



Universiteit
Leiden
The Netherlands

No effect of the prothrombin G20210A mutation on protein C activation in a large kindred with type I protein C deficiency

Vossen, C.Y.; Strandberg, K.; Stenflo, J.; Rosendaal, F.R.; Callas, P.W.; Long, G.L.; Bovill, E.G.

Citation

Vossen, C. Y., Strandberg, K., Stenflo, J., Rosendaal, F. R., Callas, P. W., Long, G. L., & Bovill, E. G. (2004). No effect of the prothrombin G20210A mutation on protein C activation in a large kindred with type I protein C deficiency. *Blood Coagulation & Fibrinolysis*, 15(7), 573-576.
Retrieved from <https://hdl.handle.net/1887/5076>

Version: Not Applicable (or Unknown)

License:

Downloaded from: <https://hdl.handle.net/1887/5076>

Note: To cite this publication please use the final published version (if applicable).

No effect of the prothrombin G20210A mutation on protein C activation in a large kindred with type I protein C deficiency

Carla Y. Vossen^{a,b}, Karin Strandberg^c, Johan Stenflo^c, Frits R. Rosendaal^{b,d}, Peter W. Callas^e, George L. Long^f and Edwin G. Bovill^a

Previously, we observed a positive association of prothrombin concentrations with thrombin generation (fragment 1 + 2) and thrombin activity (fibrinopeptide A), but no association with protein C activation peptide levels. We further evaluated a potential beneficial effect of increased prothrombin concentrations on activated protein C generation by assessing the plasma concentration of activated protein C in complex with protein C inhibitor (APC-PCI). Blood samples were used from 195 family members of a large French-Canadian kindred with type I protein C deficiency due to a 3363C insertion in the protein C gene. We utilized a new and highly sensitive assay for measuring the concentration of APC-PCI complex as a measure of the level of activation of protein C. Means of the plasma concentrations of the APC-PCI complex were compared among carriers and non-carriers of the prothrombin G20210A mutation. Protein C activity levels were positively associated with APC-PCI complex plasma concentrations; however, APC-PCI complex levels were not different for carriers of the prothrombin G20210A mutation than for non-carriers. Thus, carriers of the

prothrombin G20210A mutation do not have increased protein C activation despite the increased thrombin generation resulting from the higher prothrombin concentrations associated with the G20210A mutation. *Blood Coagul Fibrinolysis* 15:573–576 © 2004 Lippincott Williams & Wilkins.

Blood Coagulation and Fibrinolysis 2004, 15:573–576

Keywords: activated protein C–protein C inhibitor, prothrombin G20210A mutation, protein C deficiency

^aDepartment of Pathology, ^eDepartment of Biostatistics and ^fDepartment of Biochemistry, University of Vermont, Burlington, Vermont, USA, ^bDepartment of Clinical Epidemiology and ^dDepartment of Hematology, Leiden University Medical Center, Leiden, The Netherlands and ^cDepartment of Clinical Chemistry, Lund University, University Hospital, Malmö, Sweden.

Sponsorship: This study was supported by NHLBI grant PHS HL46703.

Correspondence and requests for reprints to Prof. E.G. Bovill, M.D., Department of Pathology, University of Vermont, Given Building #E208, 89 Beaumont Avenue, Burlington, VT 05405, USA.

Tel: +1 802 656 0359; fax: +1 802 656 8892; e-mail: Edwin.Bovill@uvm.edu

Received 26 May 2004 Revised 29 June 2004

Accepted 1 July 2004

Introduction

Protein C is a vitamin K-dependent (molecular weight, 62 000) zymogen for a serine protease that downregulates the hemostatic system through the proteolytic inactivation of activated factor V (FVa) and activated factor VIII. Protein C deficiency was first associated with thrombophilia in 1981 [1]. Heterozygous deficiency of protein C has a prevalence of one in 200 in the general population [2]. The incidence of symptomatic disease in penetrant families is considerably higher than in the general population [3–6]. The apparent variable penetrance of thrombotic disease among families with protein C deficiency has been attributed to the co-segregation of additional risk factors. Two likely candidates are the highly prevalent risk factors for thromboembolic disease, factor V Leiden and the prothrombin G20210A polymorphism [7,8].

