

An Unknown Genetic Defect Increases Venous Thrombosis Risk, through Interaction with Protein C Deficiency

Sandra J. Hasstedt,¹ Edwin G. Bovill,² Peter W. Callas,³ and George L. Long⁴

¹Department of Human Genetics, University of Utah, Salt Lake City; and Departments of ²Pathology, ³Biometry, and ⁴Biochemistry, University of Vermont, Burlington

Summary

We used two-locus segregation analysis to test whether an unknown genetic defect interacts with protein C deficiency to increase susceptibility to venous thromboembolic disease in a single large pedigree. Sixty-seven pedigree members carry a His107Pro mutation in the protein C gene, which reduces protein C levels to a mean of 46% of normal. Twenty-one carriers of the mutation and five other pedigree members had verified thromboembolic disease. We inferred the presence in this pedigree of a thrombosis-susceptibility gene interacting with protein C deficiency, by rejecting the hypothesis that the cases of thromboembolic disease resulted from protein C deficiency alone and by not rejecting Mendelian transmission of the interacting gene. When coinherited with protein C deficiency, the interacting gene conferred a probability of a thrombotic episode of ~79% for men and ~99% for women, before age 60 years.

Introduction

Normal coagulation has been described as a “cascade” or “waterfall” in which a series of precisely regulated enzymatic reactions convert a set of proenzymes into their active forms (Mann et al. 1995). Bleeding disorders result from deficiencies of certain components in the cascade; for example, factor VIII deficiency causes hemophilia A (Reiner and Davie 1995). In contrast, deficiencies of other components, such as protein C, increase the risk of venous thromboembolic disease (Florell and Rodgers 1997).

Protein C is a vitamin K–dependent glycoprotein that,

when activated, cleaves the procoagulant protein factor V, thereby inactivating it (Esmon 1993). The protein C gene has been isolated, characterized, and assigned to chromosome 2 (Beckmann et al. 1985; Foster et al. 1985; Plutzky et al. 1986; Kato et al. 1988). At least 160 mutations causing protein C deficiency have been identified (Reitsma 1996).

Other common causes of inherited venous thromboembolic disease include activated–protein C resistance, deficiencies of protein S and antithrombin (Florell and Rodgers 1997), and elevated plasma prothrombin. Activated–protein C resistance usually is due to an Arg506Gln mutation in factor V (factor V_{Leiden}), which eliminates the activated–protein C cleavage site (Dahlbäck et al. 1993; Dahlbäck 1994). As with protein C deficiency, many mutations have been identified as causes of protein S deficiency (Simmonds et al. 1996) and antithrombin deficiency (Lane et al. 1997). A G20210A prothrombin mutation in the 3′ UTR of the prothrombin gene is one cause of elevated plasma prothrombin (Poort et al. 1996).

Despite the fact that inherited venous thromboembolic disease has been attributed to single genetic defects, the interaction of two genetic defects often may be responsible (Miletich et al. 1993). Since its identification, factor V_{Leiden} has been found to be common among individuals whose venous thromboembolic disease previously had been attributed solely to deficiencies of protein C (Koeleman et al. 1994; Gandrille et al. 1995; Brenner et al. 1996), protein S (Koeleman et al. 1995; Zöller et al. 1995a), or antithrombin (van Boven et al. 1996). Other pairs of genetic defects also have been reported for thrombosis patients (Gandrille et al. 1988; Berruyer et al. 1994; Zöller et al. 1995b; Beauchamp et al. 1996; Züger et al. 1996). In this study, we used two-locus segregation analysis to test for evidence of a genetic defect that interacts with protein C deficiency to increase the risk of venous thromboembolic disease in a single large pedigree with 283 studied members.

