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Polymorphisms in the protein C gene as risk factor for venous thrombosis

Pomp ER, Doggen CJM, Vos HL, Reitsma PH, Rosendaal FR.

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SUMMARY

Background/Objectives: Protein C is an important inhibitor of blood coagulation. We investigated the effect of two polymorphisms within the promoter region of the protein C gene (C/T at -2405 and A/G at -2418) on risk of venous thrombosis and on plasma protein C levels. In addition the combined effect of the two polymorphisms with factor V Leiden and oral contraceptive use was investigated. Previous studies on these polymorphisms were small and were not able to investigate synergistic effects.

Patients/Methods: In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), protein C levels were determined in 2043 patients with venous thrombosis and 2857 controls, and the two polymorphisms in 4285 patients and 4863 controls.

Results: The CC/GG genotype was associated with the lowest protein C levels. Compared to carriers of the TT/AA genotype - a genotype associated with higher protein C levels - the risk of venous thrombosis in CC/GG carriers was 1.3-fold increased (CI95 1.09-1.48). The combination of factor V Leiden with the CC/GG genotype led to a 4.7-fold increased risk, compared to non-carriers with the TT/AA genotype. Oral contraceptive use together with the CC/GG genotype resulted in a 5.2-fold increased risk.

Conclusions: The CC/GG genotype is associated with lower levels of protein C and an elevated risk of venous thrombosis compared to the TT/AA genotype. There is no clear synergistic effect of the CC/GG genotype with factor V Leiden or oral contraceptive use on thrombotic risk.
INTRODUCTION

Activated protein C is a vitamin-K dependent natural anticoagulant. The anticoagulant effect of protein C is a result of the selective inactivation of coagulation factors V and VIII, with protein S serving as a cofactor.\(^1\)

The crucial role of protein C as an anticoagulant has been shown in many studies. Patients born with a homozygous protein C deficiency often have a severe form of thrombosis, called purpura fulminans. In the early 1980s, several studies showed that heterozygous deficiencies, with fifty percent reduced protein C levels resulted in an increased risk of venous thrombosis. Many families have been reported in the literature with recurrent thrombotic events due to this type of protein C deficiency. More recently, the thrombotic risk associated with low protein C levels was confirmed in the Leiden Thrombophilia Study (LETS). In this case-control study, including 474 patients and control subjects, the risk of venous thrombosis appeared to be four times higher in persons with protein C levels below 65% compared to individuals with a protein C level equal to or above 85%.\(^6\)

Age, sex and lifestyle or biochemical factors such as body mass index, LDL-cholesterol, HDL-cholesterol, triglycerides, oral contraceptive use and cigarette smoking may influence protein C levels. Protein C levels can also be influenced by genetic factors. An analysis within the LETS showed a genetic variant in the promoter region of the protein C gene which was associated with low protein C levels and an increased risk of deep venous thrombosis of the leg. Individuals with the homozygous CGT genotype of the polymorphisms at -2405, -2418 and -2583 (in LETS defined as polymorphisms at -1654, -1641 and -1476) were found to have a 1.5-2 fold greater risk of venous thrombosis than individuals with the homozygous TAA genotype. Two out of three polymorphisms investigated in the LETS (2405C/T and 2418A/G) were considered as functionally different and were evaluated again in a study including 242 patients with deep venous thrombosis and 394 healthy individuals. This study confirmed the link between these protein C gene polymorphisms and circulating protein C levels. Both studies did not evaluate the relationship between protein C levels and the risk of venous thrombosis.

Due to the relatively small number of participants in these previous studies, the risk estimates from these studies were imprecise and the joint effect of the protein C polymorphisms with other risk factors for venous thrombosis could not be investigated. The factor V Leiden mutation, which is the most common known cause of inherited thrombophilia, causes activated protein C resistance. Oral contraceptive use, another important risk factor for venous thrombosis, also contributes to activated protein C resistance. Since a previous study showed that deficiency of protein C and the factor V Leiden mutation had synergistic effects, as does the
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combination of factor V Leiden and oral contraceptive use, it is of interest to investigate the risk of venous thrombosis in individuals with factor V Leiden or oral contraceptive use and the genotype associated with low protein C levels.