Since 1985, we have studied a large kindred of French-Canadian descent with an eight-fold increased risk of venous thrombosis and early onset of disease, associated with a 3363C insertion mutation in the protein C gene [3]. Segregation analysis suggested that the increased risk of thrombosis found in this kindred

resulted from the interaction between the protein C 3363C insertion and another unknown genetic defect [9]. Currently, we have identified 787 family members, of whom 450 have been tested for the protein C 3363C insertion. Factor V Leiden was found in only four individuals, and the G20210A prothrombin polymorphism was found in 13%. We found, however, no association between the G20210A prothrombin polymorphism and increased thromboembolic disease, despite the unusually high prevalence in this kindred, including a number of individuals with both protein C deficiency and the G20210A polymorphism [10].

Since increased thrombin generation has been associated with a higher prothrombin concentration *in vivo* [11,12], we postulated a potential beneficial effect of increased thrombomodulin-mediated activated protein C generation in carriers of the prothrombin G20210A mutation. This hypothesis was not supported in a small preliminary study in which we correlated plasma concentrations of the protein C activation peptide, prothrombin fragment 1 + 2 and fibrinopeptide A with prothrombin concentration. We observed a positive association of prothrombin concentrations with throm-

bin generation (fragment 1 + 2) and thrombin activity (fibrinopeptide A), but no association with protein C activation peptide levels [11]. In the present study we have further evaluated this hypothesis in a larger sample of the family, by assessing the plasma concentration of the complex of activated protein C combined with protein C inhibitor (APC–PCI). Protein C inhibitor is a molecular weight 57 000 serine protease inhibitor with a plasma concentration of 90 nmol/l [13,14]. Plasma concentrations of APC–PCI in part reflect the degree of activation of the protein C system. Previously described assays for APC–PCI have not been sensitive enough to accurately measure the full range of concentrations of the complex in healthy individuals. In this study we have utilized a new and highly sensitive assay for measuring the concentration of APC–PCI complex as a measure of the level of activation of protein C [15].

Methods

Participants

Blood samples were collected from 201 family members of a large French-Canadian kindred with type I protein C deficiency, including spouses of family members who have children. All samples were collected in 2002 into sodium citrate pH 4.3 Stabilyte tubes (Biopool, Umeå, Sweden). The ascertainment and evaluation of the family members was previously described [3]. All subjects completed questionnaires regarding general demographic information, current health status, current medication, obstetric history, and personal history with regard to events (venous as well as arterial thrombosis and haemorrhages) and risk factors for venous thrombosis (i.e. surgeries, hospital admissions, bed rest, plaster cast). Completed forms were stored with only the patient identifier codes to protect patient confidentiality. All participating subjects gave informed consent. This study was approved by the Human Experimentation Committee of the University of Vermont College of Medicine.

Six individuals using oral anticoagulants at the time of the blood draw had levels of APC–PCI complex ranging from 0.01 to 0.03 µg/l. These individuals were excluded from all calculations.

Laboratory methods

We measured protein C activity levels by performing a clot-based functional assay using a kit provided by Diagnostica Stago (Parsippany, New Jersey, USA) [16]. The inter-assay coefficient of variation of this assay was 5.5%. The presence of the 3363C insertion in the protein C gene was determined by amplification of genomic DNA using a mutagenic oligonucleotide primer that in concert with the inserted C mutation creates a *Bgl*I cleavage site. The product was digested with *Bgl*I and analysed on a 2% agarose gel [17]. The prothrombin G20210A allele was detected by amplifica-

tion of genomic DNA with a mutagenic primer resulting in a *Hind*III cleavage site when the A-allele was present [18].

Concentrations of the APC–PCI complex were measured by a previously described assay [15]. Samples were incubated with monoclonal biotinylated capture antibody M36, which recognizes a conformation-dependent neo-epitope in APC–PCI complexes [15,19]. The mean level of APC–PCI complexes for a reference group, consisting of Swedish healthy individuals ($n = 80$; mean age, 42 years; 20 men and 60 women), was 0.13 µg/l (range, 0.07–0.26 µg/l) [20]. The functional detection limit (intra-assay coefficient of variation < 20%) in Stabilyte plasma is 32 ng/l (unpublished data).