Subjects and Methods

The sample used in the analysis consisted of a single large pedigree, which was ascertained through a 13-year-

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Address for correspondence and reprints: Dr. Sandra J. Hasstedt, University of Utah, Department of Human Genetics, 15 N 2030 E, Room 2100, Salt Lake City, UT 84112-5330. E-mail: sandy@sapporo.genetics.utah.edu

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old boy who was suspected to have had a venous thrombotic episode. Through questioning, his mother reported her own experience with a venous thrombotic episode, at age 17 years; her low protein C level prompted us to study her relatives, making her the true proband of the pedigree. We studied all available descendants of the proband's paternal grandparents, up to six generations in some lines, and verified a high incidence of venous thromboembolic disease in pedigree members (Bovill et al. 1989); the pedigree has been extended to include descendants of the proband's paternal grandmother's sister. The family resides primarily in the Northeast Kingdom region of Vermont. Both grandparents were part Abenaki American Indian and part French Canadian, and marriages among their descendants have occurred primarily within those communities. This study was approved by the Human Experimentation Committees of the University of Vermont College of Medicine and Beth Israel Hospital, Boston.

Each subject in the study completed a questionnaire and an interview, thereby providing information on thrombotic risk factors and the occurrence of venous thrombotic episodes. We classified subjects as having verified thromboembolic disease if they reported being hospitalized and treated for deep venous thrombosis; objective, corroborative tests were available in >90% of the cases. We classified subjects as having equivocal thromboembolic disease if they reported either an unhospitalized thrombotic episode or superficial thrombophlebitis. In the absence of any evidence of venous thrombotic episodes, subjects were classified as unaffected. Plasma concentrations of protein C were determined by immunoassay and were normalized to the mean of a normal plasma pool composed of 54 control subjects (Bovill et al. 1989).

We used likelihood analysis (Elston and Stewart 1971) to test for evidence of a genetic defect that interacts with protein C deficiency to increase susceptibility to venous thrombosis. Likelihoods were approximated (Hasstedt 1993) by use of the Pedigree Analysis Package (Hasstedt 1994) and the maxima obtained with the Nonlinear Programming-Systems Optimization Laboratory (Gill et al. 1986). Correction was made for ascertainment, by division of the likelihood of the pedigree by the probability of the proband's phenotype. Hypothesis testing was performed by comparison of the likelihood of a submodel to the likelihood of a more general model. Under certain conditions, -2 multiplied by the natural logarithm of the ratio of the likelihoods has an asymptotic χ^2 distribution.

We assumed that an unmeasurable quantitative liability variable underlies thrombosis susceptibility (Morton and MacLean 1974). The liability variable was distributed as a mixture of normal densities, one for each genotype, with the mixture proportions equal to the ge-

notype frequencies. The population incidence for each age interval determined a point on the liability scale such that the weighted sum of the areas between adjacent points, under the normal curves for all genotypes, equaled the venous thrombosis incidence for that age interval, with younger ages corresponding to higher liability. For an affected individual, the probability of a particular genotype equaled the integral of the appropriate normal curve between the two points, on the liability scale, bracketing the individual's age at first thrombotic episode. For an unaffected individual, the probability of a particular genotype equaled the integral of the appropriate normal curve from $-\infty$ to the point, on the liability scale, marking the upper end of the age range bracketing the age when the individual was last known to be free of thrombosis.

The genetic model included two unlinked major loci, polygenes, and random environmental factors specific to the individual. We assumed that each major locus had two alleles in Hardy-Weinberg equilibrium. The first major locus represented the protein C gene, with the genotypes defined by mutation analysis; the allele frequency at this locus was fixed at .0025, to correspond to a prevalence of protein C deficiency of 1/200 (Miletich et al. 1987). The second major locus represented an unknown interacting gene.

The parameters of the model included the frequency, q , of the interacting genetic defect; the dominance, d_m and d_f , of the interacting genetic defect, in males and females, respectively; displacement t_{cm} and t_{cf} , for protein C in males and females, respectively; displacement t_{im} and t_{if} , for the interacting genetic defect in males and females, respectively; polygenic heritability, h^2 ; and parent-to-offspring transmission probabilities τ_1 , τ_2 , and τ_3 , for the three genotypes of the interacting gene (Boyle and Elston 1979; Lalouel et al. 1983). Displacement equaled the difference in within-genotype SDs between the mean liabilities of the two types of homozygotes at one locus. We assumed additivity of displacement across loci; that is, the displacement for both loci together equaled the sum of the displacement for the protein C gene and the displacement for the interacting gene. Dominance equaled the difference between the mean liability of heterozygotes and the mean liability of unaffected homozygotes, relative to the displacement at the locus; dominance at the protein C locus was fixed at 1. Polygenic heritability equaled the proportion of the variance within major-locus genotypes that was due to polygenic inheritance. Mendelian inheritance specified $\tau_1 = 1$, $\tau_2 = \frac{1}{2}$, and $\tau_3 = 0$.

