# Familial thrombophilia

## Resistance to activated protein C and protein S deficiency

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# FAMILIAL THROMBOPHILIA

## Resistance to activated protein C and protein S deficiency

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Abstract

Inherited resistance to activated protein C (APC-resistance) and protein S deficiency are associated with functional impairment of the protein C anticoagulant system, resulting in lifelong hypercoagulability and increased risk of thrombosis. APC-resistance is the most common genetic cause of thrombosis being present in 20% to 60% of thrombosis patients.

A linkage study was performed in a large thrombophilic family with independent inheritance of APC-resistance and protein S deficiency. APC-resistance was found to co-segregate with two neutral polymorphisms in the factor V gene. A point mutation changing Arg506 to a Gln in the factor V gene was the cause of APC-resistance in the family. The mutation (FV:Q506) is localised in one of the APC-cleavage sites of factor V, rendering mutated factor Va resistant to cleavage by activated protein C (APC). The factor V mutation was analysed in 308 members from 50 thrombosis-prone families with inherited APC-resistance. In 94% (47/50) of APC-resistant families the same factor V gene mutation was identified. The magnitude of thrombotic risk was dependent on the factor V genotype.

We investigated 327 individuals in 18 thrombosis-prone families with inherited deficiency of free protein S. Deficiency of free protein S was caused by equimolar relationship between total protein S and ß-chain containing isoforms of C4BP. Moreover, type I deficiency (low free and total protein S) and type III deficiency (low free but normal total protein S) coexisted in 14 out of 18 families, demonstrating the two types to be phenotypic variants of the same genetic disease. Deficiency of free protein S was a strong risk factor for thrombosis in these families. However, thrombophilia penetrance was highly variable. The FV:Q506 mutation causing APC-resistance was identified as an additional genetic risk factor in 39% (7/18) of the families. Thus, familial thrombophilia is a multiple genetic disorder.

Biochemically affected family members had higher levels of prothrombin fragment F1+2 than their normal relatives. The results demonstrate that individuals with APC-resistance or protein S deficiency have an imbalance between pro- and anti-coagulant forces, resulting in increased thrombin generation and hypercoagulability.





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Protein S, protein C, factor V, C4b-binding protein, blood coagulation, thrombosis, mutation

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# FAMILIAL THROMBOPHILIA

## Resistance to activated protein C and protein S deficiency

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Malmö 1996

*To my wife Jane*

Acti labores jucundi

Marcus Tullius Cicero

106-43 A.C.

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## List of articles

This thesis is based on the following papers, referred to in the text by their respective Roman numerals:

I. Zöller, B., Dahlbäck, B.
Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis.
*Lancet* 1994; **343**: 1536-38.

II. Zöller, B., Svensson, P.J., 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.
*Journal of Clinical Investigation* 1994; **94**: 2521-24.

III. Zöller, B., Xuhua H., Dahlbäck, B.
Homozygous APC-resistance combined with inherited type I protein S deficiency in a young boy with severe thrombotic disease.
*Thrombosis and Haemostasis* 1995; **73**: 743-745.

IV. 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* 1995; **85**: 3518-3523.

V. Zöller, B., Garcia de Frutos, P., Dahlbäck, B.
Evaluation of the relationship between protein S and C4b-binding protein isoforms in hereditary protein S deficiency demonstrating type I and type III deficiencies to be phenotypic variants of the same genetic disease.
*Blood* 1995; **85**: 3524-3531.

VI. Zöller, B., Holm J., Svensson P.J., Dahlbäck, B.
Elevated levels of prothrombin activation fragment 1+2 in plasma from patients with heterozygous Arg506 to Gln mutation (APC-resistance) and/or inherited protein S deficiency.
*Thrombosis and Haemostasis* 1996 (In press).

**Abbreviations**

| | |
|---|---|
| ADP | Adenosine diphosphate |
| APC | activated protein C |
| APC-resistance | resistance to activated protein C |
| APTT | activated partial thromboplastine time |
| AT-III | antithrombin III |
| bp | base pairs |
| cDNA | complementary DNA |
| CI | Confidence interval |
| C4b | complement protein 4b |
| C4BP | C4b-binding protein |
| C4BPß+ | ß chain containing isoforms of C4BP |
| C4BPß- | ß chain lacking isoforms of C4BP |
| DNA | deoxyribonucleic acid |
| EGF | epidermal growth factor |
| $F_{1+2}$ | prothrombin activation fragment 1+2 |
| FV, FVII etc. | coagulation factor V, coagulation factor VII etc. |
| FVa, FVIIa etc. | the activated forms of coagulation factor V, coagulation factor VII, respectively |
| Gla | γ-carboxyglutamic acid |
| HC II | heparin cofactor II |
| HMWK | high-molecular weight kinogen |
| Kb | kilobasepairs |
| $K_d$ | constant of dissociation |
| kDA | kiloDalton |
| PAI-1 | plasminogen activator inhibitor 1 |
| PC | protein C |
| PCI | protein C inhibitor |
| PS | protein S |
| SHBG | sex hormone binding globulin |
| TF | tissue factor |
| TFPI | tissue factor pathway inhibitor |
| t-PA | tissue-type plasminogen activator |
| vWF | von Willebrand factor |

Amino acid residues are abbreviated in accordance with the 1983 Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (Eur. J. Biochem 1984: 138; 9-37), using either the three-letter code or the one-letter code.

**Popularised summary in Swedish (sammanfattning på svenska)**

Venös tromboembolism (blodpropp i ben och lungor) är en av våra vanligaste sjukdomar. Det har beräknats att ca 2.5-5% av befolkningen någon gång drabbas av venös tromboembolism. Normalt föreligger en balans mellan koagulationssystemet (blodlevringssystemet), som motverkar blödning, och antikoagulationssystemet som motverkar trombos (blodpropp). En nyckelroll spelar det s.k. protein C antikoagulationssystemet. Enzymet protein C hämmar koagulationen tillsammans med sin medhjälpare protein S (S står för Seattle där proteinet upptäcktes). Ärftlig brist på protein S eller protein C medför en livslång ökad risk att drabbas av venös tromboembolism. Trombos uppstår p.g.a. en rubbningen i balansen mellan koagulations- och antikoagulations-systemen. Dessa rubbningar kan vara såväl förvärvade (t.ex. graviditet, p-piller, kirurgi, malignitet och fetma) som genetiska (ärftliga). Förvärvade riskfaktorers betydelse för uppkomst av trombossjukdom är välkänt sedan länge. Genetiska riskfaktorer, d.v.s. brist på protein S, protein C och antitrombin III, har tidigare endast kunnat identifieras hos 5-10% av alla patienter med trombos. Situationen har emellertid radikalt ändrats i och med upptäckten av en ny ärftlig rubbning som kallas för resistens mot aktiverat protein C (APC-resistens). Tillståndet upptäcktes och beskrevs 1993 av professor Björn Dahlbäck, Universitetssjukhuset MAS, Malmö. Vid APC-resistens kan inte protein C hämma koagulationen (blodlevringen) på ett normalt sätt, varför patienter med APC-resistens har en livslång ökad risk att drabbas av venös tromboembolism. Upptäckten av APC-resistens har givit nya kunskaper om trombossjukdomens etiologi (orsak). APC-resistens hittas i upp till 20 till 60% av patienter med venös tromboembolism. Trombos är således inget som slumpvis drabbar människor. Istället är det ofta genetiskt predisponerade individer som drabbas av trombos i samband med exposition för riskfaktorer som t.ex. p-piller, kirurgi och graviditet. Denna nyvunna kunskap ger nya möjligheter att minska incidensen av venös tromboembolism i befolkningen.

Avhandlingen grundar sig på sex vetenskapliga uppsatser, vilka samtliga handlar om ärftlig APC-resistens och/eller protein S brist. Båda dessa två tillstånd beror på en medfödd defekt i ett av de skyddssystem, protein C antikoagulations systemet, som förhindrar att blodet spontant koagulerar (levrar sig) i blodådrorna. I första delarbetet genomfördes en s.k. genetisk kopplingsstudie i en stor familj med ärftlig APC-resistens och protein S brist. Vi kunde visa att APC-resistens fenomenet beror på en defekt i koagulations faktor V. Ett abnormt utfall i APC-resistens testet följde nedärvningen av två neutrala genetiska markörer i genen för koagulations faktor V. Vi kunde dessutom konfirmera att APC-resistens beror på en punktmutation (arginin 506 till glutamin) i klyvningsstället för aktiverat protein C (APC). Mutationen gör att aktivt faktor V (FVa) inte bryts ned på normalt sätt. Då FVa påskyndar koagulations processen, leder detta till en bristfällig nedreglering av koagulationen och en livslång ökad blodproppsbenägenhet.

I andra delarbetet undersöktes den ovan beskrivna mutationen hos 308 individer tillhörande 50 trombosbenägna familjer med ärftlig APC-resistens. Det visade sig att 47 av 50 familjer (94%) hade en och samma mutation i faktor V genen som förklaring till APC-resistens fenomenet. I en minoritet av familjer och patienter med APC-resistens kunde således ingen mutation identifieras. Då APC-resistens testet inte var specifikt för faktor V mutationen måste tillsvidare APC-resistens testet kompletteras med DNA-baserad analys. Vidare undersöktes symptomen vid APC-resistens. Individer med APC-

resistens anlaget i dubbel uppsättning (homozygoter) hade större risk att drabbas av trombos än individer med APC-resistens i enkel uppsättning (heterozygoter). Vid 33-års ålder hade 8% av normala släktingar, 20% av heterozygoter och 40% av homozygoter drabbats av en eller flera tromboser. Det har i en fall-kontrollstudie från Holland beräknats att heterozygoter har en c:a 5-10 gånger ökad risk att drabbas av trombos jämfört med normala individer. Risken för homozygoter är c:a 50-100 gånger ökad. Även om den relativa risken att utveckla trombos är ökad kommer inte alla individer med APC-resistens att drabbas av trombos, vilket tydligt framgår av våra familjestudier. Trombosrisken hos APC-resistenta individer påverkas av andra genetiska eller förvärvade riskfaktorer. Den höga förekomsten av trombos även bland släktingar som saknar mutationen gör det sannolikt att ytterligare genetiska riskfaktorer finns i trombosbenägna familjer med APC-resistens. I 63% av fallen utlöstes den första trombosen hos APC-resistenta individer av en förvärvad riskfaktor som t.ex. kirurgi, p-piller och graviditet. Detta visar att det ofta behövs flera samverkande genetiska och/eller förvärvade riskfaktorer för att utlösa trombos. Resultaten från vår familjestudie stämmer bra överens med publicerade fall-kontrollstudier. I en fall-kontrollstudie från Malmö/Göteborg har det visats att 60% av kvinnor som drabbas av blodpropp under graviditet, samt 30% av kvinnor som får blodpropp i samband med p-piller har APC-resistens. I två Holländska fall-kontrollstudier har man visat att heterozygota kvinnor som använder p-piller har en 35 gånger ökad risk att drabbas av blodpropp, samt att homozygota kvinnor som använder p-piller har en flera hundra gånger ökad risk att drabbas av trombos. Alla individer med ärftlig trombosbenägenhet bör därför koagulationsutredas innan operation, graviditet och p-piller förskrivning.

Trombossjukdom innan puberteten är mycket ovanligt även bland patienter med ärftliga rubbningar. I tredje delarbetet beskrevs en 10-årig pojke som drabbades av trombos i hela höger ben. Pojken hade förutom ärftlig brist på fritt protein S en mycket uttalad APC-resistens, som partiellt kunde normaliseras efter tillsats av faktor V, men ej av protein S. Pojken hade APC-resistens anlaget i dubbel uppsättning, d.v.s. pojken var homozygot för faktor V mutationen. Protein S bristen och APC-resistensen nedärvdes oberoende av varandra i familjen. Protein S är ett viktigt protein som skyddar mot trombossjukdom och brist på protein S medför en livslång ökad risk för trombos. Pojken hade således multipla genetiska riskfaktorer som orsaker till trombosen.

I fjärde delarbetet undersöktes 327 individer från 18 trombosbenägna familjer med ärftlig protein S brist. Brist på fritt protein S var en stark riskfaktor för trombos i dessa familjer. Sextio fyra av 136 (47%) individer med protein S brist hade haft trombos jämfört med 7% (14/191) av deras normala släktingar. Vid 45-års ålder hade 12% av de normala släktingarna haft trombos jämfört med 50% av släktingarna med protein S brist. Medelåldern vid första trombostillfället var 32.5 år med en spridning från 10 till 81 år. Djup trombos i benen var det vanligaste symptomet, men även lungemboli (propp i lungan) och tromboflebiter (ytliga blodproppar) i benen var vanligt. Mycket svåra venösa tromboser i tarmen (mesenterial kärlen) och hjärnan (sinus sagittalis superior) förekom hos fyra individer. Arteriell trombos tenderade även att vara överrepresenterad hos individer med protein S brist och då framför allt hos rökare. Nio (6.6%) individer med protein S brist hade haft arteriell trombos jämfört med 3 (1.6%) av deras normala släktingar. Behandling med blodförtunnande medicin (dicumarol eller warfarin) var mycket effektiv. Ingen av de 55 individer med protein S brist som

behandlats med blodförtunnande medicin hade trombosrecidiv under den tid de var adekvat behandlade.

Det förelåg en stor variation i trombosbenägenhet även mellan individer med protein S brist. Den yngste insjuknade vid 10 års ålder och den äldste fick första trombosen vid 81 års ålder. En individ med protein S brist som är 76 år har fortfarande inte drabbats av trombos. Vi undersökte därför ifall ytterligare genetiska riskfaktorer för trombossjukdom nedärvdes i dessa familjer. Samtidig ärftlighet för APC-resistens (mutation av arginin$^{506}$ till glutamin i faktor V genen) förelåg i totalt 7 av de 18 (39%) trombosdrabbade protein S brist familjerna. Detta tyder på en selektion av familjer med mer än en genetisk defekt som orsak till trombosbenägenheten. I de 7 familjerna med samtidig ärftlighet för APC-resistens hade hela 72% av individer med kombinerade genetiska defekter haft trombos jämfört med 19% av individer med isolerad protein S brist eller APC-resistens. Även förvärvade riskfaktorer påverkar trombosrisken hos individer med protein S brist. Övervikt var en riskfaktor för trombos i dessa familjer. Första trombosen utlöstes av en förvärvad riskfaktor i 48% (31/64) av de symptomatiska patienterna med protein S brist. De vanligaste riskfaktorerna var p-piller, graviditet, trauma, kirurgi och immobilisering. Trombos förekom vid 13% (19/146) av alla graviditeter hos kvinnor med protein S brist jämfört med 4% (6/163) hos kvinnliga släktingar utan protein S brist. Detta delarbete visar att venös tromboembolism orsakas av en samverkan av multipla genetiska och förvärvade faktorer. Troligen finns ytterligare idag okända genetiska riskfaktorer i trombosbenägna familjer med ärftlig protein S brist.

I femte delarbetet belystes mekanismen för ärftlig brist på fritt protein S. De 327 individerna från de 18 protein S-brist familjerna i delarbete nummer fyra undersöktes. Protein S föreligger normalt i blodet till 40% som fria molekyler medan resterande 60% återfinns bundet till ett annat protein kallat C4b-bindande protein (C4BP). Endast de fria protein S molekylerna fungerar tillsammans med protein C som koagulationshämmare. Tre olika huvudtyper av ärftlig protein S brist har beskrivits. Vid typ I brist är plasma koncentrationen låg för såväl fritt som totalt protein S. Typ II brist är reserverat för funktionell protein S brist med normala koncentrationer av fritt och totalt protein S antigen. Vid typ III protein S brist föreligger selektiv låg koncentration av fritt protein S med normal total protein S koncentration. Hela 40% (47/117) av individer med brist på fritt protein S hade normal koncentration av totalt protein S, s.k. typ III brist. I 14 av 18 familjer förelåg samtidig ärftlighet av typ I och typ III protein S brist. Typ I och III är således endast olika fenotyper (uttryck) av samma genetiska sjukdom. Brist på fritt protein S ärvdes autosomalt dominant och korrelerade bättre än brist på totalt protein S till förekomst av venös tromboembolism. Den låga koncentrationen av fritt protein S orsakades av att allt protein S var bundit till de former av C4BP som kan binda protein S (β-kedje innehållande C4BP, förkortat C4BPβ+). Protein S och C4BPβ+ föreligger nämligen i ekvimolära (lika) mängder i plasma hos individer med brist på protein S. Vi kunde även visa att warfarin behandling sänker plasma koncentrationen av C4BPβ+, trots att C4BP inte innehåller γ-karboxiglutaminsyra. Mekanismen är än så länge okänd men medieras troligen via den k-vitamin beroende protein S molekylen, vars koncentration också sänks av warfarin behandling.

I sjätte delarbetet undersöktes protrombinfragment 1+2 ($F_{1+2}$) hos 205 individer från 34 blodproppsbenägna familjer med arginin$^{506}$ till glutamin mutationen i faktor V (APC-resistens)

och/eller ärftlig protein S brist. $F_{1+2}$ koncentrationen i plasma är en markör på ökad koagulations-aktivering, d.v.s. en överaktivitet i koagulationssystemet. Individer med heterozygot faktor V mutation (APC-resistens) och/eller ärftlig protein S brist uppvisade ökade halter i plasma av $F_{1+2}$ jämfört med deras normala släktingar. Detta tyder på en ökad koagulationsaktivering hos individer med APC-resistens och/eller protein S brist. Denna överaktivitet i koagulationssystemet kan förklara den ökade trombosrisken hos individer med APC-resistens och/eller protein S brist. Intressant nog så normaliserades och t.o.m. sänktes $F_{1+2}$ koncentrationen hos warfarin (en blodförtunnande medicin) behandlade patienter. $F_{1+2}$ koncentrationen korrelerar således till den kliniskt goda blodpropps-förebyggande effekten av warfarin. Detta kan vara en användbar kunskap i utprovandet av nya behandlingsmetoder för individer med APC-resistens eller protein S brist. Sammanfattningsvis föreligger en ökad aktivitet i koagulationssystemet hos individer med APC-resistens och/eller protein S brist. Denna överaktivitet kan normaliseras genom behandling med warfarin.

Sammanfattning

Resistens mot aktiverat protein C (APC-resistens), som är den vanligaste orsaken till venös tromboembolism i Sverige, beror på en punktmutation i koagulations faktor V genen. Mutationen ändrar aminosyran arginin$^{506}$ till en glutamin varvid aktivt faktor V inte bryts ned på ett normalt sätt av aktiverat protein C (APC). Detta leder till en bristfällig hämning av koagulationssystemet och en ökad trombosrisk.

Trombosrisken tycks livslång för bärare av APC-resistens anlaget. Risken att drabbas av trombos är större för homozygoter än för heterozygoter för faktor V mutationen. Många individer med t.o.m. homozygot APC-resistens kommer dock aldrig att drabbas av trombos. Såväl andra genetiska faktorer som exposition för förvärvade riskfaktorer, som t.ex. kirurgi, trauma, p-piller och graviditet, är av betydelse för utvecklandet av trombos hos individer med APC-resistens.

Protein S brist är ett annat ärftligt tillstånd som medför en mycket hög risk för trombos. Vid 45-års ålder hade 50% av patienter med protein S brist drabbats av venös tromboembolism. Trombosförekomsten är emellertid mycket varierande, även inom samma familj. Hos 39% av trombosdrabbade familjer med protein S brist föreligger samtidig ärftlighet för en annan rubbning, nämligen APC-resistens. Risken att drabbas av blodpropp för individer med kombinerade genetiska defekter är mycket stor. Emellertid tycks även exposition för riskfaktorer som övervikt, p-piller, graviditet, kirurgi och trauma vara av betydelse för utvecklandet av trombossjukdom.

Brist på fritt protein S ärvs autosomalt dominant och beror på att nästan allt protein S är bundet till β-kedje innehållande isoformen av C4b-bindande protein. Typ I och typ III protein S brist är samma genetiska sjukdom då de nedärvs i samma familjer. Det är således nödvändigt att alltid utvärdera fritt och inte endast totalt protein S vid utredning av trombospatienter.

Individer med heterozygoti för faktor V mutationen (APC-resistens) och/eller ärftlig protein S brist har en ökad koagulationsaktivering med förhöjda koncentrationer av protrombinfragment 1+2, som en förklaring till den livslångt ökade trombosrisken. Den ökade koagulationsaktiviteten kan hämmas med blodförtunnande medicin.

## Introduction

Venous thrombosis is a relatively new disease first described in the thirteenth century (Dexter, 1973). The incidence of venous thromboembolism has increased during recent centuries, perhaps due to the adoption of more sedentary life-styles, and is now the third most common cardiovascular disease after acute ischaemic heart disease and stroke (Dexter, 1973; Goldhaber, 1994). The overall incidence of venous thrombosis has been estimated to be 2.5% to 5% (Gjöres, 1956; Coon *et al.*, 1973). The pathogenic risk factors for venous thrombosis, as described by Virchow more than a century ago, are related to disturbances in the blood flow, in the vessel wall or in the constituents of blood. Aetiological factors predisposing to thrombosis shift the balance between pro- and anti-coagulant forces in a procoagulant direction. In principle these thrombotic risk factors may be circumstantial or genetic. Circumstantial risk factors such as surgery, pregnancy, malignancy or oral contraceptives, are well established as common aetiological factors associated with venous thromboembolism (Hirsh *et al.*, 1986). The occurrence of familial thrombosis was described already in the beginning of the century (reviewed in Jordan & Nandorff, 1956), but was not accorded much attention until after 1965 when inherited antithrombin III deficiency was found to be a cause of familial thrombophilia (Egeberg, 1965). After the discovery and elucidation of the protein C anticoagulant pathway, deficiencies of protein C and protein S were found to be associated with familial thrombophilia (Griffin *et al.*, 1981; Comp & Esmon, 1984). However, genetic defects were only found in a few per cent of all thrombosis patients (Heijboer *et al.*, 1990; Malm *et al.*, 1992). The discovery of inherited resistance to activated protein C (APC resistance) as a thrombosis risk factor (Dahlbäck *et al.*, 1993), caused by a single point mutation in the factor V gene (Bertina *et al.*, 1994), has dramatically improved our understanding of the pathogenesis of venous thrombosis. We now identify APC-resistance in 20-60% of thrombosis patients (Griffin *et al.*, 1993; Koster *et al.*, 1993; Svensson & Dahlbäck, 1994). Thus, thrombosis belongs to the group of common diseases which affect genetically susceptible individuals due to the concerted actions of genetic and circumstantial risk factors (King *et al.*, 1993).

In the studies upon which this thesis is based, the molecular mechanisms and the clinical manifestations of inherited protein S deficiency and/or APC-resistance were characterised. As a background to the work, the following sections provide a review of the coagulation and anticoagulation systems, the epidemiology and aetiology of venous thrombosis.

## Haemostasis

Haemostasis is the physiological defence mechanism that prevents blood loss after vascular damage. In response to vascular injury platelets are activated. The platelets adhere, aggregate and form a platelet plug at the site of injury, which temporarily stops the blood flow (primary haemostasis). In parallel, the blood coagulation cascade is activated resulting in formation of a fibrin matrix stabilising the platelet plug. These vital but potentially hazardous reactions of the haemostatic system are carefully controlled by various plasma protease inhibitors and anticoagulant systems, in order to maintain blood fluidity without pathological thrombus formation.

**Endothelium and platelets**

It was shown over 200 years ago that blood in isolated veins does not clot for many hours (Thomas, 1994). The non-thrombogenicity of intact endothelium is necessary for vascular patency and the normal function of blood vessels. This antithrombotic property of normal endothelium is in part due to the lack of factors on the surface that can trigger coagulation and to the presence of several anticoagulant molecules (Pearson, 1994). Endothelial cells are lined with the integral membrane glycoprotein, thrombomodulin (TM), which in complex with thrombin activates the protein C anticoagulant system (Esmon, 1995). Cell surface heparin-like glucosaminoglycans accelerates the antithrombin III mediated inactivation of thrombin and other serine protease clotting enzymes (Broze & Tollefsen, 1994). The endothelium also synthesises and secretes a number of other factors with anticoagulant (prostacyclin, nitric oxide, TFPI, t-PA, protein S) or procoagulant effects (TF, vWF, PAI-1, platelet-activating factor) (Pearson, 1994). The expression of these factors may be modulated in response to inflammatory stimuli by tumour necrosis factor and interleukin 1, converting the endothelium from an anticoagulant to a procoagulant surface.

In contrast to intact endothelium, injured endothelium with exposure of subendothelial tissue is highly thrombogenic, resulting in an appropriate activation of the haemostatic system. Exposure to collagen, vitronectin and tissue factor activates platelets and coagulation (Sixma, 1994). Platelets may also be activated by the minute amounts of thrombin generated at the trauma site (reviewed in Majerus, 1994a). Activated platelets expose receptors (glycoprotein Ib, glycoprotein IIb/IIIa) on their surfaces that interact with 'contact promoting proteins' (von Willebrand factor, fibrinogen, fibrinonectin, thrombospondin), resulting in adherence to the trauma site and also to each other (platelet aggregation). More platelets are recruited by the release of ADP, serotonin and thromboxane A2. The formed platelet plug (primary haemostasis) stops blood flow temporarily but needs further strengthening by the formation of a fibrin matrix. It is therefore highly appropriate that activated platelets expose negatively charged phospholipid on which coagulation may occur. Further, activated platelets release several coagulation factors from their alpha granules, which may serve to increase the local concentrations of critical proteins in the wound (factor V, fibrinogen, von Willebrand factor, high molecular weight kinogen). They also release a number of other proteins and peptides (platelet-derived growth factor, platelet factor 4, β thromboglobulin, thrombospondin and P-selectin) (reviewed in Majerus, 1994a).

**The blood coagulation cascade**

Blood coagulation involves the stepwise participation of a large number of plasma proteins resulting in progressive amplification of the triggering signal and explosive thrombin generation (Fig. 1). The serine protease thrombin then converts fibrinogen to an insoluble fibrin matrix which consolidates and stabilises the haemostatic plug (MacFarlane, 1964; Davie & Ratnoff, 1964). Although the original cascade model of blood coagulation has been somewhat modified, it is still largely valid (reviewed in Furie & Furie, 1988; Davie *et al.*, 1991; Furie & Furie, 1992; Davie, 1995). The complex series of biochemical reactions takes place on phospholipid surfaces of platelets, white blood cells and endothelial cells, and involve zymogen-to-enzyme conversions by limited proteolysis,

accelerated by the non-enzymatic cofactors. The names of the coagulation proteins, and their properties and chromosomal locations are summarised in Table 1.

All the active coagulation enzymes are trypsin-like serine proteases that cleave arginyl bonds with a much higher specificity than does trypsin. Several of the serine proteases and the non-enzymatic cofactor protein S require vitamin K for the normal synthesis of their zymogen (Tables 1 and 2) (reviewed in Stenflo & Dahlbäck, 1994). Vitamin K is necessary for carboxylation of the amino terminal glutamic acid residues to γ-carboxyglutamic acid (Gla). The Gla residues are present in the Gla domain of prothrombin, in factors VII, IX and X, as well as in the anticoagulant proteins C and S. The Gla residues are essential for calcium ($Ca^{2+}$) dependent membrane binding of the vitamin K-dependent coagulation factors. Binding of calcium to the Gla domain induces conformational changes exposing hydrophobic side chains necessary for membrane binding of Gla-containing proteins (Sunnerhagen *et al.,* 1995). Inhibition of γ-carboxylation by vitamin K-antagonistic drugs such as warfarin down-regulate the entire coagulation cascade by reducing $Ca^{2+}$ binding and membrane interaction of Gla domains. Calcium is also essential for another central feature of the coagulation cascade: the organisation of reactants into multimolecular complexes on natural surfaces. The multimolecular complexes allow optimal and favourable steric orientation of the reactants. Each enzyme is virtually inactive without the appropriate cofactor-protein and the appropriate membrane surface. Coagulation factors V and VIII are such cofactors (Kane & Davie, 1988; Jenny *et al.,* 1994; Fay, 1993; Sadler & Davie, 1994). They are activated by proteolytic cleavage by thrombin or factor Xa. The active forms of coagulation factors V and VIII (FVa and FVIIIa) bind to negatively charged phospholipids on activated platelets, and function as receptor sites for the serine proteases factor Xa and IXa, respectively. The 'prothrombinase' (factor Xa, factor Va, phospholipid and $Ca^{2+}$) and the 'tenase' complexes (factor IXa, factor VIIIa, phospholipid and $Ca^{2+}$) activate prothrombin and factor X, respectively, over 100,000 times more rapidly than does the respective enzyme alone. Factor Va and VIIIa each accounts for a 1000-fold increase of the activity of the tenase and prothrombinase complex. Protein C mediated degradation of factor Va and VIIIa results in down regulation of the tenase and prothrombinase complexes and generalised inhibition of the coagulation cascade (Dahlbäck & Stenflo, 1994).

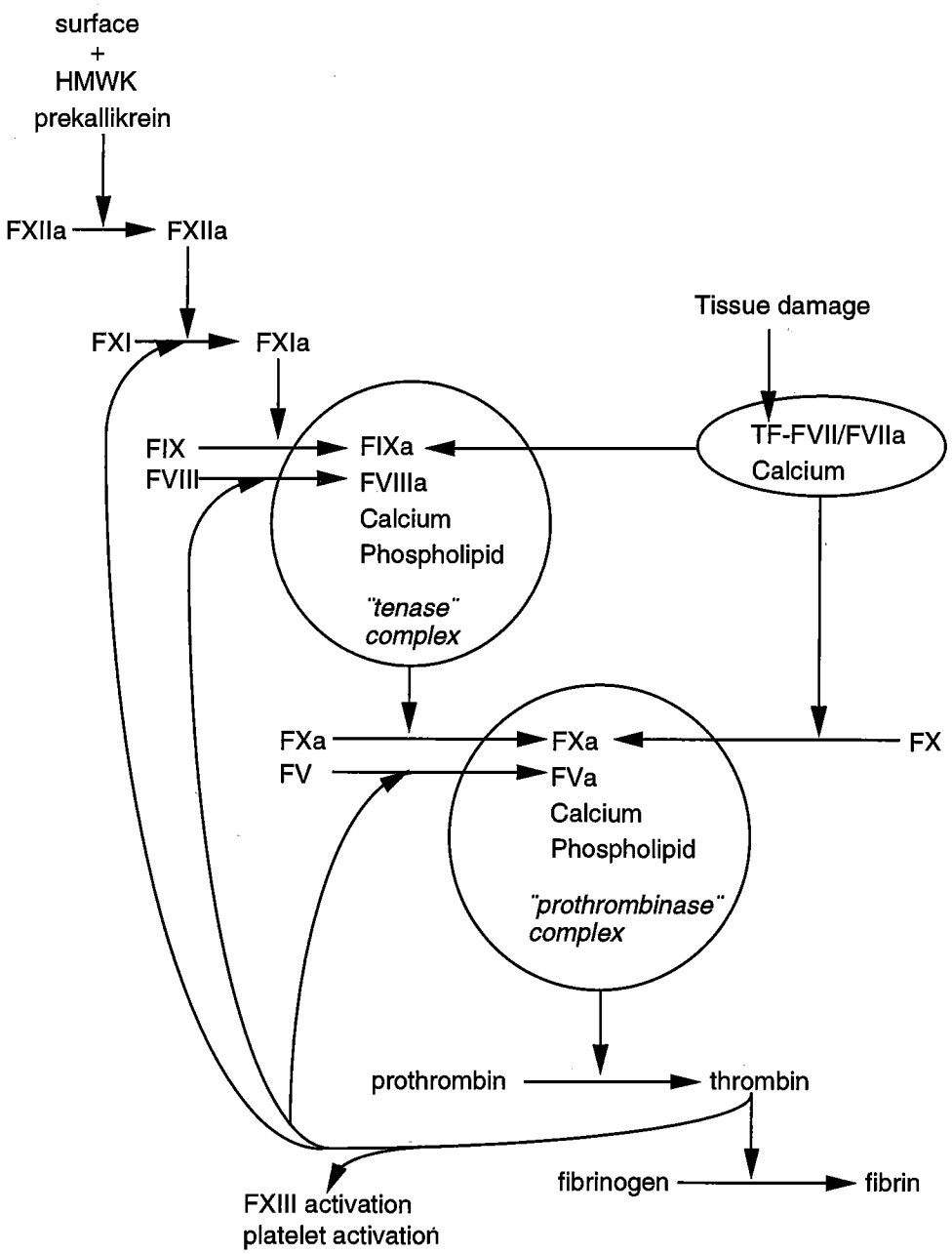


**Fig. 1.** Schematic representation of the coagulation cascade. The intrinsic pathway is initiated by exposure of the contact factors [HMWK (high-molecular weight kinogen), factor XII and prekallikrein] to a negatively charged surface (e.g. connective tissue, collagen, glass or kaolin), resulting in activation of factor XI by factor XIIa. The extrinsic pathway is initiated by tissue damage and the resultant exposure of tissue factor to factor VII/VIIa.

**Table 1. Blood coagulation factors.**

| Component | Molecular Weight | Plasma Concentration µg/mL (T1/2) | Chromosome Location | Gene (Kb) | mRNA (Kb) | Exon (n) | Function |
|---|---|---|---|---|---|---|---|
| Fibrinogen | 340,000 | 3000 (3-4 days) | | | | | Structural |
| α chain | 66,500 | | 4q23-q32 | 5.4 | | 5 | |
| β chain | 52,000 | | 4q23-q32 | 8.2 | | 8 | |
| γ chain | 46,500 | | 4q23-q32 | 8.4 | | 9 | |
| prothrombin* | 72,000 | 100 (72 h) | 11p11-q12 | 21 | 2.1 | 14 | Protease zymogen |
| Factor V | 330,000 | 10 (15 h) | 1q21-25 | ≥80 | 7.0 | 25 | Cofactor precursor |
| Factor VII* | 50,000 | 0.5 (4 h) | 13q34 | 12.8 | 2.4 | 8 | Protease zymogen |
| Factor VIII | 330,000 | 0.1 (8-12 h) | Xq28 | 186 | 9.0 | 26 | Cofactor precursor |
| Factor IX* | 55,000 | 5 (12-24 h) | Xq26-Xq27 | 34 | 2.8 | 8 | Protease zymogen |
| Factor X* | 59,000 | 10 (50 h) | 13q34-qter | 25 | 1.5 | 8 | Protease zymogen |
| Factor XI | 160,000 | 5 (60 h) | 4q35 | 23 | | 15 | Protease zymogen |
| Factor XII | 80,000 | 30 (50 h) | 5q33-qter | 12 | 2.4 | 14 | Protease zymogen |
| Factor XIII | 320,000 | 30 (4-7 days) | | | | | Protransglutaminase |
| a subunit | 75,000 | 11 | 6p25.1-p24.3 | >160 | | 15 | |
| b subunit | 80,000 | 21 | 1q31-q32.1 | 28 | | 12 | |
| Prekallikrein | 80,000 | 30-40 | 4q35 | | | 15 | Protease zymogen |
| HMWK | 120,000 | 80 | 3q27 | 27 | | 11 | Cofactor/activation |
| Tissue factor | 37,000 | 0.0 | 1p21-22 | 12.4 | 2.1 | 6 | Cofactor/initiator |
| vWF | >1.5 million 220 000 subunits | 10 (24 h) | 12p12-pter | 180 | 8.5 | 52 | Adhesion |

*, Vitamin K dependent protein containing γ-carboxyglutamic acid (Gla), HMWK (high-molecular weight kininogen). Data are compiled from (Furie & Furie, 1988; Davie, 1995; Stamatoyannopoulus *et al.*, 1994; Bloom *et al.*, 1994; Mckusick *et al.*, 1994). The latter reference is also available on internet (http://gdbwww.gdb.org/omim/docs/omimtop.html).

The blood coagulation cascade may be triggered by two independent mechanism, one known as the extrinsic pathway because it involves an extravascular factor (tissue factor), the other as the intrinsic pathway because all of the components are contained in the blood (Fig. 1). The extrinsic pathway is considered the 'prima ballerina' in the initiation of coagulation in vivo (Nemerson, 1988; Rapaport & Rao, 1995; Broze, 1995). The pathway is initiated when tissue factor comes into contact with blood. Tissue factor is an integral membrane glycoprotein in the adventitia, and comes into contact with blood only after vascular injury. Factor VII binds tightly to the extracellular domain of tissue-factor in the presence of calcium, which results in activation of factor VII on the membrane surface. The serine protease factor VIIa is generated by cleavage of the peptide bond Arg152-Ile. The precise mechanism is not known, and several proteases may be involved, e.g. factor Xa, factor IXa, factor VIIa or thrombin. The factor VIIa-tissue factor complex then catalyses not only the activation of factor X but also factor IX. The preferred substrate for FVIIa-tissue factor complex depends on the conditions, e.g. the calcium and tissue factor concentrations and the organisation of tissue factor on the phospholipid membrane (Bach *et al.*, 1986; Komiyama *et al.*, 1990; Rao *et al.*, 1992). At low concentrations of tissue factor, factor IX is a better substrate than factor X for tissue factor-factor VIIa complex, probably reflecting physiological conditions (Marlar *et al.*, 1982; Bom *et al.*, 1990).

The intrinsic pathway is initiated upon exposure of FXII, FXI, prekallikrein and high molecular weight kinogen (HMWK) in the blood to a negatively charged surface, i.e. connective tissue and collagen in vivo or glass and kaolin in the test tube. The activation of factor XII is followed

by a sequential activation of factors XI, IX and X. The blood coagulation proceeds on cell surface membranes of stimulated cells. Factor IXa and VIIIa both bind to phospholipid surfaces and form the tenase complex consisting of factor IXa, factor VIIIa, $Ca^{2+}$ and phospholipids. This complex converts factor X bound to the cell membrane to factor Xa. Although the contact phase initiates coagulation in vitro via the intrinsic pathway, it appears to be of little or no significance for the initiation in vivo. Patients with deficiency of factor XII, pre-kallekrein and high molecular weight kinogen rarely have bleeding symptoms (reviewed in Rizza, 1994), while patients with factor XI deficiency have only a mild bleeding tendency usually manifest only after injury or surgery. An explanation of the difference between the asymptomatic factor XII deficiency and the mild bleeding symptoms of factor XI deficiency, may be related to the fact that thrombin in a positive feedback loop activates factor XI (Gailani & Broze, 1991; Naito & Fujikawa, 1991). However, the intrinsic pathway is important to maintain fibrin formation once coagulation is initiated, suggested by the severe bleeding tendency of patients with inherited FVIII or FIX deficiency (haemophilia A and B). An explanation of this was provided when factor VIIa in complex with tissue factor was found to activate factor IX (Osterud & Rapaport, 1977).