Statistical methods

SPSS (SPSS Inc., Chicago, Illinois, USA) was used to calculate the mean and 95% confidence intervals (CIs) (mean $\pm 1.96 \times$ standard error) of the levels of the APC–PCI complex. Correlation analysis for levels of the APC–PCI complex and protein C activity was performed by calculating Pearson's correlation coefficient, and its non-parametric equivalent the Spearman's rank correlation coefficient. Pearson correlation coefficients were similar to the Spearman's rank correlation coefficients, but because the data were not normally distributed only the latter are presented. For each Spearman's rank correlation coefficient, we calculated the 95% CI [21]. Correlations were calculated without accounting for the family structure. However, the heritability of APC–PCI and protein C were both low enough to perform analyses that do not account for family structure [22].

Results

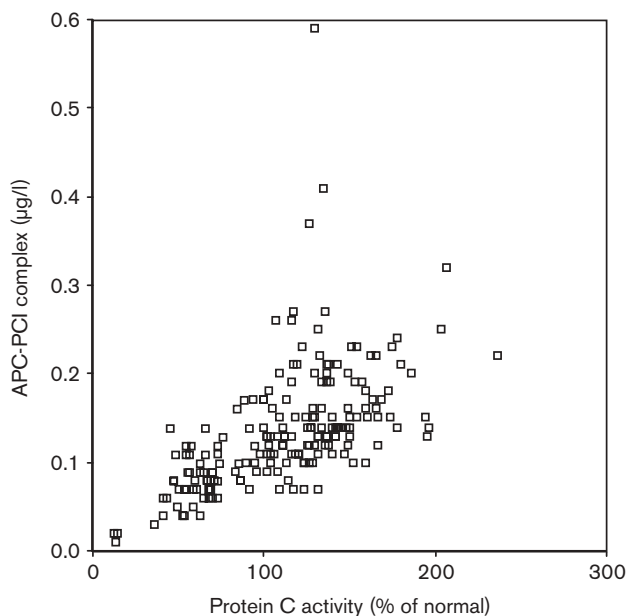
APC–PCI levels and information on carriership of the prothrombin G20210A mutation were available for 55 family members with the protein C 3363C insertion and 140 family members without this mutation (35 were spouses). Of these 195 individuals, 83 were men (43%), 19 (10%) had experienced a venous thrombosis in the past, and the prothrombin mutation was present in 24 of the family members (12%). The mean age at the blood draw was 41 years (range, 10–78 years).

Table 1 shows that family members with the protein C mutation had lower plasma concentrations of APC–PCI complex than individuals without the mutation. Protein C activity levels correlated highly with APC–PCI complex levels ($n = 195$; $r_s = 0.69$; 95% CI, 0.61–0.76). Figure 1 shows the scatterplot for the protein C activity levels and the APC–PCI complex levels with the exclusion of two subjects with APC–PCI complex concentrations > 1 µg/l. The exclusion of these two individuals did not change the correlation coefficient ($n = 193$; $r_s = 0.68$; 95% CI, 0.59–0.75). The correlation

Table 1 Activated protein C and protein C inhibitor (APC-PCI) complex levels ($\mu\text{g/l}$) of the family members

	<i>n</i>	APC-PCI complex	
		Mean (95% confidence interval)	Range
Protein C 3363C insertion	55	0.08 (0.07–0.09)	0.01–0.17
+ PT G20210A	11	0.08 (0.07–0.10)	0.03–0.13
– PT G20210A	44	0.08 (0.07–0.09)	0.01–0.17
No protein C 3363C insertion	140	0.18 (0.15–0.21)	0.07–2.20
+ PT G20210A	13	0.19 (0.14–0.23)	0.10–0.37
– PT G20210A	127	0.18 (0.14–0.24)	0.07–2.20

PT, prothrombin.

Fig. 1

Scatterplot of activated protein C and protein C inhibitor (APC-PCI) complex levels and protein C activity levels for 193 family members with or without the protein C 3363C mutation (excluding two individuals with APC-PCI complex levels above 1 $\mu\text{g/l}$).

was almost similar in family members with the protein C 3363C insertion ($n = 55$; $r_s = 0.50$; 95% CI, 0.26–0.68) and family members without the protein C 3363C insertion ($n = 140$; $r_s = 0.44$; 95% CI, 0.29–0.57).

The levels of APC-PCI complex were not different between carriers and non-carriers of the prothrombin G20210A mutation (Table 1), those with or without a history of venous thrombosis, or between men and women (data not shown).