To test the hypothesis that protein C deficiency alone determines thrombosis susceptibility, we compared the likelihood maximized by restricting $q = 0$, $d_m = 0$, $d_f = 0$, $t_{im} = 0$, $t_{if} = 0$, $\tau_1 = 1$, $\tau_2 = \frac{1}{2}$, and $\tau_3 = 0$ with the likelihood maximized by restricting $\tau_1 = 1$, $\tau_2 = \frac{1}{2}$,

Table 1
Number of Pedigree Members with Verified or Equivocal Thrombosis and Population Incidence (per 100,000) of Verified Thrombosis, by Sex and by Age at First Thrombotic Episode

AGE AT FIRST EPISODE (YEARS)	NO. OF PEDI-GREE MEMBERS WITH VERIFIED THROMBOSIS		NO. OF PEDI-GREE MEMBERS WITH EQUIVOCAL THROMBOSIS		POPULATION INCIDENCE (PER 100,000) OF VERIFIED THROMBOSIS ^a	
	M	F	M	F	M	F
	10-19	0	4	2	0	2
20-29	2	6	0	4	17	13
30-39	2	3	4	4	30	19
40-49	4	0	2	2	31	8
50-59	3	0	1	0	49	33
60-69	1	0	0	2	174	115
70-79	0	1	0	0	276	210
80+	0	0	0	0	334	327

NOTE.—M = male; F = female.

^a Data from the study by Anderson et al. (1991); also see figure 2.

^b Set at 1/100,000 in the analysis, because there were patients in the pedigree in that sex and age category.

and $\tau_3 = 0$. To test Mendelian transmission and environmental nontransmission of the interacting genetic defect, we compared the likelihoods maximized by restricting $\tau_1 = 1$, $\tau_2 = \frac{1}{2}$, $\tau_3 = 0$, and $1 - q = \tau_1 = \tau_2 = \tau_3$, respectively, with the likelihood maximized without constraints. We inferred an interacting genetic defect by rejecting the hypotheses of thrombosis susceptibility being determined by protein C deficiency alone or by protein C deficiency in conjunction with a nontransmitted environmental factor, while not rejecting the hypothesis of Mendelian transmission of an interacting genetic defect. The tests of recessivity and dominance compared the likelihoods maximized by restricting $d_m = d_f = 0$ and $d_m = d_f = 1$ with the likelihood maximized when d_m and d_f were unrestricted, while τ_1 , τ_2 , and τ_3 were restricted to 1, $\frac{1}{2}$, and 0, respectively.

Results

Table 1 presents the sex and age distributions of 26 pedigree members with verified and 21 pedigree members with equivocal thrombotic episodes. In the segregation analysis, individuals with verified thrombotic episodes were classified as “affected,” and individuals with equivocal thrombotic episodes were classified as “unknown.” Age at first thrombotic episode was within the range 17–76 years, with a mean age of 35 years.

Table 1 also includes the sex- and age-specific incidences of venous thrombosis in the general population. The segregation analysis constrained the genetic model to correspond to these incidence figures. Although thrombotic episodes in the general population usually occur at age ≥ 60 years, all thrombotic episodes in the

pedigree occurred earlier, with two exceptions, at ages 61 years and 76 years.

Sixty-seven pedigree members, including the proband but not her son, carry a His107Pro mutation in the protein C gene (Tomczak et al. 1994); 64 carriers were identified through mutation screening, and the other 3 carriers were their ancestors. This analysis ignored a Thr298Met mutation in the protein C gene (Tomczak et al. 1994), which was found in the wife of a His107Pro mutation carrier and in two of their daughters. Figure 1 shows the distribution, by disease status, of protein C levels in the 61 measured His107Pro mutation carriers. Protein C levels were within the ranges 4%–69% of normal in 20 verified thrombosis patients, 25%–69% of normal in 4 equivocal thrombosis patients, and 16%–109% of normal in 37 unaffected mutation carriers. Warfarin therapy accounts for some of the very low protein C measurements. Protein C levels met or exceeded the lower limit of the normal range (66% of normal) in 7 of the His107Pro mutation carriers.

