Resistance to Activated Protein C as an Additional Genetic Risk Factor in Hereditary Deficiency of Protein S

By Bengt Zöller, Ann Berntsdotter, Pablo Garcia de Frutos, and Björn Dahlbäck

Inherited resistance to activated protein C (APC), which is caused by a single point mutation in the gene for factor V, is a common risk factor for thrombosis. In this study, the prevalence of APC resistance in 18 unrelated thrombosis-prone families with inherited protein S deficiency was investigated to determine its role as additional genetic risk factor for thrombosis. In addition, a detailed evaluation of the clinical manifestations in these families was performed. Venous thrombotic events occurred in 47% of the protein S-deficient patients (64/138) and in 7% of relatives without protein S deficiency (14/191). As estimated from Kaplan-Meier analysis, 50% of protein S-deficient family members and 12% of those without protein S deficiency had had manifestation of venous thromboembolism at the age of 45 years. The age at the first thrombotic event ranged from 10 to 81 years (mean, 32.5 years) and a large intrafamilial and interfamilial variability in expression of thrombotic symptoms was seen. The factor V gene mutation related to APC resistance was present in 6 (38%) of 16 probands available for testing; in total, the mutation was found in 7 (39%) of the 18 families. In family members with combined defects, 72% (9/13) had had thrombosis as compared with 19% (4/21) of those with only protein S deficiency and 19% (4/21) of those with only the factor V mutation. In conclusion, APC resistance was found to be highly prevalent in thrombosis-prone families with protein S deficiency and was an additional genetic risk factor for thrombosis in these families. The results suggest thrombosis-prone families with protein S deficiency often to be affected by yet another genetic defect.

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PATIENTS AND METHODS

Protein S-deficient families and controls. The study included 327 members from 18 unrelated families with hereditary protein S deficiency. Protein S deficiency was found in 136 (117 nonanticoagulated and 19 anticoagulated) relatives. A detailed description of patients and the normal and anticoagulated control populations are given in a separate report. Plasma levels of antithrombin III, plasminogen, and protein C were normal in all family members (data not shown).

Laboratory methods. Blood sampling and routine coagulation methods, including measurements of free and total protein S, were performed as described. Protein C, antithrombin III, and plasminogen were determined with Coatest protein C, Coatest antithrombin, and Coatest plasminogen (Chromogenix, Möln达尔, Sweden), respectively. The APC-resistance test is a modified activated partial thromboplastin time (APTT) reaction, in which the anticoagulant response to APC is measured. Results were expressed as APC ratios (clotting time obtained using the APC/CaCl2 solution divided by clotting time obtained with CaCl2). Family members with confirmed APC ratios ≥2.0 were considered to be APC resistant. Preparation of genomic DNA from EDTA blood and determination of the FV gene point mutation (G to A at position 1691), which causes APC resistance, was performed as described.

Statistical methods. A probability less than .05 was considered significant. Laboratory data were expressed as mean ± standard deviation (SD). N is the number of samples in each group. The Pearson's correlation coefficient was calculated (r). The unpaired Student's t-test was used for comparing mean values. Contingency table analyses.
Table 1. Clinical Manifestations in Symptomatic Relatives

<table>
<thead>
<tr>
<th></th>
<th>Normal Protein S</th>
<th>Protein S Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients</td>
<td>14 (100)</td>
<td>64 (100)</td>
</tr>
<tr>
<td>Total no. of patients with a certain thrombotic symptom*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVT</td>
<td>7 (50)</td>
<td>521 (81)</td>
</tr>
<tr>
<td>PE</td>
<td>5 (36)</td>
<td>17 (27)</td>
</tr>
<tr>
<td>STP</td>
<td>9 (64)</td>
<td>26 (41)</td>
</tr>
<tr>
<td>Recurrences</td>
<td>7 (50)</td>
<td>41 (64)</td>
</tr>
<tr>
<td>First symptom in affected family members</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVT</td>
<td>3 (21)</td>
<td>35 (55)</td>
</tr>
<tr>
<td>PE</td>
<td>1 (7)</td>
<td>4 (6)</td>
</tr>
<tr>
<td>STP</td>
<td>7 (50)</td>
<td>16 (25)</td>
</tr>
<tr>
<td>Combined DVT + PE</td>
<td>2 (14)</td>
<td>7 (11)</td>
</tr>
<tr>
<td>Combined DVT + STP</td>
<td>1 (7)</td>
<td>2 (3)</td>
</tr>
</tbody>
</table>