In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), a large population-based case-control study, we investigated the two polymorphisms within the protein C gene (2405C/T and 2418A/G) as risk factors for venous thrombosis. We also investigated the influence of genotypic variation on plasma protein C levels and the effect of protein C levels on the risk of venous thrombosis. In addition, the joint effect of the low protein C genotype with the factor V Leiden mutation and oral contraceptive use was investigated.

METHODS

Study Design

The MEGA study included consecutive patients with a first diagnosis of venous thrombosis. Patients were selected from the files of the anticoagulation clinics in Amsterdam, Amersfoort, The Hague, Leiden, Rotterdam and Utrecht between March 1999 and September 2004. In the Netherlands, anticoagulation clinics monitor anticoagulation treatment in all patients in a geographically well-defined area. Patients between the age of 18 and 70 with a deep venous thrombosis of the leg, a pulmonary embolism or a combination of these diagnoses were included in our study. For practical reasons, patients with severe psychiatric problems or those unable to speak Dutch were considered as ineligible.

Partners of patients were asked to participate as control subjects. From January 2002 until September 2004, additional control subjects were recruited by using the random digit dialing (RDD) method. The random control subjects were frequency matched on age and sex to the patients that provided a blood sample. Only control subjects without a history of venous thrombosis were included and the same exclusion criteria were applied as for the patients.

All participants were asked to fill in a questionnaire about possible risk factors for venous thrombosis. Most questions referred to a period of 12 months prior to the index date, i.e. the date of diagnosis of the thrombosis of the patient for patients and partners and the date of filling in the questionnaire for the random control subjects. Of the variables known to influence protein C levels we collected information on age, sex, body mass index (weight/height², kg/m²), smoking and oral contraceptive use.
Blood collection

At least three months after withdrawal of anticoagulation the patients and their partners were asked to visit the anticoagulation clinic after an overnight fast, and a blood sample was drawn. Only in case of continuous use of anticoagulants for more than one year a blood sample was taken during anticoagulation therapy. From December 1999 onwards, we obtained self-administered buccal swabs by mail when participants were unable or unwilling to come for a blood draw. From June 2002 onwards, blood draws were no longer performed in patients and their partners, and the study was restricted to DNA collection by buccal swabs sent by mail. The random controls were invited for a blood draw within a few weeks after the questionnaire was sent. Within this group buccal swabs were sent when someone refused the blood draw.

Of the 6237 eligible patients, 276 died soon after the venous thrombosis. Of the remaining 5961 patients 4957 participated (83%). A blood sample was provided by 2349 patients, a buccal swab was obtained from an additional 1940 patients. Genotyping was successful in 4285 patient samples for the 2405C/T polymorphism (rs1799808) and in all 4289 samples for the 2418A/G polymorphism (rs1799809). Numbering of the polymorphisms was according to GB:AF378903. Protein C levels were successfully measured in 2347 out of 2349 blood samples.

Of the 4957 participating patients, 3581 had an eligible partner. One partner died soon after the request for participation. Of the remaining 3580 partners, 2917 participated (81%). An additional 173 control subjects were included of whom the partner was excluded for the final patient analysis, 121 partners were included of whom the patient had a deep venous thrombosis of the arm and 20 partners of non-participating patients were included, resulting in a total of 3231 partners. Within the partner group, 1465 blood samples and 1377 buccal swabs were obtained. Genotyping was successful in 2840 partners for the 2405C/T polymorphism and in all 2842 samples for the 2418A/G polymorphism. Protein C levels were successfully measured in 1463 out of 1465 blood samples.

Of the 4350 eligible RDD control subject, four died before they were able to participate. Of the remaining 4346 persons, 3000 participated (69%). A blood sample was provided by 1437 RDD control subjects, a buccal swab by 586 RDD controls. The 2405C/T and 2418A/G polymorphisms were successfully determined in all 2023 DNA samples and protein C levels in 1436 out of 1437 blood samples.