Twenty-one mutation carriers and five other pedigree members suffered verified thrombotic episodes. Figure 2 presents the distribution of 24 verified thrombosis patients, by age at first thrombotic episode, percentage of normal protein C level, and mutation status; the other two verified thrombosis patients lacked protein C measurements. Thrombotic episodes in the pedigree members

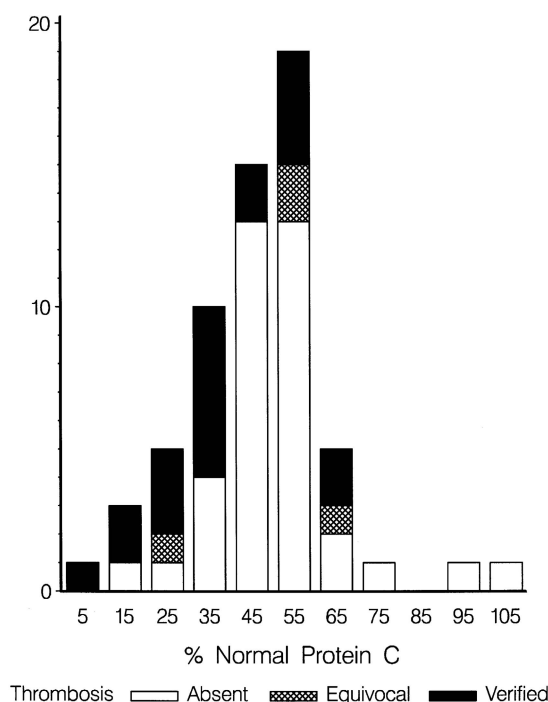


Figure 1 Distribution of protein C levels among carriers of the His107Pro protein C mutation, by thrombosis diagnosis.

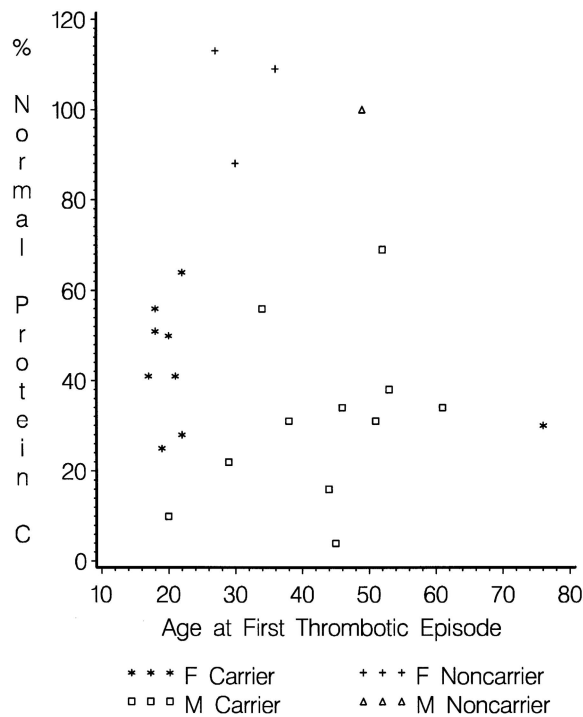


Figure 2 Distribution of protein C levels among male and female carriers and noncarriers of the His107Pro protein C mutation, by age at first thrombotic episode.

tended to occur earlier in women than in men and were precipitated by pregnancy or oral contraceptive use (Trauscht-Van Horn et al. 1992).