With the formation of factor Xa, the intrinsic and extrinsic pathways converge in the common pathway. Factor Xa, factor Va, calcium and phospholipids forms the 'prothrombinase' complex, which converts membrane-bound prothrombin to thrombin. Thrombin is then released from the cell surface and, in the last step in the coagulation cascade, converts soluble fibrinogen to insoluble fibrin by limited proteolysis. Cleavage of a arginyl-glycine peptide bond in each of the two $\alpha$ chains and in each of the two $\beta$ chains of fibrinogen results in the release of four fibrinopeptides. The N-terminal residues then bind to adjacent fibrin monomers, which result in linear and side-by-side polymerisation (Doolittle, 1994). Thrombin also catalyses the activation of factor XIII. Factor XIIIa is a calcium dependent transglutaminase that cross-links fibrin monomers by forming $\varepsilon$-amino ($\gamma$-glutamyl)-lysine bonds. The importance of cross-linking is illustrated by the fact that factor XIII deficiency is associated with a life-long severe bleeding tendency, which may present already at birth as prolonged bleeding from the umbilical stump (Rizza, 1994).

Thrombin is a key enzyme that not only forms fibrin and activates Factor XIII, but also activates platelets, which provide negatively charged surfaces necessary for the coagulation process. Moreover, thrombin activates factors XI, VIII and V, which in a positive feedback further increase thrombin generation. However, thrombin has dual functions with both pro- and anti-coagulant properties. If thrombin is formed at intact endothelium, it immediately binds to thrombomodulin and initiates the protein C anticoagulant system, which will down-regulate its own production (Dahlbäck & Stenflo, 1994; Esmon, 1995; Esmon & Schwarz, 1995).

**Regulation of the blood coagulation cascade**

It is important that the coagulation cascade activated in response to local vessel injury does not become generalised, which would result in an abnormal thrombus formation. The coagulation cascade is therefore carefully regulated by several mechanisms, some of which have been identified. One of the most important mechanisms is the protein C anticoagulant system, described in detail in the following paragraph. A summary of the names and properties of the proteins involved in the regulation of the coagulation cascade is presented in Table 2.

**Table 2. Regulators of blood coagulation.**

| Component | Molecular Weight (kD) | Plasma Concentration µg/mL (T1/2) | Chromosome Location | Gene (Kb) | mRNA (Kb) | Exon (n) | Function |
|---|---|---|---|---|---|---|---|
| Protein C* | 56,000 | 3-5 (6-8h) | 2q14-q21 | 11 | 1.8 | 9 | Protease zymogen |
| Protein S* | 75,000 | 20-25 | 3q11.2 | <80 | 3.5 | 15 | Cofactor |
| Thrombomodulin | 60,300 | 0.02 | 20p11.2 | | 3.7 | 1 | Receptor |
| C4BP | 570,000 | 130 | | | | | Complement |
| α chain | 70,000 | | 1q32 | 40 | | 12 | |
| β chain | 45,000 | | 1q32 | | 0.9 | | |
| Antithrombin III | 58,000 | 150 (61-92h) | 1q23-25 | 19 | 1.5 | 7 | Protease inhibitor |
| Heparin cofactor | 66,000 | | 22q11 | 16 | 2.2 | 5 | Protease inhibitor |
| TFPI-1 | 34,000 (40,000) | 0.1 | 2q31-q32.1 | 85 | 1.4, 4.0 | 9 | Protease inhibitor |
| Plasminogen | 92,000 | 200 | 6q26-q27 | 52.5 | 2.7 | 19 | Protease zymogen |
| t-PA | 70,000 | 0.005 | 8p12-q11.2 | >36 | 2.5 | 14 | Protease |
| u-PA | 54,000 | 0.008 | 10 | 6.4 | | 11 | Protease |
| PAI-1 | 52,000 | 0.02 | 7q21.3-q22 | 12.2 | | 9 | Protease inhibitor |
| PAI-2 | 47,000 (60,000) | <0.005 | 18q21-23 | 16.5 | | 8 | Protease inhibitor |
| PCI | 57,000 | 5 | 4q35 | | | | Protease inhibitor |
| Antiplasmin | 70,000 | 70 | 18p11.1-q11.2 | 16 | | 10 | Protease inhibitor |
| HRG | 75,000 | 0.1 | 3q28-q29 | | | | Unknown function |

*; Vitamin K dependent protein containing γ-carboxyglutamic acid (Gla). Data are compiled from Furie & Furie, 1988; Davie, 1995; Stamatoyannopoulus *et al.*, 1994; Bloom *et al.*, 1994; Mckusick *et al.*, 1994. The latter reference is available on internet (http://gdbwww.gdb.org/omim/docs/omimtop.html).

The enzymes participating in the coagulation cascade are serine proteases and thus controlled by serpins (serine protease inhibitors). The most important serpin is antithrombin III, as demonstrated by the increased risk for thrombosis observed in patients with inherited antithrombin III deficiency (reviewed in Lane *et al.*, 1994; Broze & Tollefsen, 1994). Antithrombin III, a 58,000 kD single chain glycoprotein, is the main inhibitor of thrombin. In addition to thrombin, it also inhibits other serine proteases of the coagulation cascade, e.g. $FX_a$, $FIX_a$, $FXI_a$, $FXII_a$, kallekrein and the fibrinolytic protease plasmin. The rate of inhibition of the protease is markedly enhanced by heparin binding to antithrombin III, which induces a conformational change at the reactive centre. Normally there is no heparin in blood but the endothelium is lined by heparin-like glucosaminoglycans that possess anticoagulant activity similar to heparin.

Heparin cofactor II is another heparin-dependent thrombin inhibitor (reviewed in Broze & Tollefsen, 1994). It is a plasma glycoprotein that consists of a single polypeptide chain. The physiological significance of heparin cofactor II is still unclear.

The FVIIa-tissue factor complex and FXa are inhibited by tissue factor pathway inhibitor (TFPI) previously named lipoprotein-associated coagulation inhibitor (LACI) or extrinsic pathway

inhibitor (EPI) (reviewed in Broze & Tòllefsen, 1994). It is not a serpin and belongs to the Kunitz family of serine protease inhibitors. Recently, a new inhibitor of the VIIa-tissue factor complex has been cloned (Sprecher *et al.*, 1994). Having considerable amino acid sequence homology to TFPI and similar domain organisation, it is called TFPI-2.

The fibrinolytic system is an important defence system capable of dissolving blood clots (reviewed in Collen & Lijnen, 1994). A proenzyme known as plasminogen is converted to the active enzyme plasmin by tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA) on the surface of fibrin. In turn, plasmin degrades insoluble fibrin to soluble fibrin degradation products resulting in clot lysis. Fibrinolysis is regulated at several points by serpins. Plasminogen activator inhibitor 1 (PAI-1) inhibits t-PA, plasminogen activator inhibitor 2 (PAI-2) inhibits u-PA, and plasmin is inhibited by $\alpha_2$-antiplasmin. The physiological importance of the fibrinolytic system is illustrated by the fact that mice transgenic for human PAI-1 develop venous thrombosis in the tail soon after birth (Erickson *et al.*, 1990), while mice with inactivated PAI-1 gene have greater resistance to venous thrombosis due to a mild hyperfibrinolytic state (Carmeliet *et al.*, 1993). Mice with combined u-PA and t-PA deficiency suffer from extensive spontaneous fibrin deposition, with its associated effects on growth, fertility and survival (Carmeliet *et al.*, 1994).

**The protein C anticoagulant pathway**

The explosive nature of the coagulation cascade is controlled by several physiologically important mechanisms, one of which is the protein C anticoagulant system (Fig. 2) (Dahlbäck & Stenflo, 1994; Esmon, 1995; Esmon & Schwarz, 1995). Thrombin generated at sites of vascular injury has procoagulant effects as it activates platelets and factors XIII, XI, V and VIII in a positive feedback reaction, and converts fibrinogen to insoluble fibrin. In contrast, thrombin generated at sites of intact vessels binds to the endothelial membrane receptor protein thrombomodulin (TM) and attains anticoagulant properties. Binding of thrombin to TM modulates the proteolytic specificity of thrombin; procoagulant properties are lost, and the ability to activate protein C is gained. Activated protein C (APC) inhibits blood coagulation by limited proteolysis of factor Va and factor VIIIa (Fig. 2). These reactions are stimulated by a cofactor, protein S (Walker, 1981). Recently, intact factor V was demonstrated to function as a synergistic cofactor to protein S in the APC-dependent degradation of FVIIIa (Shen & Dahlbäck, 1994). This new cofactor activity of intact FV has both been confirmed (Varadi *et al.*, 1994) and questioned (Pötzsch *et al.*, 1995). The major physiological importance of the protein C anticoagulant system is most dramatically illustrated by the severe thromboembolic disorder which affects individuals with homozygous protein C deficiency already in the neonatal period (Seligsohn *et al.*, 1984).

**Protein C and thrombomodulin**

Protein C (reviewed in Stenflo, 1988; Dahlbäck & Stenflo, 1994) was first isolated from bovine plasma in 1976 and identified as a previously unknown vitamin K-dependent protein (Stenflo, 1976). Activated protein C was found to be identical to the anticoagulant autoprothrombin II described in 1960, which facilitated the elucidation of the function of protein C (Marciniak, 1972; Seegers *et al.*,

1976). Protein C is a zymogen to a serine protease that after activation possesses potent anticoagulant activity (Kisiel *et al.,* 1977; Kisiel, 1979). It is mainly synthesised in the liver. The precursor contains 461 amino acids, but a 42 amino acid long preproleader sequence is removed prior to secretion. In the Golgi apparatus, protein C is cleaved between Arg157 and Thr158, after which Arg 157 and Lys 156 are removed by an enzyme with carboxypeptidase B-like activity. The heavy (262 aa) and light (155 aa) chains are linked by a disulphide bridge. The mature protein C molecule is a multimodular protein containing an amino terminal Gla module (residues 1-45), two epidermal growth factor (EGF)-like modules (residues 46-91, 92-136) and a serine protease module (residues 137-419). The protein C gene consists of nine exons and eight introns encompassing 11 kb genomic DNA (Foster *et al.,* 1985; Plutsky *et al.,* 1986). The protein C gene has been localised to humane chromosome 2q14-q21 (Rocchi *et al.,* 1986; Long *et al.,* 1988; Kato *et al.,* 1988; Patracchini *et al.,* 1989).

Protein C is converted to the active serine protease, activated protein C (APC), through proteolytic cleavage by thrombin bound to thrombomodulin (Esmon & Owen, 1981; Owen & Esmon, 1981; Esmon, 1995). Thrombin cleaves a peptide bond between Arg169 and Leu170 in the heavy chain of protein C, which releases a dodecapeptide (the activation peptide) from the heavy chain. The conversion of protein C to APC is slow and negligible without the participation of thrombomodulin (TM) . TM is a 557 amino acid multimodular integral membrane glycoprotein expressed on endothelial cells (Esmon, 1995). It consists of a lectin-like module, six EGF-like modules, a Ser/Thr-rich region, a transmembrane module and a short cytoplasmatic tail (Fig. 2). Thrombin binds with high affinity to the fifth and sixth EGF-modules. Thrombin bound to TM loses all its procoagulant capacity, and instead activates protein C to APC. The fourth EGF domain is required for TM to accelerate activation of protein C, and together the EGF modules 4-6 express full cofactor activity. A sulphated glycosaminoglycan in the Ser/Thr rich region also contributes to the binding of thrombin to TM. Thrombin bound to TM is 20,000-fold more effective than thrombin alone in activating protein C.

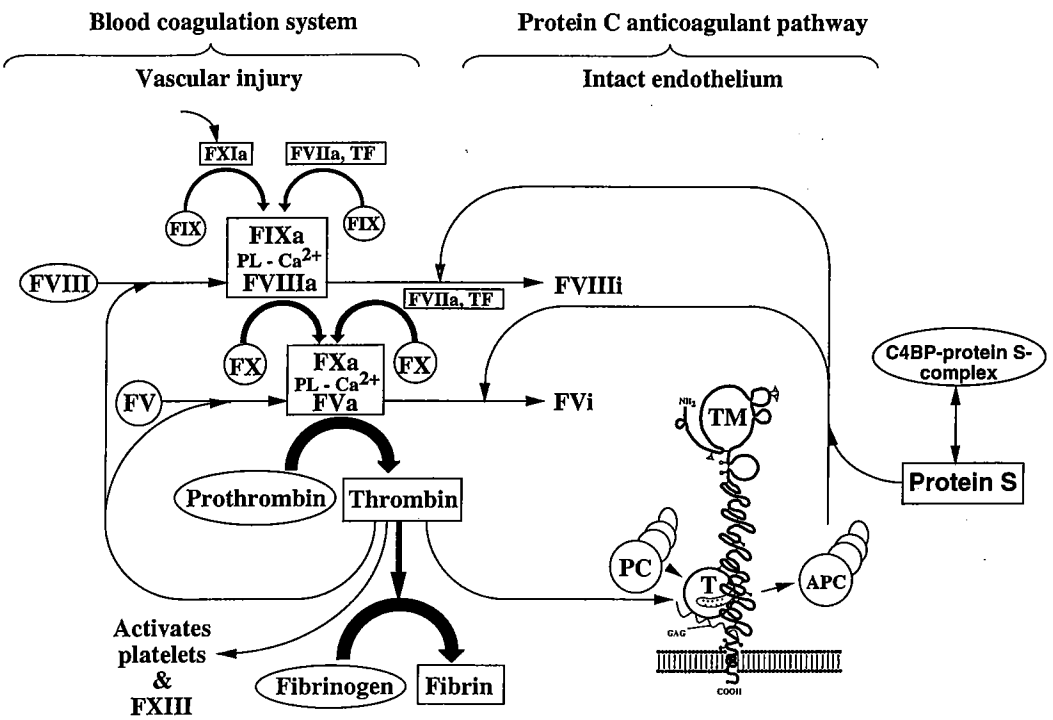


**Fig. 2.** Schematic representation of the coagulation cascade and the protein C anticoagulant system. Thrombin generated at sites of vascular damage converts fibrinogen to fibrin, and activates platelets, FXIII, FXI, FV and FVIII. Thrombin (T) formed at sites of intact endothelium binds to the membrane protein thrombomodulin (TM). Thrombin bound to TM has no procoagulant properties; instead it activates protein C. APC, together with its cofactor protein S, degrades the activated forms of factors V and VIII. Thrombin cleavage of protein S results in the loss of APC cofactor activity (not shown). TF (tissue factor) triggers the reactions involving factor VII (Modified from Zöller *et al.*, 1995c).

The catalytic region of protein C is homologous to other serin proteases. The catalytic triad in activated human protein C consists of His 211, Asp 257 and Ser 360. The specificity of APC is very limited, and its only known substrates are factors Va and VIIIa. However, protein C may well have other functions and a protein C receptor that can interact with both protein C and APC has been described (Fukudome & Esmon, 1994). The function of this interaction is unknown, but as the protein C receptor is homologous to the CD1/MHC superfamily, a role in the modulation of inflammatoric response has been suggested (Esmon, 1995). In vivo, APC blocks the lethal effects of Escherichia coli infusion in baboons (Taylor *et al.,* 1987). Moreover, a profibrinolytic effect of APC has been reported in several studies (Comp & Esmon, 1984; Taylor & Lockhart, 1985; Sakata *et al.,* 1986). However, patients with homozygous protein C deficiency have normal fibrinolytic activity, which argues against APC fulfilling an important function in the fibrinolytic system (Aznar *et al.,* 1986).

Once formed, APC is slowly inhibited by protein C inhibitor (PCI), α1-antitrypsin and by α2-macroglobulin (reviewed in Dahlbäck & Stenflo, 1994). The long half-life of APC in the circulation (15-20 minutes) is a prerequisite for its function as a circulating anticoagulant (Comp *et al.,* 1982; Comp, 1984). Complex formation between PCI and activated protein C is enhanced by heparin or other glycosaminoglycans, whereas the inhibition by α1-antitrypsin and α2-macroglobulin is heparin independent. The physiological significance of inhibition of APC is not yet known, but is probably important for the regulation of the protein C anticoagulant pathway.

**Protein S and C4b-binding protein**

Protein S was first purified from human plasma and was named after the place of its discovery, S for Seattle (Discipio *et al.,* 1977). Human protein S is a single chain glycoprotein with a molecular weight of 75 kD. Its normal concentration in plasma is 20-25 mg/mL. It is synthesised in several tissues: in the liver, by endothelial cells, by Leydigs cells in the testis, in megacaryocytes and in the brain (Schwarz *et al.,* 1985; Fair & Marlar, 1986; Fair *et al.,* 1986; Stern *et al.,* 1986a; Ogura *et al.,* 1987; Malm *et al.,* 1994; He *et al.,* 1995; Stitt *et al.,* 1995). The primary structure of human protein S has been determined by cDNA cloning (Lundwall *et al.,* 1986; Hoskins *et al.,* 1987; Ploos van Amstel *et al.,* 1987b). Protein S is synthesised as a 676 amino acid long precursor protein. Prior to secretion, a leader sequence of 41 amino acid residues is cleaved off. It is cleaved off at two sites located at Ala-18 and Arg-1. Residues -41 to -18 constitute the signal peptide necessary for transportion over the endoplasmic reticulum. The propeptide (residues Asn-17 to Arg-1) is the recognition site for the vitamin K-dependent carboxylase, which converts glutamic acids to γ-carboxyglutamic acid residues. There are two protein S genes (PSα and PSβ) in the human genome, and they are both located on chromosome 3q11.2 close to the centromere (Ploos van Amstel *et al.,* 1987a; Watkins *et al.,* 1988; Long *et al.,* 1988). Only the PSα gene is expressed, whereas the PSβ gene is a silent pseudogene containing multiple base changes, stop codons and frameshifts (Edenbrandt *et al.,* 1990; Ploos van Amstel *et al.,* 1990; Schmidel *et al.,* 1990; Ploos van Amstel *et al.,* 1988). The two protein S genes manifest 97% sequence identity between the exons. The active protein S gene spans over 80 kb and comprises 15 exons and 14 introns. Like the other vitamin K dependent coagulation factors, the

protein S molecule is composed of several discrete domains or modules. The 5' part of the molecule encoded by exons I-VIII is homologous to the other vitamin K-dependent coagulation proteins (except for the thrombin-sensitive loop). The 3' part of exon I codes for a signal peptide, exon II for a propeptide and the Gla module, exon III for the hydrophobic region, exon IV for a thrombin-sensitive module and exons V to VIII for EGF-like modules. The 3' part of the molecule is homologous with sex hormone-binding globulin and is encoded by exons IX to XV (amino acid 243-635) (Gershagen *et al.*, 1987; Baker *et al.*, 1987; Gershagen *et al.*, 1991).

The Gla module (residues 1-37) binds multiple calcium ions and has high affinity for negatively charged phospholipid membranes (Schwalbe *et al.*, 1989; Schwalbe *et al.*, 1990). A hydrophobic region (residues 38-46) connects the Gla module and the thrombin-sensitive module (residues 46-75). The thrombin-sensitive region is sensitive to proteolysis by thrombin, though the Gla module remains linked to the rest of the molecule via a disulphide bond. The APC cofactor function is lost upon thrombin cleavage, suggesting that the thrombin-sensitive module is essential for APC cofactor function and interaction with APC (Dahlbäck, 1983a; Dahlbäck *et al.*, 1986; Suzuki *et al.*, 1983; Walker, 1984a). The four EGF-like modules (residues 76-242) contain two post translationally modified residues, Hya (β-hydroxyaspartic) and Hyn (β-hydroxyasparagine) (Stenflo *et al.*, 1987). The first EGF-like module harbours one Hya, and the three following modules harbour Hyn. Very high affinity calcium binding sites important for native conformation and protease resistance are contained within the Hyn-containing EGF-like modules (Dahlbäck *et al.*, 1990b). However, the functional significance of Hya and Hyn is unknown. Recombinant protein S with Asp and Asn instead of Hya and Hyn, expresses full APC cofactor function and C4BP binding (Nelson *et al.*, 1991). The C-terminal half of protein S (residues 243-635) is homologous to SHBG (sex hormone binding globulin) and is therefore named the SHBG domain or the SHBG-like module. This part of the molecule contains three potential glycosylation sites at Asn 458, Asn 468 and Asn 489. The SHBG-like module contains two internal disulphide bonds in the form of small disulphide loops (Cys 408-Cys 434 and Cys 597-Cys 625). These two loops have been suggested to be involved in the binding of protein S to C4b-binding protein (Walker, 1989; Nelson & Long, 1992; Chang *et al.*, 1992; Fernández *et al.*, 1993; Chang *et al.*, 1994).

Protein S functions as a cofactor to APC in the inactivation of factors Va and VIIIa (Dahlbäck & Stenflo, 1994). The vitamin K-dependent protein S has a very high affinity for negatively charged phospholipids (Kd 7x10-8M), and it increases the affinity of APC for this type of membrane (Walker, 1981; Walker *et al.*, 1987; Walker, 1984b). It has been proposed that protein S augments binding of APC to membrane surfaces, abrogating the protective effects of factor Xa and IXa, respectively (Harris & Esmon, 1985; Stern *et al.*, 1986b; Regan *et al.*, 1994). Thus, the function of protein S might be to make factor Va and VIIIa available for inactivation by APC (Solymoss *et al.*, 1988). However, the level of APC cofactor activity is rather low in systems using purified components (Bakker *et al.*, 1992). A possible explanation for this puzzling phenomenon is that intact factor V and protein S function as synergistic cofactors in the degradation of factor VIII (Shen & Dahlbäck, 1994; Váradi *et al.*, 1995). However, an APC independent anticoagulant function of protein S has also been demonstrated (Heeb *et al.*, 1993; Heeb *et al.*, 1994; Hackeng *et al.*, 1994; van't Veer

*et al.,* 1995; Koppelman *et al.,* 1995). Protein S has been shown to inhibit the 'prothrombinase' activity directly without the help of APC (Heeb *et al.,* 1993; Heeb *et al.,* 1994; Hackeng *et al.,* 1994), and to inhibit the intrinsic factor X activating complex by binding to factor VIII (Koppelman *et al.,* 1995). The exact anticoagulant mechanism of protein S therefore is still somewhat unclear. Other functions than anticoagulant properties have been ascribed to protein S. Protein S has been reported to function as a potent mitogen to cultured smooth muscle cells (Gasic *et al.,* 1992). More recently, protein S has been demonstrated to be expressed in the brain (He *et al.,* 1995; Stitt *et al.,* 1995). Receptors have been identified for protein S and its relative Gas 6 (Stitt *et al.,* 1995). The physiological function of this is not yet understood.

Protein S exists in two forms in human plasma, as free protein (30-40%) and in non-covalent 1:1 complex with C4b-binding protein (C4BP) (Dahlbäck & Stenflo, 1981). Only the free protein S is active as APC-cofactor (Dahlbäck, 1986), and selective deficiency of free protein S is associated with an increased risk of thrombosis (Comp & Esmon, 1984; Comp *et al.,* 1984). C4BP functions as a regulator of the classical complement pathway and has a plasma concentration of approximately 150 mg/mL. It down-regulates the complement pathway by functioning as a cofactor to factor I in the degradation C4b and by accelerating the decay of C3-convertase (the C4bC2a complex). The function of the complex between protein S and C4BP is not known, and in bovine and rabbit plasma no complex has been demonstrated (Dahlbäck, 1986; He & Dahlbäck, 1994; Hillarp *et al.,* 1994). However, protein S may localise C4BP to negative charged phospholipid membranes to provide local down-regulation of the complement system (Schwalbe *et al.,* 1990).

C4BP is a large multimeric protein (570 kD) that exists in different isoforms (Dahlbäck & Stenflo, 1994) (Fig. 3). It is composed of 6 or 7 α-chains (70 kD) and 1 or no β-chain (45 kD) (Chung *et al.,* 1985; Hillarp & Dahlbäck, 1988; Dahlbäck & Stenflo, 1994). The C4BP molecule is a spider-like molecule with the α-chains (549 amino acids) radiating from a central body (Fig. 3). The β-chain is much shorter (235 amino acids) but both the α- and β-chains are composed of short consensus repeats (SCRs). Only the β-chain containing isoform of

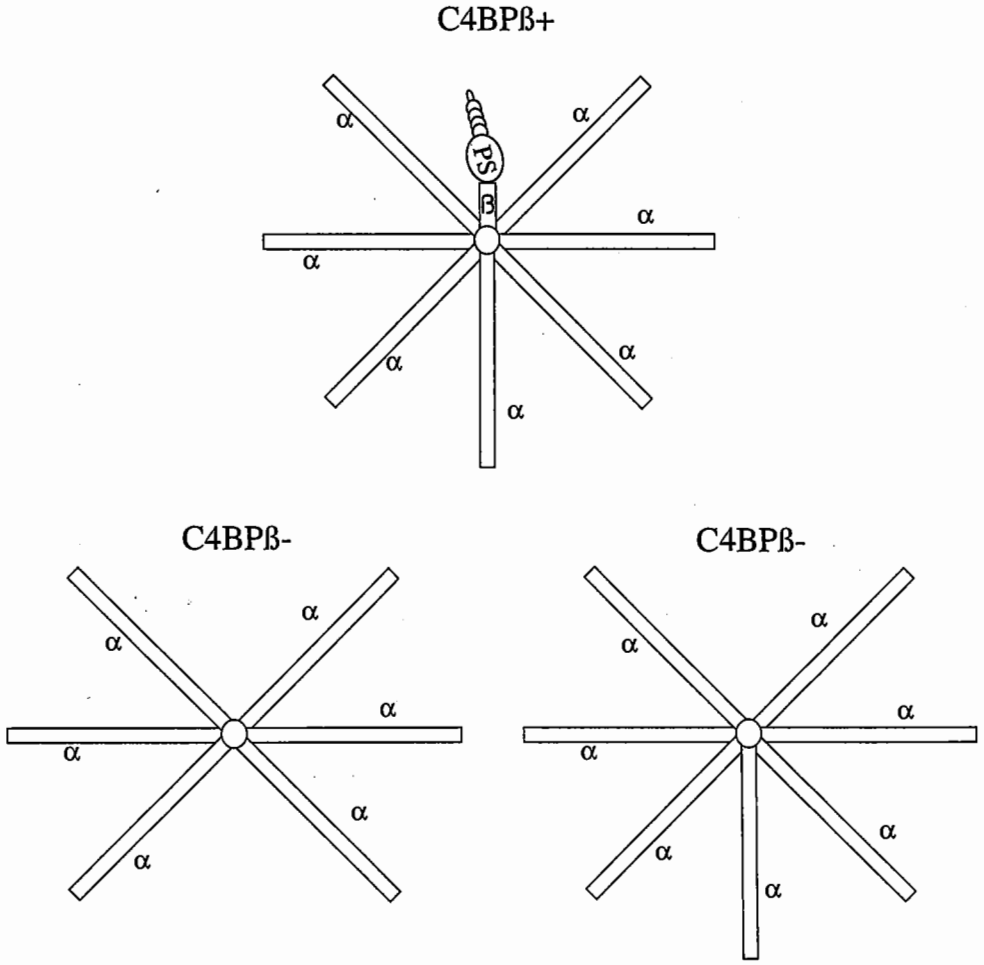


**Fig. 3.** Schematic representation of the different isoforms of C4b-binding protein (C4BP). The spider-like C4BP molecule contains two types of subunits, 6 or 7 α-chains and one or no β–chain. The subunits are linked by disulphide bridges involving cysteine residues in the COOH-terminal ends of the subunits. Only the β-chain-containing isoform of C4BP (C4BPβ+) binds protein S (PS), as the protein S binding site is localised on the β-chain. C4BPβ- denotes C4BP isoforms lacking the β-chain and unable to bind protein S.

C4BP binds protein S, as protein S interacts with the β-chain (Hillarp & Dahlbäck, 1988; Hillarp *et al.*, 1989; Härdig *et al.*, 1993; Fernandez & Griffin, 1994). The C4b binding site is located on the α-chain and is independent of protein S binding. The non-covalent protein S-C4BP interaction is of high affinity, with a $K_d$ of approximately $10^{-7}$ M in the absence of calcium and 5 x$10^{-10}$ M in its presence (Dahlbäck, 1983b; Schwalbe *et al.*, 1990; Dahlbäck *et al.*, 1990a; Nelson & Long, 1991; Nelson & Long, 1992). Different mechanisms have been suggested for the regulation of free protein S. As C4BP and protein S occurs in plasma of similar concentrations, a third component regulating the complex formation in vivo has been speculated upon (Dahlbäck *et al.*, 1990a). However, the concentration of the β-chain-containing isoform of C4BP (C4BPβ+) is lower than the total C4BP concentration (C4BPt), and free protein S has been found to represent the molar excess of total protein S over C4BPβ+ (Griffin *et al.*, 1992). This finding holds true not only for healthy persons, but also during acute-phase response where the concentration of C4BPt increases (Saeki *et al.*, 1989; de Frutos *et al.*, 1994). The expression of α- and β-chains is differentially regulated with a higher expression of C4BP isoforms lacking the β-chain (C4BPβ-), which ensures molar excess of protein S and a stable level of free anticoagulant protein S (Garcia de Frutos *et al.*, 1994). Consistent with this view is the finding of increased levels of free protein S in patients with inherited C4BP deficiency (Comp *et al.*, 1990). The mechanism responsible for the selective inherited deficiency of free protein S was previously unknown but was elucidated in one of the present studies (Zöller *et al.*, 1995a).

**Factor Va and VIIIa inactivation by activated protein C**

The activated forms of factor V and VIII (FVa and FVIIIa) are substrates for APC (Fig. 2). Factors V and VIII (FV and VIII) are high molecular weight glycoproteins (300 kD) with similar structures and functions (Kane & Davie, 1988; Jenny *et al.*, 1994; Fay, 1993; Sadler & Davie, 1994). The concentration of FV in plasma is 50-100-fold higher than that of FVIII (Table 1). Unlike FV, FVIII circulates bound to another protein, von Willebrand factor (vWF). The factor VIII gene encompasses 186 kb at chromosome Xq27.3 (Purello *et al.*, 1985). It comprises 26 exons encoding a mRNA of 9 kb (Toole *et al.*, 1984; Gitschier *et al.*, 1984). The factors V gene maps to chromosome 1q21-q25 and spans more than 80 kb (Wang *et al.*, 1988; Dahlbäck *et al.*, 1988; Cripe *et al.*, 1992). The factor V gene contains 25 exons and 24 introns. The complete cDNA of human FV codes for a 2224 amino acid long protein including a 28 amino acid leader peptide (Jenny *et al.*, 1987). Both factor VIII and factor V contain three A-modules of approximately 350 amino acids and two C-modules of approximately 150 amino acids (factor V shown in Fig 4). These modules share a 40% identity between the two factors (Kane *et al.*, 1987; Kane & Davie, 1986; Jenny *et al.*, 1987). At the amino terminal are located two A-modules. The second and third A-modules of FV and FVIII are separated by a connecting region (B-domain) of 836 and 980 amino acids, respectively (Fig. 5). Unlike the rest of the molecule, the B domain does not share any significant similarity between the two proteins, except for a high content of serine and threonine with many potential N-linked glycosylation sites (Asn-X-Ser/Thr). FVIII and FV are activated by limited proteolysis by thrombin or factor Xa (Fig 5). During activation by thrombin at least three peptide bonds are cleaved in each molecule. Activation converts FV to a heterodimer, and FVIII to a heterotrimer, the subunits of which are linked by non-

covalent $Me^{2+}$-dependent bonds. FVa and FVIIIa then bind to negatively charged phospholipid and together with calcium ions function as receptors/cofactors for factor Xa and IXa, respectively. The macro-molecular complexes 'tenase' (negatively charged phospholipids, calcium, FIXa and FVIIIa) and 'prothrombinase' (negatively charged phospholipids, calcium, FXa and FVIa) activates factor X and prothrombin, respectively, 100,000-fold more rapidly than do the respective factors alone. The rate increase is due to a lowering of the $K_m$ for the substrates mediated by phospholipids, and by an increased $V_{max}$ for FX or prothrombin activation mediated by factors FVIIIa and FVa, respectively. The latter effect accounts for a 1000-fold increase in the rate of factor X or prothrombin activation.

The powerful anticoagulant effect of APC is due to degradation of FVIIIa and FVa, resulting in inhibition of the tenase and prothrombinase activity, respectively. APC efficiently cleaves only FVa and FVIIIa, while intact factor V and VIII are poor substrates for APC. The specificity for FVa and FVIIIa is demonstrated in infusion experiments with APC. Infusion with APC in animals prolongs the activated partial thromboplastin time without affecting the plasma levels of intact FV and FVIII or the bleeding time (Comp *et al.,* 1982; Comp, 1984). Three peptide bonds are cleaved in the heavy chain of FVIIIa by APC: $Arg^{336}$-$Met^{337}$, $Arg^{562}$-$Gly^{563}$ and $Arg^{740}$-$Ser^{741}$ (Fay *et al.,* 1991). The cleavage between $Arg^{562}$ and Gly correlates best with loss of FVIIIa activity. Three peptide bonds are also cleaved in FVa by APC (Fig. 5) (Kalafatis *et al.,* 1994; Kalafatis *et al.,* 1995). First the bond between $Arg^{506}$-$Gly^{507}$ is cleaved, which facilitates cleavage at position $Arg^{306}$ and $Arg^{679}$ necessary for complete inactivation of FVa (Fig. 5). A point mutation changing $Arg^{506}$ to Gln results in resistance to activated protein C and is the most common genetic risk factor for thrombosis (Bertina *et al.,* 1994; study I, Zöller & Dahlbäck, 1994).

That the protein C system is of the utmost importance for vascular patency is demonstrated by the fact that individuals born with homozygous protein C deficiency without detectable protein C activity suffer from fatal massive thrombosis already in the neonatal period (Seligsohn *et al.,* 1984). Heterozygous deficiency of protein C or S is associated with an increased risk of thrombosis in middle-age (Griffin *et al.,* 1981; Comp & Esmon, 1984). Several in vivo experiments in animals have shown the potent anticoagulant action of the protein C system. In a rabbit microarterial model of thrombosis, APC was found to exert powerful and lasting antithrombotic effects (Arnljots *et al.,* 1994). In a subsequent study, protein S was demonstrated to potentiate the effect of APC in the same thrombosis model without any haemorrhagic side effects (Arnljots & Dahlbäck, 1995). APC infusion was also found to inhibit platelet-dependent thrombosis in a baboon arterial thrombosis model (Gruber *et al.,* 1990).

## Thrombosis

The formation of a clotted mass of blood in the non-interrupted vascular system is known as thrombosis. The mass itself is known as a thrombus. Thrombosis may be regarded as the consequence of inappropriate activity of the normal haemostatic process. A thrombus is composed of blood components containing platelets, white cells and fibrin. Thrombi are of critical importance for two reasons: they cause obstruction of the vessels and they may break loose to create an embolus that is carried away and causes obstruction at a distant site. As a background to the present investigation the epidemiology and risk factors for thrombosis will be reviewed.

## Epidemiology of deep venous thrombosis and pulmonary embolism

Venous thromboembolism is a major medical problem, constituting the third most common cardiovascular disease after acute ischaemic heart disease and stroke (Goldhaber, 1994). The overall incidence has been estimated to be 2.5% to 5%, though the diagnostic criteria in these studies were not optimal (Gjöres 1956, Coon *et al.*, 1973). In the USA, pulmonary embolism and deep venous thrombosis account for 250,000 hospitalisations annually, corresponding to an incidence of 1 per 1000, and an annual death rate of 50,000 (Gillum, 1987; Goldhaber, 1994). In Sweden, the incidence of thrombosis may be even higher. In a prospective study the annual incidence of phlebography verified deep-vein thrombosis in the city of Malmö was 1.6 per 1000 inhabitants (Nordström *et al.*, 1992).

The risk of thrombosis increases exponentially with age, and venous thromboembolism in elderly institutionalised patients is a major problem (Taubman & Silverstone, 1986; Nordström *et al.*, 1992; Kniffin *et al.*, 1994). Both acquired and inherited disorders are important aetiological factors in the development of thrombosis (Table 3). Venous thromboembolism occurs more often in Western communities than in Asian and African populations (Woo *et al.*, 1988; Thomas *et al.*, 1960). This difference has been speculated to be due to differences in diet, exercise and bowel habits (Goldhaber, 1994). However, recent data suggest that the observed difference in thrombotic incidence may in part have a genetic explanation, e.g. lower prevalence of inherited APC-resistance in Asians than in Caucasians (Zöller & Dahlbäck, 1995; Takamiya *et al.*, 1995). In Caucasians, APC-resistance has recently been found in 20 to 60% of thrombosis patients, demonstrating that the importance of genetic factors in the pathogenesis of thrombosis was previously underestimated. Interestingly, among the rare Chinese patients with venous thromboembolism, 48% (25 of 52 unselected cases) had deficiencies of protein S, protein C or antithrombin III, which are rare disorders both in Chinese population and in Western populations (Liu *et al.*, 1994). Thus, genetic factors are equally as important in the pathogenesis of thrombosis in Chinese patients as in Caucasians. It was hypothesised that the lower thrombotic incidence in Asians simply reflects the lower prevalence of inherited prothrombotic disorders. The hypothesis that the lack of APC-resistance in non-Caucasians may partly explain the rarity of thromboembolic disease in non-Western communities has recently been confirmed (Rees *et al.*, 1995). No carriers of the FV:$Q^{506}$ mutation (APC-resistance) were found in indigenous populations in Asia, Africa/middle east, Australasia, America (Indians), Pakistan, Sri Lanka or Gujerati.

## Hypercoagulable states

Hypercoagulable states are a collection of clinical conditions in which the balance between pro- and anti-coagulant forces is shifted in favour of coagulation (Hirsh *et al.*, 1986; Schafer, 1985). In principle all hypercoagulable states are due to disturbances in the blood flow, in the vessel wall or in the constituents of blood, as described by Virchow more than a century ago. The hypercoagulable states are divided into two main groups: those with a hereditary basis and those resulting from circumstantial risk factors or acquired conditions (Table 3).