The 2405C/T and 2418A/G polymorphisms were determined by 5’nuclease (Taqman; Applied Biosystems, Foster City, CA) assays using a standard PCR reaction mix (Eurogentec, Seraing, Belgium) and allele-specific fluorescent probes equipped with a minor groove binding moiety (Applied Biosystem). A detailed description
of blood collection and DNA analysis for the factor V Leiden (G1691A) mutation in the MEGA study has been published previously\textsuperscript{17}. For practical reasons, we only included 4285 patients and 4863 control subjects in the analyses with complete data for both polymorphisms. Measurement of protein C level was done with a chromogenic assay on a STA-R coagulation analyzer following the instructions of the manufacturer (Diagnostica Stago, Asnières, France). The mean intra- and inter-assay coefficients of variation were 1.4 % and 3.5 %. In the analyses with protein C levels, individuals using oral anticoagulation or with protein C deficiency (protein C levels below 66%, according to the clinical cut-off value) were excluded, resulting in 2043 patients and 2857 control subjects. In this excluded group, frequencies of the genotypes were comparable to those in the general population.

All participants gave written informed consent. The study was approved by the Medical Ethics Committee, Leiden University Medical Center, The Netherlands.

Statistical analysis

We pooled the control groups and calculated unmatched odds ratios (ORs). ORs and 95% confidence intervals (CI\textsubscript{95}) were calculated according to the method of Woolf\textsuperscript{18}. With a multiple logistic regression model ORs were adjusted for age (continuous) and sex (categorical). SPSS for Windows version 14.0.1 (SPSS Inc, Chicago, Ill) was used for all statistical analyses.

RESULTS

In the present analyses 4285 patients and 4863 control subjects were included. Mean age of patients was 48.5 years (5\textsuperscript{th}-95\textsuperscript{th} percentiles, 26.1-67.7) and control subjects were on average 47.9 years old (5\textsuperscript{th}-95\textsuperscript{th} percentiles, 26.8-66.7). In the patients 58\% (n=2491) were diagnosed with a deep venous thrombosis of the leg, 33\% (n=1398) with a pulmonary embolism and 9\% (n=396) with both. Fifty-four percent of patients (n=2324) and 53\% of control subjects (n=2590) were women. Within the patient group with measured protein C levels, 281 out of 2347 patients (12\%) were using oral anticoagulation compared to 28 out of 2899 (1\%) in the control group at the time of blood draw. An additional 23 patients and 14 control subjects were potentially protein C deficient (protein C levels below 66\%). When participants who used oral anticoagulation therapy or who were potentially protein C deficient were excluded, protein C levels were 118.2\% (5\textsuperscript{th}-95\textsuperscript{th} percentiles, 87.0-160.0\%) in patients and 117.9\% (5\textsuperscript{th}-95\textsuperscript{th} percentiles, 88.0-155.0\%) in control subjects.
The genotype distributions of the 2405C/T and 2418A/G polymorphisms in the overall group are presented in table 1. The distribution of both polymorphisms did not differ from Hardy-Weinberg equilibrium in control subjects. The T allele was present in 34% of patients and 35% of control subjects. Compared to the CC genotype, the TT genotype had a reduced risk of venous thrombosis (OR 0.85, CI95 0.74-0.97). This protective effect disappeared after adjustment for the 2418A/G polymorphism (OR 1.03, CI95 0.87-1.23). For the 2418A/G polymorphism, the G allele was slightly more frequent in patients (47%) than in control subjects (43%). The GG genotype had a small increase in risk of venous thrombosis compared...
to the AA genotype (OR 1.29, CI95 1.14-1.45). After adjustment for the 2405C/T polymorphism the risk remained 1.3-fold increased (OR 1.31, CI95 1.13-1.53).