An additional 257 pedigree members and spouses with no history of thrombosis were classified as unaffected, resulting in a total sample size of 283 for the segregation analysis (table 2). An earlier version of the complete pedigree has been published elsewhere (Bovill et al. 1989); figure 3 shows a selected branch, to illustrate the observed inheritance patterns. Individuals 1 and 2 both transmitted the His107Pro mutation; yet, thrombotic episodes occurred only among the offspring of individual 1, who also was affected. Individual 5 experienced a thrombotic episode in the absence of the His107Pro mutation, but four offspring have been free of thrombosis, although they are still young enough to remain at risk. As does figure 1, figure 3 shows that not all His107Pro mutation carriers experience a thrombotic episode. As does figure 2, figure 3 shows thrombotic episodes occurring primarily, but not exclusively, in mutation carriers. In addition, figure 3 illustrates that thrombotic episodes never occur in an individual whose parents had no history of thrombotic episodes, which also is true of the complete pedigree.

We inferred that a thrombosis-susceptibility gene in-

teracts with protein C deficiency, by rejecting the hypotheses that thrombosis susceptibility results from protein C deficiency alone ($\chi^2_{(5)} = 27.72, P = .000041$) or that a nontransmitted environmental factor interacts with protein C deficiency ($\chi^2_{(3)} = 19.57, P = .00021$), while not rejecting Mendelian transmission of an interacting genetic defect ($\chi^2_{(3)} = 1.47, P = .69$). The last χ^2 statistic indicates that the transmission probabilities, estimated as $\tau_1 = .990 \pm .019, \tau_2 = .371 \pm .122,$ and $\tau_3 = 0$, do not differ significantly from Mendelian values. We inferred dominant inheritance by rejecting the hypothesis of recessive inheritance ($\chi^2_{(2)} = 24.58, P = .0000046$) but not of dominant inheritance ($\chi^2_{(2)} = 0.00, P > .05$). Table 3 summarizes all the models tested.

The parameter estimates for the inferred dominant model were $q = .00033 \pm 0.00027, t_{Cm} = 2.80 \pm 0.53, t_{Cf} = 2.15 \pm 0.53, t_{Im} = 1.13 \pm 0.57, t_{If} = 3.55 \pm 0.57,$ and $h^2 = .083 \pm .271$. Table 4 gives the penetrances that correspond to the parameter estimates. In the men, protein C deficiency exerted the larger effect, with an estimated penetrance, by age 60 years, of 37%, compared with 2% for the interacting genetic defect. In the women, the interacting genetic defect had the larger effect, with an estimated penetrance, by age 60 years, of 61%, compared with 13% for protein C deficiency. Protein C deficiency interacting with the unknown genetic defect resulted in a penetrance, by age 60 years, of 79% in men and 99% in women.

Discussion

Homozygosity for protein C deficiency causes massive venous thrombosis and purpura fulminans in newborns (Marlar et al. 1989). Nevertheless, heterozygosity for protein C deficiency does not appear to increase the risk of venous thrombosis in individuals identified either through a homozygous relative (Branson et al. 1983; Estellés et al. 1984; Seligsohn et al. 1984; Marciniak et al. 1985) or through population screening (Miletich et

Table 2

Number of Pedigree Members, by Affection and Carrier Status, and Age Range of Unaffecteds

GROUP	NO. OF PEDIGREE MEMBERS			AGE OF UNAFFECTEDS (YEARS)	
	Studied	Affected	Carrier	Minimum	Maximum
Generation:					
II	6	4	2	41	65
III	30	11	18	36	81
IV	116	10	34	11	57
V	108	1	13	1	38
VI	7	0	0	3	15
Spouses	16	0	0	35	84
Total	283	26	67

