Massardier E, Borg JY, Woimant F, Soria C. Frequency of protein Z deficiency in patients with ischaemic stroke. *Lancet* 2001; 357:933-934.
34. Lopaciuk S, Bykowska K, Kwiecinski H, Czlonkowska A, Kuczynska-Zardzewialy A. Protein Z in young survivors of ischemic stroke. *Thromb Haemost* 2002; 88:536.
35. Fedi S, Sofi F, Brogi D, Tellini I, Cesari F, Sestini I et al. Low protein Z plasma levels are independently associated with acute coronary syndromes. Thromb Haemost 2003 ; 90 :1173-1178.
36. Le Cam-Duchez V, Soria C, Dit Sollier CB, Borg JY, Coudert M, Montalescot G et al. Rare genotypes of protein Z gene are a risk factor for premature myocardial infarction but not protein Z plasma level. Thromb Haemost 2009 ; 102:131-136. doi: 10.1160/TH09-01-0007.