In table 2 protein C levels for the various combinations of the two polymorphisms are presented. Of the nine possible genotypes, six were frequently observed, two were rare and one was not observed. Within the six frequent genotypes, the CC/GG genotype was associated with low mean protein C level in control subjects (112%), the CC/AG and CT/AG genotypes had intermediate protein C levels (respectively, 118 and 117%) and the CC/AA, CT/AA, TT/AA genotypes were associated with high protein C levels (121 to 123%). A similar pattern was found in patients.

Table 3 presents the association of the six frequent genotypes with the risk of venous thrombosis. We chose one of the homozygous genotypes with a high protein C level (TT/AA) as the reference group. Compared to the TT/AA genotype the CC/GG genotype was associated with the highest increase in risk (OR 1.27, CI95 1.09-1.48). Factors that are known to influence protein C levels did not account for this increased risk; after additional adjustment for body mass index, smoking and oral contraceptive use the risk estimate remained unchanged (OR 1.33, CI95 1.13-1.56).

To verify whether the effect of the CC/GG genotype on the risk of venous thrombosis was in fact mediated via protein C levels, we also investigated whether a decrease in protein C levels was associated with an increased risk of venous thrombosis. Variations of the PC levels within the normal range were however not clearly associated with the risk of venous thrombosis. With protein C as a continuous variable in the logistic model no increased risk was observed (OR 0.99, CI95 0.99-0.99). With protein C as a categorical variable only protein C levels below 81% (compared to protein C levels between 111 and 120%) appeared to be associated

| Table 3. Relative risk of venous thrombosis according to genotype |
|-----------------------------------|----------------|----------------|----------------|
| Genotype        | Patients | Control subjects | OR (CI95)*   |
| TT/AA         | 482      | 612               | 1 (ref.)*    |
| CT/AA         | 561      | 764               | 0.93 (0.79-1.10) |
| CC/AA         | 187      | 199               | 1.19 (0.95-1.51) |
| CT/AG         | 1342     | 1438              | 1.19 (1.03-1.36) |
| CC/AG         | 777      | 914               | 1.08 (0.93-1.26) |
| CC/GG         | 923      | 924               | 1.27 (1.09-1.48) |

OR, odds ratio; CI95, 95% confidence interval; ref., reference category
*adjusted for age and sex
† The TT/AA genotype was chosen as reference category, because it was associated with high protein C levels, was highly prevalent and was reported as reference category in previous studies thereby facilitating comparison between study results.
with a moderately, but not significant increased risk of venous thrombosis (OR <75% 1.52, CI95 0.75-3.07; OR 76-80% 1.24, CI95 0.71-2.17).

In table 4 the joint effect of factor V Leiden and the CC/GG protein C genotype is presented. The TT/AA genotype combined with factor V Leiden resulted in a 4.0-fold increased risk (OR 3.96 CI95 2.54-6.18) compared to TT/AA carriers without the mutation. Relative to TT/AA carriers without the factor V Leiden mutation, the joint effect of factor V Leiden and the GG/CC genotype led to a 4.7-fold increased risk (OR 4.65, CI95 3.24-6.68).

The joint effect of the CC/GG genotype together with oral contraceptive use in women younger than 50 is also presented in table 4. The TT/AA genotype combined with oral contraceptive use resulted in a 4.3-fold increased risk of venous thrombosis, compared to non-users with the TT/AA genotype (OR 4.33, CI95 2.67-7.01). The CC/GG genotype together with oral contraceptive use was associated with a 5.2-fold increased risk (OR 5.16, CI95 3.32-8.00) compared to TT/AA carriers without oral contraceptive use.