**Table 3. Hypercoagulable states.**

| **Primary hypercoagulable states** (Genetic risk factors) | **Secondary hypercoagulable states** (Circumstantial risk factors) |
|---|---|
| Antithrombin III deficiency | Advanced age |
| Cystathionine β-synthase deficiency | Behcet's syndrome |
| Protein C deficiency | Heart disease |
| Protein S deficiency | Hyperviscosity |
| Resistance to activated protein C | Immobilisation |
| | Lupus anticoagulans |
| **Other potential or suggested candidate genes** | Malignancy |
| Dysfibrinogenaemia | Myeloproliferative disorders |
| $\beta_2$-glycoprotein I | Nephrotic syndromes |
| Factor XII deficiency | Obesity |
| Heparin cofactor II | Oral contraception |
| HRG | Paroxysmal nocturnal haemoglobinuria |
| PAI-1 | Pregnancy and puerperium |
| Plasminogen deficiency | Trauma and surgery |
| TFPI | Varicose veins |
| Thrombomodulin | |
| t-PA | |

HRG, histidine-rich glycoprotein; PAI-1, plasminogen activator inhibitor 1; TFPI, tissue factor pathway inhibitor; t-PA, tissue plasminogen activator.

More than a century ago Trousseau described the syndrome of recurrent migratory thrombophlebitis in patients with cancer. A large number of acquired risk factors have since been recognised, which give rise to the secondary hypercoagulable states found in a large proportion of thrombosis patients (Table 3) (Schafer, 1985; Hirsh *et al.*, 1986). Though thrombosis is often familial, genetic defects of the anticoagulant factors, such as deficiency of antithrombin III, protein C or protein S, have been found in only a few percent of all thrombosis patients (Table 4). The discovery of inherited resistance to activated protein C (APC resistance) (Dahlbäck *et al.*, 1993), caused by a single point mutation in the factor V gene (Bertina *et al.*, 1994), has dramatically improved our understanding of the pathogenesis of venous thrombosis. Findings in recent studies suggest that multiple risk factors are needed for development of thrombosis, and that acquired and genetic risk factors act in concert in the pathogenesis of thrombosis. Whether genetically susceptible individuals develop thrombosis depends on the interaction of genetic and acquired risk factors or such circumstantial risk factors as sedentary life-stile, pregnancy, inflammation, immobilisation or surgery (Table 3).

**Hypercoagulable states are recognised by increased thrombin generation**

Different peptides, enzymes and enzyme-inhibitor complexes are liberated when the coagulation system is activated in vivo (Mannucci, 1994; Bauer, 1994). Sensitive assays have been developed for several of these markers, and some are available as commercial kits using either polyclonal or monoclonal antibodies. Only three markers commonly encountered will be discussed here. The fragment 1+2 ($F_{1+2}$) is cleaved off from the amino terminal end of human prothrombin when activated by factor Xa. The concentration in plasma of $F_{1+2}$ reflects the degree of activation of the common coagulation pathway. Thrombin in turn converts fibrinogen into the fibrin releasing fibrinopeptides A (FPA) and B (FPB). Thrombin is inactivated by forming a stable complex with antithrombin III (TAT). Commercial immunochemical assays are available for the measurement of FPA, $F_{1+2}$ and TAT, and have been used in a number of studies. Though healthy controls and patients overlap, several studies have shown the levels of markers of coagulation activation to be increased both in conjunction with acquired (Bauer *et al.,* 1987; Kienast *et al.,* 1993; Rahr *et al.,* 1994; Mari *et al.,* 1995) and inherited hypercoagulability (Bauer *et al.,* 1988; Mannucci *et al.,* 1992; Demers *et al.,* 1992a; Zöller *et al., 1996,* paper VI).

**Primary hypercoagulable states**

The familial thrombophilia syndrome was described already in the beginning of the century (reviewed in Jordan & Nandorff, 1956), but was not accorded much attention until after 1965 when inherited antithrombin III deficiency was first described (Egeberg, 1965). Several genes have been suggested to be involved in the pathogenesis of thrombosis but only antithrombin III, protein C, protein S and factor V (APC-resistance) have been associated with the dominant familial thrombophilia syndrome (Bertina, 1988; Allaart & Briët, 1994; Dahlbäck *et al.,* 1993). Of these defects, APC-resistance is much more common than any other genetic defect, both among thrombosis patients and healthy individuals (Table 4). However, deficiencies of antithrombin III, protein S and protein C also occur at relatively high prevalence in the general population (Table 4) (Tait *et al.,* 1995; Koster *et al.,* 1995a), suggesting that thrombosis-prone families with these defects often have yet another genetic defect predisposing to thrombosis (Koeleman *et al.,* 1994; Zöller *et al.,* 1995; van Boven *et al.,* 1995). Before describing these important genetic risk factors for thrombosis, a summary of the other candidate genes is given.

*Dysfibrinogenaemia* is detected by a prolonged thrombin time, a prolonged reptilase time and by a lower fibrinogen level that is lower in chronometric than in immunological determinations. Dysfibrinogenaemia is a very rare disorder found in approximately 0.8% of thrombosis patients (Haverkate & Samama, 1995). No more than 250 cases have been reported to date; and of these, only 20% were characterised by predisposition to thrombosis, whereas 25% were characterised by haemorrhagic diathesis instead, and the remaining 55% were asymptomatic. The clinical manifestations depend on the associated molecular fibrinogen defect. Family studies of the relatives of 26 thrombotic probands with dysfibrinogenaemia showed 20% (20/99) of biochemically affected relatives to have had thrombosis, as compared to none of 88 relatives without the defect. This

suggests that, although dysfibrinogenaemia is associated with familial thrombophilia, it is a very uncommon cause of thrombosis.

*Homocystinuria* is a very rare autosomal recessive disorder usually caused by cystathionine β-synthase deficiency (Schimke *et al.*, 1965; McCully, 1969; Harker *et al.*, 1974; Newman & Mitchell, 1984; Bertina, 1988). The incidence of cystathionine β-synthase deficiency have ranged from 1/60,000 to 17/50,000. It is characterised by venous and arterial thrombosis, mental retardation, ectopia lentis and skeletal abnormalities. So far it is not known whether heterozygous cystathionine β-synthase deficiency is also associated with an increased risk of venous thrombosis (Allaart & Briët, 1994).

**Table 4. Prevalence of antithrombin III, protein C and protein S deficiency and APC-resistance in healthy controls, and in thrombosis patients.**

| | ATIII | Protein C | Protein S | APC-resistance |
|---|---|---|---|---|
| **Healthy controls** | | | | |
| Tait *et al.*, 1991 | 0.3% | - | - | - |
| Miletich *et al.*, 1987 | - | 0.4% | - | - |
| Tait *et al.*, 1995 | - | 0.2% | - | - |
| Koster *et al.*, 1993 | - | - | - | 5% |
| Svensson & Dahlbäck, 1994 | - | - | - | 7% |
| Koster *et al.*, 1995a | 0.2-1.9% | 0.4-1.5% | 0.7-2.3% | - |
| **Patients with deep venous thrombosis** | | | | |
| Gladson *et al.*, 1988 | - | 4.3% | 5.0% | - |
| Ben Tal *et al.*, 1989 | 7.5% | 5.6% | 2.8% | - |
| Heijboer *et al.*, 1990 | 1.1% | 3.2% | 2.2% | - |
| Tabernero *et al.*, 1991 | 0.5% | 1.5% | 1.5% | - |
| Malm *et al.*, 1992 | 0.7% | 2.3% | 2.1% | - |
| Koster *et al.*, 1993 | - | - | - | 21% |
| Svensson & Dahlbäck, 1994 | - | 1.9% | 2.9% | 40% |
| Cadroy *et al.*, 1994 | - | - | - | 19% |
| Koster *et al.*, 1995a | 1.1-4.2% | 2.7-4.6% | 1.1-3.1% | - |

*Factor XII* deficiency has been shown in several studies to be associated with thrombosis (Goodnough *et al.*, 1983; Rodeghiero *et al.*, 1992; Lämmle *et al.*, 1991). However, family studies have failed to confirm the existence of correlation between factor XII deficiency and familial thrombophilia.

*Heparin cofactor II* is an other candidate gene. Families with heparin cofactor II deficiency and thrombosis have been described (Sie *et al.*, 1985; Tran *et al.*, 1985; Matsuo *et al.*, 1992). However, many patients with the deficiency manifest no thrombotic symptoms (Bertina *et al.*, 1987). *β2-glycoprotein I* (apolipoprotein H) is an inhibitor of contact activation and platelet dependent prothrombinase activity, and is a cofactor for the binding of antiphospholipid antibodies to negatively-charged phospholipids (McNeil *et al.*, 1990). However, the prevalence of β2-glycoprotein I deficiency was found to be similar in thrombosis patients and in healthy controls (Bancsi *et al.*, 1992). For another interesting candidate gene TFPI, a Kunitz-type proteinase inhibitor of the extrinsic pathway, no evidence of a relationship between inherited deficiency and thrombosis has yet been reported.

*Impairment of the fibrinolytic system* has been suggested to be associated with thrombophilia. Inherited deficiency of plasminogen, the precursor to the fibrin-digesting enzyme plasmin, has been reported in several cases of thrombophilia (Aoki *et al.,* 1978; Girolami *et al.,* 1986; Dolan *et al.,* 1988; Robbins, 1988). However, only in a minority of family members was any relationship found between low plasminogen levels and thrombotic symptoms (Tosetto *et al.,* 1994; Allaart & Briët, 1994). Whether plasminogen deficiency is an inherited cause of thrombosis is therefore still uncertain. An increased level of plasminogen activator inhibitor 1 (PAI-1) with decreased fibrinolytic activity has been reported as a cause of familial thrombophilia in three families (Johansson *et al.,* 1978; Jörgensen *et al.,* 1982; Stead *et al.,* 1983; Nilsson & Tengborn, 1983; Pizzo *et al.,* 1986; Jörgensen & Bonnevie-Nielsen, 1987). However, re-evaluation of the North American and the Swedish kindreds showed deficiency of protein S to be the main cause of thrombosis in these two families (Bolan *et al.,* 1993; Zöller & Dahlbäck, 1993). Other studies have failed to show the existence of a direct genetic relationship between thrombophilia and deficiency of t-plasminogen activator (t-PA), increased levels of PAI-1 (Engesser *et al.,* 1989) or histidine-rich glycoprotein (reduces plasminogen binding to fibrin) (Engesser *et al.,* 1987b). Thus, there exists no unequivocal evidence that the increase in the level of PAI-1 found in thrombosis patients is genetically determined (Malm *et al.,* 1992; Juhan-Vague *et al.,* 1987) or that genetic defects of the fibrinolytic system in humans are associated with an increased risk of venous thrombosis. However, a 4G allele of a common 4/5-guanine-tract (4G/5G) polymorphism in the promotor region in the PAI-1 gene has recently been suggested to be a risk factor for myocardial infarction (Eriksson *et al.,* 1995), though this was not confirmed in a large multicentre study (Ye *et al.,* 1995). The homozygous form of the 4G allele is associated with increased PAI-1 antigen levels due to reduced affinity for a transcriptional repressor protein (Eriksson *et al.,* 1995; Ye *et al.,* 1995). It remains to be determined whether this polymorphism is related to venous disease.

As *thrombomodulin* is an endothelial cell membrane protein, there are difficulties in measuring its activity in plasma. Instead, the thrombodulin gene has been screened for mutations. A dimorphism, $Ala^{455}$ to Val, was found, though it was unrelated to thrombosis (van der Welden *et al.,* 1991). Recently, a point mutation in the TM gene, which predicts replacement of $Asp^{468}$ with a Tyr, was identified in a 45-year-old man with thromboembolic disease (Öhlin & Marlar, 1995). Whether this mutation affects the TM function or the level of protein expression, and whether it is a risk factor for thrombosis remains to be elucidated.

**Antithrombin III deficiency**

Deficiency of antithrombin was first described in 1965 (Egeberg, 1965). It is inherited as an autosomal dominant trait (Thaler & Lechner, 1981; Hirsh *et al.,* 1989; Demers *et al.,* 1992b; Lane *et al.,* 1994). The prevalence of antithrombin III deficiency in 4000 healthy blood donors has been reported to be 1/350 (Tait *et al.,* 1991). Its frequency in thrombosis patients various from 1% to 8% (Table 4). Patients with antithrombin III deficiency usually present with thrombosis at an early age, but seldom in the first decade of life. The thrombotic risk increases between the ages of 15 and 30 years. By the age of 55, 85% of antithrombin-deficient patients have developed thrombosis. Thus, the thrombotic risk appears to be higher for antithrombin III deficiency than for deficiency of protein S or protein C (Finazzi & Barbui, 1994). The most common manifestations of thrombosis are deep leg and iliac vein thrombosis, although pulmonary embolism is also common. However, superficial thrombophlebitis is less common in antithrombin III deficiency than in deficiency of protein C or protein S. The first thrombotic event is triggered by a circumstantial risk factor in approximately 50% of cases. The risk of thrombosis in antithrombin III-deficient patients in conjunction with pregnancy or oral contraception is even higher than in conjunction with protein C or S deficiency (Conard *et al.,* 1990; De Stefano *et al.,* 1994b; De Stefano *et al.,* 1994a; Pabinger *et al.,* 1994b). Forty per cent of pregnancies in antithrombin III-deficient gravidae are complicated by thrombosis.

In many cases, the mutation in the antithrombin gene causing the deficiency has been identified and a database has been published and updated (Lane *et al.,* 1993). Two main types of antithrombin deficiency exist, but the formerly used classification has been revised (Lane *et al.,* 1993). Type I (classic) deficiency is characterised by low functional and immunological antithrombin III levels. Type II deficient patients have a variant antithrombin III molecule. Three different types of type II deficiency exist, depending on whether the mutation affects the function of the reactive site $Arg^{393}$-$Ser^{394}$ (reactive site defects, RS), or the N-terminal heparin binding site (heparin binding site defects, HBS), or results in a pleiotropic effect (PE). RS and HBS type II defects are both characterised by normal immunological levels but low functional (variant) antithrombin III levels. The pleiotropic variants were previously classified as type I deficiency because the immunological level of antithrombin III is decreased. However, as patients with pleiotropic variants have low levels of an abnormal antithrombin III molecule circulating in plasma, these variants are now classified as type II deficiency. The thrombotic risk is similar for all subtypes except type II HBS antithrombin III deficiency. Heterozygous type II HBS deficiency is associated with a much lower incidence of thrombosis than are the other types (Finazzi *et al.,* 1987), unless complicated by an additional genetic risk factor (Ireland *et al.,* 1995a). Many thrombotic patients with heparin binding sites defects have been found to be homozygotes, who presented with arterial or venous thrombosis at an early age (Olds *et al.,* 1992; Chowdhury *et al.,* 1994).

Although antithrombin III deficiency is associated with a high risk of thrombosis, the clinical manifestations vary even within the same family. This might reflect the presence of other genetic risk factor(s) for thrombosis. Indeed, three studies have shown APC-resistance to be an additional genetic risk factor in several antithrombin III deficient families (van Boven *et al.,* 1995; Radtke *et al.,* 1995; Ireland *et al.,* 1995a). Twenty-nine per cent (5/17) of probands with antithrombin type II HBS

deficiency were found to be carriers of the FV:$Q^{506}$ allele causing APC-resistance (Ireland *et al.*, 1995a). Thirteen per cent (13/111) of families with antithrombin deficiency (type I or II, RS or PE) were found to have the FV:$Q^{506}$ (van Boven *et al.*, 1995). The median age at onset of thrombosis was only 16 years (n=11), as compared to 26 years (n=15) in twelve families with combined defects, demonstrating the additive effect of APC-resistance and antithrombin III deficiency (van Boven *et al.*, 1995). Except in one family, where crossing over was documented, the antithrombin III deficiency and FV:$Q^{506}$ either co-segregated or segregated independently (van Boven *et al.*, 1995), depending on the regional proximity of the factor V (1q21-25) (Wang *et al.*, 1988) and the antithrombin III (1q23-25) (Bock *et al.*, 1985) gene loci.

**Protein C deficiency**

Soon after the elucidation of the anticoagulant properties of protein C, hereditary deficiency of protein C was found to be associated with venous thromboembolism (Griffin *et al.*, 1981). Heterozygous protein C deficiency is found in 2-5% of thrombosis patients (Table 4). The thrombophilic tendency of heterozygous protein C deficiency has been confirmed in a large number of thrombophilic families (Bertina *et al.*, 1982; Broekmans *et al.*, 1983; Horellou *et al.*, 1984; Broekmans & Conard, 1988; Bovill *et al.*, 1989; Reitsma *et al.*, 1991; Allaart *et al.*, 1993). In heterozygotes, the protein C concentration is about 50%, but 15% of protein C deficient patients, as determined with gene analyses, may have protein C levels within normal range (Allaart *et al.*, 1993). In thrombosis-prone families with protein C deficiency, approximately 50% of the affected members have suffered a thrombotic episode by the age of 30-45 years, suggesting an autosomal dominant mode of inheritance and protein C deficiency to be a strong risk factor for thrombosis. This interpretation was challenged by the identification of protein C deficiency in 0.3% of healthy blood donors who had no history of familial thrombophilia (Miletich *et al.*, 1987). In these families, only individuals with homozygous or compound heterozygous protein C deficiency had severe thrombotic disease, suggesting a recessive mode of inheritance (Seligsohn *et al.*, 1984). The molecular basis of the difference between dominant and recessive protein C deficiency has been enigmatic, especially as the same mutation was identified in both types of families (Reitsma *et al.*, 1991; Reitsma *et al.*, 1993). It has been proposed that thrombosis-prone families with protein C deficiency may suffer from additional genetic risk factors of thrombosis (Miletich *et al.*, 1993). This concept derived support from the demonstration of APC-resistance as an additional genetic risk factor in 19% (9/48) of Dutch thrombosis-prone protein C-deficient families (Koeleman *et al.*, 1994). In six families, two locus linkage analysis supported the assumption that the FV and protein C genes were the two loci responsible for thrombophilia. That APC-resistance is overrepresented among thrombotic patients with protein C deficiency has been verified by several other research groups (Gandrille *et al.*, 1995c; Hallam *et al.*, 1995; Pabinger *et al.*, 1995).

Two types of protein C deficiency have been described; in type I deficiency both protein C antigen and functional activity are reduced, whereas only the functional activity is reduced in type II deficiency (Broekmans & Conard, 1988; Dahlbäck & Stenflo, 1994). The genetic defect has been identified in many cases, and a mutation database is now available (Reitsma *et al.*, 1993; Reitsma *et*

*al.,* 1995a). Of a total of 132 different single-base pair substitutions, 42 (32%) occur in CpG dinucleotides and are C–>T or G–>A transitions, compatible with a model of methylation-mediated deamination. All mutations causing type II deficiency are missense mutations, while some of the mutations causing type I deficiency are nonsense mutations. Recently, three polymorphisms in the protein C promotor affecting the plasma concentration of protein C were described (Spek *et al.,* 1995). These polymorphisms are not associated with protein C deficiency, as they have only a moderate effect on the protein C concentration. However, individuals with the homozygous CGT genotype had a mean protein C concentration of 94%, and were at 1.6 times higher risk of thrombosis than individuals with the homozygous TAA genotype, who had a mean plasma protein C concentration of 116%.

During the initiating phase of oral anticoagulant therapy, individuals with protein C deficiency are at risk of developing skin necrosis (Rose *et al.,* 1986; Broekmans & Conard, 1988). This is believed to be caused by a temporary imbalance between pro- and anti-coagulant forces, which is the result of a shorter half-life of protein C ($t_{1/2} \approx 8$ h) as compared to those of factors IX, X, and prothrombin. High initial doses of coumarin should therefore be avoided (Broekmans & Conard, 1988).

In contrast to heterozygous protein C deficiency, homozygous protein C deficiency presents already in the neonatal period with disseminated intravascular coagulation, and massive and fatal (if untreated) thrombosis soon after birth (Seligsohn *et al.,* 1984; Sills *et al.,* 1984; Marlar *et al.,* 1988). The patients have no detectable protein C activity, while the protein C antigen level may or may not be detectable. The three most prominent symptoms are large purpuric (necrotic) skin lesions, CNS and central ophthalmic thrombosis. Treatment with fresh frozen plasma, or more preferable with protein C concentrate, may resolve the symptoms if initiated soon after onset of the lesions (Dreyfus *et al.,* 1991).

**Protein S deficiency**

In 1984, deficiency of protein S was found to be associated with familial thrombophilia (Comp & Esmon, 1984; Comp *et al.,* 1984; Schwarz *et al.,* 1984). The thrombophilic tendency of patients with hereditary protein S deficiency has then been confirmed in numerous studies (e.g. Broekmans *et al.,* 1985; Bertina, 1985; Sas *et al.,* 1985; Pabinger *et al.,* 1986; Comp *et al.,* 1986; Mannucci *et al.,* 1986; Kamiya *et al.,* 1986; Engesser *et al.,* 1987a; Briët *et al.,* 1988; Boyer-Neumann *et al.,* 1988; Iijima *et al.,* 1989; Schwarz *et al.,* 1989; Mannucci *et al.,* 1989; Sie *et al.,* 1989; Chafa *et al.,* 1989; Allaart *et al.,* 1990; Girolami *et al.,* 1990; Zöller *et al.,* 1995; Zöller *et al.,* 1995a). Protein S deficiency is found in 1-7% of patients with thromboembolic disease (Broekmans *et al.,* 1986; Gladson *et al.,* 1988; Ben-Tal *et al.,* 1989; Heijboer *et al.,* 1990; Tabernero *et al.,* 1991; Malm *et al.,* 1992). According to a recent report, the prevalence if protein S deficiency in the general population is as high as 0.7 to 2.3% (Koster *et al.,* 1995a). Unlike deficiency of protein C or antithrombin, protein S deficiency was not found to be a significant risk factor for thrombosis in a large case-control study (Koster *et al.,* 1995a). However, in selected families protein S deficiency is a clear risk factor for thrombosis (Engesser *et al.,* 1987a; paper IV, Zöller *et al.,* 1995). In a Dutch

study, 55% of protein S deficient patients had had thrombosis (Engesser *et al.,* 1987a), and age at first thrombosis ranged from 15 to 68 years (mean, 28 years). The most common manifestations were deep venous thrombosis (74%), pulmonary embolism (38%) and superficial thrombophlebitis (72%), although less common manifestations such as occlusion of the axillary, mesenteric or cerebral veins also occurred. A number of case reports have documented that these severe thrombotic complications are not rare among protein S deficient patients (Sas *et al.,* 1985; Broekmans *et al.,* 1987; Clark *et al.,* 1991; Cros *et al.,* 1990; Roos *et al.,* 1990; Koelman *et al.,* 1992). In the Dutch study, no patient had symptoms of arterial thrombosis before the age of 50 years (Engesser *et al.,* 1987a). However, the occurrence of arterial thrombosis has been documented in several other reports (Mannucci *et al.,* 1986; Allaart *et al.,* 1990; Girolami *et al.,* 1989; Sie *et al.,* 1989). In our study, we found that arterial thrombosis in protein S deficient patients rarely occurred before 50 years of age, but was a common finding in patients over 50 (paper IV, Zöller *et al.,* 1995). Warfarin-induced skin necrosis may occur in conjunction with protein S deficiency (Grimaudo *et al.,* 1989; Craig *et al.,* 1990; Proby *et al.,* 1990; Goldberg *et al.,* 1991), but less commonly than it does in conjunction with protein C deficiency (Engesser *et al.,* 1987a; Zöller *et al.,* 1995); the difference in frequency is probably due to the half-life of protein S being longer than that of protein C. The penetrance of thrombotic symptoms in protein S deficiency is highly variable, even within the same family. Recently, we have found APC-resistance to be an additional genetic risk factor in a high proportion of thrombosis-prone families with protein S deficiency (paper IV, Zöller & Dahlbäck, 1994; Zöller *et al.,* 1995b; Zöller *et al.,* 1995). This has also been found by others (Pabinger *et al.,* 1995; Koeleman *et al.,* 1995).

In plasma, 60-70% of protein S is bound to C4b-binding protein (C4BP), a regulatory protein of the classical complement pathway (Dahlbäck & Stenflo, 1994). Only the free form of protein S is active as a cofactor to APC. Some protein S deficient patients have low plasma levels of free protein S, whereas their total protein S levels are normal (Comp *et al.,* 1984; Comp *et al.,* 1986; Iijima *et al.,* 1989; Lauer *et al.,* 1990; Malm *et al.,* 1992). This is referred to as type III deficiency (nomenclature proposed by Bertina at the ISTH subcommittee meeting, 1991). Others present with decreased plasma levels of both free and complexed protein S (Comp & Esmon, 1984; Bertina, 1985; Briet *et al.,* 1988). This variant is referred to as type I. Type II is characterised by a functional defect of protein S in individuals with normal protein S antigen levels (Mannucci *et al.,* 1989; Maccaferri *et al.,* 1991). Recently, it has been shown that many individuals previously classified as having type II deficiency do not have a defect in their protein S gene. Instead they suffer from APC-resistance (Faioni *et al.,* 1993; Faioni *et al.,* 1994; Cooper *et al.,* 1994). The reason for this misclassification is that the mutated factor molecule (FV:$Q^{506}$), which is present in APC-resistant plasma, affects coagulation-based functional assays for protein S.

The molecular difference between type I and type III protein S deficiency has until recently remained elusive. In the course of characterising thrombosis-prone families with protein S deficiency, we found coexistence of type I and type III deficiency in 14 of 18 families (paper V, Zöller *et al.,* 1995a). This suggested the two types of protein S deficiency to be phenotypic variants of the same genetic disease. We could also show a low concentration of free protein S (16±10 nmol/L) to be the result of equimolar concentrations of total protein S (215±50 nmol/L) and β chain containing C4b-

binding protein (228±51 nmol/L).

Genetic studies of protein S deficiency have been complicated by the existence of two homologous genes for protein S on chromosome 3. The nucleotide sequences of the active protein S α-gene (PSα) and the inactive protein S-β pseudogene (PSβ) are 97% and 95.4% identical in coding and non-coding parts, respectively. All reported protein S variants are summarised in Table 5. Protein S Heerlen is not associated with thrombosis (Bertina *et al.*, 1990). It is characterised by a $Ser^{460}$ to Pro substitution in the consensus sequence for N-linked glycosylation. It has reduced molecular weight, suggesting that $Asn^{458}$ is glycosylated in normal protein S (Bertina *et al.*, 1990). Two large deletions of the protein S α-gene have been found to be associated with protein S deficiency (Ploos van Amstel *et al.*, 1989a; Schmidel *et al.*, 1991). A mutation in the protein S ß-pseudogene has been linked to protein S deficiency (Ploos van Amstel *et al.*, 1989b). Protein S deficiency in an Italian family has been linked to the protein S gene, using a common polymorphism in the protein S α-gene (Diepstraten *et al.*, 1991; Marchetti *et al.*, 1993). Recently, several point mutations in the protein S gene were described (Yamazaki *et al.*, 1993; Hayashi *et al.*, 1994; Reitsma *et al.*, 1994; Borgel *et al.*, 1994; Gómez *et al.*, 1995a; Gandrille *et al.*, 1995b; Gómez *et al.*, 1995b; Yamazaki *et al.*, 1995) (Table 5). However, the deleterious effect of one of them is controversial (Yamazaki *et al.*, 1993; Hayashi *et al.*, 1994). It has been reported to be common among the normal Japanese population (1.65%) and not to be associated with defect protein S function, and is therefore classified as a polymorphism rather than a deleterious mutation in Table 5. Using denaturing gradient gel electrophoresis in screening of exons II, IV, V, VIII, X and XV of the PSα gene from 100 protein S deficient patients, a French group found 15 novel point mutations and three polymorphisms (Gandrille *et al.*, 1995b). Three of the mutations were associated with true type II phenotypes (Arg-2 to Leu, Arg-1 to his and Thr-103 to Asn). Using another strategy with direct sequencing of all exons in the protein S gene, a Dutch group identified a deleterious mutation in approximately 50% of probands (Reitsma *et al.*, 1994; Gómez *et al.*, 1995a; Gómez *et al.*, 1995b). At present it is unclear why the yield is low, as compared with protein C deficiency (Reitsma *et al.*, 1991). Several explanations have been proposed: the mutations may be outside the sequenced region (i.e. in the poorly characterised promotor region), gene conversion taking place between the active protein S α-gene and the protein S β-pseudogene or unknown polymorphisms in the sequence recognition by primers may result in hemizygous amplification (Reitsma *et al.*, 1994; Gómez *et al.*, 1995a; Gómez *et al.*, 1995b). A fourth possibility is that, in a subgroup of protein S deficient families, protein S deficiency is not caused by a mutation in the protein S gene. Thus, it will be important to do linkage studies in families where no mutation is found, to determine whether the deficiency is linked to the protein S gene or not.

Interestingly, the same G->A transition occurred in conjunction with type III deficiency in a French patient and with type I deficiency in two Dutch families, providing further evidence that type I and III deficiencies are the same genetic disease (Zöller *et al.*, 1995a). Moreover, in four of six Dutch families where segregation analysis were performed, four patients with total protein S concentrations within the normal range were identified (Gómez *et al.*, 1995b). An analysis of all reports of patients with type III deficiency provided further support for the idea that types I and III are in fact the same disease (paper V). In most reported families, both type I and type III patients were represented.

Moreover, in type III deficient patients, the total protein S concentration was usually in the lower interval of the reference range, or only a few family members were investigated (Comp *et al.*, 1984; Comp *et al.*, 1986; Iijima *et al.*, 1989; Lauer *et al.*, 1990; Malm *et al.*, 1992). Thus, findings in previously published studies support our hypothesis that type I and III deficiencies are only different phenotypic expression of the same genetic disease (paper V, Zöller *et al.*, 1995a).

**Table 5. Polymorphisms and mutations in the protein S gene (PSα).**

| Codon (exon) | | Predicted mutation | Module (and RFLP) | Type of deficiency | Families (Co-segregation) | References |
|---|---|---|---|---|---|---|
| **Polymorphisms** | | | | | | |
| 35 (II) | CCG->CTG | Pro->Leu | Gla | - | - | Gandrille *et al.*, 1995b |
| Int-2 | +5, G->A | None | Intron 2 | - | - | Reitsma *et al.*, 1994 |
| 155 (VI) | AAG->GAG | Lys->Glu | EGF2 | II? | 3 (No) | Yamazaki *et al.*, 1993; Hayashi *et al.*, 1994 |
| 303 (X) | ATC->ATT | Ile->Ile | SHBG | - | - | Gandrille *et al.*, 1995b |
| 344 (X) | ATG->GTG | Met->Val | SHBG | - | - | Gandrille *et al.*, 1995b |
| 460 (XIII) | TCC->CCC | Ser->Pro | SHBG | - | - | Bertina *et al.*, 1990 |
| 626 (XV) | CCA->CCG | Pro->Pro | SHBG (BstXI) | - | - | Diepstraten *et al.*, 1991 |
| **Large deletions or insertions** | | | | | | |
| Gross deletion of the middle portion | | | | I | 1 (Yes) | Ploos van Amstel *et al.* 1989a |
| 5.3 kb deletion including exon XIII | | | | I | 2 (Yes) | Schmidel *et al.*, 1991 |
| **Probably deleterious point mutations** | | | | | | |
| -25 (I) | ins T | Frameshift | propeptide (+DdeI) | I | 2 (Yes) | Reitsma *et al.*, 1994; Gómez *et al.*, 1995b |
| -2 (II) | CGT->CTT | Arg->Leu | propeptide | II | 2 (Yes) | Gandrille *et al.*, 1995b |
| -1 (II) | CGT->CAT | Arg->His | propeptide | II | 1 (Yes) | Gandrille *et al.*, 1995b |
| 22 (II) | TGC->TGA | Cys->stop | Gla | I | 1 (Yes) | Gandrille *et al.*, 1995b |
| 26 (II) | GAA->GCA | Glu->Ala | Gla | I | 2 (Yes) | Gandrille *et al.*, 1995b |
| 31 (II) | TTT->TGT | Phe->Cys | Gla | I | 1 (No) | Gandrille *et al.*, 1995b |
| 37 (II) | ACG->ATG | Thr->Met | Gla | I | 1 (No) | Gandrille *et al.*, 1995b |
| 43 (III) | del A | Frameshift | Hydrophobic | I | 1 (Yes) | Gómez *et al.*, 1995a |
| 49 (IV) | CGC->CAC | Arg->His | Thrombin-sensitive | I | 1 (No) | Gandrille *et al.*, 1995b |
| 82 (V) | CCT->CC | Frameshift | EGF1 | I | 1 (No) | Borgel *et al.*, 1994 |
| 103 (V) | ACT->AAT | Thr->Asn | EGF1 | II | 1 (No) | Gandrille *et al.*, 1995b |
| Int-10 | +5, G->A | None | Intron 10 (-MaeII) | I+III | 3 (Yes) | Gandrille *et al.*, 1995b; Reitsma *et al.*, 1994 |
| 204 (VIII) | GAT->GGT | Asp->Gly | EGF4 | I | 1 (No) | Gandrille *et al.*, 1995b |
| 208 (VIII) | GAG->AAG | Glu->Lys | EGF4 | I | 1 (No) | Gandrille *et al.*, 1995b |
| 224 (VIII) | TGC->TGG | Cys->Trp | EGF4 | I | 1 (Yes) | Gandrille *et al.*, 1995b |
| 224 (VIII) | TGC->CGC | Cys->Arg | EGF4 | I | 1 (No) | Gandrille *et al.*, 1995b |
| 261 (IX) | del T | Frameshift | SHBG | I | 1 (Yes) | Gómez *et al.*, 1995b |
| 267 (IX) | del G | Frameshift | SHBG | I | 1 (No) | Gómez *et al.*, 1995b |
| 335 (X) | GAT->AAT | Asp->Asn | SHBG | I | 1 (Yes) | Gandrille *et al.*, 1995b |
| 340 (X) | GGT->GTT | Gly->Val | SHBG | I | 1 (Yes) | Gómez *et al.*, 1995b |
| 467 (XIII) | GTA->GGA | Val->Gly | SHBG (-NspI) | I | 2 (Yes) | Gómez *et al.*, 1995b |
| 522 (XIV) | CAG->TAG | Gln->stop | SHBG | I | 1 (Yes) | Yamazaki *et al.*, 1995 |
| 565 (XV) | ins T | Frameshift | SHBG | I | 1 (Yes) | Gómez *et al.*, 1995b |
| 578 (XV) | ins C | Frameshift | SHBG | I | 1 (Yes) | Gómez *et al.*, 1995b |
| 636 (XV) | TAA->TAT | stop->Tyr elongation of 13 aa | SHBG (-AflII) | I | 2 (Yes) | Reitsma *et al.*, 1994 |

**Resistance to activated protein C due to a factor V gene mutation is a novel cause of thrombophilia**

In normal plasma, clotting time is prolonged by addition of activated protein C (APC), because APC cleaves and inactivates factors VIIIa and Va. In 1993, Dahlbäck and co-workers reported three families with an inherited poor anticoagulant response to activated protein C (APC-resistance) (Dahlbäck *et al.,* 1993). Addition of increasing concentrations of exogenous APC failed to prolong the clotting time in an APPT assay (Fig. 4). The APC-resistance phenomenon co-segregated with thrombosis in the families, demonstrating a new genetic cause of familial thrombophilia.

A simple APC-resistance test was developed to measure the anticoagulant effect of exogenous APC in individual patient plasma (Dahlbäck *et al.,* 1993). The APC-resistance test is a modified APTT-reaction performed with vs. without the addition of a carefully standardised amount of APC to patient plasma. The result is expressed as an APC-ratio (i.e. the quotient of clotting time obtained using the APC/$CaCl_2$-solution divided by clotting time obtained with $CaCl_2$) (Svensson & Dahlbäck, 1994). In a consecutive series of 104 patients with venous thrombosis, 40% were found to manifest APC-resistance, as compared with only 7% of healthy controls (Svensson & Dahlbäck, 1994). The results demonstrated APC-resistance not only to be very frequent in thrombosis patients, but also to be highly prevalent in the general population. APC-resistance was 10 times more prevalent than any of the other known genetic defects. Investigation of relatives of the APC-resistant probands confirmed an autosomal dominant pattern of inheritance of the APC-resistance phenotype, and showed relationship to exist between APC-resistance and familial thrombophilia (Svensson & Dahlbäck, 1994). Other laboratories came to the same conclusion that APC-resistance is by far the most prevalent cause of venous thrombosis (Griffin *et al.,* 1993; Koster *et al.,* 1993; Faioni *et al.,* 1993; Halbmayer *et al.,* 1994a). APC-resistance was found in 52% to 64% of a group of highly selected young American thrombosis patients (Griffin *et al.,* 1993). Among 301 Dutch consecutive outpatients with thrombosis before the age of 70 years, APC resistance was found in 21% of cases. The corresponding frequency in matched controls was 4.7%. The matched odds ratio was calculated to be 6.6 (Koster *et al.,* 1993). The Dutch group also observed that there was an inverse relationship between the degree of anticoagulant response to APC and the thrombotic risk. Individuals with an APC-ratio less than 1.5 were at the highest risk of thrombosis (odds ratio 12).

