

Resistance to Activated Protein C Caused by the R⁵⁰⁶Q Mutation in the Factor V Gene as a Basis of Venous Thrombosis

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Procoagulant factors, which activate platelets and initiate blood coagulation, are exposed upon vascular damage.^(1,2) In a series of sequential proteolytic reactions, proenzymes are activated, culminating in the formation of thrombin. Several of the reactions are greatly enhanced by non-enzymatic cofactors, which together with the enzymes are assembled into highly efficient and specific proteolytic complexes on the surface of procoagulant phospholipids.⁽³⁾ The protein C anticoagulant pathway regulates the activity of the procoagulant cofactors, factor Va (FVa) and factor VIIIa (FVIIIa).⁽⁴⁻⁷⁾ Protein C, which is a zymogen to a serine protease, is activated by thrombin when it is bound to the endothelial cell membrane protein thrombomodulin.⁽⁷⁾ In the degradation of FVa and FVIIIa by activated protein C (APC), vitamin K-dependent protein S functions as a non-enzymatic cofactor.^(5,6,8) In human plasma the concentration of protein S is approximately 0.3 μ M (20–25 mg/l), and it circulates both as free protein (approximately 40%) and bound to C4b-binding protein, a regulator of the complement system.⁽⁵⁾ Only the free form of protein S is active as an APC-cofactor. Recently, intact FV was discovered to be a second APC-cofactor.^(9,10) Thus, FV appears to function as an anticoagulant protein under normal conditions. It is converted into a potent procoagulant cofactor upon activation of the coagulation system. Just like the transformation that thrombin undergoes upon its binding to thrombomodulin, this dual capacity of FV is an ingenious means of balancing pro- and anti-coagulant forces.

Heterozygous deficiency of protein C is associated with thromboembolic diseases.^(5,6) In its homozygous form, protein C deficiency manifests very severe, often fatal, thrombosis already in the neonatal period. The association between protein S deficiency and venous thrombosis supports the concept that protein S is a physiologically important natural anticoagulant. Many mutations causing quantitative or qualitative deficiencies of protein C and protein S have been described.^(11,12)

APC-Resistance as a Basis of Venous Thrombosis

Until recently, fewer than 10% of venous thrombosis patients have been found to carry genetic defects of coagulation inhibitors such as antithrombin III, protein S or protein C.^(5,13) In 1993, this changed with the description of APC-resistance as a previously unrecognized cause of thrombophilia.⁽¹⁴⁾ In normal plasma, the clotting time increases with increasing concentrations of added APC, whereas a poor anticoagulant response to APC is manifested in APC-resistant plasma. The APC-resistance test is now a standard assay, which is used in the evaluation of thrombosis patients. In its most common form, the assay is a modification of a standard activated partial thromboplastin time (APTT) reaction. Two APTT reactions are performed, one of which includes a carefully standardized amount of added APC, and results are expressed as the ratio of the values obtained with the two APTT tests.^(15,16) Among patients suffering from venous

thromboembolism, the APC-resistance phenotype is the most common laboratory abnormality found.^(13,15-20)

The R⁵⁰⁶Q Mutation in the Factor V Gene as a Cause of APC-Resistance

Isolated FV from normal plasma was found to be able to correct the APC-resistance phenotype which led to the identification of the molecular mechanism of APC-resistance.⁽⁹⁾ In more than 90% of patients with inherited APC-resistance, a single point mutation in the FV gene—a G to A substitution at nucleotide position 1691 predicting replacement of arginine (R) at position 506 with a glutamine (Q)—is found.⁽²¹⁻²⁵⁾ Arginine 506 constitutes one of three APC-cleavage sites in the heavy chain of normal FVa, and the mutation leads to a reduction in the rate of inactivation of FVa because APC cleaves less efficiently at arginine 306 and arginine 679.⁽²⁶⁻³⁰⁾ The mutation does not affect the activation of FV to FVa, and FVa:Q⁵⁰⁶ expresses normal procoagulant activity. The hypercoagulability associated with APC-resistance is due to stabilization of the prothrombinase complex and increased thrombin formation as a result of the decreased rate of APC-mediated degradation.⁽³¹⁾