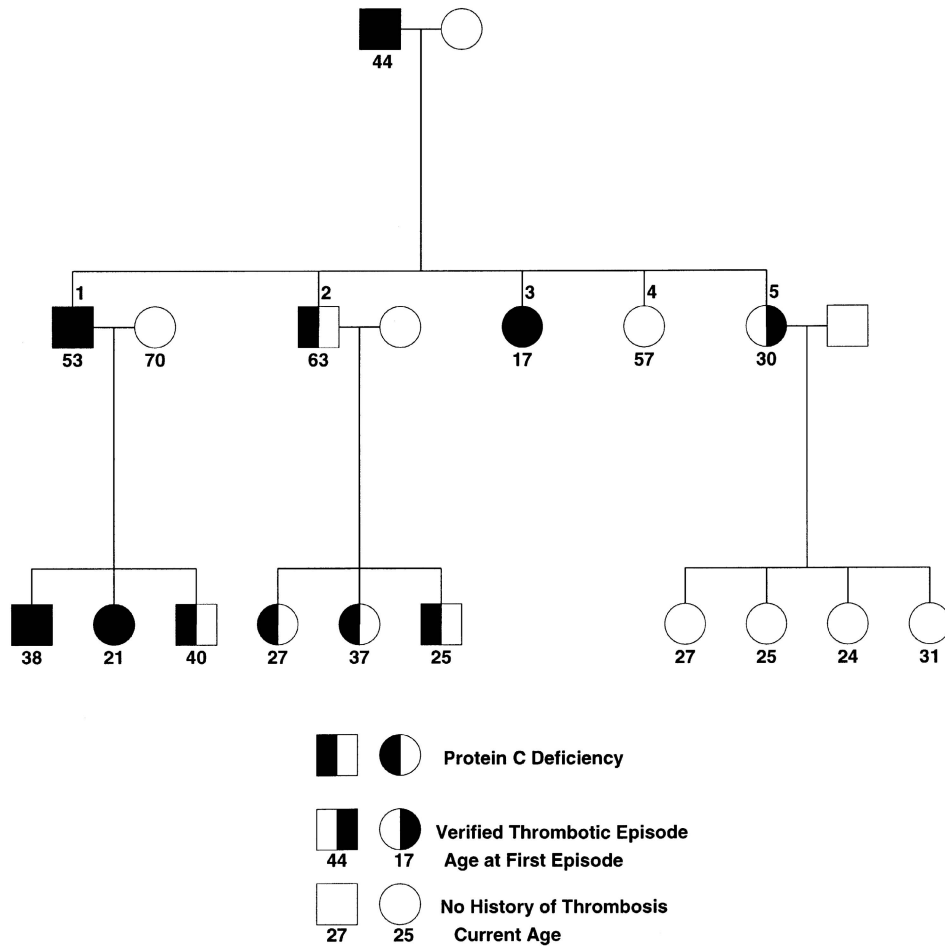


Figure 3 Selected descendants of the parents of the proband (individual 3). Unaffected individuals whose current age is not given were not studied.

al. 1987; Tait et al. 1995; McColl et al. 1996). On the other hand, heterozygosity is frequent in venous thrombosis patients (Gladson et al. 1988; Koster et al. 1995), and venous thrombosis is frequent in the heterozygous relatives of heterozygous venous thrombosis patients (Griffin et al. 1981; Broekmans et al. 1983; Horellou et al. 1984; Pabinger and Schneider 1996). These apparent inconsistencies are resolved if venous thrombotic episodes occur rarely in individuals with a single genetic defect but frequently in individuals with two genetic defects (Miletich et al. 1993; Florell and Rodgers 1997; Seligsohn and Zivelin 1997). Nevertheless, our inferred model suggests a slightly increased risk from a single genetic defect, but the estimates were based on small numbers.

The inferred model assumed that the effect due to the coinheritance of an interacting genetic defect in conjunction with the His107Pro mutation in the protein C gene equaled the sum of the effects due to each of the

genes alone. The additivity occurred on an unmeasurable quantitative liability scale assumed to underlie thrombosis susceptibility (Morton and MacLean 1974). Additivity on the liability scale produces an interactive effect on the penetrances, but to a degree that is less than multiplicative (Risch et al. 1993). The effect of the additivity assumption was to produce a penetrance for the two defects combined that was substantially higher in younger patients but only slightly higher in older patients than that for either defect alone. Nevertheless, caution is advised in interpretation of the penetrance estimates, since the validity of the additivity assumption is unknown, the incidence figures (which constrained the penetrance estimates) derive from a population different than the pedigree, and only a small number of thrombosis patients contributed to the penetrance estimates. The eventual identification of carriers of the interacting genetic defect will allow refinement of the penetrance estimates.

