molecular basis of disease
Haematologica 1995; 80:344-356

RESISTANCE TO ACTIVATED PROTEIN C DUE TO MUTATED FACTOR V AS A NOVEL CAUSE OF INHERITED THROMBOPHILIA

Valerio De Stefano, Giuseppe Leone
Divisione di Ematologia, Istituto di Semeiotica Medica, Università Cattolica, Rome, Italy

ABSTRACT

Inherited resistance to activated protein C (APC) was recently recognized as a novel cause underlying venous thrombophilia. In most cases APC-resistance is due to a single point mutation in the factor V gene leading to a replacement of Arg506 with Gln (factor V Leiden). Amino acid substitution occurs at one of the APC cleavage sites of factor Va, rendering it resistant to APC inactivation. Plasma anticoagulant response to exogenous APC as a simple diagnostic assay of APC- resistance shows good sensitivity and specificity as compared to gene analysis, yet standardization of the results needs to be improved. The APC-resistance trait is present in 2%-6% of the general population and was found to be associated with venous thrombophilia in about 20% of patients with unexplained thrombosis. Clinical features are substantially similar to other congenital plasma abnormalities predisposing to thrombosis (antithrombin III, protein C, protein S deficiencies); yet the overall clinical penetrance of the defect seems lower, at least for the heterozygous condition. Preliminary data suggest a higher risk of thrombosis in APC-resistant homozygous individuals or in patients exhibiting APC-resistance together with other thrombophilic genetic defects. To date, genetically determined APC-resistance does not seem to play a significant role in the development of arterial thrombotic disease.

Key words: thrombophilia, APC resistance, mutated factor V

Familial thrombophilia was recognized as a distinct nosographic entity in 1956 by Jordan and Nandorff, who reported on 43 kindreds with thrombotic diathesis.[1] Since then, a number of inherited alterations of coagulation mechanisms underlying thrombophilia have been identified.[2] The natural anticoagulant systems mainly involved in the pathogenesis of inherited thrombophilia are antithrombin III and the protein C pathway. Antithrombin III inactivates thrombin and factor Xa and, to a lesser extent, other serine proteases (VIIa, IXa, XIa, XIIa); its action is enhanced by heparin.[3] Circulating protein C is converted to its activated form by thrombin complexed with thrombomodulin (an endothelial receptor). Activated protein C (APC) inactivates factor VIIIa and factor Va by limited proteolysis; the anticoagulant effect of APC requires the presence of the cofactor protein S in its unbound form[4] (Figure 1).

The key role of these coagulation inhibitors in preventing thrombosis is clearly confirmed by the clinical features associated with the inherited deficiencies or abnormalities of antithrombin III, protein C, protein S. A history of thrombosis is present in 50% of affected individuals. Half of the thrombotic episodes develop without any evident associated risk factor (pregnancy, surgery, oral contraceptives), and the thrombotic onset occurs before 40 years of age in 80% of symptomatic patients.[2,5,6]

In a series of consecutive patients with deep vein thrombosis, an isolated deficiency of antithrombin III, protein C, protein S was reported in 6.8% of the cases;[7] in selected series of thrombotic patients referred to specialized centers the prevalence of these defects taken together was 9.2% of the cases (cumulative analysis of 1705 cases, range 3-21%).[8-11]

Thus, a biochemical basis for thrombophilia

Correspondence: Dr. Valerio De Stefano, Istituto di Semeiotica Medica, Università Cattolica, largo Gemelli 8, 00168 Rome, Italy. Fax international +39.6.3051343.
Received April 4, 1995; accepted June 8, 1995.

related to the above considered defects can be identified only in a relatively low number of individuals with thrombotic tendency; a laboratory search for other inherited causes of thrombophilia (defect of heparin cofactor II, abnormalities in fibrinogen or in the fibrinolytic system) offers only a slight increase in the diagnostic yield.[2]

Plasma resistance to activated protein C

In 1993 Dahlback *et al*. reported the case of a middle-aged man with a history of recurrent venous thrombosis.[12] The effect of exogenous activated protein C (APC) was measured in an activated partial thromboplastin time (aPTT)-derived assay. In the normal response, addition of APC induced a prolongation of aPTT because of cleavage and inhibition of factors Va and VIIIa; in the proband the prolongation of aPTT in the presence of APC was much shorter than in control plasma. Most of the proband's relatives manifested poor anticoagulant response to activated protein C (APC-resistance), so that a genetic basis for the alteration could be hypothesized; other two kindreds with APC-resistance were also identified. The distribution of the alteration over the family members suggested an autosomal dominant mode of inheritance. A history of thrombosis was present in 8 of the 20 individuals with APC-resistance identified in the three kindreds, and in none of the 18 relatives with a normal response to activated protein C.[12] Thus, a novel inherited plasma alteration apparently associated with thromboembolic manifestations was identified.

Some possible explanations for the poor anticoagulant response to APC, such as the presence of an autoantibody to protein C, a fast-acting inhibitor of APC, a previously unrecognized protein S functional deficiency, a factor VIII mutation resulting in APC-resistant molecules, were ruled out by the evidence of the inherited nature of the defect and/or by experimental work. In fact, the poor response of proband plasma to APC was corrected by the addition of normal plasma but not by depletion of immunoglobulins or addition of purified protein S; moreover, analysis of factor VIII-von Willebrand factor DNA polymorphism in the index family argued against linkage of the APC-resistance to a factor VIII genotype.[12]

The possibility that APC-resistance was caused by an abnormal factor V molecule was explored by adding purified normal factor V to abnormal plasma, which induced a dose-dependent increase in anticoagulant response to APC.[13] These results led to the tentative conclusion that the factor V molecule contains both procoagulant and anticoagulant (APC-cofactor) activities.[13] To elucidate this hypothesis further, factor VIIIa degradation by APC was studied in a purified system employing factor V and protein S; the efficiency of APC action on factor VIIIa was greater in the presence of both factor V and protein S than protein S alone, suggesting that factor V cooperates with protein S in increasing the degradation of factor VIIIa by APC[14] (Figure 1). Moreover, factor V (but not activated factor V) has been reported to enhance the proteolytic degradation of factor VIII by APC, even in the absence of protein S.[15]

Molecular basis of APC-resistance

Another approach to investigating the role of abnormal factor V or factor VIII molecules as the cause of APC-resistance employed plasma from affected individuals as the source of factor V or factor VIII in deficient plasmas; patient factor V but not patient factor VIII rendered the substrate plasma resistant to APC.[16] That was confirmed by adding the factor V purified from patient's plasma or normal plasma to factor V-deficient plasma. The APC-resistance phenomenon was obtained only in the former case.[16]