Discussion

The prothrombin G20210A variant is clearly associated with an increased risk for venous thromboembolic

disease [18,23–25], as are the higher plasma levels of prothrombin associated with the A-allele [8,18,26,27]. Thrombin generation, as reflected by prothrombin fragment 1 + 2 plasma concentration, varies directly with prothrombin concentration [11,12]. This latter observation fits well with the increased risk of thrombosis associated with the mutation and raises questions with respect to the finding that the mutation does not confer risk in the presence of protein C deficiency in this French-Canadian family [11]. However, the findings of the present study do not support our hypothesis of increased protein C activation resulting from higher prothrombin concentrations associated with the prothrombin G20210A polymorphism. It is possible that the plasma concentration of APC-PCI does not reflect APC production, but the positive correlation between plasma protein C levels and APC-PCI does not support this explanation. The only described situation in which APC-PCI does not reflect APC production is when PCI has been depleted, such as in seriously ill septic or DIC patients [28]. Thus, our findings suggest that if the observed higher levels of thrombin generation and activity compensate for the impaired protein C pathway in this thrombophilic family, it must be by an alternative mechanism.

Thrombin plays multiple roles in coagulation, fibrinolysis, platelet activation, cell growth, peripheral blood cell activation, anticoagulation, vascular endothelium and cell migration. Thus, the interaction of the prothrombin G20210A polymorphism with protein C deficiency in this thrombophilic kindred may not directly involve the protein C system. A recently described thrombin-mediated endothelial cell-dependent mechanism for FVa inactivation is a possible alternative mechanism [29]. However, as is the case with this multifunctional protein, thrombin has also been shown to inhibit the inactivation of FVa by activated protein C in purified systems [30]. Thus, we are left with an apparently paradoxical interaction of two well-established risk factors and an opportunity to learn more about the tonic thrombohaemorrhagic balance first postulated by Åstrup in 1958 [31].

Acknowledgements

The authors thank Julia Valliere and Shelly Naud for their contributions to this study.