The prevalence of the FV:Q⁵⁰⁶ allele is between 2% and 15% in Western societies, but it varies widely depending on the geographic location and the ethnic background of the population.^(21,32-35) The highest frequencies of heterozygosity for FV:Q⁵⁰⁶ have been observed in certain regions of southern Sweden⁽³⁶⁾ and in Greece.⁽³⁵⁾ The FV:Q⁵⁰⁶ allele is most probably the result of a founder effect.⁽²¹⁾ In this respect, it is noteworthy that the mutated allele is not found in certain populations, such as the Japanese, Chinese, and the aboriginal inhabitants of Australia, Africa and America. It is tempting to speculate that the relatively low incidence of thrombosis in some of these societies is related to the absence in these populations of the FV:Q⁵⁰⁶ allele. It is also tempting to speculate that the high prevalence of the FV:Q⁵⁰⁶ allele in some populations is due to a survival advantage conferred by the mutation during evolution. Possibly, a lower bleeding tendency after trauma or pregnancy may have been the positive survival factors that have been involved in spreading the mutated allele in the population.

Laboratory Determination of APC-Resistance and FV:Q⁵⁰⁶

So far, practical clinical experience has mainly been obtained with the APTT-based test for APC-resistance.⁽¹⁴⁾ The APTT-reaction is run in the presence and absence of a carefully standardized amount of APC, and the two clotting times are converted to an APC-ratio. If the assay is always done on the same instrument and also in other respects performed under strictly standardized conditions, the resulting APC-ratios can be used as they are.^(15,18) However, different instruments give different clotting times and APC-ratios obtained on one type of instrument cannot be directly compared with those from another type.⁽³⁷⁾ The quality of the results of the APC-resistance test depends on strict standardization. When all variables are strictly controlled, the APC-resistance test gives good discrimination between normal and APC-resistant individuals.^(15,18) The sensitivity and specificity of the APC-resistance test for FV:Q⁵⁰⁶ are 85–90% or better.^(21,25) Variation in plasma levels of protein C has no influence on the APC-ratio because a standardized amount of APC is added. In addition, variation of the endogenous free

protein S level within the range expected for normals and heterozygous protein S deficiency has no or only a very minor influence on the APC response in the APTT-based assay.^(15,38) For analysis of plasmas from individuals receiving oral anticoagulants, or heparin, the “classical” APC-resistance test is not reliable. The same is true if the plasmas are derived from individuals with other coagulation defects, such as lupus anticoagulants or coagulation factor deficiencies.^(15,38) In order to allow analysis of such plasmas, a modified APC-resistance, in which sample plasma is prediluted with factor V deficiency plasma before assay, was developed.^(39,40) This modification gives valid results not only for patients on oral anticoagulation but has been found also to provide an improved discrimination for the FV:Q⁵⁰⁶ mutation. In addition, this procedure makes the APC-resistance quite insensitive to differences in plasma handling before analysis. For some time this modification has been evaluated in our laboratory, using plasmas from individuals with or without oral anticoagulant therapy, and sensitivity and specificity for the presence of the FV:Q⁵⁰⁶ allele of 100% was obtained, irrespective of the plasma origin.

The codon for Arg⁵⁰⁶ is positioned close to the exon-intron boundary in exon 10 of the factor V gene. Determination of the G to A mutation involves amplification of this nucleotide region either from genomic DNA or from mRNA. The detection of the point mutation can be made in many ways, e.g., by nucleotide sequencing, by different hybridization techniques, by restriction enzyme cleavage or by allele-specific amplification. The methodology can be optimized to allow analysis of a large number of samples every day. The rate limiting step is usually the preparation of patient DNA, even though rather simple extraction procedures can be used. It is important to recognize the risk of contamination of PCR-based assays, and hence it is of utmost importance to organize the work carefully and include both positive and negative controls.