Linkage studies in two large kindreds supported the hypothesis that APC-resistance was related to an abnormal factor V gene product.[17,18] The locus for the factor V gene has been mapped to chromosome 1 (1q21-25);[19] APC-resistance cosegregated with a factor V gene polymorphism in one of the kindreds[18] and with a microsatellite marker located in the 1q21-25 region in the other kindred.[17]

Factor V can be activated by means of limited proteolysis by thrombin and factor Xa.[20] Inacti-

vation of human factor Va requires binding of factor Va to a membrane surface[21] and cleavage by APC in the heavy-chain region at Arg506-Gly507,[20,22] with subsequent loss of the binding sites for factor Xa and prothrombin;[23] cleavage of the light chain takes place at Arg1765-Leu1766, without loss of biologic activity.[20] Activated protein C binds to a high affinity binding site in the light-chain region corresponding to the amino acid residues Arg1865-Ile1874.[24,25]

On the basis of these data, a search for a factor V mutation involving the putative APC-binding site (Arg1865-Ile1874) and/or the putative cleavage site (Arg506-Gly507) was conducted; two individuals, supposedly homozygous for APC-resistance because both parents were affected, were proven to be homozygous for a guanine to adenine substitution at nucleotide 1691 (1691G→A).[17] This mutation predicts the replacement of Arg506 (CGA) by Gln (CAA) and was named Factor V Leiden (FV Q506) from the discovering center; it was found to cosegregate with APC-resistance in a large kindred.[17] Factor V mutated at the primary cleavage site can be expected to be resistant to degradation by APC and maintain normal procoagulant activity. Accordingly, the factor Va formed by activation of factor V Leiden with factor Xa was shown to be resistant to APC-inactivation;[17] paradoxically, APC- inactivation occurred normally if factor V Leiden was activated by the addition of α-thrombin.[17] This latter point requires further elucidation.

However, these data can explain the correction of plasma APC-resistance by the addition of normal factor V. It is possible that mutated factor Va is inactivated by APC at a lower rate than normal factor Va, resulting in a higher rate of thrombin formation. Consequently, the greater thrombin formation induces elevated activation of factor VIII and factor V, increasing coagulation activation with further consumption of factor V and then factor V-dependent APC- cofactor activity[26,27] (Figure 1). Thus, the addition of normal factor V could lead to increased APC-inactivation of factor VIIIa due to the APC-cofactor activity of factor V, resulting in a decreased rate of coagulation activation. On the other hand, an excess of normal factor V does not induce hypercoagulability because factor Va, derived mostly from normal molecules, is inactivated by APC at normal rates.[26,27] The cofactor activity of factor V to the APC action does not seem to be affected by the Q506 mutation.[26,27]

In the Dutch study the factor V Leiden mutation was found in 56 out of 70 (80%) subjects classified as APC-resistant by means of the aPTT-derived assay; of the 56 affected individuals, 6 were homozygotes and 50 heterozygotes.[17] The remaining 14 subjects, with a normal factor V cleavage site, had an anticoagulant response to APC that was only marginally reduced.[17]

That the factor V Q506 mutation was the cause of APC-resistance was confirmed nearly contemporaneously by at least other four independent centers, which reported the presence of factor V Leiden in 85-100% of APC-resistant individuals.[18,28-31] As a rule, the search for the Q506 mutation failed only in individuals with slight plasma APC-resistance;[30,31] nevertheless, some familial cases with APC-resistance, but without the mutation have been reported.[31] Obviously, some hitherto unrecognized molecular alterations leading to APC- resistance cannot be ruled out.

Methodological problems and conditions influencing the assay

In spite of the simplicity of execution of the APC-resistance plasma assay as designed by Dahlback *et al.*,[12] a number of methodological problems have arisen.

Expression of the results

In the original report[12] the results of the APC-resistance assay were expressed as prolongation of aPTT in the presence of APC as compared to aPTT values in the absence of APC. The ratio of the aPTT value in the presence of APC to the same parameter in the absence of APC (APC-sensivity ratio, APC-SR) has since been more extensively employed to express the results of the assay.[32-34] The plasma APC-sensivity ratio of the patient sample can be normalized to the ratio obtained in the same test run with a reference plasma (normalized APC-sensivity ratio, n-

APC-SR)[17] (Table 1). This latter expression of the results avoids variations related to different concentrations of calcium chloride or APC and to different batches of aPTT activator or APC.[35]

Based on extensive investigation of healthy individuals and thrombotic patients, n-APC-SR ranges were proposed for non carriers (n-APC-SR ≥0.70), heterozygotes (n- APC-SR 0.45-0.70) and homozygotes (n-APC-SR < 0.45) for factor V Leiden mutation;[17,35] according to the authors, this approach received full confirmation by DNA analysis. Conversely, the use of a simple, non normalized APC-SR can result a false positive or negative result in some cases due to the presence of factor V Leiden.[28-31]

Poor assay reproducibility on samples obtained by different blood drawings was claimed by some authors using a commercial kit.[36,37] In other studies a second assay was reported to be confirmatory in all new samples, with a high correlation being observed between the APC-SRs obtained in the two determinations.[32] Most of the first reports were based on results obtained from frozen samples; their conclusions could be biased by the fact that significantly lower APC-sensivity ratio values have been observed in frozen samples as compared to fresh samples, regardless of the storage temperature.[38-40] Yet the APC-resistance assay was reportedly unaffected by the duration of freezing[33,40,41] or by subsequent cycles of freezing and thawing.[34,35]

A falsely decreased APC-ratio may also be due to platelet contamination, so that careful centrifugation is required to obtain platelet poor plasma.[42]

A multicenter investigation employing 35 coagulation instruments from 9 different manufacturers revealed a large interlaboratory variation in the mean APC-SR of the samples investigated (range 2.70-3.56); a sample with mild APC-resistance displayed an APC-SR between 1.55 and 2.42 but was nonetheless classified as the lowest APC-responder in all laboratories regardless of the instrument employed.[43] In our laboratory the results obtained on four different coagulation instruments in a simultaneous session run showed that the use of n-APC-SR allows narrow between-instrument variation with respect to the APC-SR; however, in a group of proven heterozygous individuals expressing mild APC-resistance, the sensitivity of the commercial assay depended on the coagulation instrument employed.[44] Thus, more information is urgently needed to establish whether different instruments require specific reagent concentrations to improve assay resolution.

Table 1. APC-sensivity plasma assay: expression of the results. APC-aPTT: aPTT assay in the presence of activated protein C; aPTT: aPTT assay in the absence of activated protein C.

