RAPID COMMUNICATION

Activated Protein C Resistance as an Additional Risk Factor for Thrombosis in Protein C-Deficient Families

By Bobby P.C. Koeleman, Pieter H. Reitsma, C.F. Allaart, and Rogier M. Bertina

Heterozygous protein C deficiency is associated with an increased risk for thrombosis. This association is restricted to a minority of protein C-deficient families, which have been defined as clinically dominant protein C-deficient. In contrast, in the clinically recessive protein C-deficient families, only the homozygous family members are (severely) affected. One possible explanation for this difference in thrombotic risk between families may be the presence of a second hereditary risk factor. A good candidate for this second risk factor is the recently identified resistance to activated protein C (APC). APC resistance, which is associated with a mutation in the FV gene (FV Leiden), is a common and strong risk factor for thrombosis. We show here that the prevalence of the FV Leiden mutation is high among symptomatic protein C-deficient probands (19%). In 6 clinically dominant prob-

The PROTEIN C anticoagulant pathway is an important downregulating mechanism of the blood coagulation cascade. Protein C is the zymogen of a serine protease, and is activated by the thrombin-thrombomodulin complex. Activated protein C (APC) inhibits the blood coagulation cascade by selective degradation of the procoagulant factors Va and VIIIa. For the optimal exertion of this anticoagulant activity, APC has to form a complex with the cofactor protein S.

The physiologic importance of protein C is most dramatically shown by patients homozygous for protein C deficiency, who usually develop widespread disseminated intravascular coagulation in the neonatal period. This syndrome is fatal unless treated adequately. The parents of these homozygous patients and other heterozygous family members are reported to be free of thrombotic symptoms. Therefore, protein C deficiency is an autosomal recessive disease with respect to the thrombotic symptoms. Also, the prevalence of heterozygous protein C deficiency found in healthy blood donors (0.1% to 0.5%) is in agreement with a recessive disorder.

It is therefore surprising that, both in family- and in population-based studies, heterozygosity for protein C deficiency has also been reported to be associated with an increased risk for thrombosis. Several studies report an increased prevalence for heterozygous protein C deficiency in groups of thrombotic patients (2% to 9%) compared with the prevalence in healthy blood donors. Furthermore, it has been shown that the heterozygous first degree relatives of symptomatic probands are more at risk for thrombosis than the nonheterozygous first degree relatives. Based on these observations, protein C deficiency should be considered as an autosomal dominant disease.

Thus, it is clear that the risk for thrombosis varies among protein C-deficient families. It is unlikely that this difference in thrombotic risk is caused by differences in the protein C gene defect, because plasma protein C levels are identical in recessive and dominant protein C-deficient families. More importantly, identical protein C gene mutations have been found in clinically recessive and in clinically dominant families. For these reasons, the thrombophilia in clinically dominant protein C-deficient families is probably caused by the combined action of heterozygous protein C deficiency and an additional risk factor(s). Because of the familial clustering of the thrombophilia, it seems likely that the other risk factor(s) is genetic.

Recently, a novel strong genetic risk factor for deep vein thrombosis has been identified. This risk factor was first reported by Svensson and Dahlbäck, who showed that in some families a poor anticoagulant response to APC (which is referred to as APC resistance) segregated with thrombophilia. Recently, it was shown that this APC resistance is a strong risk factor for venous thrombosis (matched odds ratio of 7) and is associated with a mutation in the factor V (FV) gene. The mutation predicts the presence of an abnormal factor V molecule (FV Leiden), in which Arg506 in one of the APC cleavage sites has been replaced by Gln. The FV Leiden mutation has a relatively high allele frequency in the normal population (2%).

Given the proposed complex genetic cause of clinically dominant protein C deficiency, we addressed the question whether APC resistance might contribute to the familial clustering of thrombosis in clinically dominant protein C-deficient families.


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by Leiden allele results in fragments of 67 and 153 bp. These fragments 
manifestations of deep venous thrombosis, pulmonary embolism, super-

tron IO of the FV gene, followed by digestion with the restriction 
rate chain reaction (F'CR) product of a normal FV allele is cleaved 
enzyme coding and flanking regions of the protein C gene followed by direct 

a~tivity.'~ Mutation analysis was performed by amplification of the 

is identified in the proband of the family and were interviewed 

certained through a symptomatic proband.”