Values are the number of patients with percentages in parentheses. Abbreviations: DVT, deep venous thrombosis; PE, pulmonary embolism; STP, superficial thrombophlebitis.

* Because one patient may have suffered from different thrombotic events, the numbers do not add up to 100.
† Two protein S-deficient patients had had superior sagittal sinus thrombosis, two had mesenteric vein thrombosis, two had axillary vein thrombosis, and one had subclavian vein thrombosis.

were performed with a standard χ² test. Fisher's exact test was used to compare two proportions for small numbers. Thrombosis-free survival curves were constructed according to Kaplan and Meier. For comparison of two curves, the log-rank test resulting in a statistic test with a χ² distribution and one degree of freedom was used. As an approximation of the relative risk, crude odds ratios (univariate) were calculated for several putative risk factors by simple crosstabulation. These odds ratios reflect the risk when the proposed risk factor is present relative to the risk when it is absent, unadjusted for other factors. A Cox proportional hazards model was used in a multivariate survival analysis, allowing several factors to be adjusted for simultaneously. The hazard ratio is the incidence rate ratio, which is assumed to be constant over time, whereas the baseline hazard is allowed to vary. The hazard ratio may be interpreted as the relative risk associated with each factor, adjusted for all other factors in the model. The incidence of first thrombotic events in protein S-deficient and normal relatives was calculated by counting patient-years of observation and dividing the number of events in each group by the sum of observation-years of all the individuals in the group.

RESULTS

Clinical data. Sixty-four (47%) of the 136 protein S-deficient family members had experienced one or more venous thrombotic events as compared with 14 (7%) of the 191 relatives without protein S deficiency (Table 1). Thrombotic episodes were associated with one or more circumstantial risk factors in 31 (48%) of the protein S-deficient patients and in 10 (71%) of the relatives without protein S deficiency (Table 2). The mean age at the first thrombotic event was 32.5 years (range, 10 to 81 years) in protein S-deficient cases and 30.5 years (range, 16 to 50 years) in those without protein S deficiency. According to a Kaplan-Meier analysis, the probability of a protein S-deficient family member to be free of venous thrombosis at 45 years of age was 0.50 (95% confidence interval [CI], 0.31 to 0.69), whereas the corresponding value for family members without protein S deficiency was 0.88 (95% CI, 0.98 to 0.99; Fig 1A). No differences in thrombosis-free-survival were observed between type I and type III protein S-deficient individuals or between men and women (results not shown). The incidence of thrombotic events (only first episodes included in the calculation) in protein S-deficient patients was 10.0 per 1,000 person-years up to 25 years of age (v 1.0 per 1,000 in normal

![Graph A](image)

![Graph B](image)

Fig 1. Kaplan-Meier analysis of all 18 protein S-deficient families showing the probability of being free of thrombosis at a certain age. (A) 136 protein S-deficient and 191 normal members. The difference between the curves is highly significant (P < .001). (B) Same analysis after exclusion of the 19 probands. The difference between the survival curves is still highly significant (P < .001).
pregnancies in the protein S-deficient individuals, thrombo-
protein S-deficient women were pregnant (130 per 1,000
ratio. However, in the proportional hazard model, only being

evidence of thrombosis was quite high in the years in which

included in the analysis (hazard ratio, 1.5; 95% CI, 0.6 to

event, compared with 3 (1.6%; age 44 to 64 years) among

overweight was found to be a significant risk factor

the 191 family members without protein

deficiency. One of the 9 protein S-deficient members with

relatives), 18.2 per 1,000 person-years (v 5.3 per 1,000 in

normal family members) between 26 and 45 years of age,

and 39.2 per 1,000 person-years (v 0.6 per 1,000 in normal

family members) between 46 and 83 years of age.