Both the APC-resistance test and the FV gene mutation analysis is required for optimal evaluation of a single patient, because the two methods provide complementary information. For economical and practical reasons this is not always possible. In our laboratory we perform the original and the modified APC-resistance tests in parallel and confirm positive samples with genotyping. This approach allows analysis of plasma from individuals on oral anticoagulant therapy and decreases the need for confirmatory genetic testing.

It is important to identify genetic risk factors in patients with venous thrombosis, but general screening for anticoagulant protein deficiencies has until now not been judged cost-effective. This is mainly due to the low prevalence of these defects. The high prevalence of APC-resistance in the population together with the clear relationship between APC-resistance and an increased risk of thrombosis call for an evaluation of whether it is worthwhile to screen for APC-resistance in association with hospitalization, surgery, usage of oral contraception, pregnancy, and other circumstantial risk situations.

Clinical Manifestations in APC-Resistance

The clinical manifestations of APC-resistance are similar to those of deficiencies of protein C, protein S or antithrombin III, deep venous thrombosis being the most common clinical problem. Thrombosis is more common among homozygotes than among

heterozygotes, and the first thrombotic event tends to occur at an earlier age in homozygotes than in heterozygotes.⁽²⁵⁾ This agrees well with findings that the risk of thrombosis is increased 5- to 10-fold in heterozygotes but 50- to 100-fold in homozygotes (as estimated from population data).^(21,41) The increased risk of thrombosis is lifelong and increases with increasing age.^(33,41)

The thrombotic tendency in APC-resistant individuals is influenced by acquired risk factors such as oral contraceptive usage, pregnancy, trauma and surgery.⁽²⁵⁾ The importance of the use of oral contraceptives^(42,43) and pregnancy^(43,44) as predisposing risk factors in FV:Q⁵⁰⁶ carriers has been shown in several studies. Carriership of the FV:Q⁵⁰⁶ allele does not seem to be a significant risk factor for arterial thrombosis.

Until recently, inherited thrombosis was regarded as a single gene disorder, but the identification of APC-resistance as an additional genetic risk factor in families with protein C deficiency and protein S deficiency of thrombosis demonstrated thrombosis to be a multi-genetic disease.^(21,24,45-48)

Management of APC-Resistant Patients

Most of our knowledge of the management of thrombophilic disorders derives from case reports, cross-sectional studies and uncontrolled clinical observations and this is true also for APC-resistance. For this reason, it is at present only possible to give general guidelines rather than specific recommendations. Symptomatic heterozygous and homozygous APC-resistant patients are probably best treated as patients with protein S, protein C or antithrombin III deficiency (reviewed in 49). Prophylactic therapy is given in risk situations, and oral contraceptive usage is avoided. Chronic anticoagulation is considered if thrombosis is recurrent and may be an option already after one episode if the patient has additional genetic risk factors, e.g., homozygosity for the FV:Q⁵⁰⁶ allele, or protein C, protein S and antithrombin III deficiency, or has suffered a life-threatening (mesenteric or cerebral) or spontaneous first thrombotic event. Heterozygous APC-resistant patients with an additional genetic defect and asymptomatic homozygous individuals are given liberal preventive therapy in risk situations. Prophylactic treatment is given to asymptomatic heterozygous individuals without family histories of thrombosis only in situations known to provoke thrombosis (e.g., major surgery). The patient is treated like an asymptomatic individual with protein S, protein C or antithrombin deficiency if there is a history of familial thrombophilia.

