PS levels cannot be said to have any relationship with the studied endothelial activation markers.

Therefore, acquired free PS deficiency may be associated with lipid profile levels and/or another variable common to lipids and PS, conferring an increased potential thrombotic risk on HIV-infected patients naive of treatment. Additionally, this free PS deficiency may have some role in the development of avascular necrosis seen in HIV-infected patients naive of any antiretroviral treatment.

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Protein Z gene polymorphisms are associated with protein Z plasma levels

R. SANTACROCE, F. CAPPUCCI,* P. DI PERNA,* F. SESSA and M. MARGAGLIONE
*Cattedra di Genetica Medica, Dipartimento di Scienze Biomediche, Università di Foggia, Foggia, Italy; and *Unita' di Ateroscleroси e Trombosi, I.R.C.C.S. 'Casa Sollievo della Sofferenza', S. Giovanni Rotondo, Italy*

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The protein Z (PZ) is a member of the coagulation cascade known by many years but its role has remained obscure until recently. PZ is a vitamin K-dependent glycoprotein with an important role in the regulation of the coagulation cascade because of PZ-dependent protease inhibitor [1]. The PZ-dependent protease inhibitor in the presence of PZ causes fast inactivation of factor Xa. Recently, reduced circulating levels of PZ have been suggested to play a role in the occurrence of bleeding [2] and deep vein thrombosis [3,4], as well as primary aborts with unexplained early fetal loss [5]. However, these data have been disputed [6–8]. A series of variants naturally occurring within the PZ gene locus have been excluded to be associated with the risk of deep vein thrombosis [9]. In a cohort of apparently healthy subjects, we have investigated the PZ gene looking for a relationship between gene polymorphisms and PZ plasma levels.

One hundred apparently healthy subjects (41 men and 59 women; median age 52.5 years, range 31–73) randomly selected from a Southern Italian general population without a history of venous thromboembolism were investigated. After approval of the local Ethics Committee, the study was carried out according to the Principles of the Declaration of Helsinki; informed consent was obtained from all the subjects.

Blood samples were collected into vacuum plastic tubes containing 3.8% trisodium citrate and centrifuged at 2000 × *g* for 15 min to obtain platelet-poor plasma. The latter was frozen and stored in small aliquots at − 70 °C until tested. PZ plasma levels were evaluated by means of an enzyme-linked

Correspondence: Maurizio Margaglione, Cattedra di Genetica Medica, Dipartimento di Scienze Biomediche, Università degli Studi di Foggia, viale Pinto, Foggia 71100, Italy.
Tel.: + 39 8 8173 3842; fax: + 39 8 8173 2188; e-mail: m.margaglione@unifg.it

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immunosorbant assay (Asserachrom Protein Z; Diagnostica Stago, Asnières, France). DNA was extracted from peripheral blood leukocytes according to standard protocols [10]. Amplifications of all coding regions of PZ gene and intron/exon boundaries were achieved using sense and antisense oligonucleotide designed on the basis of known sequences of PZ gene locus (GenBank accession number AF440358). Oligonucleotide custom synthesis service was from Life Technologies (Paisley, UK). Polymerase chain reaction was carried out on 50 μL volume samples, in a Perkin Elmer-Cetus thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Each sample contained 0.1 μg of genomic DNA, 10 pmol of each primer, 125 μM of dNTP, 5 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 1 U Taq polymerase. The solution was overlaid with 50 μL of mineral oil and, after an initial denaturation step (3 min at 95 °C), it was put through 30 cycles each consisting of 1 min at 95 °C, 1 min at 56–60 °C and 2 min at 72 °C. Amplified DNA fragments were then subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 310 Genetic Analyzer sequencer (PE Biosystems, Foster City, CA, USA). All the analyses were performed according to the Statistical Package for Social Science (SPSS 10.0 for Macintosh, Chicago, IL, USA). The significance of any difference in means was evaluated by non-parametric test. The allele frequencies were estimated by gene counting, and genotypes were scored. The observed numbers of each PZ genotype were compared with those expected for a population in Hardy–Weinberg equilibrium using a χ^2 test. Statistical significance was taken as $P < 0.05$.

The mean (± SD) PZ plasma concentration was 1.36 ± 0.61 μg mL^{-1} with a total range of 0.21–3.44 μg mL^{-1} and a median value of 1.33 μg mL^{-1}. PZ plasma levels were significantly higher in men (mean ± SD 1.54 ± 0.70 μg mL^{-1}) than in women (mean ± SD 1.24 ± 0.52 μg mL^{-1}; Mann–Whitney *U*-test: $P = 0.035$). The entire PZ gene was investigated for polymorphisms by means of direct cycle sequence analysis. Polymorphisms detected, allele and genotype frequencies found are shown in Table 1. Among the 14 common or rare gene variants previously detected within the PZ gene locus [9], most were not found in the setting analyzed. In addition, three novel polymorphisms were identified (Table 1). Two of the three new polymorphisms within the intron A, *a224g* and *g277a*, were in complete linkage disequilibrium. A significant association with circulating PZ levels was shown for the intron A *g*−103a and the intron F *g79a* polymorphisms (Table 1). Actually, heterozygous carriers of the intron A *a*−103 allele, as well as heterozygous subjects with the intron F *a79* allele, showed mean PZ levels (1.00 and 1.15 μg mL^{-1}) lower than individuals with the *gg* genotype (1.48 and 1.53 μg mL^{-1}; *post hoc* Sheffé test for multiple comparisons: $P = 0.005$ and $P = 0.015$, respectively). In those presenting with the *aa* genotype, the plasma concentration was further reduced (0.88 and 0.84 μg mL^{-1}; *post hoc* Sheffé test for multiple comparisons: $P =$ ns and $P = 0.004$, respectively). A subject carried the intron G *c24* allele and showed lower levels (0.78 μg mL^{-1}) than subjects with the *tt* genotype (1.37 ± 0.61 μg mL^{-1}). None of the remaining PZ gene polymorphisms was associated with circulating levels of PZ (Table 1).