References

- Griffin JH, Evatt B, Zimmerman TS, Kleiss AJ, Wideman C. Deficiency of protein C in congenital thrombotic disease. *J Clin Invest* 1981; **68**:1370–1373.
- Miletich J, Sherman L, Broze G, Jr. Absence of thrombosis in subjects with heterozygous protein C deficiency. *N Engl J Med* 1987; **317**:991–996.
- Bovill EG, Bauer KA, Dickerman JD, Callas P, West B. The clinical spectrum of heterozygous protein C deficiency in a large New England kindred. *Blood* 1989; **73**:712–717.
- Allaart CF, Poort SR, Rosendaal FR, Reitsma PH, Bertina RM, Briët E. Increased risk of venous thrombosis in carriers of hereditary protein C deficiency defect. *Lancet* 1993; **341**:134–138.
- Bertina RM, Broekmans AW, van der Linden IK, Mertens K. Protein C deficiency in a Dutch family with thrombotic disease. *Thromb Haemost* 1982; **48**:1–5.
- Simioni P, Sanson BJ, Prandoni P, Tormene D, Friederich PW, Girolami B, et al. Incidence of venous thromboembolism in families with inherited thrombophilia. *Thromb Haemost* 1999; **81**:198–202.
- Koeleman BP, Reitsma PH, Allaart CF, Bertina RM. Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. *Blood* 1994; **84**:1031–1035.
- Makris M, Preston FE, Beauchamp NJ, Cooper PC, Daly ME, Hampton KK, et al. Co-inheritance of the 20210A allele of the prothrombin gene increases the risk of thrombosis in subjects with familial thrombophilia. *Thromb Haemost* 1997; **78**:1426–1429.
- Hasstedt SJ, Bovill EG, Callas PW, Long GL. An unknown genetic defect increases venous thrombosis risk, through interaction with protein C deficiency. *Am J Hum Genet* 1998; **63**:569–576.
- Bovill EG, Hasstedt SJ, Callas PW, Valliere JE, Scott BT, Bauer KA, Long GL. The G20210A prothrombin polymorphism is not associated with increased thromboembolic risk in a large protein C deficient kindred. *Thromb Haemost* 2000; **83**:366–370.
- Bovill EG, Bauer KA, Callas PW, Valliere JE, Scott BT, Long GL. Prothrombin concentration has a positive correlation with thrombin generation but, a seemingly paradoxical, negative correlation with protein C activation in a large protein C deficient kindred [Abstract]. *Thromb Haemost* 1999; **82**:216a.
- Bauer KA, Humphries S, Smillie B, Li L, Cooper JA, Barzegar S, et al. Prothrombin activation is increased among asymptomatic carriers of the prothrombin G20210A and factor V Arg506Gln mutations. *Thromb Haemost* 2000; **84**:396–400.
- Suzuki K, Nishioka J, Hashimoto S. Protein C inhibitor. Purification from human plasma and characterization. *J Biol Chem* 1983; **258**:163–168.
- Suzuki K. Activated protein C inhibitor. *Semin Thromb Hemost* 1984; **10**:154–161.
- Strandberg K, Kjellberg M, Knebel R, Lilja H, Stenflo J. A sensitive immunochemical assay for measuring the concentration of the activated protein C-protein C inhibitor complex in plasma: use of a catcher antibody specific for the complexed/cleaved form of the inhibitor. *Thromb Haemost* 2001; **86**:604–610.
- Sala N, Owen WG, Collen D. A functional assay of protein C in human plasma. *Blood* 1984; **63**:671–675.
- Tomczak JA, Ando RA, Sobel HG, Bovill EG, Long GL. Genetic analysis of a large kindred exhibiting type I protein C deficiency and associated thrombosis. *Thromb Res* 1994; **74**:243–254.
- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; **88**:3698–3703.
- Strandberg K, Astermark J, Björgell O, Becker C, Nilsson PE, Stenflo J. Complexes between activated protein C and protein C inhibitor measured with a new method: comparison of performance with other markers of hypercoagulability in the diagnosis of deep vein thrombosis. *Thromb Haemost* 2001; **86**:1400–1408.
- Strandberg K, Svensson A, Stenflo J. Stabilite tubes that contain strongly acidic citrate prevent in vitro complex formation between activated protein C and protein C inhibitor. *Thromb Haemost* 2003; **89**:947–949.
- Zar JH. *Biostatistical analysis*, 4th edition. Upper Saddle River, NJ: Prentice Hall; 1999.
- Vossen CY, Hasstedt SJ, Rosendaal FR, Callas PW, Bauer KA, Broze GJ, et al. Heritability of plasma concentrations of clotting factors and measures of a prethrombotic state in a protein C-deficient family. *J Thromb Haemost* 2004; **2**:242–247.
- Hillarp A, Zoller B, Svensson PJ, Dahlbäck B. The 20210 A allele of the prothrombin gene is a common risk factor among Swedish outpatients with verified deep venous thrombosis. *Thromb Haemost* 1997; **78**:990–992.
- Leroyer C, Mercier B, Oger E, Chenu E, Abgrall JF, Fêrec C, Mottier D. Prevalence of 20210 A allele of the prothrombin gene in venous thromboembolism patients. *Thromb Haemost* 1998; **80**:49–51.
- Margaglione M, Brancaccio V, Giuliani N, D'Andrea G, Cappucci G, Iannaccone L, et al. Increased risk for venous thrombosis in carriers of the prothrombin G → A20210 gene variant. *Ann Intern Med* 1998; **129**:89–93.
- Simioni P, Tormene D, Manfrin D, Gavasso S, Luni S, Stocco D, Girolami A. Prothrombin antigen levels in symptomatic and asymptomatic carriers of the 20210A prothrombin variant. *Br J Haematol* 1998; **103**:1045–1050.
- Kyrle PA, Mannhalter C, Béguin S, Stümpflen A, Hirschl M, Weltermann A, et al. Clinical studies and thrombin generation in patients homozygous or heterozygous for the G20210A mutation in the prothrombin gene. *Arterioscler Thromb Vasc Biol* 1998; **18**:1287–1291.
- Gruber A, Griffin JH. Direct detection of activated protein C in blood from human subjects. *Blood* 1992; **79**:2340–2348.
- Hockin MF, Kalafatis M, Cawthern KM, Simioni P, Mann KG. A thrombin mediated endothelial cell dependent mechanism for factor Va inactivation [Abstract]. *Blood* 1998; **92**:183a.
- Smirnov MD, Safa O, Esmon NL, Esmon CT. Inhibition of activated protein C anticoagulant activity by prothrombin. *Blood* 1999; **94**:3839–3846.
- Åstrup T. The haemostatic balance. *Thromb Diath Haemorrh (Stutt)* 1958; **2**:347–357.