Gene frequencies of the two trait loci were set equal to the estimates 
disequilibrium between trait locus and mutant allele was assumed. 
mutations were used as markers, and additional to linkage, linkage 

zygosity for the mutation. Penetrance values for the heterozygous 
genotypes are directly based on the family data. Penetrances for the 

were estimated from the family data as the ratio of the number 
suspects with genotype g divided by the total number of subjects with genotype g. The penetrances of the homozygous geno-
types were given an appropriate value based on available data.14,19

We calculated the likelihoods for four assumptions: (1) assuming 
linkage and linkage disequilibrium between each trait locus and one 
marker locus; (2 and 3) assuming that only one trait locus is linked 
and in linkage disequilibrium with one marker locus; and (4) assum-

ing that both trait loci are unlinked and in linkage equilibrium with 
the marker loci. The lod scores were calculated as the odds in favor 
over assumption (4), i.e., no linkage and linkage equilibrium between 
both trait loci and marker loci.

Statistical methods. P values were calculated with a standard \( \chi^2 \) 
test.

RESULTS

Probands. DNA of 48 unrelated probands with clinically 
dominant protein C deficiency, confirmed by protein C gene 
analysis, was available for analysis. The FV Leiden mutation 
was identified in 9 of these 48 symptomatic protein C-defi-
cient probands (19%). Of these probands, 1 has the 

Phe-Leu, 1 the Gln-stop, 2 the Gln-stop, 1 the Cys-Arg, 
2 the Arg-Cys, and 2 the Ile-Met protein C gene muta-

tion.14

Families. DNA of the family members of 18 protein C-
deficient probands was available for study. All subjects who 
had experienced a thrombotic episode and/or subjects who 
had the protein C gene mutation were screened for the FV 
Leiden mutation. Individuals with the FV Leiden mutation 
were found in 6 families. In these families, the asymptomatic 
non-protein C-deficient members were also tested for the 
FV Leiden mutation. The pedigrees of these families are 
given in Fig 1. The FV Leiden mutation was not present in 
the proband of 2 of these families, both having the 230Arg-
Cys protein C gene mutation (pedigree pc-87 and pc-109). 
In pedigree pc-87, only 2 individuals, an uncle and aunt of 
the proband, were identified with both the defects. In pedi-
gree pc-109, the FV Leiden mutation was not segregating 
in the two proband lineages.

Table 2 shows the phenotypes of all available members 
of these 6 families. A significantly higher percentage of the 
family members with both the protein C gene mutation and 
the FV Leiden mutation had developed thrombosis (73%), 
compared with family members with either the protein C 
gene mutation or the FV Leiden mutation (36% and 10%, 
respectively; \( P < .001 \) for both groups). Of the subjects 
lacking both the mutations, only 7% had experienced a 
thrombotic episode.

The results given above are biased by the inclusion of 
sibships in which the FV Leiden mutation is not segregating. 
Therefore, we repeated the analysis using only those sibships 
in which both defects were segregating. In these sibships, 
each individual has an equal chance of inheriting both the 
defects. Table 3 shows the number and percentage of symp-
tomatic individuals for the different genotypes in these sib-
ships. Among subjects with only the protein C gene muta-
tion, the percentage of thrombotic cases is 31%. The 
percentage of thrombotic cases among subjects with exclu-
sively the FV Leiden mutation is 13%. Again, these percent-
ages are significantly different from the percentage of throm-
botic cases among subjects having both defects (73%; both 
groups \( P < .001 \)). These differences remain when the pro-

Table 1. The Two Locus Segregation Model

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>FV Gene</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Protein C Gene</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>12</td>
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<td>22</td>
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Shown are the penetrances of the two locus model in which the 
protein C gene and the FV gene are assumed to be the two trait loci 
responsible for the thrombophilia. The presence of the mutation is 
represented as a genotype: 11 represents the normal genotype, 12 
represents heterozygosity for the mutation, and 22 represents 
 homozygosity for the mutation. Penetrance values for the heterozygous 
genotypes are directly based on the family data. Penetrances for the 

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bands are excluded from the analysis to correct for an ascertainment bias (respectively, $P < .005$ and $P \approx .001$).

The median age at the first thrombotic episode was also calculated in these sibships. The probands were excluded to correct for an ascertainment bias. The median age at the first thrombotic episode is 30 years for the 12 subjects having both the defects (range, 19 to 71 years) and 36 years for the 5 subjects with only the protein C gene mutation (ie, 28, 35, 36, 54, and 64 years). The 2 symptomatic subjects with only the FV Leiden mutation were 41 and 29 years at the first thrombotic episode.