According to previous studies [6], PZ concentrations showed a wide range and were different from those measured in some studies [5,7,11] but in agreement with others [6,8,12], including a different Italian setting [13]. Genetic factors may be an important determinant of the wide normal range of PZ plasma concentrations [14]. A series of common and rare gene variants has been reported in the PZ locus [9]. Most of polymorphisms previously reported were not found, whereas we identified three new common gene variants and few rare alleles. Interestingly, the intron A *g*−103a, as well as the intron F *g79a* polymorphism, was found to be associated with PZ plasma levels, carriers of the rare allele showing lower concentrations. These data are in agreement with recent results obtained investigating the intron F *g79a* polymorphism [15]. The rare intron G *c24* allele

Table 1 Mean protein Z (PZ) plasma levels according to gene polymorphisms identified

Gene polymorphism	Allele frequencies	PZ ag levels
Intron A a224g*	a 90%	aa [$n = 82$] 1.36 (0.61)
	g 10%	ag [$n = 18$] 1.40 (0.64)
Intron A g277a*	a 90%	aa [$n = 82$] 1.36 (0.61)
	g 10%	ag [$n = 18$] 1.40 (0.64)
Intron A g-103a†	g 86%	gg [$n = 76$] 1.48 (0.63)
	a 14%	ga [$n = 20$] 1.00 (0.34)‡
		aa [$n = 4$] 0.88 (0.39)
Exon 4 ins/del	Del 97.5%	Del/Del [$n = 95$] 1.36 (0.59)
	Ins 2.5%	Del/Ins [$n = 5$] 1.33 (1.00)
Intron F g79a	g 67%	gg [$n = 63$] 1.53 (0.60)
	a 23%	ga [$n = 28$] 1.15 (0.57)§
		aa [$n = 9$] 0.84 (0.34)¶
Intron G t24c	t 99.5%	tt [$n = 99$] 1.37 (0.61)
	c 0.5%	tc [$n = 1$] 0.78
Exon 8 Arg255His	Arg 94.5%	Arg/Arg [$n = 89$] 1.39 (0.63)
	His 5.5%	Arg/His [$n = 11$] 1.18 (0.46)

Mean (SD) PZ plasma levels are expressed in μg mL^{-1}. *The intron A a224g and the intron A g277a were in complete linkage disequilibrium. †A rare allele (−103t) was found in one heterozygous subject. Ins/del: insertion/deletion. ‡$P < 0.01$ vs. the gg genotype; §$P < 0.02$ vs. the gg genotype; ¶$P < 0.01$ vs. the gg genotype.

was found in a subject with reduced PZ concentrations. However, the low prevalence of the intron G *c24* allele did not allow for any statistical comparison. None of these gene polymorphisms is likely to have a direct effect on the gene expression. Thus, it is conceivable that they are in linkage disequilibrium with unknown allelic variants that modulate the PZ gene expression. Nonetheless, these findings add strength to the importance of common genetic variations within the PZ locus in the modulation of plasma concentrations and help to explain, to some extent, the wide range observed in the general population as well as inconsistencies in mean plasma levels observed in different populations. Of the remaining polymorphisms and rare allelea identified, none was found to be associated with PZ plasma levels.

In conclusion, results of the present investigation confirm the wide range of PZ plasma values and show that genetic factors within the PZ locus may explain, at least in part, this interindividual variability.

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Tissue factor expression and $P2Y_{12}$ gene polymorphism

J-L. RENY, P. GAUSSEM, V. REMONES, J. EMMERICH and P. FONTANA
Service d'Hématologie Biologique A, Hôpital Européen Georges Pompidou, Inserm Unité 428 and Université Paris V, Paris, France

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Platelets are critical mediators of primary hemostasis through adhesion, aggregation and subsequent thrombus formation induced by collagen, von Willebrand factor, thrombin, and other factors exposed at site of vascular injury. In addition to their crucial role in primary hemostasis, activated-platelets contribute to the generation of thrombin which provides fibrin for the stabilization of the newly formed thrombus [1].

Correspondence: Pascale Gaussem, Service d'Hématologie Biologique A, Hôpital Européen Georges Pompidou, Inserm Unité 428 and Université Paris V, Paris, France.
Tel.: +33 1 5609 3936; fax: +33 1 5609 3913; e-mail: pascale.gaussem@egp.ap-hop-paris.fr

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Leon *et al*. recently presented results on the contribution of platelet ADP receptors to the initiation of intravascular coagulation [2]. The authors showed that both the $P2Y_1$ and $P2Y_{12}$ ADP receptors were implicated in the exposure of tissue factor (TF) in collagen-activated whole blood, emphasizing the role of platelet activation in this situation. We recently described a $P2Y_{12}$ gene polymorphism associated with ADP-induced maximal platelet aggregation [3], the H2 haplotype being associated with a higher aggregation response in healthy subjects. Moreover, we found a significant association between the $P2Y_{12}$ H2 haplotype and peripheral vascular disease in a case–control study [4]. In view of the results presented by Leon *et al*., we hypothesized that the H1/H2 polymorphism could be associated with collagen-induced platelet aggregation response, basal TF expression in monocytes (TF mRNA), plasma