	Ref.	*Range of controls*	*APC-resistance diagnosis*
aPTT prolongation (APC-aPTT minus aPTT)	12	17-269 sec.	< 23 sec.
	33	40-180 sec.	< 40 sec
APC-sensivity ratio, APC-SR (APC-aPTT/aPTT)	33	2.15-6.20	< 2.15
	34	1.35-4.10	< 2.17
normalized APC-SR, n-APC-SR (patient APC-SR/control APC-SR)	17	> 0.84	< 0.84*

**genetic analysis revealed FV Q506 mutation only in patients with n-APC-SR < 0.70.*

Influence of lupus anticoagulant on the APC-resistance assay

Interference of antiphospholipid antibodies (anticardiolipin antibodies and/or lupus anticoagulant) with the APC-resistance test has been repeatedly confirmed, since false positive APC-resistance is observed as a result of a decrease in APC-SR.[45-50] This reflects the interference of antiphospholipid antibodies with the protein C pathway, as was already demonstrated by previous *in vitro* studies.[51] Recently Bokarewa *et al.*[52] showed that the addition to normal plasma of IgG fractions isolated from patients with antiphospholipids can simulate the APC-resistance phenomenon.

In this study APC-resistance was induced only by IgG fractions with both anticardiolipin and lupus anticoagulant activity.[52] The underlying mechanism was postulated to be a negative interaction between the IgG and the APC-binding sites on factor Va and factor VIIIa molecules. Currently, it appears that 58% to 100% of patients with antiphospholipids expressing lupus

anticoagulant activity show an APC-SR compatible with APC-resistance,[45,46,49,50] whereas patients with the isolated presence of anticardiolipin antibodies present a positive APC- resistance test in a percentage ranging from 18% to 64%.[45,48,50] A modified assay able to detect factor V Leiden even in lupus anticoagulant samples has been described; it employs test plasma diluted in factor V-deficient plasma and tissue factor as clotting activator.[53]

Influence of anticoagulant treatment on the APC-resistance assay

An APC-resistance plasma assay is not possible with heparinized samples unless a preliminary step with a heparin-inactivator is included; as expected, the presence of heparin, even at low concentrations, induces a prolongation of aPTT with or without APC that renders the assay unreliable.[35]

The APC-resistance assay is influenced by reductions in the concentration of factors II, X, IX and VIII that lead to increased APC-SR values.[35] Therefore it must be pointed out that patients on oral anticoagulant treatment cannot be examined for APC-resistance; however, modified assays based on dilution of test plasma in factor V-deficient plasma have been reported to be highly reliable in patients on oral anticoagulants and more sensitive than the original procedure.[53-57]

Interference of APC-resistance on protein S or protein C functional determination

Protein S functional determination was demonstrated to be influenced by the presence of genetically determined APC-resistance in two commercial assays based on APC-inactivation of factor V as tested by a PT-derived or an aPTT-derived system, respectively.[58-62] In both tests performed on APC-resistant individuals at the plasma dilutions recommended by the manufacturers a spurious low level of protein S was found in 75 to 100% of the assays with the IL test, and in 41 to 46% of the assays with the Staclot test;[59-62] it was concluded that both tests are sensitive to the presence in the patient plasma of the mutant factor V, which is resistant to inactivation by protein C.

The presence of the mutant factor V was reportedly misdiagnosed as a protein C functional deficiency in one kindred because of spurious low levels of protein C detected by a coagulometric assay.[63]

Conditions other than thrombotic disease investigated

The concentration of protein S was not found to influence APC-SR when values exceeded 20%, so that misdiagnosis of APC-resistance in individuals heterozygous or protein S deficiency should be unlikely;[35] accordingly, patients with a known inherited protein S deficiency have been reported to have a normal anticoagulant response to APC.[32,33,64]

Factor V levels do not significantly influence the APC-resistance assay, even at very low concentrations.[35,65] Nevertheless, a poor response to activated protein C was described in homozygous factor V-deficient individuals, but not in heterozygous patients.[66] In a kindred with factor V deficiency examined in our laboratory two homozygous subjects displayed an n-APC-SR in the lower range of the control group (0.83 and 0.73, respectively), whereas their parents (with half- levels of factor V) had clearly normal n-APC-SR values (unpublished data).

Pregnancy[67,68] or oral contraceptives[69,70] induce an acquired APC-resistance by a mechanism that remains to be clarified. In this regard, it is of interest that women have repeatedly been found to have slightly lower APC-SR ratios than men,[34,69,71] but conflicting results have also been reported.[31,72]

Clinical relevance of APC-resistance as underlying cause of thrombotic disease

APC-resistance and venous thromboembolism

In the first kindred reported, out of 14 individuals with APC-resistance over three generations, 5 had a history of venous thrombosis.[12] In the first investigations on patient cohorts with venous thrombotic disease the prevalence of APC-resistance ranged from 21 to 64%,[32-34] with a frequency 2 to 7 times greater than congenital deficiencies of antithrombin III, protein C and protein S taken together. Family studies sugge-

sted an association between the APC-resistance trait and an increased risk for thrombosis.[32] An overall review of the patient series with venous thromboembolism reported so far (even in abstract form) showed quite a large prevalence range of APC resistance (4-64%); however, if only the larger series (over 100 patients) are considered, the APC-resistance prevalence appeared to be 10% in one report[41] and 21 to 33% in four others[32,34,58,78] (Table 2a). These discrepancies could be due mainly to different selection criteria: e.g. exclusion of patients over 45 years old with thrombosis, those on oral anticoagulants, and/or those with an established acquired or inherited cause of thrombosis. Another cause for the discrepancy could be related to different ways of expressing the results of the plasma assay. It is noteworthy that the first two large patient series defined by the investigators as *consecutive* were somewhat selected because patients taking oral anticoagulants were not studied,[32,34] and those over 70 with thrombosis were excluded.[34] Cumulative analysis showed a prevalence of 5% in 176 truly consecutive patients (data available only in abstract form), and of 20% in 1184 selected patients (Table 2a). Genetic analysis confirmed the prevalence of the APC-resistance/factor V Leiden trait was about 20% in thrombotic patients investigated either consecutively or after selection (Table 2b). Interestingly, the prevalence of APC-resistance in thrombotic patients was reported to be independent of the age at first thrombosis (23%, 30/129, in patients with thrombotic onset before 45 vs. 19%, 34/172, in the group with onset after 45).[34] We found a similar result in patients selected after exclusion of other causes of thrombophilia; APC-resistance was present in 28%, 26/93, of patients with onset before 45 and in 28%, 7/25, of those with onset after 45 (unpublished data). In a prospective study performed on healthy men (Physicians' Health Study), the prevalence of factor V Leiden in the subjects who developed venous thromboembolism after 40 years of age was 11.6%.[80] That is in sharp contrast to congenital deficiencies of antithrombin III, protein C and protein S, which are very rarely associated with thrombotic onset after 40-45 years of age.[8-11]

Prevalence of APC-resistance in the general population

The high prevalence of APC-resistance in patients with venous thromboembolism in part reflects the distribution of the abnormal factor V allele in the general population; among healthy controls 2.5% were found to be APC-resistant (cumulative analysis of 2120 subjects) (Table 3a). As a rule, control populations from Southern Europe (France, Italy, Austria, Spain) seem to have a lower prevalence of APC-resistance than controls from Northern Europe (Netherlands, United Kingdom, Sweden) (1.3-2.8% vs. 4.6-6.9%). Studies on healthy controls performed by factor V gene analysis support these data, showing the frequency of indivi-

Table 2.
A) Prevalence of APC-resistance in patients with venous thrombotic disease. Studies not validated by factor V gene analysis.