Protein S deficiency was a strong risk factor for thrombo-
sis in these families (P < .001; Table 3). Malignancy, being

overweight, and smoking appeared to be associated with

increased risk of thrombosis as judged by the crude odds

incidence of thrombosis than family members with either of

subjects having both defects (n = 13; range, 10 to 55 years),

47 years for subjects with only protein S deficiency (n = 4;

range, 30 to 65 years), and 27 years for those with only

the FV gene mutation (n = 4; range, 21 to 31 years). The

mean age at the first thrombotic episode was 31 years for

subjects having both defects (n = 13; range, 10 to 55 years).

Moreover, thrombotic events occurred in 19 (13%) of 146 pregnancies in protein S-deficient women and in 6 of 163 (4%) pregnancies in women without protein S deficiency (P < .01). In 8 of the 19 events associated with pregnancies in the protein S-deficient individuals, thrombo-
sis occurred in the postpartum period as compared with 4 of 6 events in women without protein S deficiency.

Nine (6.6%) of the 136 protein S-deficient family mem-
bers (age 41 to 74 years) had had an arterial thrombotic event, compared with 3 (1.6%; age 44 to 64 years) among

the 191 family members without protein S deficiency (P < .05). When only patients older than 50 years were consid-
ered, 9 (21%) of 42 protein S-deficient relatives had had

arterial thrombosis, compared with 3 (6.7%) of 45 individu-

als without protein S deficiency (P < .05). Eight of the 9

protein S-deficient patients with arterial thrombosis were

smokers. In this context, it is interesting that smoking in

itself lowers the plasma concentration of protein S, which

may be particularly harmful in individuals with protein S
deficiency.25 One of the 9 protein S-deficient members with

arterial thrombosis was found to be heterozygous for the FV
gene mutation.

Coumarine-induced skin necrosis had not occurred in any

of the 55 protein S-deficient patients who had been treated with oral anticoagulant for longer or shorter periods. More-

over, no thrombotic events had occurred during adequate

oral anticoagulation (warfarin) of protein S-deficient family

members, even in the 11 individuals with combined APC

resistance and protein S deficiency (see below). However, 4

patients had recurrences of venous thrombosis during tempo-

rary discontinuation of treatment.

**APC resistance, an additional risk factor of thrombosis.**

DNA samples were available from 294 family members. The Arg506 to Gln mutation in the FV gene was tested for in

these 294 individuals and found to be present in 40, which

included 6 (38%) of the 16 investigated probands. In family

members without the FV gene mutation, APC ratios were

slightly, but significantly, lower in the protein S-deficient

group than in the group without protein S deficiency (Fig

2). Among those with the FV gene mutation, there was no

difference in APC ratios between those with and without

protein S deficiency. When all 18 families (294 members)

were included in analysis of phenotypic expression, a sig-

nificant difference in thrombotic frequency (P = .041) but

not in thrombosis-free survival (P = .900) was found be-

tween family members with only protein S deficiency and

those with combined defects (Table 4A). To evaluate the

influence of the FV gene mutation more stringently, only

the 7 families having both defects (families 2, 6, 8, 11, 12,

and 18) were included in the analysis. Individuals with

combined defects were found to have significantly higher

incidence of thrombosis than family members with either of

the two defects (P = .001), the differences being signifi-

cantly greater even after exclusion of probands from the

analysis (P = .034 and P = .028, respectively; Table 4B). In

these families, the mean age at the first thrombotic episode was 31 years for

subjects having both defects (n = 13; range, 10 to 55 years),

47 years for subjects with only protein S deficiency (n = 4;

range, 30 to 65 years), and 27 years for those with only

the FV gene mutation (n = 4; range, 21 to 31 years). The

thrombosis-free survival curve of individuals with both de-

fects was significantly different from those of individuals

with either protein S deficiency or the FV gene mutation

(Fig 3). There was no significant difference between those

with only the FV gene mutation and those with isolated

protein S deficiency. Noteworthy, three family members

were homozygous for the FV gene mutation, with normal

protein S being asymptomatic (ages 14 and 8 years, respec-

atively) and I with protein S deficiency having had thrombosis

at 10 years of age.