It was shown that a crude protein fraction of normal plasma corrected the APC-resistance, whereas the corresponding fraction obtained from APC-resistant plasma was without effect (Dahlbäck & Hildebrand, 1994). The protein was purified and identified as the intact form of factor V, suggesting APC-resistance to be caused by a defect in the factor V molecule. This hypothesis derived further support from linkage between a neutral polymorphism in the factor V gene and the APC-resistance phenotype in two families (Bertina *et al.,* 1994; paper I, Zöller & Dahlbäck, 1994). Two other research groups independently came to the same conclusion that APC-resistance is caused by a defect in the factor V molecule (Bertina *et al.,* 1994; Sun *et al.,* 1994). In plasma mixing experiments, they found APC-resistance to be corrected by all coagulation factor deficient plasmas except that deficient in factor V. Bertina and co-workers were also the first to report APC-resistance to be caused

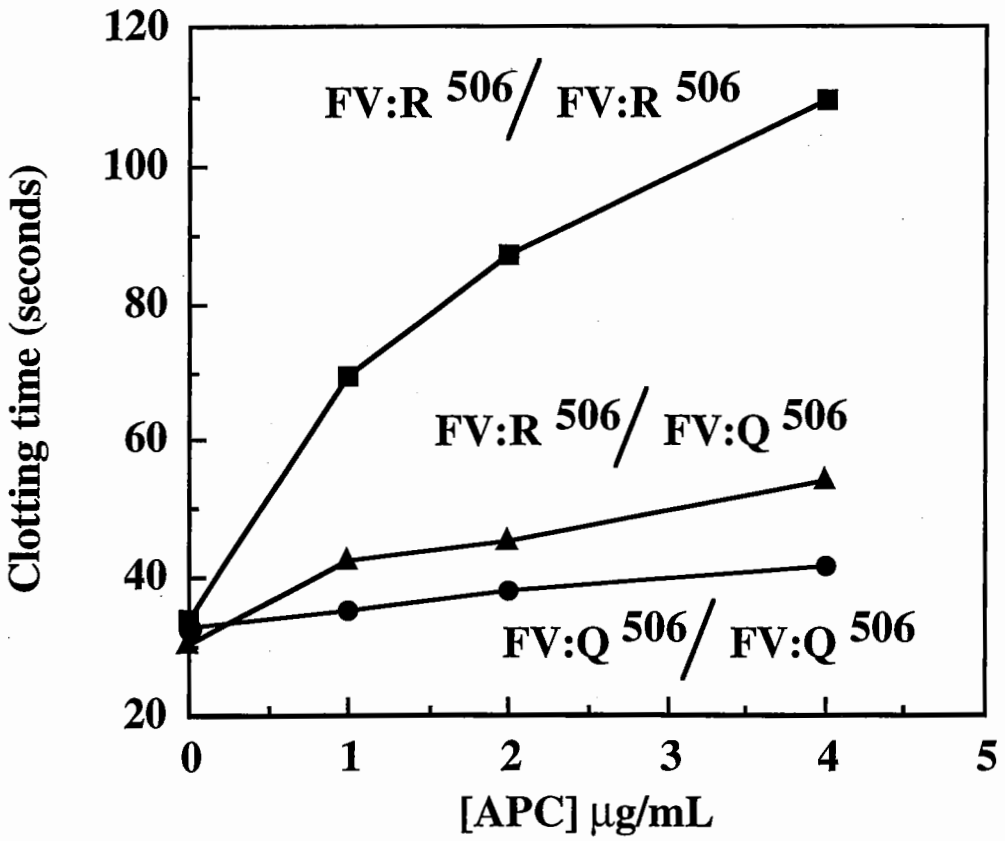


**Fig 4.** APC resistance in a patient with heterozygosity ($FV:R^{506}/FV:Q^{506}$) and a patient with homozygosity ($FV:Q^{506}/FV:Q^{506}$) for the $FV:Q^{506}$ allele (factor V Leiden). The heteterozygote is a middle-aged man with recurrent venous thrombosis whose disease was the first case of APC-resistance to be described (Dahlbäck *et al.*, 1993), and the homozygote is a previously described healthy women with pronounced APC-resistance (Dahlbäck & Hildebrand, 1994). A distinct dose-dependent prolongation of clotting time was observed in the normal control plasma ($FV:R^{506}/FV:R^{506}$). The heterozygous plasma responds poorly to the anticoagulant activity of APC, and the homozygous plasma is almost completely resistant to APC (from Zöller *et al.*, 1995c).

by a point mutation in the factor V gene (Bertina *et al.*, 1994). The mutation is a substitution of G with an A at nucleotide position 1691 predicting replacement of arginine (R)$^{506}$ with a Gln (Q). Almost simultaneously, two other laboratories reported relationship to exist between the FV:Q$^{506}$ mutation, also known as factor V Leiden, and APC-resistance (Greengard *et al.*, 1994b; Voorberg *et al.*, 1994). Presence of the same mutation was confirmed in Swedish families with APC-resistance (papers I and II; Zöller & Dahlbäck, 1994; Zöller *et al.*, 1994). R$^{506}$ is located in one of three APC cleavage sites in the heavy chain of factor Va. FVa:Q$^{506}$ is not cleaved at position 506, but is slowly though completely inactivated by APC-mediated cleavages at R$^{306}$ and R$^{679}$ (Kalafatis *et al.*, 1994; Kalafatis *et al.*, 1995) (Fig. 5). The rate of inactivation of FVa:Q$^{506}$ by APC is 10-20-fold lower than that of degradation of FVa:R$^{506}$, as cleavage at R$^{506}$ only facilitates the cleavage at R$^{306}$ and R$^{679}$ necessary for complete inactivation of factor Va (Kalafatis *et al.*, 1995; Aparicio & Dahlbäck, 1995; Heeb *et al.*, 1995). A recent study by Nicolaes and co-workers contradicts these observations (Nicolaes *et al.*, 1995). They suggest that cleavage at R$^{506}$ affects the cofactor activity of normal FVa. At low FVa concentrations cleavage at R$^{506}$ occurs at a rate that is 20-fold higher than the rate of cleavage at R$^{306}$. This results in the accumulation of a FVa molecule that is cleaved at R$^{506}$ and that exhibits 40% cofactor activity at high FXa concentration (5 nM), and virtually no cofactor activity at low FXa concentration (0.3 nM). Subsequent cleavage at R$^{306}$, which may occur both in the cleaved FVa molecule and in intact FVa, results in a complete loss of cofactor activity (Nicolaes *et al.*, 1995).

### Laboratory diagnosis of APC-resistance and the FV:Q$^{506}$ allele

The original APC-resistance test used to measure the anticoagulant action of activated protein C was based on an activated thromboplastin time (APTT) assay (Dahlbäck *et al.*, 1993; Svensson & Dahlbäck, 1994). The test consists of measurements of two APTT tests, one performed in the presence of a carefully standardised amount of APC (APTT+APC), the other without the addition of APC (APTT-APC). The test result is then expressed as an APC-ratio (i.e. the quotient of APTT+APC divided by APTT-APC). In normal plasma, added APC degrades both FVa and FVIIIa, which delays the thrombin burst and prolongs the clotting time. The mutated FV:Q$^{506}$ molecule is degraded more slowly than normal factor V (Kalafatis *et al.*, 1995; Heeb *et al.*, 1995; Aparicio & Dahlbäck, 1995) (Fig. 5). The prothrombinase complex is therefore inadequately inhibited, which results in a shorter clotting time and a lower APC-ratio. Careful standardisation of the test procedure is essential. The test results depend on the choice of APTT-reagent, the $CaCl_2$ concentration, the citrate concentration in the sample, the concentration and quality of APC, instrumentation and sample handling (freezing and thawing, storage, centrifugation and platelet contamination) (Rosén *et al.*, 1994; de Ronde & Bertina, 1994; Dahlbäck, 1995; Girolami *et al.*, 1994; Legnani *et al.*, 1994; Sidelmann *et al.*, 1995; Lindberg *et al.*, 1995). However, even if the test is carefully standardised the results are affected by the use of oral anticoagulants, heparin in the sample, lupus anticoagulants (de Ronde & Bertina, 1994; Hampton *et al.*, 1994; Bokarewa *et al.*, 1994; Ehrenforth *et al.*, 1995; Martorell *et al.*, 1995; Halbmayer *et al.*, 1994b; Gschwandtner *et al.*, 1995), and by the levels of factor X, prothrombin, factor VIII, factor V and factor IX (de Ronde & Bertina, 1994; Simioni & Girolami, 1994; Colucci *et al.*, 1994). Gender, oral contraception and pregnancy have also been reported to be factors affecting the APC-ratio

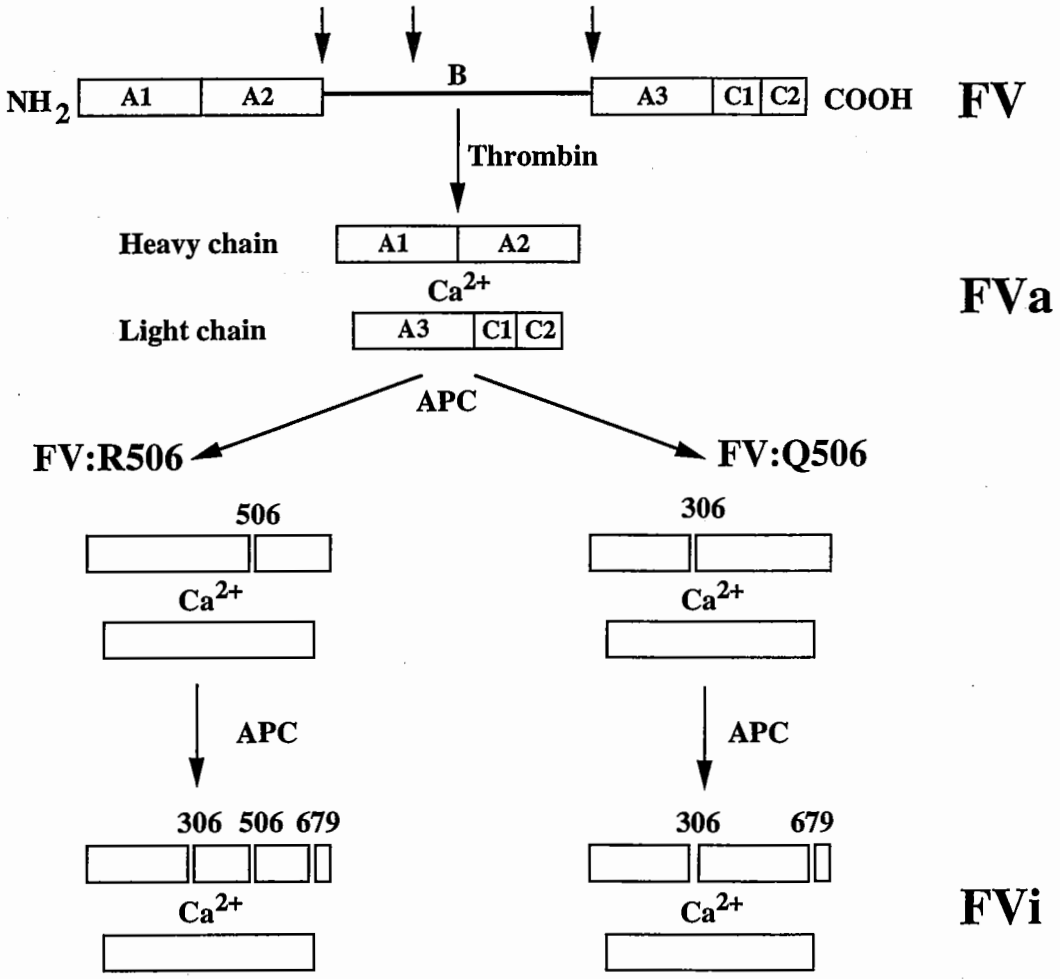


**Fig 5.**. Schematic models of the activation and inactivation of mutant (FV:$Q^{506}$) and normal factor V (FV:$R^{506}$). Factor V is composed of three homologous A-modules, one B-region and two homologous C-modules. Upon activation of factor V, thrombin cleaves three peptide bonds in the B domain, as indicated by arrows. During inactivation of normal FVa, APC first cleaves at $R^{506}$, which results in the exposure of additional cleavage sites at $R^{306}$ and $R^{679}$, which in turn are necessary for complete inactivation of FVa (Kalafatis *et al.*, 1994; Kalafatis *et al.*, 1995; Heeb *et al.*, 1995; Aparicio & Dahlbäck, 1995). In contrast, mutated FVa:$Q^{506}$ is not cleaved at position 506, while cleavages at sites 306 and 679 occur at a 10-20-fold reduced rate (from Zöller *et al.*, 1995c).

(Henkens *et al.,* 1995; Cumming *et al.,* 1995). Many modifications of the original test have been published (Faioni *et al.,* 1993; Bokarewa & Blombäck, 1994; Vasse *et al.,* 1994; Kraus & Wagner, 1994; de Ronde & Bertina, 1994; Rosén *et al.,* 1994; Jorquera *et al.,* 1994; Trossaërt *et al.,* 1994; Denson *et al.,* 1995; Le *et al.,* 1995).

Apart from economic and technical aspects of the different assays, the clinical usefulness of a functional APC-resistance test is determined by its sensitivity and specificity in the detection of FV:$Q^{506}$ allele carriers. The APC-test developed by the Dutch group is more sensitive and specific for the mutation than the original APC-resistance test and most commonly used tests (de Ronde & Bertina, 1994; Zöller *et al.,* 1994; Lindberg *et al.,* 1995), which is probably related to the choice of APTT reagents. Although the effect of heparin may be neutralised with Hepzym®, the results of Dutch and other APTT-based APC-resistance tests are affected by oral anticoagulants and to a varying degree by lupus anticoagulants, which disqualifies many thrombosis patients from testing. However, a modification of the APC-resistance test with predilution of patient plasma in factor V deficient plasma seems to overcome some of these problems (Jorquera *et al.,* 1994; Trossaërt *et al.,* 1994; Tosetto & Rodeghiero, 1995; Denson *et al.,* 1995). The specificity and sensitivity for the factor V FV:$Q^{506}$ mutation appears to be around 100% for these assays. Recently, new non-clotting, chromogenic assays have been reported that appear promising (Rosén *et al.,* 1995; Varadi *et al.,* 1995). However, as APC-resistance is a life-long condition, DNA-based verification of the FV:$Q^{506}$ mutation will have its place in the diagnostic arsenal even in the future.

DNA based verification of the FV:$Q^{506}$ allele, a G to A transition at position 1,691 in exon 10 of the factor V gene, may be performed in several ways (Dahlbäck, 1995). All methods include PCR amplification of the nucleotide region near the mutation, either from mRNA or from genomic DNA. After amplification, the mutation may be detected by direct sequencing, allele specific hybridisation (Bertina *et al.,* 1994), restriction enzyme cleavage (RFLP) (Bertina *et al.,* 1994) or single-stranded conformational polymorphism (SSCP) (Jaksch *et al.,* 1995). Alternative, methods are allele specific amplification or the new isothermal amplification procedure, NASBA, together with ELGA (enzyme linked gel assay) (Reitsma *et al.,* 1995b). The RFLP method used in the present investigation is based on the fact that the FV:$Q^{506}$ mutation destroys the recognition sequence for the restriction enzyme Mnl1. The DNA based methods are more laborious than the functional assays. They are sensible for contamination and require experience in DNA technology. DNA based diagnosis is nevertheless important, as homozygotes for the factor V gene defect are at higher thrombotic risk than heterozygotes, and as the specificity and sensitivity of the clotting based APC-resistance test for the mutation, which is around 85% to 90% is insufficient (Zöller *et al.,* 1994).

Another important question is whether APC-resistance may have other genetic causes than the FV:$Q^{506}$ mutation. With the original APC-test, several individuals with low APC-ratios but without the FV:$Q^{506}$ mutation were identified in a study of 50 families with inherited APC-resistance (Zöller *et al.,* 1994). There was a tendency for APC-ratios to be lower in thrombotic than in asymptomatic family members. However, it is still unclear whether APC-resistance without the factor V mutation is a risk factor for thrombosis, even if a tendency for APC-ratios to be lower in thrombosis patients than in healthy controls has been confirmed (Bertina *et al.,* 1995). To sum up thus far, no

convincing evidence has yet been found of other genetic causes of APC-resistance than the $Arg^{506}$ to Gln mutation.

The presence of APC-resistance has been reported to affect the results of several clotting-based functional protein S and protein C tests (Faioni *et al.,* 1993; Faioni *et al.,* 1994; Cooper *et al.,* 1994; Ireland *et al.,* 1995b). APC-resistant patients may therefore have been misclassified as cases of functional (type II) protein C or protein S deficiency. New and more specific assays are needed for detection of functional protein S or C deficiency.

**Epidemiology of APC-resistance**

A number of studies using either the functional APC-resistance test or DNA based tests have shown APC-resistance or the FV:$Q^{506}$ allele to be associated with an increased risk of venous thrombosis (Table 6). However, there is very wide variation in the prevalence of APC-resistance/FV:$Q^{506}$ allele (range 4-64%). This may in part be explained by differences in the selection criteria, and in part by differences in the prevalence of the FV:$Q^{506}$ allele between countries. The study in which a 64% prevalence of APC-resistance was found included highly selected young patients with unexplained thrombophilia (Griffin *et al.,* 1993). In consecutive outpatients with thrombosis, the prevalence of APC-resistance/FV:$Q^{506}$ allele is lower (17.6-21%) (Koster *et al.,* 1993; Bertina *et al.,* 1994; Rosendaal *et al.,* 1995). Nevertheless, even in these three studies APC-resistance/FV:$Q^{506}$ allele was found to be a strong risk factor for venous thrombosis. The odds ratio for homozygous individuals was calculated to be 30-140, and that for heterozygous individuals to be 6.5-8.0, depending on age (Rosendaal *et al.,* 1995). The prevalence of the FV:$Q^{506}$ allele among venous thrombosis cases was even lower (11.6%) in the only cohort study performed so far, where 14,916 apparently healthy men had been followed for a mean duration of 8.6 years (Ridker *et al.,* 1995). The relative risk of venous thrombosis for FV:$Q^{506}$ carriers was calculated to be 2.7 ($p=0.008$), though the risk of thrombosis increased significantly with age. The relative risk of primary thrombosis was 7.0 for subjects more than 60 years of age. This is in agreement with findings in a Dutch study where the estimated incidence of thrombosis increased with age in heterozygous subjects (Rosendaal *et al.,* 1995). This is noteworthy, as thrombosis in the elderly is a major medical problem associated with high recurrence and mortality rates (Kniffin *et al.,* 1994; Anderson *et al.,* 1991).

Selection criteria aside, the prevalence of APC-resistance/FV:$Q^{506}$ allele in patients with venous thrombosis also reflects the frequency of the FV:$Q^{506}$ allele in the general population (Table 6). Prevalences of 0-15% among healthy controls have been found in several studies. The prevalence appears to be highest in Caucasians in Europe and America, whereas the condition is virtually non-existent in other races (Table 6). The finding of linkage disequilibrium of the FV:$Q^{506}$ mutation with the common Hinf1 allele of factor V (cytosine at nucleotide 2,298) polymorphism suggested a founder effect to be involved in the spread of this allele in the population (Bertina *et al.,* 1994). Linkage disequilibrium, and the confined geographic area, suggest that mutation has occurred once, and that all cases are probably inherited rather than spontaneous mutations (Rees *et al.,* 1995). The high prevalence of the allele in the population suggests that it has conferred some genetic advantage. The advantage of carrier status with a slight hypercoagulability may be the reduction of menstrual blood

loss and postpartum haemorrhage, thus protecting against iron deficiency, though this has yet to be demonstrated. Interestingly, in Japan no FV:$Q^{506}$ allele was found among 192 healthy individuals, suggesting APC-resistance to be very rare in Japan (Takamiya *et al.*, 1995). In a large study of 1,690 unrelated individuals from twenty-four populations, no FV:$Q^{506}$ mutation was found in 1,600 chromosomes from Africa, Southeast Asia, Australasia and the Americas (Indians) though the allele frequency was 0.6% in Minor Asia (Rees *et al.*, 1995). In all likelihood, the high incidence of venous thromboembolism in Caucasians is in part due to the high prevalence of the FV:$Q^{506}$ allele.

**Table 6. Prevalence of APC-resistance or the FV:Q$^{506}$ allele among patients with venous thrombosis and in the healthy population**

| Country and authors | Venous thrombosis n APC-resistant/n tested (% APC-resistant) | | Healthy controls n APC-resistant/n tested (% APC-resistant) | |
|---|---|---|---|---|
| **Studies not validated by factor V gene analysis** | | | | |
| Austria (Halbmayer *et al.*, 1994a) | 7/40 | (17.5%) | 1/50 | (2%) |
| Finland (Hakala *et al.*, 1995) | 30/173 | (17%) | 2/120 | (1.7%) |
| Germany (Vogel *et al.*, 1995) | 227/984 | (23%) | - | |
| Greece (Gerotziafas *et al.*, 1995) | 11/42 | (26%) | - | |
| Italy (Faioni *et al.*, 1993) | 35/106 | (33%) | 0/40 | (0%) |
| Italy (De Stefano *et al.*, 1995) | 33/118 | (28%) | - | |
| Italy (Legnani *et al.*, 1994) | 26/261 | (10%) | - | |
| Italy (Simioni *et al.*, 1994) | - | | 0/55 | (0%) |
| Italy (Tosetto *et al.*, 1994) | 2/20 | (10%) | 20/1212 | (1.6%) |
| Netherlands (Koster *et al.*, 1993) | 64/301 | (21%) | 14/301 | (4.7%) |
| Poland (Lopaciuk & Bykowska, 1994) | 9/72 | (12.5%) | 1/110 | (0.9%) |
| Portugal (Ferrer-Antunes *et al.*, 1995) | 10/66 | (15%) | - | |
| Spain (Cadroy *et al.*, 1994) | 9/48 | (19%) | 1/75 | (1.3%) |
| Spain (Borell *et al.*, 1994) | 3/72 | (4.2%) | 3/107 | (28%) |
| Sweden (Svensson & Dahlbäck, 1994) | 41/104 | (39%) | 9/130 | (6.9%) |
| USA (Cushman *et al.*, 1994) | 4/21 | (19%) | 2/39 | (5.1%) |
| USA (Griffin *et al.*, 1993) | 16/25 | (64%) | 2/35 | (5.7%) |
| **Studies validated by factor V gene analysis** | | | | |
| Africa/Middle-East (Rees *et al.*, 1995) | - | | 0/306 | (0%) |
| America, Indians (Rees *et al.*, 1995) | - | | 0/146 | (0%) |
| Asia Minor (Rees *et al.*, 1995) | - | | 2/180 | (1.1%) |
| Asia, Southeast (Rees *et al.*, 1995) | - | | 0/272 | (0%)- |
| Australasia (Rees *et al.*, 1995) | - | | 25/168 | (0%) |
| Australia (Ma *et al.*, 1994) | 7/30 | (23%) | - | |
| Brazil (Arruda *et al.*, 1995) | 8/78 | (10%) | 2/100 | (2%) |
| Canada, Quebec (Liu *et al.*, 1995) | - | | 8/90 | (8.9%) |
| Canada, Hamilton (Lee *et al.*, 1995) | - | | 13/229 | (5.7%) |
| Canada, Portland (Liu *et al.*, 1995) | - | | 15/197 | (7.6%) |
| France (Alhenc Gelas *et al.*, 1994) | 14/87 | (16%) | - | |
| France, Nancy (Soubrier *et al.*, 1995) | - | | 32/373 | (8.6%) |
| France, Paris (Soubrier *et al.*, 1995) | - | | 5/229 | (2.2%) |
| Germany (Aschka *et al.*, 1995) | 6/32 | (19%) | 10/117 | (8.5%) |
| Greece (Rees *et al.*, 1995) | - | | 25/187 | (13%) |
| Italy (Rees *et al.*, 1995) | - | | 0/47 | (0%) |
| Japanese (Takamiya *et al.*, 1995) | - | | 0/192 | (0%) |
| Netherlands (Bertina *et al.*, 1994) | 53/301 | (17.6%) | 6/301 | (2%) |
| Netherlands (Rosendaal *et al.*, 1995) | 92/471 | (19.5%) | 14/474 | (3.0%) |
| Netherlands (Voorberg *et al.*, 1994) | 10/27 | (37%) | - | |
| Sweden, Kristianstad (unpublished) | - | | 37/247 | (15%) |
| Sweden, Malmö (Holm *et al.*, 1995) | - | | 11/101 | (11%) |
| UK (Beuchamp et al., 1994) | - | | 5/144 | (3.5%) |
| USA (Ridker *et al.*, 1995) | 14/121 | (11.6%) | 42/704 | (6.0%) |
| USA, Portland (Liu *et al.*, 1995) | - | | 15/197 | (7.6%) |

**Clinical manifestations of APC resistance**

A study of 211 individuals in 34 different APC-resistant families demonstrated unequivocal correlation between familial thrombosis and APC-resistance (Svensson & Dahlbäck, 1994). However, after exclusion of the probands, the thrombotic risk appeared lower than for patients with inherited deficiencies of protein C, protein S or antithrombin III (Thaler & Lechner, 1981; Engesser *et al.*, 1987a; Broekmans & Conard, 1988). The relative risk of thrombosis has been reported to be increased in patients with very low APC-ratios (Koster *et al.*, 1993). This was explained when the molecular cause of APC-resistance was elucidated (Bertina *et al.*, 1994; Majerus, 1994b). Homozygous individuals were found to be characterised both by a higher risk of thrombosis and by a lower APC-ratio than individuals heterozygous for the factor FV:$Q^{506}$ gene defect. Heterozygosity and homozygosity for FV:$Q^{506}$ were estimated to be associated with a 5-10-fold and 50-100-fold increased risk of thrombosis, respectively. In a subsequent study of 50 families with inherited APC-resistance, a clear difference in thrombotic incidence was observed between homozygotes, heterozygotes and individuals lacking the FV:$Q^{506}$ mutation (Zöller *et al.*, 1994). Deep venous thrombosis was the most common manifestation, although pulmonary embolism and superficial thrombosis also occurred. Rare thrombotic manifestations, such as Budd-Chiari syndrome have also been reported in association with APC resistance (Denninger *et al.*, 1995; Mahmoud *et al.*, 1995; Levoir *et al.*, 1995). The multifactorial aetiology of thrombosis was illustrated by the fact that the first thrombotic episode was associated with a circumstantial risk factor in 63% (32/51) of FV:$Q^{506}$ carriers (paper II, Zöller *et al.*, 1994).

The presence of APC-resistance has been reported in several young patients with arterial thrombosis (Lindblad *et al.*, 1994; Holm *et al.*, 1994; Simioni *et al.*, 1995). However, only two case-control studies have shown relationship to exist between APC-resistance/ FV:$Q^{506}$ allele and arterial thrombosis. In young Austrian patients with stroke, the prevalence of APC-resistance (20%) was similar to that observed in patients with venous thrombosis, but the study was not validated by factor V gene analysis (Halbmayer *et al.*, 1994a). In German subjects with coronary heart disease the FV:$Q^{506}$ allele was significantly overrepresented compared with controls (März *et al.*, 1995). Other case-control studies have failed to confirm these two results (Table 7). In a large cohort study of apparently healthy men, no relationship between myocardial infarction or stroke and heterozygosity for FV:$Q^{506}$ was observed (Ridker *et al.*, 1995). Moreover, in a study of 308 subjects in 50 Swedish thrombosis-prone families with inherited APC-resistance, no significant relationship was found between arterial disease and the FV:$Q^{506}$ allele (paper II, Zöller *et al.*, 1994). Thus, APC-resistance does not appear to be an important risk factor for the development of arterial thrombosis. However, it is possible that in special situations homozygous FV:$Q^{506}$ allele may contribute to severe arterial thrombosis (Lindblad *et al.*, 1994; Holm *et al.*, 1994).

**Table 7. Prevalence of APC-resistance or the FV:Q$^{506}$ allele among patients with arterial thrombosis and in the healthy population.**

| Country and authors | Arterial thrombosis n APC-resistant/ n tested (% APC resistant) | Healthy controls n APC-resistant/ n tested (% APC resistant) |
|---|---|---|
| **Studies not validated by factor V gene analysis** | | |
| Austria (Halbmayer *et al.*, 1994a) | 6/30 (20%) | 1/50 (2%) |
| Finland (Hakala *et al.*, 1995) | 15/200 (7.5%) | 2/120 (1.7%) |
| Germany (Thiel *et al.*, 1995) | 2/55 (3.6%) | - |
| Norway (Eritsland *et al.*, 1995) | 12/546 (2.2%) | - |
| Switzerland (Biasiutti *et al.*, 1995) | 3/134 (2.2%) | 2/100 (2%) |
| USA (Cushman *et al.*, 1994) | 2/44 (4.5%) | 2/39 (5.1%) |
| **Studies validated by factor V gene analysis** | | |
| Australia (van Bockxmeer *et al.*, 1995) | 11/222 (5.0%) | 5/126 (4.0%) |
| Finland (Syrjälä *et al.*, 1995) | 5/99 (5%) | ?/148 (3%) |
| Finland (Kontula *et al.*, 1995) | 16/358 (4.5%) | 4/137 (2.9%) |
| France, Lille (Emmerich *et al.*, 1995) | 4/96 (4.2%) | 1/148 (0.7%) |
| France, Strasbourg (Emmerich *et al.*, 1995) | 22/207 (10.6%) | 17/193 (8.8%) |
| France, Toulouse (Emmerich *et al.*, 1995) | 3/143 (2.1%) | 6/207 (2.9%) |
| Germany (Heinrich *et al.*, 1995) | 6/91 (6.6%) | 14/222 (6.3%) |
| Germany (März *et al.*, 1995) | 21/224 (9.4%) | 8/196 (4.1%) |
| Sweden, Malmö (Holm *et al.*, 1995) | 18/101 (18%) | 11/101 (11%) |
| UK (Catto *et al.*, 1995) | 16/386 (4.1%) | 14/247 (5.6%) |
| UK (Forsyth & Dolan, 1995) | 1/45 (2.2%) | - |
| UK, Belfast (Emmerich *et al.*, 1995) | 5/197 (2.5%) | 10/178 (5.6%) |
| UK (Samani *et al.*, 1994) | 2/60 (3.3%) | - |
| USA (Ridker *et al.*, 1995) | 32/583 (5.5%) | 42/704 (6.0%) |

### Familial thrombophilia is the result of multiple genetic defects

It is noteworthy that more than one genetic defect segregated in the two families where the APC-resistance phenotype was linked to the factor V gene (Bertina *et al.*, 1994; Zöller & Dahlbäck, 1994). The Dutch family had protein C deficiency, whereas the Swedish family carried a protein S deficiency in addition to APC-resistance. These reports were the first accounts of familial thrombophilia being caused by two genetic defects. This concept derived further support from the identification of APC-resistance in 19% (9/48) of symptomatic protein C deficient probands (Koeleman *et al.*, 1994) and in 39% (7/18) of Swedish thrombosis-prone protein S deficient families (paper IV, Zöller *et al.*, 1995). In these families, the thrombotic risk was significantly higher in patients with combined genetic defects than in individuals with isolated defects. The youngest patient to suffer from thrombosis in these two studies was a 10-year-old protein S deficient boy, who had a combination of protein S deficiency and APC-resistance due to homozygosity for the FV:Q$^{506}$ mutation (paper III, Zöller *et al.*, 1995b). The concept of thrombophilia as a multiple genetic disorder has been confirmed by several findings in other studies (Koeleman *et al.*, 1995; Gandrille *et al.*, 1995c; Hallam *et al.*, 1995; Pabinger *et al.*, 1995; Ireland *et al.*, 1995a; van Boven *et al.*, 1995; Gandrille *et al.*, 1995a; Radtke *et al.*, 1995).

**Influence of APC-resistance on acquired hypercoagulable states**

The secondary hypercoagulable states listed in Table 3 are well known risk factors for thrombosis. However, only a fraction of people suffer from thrombosis when exposed to these acquired or circumstantial risk factors. Thrombosis is often provoked by a circumstantial risk factor in patients with deficiencies of protein S, protein C or antithrombin III, and in APC-resistant patients (Zöller *et al.,* 1994), but only APC-resistance is highly prevalent in the general population. It is therefore unsurprising that APC-resistance has been found to be an important risk factor for development of thrombosis when it occurs in conjunction with a circumstantial risk factor such as oral contraception, pregnancy, surgery or old age. However, more data are needed to determine whether general screening for APC-resistance is warranted before exposure to secondary hypercoagulable states or circumstantial risk factors.

*Advanced age* . Advanced age is a risk factor for thrombosis (Schafer, 1985; Hirsh *et al.,* 1986; Taubman & Silverstone, 1986; Nordström *et al.,* 1992). Even healthy centenarians are characterised by a hypercoagulable state with increased levels of the activation peptides of prothrombin, factor IX, factor X, and thrombin-antithrombin complexes (Mari *et al.,* 1995). Thus, it is unsurprising that the incidence of venous thromboembolism increases exponentially with age. The risk of thrombosis is especially high in hospitalised elderly patients (Taubman & Silverstone, 1986). The prognosis is also worse in the elderly; 1-year mortality associated with pulmonary embolism and deep venous thrombosis was 39% and 21%, respectively (Kniffin *et al.,* 1994). Recently, a large prospective study of apparently healthy men demonstrated the FV:$Q^{506}$ mutation to be a risk factor for venous thromboembolic disease, in particular after the age of 60 (Ridker *et al.,* 1995). This is in agreement with another recent observation that the risk of thrombosis in heterozygous APC-resistant individuals increases with age (Rosendaal *et al.,* 1995).

*Pregnancy* is a recognised risk factor for venous thrombosis (Schafer, 1985; Hirsh *et al.,* 1986). Deep venous thrombosis complicates approximately 0.018% to 0.29% of pregnancies (Sipes & Weiner, 1990), and pulmonary embolism accounts for 15% of deaths among gravidae in Sweden (Högberg, 1986). The hypercoagulability observed in pregnancy and the puerperium may be related to venous stasis, increased levels of procoagulant factors (fibrinogen, FVII, FVIII, FIX), decreased levels of anticoagulant factors (antithrombin III, free protein S and fibrinolytic activity) and a decreased response to activated protein C (Hirsh *et al.,* 1986; Malm *et al.,* 1988; Cumming *et al.,* 1995). A Swedish study identified APC-resistance as a major risk factor in 60% of women with thrombosis during pregnancy (Hellgren *et al.,* 1995). That APC-resistance is a risk factor for thrombosis in association with pregnancy has also been suggested by findings in several other studies (Cook *et al.,* 1994; Zöller *et al.,* 1994; De Stefano *et al.,* 1995; Conard *et al.,* 1995; Beufé *et al.,* 1995).

*Oral contraception* has been debated as a risk factor for thrombosis since the first report on thrombosis associated with the use of oral contraceptives (reviewed in Schafer, 1985; Hirsh *et al.,* 1986). Oestrogens in oral contraceptives produce many of the changes that occur during pregnancy, e.g. they decrease protein S (Malm *et al.,* 1988), antithrombin III and fibrinolytic activity, and increase the concentration of a number of coagulation factors. Oral contraceptive usage has also

recently been demonstrated to decrease the response to activated protein C (Osterud *et al.*, 1994; Henkens *et al.*, 1995). It is therefore logical that the thrombotic risk appears to be related to the oestrogen content of the oral contraceptive, lower thrombotic risk being associated with low-dose oestrogen pills. In a recent review, it was suggested that oral contraceptives are associated with an almost three-fold increase in the risk of venous thrombosis (Koster *et al.*, 1995b). However, the problem is that a switch to alternative modes of contraception may result in more unwanted pregnancies, and pregnancy itself is of course a risk factor for thrombosis (Koster *et al.*, 1995b). In fact, the only randomised controlled trial yet performed showed no difference in thrombotic risk to exist between oral contraceptives users and non-users (Fuertes de la Haba *et al.*, 1971). A Dutch study demonstrated oral contraceptive usage and APC-resistance to be additive risk factors for venous thrombosis (Vandenbroucke *et al.*, 1994). Women heterozygous for the FV:$Q^{506}$ allele are at 35-fold greater risk of developing thrombosis if they take oral contraceptives. Homozygous women taking oral contraceptives may be at a several hundred-fold increased risk (Rosendaal *et al.*, 1995). A Swedish study confirmed APC-resistance to be a major risk factor for developing thrombosis in association with the use of oral contraceptives. Thirty per cent of Swedish women with thrombosis associated with oral contraceptive usage are APC-resistant (Hellgren *et al.*, 1995). An even higher proportion (48%) was found in young Italian women with thrombosis in association with oral contraception (Pini *et al.*, 1995).

*Surgery and trauma* induce a number of thrombogenic changes which include (Schafer, 1985; Hirsh *et al.*, 1986): release of tissue thromboplastin into the blood, stasis due to immobilisation, local vessel damage and reduced fibrinolytic activity. Hence the associated thrombotic risk depends on several factors such as the site and extent of surgical trauma, duration of the operative procedure and the length of time the patient remains immobilised. Thrombotic risk is particularly high in conjunction with orthopaedic surgery of the lower limb or trauma to the pelvis or lower limb. Moreover, a Danish group found 30% of patients who develop thrombosis after knee surgery to be APC-resistant, suggesting APC-resistance to be a major risk factor for postoperative thrombosis (Fauno *et al.*, 1995).

## The present investigation

The aim of my work has been to characterise the molecular cause of inherited protein S deficiency and to study the clinical manifestations associated with this hypercoagulable disorder. In the autumn of 1992 I started to investigate all protein S deficient families in the files of the Department for Coagulation Disorders, Malmö General Hospital. As patient samples were being analysed it became obvious that thrombophilic families with protein S deficiency were often affected by yet another hypercoagulable genetic defect, i.e. APC-resistance, thus demonstrating that thrombophilia is a multiple gene disorder. Therefore in 1994 I became involved in research about APC-resistance taking place at our laboratory. My thesis therefore covers inherited APC-resistance as much as protein S deficiency, two independent causes of familial thrombophilia.

### Linkage between inherited resistance to activated protein C and factor V gene mutation in a large thrombophilic family with independent protein S deficiency

(Study I)

In 1993, a poor anticoagulant response to activated protein C (APC) was described as a novel mechanism for familial thrombophilia (Dahlbäck *et al.*, 1993). Addition of exogenous APC to plasma from a middle-aged man with recurrent venous thrombosis did not result in the expected prolongation of the clotting time (Dahlbäck *et al.*, 1993). The APC-resistance phenotype was inherited as an autosomal dominant trait and co-segregated with thrombosis. In several studies, APC-resistance was found to be the most common genetic risk factor associated with venous thrombosis (Griffin *et al.*, 1993; Koster *et al.*, 1993; Svensson & Dahlbäck, 1994; Halbmayer *et al.*, 1994a). APC-resistance was found in 20-60% of thrombosis patients but in only 2-7% of healthy controls. Dahlbäck demonstrated a crude protein fraction of normal plasma to correct the APC-resistance, whereas corresponding fraction from APC-resistant plasma was without effect (Dahlbäck & Hildebrand, 1994). The protein was purified and identified as the intact form of factor V, suggesting APC-resistance to be caused a molecular defect in factor V (Dahlbäck & Hildebrand, 1994). As APC-resistant plasma contained normal levels of factor Va procoagulant activity, a selective defect in the novel anticoagulant function of intact factor V was suggested (Dahlbäck & Hildebrand, 1994; Shen & Dahlbäck, 1994).