	Ref.	*n pts.*	*selection criteria*	*n APC-resistant individuals*	*kindreds*
Griffin et al	(33)	25	yes	16 (64%)	n.i.
Svensson et al	(32)	104	yes	34 (33%)	34/45
Koster et al	(34)	301	yes	64 (21%)	10/11
Legnani et al	(41)	261	yes	26 (10%)	n.i.
Faioni et al	(58)	106	yes	35 (33%)	n.i.
Halbmayer et al	(59)	40	yes	7 (17%)	2/2
Cadroy et al	(71)	84	yes	9 (19%)	n.i.
Tosetto et al	(72)*	20	no	2 (10%)	n.i.
Cushman et al	(73)	37	yes	9 (24%)	n.i.
Lopaciuk et al	(74)*	72	yes	9 (12%)	n.i.
Borrell et al	(75)*	72	no	3 (4%)	n.i.
Cumming et al	(76)*	36	yes	3 (8%)	n.i.
Pickering et al	(77)*	84	no	5 (5%)	n.i.
De Stefano et al	(78)	118	yes	33 (28%)	15/15
Total					
consecutive patients	176			10 (5%)	
selected patients	1184			245 (20%)	

*n.i.: not investigated; * : abstract paper.*

B) Prevalence of APC-resistance in patients with venous thrombotic disease. Studies validated by factor V gene analysis.

Bertina et al	(17)	301	yes	53 (17%)	
Alhenc-Gelas et al	(28)	87	no	14 (16%)	
Voorberg et al	(30)	27	no	10 (37%)	
Ma et al	(79)*	30	yes	7 (23%)	
Ridker et al	(80)	121	yes	14 (11%)	
Total					
consecutive patients	114			24 (21%)	
selected patients	452			74 (16%)	

**: abstract paper.*

Table 3.
A) Prevalence of APC-resistance in the healthy population. Studies not validated by factor V gene analysis.

	Ref.	n individuals tested	Country	n APC-resistant individuals	
Svensson et al	(32)	130	Sweden	9	(6.9%)
Koster et al	(34)	301	Netherlands	14	(4.6%)
Halbmayer et al	(59)	50	Austria	1	(2.0%)
Simioni et al	(64)	55	Italy	0	
Cadroy et al	(71)	75	France	1	(1.3%)
Tosetto et al	(72)*	1212	Italy	20	(1.6%)
Cushman et al	(73)	39	USA	2	(5.1%)
Lopaciuk et al	(74)*	110	Poland	1	(0.9%)
Borrell et al	(75)*	107	Spain	3	(2.8%)
Cumming et al	(76)*	41	United Kingdom	2	(4.9%)
Total		2120		53	(2.5%)

*: abstract paper.

B) Prevalence of APC-resistance in the healthy population. Studies validated by factor V gene analysis.

Bertina et al	(17)	164	Netherlands	6	(3.6%)
De Ronde et al	(35)	100	Netherlands	4	(4.0%)
Ridker et al	(80)	704	USA	42	(6.0%)
Catto et al	(81)*	247	United Kingdom	14	(5.6%)
Beauchamp et al	(82)	144	United Kingdom	5	(3.4%)
Emmerich et al	(83)	168	Belfast (UK)	10	(5.9%)
ibidem		147	Lille (F)	1	(0.6%)
ibidem		176	Strasbourg (F)	17	(9.6%)
ibidem		201	Toulouse (F)	6	(2.9%)
Witt et al	(84)*	196	Germany	8	(4.3%)
Prohaska et al	(85)*	202	Germany	16	(7.9%)
Kontula et al	(86)	137	Finland	4	(2.9%)
Soubrier et al	(87)	229	Paris (F)	5	(2.2%)
ibidem		373	Nancy (F)	32	(8.6%)
Total		3188		170	(5.3%)

*abstract paper.

duals with an abnormal genotype to be 0.6-2.9% in France and 3.4-5.9% in the United Kingdom and the Netherlands; in the U.S. the prevalence among (white) individuals is 6.0%. However, a remarkable cluster of factor V Leiden carriers (8.6-9.6% of the overall population) has been reported in Central Europe, namely in Alsace (Table 3b). A founder effect was demonstrated in the Dutch population in carriers and non-carriers of the Leiden mutation.[17]

Linkage to a genetic advantage of some kind could be hypothesized to explain the fact that the abnormal factor V allele is maintained at such a frequency in the general population; so far only speculative explanations have been attempted.[26,27,34]

Association of APC-resistance with other congenital plasma deficiencies

The high frequency of APC-resistance in thrombotic patients and in healthy controls raises the question of whether this mutation may be associated with other thrombophilic traits, possibly accounting for different degrees of clinical penetrance as noticed, for instance, in protein C deficiency.[89,90]

Support for this hypothesis was provided by the presence of two independently inherited deficiencies, of protein C and protein S, respectively, in the first two kindreds extensively analyzed for the factor V Leiden mutation.[17,18] In both families clinical penetrance of the thrombotic tendency was more severe in individuals having both defects than in those with only one.[17,18] A factor V Leiden mutation was found in from 10 to 19% of protein C-deficient probands.[91,92] Analysis of 77 individuals showed a significantly higher prevalence of thrombotic disease (73%) in subjects with both mutations than in those with only protein C deficiency or factor V mutation (31 and 13%, respectively).[91] According to other reports, the association of APC-resistance with other thrombophilic plasma abnormalities (antithrombin III, protein C, protein S, heparin cofactor II deficiencies or reduced fibrinolytic activity) is not uncommon;[31,33,36,67,73,74,93] several studies on patient cohorts previously diagnosed as having a thrombophilic plasma abnormality displayed a prevalence of APC-resistance ranging from 5 to 31%.[33,67,73] Yet Simioni *et al.*[64] using plasma did not find the APC-resistance trait assay in any of 113 individuals belonging to 23 families with antithrombin III, protein C or protein S deficiency. In our experience genetic analysis failed to find factor V Leiden in thrombotic probands belonging to 12 different kindreds with antithrombin III or protein C deficiency identified in our center (Lane and Ireland, personal communication). Moreover, in two large series (n=405) of patients unselected with respect to a previous diagnosis of plasma abnormalities, an associated protein C deficiency was found in only one of 98 individuals identified as APC-resistant (1%),[32,34] with a frequency of double deficiencies comparable to that found in other series.[7,9] Thus, the true frequency of com-

bined APC-resistance and other thrombophilic defects remains to be established and is probably strongly influenced by various factors.