Table 3
Parameter Estimates and χ^2 Statistics for Each Model

PARAMETER	PARAMETER VALUE, BY MODEL FOR INTERACTING GENETIC DEFECT ^a					
	None	Recessive	Dominant	Codominant	Environmental	General
q	[0]	.0804	.0003	.0003	.1120	.0003
τ_1	[1]	[1]	[1]	[1]	[1 - q]	.990
τ_2	[.5]	[.5]	[.5]	[.5]	[1 - q]	.371
τ_3	[0]	[0]	[0]	[0]	[1 - q]	(0)
d_m	[0]	[0]	[1]	(1)	.819	(1)
d_f	[0]	[0]	[1]	(1)	(1)	(1)
t_{Cm}	.707	1.068	2.799	2.799	4.186	2.803
t_{Cf}	1.671	1.656	2.151	2.151	1.676	2.016
t_{im}	[0]	2.394	1.135	1.135	4.726	1.132
t_{if}	[0]	.097	3.549	3.549	(1)	2.921
h^2	(1)	(1)	.083	.083	(1)	.022
χ^2	27.72*	24.58*	.00	1.47	19.57*	...
df	5	2	2	3	3	...
Comparison model	Codominant	Codominant	Codominant	General	General	...

^a Square brackets indicate fixed parameter values. Parentheses indicate boundary parameter estimates.

* $P < .0005$.

There are many candidates for the inferred interacting gene. Factor V_{Leiden} (Koeleman et al. 1994; Gandrille et al. 1995; Brenner et al. 1996) and dysfibrinogenemia (Gandrille et al. 1988) reportedly increase venous thrombotic risk, through interaction with protein C deficiency. However, we ruled out factor V_{Leiden} , through mutation screening, and failed to find dysfunctional fibrinogen during functional analysis. In addition, we ruled out protein S, antithrombin, plasminogen activator inhibitor-1, and plasminogen activator inhibitor-3 as candidates for interaction with protein C deficiency in this pedigree, by finding normal levels in affected pedigree members. Nevertheless, numerous other genes for protein products that contribute to the coagulation cascade remain as candidates (Miletich et al. 1993).

If none of the candidate genes prove to be the gene interacting with protein C deficiency in this pedigree, we

can localize the gene by using linkage analysis. However, a sample size with sufficient power is more difficult to obtain for analysis of a two-gene disease than for analysis of a single-gene disease. For only 13 of the 67 pedigree members who carry the His107Pro mutation, the probability of also carrying the interacting genetic defect is $>50\%$. We have begun to extend the pedigree to descendants of the siblings of the proband's paternal grandmother. However, if each grandparent contributed one genetic defect, only their common descendants can inherit both defects. We have attributed the His107Pro mutation in the protein C gene to the proband's grandmother by detecting the mutation in eight descendants of her sister. However, the source of the interacting genetic defect remains indeterminate at this point, despite the testing of 34 members of this branch of the pedigree. Three verified venous thrombotic episodes among the sister's descendants suggest but do not confirm that the interacting gene also derived from the grandmother.

In summary, we inferred a gene that interacts with protein C deficiency to increase the risk of venous thrombosis in a large pedigree. The interacting gene remains to be identified, but this study justifies initiation of a search for the gene by the testing of candidate loci and the performance of a genomewide screen.

Table 4
Penetrance of Verified Thrombosis, by Genotype, Sex, and Age at First Thrombotic Episode

AGE AT FIRST EPISODE (YEARS)	PENETRANCE (%), BY PRESENCE (+) OR ABSENCE (-) OF PROTEIN C DEFICIENCY/INTERACTING GENETIC DEFECT							
	-/-		+/-		-/+		+/+	
	M	F	M	F	M	F	M	F
10-19	.00	.00	.40	.01	.00	1.23	6.42	46.16
20-29	.00	.00	4.13	.76	.03	15.17	27.38	86.91
30-39	.00	.00	13.08	3.79	.27	35.27	50.48	96.19
40-49	.03	.02	24.86	7.49	.96	48.59	67.57	98.28
50-59	.09	.05	37.46	13.20	2.36	61.07	79.25	99.25
60-69	.32	.19	52.76	22.72	5.53	74.21	88.57	99.75
70-79	.83	.52	65.69	34.14	10.38	83.88	93.81	99.92
80+	1.70	1.19	75.13	45.67	16.21	90.13	96.51	99.97

NOTE.—M = male; F = female.

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