In an attempt to elicit further evidence that APC-resistance is caused by a defect in the factor V gene, a linkage study was performed in a large thrombophilic family with independent inheritance of APC-resistance and protein S deficiency (Zöller & Dahlbäck, 1994) (Fig. 6A). A sequence of 1188 base pairs in exon 13 of the factor V gene was amplified from genomic DNA (Fig. 6B) (nucleotides 2,066 to 3,254 of the factor V cDNA; sequence from Genebank). Within this sequence are located two neutral dimorphisms, neutral in the sense that they do not change any amino acid in the mature factor V molecule (Shen *et al.*, 1993). However, they do affect the cleavage sites for the restriction enzymes Taq 1 (G or A at position 2,391) and EcoR1 (A or G 2,379), respectively (Fig. 6B). The genotype could therefore be assessed with agarose electrophoresis of Taq 1 and EcoR1 cleaved PCR products. Fourteen family members were informative for these two markers, and 100% linkage of the a-allele (Taq 1) and the I-allele (EcoR1) with the APC-resistance phenotype was observed (Fig. 6A). A lod

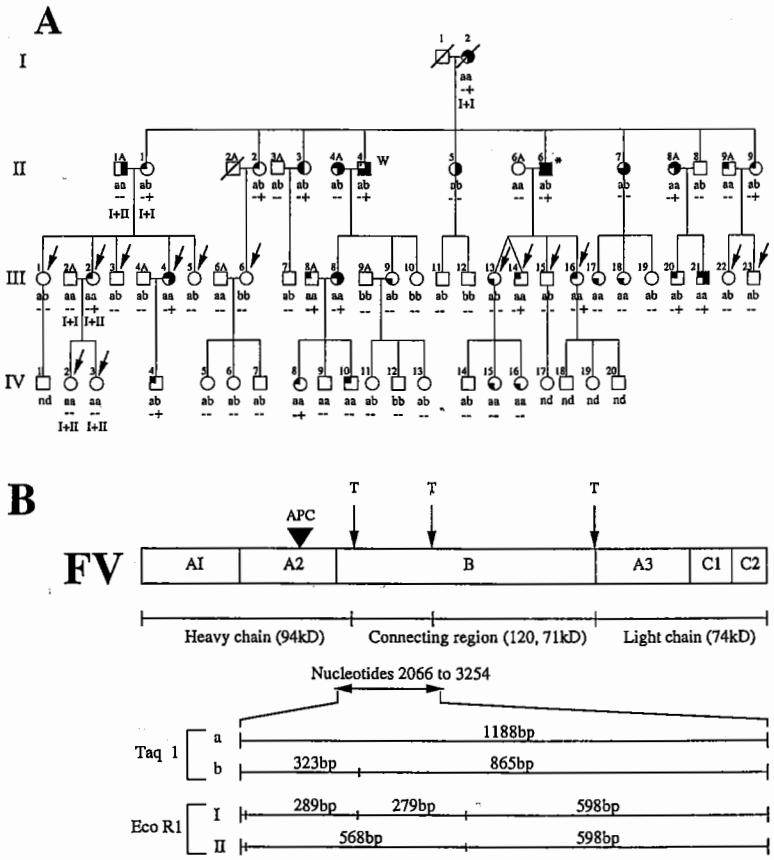


**Figure 6.** Pedigree demonstrating linkage between APC-resistance and the factor V gene. **A.** A large kindred with thrombophilia was found to have independent inheritance of APC-resistance and protein S deficiency. The proband II:6, who had both defects and severe thromboembolism, is indicated with an asterisk. Filled lower left quadrants denote protein S deficiency and filled upper left quadrants APC-resistance. Filled right half of the symbol denotes thrombosis. Individual II:4 was on oral anticoagulation (denoted with W) which made it difficult to diagnose APC-resistance. The a-allele contained no Taq 1 cleavage site, whereas the b-allele was cleaved by Taq 1. The Eco R1 polymorphism (alleles I and II) is shown in only a few cases because all other family members investigated were found to be homozygous for the I allele. The arrows indicate informative family members. The + and - signs indicate presence or absence of the G to A mutation at nucleotide position 1,691, changing $Arg^{506}$ to Gln. (nd, not determined). **B.** Schematic model of factor V. The 2,196 amino acid residues long protein contains two types of homologous repeats (A1-A3 and C1-C2) and a large B-domain. Factor V is activated by thrombin (T) through limited proteolysis, factor Va being the resulting complex between the 94 kDa heavy chain and the 74 kDa light chain. The B-domain, which is encoded by exon 13, is not part of factor Va. The amplified segment of exon 13 is shown. Taq 1 a and b and Eco R1 I and II represent polymorphic alleles. The different cleavage products of the amplified segment are shown. The arrow head indicates the APC-cleavage site at $Arg^{506}$. Modified from Zöller & Dahlbäck, 1994.

score of 3.9 was calculated at a recombination fraction of 0.0, which is consistent with close linkage. During our search for the causal mutation and after submission of the paper, we learnt that a G to A mutation at nucleotide 1,691 in the factor V, changing $Arg^{506}$ to Gln ($FV:Q^{506}$ or factor V Leiden) had been identified by Bertina and co-workers (reported first at the second Pine Ridge conference at Snowbird, Utah, April 1994). The mutation results in loss of one of the APC-cleavage sites of FV, and a FVa molecule that is not properly inactivated by APC (Bertina *et al.,* 1994). Bertina and co-workers had found that plasma deficient in factor V were APC-resistant, and also found a close linkage to a microsatelite marker located near the FV gene (Bertina *et al.,* 1994). The mutation results in a loss of a MnL1 cleavage site (Bertina *et al.,* 1994), which allows detection of the mutation with agarose gel electrophoresis of the MnL1 cleaved PCR product. We found all genetically related APC-resistant family members to be heterozygous for the $FV:Q^{506}$ allele. However, of the four APC-resistant individuals who had married into the family (II:4A, II:8A, II:9A and III:8A), only two had the same mutation (Fig. 6A). This suggested that the cause of APC-resistance is heterogeneous, a concept that was further investigated in study II.

Interestingly, the two most severely affected individuals in the family had combined APC-resistance and protein S deficiency (II:4 and II:6 in Fig. 6). This family, together with a Dutch family with combined protein C deficiency and APC-resistance (Bertina *et al.,* 1994), provided the first evidence that familial thrombophilia is often associated with more than one genetic defect. The concept of APC-resistance as an additional genetic risk factor in families with inherited protein S deficiency was further investigated in studies III and IV.

**Identification of the same factor V gene mutation in 47 of 50 thrombosis-prone families with inherited resistance to activated protein C** (study II)

Presence of the APC-resistance phenotype is reflected in poor anticoagulant response to APC in an APT-time based clotting assay (Dahlbäck *et al.,* 1993; Svensson & Dahlbäck, 1994). The clotting time is determined with vs. without the addition of APC. The result is preferably expressed as a ratio (the APC-ratio, i.e. the quotient of the clotting time with the addition of APC divided by the clotting time obtained without the addition of APC). With carefully standardised procedures the APC-resistance test yields stable and reproducible results (Dahlbäck, 1995). To determine whether all inherited cases of APC-resistance are caused by the same factor V mutation, the $FV:R^{506}$ allele was investigated in 308 individuals from 50 thrombosis-prone families with inherited APC-resistance. Families with at least two members with an APC-ratio of 2.0 or less were included in the study (Svensson & Dahlbäck, 1994). In this study we used a modified version of the method used for determination of the mutation in study I (Zöller & Dahlbäck, 1994). A slightly longer fragment of exon 10 was amplified with inclusion of a second MnL1 site unaffected by the mutation.

The $FV:R^{506}$ allele DNA was found in 94% (47/50) of the families. Of 33 APC-resistant index cases available for analysis, 24 were heterozygous and 5 homozygous for the factor V mutation. Of the 308 family members investigated, 146 were normal, 144 were heterozygotes and 18 homozygotes for the factor V gene mutation. Perfect co-segregation of low APC-ratio and the mutation was seen in 40 families, while in 7 families 21% (12/57) of the APC-resistant members lacked the mutation (range

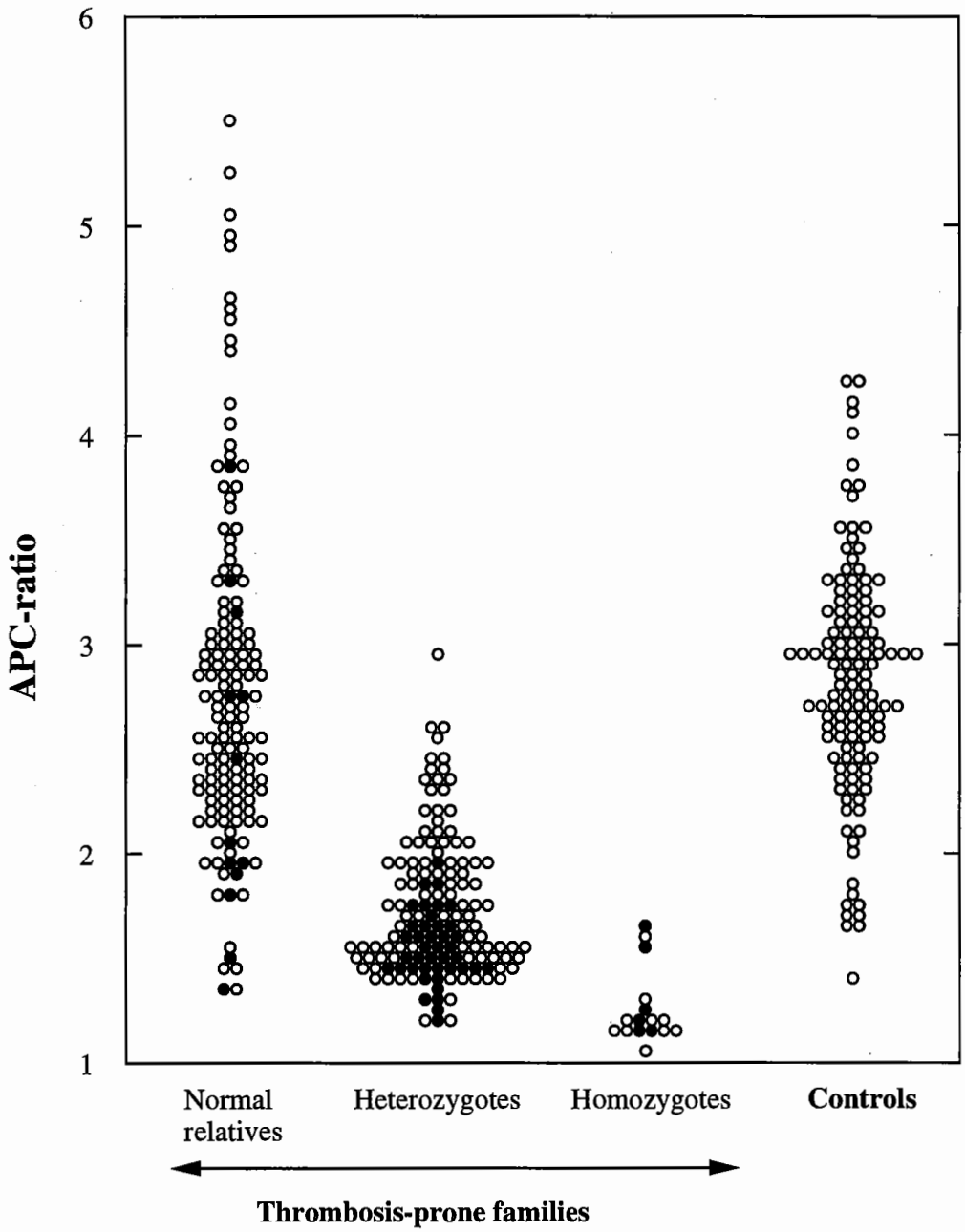


**Figure 7.** Relationships between the FV:$Q^{506}$ allele and APC-ratios in families with APC resistance (Zöller *et al.,* 1994). Filled circles denote family members with a history of thrombosis. Differences in APC-ratios (number, mean±SD) between normals (n=143, 2.8±0.8), heterozygotes (n=142, 1.7±0.3) and homozygotes (n=16, 1.3±0.2) were highly significant ($p<0.001$). Were diagnosis of the factor V mutation were to be based on an APC-ratio ≤ 2.0, the sensitivity and specificity for the FV:$Q^{506}$ allele would be 85% and 87%, respectively. Modified from Zöller *et al.,* 1994.

1.3-2.0). In the remaining 3 families, APC-resistance was not at all linked to the mutation, suggesting other acquired or inherited causes of APC-resistance in a minority of APC-resistant cases. The APC-ratios were low in all the homozygous cases, whereas there was an overlap in APC-ratios between heterozygotes and normals (Fig. 7). Were diagnosis of the factor mutation to be solely based on the APC-ratio, 15% of heterozygotes would not be identified and 13% of normals would be classified as factor V mutants. Thus, the APC-resistance test is not specific for the FV:$Q^{506}$ allele, as also found by several other groups (Voorberg *et al.*, 1994; Liu *et al.*, 1995). Until a more specific test is developed, it is necessary to confirm a poor anticoagulant response to activated protein C by DNA-based FV gene analysis.

The factor V genotype was found to be the major determinant of thrombophilia penetrance. Forty-four per cent (8/18) of homozygotes, 30% (43/144) of heterozygotes and 10% (14/146) of family members without the FV:$Q^{506}$ mutation had experienced one or more venous thrombotic event. Homozygotes were affected at an earlier age than heterozygotes [25 yrs. (range 10-40) vs. 36 yrs. (range 18-71)], which was reflected in a significant difference in thrombosis-free survival curves between the subgroups (Fig. 8). By the age of 33 years, 8% of those not carrying the mutation, 20% of heterozygotes and 40% of homozygotes had had at least one manifestation of venous thromboembolic disease. Deep venous thrombosis was the most common manifestation, although pulmonary embolism and superficial thrombophlebitis also occurred (Table 8). However, pulmonary embolism tended to occur less often in heterozygotes with thrombosis (19%) than in either homozygotes (50%) or in patients with antithrombin III (40%), protein S (31%) or protein C deficiency (49%) (Thaler & Lechner, 1981; Broekmans & Conard, 1988; Engesser *et al.*, 1987a; Zöller *et al.*, 1995) (Table 8). Superficial thrombophlebitis also tended to occur less often in symptomatic APC-resistant patients than in protein S or protein C deficient patients, though more often than in those with antithrombin deficiency. Severe thrombosis at uncommon sites such as the mesenteric or cerebral veins may occur in patients with protein S, protein C or antithrombin III deficiency. However, in these 50 APC-resistant families the only case of thrombosis at an unusual site in FV:$Q^{506}$ allele carriers was a central retinal vein thrombosis. Thus, it appears that among heterozygous APC-resistant patients not only is there less thrombophilia penetrance than among those with heterozygous deficiency of protein C, protein S or antithrombin III, but the clinical manifestations are also less severe. The contrast is even more striking between homozygous FV:$Q^{506}$ carriers and patients with homozygous deficiency of protein S or C. The oldest APC-resistant homozygote still free of thrombosis was 68 years of age, whereas untreated patients with homozygous protein S or C deficiency die of massive thrombosis already in the neonatal period (Marlar *et al.*, 1988). The relatively mild thrombotic disease encountered even in homozygous individuals with APC-resistance might be explained by that the APC-resistance is not complete. APC-mediated inactivation of mutated FVa by proteolytic cleavage at $Arg^{306}$ and $Arg^{679}$ still occurs, though at a 10-20 times slower rate (Kalafatis *et al.*, 1995; Heeb *et al.*, 1995; Aparicio & Dahlbäck, 1995) (Fig. 5). Moreover, ability to inactivate FVIIIa is unaffected by the factor V gene mutation.

Four per cent (6/144) of the heterozygotes and 6% (1/18) of the homozygotes had had arterial thrombosis, as compared to 1% (2/146) of their normal relatives. Our findings are in agreement with

those of the majority of case-control studies showing no relationship to exist between arterial thrombosis and APC-resistance (Table 8). Thus, the pathogenesis of arterial thrombosis appears to be completely different from that of venous thrombosis.

**Table 8. Clinical manifestations in thrombosis patients; comparison of FV:$Q^{506}$ heterozygotes and homozygotes among 50 thrombosis-prone families with inherited APC-resistance (Zöller *et al.*, 1994) and patients with inherited protein S (Engesser *et al.*, 1987a; Zöller *et al.*, 1995), protein C (Broekmans & Conard, 1988) or antithrombin III (Thaler & Lechner, 1981) deficiency.**

| | FV:$Q^{506}$ genotype | | | | |
|---|---|---|---|---|---|
| | Heterozygotes (N=43) % | Homozygotes (N=8) % | Protein S (N=103) % | Protein C (N=116) % | Antithrombin III (N=120) % |
| DVT | 84 | 100 | 79 | 82 | 91 |
| PE | 19 | 50 | 31 | 49 | 40 |
| STP | 19 | 12 | 52 | 47 | 8 |
| Recurrence | 40 | 50 | 69 | 70 | 60 |

Since a patient may have suffered from several different thrombotic events, the numbers do not add up to 100%. DVT denotes deep venous thrombosis, PE pulmonary embolism and STP superficial thrombophlebitis. Modified from Hillarp *et al.*, 1996.

**Table 9. Risk factors associated with the first thrombotic episode[a].**

| | Normals No. | Heterozygotes No. | Homozygotes No. |
|---|---|---|---|
| Pregnancy or postpartum | 2 | 12 | 1 |
| Oral contraceptives | 2 | 8 | 3 |
| Trauma and surgery | 4 | 8 | 2 |
| Immobilisation | 2 | 1 | - |
| Other risk factors[b] | 1 | 1 | 1 |
| Protein S deficiency | 3 | 3 | 1 |
| Total number of patients with risk factor | 8 | 25 | 7 |

a Some patients were exposed for several risk factors.
b Malignancy, varicose, and oedema. Modified from Zöller *et al.*, 1994.

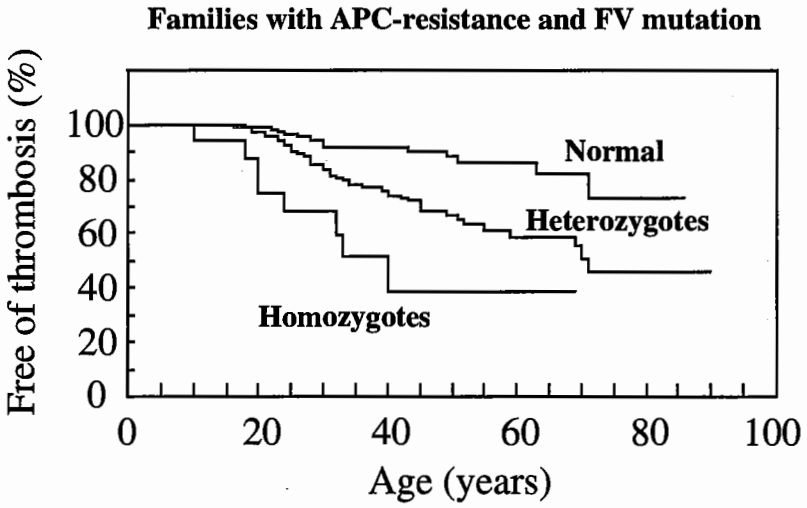


**Figure 8.** Thrombosis-free survival curves of the different FV:$Q^{506}$ genotypes. The age-related probability of remaining free from thrombotic events, for 146 normals, 144 heterozygotes and 18 homozygotes, shown in Kaplan-Meier curves. At the age of 33 years, 8% of normals, 20% of heterozygotes and 40% of homozygotes had had venous thrombotic events. Differences between normals and heterozygotes and between heterozygotes and homozygotes were highly significant, $p<0.001$ and $p=0.01$. Modified from Zöller *et al.*, 1994.

Many APC-resistant patients, even in thrombosis-prone families, will never suffer from thrombosis (Fig. 8). The penetrance of thrombosis is highly variable. Both genetic and acquired or circumstantial risk factors may modulate the penetrance in APC-resistant individuals. The first thrombotic episode was associated with a circumstantial risk factor in 57% (8/14) of normals, in 58% (25/43) of heterozygotes and in 88% (7/8) of homozygotes. The most common circumstantial risk factors were pregnancy, surgery, trauma and oral contraceptive usage (Table 9). Heterozygous women tended to have their first thrombosis at an earlier age than heterozygous men (34±14 vs. 38±15 years), although the difference was not statistically significant. This tendency may be related to exposure to pregnancy and oral contraceptives. Exposure to circumstantial risk factors appears to be an important determinant of the development of thrombosis, as also reported by others (Vandenbroucke *et al.*, 1994; Hellgren *et al.*, 1995). Future studies will determine whether screening before exposure to oral contraceptives, pregnancy or surgery is warranted.

Other genetic defects may also be contributing risk factors. As many as 10% of family members without the mutation had had manifestations of thrombosis. A few patients in these families also had protein S deficiency as a contributing risk factor. Moreover, it is possible that yet other unidentified risk factors are responsible for segregation in these families just as in thrombosis-prone families with protein C or S deficiency (Koeleman *et al.*, 1994; Zöller *et al.*, 1995; Koeleman *et al.*, 1995). The presence or absence of additional genetic risk factors may explain why only in some families is APC-resistance associated with thrombophilia, while other APC-resistant families are asymptomatic. In this context it is noteworthy that heterozygotes with a history of thrombosis had significantly lower APC-ratios than those without thrombosis ($P<0.001$, 1.5±0.2 vs. 1.8±0.4), and none of the heterozygotes with APC-ratios >2.0 had had thrombosis. Normal relatives with a history of thrombosis had significantly lower APC-ratios than those without thrombosis even if the protein S deficient cases were excluded ($P<0.05$, 2.4±0.7 vs. 2.9±0.8). Moreover, when comparing APC-resistant and non-APC-resistant family members lacking the mutation, a significant difference in thrombosis-free survival was found (not shown). This suggests that, even without the $Arg^{506}$ mutation, APC-resistance may be a risk factor for thrombosis. The cause of this is not clear, but it may be the presence of other segregating genetic defects that affect both the APC-ratio and the risk of thrombosis. However, the phenomenon may also be an acquired postthrombotic effect. The same tendency for APC-ratios to be lower among thrombosis patients than among controls has been observed in a large Dutch study (Bertina *et al.*, 1995). Thus, the importance of a low APC-ratio without the factor V gene defect remains an open question.

**Homozygous APC-resistance combined with inherited type I protein S deficiency in a young boy with severe thrombotic disease** (paper III)

Childhood thrombosis is a rare but severe disorder that occurs with an annual incidence of 0.7 per 100,000 (Andrew *et al.*, 1994). Based on the observation in study I that thrombophilia might be associated with combined genetic defects, APC-resistance was assessed in the youngest protein S deficient patient with thrombosis in our laboratory (Zöller *et al.*, 1995b). At the age of ten, this patient suffered from severe thrombosis of his right leg extending to the pelvic veins after a minor blunt

trauma to the right leg. Among published reports, it is very uncommon to read of such early debut of thrombosis in patients with inherited protein S deficiency (Engesser *et al.,* 1987a). In the study by Engesser and co-workers, the youngest individual with thrombosis was 15 years old. Plasma drawn in a warfarin-free interval not only manifested low free and total protein S antigen concentrations, but also very poor anticoagulant response to activated protein C. Both traits were inherited independently in his family. Addition of the APC cofactor protein S to his plasma was without effect, while addition of FV partially corrected his poor response to activated protein C in a manner similar to that in an unrelated patient with the homozygous FV:$Q^{506}$ allele (Fig. 9). DNA analysis confirmed homozygosity for the FV:$Q^{506}$ mutation.

The explanation for the partial correction of APC-resistance by the addition of factor V but not by the addition of protein S is unclear. The partial correction of APC-resistance by added factor V may be the result of an increased APC-mediated degradation of factor VIIIa, because intact factor V is an APC-cofactor (Shen & Dahlbäck, 1994). Were this the explanation, however, added protein S would be expected to correct the APC-resistance, which was not the case. Another possibility is that an excess of normal factor V results in competitive inhibition of activation of mutated factor, assuming mutated and normal factor V to be activated at equal rates. The normal factor Va molecules are then degraded at a normal rate by APC which would result in correction of the APC-resistance. There is no available evidence to support a third possibility that mutated factor Va might be a competitive inhibitor to APC in degradation of factor VIIIa and factor Va.

This is the first description of a patient with combined homozygosity for APC-resistance and another inherited prothrombotic disorder. Interestingly, even in this case with multiple genetic defects thrombosis was provoked by a circumstantial risk factor, demonstrating how multiple acquired and genetic risk factors may act in concert in the development of venous thrombosis. The case illustrates the concept of multiple genetic defects as a cause of thrombophilia, and emphasises the need of complete evaluation of thrombosis patients in order to determine whether multiple risk factors exist. However, this family was too small to allow evaluation of APC-resistance as a determinant of the manifestations of protein S deficiency. This was investigated further in study IV.

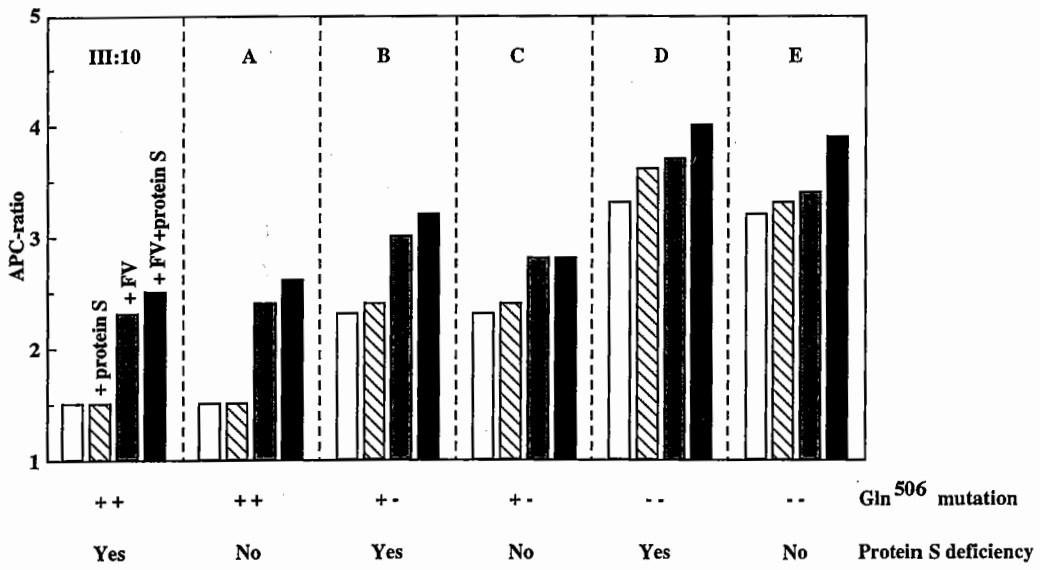


**Figure 9.** Response to activated protein C with vs. without the addition of protein S, factor V or factor V + protein S. The response of the propositus is compared with that of unrelated individuals having the following genetic defects: homozygous factor V mutation with normal protein S (A), heterozygous factor V mutation with protein S deficiency (B), heterozygous factor V mutation with normal protein S (C), negative for factor V mutation but with protein S deficiency (D), and that of normal controls (E). The APC-ratios were slightly different from those seen in figure 1 because another instrument was used to monitor clotting. The individual in A is a healthy woman (46 years of age) with no history of thrombosis. Modified from Zöller *et al.*, 1995b.

**Resistance to activated protein C is an additional genetic risk factor in hereditary deficiency of protein S** (paper IV)

Inherited deficiency of vitamin K-dependent protein S, is a well-established cause of familial thrombophilia (Comp & Esmon, 1984; Comp *et al.,* 1984; Schwarz *et al.,* 1984; a; Sas *et al.,* 1985; Bertina 1985; Comp *et al.,* 1986; Kamiya *et al.,* 1986; Mannucci *et al.,* 1986; Pabinger *et al.,* 1986; Engesser *et al.,* 1987a). Protein S functions as a cofactor to APC in the degradation of factors Va and VIIIa. In plasma protein S exists either as free protein (30-40%) or as part of a bimolecular, non-covalent high affinity complex with C4b-binding protein (C4BP) (Dahlbäck & Stenflo, 1981; Dahlbäck, 1983b). Only the free form of protein S is functionally active as a cofactor to APC (Bertina, 1985; Dahlbäck, 1986). Three different types of protein S deficiency have been described. In type I protein S deficiency, both total and free protein S is decreased. As with the nomenclature for protein C and antithrombin III deficiency, type II is reserved for functionally abnormal protein S molecules characterised by decreased functional protein S deficiency combined with normal levels of total and free protein S. Type III deficiency is characterised by deficiency of free protein S occurring in conjunction with normal total protein S. However, in many clinical studies on inherited protein S deficiency, only total protein S and not the active free fraction of protein S has been determined (type I). In our study we investigated both free and total protein S concentrations in a total of 327 members of 18 unrelated protein S deficient families, which is the largest study of inherited protein S deficiency yet performed.

Multivariate survival analysis showed deficiency of free protein S to be a strong and independent risk factor for thrombosis, with a hazard ratio of 6.8 (CI 95% 3.7-12.5). According to Kaplan-Meier analysis, 50% of protein S-deficient family members and 12% of those without protein S deficiency had had one or more thrombotic event by the age of 45 years (Fig. 10). Venous thrombotic events were frequent, having occurred in 47% (64/136) of the protein S deficient patients and in 7% (14/91) of family members without protein S deficiency. The most common clinical manifestations in the 64 protein S deficient patients with venous thrombosis were deep venous thrombosis (81%), pulmonary embolism (27%) and superficial thrombophlebitis (41%) in various combinations. Sixty-four per cent of thrombotic protein S deficient patients had had recurrent thrombotic events. Seven protein S-deficient patients had had thrombosis at unusual sites; the superior sagittal sinus (n=2), the mesenteric vein (n=2), the axillary vein (n=2), or the subclavian vein (n=1). The clinical manifestations were similar to those found in previously published studies of hereditary protein S deficiency (Engesser *et al.,* 1987a). In Table 8, the results from this study are added to those from a Dutch study and compared with other primary hypercoagulable states. Except that antithrombin III deficient patients and to some extent APC-resistant patients tended to have fewer superficial thrombophlebitis episodes, the manifestations were similar.

The occurrence of arterial thrombosis in protein S deficient patients have been reported in a number of case reports (Mannucci *et al.,* 1986; Girolami *et al.,* 1989; Sie *et al.,* 1989; Allaart *et al.,* 1990). In a Dutch study no protein S deficient patients had had arterial thrombosis before age of 50 years (Engesser *et al.,* 1987a). Our study confirms the impression that arterial thrombosis at an early age is uncommon among protein S deficient patients. Only one patient had had thrombosis before the age of

50 years. However, arterial thrombosis was significantly overrepresented among relatives with protein S deficiency. The risk of arterial thrombosis appears high, especially after the age of 50 years as 21% (9/42) of family members older than 50 years had had arterial thrombosis. Smoking is an important factor in the development of arterial thrombosis in protein S deficient patients, as 89% (8/9) of these with arterial thrombosis were smokers.

Mean age at first thrombotic event was 32.5 years with a range of 10 to 81 years, which is a wider range than that found in a previous study (15-68 years) (Engesser *et al.,* 1987a). The highly variable penetrance of thrombotic symptoms is also evident from the thrombosis-free survival curves in Figure 10. In order to reduce the thrombotic incidence in patients with protein S deficiency, it is important to establish which factors modulate the penetrance of thrombosis in protein S deficient patients. As judged from crude odds ratios several factors such as overweight, smoking, pregnancy and childbirth and malignancy appeared to be associated with an increased risk for thrombosis in the 18 families, though in multivariate analysis only overweight was a risk factor. According to published reports, overweight is a well established risk factor for thrombosis and thus may well be an additional risk factor in protein S deficient families (Hirsh *et al.,* 1986; Schafer, 1985).

In the multivariate analysis of thrombosis-free survival, the relationship between thrombosis and circumstantial or acquired risk factors may have been underestimated. The thrombotic risk in pregnant protein S deficient women was very high (13%), as compared to the reported frequency of 0.018% to 0.29% in normal pregnancies (Sipes & Weiner, 1990). The importance of circumstantial risk factors in the pathogenesis of thrombosis in protein S deficient patients was demonstrated by the fact that 48% of the first thrombotic episodes were associated with circumstantial risk factor, e.g. pregnancy, oral contraception usage, trauma, surgery or immobilisation. It is well known from published reports that thrombosis is provoked by a circumstantial risk factor in approximately 50% of patients with inherited deficiency of antithrombin III, protein S or protein C (Thaler & Lechner, 1981; De Stefano *et al.,* 1994a; Pabinger *et al.,* 1994a). This reflects the multifactorial origin of thrombosis even in families with an inherited disorder. Our results confirm that antithrombotic prophylaxis is to be recommended for protein S deficient patients exposed to thrombotic risk factors (Bauer, 1995). Indeed, this has been shown to decrease the incidence of thrombotic events in patients with inherited thrombophilic disorders (De Stefano *et al.,* 1994a).

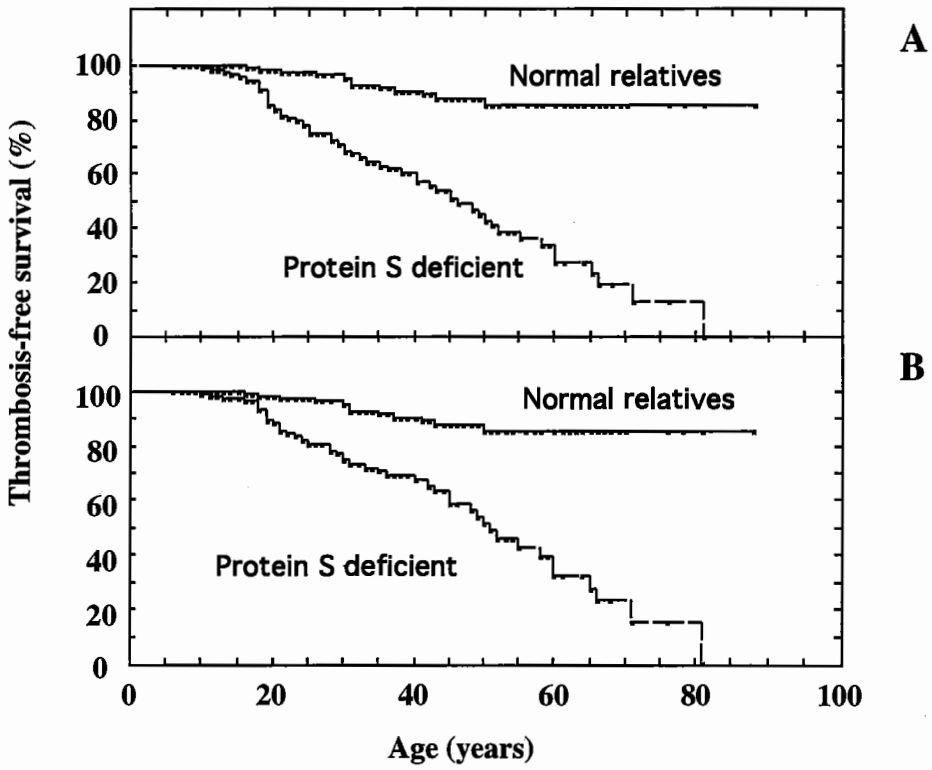


**Figure 10.** Kaplan-Meier analysis of all 18 protein S deficient families showing the probability of being free of thrombosis at a certain age. A, 136 protein S deficient and 191 normal family members. The difference between the curves is highly significant ($p<0.001$). B, the same analysis after exclusion of the 19 probands; the difference between the survival curves is still highly significant ($p< 0.001$). Modified from Zöller *et al.*, 1995.

However, the penetrance of thrombosis may also be affected by other genetic risk factors than protein S deficiency, as suggested by the high thrombotic incidence even among relatives without protein S deficiency. The youngest patient, with thrombosis at the age of 10 years, had combined protein S deficiency and homozygous FV:$Q^{506}$ mutation (APC-resistance). In all, 38% (6/16) of probands available for testing were carriers of the FV:$Q^{506}$ mutation. The mutation was found to segregate in seven (39%) of the 18 families. In the seven families where both defects occurred, 72% (13/18) of individuals with combined defects had had thrombosis, as compared with 19% (4/21) of patients with isolated protein S deficiency or the factor V mutation. Thrombosis also occurred at a younger age in individuals with combined defects, as reflected in the thrombosis-free survival curves (Fig. 11). Our results demonstrate that both genetic and circumstantial risk factors affect the penetrance of thrombosis in patients with inherited protein S deficiency. Thrombosis-prone families with protein S deficiency are often affected by yet another genetic defect. It is likely that other as yet unknown genetic factors are responsible for segregation in some of the protein S deficient families where no APC-resistance was found. In recent large Dutch case-control study, protein S deficiency was also common among healthy controls (0.7-2.3%) and was not a significant risk factor for thrombosis (Koster *et al.,* 1995a). This confirms the hypothesis that thrombosis-prone protein S deficient families are affected by one or more genetic defects other than protein S deficiency (Koster *et al.,* 1995a). That APC-resistance is a common and important risk factor in thrombotic patients with protein S, protein C or antithrombin III deficiency has been demonstrated in several other studies (Koeleman *et al.,* 1994; Koeleman *et al.,* 1995; van Boven *et al.,* 1995; Hallam *et al.,* 1995; Gandrille *et al.,* 1995c; Pabinger *et al.,* 1995). It is therefore possible to generalise the concept of familial thrombophilia as a multiple gene disorder (Bertina *et al.,* 1995).

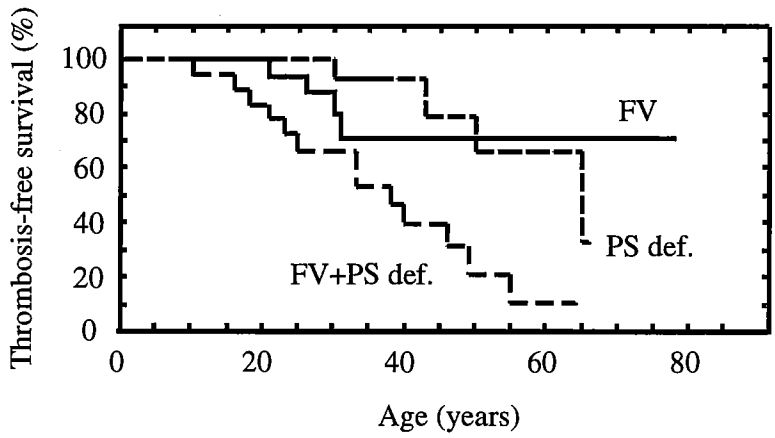


**Figure 11.** APC-resistance as an additional risk factor for thrombosis in protein S deficiency. APC resistance was found in 39% (7/18) of families with protein S deficiency. Thrombosis-free survival curves in 21 family members with only APC resistance (FV:$Q^{506}$), 21 with only protein S deficiency, and in 18 with both defects. The differences between those having either the FV gene mutation or protein S deficiency and those with combined defects were significant (p=0.008 and p=0.002). The differences were significant even after exclusion of the probands (not shown). There was no significant difference between those with only the factor V gene mutation and those with isolated protein S deficiency (p=0.47). Modified from Zöller *et al.*, 1995.