APC-resistance and arterial thromboembolism

Lindblad *et al.*[94] first reported a case of a 32-year-old man with arterial occlusion and APC-resistance who died from pulmonary embolism after a vascular reconstruction operation. Two other young patients with myocardial infarction were reported to be homozygous for APC-resistance.[95] In a study by Halbmayer *et al.*[59] the prevalence of APC-resistance in arterial thrombosis patients was comparable to that observed in subjects with venous thromboembolism (20%), whereas according to Cushman *et al.*[73] the prevalence of this defect in arterial patients did not differ from that of the control group (4%) (Table 4a). All the studies conducted so far utilizing factor V gene analysis in patients with ischemic stroke[80,86] or myocardial infarction[80,83,86,88] have reported that the frequency of the factor V Leiden mutation did not differ from that of the control group (Table 4b). Thus, at present APC- resistance does not seem to be a risk factor for arterial thrombotic disease, at least in heterozygous individuals.

Clinical manifestations in individuals with APC-resistance

A study by Svensson *et al.*[32] on 211 individuals with APC-resistance belonging to 34 different kindreds stated that the overall rate of thrombotic disease was 23% (49/211). Excluding the index patients and considering only the individuals diagnosed during family investigation, the rate of thrombosis was considerably lower (8%, 15/177); nevertheless, thrombosis-free survival in these subjects was significantly different from that of the unaffected relatives, confirming that APC-resistance is a risk factor for thrombosis. The overall probability of thrombosis at age 45 was 41%.[32] In a subsequent investigation validated by factor V gene analysis the authors were able to confirm these data, since 31% of the affected individuals (51/162) presented a history of thrombosis; after exclusion of the index patients, the rate of thrombosis was 16% (22/133).[31] In our experience with 40 individuals

Table 4.

A) Prevalence of APC-resistance in patients with arterial thrombo tic disease. Studies not validated by factor V gene analysis. Presence of lupus anticoagulant was ruled out only in the study by Halbmayer et al (59).

	Ref.	*n pts.*	*selection criteria*	*APC-resistant individuals*	*kindreds*
Halbmayer et al	(54)	30	yes	6 (20%)	1/1
Cushman et al	(67)	44	yes	2 (4%)	n.i.
Total		74		8 (10%)	

B) Prevalence of APC-resistance in patients with arterial thrombo- tic disease. Studies validated by factor V gene analysis.

Ridker et al	(80)	583	yes	32 (5%)	
Catto et al	(81)*	348	not specified	15 (4%)	
Emmerich et al	(83)	609	yes	34 (5%)	
Witt et al	(84)*	224	yes	21 (9%)	
Prohaska et al	(85)*	300	not specified	21 (7%)	
Kontula et al	(86)	358	yes	16 (4%)	
Samani et al	(88)	60	yes	2 (3%)	
Total		2482		141 (6%)	

**: abstract paper.*

with APC-resistance belonging to 15 unrelated kindreds, 20 (50%) showed a history of thrombosis; after exclusion of the index patients, the percentage of relatives having suffered thrombosis was 20% (5/25). In a large multicenter Italian survey, individuals with antithrombin III, protein C or protein S deficiencies taken together demonstrated a history of thrombosis in 54% of the cases; this percentage dropped to 33% after exclusion of the index patients.[6] Thus, APC-resistance is probably somewhat less thrombogenic than antithrombin III, protein C and protein S deficiencies. In a direct analysis the thrombosis-free survival of APC-resistant subjects at age 45 compared favorably to that found in the other plasma abnormalities (70% vs. 57%, 47% and 44% for antithrombin III, protein C and protein S deficient subjects, respectively).[96] The most frequent clinical manifestation is deep vein thrombosis of the lower limbs, with or without pulmonary embolism,[32,96] as in the other inherited plasma defects predisposing to thrombosis.[6] In our series this clinical condition accounted for about 70% of first events; splanchnic vein thrombosis was quite rare, in agreement with other series.[32,96,97] Perhaps these data were biased by the exclusion of individuals on long-term oral anticoagulants from the APC- resistance as-

say, probably underscoring the rather severe clinical manifestations found in the study group.

In a series of 51 patients arterial thrombosis occurred in 7 subjects (13%), but only in one before age 45.[31] In 62% of the 51 cases the first thrombotic episode was associated with a triggering factor.[31] We found a higher percentage (80%) of clinical onset related to triggering situations (in most cases pregnancy or oral contraceptives) in a series of 20 APC-resistant patients with proven inheritance. Thus, the percentage of spontaneous episodes seems to be lower than in antithrombin III, protein C and protein S deficiencies, where thrombosis can develop in about half of the cases with no known associated risk factor.[6] This would be in agreement with the supposedly lower clinical penetrance of APC-resistance.

From an analysis of the outcome of 57 pregnancies in 27 APC-resistant women, we established a prevalence of thrombotic complications during pregnancy and puerperium of 28%, a rate 400 times higher than that found in the general population; most of the episodes (62%) occurred during puerperium.[78]

The relevance of concomitant risk factors as triggering causes of thrombosis in APC-resistance can also be inferred from a recent study demonstrating an increased trombotic risk in affected women using oral contraceptives.[98] It would therefore appear that once the APC-resistance trait has been diagnosed, special care must be taken to prevent thromboses associated with risk situations by adopting measures previously shown to modify the natural outcome of other plasma abnormalities.[6]

The homozygous condition for mutated factor V has been reported to carry a greater thrombotic risk than the heterozygous state: in homozygotes the thrombosis-free survival curve showed a probability of thrombosis at age 33 double that of heterozygotes (40% vs. 20%). A history of thrombosis was present in 44% of homozygotes and in 30% of heterozygotes.[31] Varying severity of clinical manifestations was reported in homozygous siblings belonging to the same kindred;[99] nevertheless, for now the homozygous state should be considered at higher risk in counselling practice.[31,98]

Conclusions

Identification of the factor V mutation underlying APC-resistance ahs led to entirely new insights into the genesis of thrombosis. This condition is now recognized to be associated with about 20% of all unexplained venous thromboses and is present in 2% to 6% of healthy individuals.