In addition to these analyses, we also investigated the combined effect of the CC/GG genotype with body mass index, since obesity is also related to activated protein C resistance. We found no synergistic effect for the combination of the CC/GG genotype and obesity (data not shown).
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DISCUSSION

In a large population-based case-control study, we investigated two polymorphisms, 2405C/T and 2418A/G, within the protein C gene as risk factors for venous thrombosis. The T allele at position 2405 was associated with a protective effect and the G allele at position 2418 was associated with a small increased risk of venous thrombosis compared to the C and A alleles at these positions. Combining these alleles into different genotypes revealed that the CC/GG genotype was associated with low protein C levels compared to the other genotypes. The CC/GG genotype was associated with a 1.3-fold increased risk of venous thrombosis compared to the TT/AA genotype, which was a genotype with relatively high protein C levels. There were only minor synergistic effects of the CC/GG genotype with the factor V Leiden mutation or oral contraceptive use.

An increased risk for the C and G alleles compared to the T and A alleles is in accordance with the findings of previous studies. The finding of low protein C levels in carriers of the homozygous CC/GG genotype is also in agreement with these studies. The G allele at 2418 in the genotype seemed to be the most important determinant of protein C levels. Carriers of genotypes without a G allele presented relatively high protein C levels, carriers of genotypes with one G allele had intermediate levels and carriers of the genotype with two G alleles had the lowest protein C level. Homozygosity or heterozygosity for the 2405C/T polymorphism was less important in the determination of protein C levels, indicating that the effect on protein C levels was mainly mediated by the 2418A/G polymorphism. To verify this, we calculated the relative risk of the 2405C/T polymorphism adjusted for the 2418A/G polymorphism. As was expected the effect of the 2405C/T polymorphism disappeared. In contrast, the relative risks of the 2418A/G polymorphism remained elevated after adjustment for the 2405C/T polymorphism. In this study 19.1% of the control subjects, who represent the general population, had the GG genotype. This suggests that 5% of all venous thrombotic disease is associated with this genotype (population attributable risk: 5.1%).

In accordance with the finding of the lowest protein C levels in the CC/GG genotype we found the highest risk of venous thrombosis for carriers of this genotype. To verify whether the effect of the CC/GG genotype on the risk of venous thrombosis was truly mediated via protein C levels, low protein C levels themselves had to be associated with an increased risk of venous thrombosis. We found a moderately increase in risk, only for protein C levels below 81%. It seems that the risk of venous thrombosis is only influenced by protein C levels in the very low range. This is supported by the findings of a case-control study in women aged 45 to 64 years that reported a 2.9-fold increased risk for levels below 81 iu/dl. In addition, we found
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a 2.9-fold increased risk of venous thrombosis (CI95 1.25-6.73) in individuals with protein C levels below 66%. Previous studies on the 2405 C/T and 2418A/G polymorphisms\textsuperscript{10,11} did not evaluate the relationship between protein C levels and the risk of venous thrombosis. In our analysis, the exclusion of individuals with oral anticoagulation therapy could have resulted in a small underestimation of risk estimates in the lower range. It is very likely that protein C levels of the excluded group were not evenly distributed throughout the categories; individuals with relatively low protein C levels were probably overrepresented in the excluded group.

We also assessed the combined effect of the CC/GG genotype with the factor V Leiden mutation or oral contraceptive use, which both lead to resistance to activated protein C\textsuperscript{12,13}. Previously, a family study showed that a higher percentage of family members with both protein C deficiency and the factor V Leiden mutation had developed thrombosis (73%), compared with family members with either protein C deficiency (36%) or the factor V Leiden mutation (10%) ($P < 0.001$ for both groups). Of the subjects lacking both the mutations, only 7% had experienced a thrombotic episode\textsuperscript{14}. In our study however, factor V Leiden together with the CC/GG genotype resulted in a 4.7-fold increased risk, which was only slightly higher than the sum of the separate effect of the CC/GG genotype and factor V Leiden. Also for the combination with oral contraceptive use or obesity no substantial synergistic effects were found.

In conclusion, the MEGA study confirmed the link between the CC/GG genotype, low protein C levels and an elevated risk of venous thrombosis. The increase in risk for the CC/GG genotype was mainly mediated by the presence of the G allele for the A/G 2418 polymorphism. CC/GG carriers, who were also factor V Leiden carriers or oral contraceptive users, did not have a substantial higher risk than expected based on the effect of each factor separately.

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