**Type I and type III protein S deficiency is the same genetic disease caused by equimolar concentrations of protein S and β chain-containing C4BP** (paper V)

In plasma, 60-70% of protein S is bound to C4b-binding protein (C4BP), a regulatory protein of the classical complement pathway (Dahlbäck & Stenflo, 1994). Only the free form of protein S is active as a cofactor to APC. Some protein S deficient patients have low plasma levels of free protein S, whereas their total protein S levels are normal (Comp *et al.*, 1984; Comp *et al.*, 1986; Iijima *et al.*, 1989; Lauer *et al.*, 1990; Malm *et al.*, 1992); this is referred to as type III deficiency (nomenclature proposed by Bertina at the ISTH subcommittee meeting 1991). Others present with decreased plasma levels of both free and complexed protein S (Comp & Esmon, 1984; Schwarz *et al.*, 1984; Bertina, 1985; Briet *et al.*, 1988); referred to as type I. Type II is characterised by a functional defect in individuals having normal protein S antigen levels (Mannucci *et al.*, 1989; Maccaferri *et al.*, 1991). Recently, it has been shown that most individuals previously classified as having type II deficiency do not have a defect in their protein S gene; instead they suffer from APC resistance (Faioni *et al.*, 1993; Faioni *et al.*, 1994; Cooper *et al.*, 1994). The reason for this misclassification is that FV:$Q^{506}$, which is present in APC-resistant plasma, affects coagulation-based functional assays for protein S.

To understand the molecular mechanisms involved in the expression of the two phenotypes of protein S deficiency, type I and type III, a detailed knowledge of the C4BP subunit structure and of the physiological regulation of the plasma levels of the C4BP isoforms and protein S is required. In plasma, C4BP is composed of 6 or 7 α chains and 1 or no ß chain, the latter containing the protein S binding site (Härdig *et al.*, 1993; Dahlbäck & Stenflo, 1994) (Fig. 3). Consequently, only the C4BP isoform that contains the ß chain (C4BPβ+) binds protein S (Hillarp & Dahlbäck, 1988; Hillarp *et al.*, 1989). Under physiological conditions, protein S-C4BP interaction is of high affinity and the equilibrium is shifted towards complex formation (Dahlbäck & Stenflo, 1994). The concentration of free protein S in plasma is equal to the molar excess of protein S over C4BPβ+ (Griffin *et al.*, 1992; Garcia de Frutos *et al.*, 1994). Thus, the concentrations of protein S and C4BPβ+ are the only variables which determine the level of free protein S. During acute phase reactions, the relative proportion of C4BP isoforms lacking the ß chain increases, which ensures stable levels of free protein S despite the high plasma levels of total C4BP (Garcia de Frutos *et al.*, 1994).

The molecular difference between type I and type III protein S deficiencies has remained elusive. In our study we evaluated the plasma concentrations of total C4BP, ß-chain-containing C4BP and free, bound and total protein S in 18 unrelated families with inherited protein S deficiency. Total C4BP (C4BPt) and C4BPβ+ plasma levels were measured with previously described enzyme-linked immunosorbent assay methods (Garcia de Frutos *et al.*, 1994). The C4BP molecule was sandwiched between a catching polyclonal antibody and a specific monoclonal antibody directed either against the β chain (C4BPβ+) or the α chain (C4BPt). Free and total protein S concentrations were determined with a previously described RIA (Malm *et al.*, 1988). The plasma level of free protein S was determined after precipitation of plasma with 5% PEG 6000 (Malm *et al.*, 1988). Bound protein S was calculated as the difference between total and free protein S (Garcia de Frutos *et al.*, 1994). We found coexistence of type I and type III deficiency in 14 out of 18 families, suggesting the two types of protein S deficiency to be phenotypic variants of the same genetic disease (Fig. 12). There was a

perfect autosomal dominant pattern of inheritance of free protein S deficiency, whereas the penetrance of total protein S deficiency was incomplete. In these families, protein S deficiency was associated with an increased risk of thrombosis, 50% of protein S deficient relatives having had one or more thrombotic event before the age of 45 years, as compared with 12% among normal relatives. There was no significant difference in thrombosis-free survival between type I and type III deficient relatives.

A review of the literature on type III deficiency confirms the impression that type I and III are simply different phenotypic expressions of the same genetic disease (Comp *et al.*, 1984; Comp *et al.*, 1986; Iijima *et al.*, 1989; Lauer *et al.*, 1990; Malm *et al.*, 1992; Marchetti *et al.*, 1993; Gouault-Heilmann *et al.*, 1994). In no study was there a large family with only type III deficient patients. In most published type III deficient families, both type I and III actually co-existed (Iijima *et al.*, 1989; Marchetti *et al.*, 1993). In same cases only very few individuals in each family have been investigated (Lauer *et al.*, 1990; Gouault-Heilmann *et al.*, 1994). Moreover, most patients previously classified as having type III deficiency have only borderline total protein S values (Iijima *et al.*, 1989; Lauer *et al.*, 1990; Gouault-Heilmann *et al.*, 1994). Our hypothesis has also been confirmed at the molecular level. The same protein S mutation caused type III deficiency in a French patient and type I deficiency in two Dutch families (Reitsma *et al.*, 1994; Gandrille *et al.*, 1995b). In a more recent study in the Netherlands, normal or borderline total protein S values (type III) were found in 4 patients from four different type I deficient families (Gómez *et al.*, 1995b). Thus, it can be concluded that the measurement of free protein S is necessary in the characterisation of protein S deficiency.

**Table 10. Mean values of protein S (PS) and C4BP in normal family members, non-anticoagulated and anticoagulated protein S-deficient family members, anticoagulated controls and normal controls.**

| | Normal members | PS deficient members | Anticoagulated PS deficient | Anticoagulated controls | Normal controls |
|---|---|---|---|---|---|
| Protein S | (n=190) | (n=117) | (n=34) | (n=40) | (n=60) |
| Total | 355±65 | 215±50 a* | 109±31 b* | 241±34 c* | 363±71 d* |
| Free | 96±23 | 16±10 a* | 4±4 b* | 44±14 c* | 108±37 d* |
| Bound | 259±55 | 198±49 a* | 105±31 b* | 197±31 c* | 255±55 d* |
| C4BP | (n=166) | (n=95) | (n=25) | | |
| C4BPß+ | 275±47 | 228±51 a* | 154±38 b* | 188±30 c* | 258±49 d* |
| C4BPt | 344±72 | 306±73 a* | 237±59 b* | 248±43 c | 331±65 d* |

Values are given as nanomoles per liter (mean ± SD). Abbreviations: a, significance versus non-anticoagulated normal members; b, significance versus non-anticoagulated protein S- deficient members; c, significance versus anticoagulated protein S- deficient members; d, significance versus anticoagulated controls. * P<0.001 (analysed with unpaired t-test, see abbreviations for groups compared). Modified from Zöller et al., 1995a.

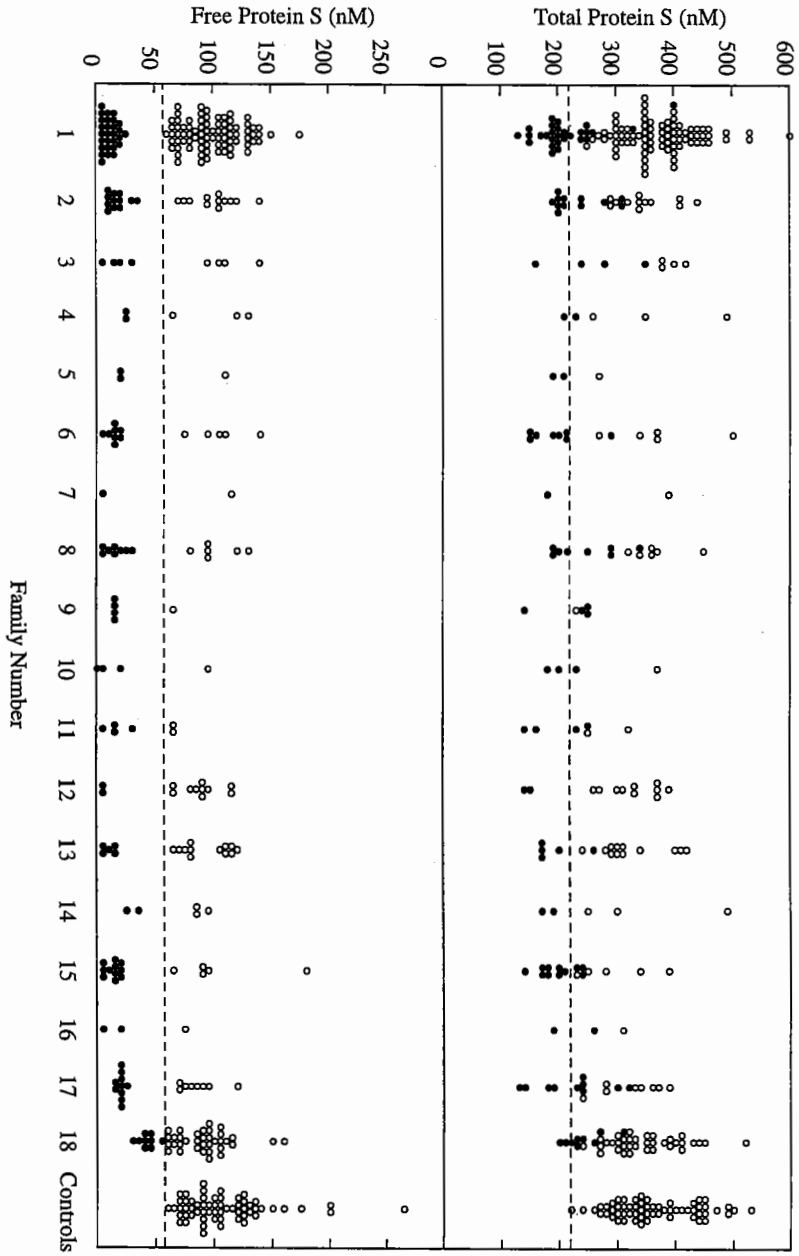


**Figure 12.** Plasma levels of free and total protein S in 307 non-anticoagulated members of 18 unrelated protein S deficient families. The dotted lines denote the lower normal reference levels for free and total protein S (56 and 219 nmol/L, respectively). Individuals with free protein S values below 56 nmol/L are considered to be protein S deficient and are denoted by filled circles. In all families, the difference between protein S deficient and normal relatives was most evident from results of measurements of free protein S. The overlap in total protein S levels between normal and protein S deficient cases illustrates the coexistence of type I and type III deficiencies in 14 of the families. Modified from Zöller *et al.,* 1995a.

Careful standardisation of the C4BP and protein S assays with purified proteins allowed determination of the molar amounts present in plasma (Garcia de Frutos *et al.,* 1994). In protein S deficient family members, the low free protein S concentration was the result of equimolar concentrations of total protein S and C4BPß+, taking into account the high affinity interaction that is known to occur between the two proteins (Table 10). In protein S deficient cases, as well as in normal individuals, there was a strong correlation between the plasma concentrations of protein S and C4BPß+, which suggests the plasma concentrations of these two proteins to be regulated by similar mechanisms (Zöller *et al.,* 1995a). The two types of protein S deficiency differ in that individuals with type III deficiency have higher plasma concentrations of both total protein S and C4BPß+ than those with type I deficiency. This may partly be an age-dependent phenomenon, because individuals with type III deficiency tended to be older than those with type I deficiency.

Interestingly, C4BPß+ and C4BPt was decreased in warfarin treated controls, and even more so in warfarin-treated protein S deficient patients (Table 10). As C4BP does not contain γ-carboxyglutamic acid, this effect is probably mediated by the vitamin K-dependent protein S. The strong correlation between total/bound protein S and C4BPß+/C4BPt in non-anticoagulated and warfarin-treated controls suggests a close regulation of the plasma concentration of the two plasma proteins. The decrease of C4BPß+/C4BPt during warfarin therapy, both in controls and protein S deficient patients, suggests that the level of protein S determines the C4BP concentration rather than the reverse. However, the mechanism responsible for this noteworthy phenomenon is still unclear.

To sum up, measuring free protein S is necessary in the elucidation of hereditary protein S deficiency. Our results demonstrate type I and type III to be simply different phenotypic variants of the same genetic disease, and that deficiency of free protein S is the result of equimolar relationship between C4BPβ+ and protein S, which in turn is the consequence of a reduced concentration of total protein S.

**Increased thrombin generation in patients with inherited APC-resistance and/or protein S deficiency** (paper VI)

The prothrombin fragment 1+2 ($F_{1+2}$), which constitutes the N-terminal part of prothrombin, is formed upon activation of prothrombin by factor Xa (reviewed in Mannucci, 1994; Bauer, 1994). The concentration of $F_{1+2}$ in plasma reflects the degree of activation of the common coagulation pathway. Several studies have shown the level of $F_{1+2}$ to be increased both in acquired and inherited hypercoagulable states (reviewed in Bauer, 1994; Mannucci, 1994).

In study VI we measured the concentrations of $F_{1+2}$ in plasma from patients with heterozygosity for the FV:$Q^{506}$ mutation to investigate whether in these individuals the hypercoagulable state is due to increased thrombin generation. $F_{1+2}$ levels were measured with a commercial ELISA (Enzygnost® $F_{1+2}$; Behring). In addition, plasma levels of $F_{1+2}$ were measured in individuals with inherited protein S deficiency, both those who were untreated and those who received warfarin. The plasma concentration of prothrombin fragment 1+2 ($F_{1+2}$) was measured in a total of 205 members of 34 thrombosis-prone families with inherited protein S deficiency and/or APC-resistance. The plasma concentration of $F_{1+2}$ was significantly higher both in 38 individuals carrying

the FV:$Q^{506}$ mutation in heterozygous state (1.7±0.7 nmol/L; mean±SD) and in 48 protein S deficient cases (1.9±0.9 nmol/L), than in 100 unaffected relatives (1.3±0.5 nmol/L). APC-resistant and protein S deficient patients had higher levels of $F_{1+2}$ even if only asymptomatic family members were included in the calculations. $F_{1+2}$ levels have been reported by age, but the age distribution was similar among the different groups. In agreement with our results, high levels of $F_{1+2}$ have been reported in a few APC-resistant individuals all belonging to the same family (Greengard *et al.*, 1994a. In contrast, $F_{1+2}$ was equally increased in stroke patients with APC-resistance (n=6) and in patients not harbouring the mutation (n=185) (Catto *et al.*, 1995), though only few APC-resistant patients were investigated.

Warfarin therapy decreased the $F_{1+2}$ levels, even in those patients with combined defects (0.5±0.3 nmol/L), a finding in agreement with the clinical antithrombotic effect. Monitoring of thrombin generation with $F_{1+2}$ may be an important tool for evaluating the effectiveness of new antithrombotic regimens in patients with APC-resistance and protein S deficiency. At the moment, no therapy is available for long-term use in asymptomatic patients with APC-resistance or protein S deficiency. Both aspirin (Szczeklik *et al.*, 1992; Yasu *et al.*, 1993) and low-dose warfarin (Holm *et al.*, 1993) have been demonstrated to be effective in reducing thrombin generation in normal individuals. However, it remains to be determined whether these regimens also reduce thrombin generation in patients with APC-resistance and protein S deficiency.

Our results suggest individuals with inherited APC-resistance or protein S deficiency to have an imbalance between pro- and anti-coagulant forces in resulting increased thrombin generation and a hypercoagulable state. Interestingly, increased thrombin generation preoperatively, measured as high levels of thrombin-antithrombin III complex (TAT), has been reported to be a significant risk factor for the development of postoperative thrombosis (Ginsberg *et al.*, 1995). Indeed, a recent study showed APC-resistance to be a major risk factor in the development of postoperative thrombosis following knee arthroplasty (Fauno *et al.*, 1995).

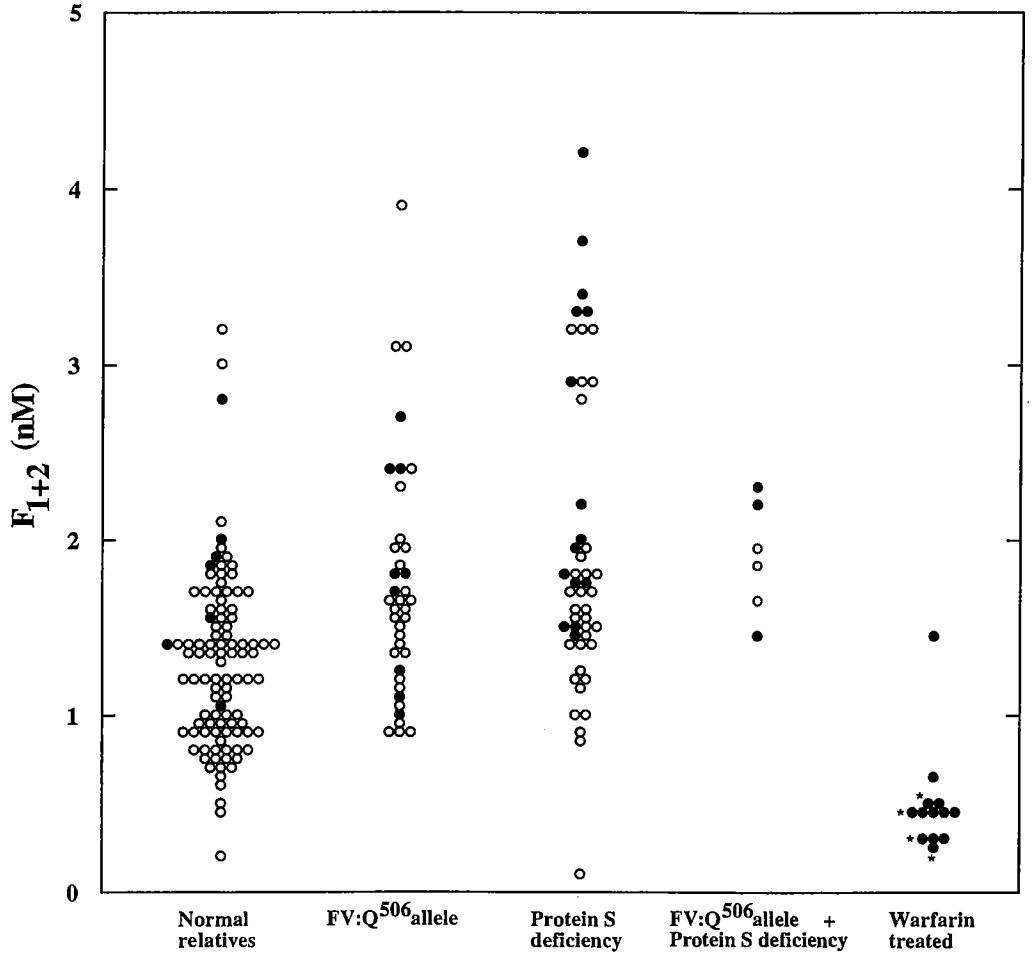


**Figure 13.** The prothrombin activation fragment $F_{1+2}$ was measured in 205 individuals from 34 thrombosis-prone families with inherited protein S deficiency and/or APC-resistance (100 normal relatives, 38 individuals with heterozygosity for the FV:Q$^{506}$ allele, 48 with protein S deficiency, 6 with combined defects, and in 13 anticoagulated protein S deficient patients of which four also had the FV:Q$^{506}$ allele). Patients having thrombotic histories are denoted by filled symbols. The four patients with combined defects in the anticoagulant group are denoted by asterisks. Modified from Zöller *et al.*, 1996.

**Future prospects**

The recent discovery of inherited APC-resistance as a major risk factor for thrombosis has enhanced our understanding of the pathogenesis of thrombosis and opened the way to new strategies for the clinical management of thrombosis patients. It is now evident that thrombosis is a polygenetic manifestation where genetic and environmental factors act in concert. Both our studies and those of others have demonstrated that thrombosis often affects genetically susceptible individuals who may or may not develop thrombosis depending on the interaction of genetic and acquired or circumstantial risk factors. It is therefore not unlikely that future studies will show that general screening for APC-resistance may be warranted before exposure to circumstantial risk factors (e.g. surgery, pregnancy, hospitalisation and oral contraceptive usage). Moreover, genetic susceptibility conferred by the hypercoagulable state associated with the factor V Arg506 to Gln mutation could also cause differences in the natural history of disease and in response to therapeutic anticoagulants. Thus, APC-resistance could be related to the high recurrence rate of pulmonary embolism (Wheeler *et al.*, 1988, Monreal *et al.* 1989), and the consistently high mortality due to pulmonary embolism (Lilienfeld *et al.* 1990, Soskolne *et al.*, 1990). APC-resistant individuals may need more intensive anticoagulant treatment. Therefore, in studies evaluating the effectiveness of different anticoagulant regimens APC-resistance should be checked for. Additional clinical research is needed to define precise guidelines for the management of the roughly 50 million APC-resistant individuals in Europe and America.

Another important issue is to determine whether other as yet unknown genetic risk factors for thrombosis may exist. The clustering of thrombosis in only a fraction of APC-resistant and protein C deficient families indicates that this might be the case. In principle there are two different approaches. One can work from the phenotype down to the genotype, as in the discovery of APC-resistance, and antithrombin III, protein C or protein S deficiency. Another possibility is to use genetic markers in linkage or association studies or directly sequence or screen candidate genes (e.g. denaturing gradient gel electrophoresis, single-strand conformation polymorphism analysis, heteroduplex analysis, chemical mismatch analysis), i.e. working from the genotype up to the phenotype. A related question is whether there exist other genetic causes of APC-resistance than the factor V Arg506 to Gln mutation. We described a few cases with APC-resistance without the factor V Arg506 to Gln mutation (study II). However, re-examination of plasma from seven of these individuals suggests that the APC-resistance was caused by an increase of several procoagulant factors, i.e. factor VIII, FIX, FX and FII (unpublished observation). It remains to be determined whether this reflects an acquired hypercoagulable state with an increased risk of thrombosis with impairment of the protein C anticoagulant system. However, this does not exclude the possibility that in rare cases other mutations in the APC-cleavage sites of factor Va and VIIIa may cause APC-resistance.

Another interesting issue is why the factor V mutation has spread and become so common among Caucasians. It might be beneficial in certain situations to have a slightly hypercoagulable state, e.g. APC-resistance might protect against excessive menstrual and postpartum bleeding and consequently against iron deficiency in fertile women. A clinically important and related issue is whether APC-resistant individuals are at a lower risk of peri- and post-operative bleeding, or are characterised by a lower frequency of bleeding complications during warfarin treatment.

In the years to come, several hundreds or thousands of new studies will elucidate the biological and clinical importance of APC-resistance. Already today, less than two years after the discovery of APC-resistance by Björn Dahlbäck, I have 299 references about APC-resistance in my database reflecting the importance of this new discovery. It is to be hoped that the recently gained knowledge of the molecular genetics of venous thrombosis together with the current development of efficient prophylactic and therapeutic protocols, will eventually result in a reduction of the morbidity and mortality due to this disease.

**Summary**

Close linkage between inherited resistance to activated protein C and two neutral polymorphisms in the factor V gene was demonstrated in a large Swedish family. A point mutation in the factor V gene changing $Arg^{506}$ in the APC cleavage site to a Gln was found in APC-resistant individuals. In the same family protein S deficiency segregated independently and both defects contributed to familial thrombophilia.

In a majority of patients the APC-resistance phenotype is associated with the same $Arg^{506}$ to Gln factor V gene mutation. Carriers are of increased risk of thrombosis as compared with normal relatives, and homozygotes are at the greatest risk. Circumstantial risk factors are important aetiological factors for the development of thrombosis in APC-resistant patients. In a minority of patients, APC-resistance is due to other acquired or genetic defects than the FV:$R^{506}$ mutation.

The combination of homozygous APC-resistance and inherited protein S deficiency was identified as the cause of severe venous thrombosis in a boy aged 10. It was shown that the addition of FV but not protein S could partially correct the poor anticoagulant response to activated protein C. The case illustrates the concept of multiple genetic defects as a cause of thrombophilia and emphasises the need of complete evaluation of thrombosis patients in order to determine whether multiple risk factors exist.

Inherited deficiency of free protein S is associated with a high risk for thrombosis. Fifty per cent of biochemically affected family members had had manifestations of thrombosis by the age of 45 years, as compared with 12% of those without protein S deficiency. APC-resistance was identified as an additional genetic risk factor in 39% (7/18) of thrombophilic protein S deficient families. In the seven families where the two defects segregated, the penetrance of thrombophilia was significantly higher in individuals with two genetic defects than in carriers of one genetic defect. The results suggest thrombosis-prone families with protein S deficiency to be affected by yet another genetic defect and support the concept that familial thrombophilia is a multiple genetic disease.

Type I and type III protein S deficiency was found to co-exist in 14 of 18 families with inherited protein S deficiency, suggesting that the two types are merely different phenotypic expressions of the same genetic disease. The importance of measuring free protein S was underscored by the fact that total protein S was within the normal range in 40% of patients deficient in free protein S. The different forms of protein S and C4BP were quantified in molar amounts, and deficiency of free protein S was shown to be caused by equimolar amounts of β chain-containing C4BP and total protein S. Moreover, C4BP was demonstrated to be decreased during warfarin treatment, especially in protein S deficient patients, suggesting that the level of the protein S concentration determines the C4BP concentration.

Prothrombin fragment 1+2 was measured in 205 individuals in 34 thrombophilic families with inherited APC-resistance and/or protein S deficiency. The level of F1+2 was higher in biochemically affected family members than in unaffected family members. The results suggest the hypercoagulability associated with APC-resistance and protein S deficiency to be due to increased thrombin generation.

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I

# Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis

*Bengt Zöller, Björn Dahlbäck*

Resistance to activated protein C (APC) is a major cause of familial thrombophilia, and can be corrected by an anticoagulant activity expressed by purified factor V. We investigated linkage between APC resistance and the factor V gene in a large kindred with familial thrombophilia. Restriction fragment length polymorphisms in exon 13 of the factor V gene were informative in 14 family members. The 100% linkage between factor V gene polymorphism and APC resistance strongly suggested a factor V gene mutation as a cause of APC resistance. A point mutation changing $Arg^{506}$ in the APC cleavage site to a Gln was found in APC resistant individuals. These results suggest factor V gene mutation to be the most common genetic cause of thrombophilia.

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See *Commentary page 1515*

Heterozygous protein C or protein S deficiency is associated with familial thrombosis,[1] and inherited resistance to activated protein C (APC) as a possible cause of thrombophilia was discovered in a family with thrombosis.[2] It is now well established that APC resistance is a major cause of venous thrombosis,[3-6] and APC resistance in different families appears to be characterised by a molecular similarity.[3] In a cohort of thrombosis patients, APC resistance was at least ten times more frequent than any of the other anticoagulant protein deficiencies (40% *vs* ≤4%) and in familial thrombophilia, it accounted for more than 50% of cases.[3] APC resistance in the general population is around 5%.[3,5]

We have isolated and characterised the protein that corrects APC resistance, and found it to be identical to factor V.[7] Factor V is pro-coagulant after activation by thrombin, whereas the novel anticoagulant cofactor activity, which we have also found in purified systems (Dr L Shen and BD, Department of Clinical Chemistry, Malmo General Hospital, Sweden), appears to be a property of unactivated factor V. Because APC-resistant plasma contains normal levels of factor V pro-coagulant activity, APC resistance may be caused by mutations in the factor V gene resulting in selective loss of the anticoagulant activity of factor V or in increased resistance to APC of mutant factor Va itself. We have investigated whether APC resistance is due to mutation in the factor V gene in a study of linkage in a large kindred with familial thrombophilia.

The APC resistance test, a modified activated partial thromboplastin time in which the anticoagulant response to standardised addition of APC is measured, was done as described.[2,3] The results were expressed as the APC ratio (clotting time with the APC/$CaCl_2$ solution divided by clotting time with $CaCl_2$). Family members with confirmed APC ratios under 2·0 were considered to be APC resistant.[3] Free and total protein S were measured with a radioimmunoassay.[3] Family members with a concentration of free protein S below the normal limit were considered to be protein S deficient. Their total protein S levels were slightly low or in the low normal range.

Genomic DNA was prepared from EDTA-blood by standard procedures. A sequence of 1188 basepairs (bp) of the factor V gene (nucleotides 2066 to 3254 of the factor V cDNA; sequence from Genebank) was amplified from genomic DNA with two primers 5'GAACTTGGATGTTAACTTCC3' and 5'GGCTTCACT-TCTTAGAGGGTG3' (figure). The conditions for polymerase chain reaction (PCR) for 40 cycles of amplification were: 60 s denaturation at 93°C, 30 s annealing at 61°C, and 180 s extension at 72°C. After amplification, the DNA was cleaved with *Taq*1 and with *Eco*R1, and subjected to agarose-gel electrophoresis. The region in exon 10 that encodes one of the APC cleavage sites in factor V was PCR amplified from genomic DNA with 5'GGG-CTAATAGGACTACTTCTAATC3' (corresponding to $Gly^{490}$-$Ile^{497}$) and 5'TCTCTTGAAGGAAATGCCCCATTA3' (derived from intron sequence provided by Dr W Kane). The PCR conditions were 5 min initial denaturation at 94°C followed by 30 cycles of 60 s denaturation at 93°C, 30 s annealing at 61°C, and 90 s

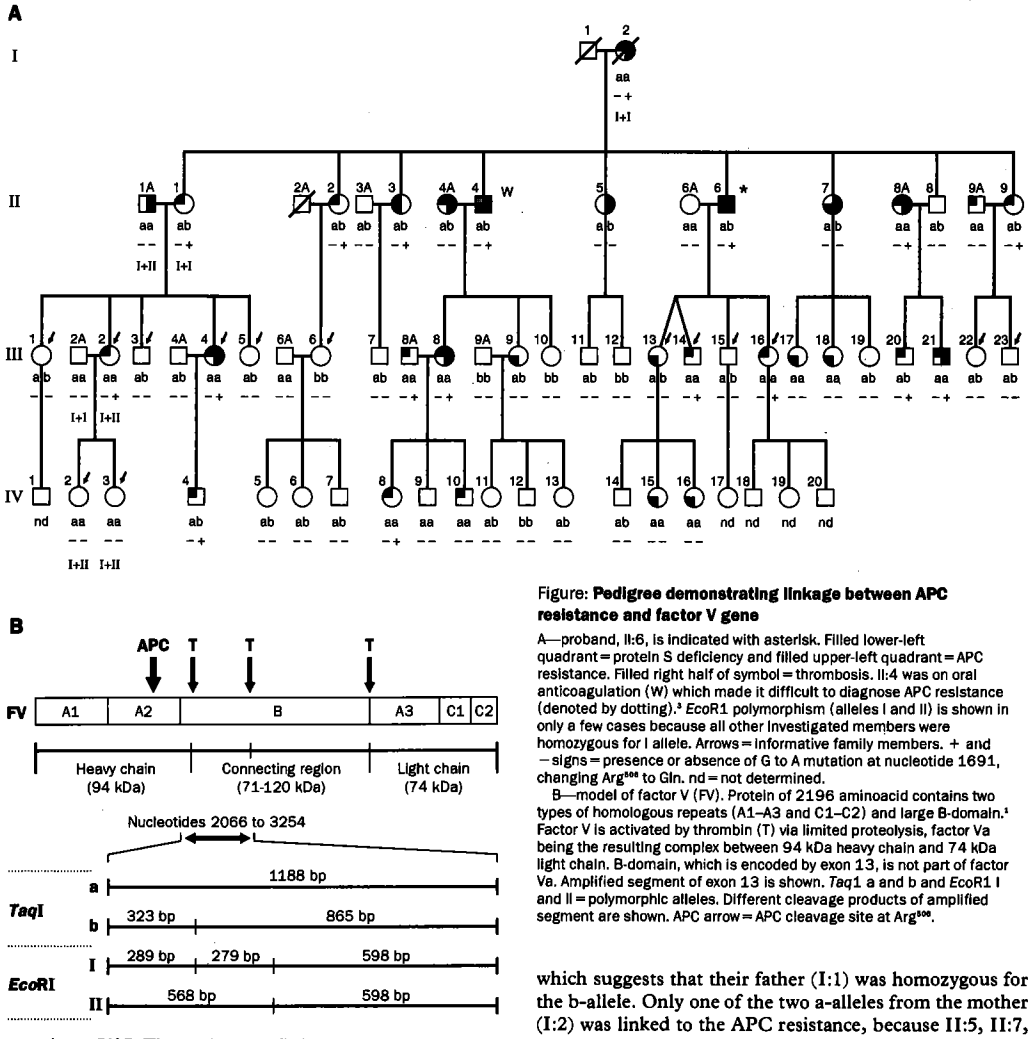


Figure: **Pedigree demonstrating linkage between APC resistance and factor V gene**

A—proband, II:6, is indicated with asterisk. Filled lower-left quadrant = protein S deficiency and filled upper-left quadrant = APC resistance. Filled right half of symbol = thrombosis. II:4 was on oral anticoagulation (W) which made it difficult to diagnose APC resistance (denoted by dotting).[3] *Eco*R1 polymorphism (alleles I and II) is shown in only a few cases because all other investigated members were homozygous for I allele. Arrows = informative family members. + and − signs = presence or absence of G to A mutation at nucleotide 1691, changing $Arg^{506}$ to Gln. nd = not determined.

B—model of factor V (FV). Protein of 2196 aminoacid contains two types of homologous repeats (A1–A3 and C1–C2) and large B-domain.[4] Factor V is activated by thrombin (T) via limited proteolysis, factor Va being the resulting complex between 94 kDa heavy chain and 74 kDa light chain. B-domain, which is encoded by exon 13, is not part of factor Va. Amplified segment of exon 13 is shown. *Taq*1 a and b and *Eco*R1 I and II = polymorphic alleles. Different cleavage products of amplified segment are shown. APC arrow = APC cleavage site at $Arg^{506}$.

extension at 72°C. The 161 bp amplified product was subjected to *Mnl*1 digestion, which produced fragments of 43 and 118 bp. A G to A mutation (nucleotide 1691) in the codon for $Arg^{506}$ resulted in the loss of this cleavage site.

Linkage was analysed with the programs MLINK and LINKMAP.[8] The logarithm of likelihood for linkage (lod score) was calculated at various recombination fractions. On the basis of segregation analysis, we assumed an autosomal dominant inheritance of a single gene with a penetrance of 1·0. The allele frequency of APC resistance was assumed to be 5% in both men and women.

Protein S deficiency and APC resistance were independently inherited (figure). The proband (II:6) had APC resistance and protein S deficiency, and a history of recurrent episodes of deep venous thrombosis. To test the hypothesis that APC resistance is caused by mutation in the factor V gene, linkage between APC resistance and a factor V gene polymorphism was investigated. Two single-nucleotide neutral polymorphisms in exon 13 (encoding the B domain) were found to affect cleavage sites for *Taq*1 (G/A at position 2391) and *Eco*RI (A/G 2379).[9] All family members in generation II had both a-allele and b-allele, which suggests that their father (I:1) was homozygous for the b-allele. Only one of the two a-alleles from the mother (I:2) was linked to the APC resistance, because II:5, II:7, and II:8 were not APC resistant. All APC resistant family members of generation III had inherited an a-allele, whereas those with a b-allele were not APC resistant. Use of the *Taq*1 polymorphism yielded 12 informative family members, all of whom manifested linkage. The *Eco*RI polymorphism was informative in 2 additional members (IV:2 and IV:3) who were not APC resistant. A healthy a+II allele of these individuals derived from the grandfather (II:1A), whereas another healthy +I allele was inherited from the father (III:2A). This suggested that the a+I allele of III:2 carried the genetic defect resulting in APC resistance. As no recombinations were seen, the probability of the observed linkage in 14 informative members occurring by chance is $0{\cdot}5^{14}$ or 1/16 384. The results suggest very close genetic linkage between APC resistance and the factor V gene, the locus of which is 1q21-25.[10] A lod score of 3·9 was calculated at a recombination fraction of 0·0 (data from all family members were used in the calculation), which is consistent with close linkage. The high frequency of APC resistance in the population was reflected by the fact that 4 family members

had married APC resistant individuals (II:4A, II:8A, II:9A, and III:8A).

After the linkage study was completed and during our search for the causal mutation, we learned that a G to A mutation at nucleotide 1691, changing $Arg^{506}$ to Gln (resulting in the loss of a *Mnl*1 cleavage site), had been identified in several individuals with APC resistance (Prof R M Bertina, Haemostasis and Thrombosis Research Centre, University Hospital, Leiden; second Pine Ridge conference at Snowbird, Utah, April, 1994). Because APC cleaves factor Va at $Arg^{506}$, such a mutation could cause APC resistance. We found individual I:2 to be heterozygous for this mutation and that family members who were APC resistant and genetically related to her also had the mutation. This does not prove the mutation to be the cause of APC resistance but supports the concept that APC resistance is due to a factor V mutation. Interestingly, the genetically unrelated family members II:8A and III:8A, who both had APC resistance, had the same mutation, whereas APC resistant individuals II:4A, her grandchild IV:10, and II:9A did not have the mutation. This demonstrates that the molecular background for APC resistance was heterogeneous.

Thus evidence from protein and DNA suggests APC resistance to be caused by one or more defects in the factor V gene, leading to increased resistance to APC of mutant factor Va itself and/or to loss of the putative anticoagulant activity of factor V. The findings suggest factor V gene mutation to be the most common genetic cause of thrombophilia.

We thank Ms Elsy Sjörin and Mrs Ing-Marie Persson for technical assistance; Dr William Kane, Duke University, for providing the factor V gene intron sequence; and Dr Mark Keating, Eccles Institute of Human Genetics, Salt Lake City, for help in calculation of the lod score. This work was supported by the Swedish Medical Council (grants 07143 and 10827) and by grants from the King Gustaf V 80th Birthday Trust, the King Gustav V and Queen Victoria Trust, the Magnus Bergwall Trust, the Albert Påhlsson Trust, the Johan Kock Trust, and Malmö General Hospital research funds.