Plasma assay can be misleading in some cases and criteria for diagnosis of APC-resistance need further improvement and standardization. In the near future a diagnostic approach utilizing factor V gene analysis will probably become much more widespread; however, simplicity of execution and the evidence of a small number of kindreds with inherited factor V-unrelated APC-resistance argue in favor of retaining the plasma assay as the first diagnostic step. Anticoagulant treatment or evidence of antiphospholipid antibodies render the plasma assay unreliable and make gene analysis the only valid diagnostic approach in such patients; modified plasma assays have been proposed to circumvent these limitations but they need to be verified in larger studies. More clinical experience is needed to establish the thrombotic risk related to APC-resistance as well as to provide guidelines for the management of affected hetero- and homozygous subjects. At present it is advisable to adopt tentative criteria similar to the ones employed for the management of other congenital thrombophilic defects (antithrombin III, protein C, protein S deficiencies), namely giving asymptomatic subjects short-term antithrombotic prophylaxis only during risk situations, and prescribing indefinite oral anticoagulation for patients who suffer spontaneous and/or recurrent major thrombotic episodes. Further clinical studies are needed to establish possible differences in this approach related to the presence of a heterozygous or homozygous genotype.

References

1. Jordan FLJ, Nandorff A. The familial tendency in thromboembolic disease. Acta Med Scand 1956; 156:267-75.
2. Mannucci PM, Tripodi A. Laboratory screening of inherited thrombotic syndromes. Thromb Haemostas 1987; 57:247-51.
3. Lane Da, Olds RR, Thein SL. Antithrombin and its deficiency states. Blood Coag Fibrinol 1992; 3:315-41.

4. Dolan G, Ball J, Preston FE. Protein C and protein S. In: Tuddenham EGD, ed. The molecular biology of coagulation. Baillièrés Clin Hematol 1989; 2:999-1042.
5. Tripodi A, Mannucci PM and the *ad hoc* Study Group. A survey of inherited thrombotic syndromes in Italy. Res Clin Lab 1989; 19:67-74.
6. De Stefano V, Leone G, Mastrangelo S, et al. Clinical manifestations and management of inherited thrombophilia; retrospective analysis and follow-up after diagnosis of 238 patients with congenital deficiency of antithrombin III, protein C, protein S. Thromb Haemostas 1994; 72:352-8.
7. Heijboer H, Brandjes DPM, Buller HR, Sturk A, ten Cate JW. Deficiencies of coagulation-inhibiting and fibrinolytic proteins in outpatients with deep-vein thrombosis. N Engl J Med 1990; 323:1512-6.
8. Tabernero MD, Tomas JF, Alberca I, Orfao A, Borrasca AL, Vicente V. Incidence and clinical characteristics of hereditary disorders associated with venous thrombosis. Am J Hematol 1991; 36:249-54.
9. Pabinger I, Brucker S, Kyrle PA, et al. Hereditary deficiency of antithrombin III, protein C and protein S: prevalence in patients with a history of venous thrombosis and criteria for rational patient screening. Blood Coag Fibrinol 1992; 3:547-53.
10. Melissari E, Monte G, Lindo VS, et al. Congenital thrombophilia among patients with venous thromboembolism. Blood Coag Fibrinol 1992; 3:749-58.
11. Malm J, Laurell M, Nilsson IM, Dahlback B. Thromboembolic disease. Critical evaluation of laboratory investigation. Thromb Haemostas 1992; 68:7-13.
12. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci USA 1993; 90:1004-8.
13. Dahlback B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. Proc Natl Acad Sci USA 1994; 91:1396-400.
14. Shen L, Dahlback B. Factor Va and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. J Biol Chem 1994; 269:18735-8.
15. Varadi K, Rosing J, Tans G, Schwarz HP. Influence of factor V and factor Va on APC-induced cleavage of human factor VIII. Thromb Haemostas 1995; 73:730-1.
16. Sun X, Evatt B, Griffin JH. Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. Blood 1994; 83:3120-5.
17. Bertina RM, Koeleman BPC, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369:64-7.
18. Zoller B, Dahlback B. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. Lancet 1994; 343:1536-8.
19. Wang H, Ridell DC, Guinto ER, MacGillivray RTA, Hamerton JL. Localization of the gene encoding human factor V gene to chromosome 1q21-25. Genomics 1988; 2:324-8.
20. Kane WH, Davie EW. Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. Blood 1988; 71:539-55.
21. Kalafatis M, Mann KG. Role of the membrane in the inactivation of factor Va by activated protein C. J Biol Chem 1993; 268:27246-57.
22. Odegaard B, Mann KG. Proteolysis of factor Va by factor Xa and activated protein C. J Biol Chem 1987; 262:11233-8.
23. Guinto ER, Esmon CT. Loss of prothrombin and of factor Xa-factor Va interactions upon inactivation of factor Va by activated protein C. J Biol Chem 1984; 259:13986-92.
24. Krishnaswamy S, Williams EB, Mann KG. The binding of activated protein C to factors V and Va. J Biol Chem 1986; 261: 9684-93.
25. Walker FJ, Scandella D, Fay PJ. Identification of the binding site for activated protein C on the light chain of factors V and VIII. J Biol Chem 1990; 265:1484-9.
26. Dahlback B. Physiological anticoagulation. Resistance to activated protein C and venous thromboembolism. J Clin Invest 1994; 94:923-7.
27. Dahlback B. Inherited thrombophilia: resistance to activated protein C as a pathogenetic factor of venous thromboembolism. Blood 1995; 85:607-14.
28. Alhenc-Gelas M, Gandrille S, Aubry M-L, Emmerich J, Fiessinger J-N, Aiach M. Unexplained thrombosis and factor V Leiden mutation. Lancet 1994; 344:555-6.
29. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet 1994; 343:1361-2.
30. Voorberg J, Roelse J, Koopman R, et al. Association of idiopathic venous thromboembolism with single-point mutation at Arg506 of factor V. Lancet 1994; 343:1535-6.
31. Zoller B, Svensson PJ, He X, Dahlback B. Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. J Clin Invest 1994; 94:2521-4.
32. Svensson PJ, Dahlback B. Resistance to activated protein C as a basis for venous thrombosis. N Engl J Med 1994; 330:517-22.
33. Griffin JH, Evatt B, Wideman C, Fernandez JA. Anticoagulant protein C pathway defective in majority of thrombophilic patients. Blood 1993; 82:1989-93.
34. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 1993; 342:1503-6.
35. De Ronde H, Bertina RM. Laboratory diagnosis of APC- resistance: a critical evaluation of the test and the development of diagnostic criteria. Thromb Haemostas 1994; 72:880-6.
36. Legnani C, Palareti G, Biagi R, Coccheri S. Activated protein C resistance in deep-vein thrombosis. Lancet 1994; 343:541-2.
37. Baker R, Thom J, van Bockxmeer. Diagnosis of activated protein C resistance (factor V Leiden). Lancet 1994; 344:1162.
38. Girolami A, Simioni P, Scarano L. Evaluation of activated protein C resistance in stored plasma. Lancet 1994; 343: 1288-9.
39. Jones DW, Winter M. Activated protein C resistance is affected by freezing and thawing (Abstract). Br J Haematol 1994; 86(suppl 1):32.
40. Trossaert M, Conard J, Horellou MH, Samama MM. Influence of storage conditions on activated protein C resistance assay. Thromb Haemostas 1995; 73:163-4.
41. Legnani C, Palareti G, Biagi R, Coccheri S. Evaluation of activated protein C resistance in stored plasma. Author's reply. Lancet 1994; 343:1289.
42. Dahlback B. Resistance to activated protein C, the Arg506 to Gln mutation in the factor V gene, and venous thrombosis. Functional tests and DNA-based assays, pros and cons. Thromb Haemostas 1995; 73:739-42.
43. Rosén S, Johansson K, Lindberg K, Dahlback B (for the APC resistance study group). Multicenter evaluation of a kit for activated protein C resistance on various coagulation instruments using plasmas from healthy individuals. Thromb Haemostas 1994; 72:255-60.
44. De Stefano V, Paciaroni K, Mastrangelo S, Bizzi B, Leone G. Instrument effect on the activated protein C-resistance plasma assay performed by a commercial kit. Thromb Haemostas, in press.