**DISCUSSION**

Deficiency of protein S is a strong risk factor of thrombo-
sis and 64 of the 136 protein S-deficient relatives had experi-
enced one or more venous thrombotic events. The incidence

of first thrombotic event increased with age, showing protein

S deficiency to be a life-long risk factor of thrombosis. The

clinical manifestations of protein S deficiency in this study

were similar to those described in an earlier report, in which

Table 3. Effects of Potential Risk Factors on the Occurrence of Venous Thrombosis in 327 Family Members

<table>
<thead>
<tr>
<th>Potential Risk Factor</th>
<th>Venous Thrombosis (Yes/No)</th>
<th>Crude OR</th>
<th>Hazard Ratio* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein S deficient</td>
<td>64/72</td>
<td>14/177</td>
<td>11.2</td>
</tr>
<tr>
<td>Female sex</td>
<td>41/136</td>
<td>37/113</td>
<td>0.9</td>
</tr>
<tr>
<td>Pregnancy and childbirth†</td>
<td>34/83</td>
<td>44/166</td>
<td>1.5</td>
</tr>
<tr>
<td>Smoking†</td>
<td>41/87</td>
<td>37/162</td>
<td>2.1</td>
</tr>
<tr>
<td>Overweight‡</td>
<td>38/63</td>
<td>40/186</td>
<td>2.8</td>
</tr>
<tr>
<td>Malignancy‡</td>
<td>3/3</td>
<td>75/246</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Proportional hazards model; all listed proposed risk factors entered.
† Indicates P < .05. Even when probands were excluded, only protein S deficiency (6.0; 95% CI, 3.3 to 10.9) and being overweight (2.0; 95% CI, 1.2 to 3.4) were significant risk factors for thrombosis.
‡ Ever had risk factor compared with never.
§ Overweight defined as body mass index >25. Body mass index = weight (kg) divided by the square of the body length (meter).
APC-RESISTANCE AND PROTEIN S DEFICIENCY

Fig 2. APC resistance in protein S-deficient families. APC ratios in family members not having protein S deficiency or the FV gene mutation (n = 140), in protein S-deficient individuals without the FV gene mutation (n = 76), in members with normal protein S having the FV gene mutation (n = 22), in protein S-deficient members also having the FV mutation (n = 15), and in healthy controls (n = 126, 2.8 ± 0.6). Thrombotic family members are indicated with solid circles. In the absence of the FV gene mutation, APC ratios were slightly lower in those having protein S deficiency than in those without protein S deficiency (3.1 ± 0.8 v 2.8 ± 0.3, P = .013). There was no significant difference in APC ratios between protein S-deficient and nondeficient carriers of the FV gene mutation (1.6 ± 0.4 v 1.6 ± 0.4).

only total protein S was measured. However, in the now described families, the median age for the first thrombosis was 45 years (50 years if probands were excluded), as compared with less than 30 years in the cited study. This finding is not explained by the different detection methods for protein S deficiency (measurements of free v total protein S) because we found no significant difference in thrombosis-free survival between patients with so-called type I and those with type III deficiency. The age at the first thrombotic event ranged between 10 and 81 years and one patient was still asymptomatic at 76 years of age. This is a wider range than found in a previous study (range, 15 to 68 years).

It is becoming evident that thrombosis-prone families with protein C deficiency often have more than one genetic defect (see below). In the general population, approximately 1 in 250 carry protein C deficiency and the risk of thrombosis in individuals with isolated protein C deficiency is not yet determined. In a recent report on thrombosis-prone families with protein C deficiency, the probability of being free of thrombosis was found to be 50% at 45 years of age and the results of this report suggest the risk of thrombosis in thrombosis-prone families with protein S deficiency to be similar. The higher thrombosis risk in previous studies with protein S deficiency may be caused by the selection of severely affected families. Epidemiologic studies are needed to elucidate the prevalence of protein S deficiency in the general population and to determine the associated risk of thrombosis.