Two locus linkage analysis. With the two locus linkage analysis, we tested whether the segregation of the two gene mutations is in agreement with the assumption that the protein C gene and the FV gene are the two trait loci responsible for the thrombosis. Therefore, the mutations are used as markers, and additional to linkage, linkage disequilibrium is assumed, because only the mutant allele must be associated with the thrombosis. The analysis was performed with the complete 6 families. The lod scores are given in Table 4. Linkage and linkage disequilibrium of both the protein C gene mutation and FV gene mutation to the trait-loci is sup-

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**Fig 1.** Pedigrees of the 6 families in which both a protein C gene mutation and the FV Leiden mutation are segregating. Thrombotic symptoms are indicated by a hatched upper half of the symbols, the presence of a protein C gene mutation by a solid lower right of the symbols, and the presence of the FV Leiden mutation by a solid lower left of the symbols. Tested healthy subjects are indicated by a dot in the center of the symbol. Probandes are indicated by an arrow. The sibships in which both the gene defects are segregating are indicated by a double line.
ported by a lod score of 19.62, representing odds of 4.2 \times 10^{19} to 1, when compared with no linkage and linkage equilibrium. The lod score for the linkage and linkage disequilibrium of the protein C gene mutation to one trait locus, and no linkage and linkage equilibrium of the FV Leiden mutation to the other trait locus, is 16.35. This lod score is 3.27 lower. Thus, a lod score of 3.27 (representing odds of 3.27 lower) represents an important risk factor for thrombosis. Nevertheless, the question remains of why only a subset of protein C-deficient individuals are at risk. One explanation is that the equilibrium of the protein C gene mutation to one trait locus, and no linkage and linkage equilibrium of the other trait locus, additional to the protein C gene. The lod score for linkage and linkage disequilibrium of the FV Leiden mutation to one trait locus, and no linkage and linkage equilibrium of the protein C gene mutation to the other trait locus is 5.95.

**DISCUSSION**

There is no doubt that heterozygous protein C deficiency represents an important risk factor for thrombosis. Nevertheless, the question remains of why only a subset of protein C-deficient individuals are at risk. One explanation is that an interaction of one or more risk factors with heterozygous protein C deficiency is necessary for an increased thrombotic risk. Until now, no evidence for this hypothesis has been found. The combination of protein C deficiency with other known thrombotic risk factors such as protein S and antithrombin deficiency are too rare to allow extensive investigation. Other genetic risk factors, with relatively high prevalences, may therefore exist. The newly described FV Leiden mutation is an example of such a risk factor. Recently, the prevalence of carriers of the FV Leiden mutation was found to be 21% among unselected patients with thrombosis and 5% in the Dutch population. These new findings led to the following questions: (1) What is the prevalence of the FV Leiden mutation among clinically dominant protein C-deficient probands? (2) Does the FV Leiden mutation increase the risk for thrombosis in carriers of a protein C gene mutation?

Evidence for an increased risk of thrombosis in carriers of both gene defects comes from 6 families in which the combined segregation of the protein C gene mutation and FV Leiden mutation could be studied. In the sibships in which both defects are segregating, a significantly higher percentage of carriers of both the defects is affected (73%) than of carriers of a single defect (31% and 13% for the protein C gene mutation and FV Leiden mutation, respectively). These differences remain significant when the probands are not included to correct for an ascertainment bias.

From the present data, no conclusion can be drawn with respect to differences in the age of onset between the different groups. Linkage analysis is less affected by an ascertainment bias, and a more appropriate method to test for an association between disease and a candidate gene in families. The results of the two locus linkage analysis described here are in favor of the assumption that the protein C gene and the FV gene are the two trait loci responsible for the thrombophilia. The highest odds for observing these 6 families are found.
when the two gene mutations are assumed to be completely linked and in linkage disequilibrium with the two trait loci involved in the thrombophilia.

Not every thrombotic case in the 6 families can be explained by the combination of the two defects. Even in the sibships in which both defects are segregating, 7 thrombotic cases occur in subjects who have only a single defect. This implies that either the single gene defect has a certain risk by itself or that yet a third risk factor is segregating in these lineages. This is further illustrated in pedigree pc-109, in which the FV Leiden mutation is not segregating in the two proband lineages. The exceptional young age of (22 and 26) and severity of the thrombosis (CVA and DVT) in the two index cases of these lineages may be an indication of the segregation of a third genetic risk factor.

In conclusion, APC resistance plays an important role in the thrombophilia in the families presented here, but the majority of our protein C-deficient families need to be further investigated for the presence of other (as yet unidentified) risk factors. With the rapid advances made in the Human Genome Project, many highly polymorphic markers are now available. This makes future single and two loci linkage analysis and other methods for detection of these unknown risk factors feasible.

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