45. Walker ID, Hickman GM, Stevenson C, McCall F. High prevalence of "APC resistance" in the presence of antiphospholipids (abstract). Br J Haematol 1994; 86 (suppl 1):2.
46. Cooper PC, Hampton KK, Abuzenadah A, Malia RG, Preston FE, Greaves M. Activated protein C resistance in antiphospholipid-containing plasma: a possible mechanism of thrombosis (abstract). Br J Haematol 1994; 86(suppl 1):2.
47. Hampton KK, Preston FE, Greaves M. Resistance to activated protein C. N Engl J Med 1994; 331:130.
48. Ruggeri M, Tosetto A, Rodeghiero F. High prevalence of APC-resistance in patients with anti-phospholipid antibodies. Relationship with cerebral vascular disease (abstract). Br J Haematol 1994; 87(suppl 1):185.
49. Halbmayer W-M, Haushofer A, Schon R, Fischer M. Influence of lupus anticoagulant on a commercially available kit for APC-resistance. Thromb Haemostas 1994; 72:645-6.
50. Bokarewa MI, Bremme K, Falk G, Sten-Linder M, Egberg N, Blomback M. Studies on phospholipids antibodies, APC- resistance and associated mutation in the coagulation factor V gene. Thromb Res 1995; 78:193-200.
51. Marciniak E, Romond EH. Impaired catalytic function of activated protein C: a new *in vitro* manifestation of lupus anticoagulant. Blood 1989; 74:2426-32.
52. Bokarewa MI, Blomback M, Egberg N, Rosén S. A new variant of interaction between phospholipid antibodies and the protein C system. Blood Coag Fibrinol 1994; 5:37-41.
53. The Le D, Griffin JH, Greengard JS, Mujumdar V, Rapaport SI. Use of a generally applicable tissue factor-dependent factor V assay to detect activated protein C-resistant factor Va in patients receiving warfarin and in patients with a lupus anticoagulant. Blood 1995; 85:1704-11.
54. Jorquera J, Montoro JM, Angeles Fernandez M, Aznar JA, Aznar J. Modified test for activated protein C resistance. Lancet 1994; 344:1162-3.
55. Trossaert M, Conard J, Horellou MH, et al. Modified APC resistance assay for patients on oral anticoagulants. Lancet 1994; 344:1709.
56. Tosetto A, Rodeghiero F. Diagnosis of APC-resistance in patients on oral anticoagulant therapy. Thromb Haemostas 1995; 73:732-3.
57. Cadroy Y, Sié P, Alhenc-Gelas M, Aiach M. Evaluation of APC resistance in the plasma of patients with Q506 mutation of factor V (Factor V Leiden) and treated by oral anticoagulants. Thromb Haemostas 1995; 73:734-5.
58. Faioni EM, Franchi F, Asti D, Sacchi E, Bernardi F, Mannucci PM. Resistance to activated protein C in nine thrombophilic families: interference in a protein S functional assay. Thromb Haemostas 1993; 70:1067-71.
59. Halbmayer WM, Haushofer A, Schon R, Fischer M. The prevalence of poor anticoagulant response to activated protein C (APC-resistance) among patients suffering from stroke or venous thrombosis and among healthy subjects. Blood Coag Fibrinol 1994; 5:51-7.
60. Faioni EM, Boyer-Neumann C, Franchi F, Wolf M, Meyer D, Mannucci PM. Another protein S functional assay is sensitive to resistance to activated protein C. Thromb Haemostas 1994; 72:648.
61. Cooper PC, Hampton KK, Makris M, Abuzenadah A, Paul B, Preston FE. Further evidence that activated protein C resistance can be misdiagnosed as inherited functional protein S deficiency. Br J Haematol 1994; 88:201-3.
62. Viganò D'Angelo S, Mazzola G, Della Valle P, Testa S, Pattarini E, D'Angelo A. Variable interference of activated protein C resistance in the measurement of protein S activity by commercial assays. Thromb Res 1995; 77:375-8.
63. Ireland H, Bayston T, Thompson E, et al. Apparent heterozygous type II protein C deficiency caused by the factor V 506 Arg to Gln mutation. Thromb Haemostas 1995; 73:731-2.
64. Simioni P, Prandoni P, Girolami A. Patients with AT III, protein C or protein S defects show no associated hereditary APC-resistance. Thromb Haemostas 1994; 72:481-2.
65. Colucci M, Ciavarella N, Giliberti MG, Semeraro N. Resistance to activated protein C (APC): influence of factor V levels. Thromb Haemostas 1994; 72:987-8.
66. Simioni P, Girolami A. Homozygous factor V-deficient patients show resistance to activated protein C whereas heterozygotes do not. Blood Coag Fibrinol 1994; 5:825-7.
67. Faioni EM. Resistenza alla proteina C attivata: una nuova causa di trombofilia ereditaria. XIII Congresso Società Italiana per lo Studio dell'Emostasi e della Trombosi, Lanciano 1994. Relazioni, pp. 99-110.
68. Vasse M, Leduc O, Borg J-Y, Chrétien MH, Monconduit M. Resistance to activated protein C: evaluation of three functional assays. Thromb Res 1994; 76:47-59.
69. Henkens CMA, Bom VJJ, Seinen AJ, van der Meer J. Resistance to activated protein C: influence of gender and oral contraceptives? (abstract) Haemostasis 1994; 24(suppl 1):143.
70. Osterud B, Robertsen R, Asvang GB, Thijssen F. Resistance to activated protein C is reduced in women using oral contraceptives. Blood Coag Fibrinol 1994; 5:853-4.
71. Cadroy Y, Sié P, Boneu B. Frequency of a defective response to activated protein C in patients with a history of venous thrombosis. Blood 1994; 83:2008-9.
72. Tosetto A, Gatto E, Masiero MT, Rodeghiero F. APC-resistance in the general population. Relationship with other coagulation variables and with venous thrombotic history. (abstract) Br J Haematol 1994; 87(suppl 1):188.
73. Cushman M, Bhushan F, Bovill E, Tracy R. Plasma resistance to activated protein C in venous and arterial thrombosis. Thromb Haemostas 1994; 72:647.
74. Lopaciuk S, Bykowska K. Prevalence of poor anticoagulant response to activated protein C in patients with a history of venous thrombosis. (abstract) Haemostasis 1994; 24(suppl 1):141.
75. Borrell M, Vallvé C, Urrutia T, et al. APC resistance in patients with thrombophilia (abstract). Haemostasis 1994; 24(suppl 1):145.
76. Cumming AM, Fildes S, Pylypczuk CC, et al. Incidence of reduced anticoagulant response to activated protein C in patients with venous thrombosis (abstract). Br J Haematol 1994; 87(suppl 1):178(abs).
77. Pickering W, Dixit M, Campbell P, Cohen H. Prevalence of activated protein C resistance and other thrombophilic defects in patients with venous thromboembolism (abstract). Br J Haematol 1994; 86(suppl 1):68(abs).
78. De Stefano V, Mastrangelo S, Paciaroni K, et al. Thrombotic risk during pregnancy and puerperium in women with APC-resistance. Effective subcutaneous heparin prophylaxis in a pregnant patient. Thromb Haemostas, in press.
79. Ma DDF, Williams BG, Aboud MR, Isbister JP. Factor V missense mutation and APC-resistance in patients with venous thromboembolism (abstract). Blood 1994; 84 (suppl 1):84a.
80. Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Eisenberg PR, Miletich JP. Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke, and venous thrombosis in apparently healthy men. N Engl J Med 1995; 332:912-7.
81. Catto AJ, Carter AM, Ireland H, et al. Incidence of the factor V Leiden gene mutation and prothrombin fragment F1+2 in acute stroke (abstract). Blood Coag Fibrinol 1994; 5:807.
82. Beauchamp NJ, Daly ME, Hampton KK, Cooper PC, Preston FE, Peake IR. High prevalence of a mutation in the factor V gene within the U.K. population: relationship to activated protein C resistance and familial thrombosis. Br J Haematol 1994; 88:219-22.
83. Emmerich J, Poirier O, Evans A, et al. Myocardial infarction,