The expression of clinical manifestations in protein S-deficient members showed a marked interfamilial and intrafamilial heterogeneity and the thrombotic tendency in relatives without protein S deficiency was higher than expected. This finding suggests additional genetic risk factors to be present. One such genetic factor is the FV gene mutation causing APC resistance, which was found in 7 of the 18 protein S-deficient families. In these families, individuals with combined protein S deficiency and APC resistance had

Table 4. Genetic Status in Relationship to Presence or Absence of Thrombosis

<table>
<thead>
<tr>
<th>Genetic Defect</th>
<th>Symptoms of Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
</tr>
<tr>
<td>(A) All 18 protein S-deficient families</td>
<td></td>
</tr>
<tr>
<td>Protein S and FV</td>
<td>13 (72)</td>
</tr>
<tr>
<td>Protein S</td>
<td>47 (46)</td>
</tr>
<tr>
<td>FV</td>
<td>4 (18)</td>
</tr>
<tr>
<td>None</td>
<td>9 (6)</td>
</tr>
<tr>
<td>(B) 7 Families in which both protein S deficiency and the FV gene mutation were present</td>
<td></td>
</tr>
<tr>
<td>Protein S and FV</td>
<td>13 (72)</td>
</tr>
<tr>
<td>Protein S</td>
<td>4 (19)</td>
</tr>
<tr>
<td>FV</td>
<td>4 (19)</td>
</tr>
<tr>
<td>None</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

The presence of protein S deficiency is indicated as protein S. The presence of the FV gene mutation is indicated as FV. Values are the number of patients with percentages in parentheses.
a higher risk for thrombosis than those carrying either of the two defects. APC resistance was recently found to be an additional risk factor for thrombosis in Dutch families with protein C deficiency, whereas no cases of APC resistance were found in Italian families with protein S, protein C, or antithrombin III deficiency. This difference may be caused by higher allele frequencies of the FV gene mutation or protein S deficiency and those with combined defects was significant (P = .008 and P = .002). There was no significant difference between those with only the FV gene mutation and those with isolated protein S deficiency (P = .471). The same analysis after exclusion of the 7 index cases. The difference between those with isolated FV gene mutation and those with combined defects was not significant (P = .055), whereas the difference between those with protein S deficiency and those with combined defects was significant (P = .032). There was no significant difference in thrombosis-free survival between those with isolated FV gene mutation and those with isolated protein S deficiency (P = .48).

**Fig 3.** The probability of being free of thrombotic events at a certain age in the 7 families having both the FV gene mutation and protein S deficiency. (A) Thrombosis-free survival curves in 21 families with the FV gene mutation, 21 with protein S deficiency, and 18 with both defects. The difference between those having only the FV gene mutation or protein S deficiency and those with combined defects was significant (P = .008 and P = .002). There was no significant difference between those with only the FV gene mutation and those with isolated protein S deficiency (P = .471). (B) The same analysis after exclusion of the 7 index cases. The difference between those with isolated FV gene mutation and those with combined defects was not significant (P = .055), whereas the difference between those with protein S deficiency and those with combined defects was significant (P = .032). There was no significant difference in thrombosis-free survival between those with isolated FV gene mutation and those with isolated protein S deficiency (P = .48).

In conclusion, although hereditary protein S deficiency is associated with a life-long increased risk of venous thrombosis, expression of clinical manifestations varies between different families depending on whether additional genetic and circumstantial risk factors are present or not. APC resistance, which is highly prevalent in the general population, was found to be an additional risk factor for thrombosis in several thrombosis-prone protein S-deficient families.

**ACKNOWLEDGMENT**

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**REFERENCES**

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Resistance to activated protein C as an additional genetic risk factor in hereditary deficiency of protein S

B Zoller, A Berntsdotter, P Garcia de Frutos and B Dahlback