References

1. Davie EW: Biochemical and molecular aspects of the coagulation cascade. *Thromb Haemost* 74:1, 1995
2. Nemerson Y: Tissue factor: Then and now. *Thromb Haemost* 74:180, 1995
3. Kane WH, Davie EW: Blood coagulation factors V and VIII: Structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. *Blood* 71:539, 1988
4. Esmon CT: The protein C anticoagulant pathway. *Arterioscler Thromb* 12:135, 1992

5. Dahlbäck B, Stenflo J: The protein C anticoagulant system, in Stamatoyannopoulos G, Nienhuis AW, Majerus PW, Varmus H (eds): *The Molecular Basis of Blood Diseases*, Philadelphia, WB Saunders, 1994, p 599
6. Dahlbäck B: The protein C anticoagulant system: inherited defects as basis for venous thrombosis. *Thromb Res* 77:1, 1995
7. Esmon CT: Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. *FASEB J* 9:946, 1995
8. Arnljots B, Dahlbäck B: Protein S as an in vivo cofactor to activated protein C in prevention of microarterial thrombosis in rabbits. *J Clin Invest* 95:1987, 1995
9. Dahlbäck 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* 81:1396, 1994
10. Shen L, Dahlbäck B: Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *J Biol Chem* 269:18735, 1994
11. Reitsma PH, Bernardi F, Doig RG, Gandrille S, Greengard JS, Ireland H, Krawczak M, Lind B, Long GL, Poort SR, Saito H, Sala N, Witt I, Cooper DN: Protein C deficiency: A database of mutations, 1995 update. *Thromb Haemost* 73:876, 1995
12. Aiach M, Gandrille S, Emmerich J: A review of mutations causing deficiencies of antithrombin, protein C and protein S. *Thromb Haemost* 74:81, 1995
13. Dahlbäck B: Physiological anticoagulation. Inherited resistance to activated protein C and venous thromboembolism. *J Clin Invest* 94:923, 1994
14. Dahlbäck 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* 90:1004, 1993
15. Svensson PJ, Dahlbäck B: Resistance to activated protein C as a basis for venous thrombosis. *N Engl J Med* 330:517, 1994
16. Dahlbäck 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 Haemost* 73:739, 1995
17. Griffin JH, Evatt BL, Wideman C, Fernandez JA: Anticoagulant protein C pathway defective in a majority of thrombophilic patients. *Blood* 82:1989, 1993
18. Koster T, Rosendaal FR, de Ronde F, Briët E, Vandenbroucke JP, Bertina RM: Venous thrombosis due to poor response to activated protein C: Leiden thrombophilia study. *Lancet* 342:1503, 1993
19. Halbmayer WM, Haushofer A, Schön 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 Coagul Fibrinolysis* 5:51, 1994
20. Dahlbäck B: Inherited thrombophilia: resistance to activated protein C as a pathogenic factor of venous thromboembolism. *Blood* 85:607, 1995
21. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH: Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 369:64, 1994

22. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt BL: Activated protein C resistance caused by Arg506 Gln mutation in factor Va. *Lancet* 343:1362, 1994
23. Voorberg J, Roelse J, Koopman R, Büller H, Berends F, ten Cate JW, Mertens K, van Mourik JA: Association of idiopathic thromboembolism with single point mutation at Arg506 of factor V. *Lancet* 343:1535, 1994
24. Zöller B, Dahlbäck B: Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet* 343:1536, 1994
25. Zöller B, Svensson PJ, He X, Dahlbäck 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* 94:2521, 1994
26. Nicolaes GAF, Tans G, Thomassen MCLGD, Hemker HC, Pabinger I, Varadi K, Schwarz HP, Rosing J: Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *J Biol Chem* 270:21158, 1995
27. Sun X, Evatt BL, Griffin JH: Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. *Blood* 83:3120, 1994
28. Kalafatis M, Bertina RM, Rand MD, Mann KG: Characterization of the molecular defect in factor VR506Q. *J Biol Chem* 270:4053, 1995
29. Heeb MJ, Kojima Y, Greengard JS, Griffin JH: Activated protein C resistance: Molecular mechanisms based on studies using purified Gln506-factor V. *Blood* 85:3405, 1995
30. Aparicio C, Dahlbäck B: Molecular mechanisms of activated protein C resistance. Properties of factor V isolated from an individual with homozygosity for the Arg506 to Gln mutation in the factor V gene. *Biochem J* 313:467, 1996
31. Zöller B, Holm J, Svensson PJ, Dahlbäck B: Elevated levels of prothrombin activation fragment 1+2 in plasma from patients with inherited APC-resistance and/or protein S deficiency. *Thromb Haemost* (in press)
32. van Bockxmeer FM, Baker RI, Taylor RR: Premature ischaemic heart disease and the gene for coagulation factor V. *Nature Medicine* 1:185, 1995
33. 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* 332:912, 1995
34. Kontula K, Ylikorkala A, Miettinen H, Vuorio A, Kauppinenmakelin RK, Hamalainen L, Palomaki H, Kaste M: Arg506Gln factor V mutation (factor V Leiden) in patients with ischaemic cerebrovascular disease and survivors of myocardial infarction. *Thromb Haemost* 73:558, 1995
35. Rees DC, Cox M, Clegg JB: World distribution of factor V Leiden. *Lancet* 346:1133, 1995
36. Holm J, Zöller B, Berntorp E, Erhardt L, Dahlbäck B: Prevalence of factor V gene mutation among myocardial infarction patients and healthy controls higher in Sweden than in other countries. *J Intern Med* 239:221, 1996
37. Rosén S, Johansson K, Lindberg K, Dahlbäck B: Multicenter evaluation of a kit for activated protein C resistance on various coagulation instruments using plasmas from healthy individuals. *Thromb Haemost* 72:255, 1994

38. de Ronde H, Bertina RM: Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. *Thromb Haemost* 72:880, 1994
39. Trossaert M, Conard J, Horellou MH, Samama MM, Ireland H, Bayston TA, Lane DA: Modified APC resistance assay for patients on oral anticoagulants. *Lancet* 344:1709, 1994
40. Jorquera JI, Montoro JM, Fernández MA, Aznar JA, Aznar J: Modified test for activated protein C resistance. *Lancet* 344:1162, 1994
41. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH: High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C Resistance). *Blood* 85:1504, 1995
42. Vandenbroucke JP, Koster T, Briët 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* 344:1453, 1994
43. Hellgren M, Svensson PJ, Dahlbäck B: Resistance to activated protein C as a basis for venous thromboembolism associated with pregnancy and oral contraceptives. *Am J Obstet Gynecol* 173:210, 1995
44. Cook G, Walker ID, McCall F, Conkie JA, Greer IA: Familial thrombophilia and activated protein C resistance: thrombotic risk in pregnancy. *Br J Haematol* 87:873, 1994
45. Koeleman BPC, Reitsma PH, Allaart RC, Bertina RM: Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. *Blood* 84:1031, 1994
46. Zöller B, Berntsdotter A, Garcia de Frutos P, Dahlbäck B: Resistance to activated protein C as an additional genetic risk factor in hereditary deficiency of protein S. *Blood* 85:3518, 1995
47. Gandrille S, Greengard JS, Alhenc Gelas M, Juhan-Vague I, Abgrall JF, Jude B, Griffin JH, Aiach M, the French network on behalf of INSERM: Incidence of activated protein C resistance caused by the ARG 506 GLN mutation in factor V in 113 unrelated symptomatic protein C-deficient patients. *Blood* 86:219, 1995
48. Hallam PJ, Millar DS, Krawczak M, Kakkar VV, Cooper DN: Population differences in the frequency of the factor V Leiden variant among people with clinically symptomatic protein C deficiency. *J Med Genet* 32:543, 1995
49. Bauer KA: Management of patients with hereditary defects predisposing to thrombosis including pregnant women. *Thromb Haemost* 74:94, 1995