Arg 506 to Gln factor V mutation, and activated protein C resistance. Lancet 1995; 345:321.
84. Witt I, Seydewitz HH, Marz W, Winckelmann BR, Wieland H. Increased frequency of the factor V Leiden variant in patients with coronary artery disease (abstract). Ann Hematol 1995; 70 (suppl 1):1.
85. Prohaska W, Mannebach H, Gleichmann U, Kleesiek K. Frequency of the factor V 1691 G→A mutation with resistance to active protein C in blood donors and patients with coronary artery disease (abstract). Ann Hematol 1995; 70(suppl 1):27.
86. Kontula K, Ylikorkala A, Miettinen H, et al. Arg506Gln factor V mutation (Factor V Leiden) in patients with ischaemic cerebrovascular disease and survivors of myocardial infarction. Thromb Haemostas 1995; 73:558-60.
87. Soubrier F, Fery I, Verdy E, et al. The frequency of the factor V gene R506Q mutation varies between regions of France. Nouv Rev Fr Hematol 1995; 37:175.
88. Samani NJ, Lodwick D, Martin D, Kimber P. Resistance to activated protein C and risk of premature myocardial infarction. Lancet 1994; 344:1709-10.
89. Miletich J, Sherman L, Broze GJ Jr. Absence of thrombosis in subjects with heterozygous protein C deficiency. N Engl J Med 1987; 317:991-6.
90. Allaart CF, Poort SR, Rosendaal FR, Reitsma PH, Bertina RM, Briet E. Increased risk of venous thrombosis in carriers of hereditary protein C deficiency. Lancet 1993; 341:134-8.
91. Koeleman BPC, 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: 1031-5.
92. Ireland H, Bayston T, Thompson E, Lane DA, Finazzi G, Barbui T. Association between protein C deficiency and factor V gene mutation (abstract). Blood Coag Fibrinol 1994; 5:806.
93. Zoller B, He X, Dahlback B. Homozygous APC-resistance combined with inherited type I protein S deficiency in a young boy with severe thrombotic disease. Thromb Haemostas 1995; 73:743-5.
94. Lindblad B, Svensson PJ, Dahlback B. Arterial and venous thromboembolism with fatal outcome and resistance to activated protein C. Lancet 1994; 343:917.
95. Holm J, Zoller B, Svensson PJ, Berntorp E, Erhardt L, Dahlback B. Myocardial infarction associated with homozygous resistance to activated protein C. Lancet 1994; 344:952-3.
96. Martinelli I, Faioni EM, Monzani ML, Mannucci PM. Inherited resistance to activated protein C (APC): clinical and laboratory features (abstract). Blood 1994; 84 (suppl 1):84a.
97. Levoir D, Emmerich J, Alhenc-Gelas M, et al. Portal vein thrombosis and factor V Arg 506 to Gln mutation. Thromb Haemostas 1995; 73:550-1.
98. Vandenbroucke JP, Koster T, Briet E, Reitsma PH, Bertina RM, Rosendaal FR. Increased risk of venous thrombosis in oral contraceptive users who are carriers of factor V Leiden mutation. Lancet 1994; 344:1453-7.
99. Greengard JS, Eichinger S, Griffin JH, Bauer KA. Brief report: variability of thrombosis among homozygous siblings with resistance to activated protein C due to an Arg→Gln mutation in the gene for factor V. N Engl J Med 1994; 331:1559-62.