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II

# Identification of the Same Factor V Gene Mutation in 47 out of 50 Thrombosis-prone Families with Inherited Resistance to Activated Protein C

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## Abstract

**Resistance to activated protein C (APC) is the most prevalent inherited cause of venous thrombosis. The APC resistance phenotype is associated with a single point mutation in the factor V gene, changing $Arg^{506}$ in the APC cleavage site to a Gln. We have investigated 50 Swedish families with inherited APC resistance for this mutation and found it to be present in 47 of them. Perfect cosegregation between a low APC ratio and the presence of mutation was seen in 40 families. In seven families, the co-segregation was not perfect as 12 out of 57 APC-resistant family members were found to lack the mutation. Moreover, in three families with APC resistance, the factor V gene mutation was not found, suggesting another still unidentified cause of inherited APC resistance. Of 308 investigated families members, 146 were normal, 144 heterozygotes, and 18 homozygotes for the factor V gene mutation and there were significant differences in thrombosis-free survival curves between these groups. By age 33 yr, 8% of normals, 20% of heterozygotes, and 40% of homozygotes had had manifestation of venous thrombosis. (*J. Clin. Invest.* 1994. 94:2521–2524.) Key words: blood coagulation • thrombophilia • protein C • protein S • factor V**

## Introduction

Blood coagulation is downregulated by the protein C anticoagulant system (1). Protein C is activated on endothelial cells by thrombin bound to thrombomodulin. Together with its cofactor, protein S, activated protein C (APC)[1] degrades the activated forms of factors VIII and V (VIIIa and Va), whereas intact factors VIII and V are poor substrates for APC.

Inherited APC resistance as a cause of thrombophilia was recently discovered in a single family (2) and is already recognized as a major cause of venous thrombosis (3–6). Intact factor V was found to correct APC resistance (7) which together with close linkage between APC resistance and the factor V gene (8, 9), suggested the molecular defect to be located in the factor V gene. A single point mutation (G to A at position 1691) has been found in many APC-resistant individuals (8–11). This mutation predicts replacement of $Arg^{506}$ in the APC cleavage site of factor Va with a Gln, which results in APC-resistant factor Va (8, 12). The prevalence in the European population of APC resistance is between 3 and 7% (3, 5).

Recently, our laboratory found intact factor V to function in synergy with protein S as cofactor to APC in a purified factor VIIIa degradation system (13). This suggests the possibility of thrombophilia being the result of other factor V gene mutations causing defects in the anticoagulant function of factor V, but as yet there are no such cases described.

We have previously described a large number of families with inherited APC resistance (2–3, 9). To elucidate whether the $Arg^{506}$ to Gln mutation was present in all of them, or if other genetic defects may also cause or contribute to the APC resistance, 308 individuals from 50 families with inherited resistance to activated protein C were investigated. The APC resistance was found to be associated with the same factor V gene mutation in 47 of the 50 families.

## Methods

*Patients and controls.* Thrombosis patients referred to the Department for Coagulation Disorders, Malmö General Hospital, were investigated for APC resistance, the diagnosis of APC resistance being based on APC ratio $\leq 2.0$ in at least two samples drawn on different occasions (3). APC resistance was judged to be inherited when APC resistance was found in two or more family members. In total, 389 individuals from 50 different families were evaluated for APC resistance. DNA samples were available from 308 family members (179 females and 129 males), including 33 (22 females and 11 males) of the 50 index cases. Table I lists the sizes of the investigated kindreds. The control group, which comprised 125 healthy volunteers, has been described previously (3).

*Methods.* Blood sampling and routine laboratory evaluation of coagulation parameters were performed as described (3). The APC resistance test is a modified activated partial thromboplastin time reaction, in which the anticoagulant response to APC is measured (2, 3). Results were expressed as APC ratios (clotting time obtained using the APC/$CaCl_2$ solution divided by clotting time obtained with $CaCl_2$). Genomic DNA was prepared from EDTA blood as described (14). The region in exon 10 that encodes an APC cleavage site in factor V was PCR amplified from genomic DNA using the two primers: 5′GGA ACA ACA CCA

Bengt Zöller and Peter J. Svensson contributed equally to this work.

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1. *Abbreviation used in this paper:* APC, activated protein C.

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*Table I. Sizes of Investigated Kindreds*

| No. of individuals* | No. of families |
|---|---|
| 2–5 | 25 |
| 6–10 | 17 |
| 11–18 | 7 |
| 79 | 1 |

* This includes individuals that were investigated for APC resistance. Descriptive statistics for the number of investigated members per family: mean, 7.8; mode, 4; median, 5.5; and $n$ = 389 individuals.

TGA TCA GAG CA3′, and 5′TAG CCA GGA GAC CTA ACA TGT TC3′ (derived from intron sequence kindly provided by Dr. W. Kane, Duke University). The PCR conditions were: 5 min initial denaturation at 94°C followed 30 cycles of 60 s of denaturation at 93°C, 30 s of annealing at 62°C, and 90 s of extension at 72°C. The 287-bp amplified product was subjected to MnlI digestion which in a normal person produced fragments of 157, 93, and 37 bp. A G to A mutation (nucleotide position 1691) in the codon for $Arg^{506}$ resulted in the loss of one cleavage site, which produced fragments of 157 and 130 bp (pattern in a homozygous individual). Analysis of a heterozygous person yielded bands of 157, 130, 93, and 37 bp. Alternatively, previously described PCR primers were used (9).

*Statistical analysis.* Student's $t$ test and the Mann–Whitney U test were used to compare normal and nonnormal distributed populations, respectively. The Kolmogorov–Smirnov two-sample test was used to compare the distribution of APC ratios in patients and controls. Thrombosis-free survival curves were constructed according to the Kaplan and Meier method (15). For comparison of two curves, the log rank test, resulting in a test statistic with a chi-squared distribution and one degree of freedom, was used (16). Laboratory data were expressed as mean±SD. A probability below 0.05 was considered significant.

## Results

*Laboratory results.* The $Arg^{506}$ to Gln mutation in the factor V gene was found in 47 of the 50 (94%) APC-resistant families. Of 308 family members, 144 were heterozygous, 18 were homozygous, and 146 lacked the mutation. Out of the 33 APC-resistant index cases that were available for genetic analysis, 24 were heterozygous, 5 homozygous, and 4 lacked the mutation. APC resistance cosegregated perfectly with the factor V gene mutation in 40 of the 47 families. In the remaining 7 families, 12 out of 57 APC-resistant individuals lacked the mutation. APC ratios in these 12 individuals ranged from 1.3 to 2.0. APC ratios were low in all homozygous cases, whereas there was an overlap in APC ratios between heterozygotes and normals (Fig. 1). Heterozygotes with a history of thrombosis had significantly lower APC ratios than those without thrombosis (1.5±0.2 vs. 1.8±0.4, $P < 0.001$), and none of the heterozygotes with APC ratio > 2.0 had had thrombosis. Moreover, relatives without the mutation but with histories of thrombosis had significant lower APC ratios than those without thrombosis, even when protein S–deficient cases were excluded from the analysis (mean±SD of 2.4±0.7 vs. 2.9±0.8, $P < 0.05$). No influence on APC ratios of age or sex was observed among normal or heterozygous relatives.

The four APC-resistant probands who lacked the mutation had severe thromboembolic disease (deep venous thrombosis, pulmonary emboli, and/or superior sagital sinus thrombosis) but no other known defect. Three of them had only slightly low

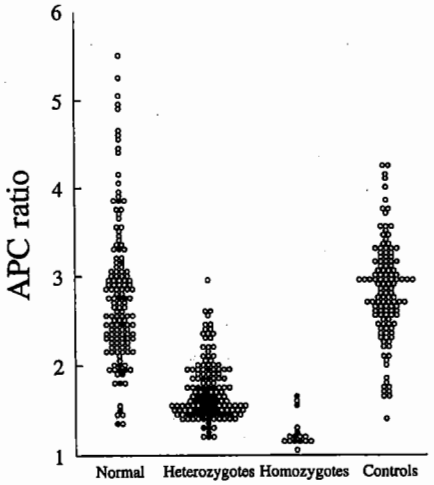


*Figure 1.* APC ratios in nonanticoagulated family members and in unrelated control subjects. Filled circles denote patients with a history of thrombosis. Plasmas were missing from two family members; five patients were excluded because of anticoagulant therapy. Differences in APC ratios (No., mean±SD) between normals ($n$ = 143, 2.8±0.8), heterozygotes ($n$ = 142, 1.7±0.3) and homozygotes ($n$ = 16, 1.3±0.2) were highly significant ($P < 0.001$). Even though the mean value of controls ($n$ = 126, 2.8±0.6) was not different from that of normal relatives, the distribution of values in the two groups appeared different. However, in a Kalmogorov–Smirnov two-sample test this difference was not significant, $P$ = 0.1.

APC ratios (1.8–2.0), whereas the fourth had an APC ratio of 1.5. DNA samples were available for analysis from several relatives in three of the four families. In one family (five relatives tested), four APC-resistant relatives were found to be heterozygotes for the mutation, even though the index case lacked the mutation. In the remaining two families, slightly low APC ratios (1.8–2.0) were found in several relatives who were negative for the factor V gene mutation; three of eight relatives in one family and one of five in the other.

Independent inheritance of protein S deficiency was found in three of the families. 5 protein S–deficient individuals were heterozygous for the factor V mutation, 1 was homozygous and 12 were normal. 3 of the 5 heterozygotes, the homozygous case and three of those without the factor V gene mutation had experienced one or more thrombotic event. The homozygous case with protein S deficiency had severe thrombosis at the age of 10 yr. No deficiencies of protein C, antithrombin III, or plasminogen were found.

*Clinical data.* 43 of 144 (30%) heterozygotes, 8 of 18 (44%) homozygotes, and 14 of 146 (10%) individuals without the mutation had experienced one or more venous thrombotic events (Table II). Significant differences in thrombosis-free survival of heterozygotes, homozygotes, and normals were found (Fig. 2). At 33 yr of age, 8% of those not carrying the mutation, 20% of heterozygotes, and 40% of homozygotes had had at least one manifestation of venous thromboembolic disease. It is noteworthy that also in the group of family members who lacked the mutation, a significant difference in thrombosis-free survival between those with and those without APC resis-

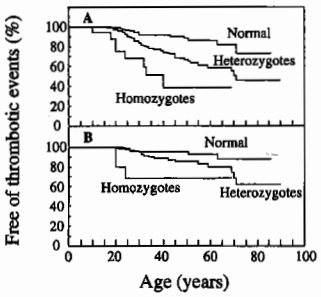


*Figure 2.* Thrombosis-free survival curves. (*A*) the probability to be free from thrombotic events at a certain age is presented in the Kaplan–Meier analysis for 146 normals, 144 heterozygotes, and 18 homozygotes. Differences between normals and heterozygotes and between heterozygotes and homozygotes were highly significant, $P < 0.001$ and $P = 0.01$, respectively. (*B*) the same analysis after exclusion of the 33 APC-resistant probands and 18 protein S–deficient family members. Differences normals vs. heterozygotes and heterozygotes vs. homozygotes were both significant, $P < 0.05$.

tance was observed, even after exclusion of the four APC-resistant probands (not shown).

The mean age at the first thrombotic event was 36 yr (range 16–71) for normals, 36 yr (range 18–71) for heterozygotes, and 25 yr (range 10–40) for homozygotes. The first thrombotic episodes were associated with circumstantial risk factors in 8 of 14 normals, in 25 of 43 heterozygotes, and in 7 out of 8 homozygotes. The most common risk factors were pregnancy, surgery, trauma, and oral contraceptives (Table III). Heterozygous women tended to have their first thrombosis at an earlier age than heterozygous men (34±14 vs. 38±15), although the difference was not statistically significant. 29 of the 85 (34%) heterozygous women and 14 of the 59 (24%) heterozygous men had had thrombosis, but there was no significant difference

*Table II. Clinical Manifestations in Symptomatic Family Members*

| | Normals | Heterozygotes | Homozygotes |
|---|---|---|---|
| Total no. of patients | 14 | 43 | 8 |
| Total no. of patients with indicated thrombotic symptom* | | | |
| DVT | 8§ | 36‡ | 8 |
| PE | 3 | 8 | 4 |
| STP | 5 | 8 | 1 |
| Recurrence | 7 | 17 | 4 |
| First episode in affected family members | | | |
| DVT | 7 | 31 | 6 |
| PE | 3 | 1 | 0 |
| STP | 4 | 7 | 0 |
| Combined DVT + PE | 0 | 4 | 1 |
| Combined DVT + PE + STP | 0 | 0 | 1 |

* Since a patient may have suffered from several different thrombotic events, the numbers do not add up to the total no. of patients. DVT, deep venous thrombosis; PE, pulmonary embolism; and STP, superficial thrombophlebitis. ‡ One heterozygote had central retinal venous thrombosis. § One patient had a superior sagital sinus thrombosis.

*Table III. Risk Factors Associated with the First Thrombotic Episode**

| | Normals | Heterozygotes | Homozygotes |
|---|---|---|---|
| | *n* | *n* | *n* |
| Pregnancy or postpartum | 2 | 12 | 1 |
| Oral contraceptives | 2 | 8 | 3 |
| Trauma and surgery | 4 | 8 | 2 |
| Immobilization | 2 | 1 | — |
| Other risk factors‡ | 1 | 1 | 1 |
| Protein S deficiency | 3 | 3 | 1 |
| Total no. of patients with risk factor | 8 | 25 | 7 |

* Some patients were exposed for several risk factors. ‡ Malignancy, varicose, and edema.

in thrombosis-free survival curves between men and women (not shown). Six heterozygotes (at age 51–74), one homozygote (at age 34) and two without the mutation (at age 55 and 64) had had an arterial thrombotic event. We have previously reported a case with severe APC resistance and arterial thrombosis (17) and more data are needed to determine whether APC resistance is associated with an increased risk of arterial thrombosis.

## Discussion

The $Arg^{506}$ to Gln mutation in the factor V gene was found in more than 90% of families with APC resistance and there were significant differences in thrombosis-free survival, as well as in APC ratios, between individuals with and without the mutation, homozygotes being more severely affected than heterozygotes. This demonstrated the factor V mutation to be a risk factor for thrombosis and to be the major cause of APC resistance. In a limited number of individuals with inherited APC resistance, the factor V gene mutation was not found, suggesting that other as yet unknown genetic defects may also cause APC resistance. Such defects appeared to be present also in some families harboring the factor V gene mutation, as the mutation was not found in all APC-resistant family members. The very low APC ratios observed in some heterozygous individuals may be the result of a combination of the putative second genetic defect and the factor V gene mutation. The presence of additional genetic defects associated with slightly low APC response was also suggested by the large group of normal relatives having APC ratios just above the lower normal limit (Fig. 1). This pattern was not observed in the controls and was not explained by age or sex differences. In this context it is also noteworthy that heterozygous family members with a history of thrombosis had lower APC ratios than those without thrombosis. Even though this is also in line with the hypothesis that these thrombosis-prone families are affected by more than one genetic defect, the possibility of lower APC ratios in thrombotic individuals being a postthrombotic phenomenon cannot be ruled out.

The APC resistance test is not a quantitative assay, but is to be regarded as a screening test. Complicated pro- and anticoagulant forces determine the APC ratio and it is easily envisioned that a number of factors may affect the results. Al-

though sample handling, choice of reagents, and instrumentation influence the APC ratio, experience from different laboratories suggest that reliable results are obtained if the assay is performed under strictly standardized conditions (3, 5, 8, 18). If diagnosis of the factor V mutation in the investigated families were to be based on an APC ratio $\leq$ 2.0 only, 15% of heterozygotes would not be identified (sensitivity 85%) and 13% of normals would be classified as factor V mutants (specificity 87%). Thus, in families with APC resistance (assuming a 50% prevalence of the factor V gene mutation) the predictive value for the factor V mutation of a positive APC resistance test is 87%, whereas the predictive value of a negative test is 85%. As DNA samples were not available from the control group we do not know whether the nine individuals with APC ratio $\leq$ 2.0 carry the mutation. In a recent report of a similar reference population, the mutation was found in the 3% of control individuals that had distinct APC resistance, whereas cases with borderline APC ratios were negative for the mutation (8, 18).

In a majority of both heterozygous and homozygous cases, thrombosis was associated with circumstantial risk factors, the most frequent being pregnancy, the use of oral contraceptives, trauma, and surgery, suggesting a combination of circumstantial and genetic risk factors to determine the risk for thrombosis. Heterozygosity for the factor V gene mutation is associated with a 5–10-fold increased risk of thrombosis (8, 18) and unless heterozygous individuals are exposed for circumstantial risk factors, thrombosis may not present until advanced age or not at all. Even though homozygosity is associated with a 50–100-fold increased risk of thrombosis, affected individuals may live healthy lives without thrombosis; the oldest homozygous person in this study being free of thrombosis was 68 yr of age. The relatively mild thrombotic tendency even in homozygous cases is in marked contrast to the severe thrombotic disease that affect individuals with homozygous protein C or protein S deficiency already in the neonatal period (19). In factor Va, several peptide bonds are cleaved by APC and even though the $Arg^{506}$ to Gln mutation is associated with APC-resistant factor Va, the resistance may not be complete. Moreover, the ability of APC to downregulate the rate of activation of coagulation at the level of factor VIIIa is unaffected by the factor V gene mutation.

In conclusion, the APC resistance phenotype is in the majority of cases associated with the same mutation in the factor V gene. Carriers have increased thrombotic risk compared to their normal relatives and homozygotes are the worst affected. In a minority of patients, APC resistance is due to an as yet unknown genetic defect.

## Acknowledgments

The expert technical assistance of Ms. Elsy Sjörin and Mrs. Ing–Marie Persson is gratefully acknowledged. We are grateful to Dr. William Kane at Duke University for providing the factor V gene intron sequence before publication.

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# Homozygous APC-resistance Combined with Inherited Type I Protein S Deficiency in a Young Boy with Severe Thrombotic Disease

III

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## Summary

Inherited resistance to activated protein C (APC) is a frequent cause of familial thrombosis. It is associated with a factor V gene point mutation replacing arginine$^{506}$ in the APC-cleavage site with a glutamine. Thrombotic events are rare during childhood even in patients with homozygous APC-resistance. We now wish to report on a case of severe venous thrombosis, in a 10-year-old boy. He was found to have pronounced APC-resistance due to homozygous factor V gene mutation in combination with inherited type I protein S deficiency. The two traits were independently inherited in the family. The APC-resistance was partially corrected by adding factor V, whereas added protein S was without effect. This is the first reported case of homozygous APC-resistance combined with another inherited prothrombotic disorder. It illustrates how multiple genetic defects may provoke thrombosis at young age and emphasizes the need of complete evaluation of thrombotic patients in order to determine whether multiple risk factors exist.

## Introduction

Blood coagulation is inhibited through degradation of cell bound factor VIIIa and factor Va by activated protein C (APC), a key component in a physiologically important anticoagulant system (1). Another anticoagulant protein, protein S, functions as cofactor to APC. The rate of the coagulation reaction depends on the balance between activation of factors VIII and V by thrombin and degradation of their activated forms by APC. Heterozygous protein C or protein S deficiency is associated with familial thrombosis (1). Homozygous deficiency of either protein has been described in few cases and is associated with purpura fulminans in the neonatal period (2) but no cases of purpura fulminans and homozygous APC-resistance have been described.

Inherited resistance to APC was discovered in a single family with thrombosis (3) and is now recognized as a major cause of venous thrombosis (4-7). In thrombosis patients, APC-resistance is at least ten times more prevalent than any of the other anticoagulant protein deficiencies and accounts for more than 50% of the cases with familial thrombophilia (7). APC-resistance is frequent in the general population (3-7%) (5, 7).

APC-resistance was found to be corrected by intact factor V, suggesting a factor V gene mutation to cause the phenotype (8). More recently, APC-resistance has been found to be associated with a single point mutation in the factor V gene (9-12), which changes arginine$^{506}$ in the APC-cleavage site to a glutamine (9-12). This renders factor Va resistant to APC (9, 13).

Three different types of protein S deficiency have been described (1), and a nomenclature for protein S deficiencies has been proposed (Bertina, ISTH subcommittee meeting, 1991). In type I deficiency, there is a parallel decrease of total and free protein S antigen concentrations. In type II deficiency, functional (clotting) protein S activity is decreased while the levels of total and free protein S antigen are normal, whereas in type III deficiency, the total protein S antigen concentration is essentially normal, but the level of free protein S antigen is low. Recently, functional protein S assays have been reported to be influenced by APC-resistance (6). This may lead to misclassification of APC-resistant individuals as being type II protein S deficient (6). However, immunologic protein S assays measuring free and total protein S are not affected by APC-resistance and the APC-resistance test is not influenced by the concentrations of free and total protein S (3, 7). Independent inheritance of protein S deficiency (decreased antigen concentrations of protein S) and APC-resistance have been reported (11). In this report we describe a 10-year-old boy with severe thrombosis, who is both homozygous for the factor V gene mutation and protein S deficient (type I).

## Methods

The APC-resistance test was performed as described (3). The results were expressed as APC-ratios (clotting time obtained using the APC/$CaCl_2$-solution divided by clotting time obtained with $CaCl_2$) and family members with APC-ratios ≤ 2.0 were considered to be APC-resistant (7). The concentrations of free and total protein S antigen in plasma were measured with a radio immune assay as described (immunological method using a polyclonal antiserum) (14). The amount of free protein S antigen was determined in 5% PEG 6000 supernatants, using a pool of normal plasma as standard (14). Family members with free protein S antigen (normal range 18-58%) below the lower normal limit were considered to be protein S deficient (Fig. 1). Their total protein S antigen levels (normal range 70-130%) were low, except in one family member (II:1), which is most consistent with type I protein S deficiency (1). Plasma and DNA was obtained from a previously described healthy individual (AS) with pronounced APC-resistance due to homozygosity for the factor V gene mutation (8). Protein S and FV were purified as described (3, 8). The effect in the APC-resistance test of factor V and protein S was investigated; protein S, factor V or factor V+ protein S (11 µl in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 2 mM $CaCl_2$) were added to 100 µl citrated plasma. The final concentrations in plasma of added protein S and FV were 10 µg/ml and 20 µg/ml (approximate plasma concentrations are 25 µg/ml and 7 µg/ml), respectively. APTT-reagent (Chromogenix, Mölndal, Sweden) (100 µl) was added and after 200 s incubation at 37° C, coagulation was initiated with 100 µl of the APC/$CaCl_2$ mixture. Genomic DNA was prepared from EDTA blood using standard procedures. Two primers, 5'GGA ACA ACA CCA TGA TCA GAG CA3' and

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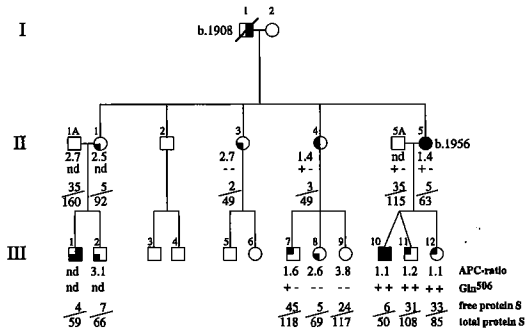


*Fig. 1* Pedigree demonstrating independent inheritance of APC-resistance and protein S deficiency. Filled lower left quadrant denote protein S deficiency and filled upper left quadrant indicate APC-resistance. Filled right half of the symbol denote thrombosis. The + and - signs indicate presence or absence of the G to A mutation in the factor V gene at nucleotide position 1691, changing arginine$^{506}$ to glutamine. The APC-ratio, free/total protein S concentrations (antigenic) are shown. nd, not determined. Reference ranges for free and total protein S were 18-58% and 70-130%, respectively

5'TAG CCA GGA GAC CTA ACA TGT TC3' were used to PCR amplify a region of exon 10 and the adjacent intron. The PCR conditions were: 5 min initial denaturation at 94° C followed by 30 cycles of 60 s of denaturation at 93° C, 30 s of annealing at 62° C, and 90 s of extension at 72° C. The 287 bp amplified product was subjected to Mnl I digestion, which in a normal person produced fragments of 157, 93 and 37 bp. A G to A mutation (nucleotide position 1691) in the codon for arginine$^{506}$ resulted in the loss of one cleavage site, which produced fragments of 157 and 130 bp (pattern in a homozygous individual). Analysis of heterozygous person yielded bands of 157, 130, 93 and 37 bp.

## Case Report

A 10-year-old boy (III:10), born 1979, was admitted to the hospital because of painful swelling of his right leg. A few days before, the boy had been kicked on the leg. Phlebography demonstrated a venous thrombus in the right calf extending to the pelvic veins. He was treated temporarily with heparin and warfarin and has been free of recurrence. After diagnosis of homozygous APC-resistance and type I protein S deficiency, he was given long-term anticoagulant therapy. Plasma drawn before the anticoagulant therapy demonstrated normal values for antithrombin III, protein C, plasminogen, C4b-binding protein, fibrinogen and plasminogen activator inhibitor 1 (PAI 1) and APTT. His mother (II:5) has had recurrent superficial thrombophlebitis (normal phlebographies) at age 21 years (during use of oral contraceptives) and 29 years (during pregnancy). She also had had one episode of suspected pulmonary embolism. Her sister (II:4) has had several episodes with painful swellings of the legs (not examined with phlebography) and has chronic swelling of the legs. A cousin (III:1) has had three episodes of objectively verified deep venous thrombosis in the legs at 15, 19 and 34 years of age. When discontinuing warfarin at age 35, he developed a mesenteric vein thrombosis. The intestine was resected and he has been on continuous warfarin therapy thereafter. He has a diagnosed protein S deficiency, but samples for DNA analysis were not available. The deceased grandfather (I:1) was suffering from deep venous thrombosis. The father of the propositus (II:5A) has not had any thrombotic events.

## Results and Discussion

Pronounced APC-resistance and low free and total protein S antigen concentrations (type I protein S deficiency) was found in the propositus (Fig. 1). His healthy dizygote twin brother and a younger sister (born 1985), had pronounced APC-resistance but normal protein S antigen concentrations. All three were found to carry the arginine$^{506}$ to glutamine mutation in homozygous form. Other relatives (II:4, II:5, III:7), with less pronounced APC-resistance, were heterozygous for the mutation. The APC-ratios correlated with the factor V genotype, but not with the plasma antigen concentrations of free or total protein S.

The APC-resistance of the propositus was partially corrected by added factor V, whereas added protein S had no effect (Fig. 2). An unrelated individual with homozygous factor V gene mutation, but without protein S deficiency, demonstrated a similar response. The less pronounced APC-resistance of heterozygous individuals with protein S deficiency was also partially corrected by factor V, but not by protein S. We have recently shown that factor V and protein S function as synergistic cofactors to APC in a purified factor VIIIa-degradation system (15). The partial correction of APC-resistance by added factor V may be the result of an increased APC-mediated degradation of factor VIIIa, because factor V is an APC-cofactor. However, if this was true added protein S would be expected to correct the APC-resistance, which is not the case. Another possibility is that an excess of normal factor V results in competitive inhibition of activation of mutated factor V, assuming mutated and normal factor V to be activated at equal rates. The normal factor Va molecules are then degraded at normal rate by APC which would lead to correction of the APC-resistance. No data on record support a third possibility of mutated factor Va being a competitive inhibitor to APC in degradation of factor VIIIa and factor Va. The observation that intact factor V has the ability to correct severe APC-resistance may suggest factor V to have a therapeutic potential in individuals with homozyogus APC-resistance (with or without other genetic defects), in particular when they are affected by severe thromboembolic complications.

The now reported case illustrates that homozygosity for the arginine$^{506}$ to glutamine factor V gene mutation with associated APC-resistance is compatible with life also when combined with inherited type I protein S deficiency although the risk of an early debut of severe thrombotic disease appears high. Homozygous protein S or protein C

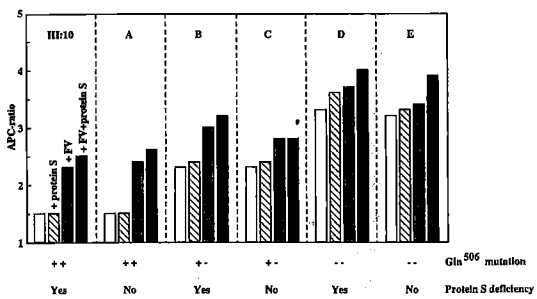


*Fig. 2* Response to activated protein C with and without additions of protein S, factor V and factor V+protein S. The response of the propositus (III:10) is compared with the response of unrelated individuals having the following genetic defects: homozygous factor V mutation with normal protein S (A), heterozygous factor V mutation with protein S deficiency (B), heterozygous factor V mutation with normal protein S (C), negative for factor V mutation but with protein S deficiency (D), normal control (E). The APC-ratios were slightly different from those seen in Fig. 1 because another instrument was used to monitor clotting. The individual in A is a healthy women (46 years of age) without history of thrombosis

deficiency is associated with purpura fulminans in the neonatal period (2). Homozygosity for the factor V gene mutation is obviously less severe (16, 17), and even when combined with protein S deficiency, as in the now presented case, thrombosis was actually provoked by a circumstantial risk factor. It will be of clinical importance to identify homozygous APC-resistant individuals and study clinical manifestations of thrombotic disease in order to established guidelines for handling of this patient group.

Due to its high prevalence, APC-resistance may often affect the clinical expression of other inherited disorders like deficiencies of protein S, protein C and antithrombin III. Patients with protein S deficiency manifest a variable expression of thrombotic manifestations (16) and APC-resistance may be a contributing risk factor in some of the protein S deficient cases with severe thrombosis (11). The now investigated family is however too small to allow accurate evaluation of the influence of APC-resistance on manifestations of protein S deficiency. However, the propositus is the youngest protein S deficient patient with thrombosis examined in our laboratory (out of 136 protein S deficient patients) and according to the literature, thrombosis in young protein S deficient individuals is rare. In a Dutch study of 71 protein S deficient patients, the age at first thrombotic event ranged from 15 to 68 years (18). APC-resistance in a thrombosis-prone protein C deficient family (9) has also been described and APC-resistance was recently demonstrated to be an additional genetic risk factor in several families with inherited protein C deficiency (17). However, only individuals with heterozygous APC-resistance and protein C deficiency were described and the range of first thrombotic event was 19-71 years for the 12 individuals with combined defects (19).

In conclusion, we report a case of severe thrombosis in a young boy with homozygous APC-resistance and inherited type I protein S deficiency. This emphasises the need to do complete evaluations of thrombotic patients to determine whether multiple risk factors exist.

*Acknowledgements*

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

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

**Inherited resistance to activated protein C (APC), which is caused by a single point mutation in the gene for factor V, is a common risk factor for thrombosis. In this study, the prevalence of APC resistance in 18 unrelated thrombosis-prone families with inherited protein S deficiency was investigated to determine its role as additional genetic risk factor for thrombosis. In addition, a detailed evaluation of the clinical manifestations in these families was performed. Venous thrombotic events had occurred in 47% of the protein S-deficient patients (64/136) and in 7% of relatives without protein S deficiency (14/191). As estimated from Kaplan-Meier analysis, 50% of protein S-deficient family members and 12% of those without protein S deficiency had had manifestation of venous thromboembolism at the age of 45 years. The age at the first thrombotic event ranged from 10 to 81 years (mean, 32.5 years) and a large intrafamilial and interfamilial variability in expression of thrombotic symptoms was seen. The factor V gene mutation related to APC resistance was present in 6 (38%) of 16 probands available for testing; in total, the mutation was found in 7 (39%) of the 18 families. In family members with combined defects, 72% (13/18) had had thrombosis as compared with 19% (4/21) of those with only protein S deficiency and 19% (4/21) of those with only the factor V mutation. In conclusion, APC resistance was found to be highly prevalent in thrombosis-prone families with protein S deficiency and was an additional genetic risk factor for thrombosis in these families. The results suggest thrombosis-prone families with protein S deficiency often to be affected by yet another genetic defect.**



IV

VITAMIN K-DEPENDENT protein S is an important anticoagulant plasma protein that functions as that of a cofactor to activated protein C (APC) in the degradation of factors Va and VIIIa.[1] Hereditary deficiency of protein S is a well-established cause of familial thrombosis.[2-15] In cohorts of thrombosis patients, between 1.5% and 7% of the patients suffer from protein S deficiency.[16-21] In human plasma, protein S exists in two forms, ie, as free protein S (30% to 40%) and in complex with C4b-binding protein.[22,23] Only free protein S is active as APC cofactor.[5,24,25] Three different types of protein S deficiency have been described[1]: type I is characterized by a reduction in both free and total protein S,[3-6] type II by a functional protein defect,[26,27] and type III by a selective deficiency of free protein S.[2,15,28] We have recently found type I and type III deficiencies to be phenotypic variants of the same genetic disease.[28a]

APC resistance is a recently described inherited pathogenetic risk factor of venous thrombosis.[29,30] It is found in 21% to 60% of thrombotic patients and in 3% to 7% of healthy controls.[31-33] In a majority of cases, APC resistance is caused by a single point mutation in the factor V (FV) gene, which renders FVa resistant to inactivation by APC.[34-39] APC resistance has recently been found to be an additional risk factor for thrombosis in several thrombosis-prone Dutch protein C-deficient families.[34,40] We have described independent inheritance of protein S deficiency and APC resistance in two families, suggesting that APC resistance may contribute to the thrombotic tendency also in protein S deficiency.[37,40a] In contrast, in Italian families with deficiency of antithrombin III, protein C, or protein S, no increased prevalence of APC resistance was found.[41]

In this study, we describe APC resistance to be an additional genetic risk factor for thrombosis in several Swedish protein S-deficient families. In addition, we provide a detailed description of the clinical manifestations of protein S deficiency.

## PATIENTS AND METHODS

*Protein S-deficient families and controls.* The study included 327 members from 18 unrelated families with hereditary protein S deficiency. Protein S deficiency was found in 136 (117 nonanticoagulated and 19 anticoagulated) relatives. A detailed description of patients and the normal and anticoagulated control populations are given in a separate report.[28a] Plasma levels of antithrombin III, plasminogen, and protein C were normal in all family members (data not shown).

*Laboratory methods.* Blood sampling and routine coagulation methods, including measurements of free and total protein S, were performed as described.[21] Protein C, antithrombin III, and plasminogen were determined with Coatest protein C, Coatest antithrombin, and Coatest plasminogen (Chromogenix, Mölndal, Sweden), respectively. The APC-resistance test is a modified activated partial thromboplastin time (APTT) reaction, in which the anticoagulant response to APC is measured.[29,33] Results were expressed as APC ratios (clotting time obtained using the APC/$CaCl_2$ solution divided by clotting time obtained with $CaCl_2$). Family members with confirmed APC ratios $\leq 2.0$ were considered to be APC resistant.[33] Preparation of genomic DNA from EDTA blood and determination of the FV gene point mutation (G to A at position 1691), which causes APC resistance, was performed as described.[37,38]

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

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**Table 1. Clinical Manifestations in Symptomatic Relatives**

| | Normal Protein S | Protein S Deficient |
|---|---|---|
| Total no. of patients | 14 (100) | 64 (100) |
| Total no. of patients with a certain thrombotic symptom* | | |
| DVT | 7 (50) | 52† (81) |
| PE | 5 (36) | 17 (27) |
| STP | 9 (64) | 26 (41) |
| Recurrences | 7 (50) | 41 (64) |
| First symptom in affected family members | | |
| DVT | 3 (21) | 35 (55) |
| PE | 1 (7) | 4 (6) |
| STP | 7 (50) | 16 (25) |
| Combined DVT + PE | 2 (14) | 7 (11) |
| Combined DVT + STP | 1 (7) | 2 (3) |

Values are the number of patients with percentages in parentheses.

Abbreviations: DVT, deep venous thrombosis; PE, pulmonary embolism; STP, superficial thrombophlebitis.

* Because one patient may have suffered from different thrombotic events, the numbers do not add up to 100%.

† Two protein S-deficient patient had had superior sagittal sinus thrombosis, two had mesenteric vein thrombosis, two had axillary vein thrombosis, and one had subclavian vein thrombosis.

**Table 2. Circumstantial Risk Factors Associated With the First Thrombotic Episodes in Symptomatic Relatives**

| | Normal Protein S | Protein S Deficient |
|---|---|---|
| Pregnancy or postpartum | 3 | 6 |
| Oral contraceptives | 3 | 8* |
| Trauma and surgery | 3 | 9 |
| Immobilization | 1 | 5 |
| Other risk factors | 1 | 4† |
| Total no. of patients with risk factor | 10 | 31 |

Some patients had had several risk factors provoking a thrombotic event. In addition to these circumstantial risk factors, the first thrombosis occurred after sedentary flight or car travelling in 7 protein S-deficient patients. Values are the number of patients.

* One protein S-deficient patient had superior sagittal sinus thrombosis at age 16 in conjunction with oral contraceptives and acute media otitis.

† Two protein S-deficient patients had hormone therapy because of malignancy and two had varicosis.

were performed with a standard $\chi^2$ test. Fischer's exact test was used to compare two proportions for small numbers. Thrombosis-free survival curves were constructed according to Kaplan and Meier.[42] For comparison of two curves, the log-rank test resulting in a statistic test with a $\chi^2$ distribution and one degree of freedom was used.[43] As an approximation of the relative risk, crude odds ratios (univariate) were calculated for several putative risk factors by simple crosstabulation. These odds ratios reflect the risk when the proposed risk factor is present relative to the risk when it is absent, unadjusted for other factors. A Cox proportional hazards model was used in a multivariate survival analysis, allowing several factors to be adjusted for simultaneously. The hazard ratio is the incidence rate ratio, which is assumed to be constant over time, whereas the baseline hazard is allowed to vary.[44] The hazard ratio may be interpreted as the relative risk associated with each factor, adjusted for all other factors in the model. The incidence of first thrombotic events in protein S-deficient and normal relatives was calculated by counting patient-years of observation and dividing the number of events in each group by the sum of observation-years of all the individuals in the group.

## RESULTS

*Clinical data.* Sixty-four (47%) of the 136 protein S-deficient family members had experienced one or more venous thrombotic events as compared with 14 (7%) of the 191 relatives without protein S deficiency (Table 1). Thrombotic episodes were associated with one or more circumstantial risk factors in 31 (48%) of the protein S-deficient patients and in 10 (71%) of the relatives without protein S deficiency (Table 2). The mean age at the first thrombotic event was 32.5 years (range, 10 to 81 years) in protein S-deficient cases and 30.5 years (range, 16 to 50 years) in those without protein S deficiency. According to a Kaplan-Meier analysis, the probability of a protein S-deficient family member to be free of venous thrombosis at 45 years of age was .50 (95% confidence interval [CI], 0.39 to 0.61), whereas the corresponding value for family members without protein S deficiency was 0.88 (95% CI, 0.83 to 0.94; Fig 1A). No differences in thrombosis-free-survival were observed between type I and type III protein S-deficient individuals or between men and women (results not shown). The incidence of thrombotic events (only first episodes included in the calculation) in protein S-deficient patients was 10.0 per 1,000 person-years up to 25 years of age (v 1.0 per 1,000 in normal

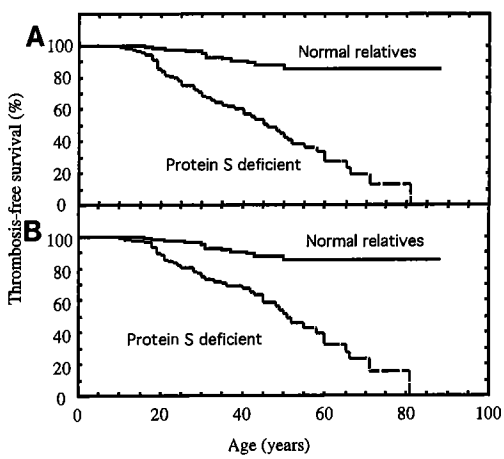


**Fig 1. Kaplan-Meier analysis of all 18 protein S-deficient families showing the probability of being free of thrombosis at a certain age. (A) 136 protein S-deficient and 191 normal members. The difference between the curves is highly significant ($P < .001$). (B) Same analysis after exclusion of the 19 probands. The difference between the survival curves is still highly significant ($P < .001$).**

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

| Potential Risk Factor | Venous Thrombosis (yes/no) in Exposed | Nonexposed | Crude OR | Hazard Ratio* (95% CI) |
|---|---|---|---|---|
| Protein S deficient | 64/72 | 14/177 | 11.2 | 6.8 (3.7-12.5)† |
| Female sex | 41/136 | 37/113 | 0.9 | 1.0 (0.4-2.5) |
| Pregnancy and childbirth‡ | 34/83 | 44/166 | 1.5 | 1.0 (0.4-2.7) |
| Smoking‡ | 41/87 | 37/162 | 2.1 | 1.2 (0.8-2.0) |
| Overweight§ | 38/63 | 40/186 | 2.8 | 2.0 (1.2-3.2)† |
| Malignancy‡ | 3/3 | 75/246 | 3.3 | 2.0 (0.6-6.4) |

* Proportional hazards model; all listed proposed risk factors entered.

† Indicates $P < .05$. Even when probands were excluded, only protein S deficiency (6.0; 95% CI, 3.3 to 10.9) and being overweight (2.0; 95% CI, 1.2 to 3.4) were significant risk factors for thrombosis.

‡ Ever had risk factor compared with never.

§ Overweight defined as body mass index >25. Body mass index = weight (kg) divided with the square of the body length (meter).

relatives), 18.2 per 1,000 person-years (*v* 5.3 per 1,000 in normal family members) between 26 and 45 years of age, and 39.2 per 1,000 person-years (*v* 0.6 per 1,000 in normal family members) between 46 and 83 years of age.

Protein S deficiency was a strong risk factor for thrombosis in these families ($P < .001$; Table 3). Malignancy, being overweight, and smoking appeared to be associated with increased risk of thrombosis as judged by the crude odds ratio. However, in the proportional hazard model, only being overweight was found to be a significant risk factor ($P < .01$). In the same model, pregnancy was not a significant risk factor of thrombosis even when only women were included in the analysis (hazard ratio, 1.5; 95% CI, 0.6 to 3.4). However, the proportional hazard model presumably underestimates pregnancy as a risk factor because the incidence of thrombosis was quite high in the years in which protein S-deficient women were pregnant (130 per 1,000 person-years). Moreover, thrombotic events occurred in 19 (13%) of 146 pregnancies in protein S-deficient women and in 6 of 163 (4%) pregnancies in women without protein S deficiency ($P < .01$). In 8 of the 19 events associated with pregnancies in the protein S-deficient individuals, thrombosis occurred in the postpartum period as compared with 4 of 6 events in women without protein S deficiency.

Nine (6.6%) of the 136 protein S-deficient family members (age 41 to 74 years) had had an arterial thrombotic event, compared with 3 (1.6%; age 44 to 64 years) among the 191 family members without protein S deficiency ($P < .05$). When only patients older than 50 years were considered, 9 (21%) of 42 protein S-deficient relatives had had arterial thrombosis, compared with 3 (6.7%) of 45 individuals without protein S deficiency ($P < .05$). Eight of the 9 protein S-deficient patients with arterial thrombosis were smokers. In this context, it is interesting that smoking in itself lowers the plasma concentration of protein S, which may be particularly harmful in individuals with protein S deficiency.[45] One of the 9 protein S-deficient members with arterial thrombosis was found to be heterozygous for the FV gene mutation.

Coumarine-induced skin necrosis had not occurred in any of the 55 protein S-deficient patients who had been treated with oral anticoagulant for longer or shorter periods. Moreover, no thrombotic events had occurred during adequate oral anticoagulation (warfarin) of protein S-deficient family members, even in the 11 individuals with combined APC resistance and protein S deficiency (see below). However, 4 patients had recurrences of venous thrombosis during temporary discontinuation of treatment.

*APC resistance, an additional risk factor of thrombosis.* DNA samples were available from 294 family members. The Arg506 to Gln mutation in the FV gene was tested for in these 294 individuals and found to be present in 40, which included 6 (38%) of the 16 investigated probands. In family members without the FV gene mutation, APC ratios were slightly, but significantly, lower in the protein S-deficient group than in the group without protein S deficiency (Fig 2). Among those with the FV gene mutation, there was no difference in APC ratios between those with and without protein S deficiency. When all 18 families (294 members) were included in analysis of phenotypic expression, a significant difference in thrombotic frequency ($P = .041$) but not in thrombosis-free survival ($P = .090$) was found between family members with only protein S deficiency and those with combined defects (Table 4A). To evaluate the influence of the FV gene mutation more stringently, only the 7 families having both defects (families 2, 6, 8, 10, 11, 12, and 18)[28a] were included in the analysis. Individuals with combined defects were found to have significantly higher incidence of thrombosis than family members with either of the two defects ($P = .001$), the differences being significant even after exclusion of probands from the analysis ($P = .034$ and $P = .028$, respectively; Table 4B). In these families, the mean age at the first thrombotic episode was 31 years for subjects having both defects (n = 13; range, 10 to 55 years), 47 years for subjects with only protein S deficiency (n = 4; range, 30 to 65 years), and 27 years for those with only the FV gene mutation (n = 4; range, 21 to 31 years). The thrombosis-free survival curve of individuals with both defects was significantly different from those of individuals with either protein S deficiency or the FV gene mutation (Fig 3). There was no significant difference between those with only the FV gene mutation and those with isolated protein S deficiency. Noteworthy, three family members were homozygous for the FV gene mutation, 2 with normal protein S being asymptomatic (ages 14 and 8 years, respectively) and 1 with protein S deficiency having had thrombosis at 10 years of age.

## DISCUSSION

Deficiency of protein S is a strong risk factor of thrombosis and 64 of the 136 protein S-deficient relatives had experienced one or more venous thrombotic events. The incidence of first thrombotic event increased with age, showing protein S deficiency to be a life-long risk factor of thrombosis. The clinical manifestations of protein S deficiency in this study were similar to those described in an earlier report, in which

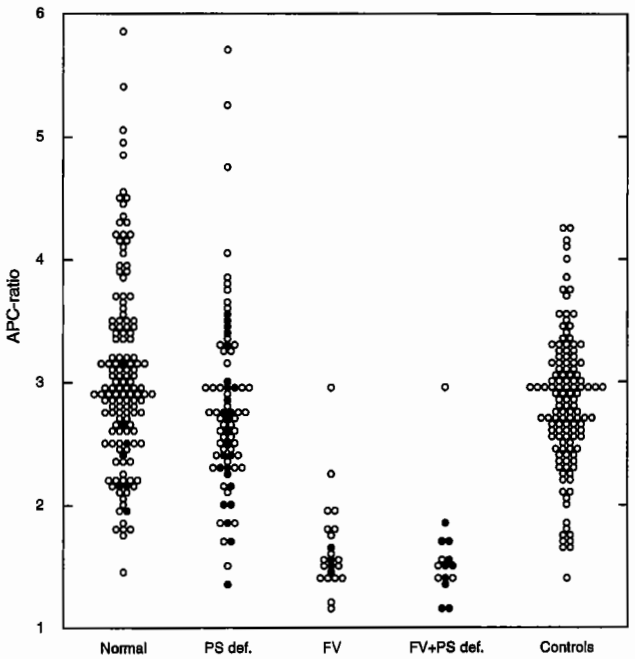


**Fig 2. APC resistance in protein S-deficient families. APC ratios in family members not having protein S deficiency or the FV gene mutation (n = 140), in protein S-deficient individuals without the FV gene mutation (n = 75), in members with normal protein S having the FV gene mutation (n = 22), in protein S-deficient members also having the FV mutation (n = 15), and in healthy controls (n = 126, 2.8 ± 0.6). Thrombotic family members are indicated with solid circles. In the absence of the FV gene mutation, APC ratios were slightly lower in those having protein S deficiency than in those without protein S deficiency (3.1 ± 0.8 *v* 2.8 ± 0.8, *P* = .013). There was no significant difference in APC ratios between protein S-deficient and nondeficient carriers of the FV gene mutation (1.6 ± 0.4 *v* 1.6 ± 0.4).**

only total protein S was measured.[11] However, in the now described families, the median age for the first thrombosis was 45 years (50 years if probands were excluded), as compared with less than 30 years in the cited study. This finding is not explained by the different detection methods for protein S deficiency (measurements of free *v* total protein S) because we found no significant difference in thrombosis-free survival between patients with so called type I and those with type III deficiency. The age at the first thrombotic event ranged between 10 and 81 years and one patient was still asymptomatic at 76 years of age. This is a wider range than found in a previous study (range, 15 to 68 years).[11]

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

| Genetic Defect | Symptoms of Thrombosis: Present | Absent |
|---|---|---|
| (A) All 18 protein S-deficient families | | |
| Protein S and FV | 13 (72) | 5 (28) |
| Protein S | 47 (46) | 55 (54) |
| FV | 4 (18) | 18 (82) |
| None | 9 (6) | 143 (94) |
| (B) 7 Families in which both protein S deficiehcy and the FV gene mutation were present. | | |
| Protein S and FV | 13 (72) | 5 (28) |
| Protein S | 4 (19) | 17 (81) |
| FV | 4 (19) | 17 (81) |
| None | 1 (2) | 43 (98) |

The presence of protein S deficiency is indicated as protein S. The presence of the FV gene mutation is indicated as FV. Values are the number of patients with percentages in parentheses.

It is becoming evident that thrombosis-prone families with protein C deficiency often have more than one genetic defect (see below). In the general population, approximately 1 in 250 carry protein C deficiency[46] and the risk of thrombosis in individuals with isolated protein C deficiency is not yet determined. In a recent report on thrombosis-prone families with protein C deficiency, the probability of being free of thrombosis was found to be 50% at 45 years of age[47] and the results of this report suggest the risk of thrombosis in thrombosis-prone families with protein S deficiency to be similar. The higher thrombosis risk in previous studies with protein S deficiency may be caused by the selection of severely affected families. Epidemiologic studies are needed to elucidate the prevalence of protein S deficiency in the general population and to determine the associated risk of thrombosis.

The expression of clinical manifestations in protein S-deficient members showed a marked interfamilial and intrafamilial heterogeneity and the thrombotic tendency in relatives without protein S deficiency was higher than expected. This finding suggests additional genetic risk factors to be present.[48] One such genetic factor is the FV gene mutation causing APC resistance, which was found in 7 of the 18 protein S-deficient families. In these families, individuals with combined protein S deficiency and APC resistance had

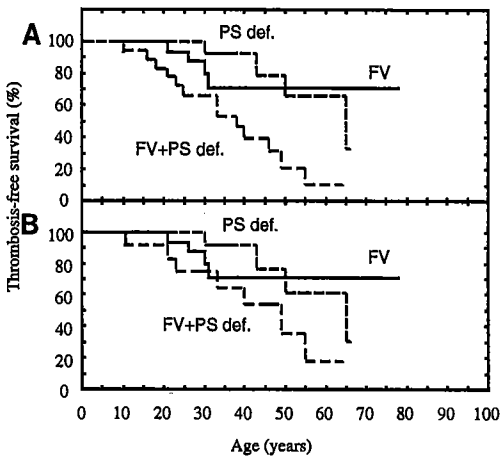


**Fig 3. The probability of being free of thrombotic events at a certain age in the 7 families having both the FV gene mutation and protein S deficiency. (A) Thrombosis-free survival curves in 21 family members with the FV gene mutation, 21 with protein S deficiency, and 18 with both defects. The difference between those having only the FV gene mutation or protein S deficiency and those with combined defects was significant ($P = .008$ and $P = .002$). There was no significant difference between those with only the FV gene mutation and those with isolated protein S deficiency ($P = .47$). (B) The same analysis after exclusion of the 7 index cases. The difference between those with isolated FV gene mutation and those with combined defects was not significant ($P = .055$), whereas the difference between those with protein S deficiency and those with combined defects was significant ($P = .032$). There was no significant difference in thrombosis-free survival between those with isolated FV gene mutation and those with isolated protein S deficiency ($P = .48$).**

a higher risk for thrombosis than those carrying either of the two defects. APC resistance was recently found to be an additional risk factor for thrombosis in Dutch families with protein C deficiency,[40] whereas no cases of APC resistance were found in Italian families with protein S, protein C, or antithrombin III deficiency.[41] This difference may be caused by higher allele frequencies of the FV Gln506 mutation in the Dutch and Swedish populations than in the Italian population.

In the families with combined defects, only 19% (4/21) of the individuals with only protein S deficiency and 19% (4/21) of those with only the FV gene mutation had had thrombosis. Thus, in these families there was no difference in severity of the thrombotic disease between individuals with only protein S deficiency and those with only FV gene mutation. In contrast, 72% (13/18) of those with combined defects had suffered from thrombosis. In this context it is noteworthy that there was no significant difference in thrombosis-free survival between relatives with isolated protein S deficiency and combined defects when all 18 families were considered. This finding would tend to suggest that many of the thrombosis-prone protein S-deficient families without the FV gene mutation have other genetic defects. This is illustrated by a high incidence of thrombosis in family no. 1[28a] in which 23 of 41 protein S-deficient and 6 of 81 nondeficient relatives had had venous thrombosis, with the age of onset of thrombosis ranging between 11 and 71 years of age. A large variability in clinical symptoms of protein S-deficient members was also observed in the other 10 families without APC resistance (3 of 39 nondeficient relatives and 22 of 47 protein S-deficient relatives having had venous thrombosis; the age range of first thrombosis in protein S-deficient cases was 13 to 66 years), which supports the idea that multiple genetic defects cause thrombophilia. The variability in expression of symptoms may also be related to the nature of the causative mutation. Thus, different mutations may be associated with different risks of thrombosis even if they have the same protein S concentration. Circumstantial risk factors also affect expression of thrombotic manifestations, as shown by the observation that 48% of first thrombotic events occurred in conjunction with circumstantial risk factors such as pregnancy, trauma, surgery, or oral contraceptives.

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

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# Evaluation of the Relationship Between Protein S and C4b-Binding Protein Isoforms in Hereditary Protein S Deficiency Demonstrating Type I and Type III Deficiencies to be Phenotypic Variants of the Same Genetic Disease

By Bengt Zöller, Pablo García de Frutos, and Björn Dahlbäck

**Type III protein S deficiency is characterized by a low plasma level of free protein S, whereas the total concentration of protein S is normal. In contrast, both free and total protein S levels are low in type I deficiency. To elucidate the molecular mechanism behind the selective deficiency of free protein S in type III deficiency, the relationship between the plasma concentrations of β-chain containing isoforms of C4b-binding protein (C4BPβ+) and different forms of protein S (free, bound, and total) was evaluated in 327 members of 18 protein S-deficient families. In normal relatives (n = 190), protein S correlated well with C4BPβ+, with free protein S (96 ± 23 nmol/L) being equal to the molar excess of protein S (355 ± 65 nmol/L) over C4BPβ+ (275 ± 47 nmol/L). In protein S-deficient family members (n = 117), the equimolar relationship between protein S (215 ± 50 nmol/L) and C4BPβ+ (228 ± 51 nmol/L), together with the high affinity of the interaction, resulted in low levels of free protein S (16 ± 10 nmol/L). Free protein S levels were distinctly low in protein S-deficient members, whereas in 47 of the protein S-deficient individuals, the concentration of total protein S was within the normal range, which fulfils the criteria for type III deficiency. The remaining 70 had low levels of both total and free protein S and, accordingly, would be type I deficient. Coexistence of type I and type III deficiency was found in 14 families, suggesting the two types of protein S deficiency to be phenotypic variants of the same genetic disease. Interestingly, not only protein S but also C4BPβ+ levels were decreased in orally anticoagulated controls and even more so in anticoagulated protein S-deficient members, suggesting that the concentration of C4BPβ+ is influenced by that of protein S. In conclusion, our results indicate that type I and type III deficiencies are phenotypic variants of the same genetic disease and that the low plasma concentrations of free protein S in both types are the result of an equimolar relationship between protein S and C4BPβ+.**



V

PROTEIN S IS A VITAMIN K-dependent plasma protein with important anticoagulant properties.[1-3] Although its mechanism of action is not fully understood, its main activity appears to be as that of a cofactor to activated protein C (APC) in the degradation of the activated forms of factors V and VIII (Va and VIIIa). The APC-cofactor activity of protein S, which is weak in purified systems, was recently found to be potentiated by factor V but not by factor Va.[4] Hereditary protein S deficiency is associated with an increased risk of thromboembolic disease.[5-17] Acquired protein S deficiency may be the result of oral anticoagulant therapy, liver disease, nephrotic syndrome, disseminated intravascular coagulation, or pregnancy.[18-24] Protein S deficiency is found in a small proportion (1.5% to 7%) of patients with thromboembolic disease.[25-30]

Protein S exists in two forms in human plasma, as free protein (30% to 40%) and as part of a bimolecular, noncovalent complex, the interacting protein being C4b-binding protein (C4BP),[31] which is a regulator of the classical complement pathway.[32] C4BP is composed of 6 or 7 α-chains and 1 or no β-chain. Different C4BP isoforms are present in plasma, but only β-chain containing C4BP (C4BPβ+) binds protein S, with the protein S binding site being located on the β chain.[33-35] The protein S-C4BP interaction is of high affinity and the equilibrium is shifted towards complex formation in vivo.[32] Because only free protein S is active as APC-cofactor,[8,31,36] it is crucially important that its plasma concentration is carefully regulated.[37] The mechanisms by which this is achieved are not fully understood. In healthy individuals, the concentration of free protein S is determined by the molar concentrations of protein S and C4BPβ+, with free protein S being equal to the molar surplus of protein S over C4BPβ+.[38] This finding holds true also during the acute-phase response, during which the α- and β-chains are differentially regulated.[39] The balance between the concentrations of C4BPβ+ and total protein S remains during the acute phase and, as a consequence, the levels of free protein S are stable. This finding suggests that the concentrations of protein S and C4BPβ+ are the only parameters that determine the concentration of free protein S.

Three different types of protein S deficiency have been described.[15] According to the nomenclature proposed by Bertina at the ISTH subcommittee meeting in 1991, type I deficiency is characterized by a parallel decrease in both total and free protein S.[6,8,9,12,15] In type II deficiency, the functional protein S activity is decreased, whereas the levels of total and free protein S are normal.[40,41] Type III deficiency is characterized by normal concentrations of total protein S but low levels of free protein S.[5,30,42-45] It was recently shown that many of the reported type II deficiency cases do not suffer from protein S gene defects and that the condition is caused by APC resistance.[46]

To elucidate the molecular mechanism of the selectively decreased levels of free protein S in type III protein S deficiency, plasma concentrations of C4BP isoforms and protein S were determined in both type I and type III protein S deficiency. The two types of protein S deficiency were found to be phenotypic variants of the same genetic disease and the low free protein S to be the result of an equimolar relationship between protein S and C4BPβ+.

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## PATIENTS AND METHODS

*Patients.* All participants gave informed consent and the study was approved by the medical ethics committee at the University of Lund. Patients were selected from the files of the Department of Coagulation Disorders, Malmö General Hospital. When the study began, 20 families with inherited deficiency of protein S were known. The diagnosis of protein S deficiency was based on free protein S antigen levels less than the normal reference range (56 to 182 nmol/L) combined with low or normal total protein S (normal range, 219 to 407 nmol/L). In patients on stable warfarin treatment, diagnosis was based on free protein S levels less than the lower range observed in an anticoagulated control group (16 to 91 nmol/L) combined with total protein S levels less than or within the range observed in anticoagulated controls (156 to 322 nmol/L). Eighteen of the 20 probands had had one or more of the following venous thrombotic events: deep venous thrombosis (n = 18), pulmonary emboli (n = 4), superficial thrombophlebitis (n = 7), and mesenteral vein thrombosis (n = 2). The mean age at the first episode was 27 years (range, 14 to 46 years). A history of arterial thrombosis was found in two probands; one had suffered a stroke at 53 years of age and the other had bilateral thrombosis of the popliteal arteries at age 56 years. One of the probands had not had thrombosis and was referred because of familial thrombophilia. One proband was deceased but his relatives volunteered to participate. The remaining probands were 7 women and 12 men. After pedigree analysis, two plus two families turned out to be related, which reduced the number of unrelated families to 18. Totally, 308 relatives were studied; the mean age of all 327 individuals was 37.5 years (range, 6 to 88 years).

The investigated subjects answered a questionnaire about their medical history that emphasized manifestations of deep venous thrombosis, pulmonary embolism, superficial thrombosis, and arterial thrombosis. Patients with thrombosis were interviewed by a physician and/or medical records were studied. The term deep venous thrombosis refers to deep leg vein thrombosis as well as to thrombosis at unusual locations such as the axillary, mesenteric, and cerebral veins. Thrombotic event refers to deep venous thrombosis, pulmonary embolism, or superficial thrombophlebitis. Only thrombotic episodes established by a physician on the basis of objective means or by physical examination were considered thrombotic events; 54 (69%) of the 78 symptomatic patients had had a thrombotic event diagnosed by an objective method. In 20 family members, protein S levels were measured during stable anticoagulant treatment only.

Of the 136 protein S-deficient patients, 64 (47%) had had one or more venous thrombotic events as compared with 14 (7%) of the normal relatives (n = 191). The clinical manifestations are presented in further detail elsewhere.[46a]

*Controls.* The healthy control group was composed of 60 volunteers. Outpatients (n = 40; mean age, 68 ± 13 years) on stable long-term oral anticoagulant treatment served as the anticoagulated control group. Seven of these individuals had had a venous thromboembolic event not related to protein S deficiency, and the remaining 33 had disorders unrelated to venous thromboembolism.

*Laboratory methods.* Blood sampling and routine coagulation methods were performed as described.[30] Protein S was measured with a radioimmunoassay as previously described.[23] The amount of free protein S was determined in 5% PEG 6000 supernatants, using a pool of normal plasmas as control.[23] The amount of bound protein S was calculated as the difference between total and free protein S.[39] Total C4BP (C4BPt) and C4BP$\beta$+ plasma levels were measured with previously described enzyme-linked immunosorbent assay (ELISA) methods[39] with the following modification: a polyclonal antibody against plasma purified C4BP was used as catching antibody (coated at a concentration of 50 $\mu$g/mL) instead of the polyclonal antiserum against C4BP tentacles.[33]

The following extinction coefficients were used: C4BPt, 14.1[47]; C4BP$\beta$+, 13.6[39]; and protein S, 9.5.[48] Proteins used as standards have been described previously.[39] The molecular weights used for molarity calculations were 75,000 for protein S and 570,000 for C4BP.

*Statistical methods.* All data were expressed as mean ± standard deviation (SD); n equals the number of samples in each group. Calculations of Pearsson's correlation coefficient ($r$), probability ($P$), and comparison by the Student's $t$-test were performed with Statview SE + Graphics software (Abacus Concepts, Inc, Berkeley, CA). $P < .05$ was considered significant.

## RESULTS

*Type I and type III deficiencies as phenotypic variants of the same genetic disease.* A total of 327 family members (177 women and 150 men) from 18 unrelated families with inherited protein S deficiency were investigated. The protein S-deficient family members were best identified from measurements of free protein S because there was a clear demarcation zone between protein S-deficient and normal members in 17 of the 18 families (Fig 1). Low levels of free protein S (<56 nmol/L) were found in 117 (49 men and 68 women) of 307 nonanticoagulated family members, with the remaining 190 (89 men and 101 women) having normal levels of free protein S (Figs 1 and 2). Decreased levels of total protein S were found in 70 of the cases with free protein S deficiency (fulfilling criteria for type I deficiency), whereas the remaining 47 patients had total protein S concentration within the reference range (fulfilling criteria for type III deficiency). If diagnosis of protein S deficiency was to be based only on the concentration of total protein S, 40% of protein S-deficient family members would have been classified as normal. In family 18, protein S-deficient members had somewhat higher mean values of both free (41 ± 7 nmol/L) and total protein S (240 ± 35 nmol/L) as compared with protein S-deficient cases of the other families.

The deficiency of free protein S was inherited as an autosomal dominant trait in all families with no exception. In contrast, total protein S was an insufficient marker of the genetic disease because several type I deficient members (low total protein S/low free protein S) had inherited the disease from a parent with type III deficiency (normal total protein S/low free protein S). Moreover, the reverse variant of type III deficient children inheriting the disease from a type I deficient parent was also observed. In 14 of the 18 families, both type I and type III deficiencies were represented (Fig 1), suggesting the two types to be phenotypic variants of the same disease.

Deficiency of free protein S was significantly associated with an increased risk for thrombosis as shown by the thrombosis-free survival curves.[46a] According to a Kaplan-Meier analysis there was no significant difference in thrombosis-free survival between type I and III deficient family members (not shown), even though 22 (31%) of the 70 type I and 23 (49%) of the 47 type III deficient patients had had venous thrombosis ($P = .06$). This difference was presumably caused by the age difference between type I and type III deficient family members (34 ± 15 $v$ 46 ± 20 years, $P < .001$).

*Equimolar concentrations of protein S and C4BP$\beta$+ in protein S deficiency resulting in low levels of free protein S.*

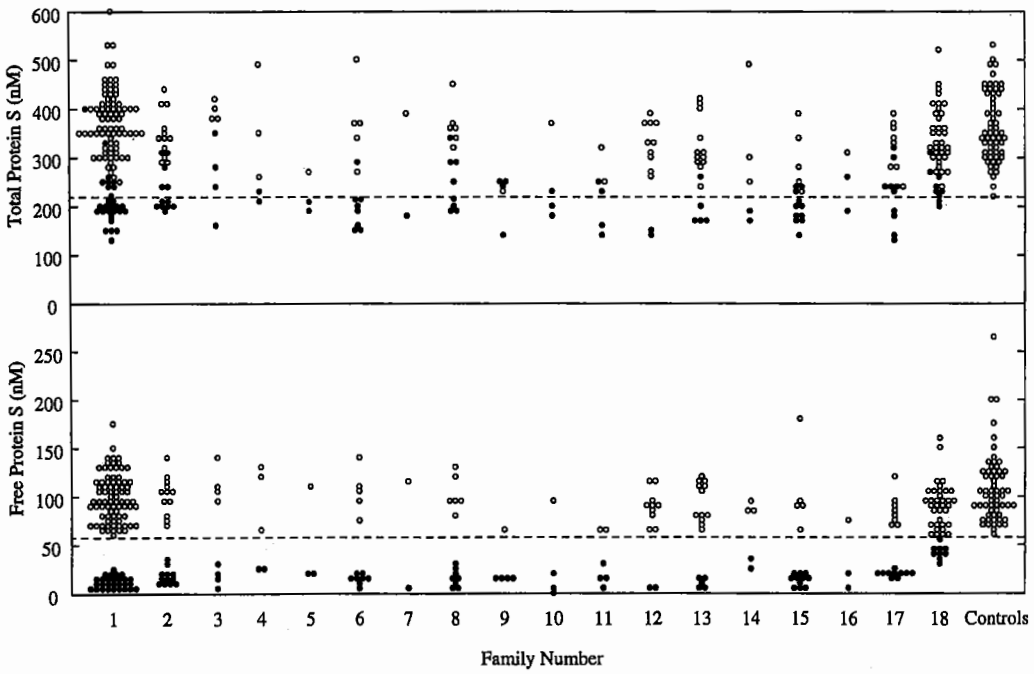


**Fig 1. Plasma levels of free and total protein S in 307 nonanticoagulated members from 18 unrelated protein S-deficient families. The lower normal reference levels for free and total protein S are marked with dotted lines (56 and 219 nmol/L, respectively). Individuals with free protein S levels less than 56 nmol/L are considered to be protein S deficient (●). In all families, the difference between protein S-deficient and normal relatives was most evident from results of measurements of free protein S. The overlap in total protein S levels between normal and protein S-deficient cases illustrates the coexistence of type I and type III deficiencies in 14 of the families.**

In normal family members as well as in controls, protein S was in molar excess over C4BP$\beta$+ (Table 1), which agrees with the concept that free protein S equals the molar surplus of protein S over C4BP$\beta$+.[38,39] In the control group, the concentrations of bound protein S and C4BP$\beta$+ were not significantly different, indicating that all protein S binding sites on C4BP$\beta$+ were occupied, whereas normal family members for unknown reasons had slightly higher C4BP$\beta$+ than bound protein S. The correlation between C4BP$\beta$+ and bound protein S was high in normal relatives and somewhat lower in protein S-deficient relatives (Table 2 and Fig 3). In this latter group, the mean total protein S concentration was 61% of normal and the molar concentrations of protein S were not significantly different from those of C4BP$\beta$+. Because most of their protein S existed in its bound state, the free protein S levels were very low (Table 1 and Fig 2). In protein S-deficient family members, the concentrations of C4BPt and C4BP$\beta$+ were 89% and 83% of the level in their normal relatives, respectively, suggesting that the C4BP concentration is influenced by the inherited protein S deficiency. In protein S-deficient members of family 18, the mean concentration of total protein S (240 ± 35 nmol/L) was slightly higher than that of C4BP$\beta$+ (221 ± 31 nmol/L), in accordance with the slightly higher levels of free protein S in this family than in the other protein S-deficient families.

*Oral anticoagulant therapy associated with low levels of protein S and C4BP$\beta$+.* The mean plasma concentrations of free and total protein S in the 36 family members that were treated with vitamin K-antagonists were considerably lower than corresponding concentrations of 40 unrelated anticoagulated outpatients (Table 1 and Fig 2). Thirty-three of the anticoagulated family members had lower levels of free protein S (range, 0 to 13 nmol/L) than the lowest level observed in anticoagulated controls (range, 16 to 91 nmol/L) and they were therefore considered to be protein S deficient. The diagnosis was confirmed during warfarin-free intervals in 14 of the family members and by pedigree analysis in another 10 patients (not shown). In one anticoagulated family member having a free protein S level equal to the lower limit of anticoagulated controls (16 nmol/L), deficiency of free and total protein S was found during a warfarin-free interval. The remaining 2 anticoagulated relatives had free protein S levels within the range of anticoagulated controls (41 and 31 nmol/L, respectively) and they were considered not to be protein S deficient. Normal protein S was found during a warfarin-free interval in one of them. The other had never had venous thrombosis and was anticoagulated because of cardiac arrhythmia. The 34 anticoagulated protein S-deficient individuals had significantly lower concentrations than nonanticoagulated protein S-deficient relatives not

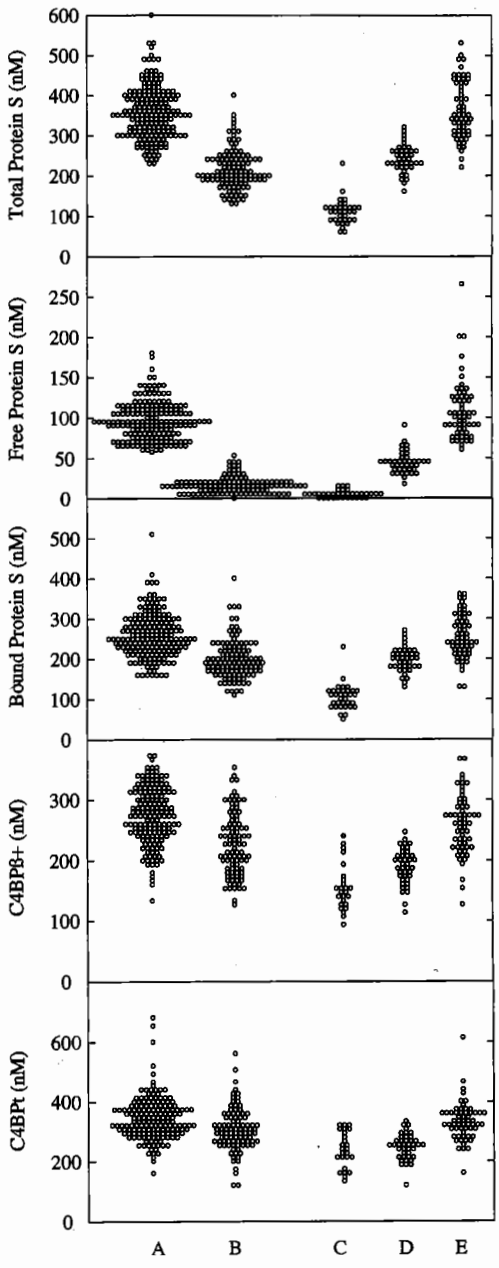


**Fig 2. Plasma levels of protein S (total, free, and bound), C4BPβ+, and C4BPt in the five studied groups: A, normal relatives (n = 190); B, nonanticoagulated protein S-deficient relatives (n = 117); C, anticoagulated protein S-deficient relatives (n = 34); D, anticoagulated control group (n = 40); and E, nonanticoagulated control group (n = 60).**

only of free and total protein S but also of C4BPβ+ (Table 1 and Fig 2).

The mean total protein S level in the anticoagulated control group was 34% lower than that of the normal control group (Table 1 and Fig 2). The concentrations of C4BPt and C4BPβ+ were also found to be decreased, in accordance with other reports in which C4BPt have been measured.[14,19,24,49] It is noteworthy that the concentrations of protein S (free, bound, and total), C4BPt, and C4BPβ+ were decreased to an even greater extent in the anticoagulated protein S-deficient patients than in the anticoagulant control group (Table 1). This difference was not caused by differences in intensity of anticoagulant therapy because no significant difference in Owren's P&P (factor II + VII + X) was observed between the anticoagulated controls and anticoagulated protein S-deficient members (22% ± 5% *v* 23% ± 14%; normal reference range, 70% to 130%; therapeutic range, 10% to 25%).

The correlation between C4BPβ+ and bound protein S, which was high in normal relatives and controls, was also strong in anticoagulated controls, but weaker in protein S-deficient relatives (Table 2 and Fig 3). In anticoagulated protein S-deficient family members there was no correlation between C4BPβ+ and bound protein S, which presumably is caused by the molar excess of C4BPβ+ over bound protein S. The correlation between free and total protein S was high in healthy relatives as well as in normal and anticoagulated controls, suggesting that the total protein S levels regulate the concentration of free protein S in normal individuals also when they are anticoagulated (Table 2 and Fig 4). The correlation between free and total protein S was weak in the protein S-deficient patients and absent in anticoagulated protein S-deficient patients.

## DISCUSSION

Type I and type III protein S deficiencies are both characterized by low plasma concentrations of free protein S, with type III being distinguished from type I in having a normal level of total protein S in plasma. We now demonstrate the two types of protein S deficiency to coexist in many families with protein S deficiency and to be inherited as a single genetic trait, suggesting that they are phenotypic variations of the same genetic disease. In normal individuals, there is a high correlation between the concentrations of protein S and C4BPβ+, with the level of free protein S being equal to the molar surplus of protein S over C4BPβ+.[38,39] From this finding it follows that essentially all C4BPβ+ molecules are occupied with protein S. In protein S deficiency, the concentration of protein S is equal to or somewhat lower than that of C4BPβ+, which results in low levels of free protein S. In the protein S-deficient families, the concentrations of total protein S in normal members overlapped with the concentrations in those with protein S deficiency. As a result, some protein S-deficient family members had total protein S within the normal range, a pattern characteristic of type III protein S deficiency. Because the concentrations of protein S and C4BPβ+ correlated, family members with type III deficiency had slightly higher concentrations not only of protein S but also of C4BPβ+ than those with type I deficiency. The high-affinity interaction between C4BPβ+

**Table 1. Mean Values of Protein S and C4BP in Normal Family Members, Nonanticoagulated and Anticoagulated Protein S-Deficient Members, Anticoagulated Controls, and Normal Controls**

| | Normal Members | Protein S-Deficient Members | Anticoagulated Protein S-Deficient | Anticoagulated Controls | Normal Controls |
|---|---|---|---|---|---|
| Protein S | | | | | |
| No. | 190 | 117 | 34 | 40 | 60 |
| Total | 355 ± 65 | 215 ± 50 a* | 109 ± 31 b* | 241 ± 34 c* | 363 ± 71 d* |
| Free | 96 ± 23 | 16 ± 10 a* | 4 ± 4 b* | 44 ± 14 c* | 108 ± 37 d* |
| Bound | 259 ± 55 | 198 ± 49 a* | 105 ± 31 b* | 197 ± 31 c* | 255 ± 55 d* |
| C4BP | | | | | |
| No. | 166 | 95 | 25 | | |
| C4BPβ+ | 275 ± 47 | 228 ± 51 a* | 154 ± 38 b* | 188 ± 30 c* | 258 ± 49 d* |
| C4BPt | 344 ± 72 | 306 ± 73 a* | 237 ± 59 b* | 248 ± 43 c | 331 ± 65 d* |

Values given as nanomoles per liter (mean ± SD).

Abbreviations: a, significance versus nonanticoagulated normal members; b, significance versus nonanticoagulated protein S-deficient members; c, significance versus anticoagulated protein S-deficient members; d, significance versus anticoagulated controls.

* $P < .001$ (analyzed with unpaired *t*-test, see abbreviations for groups compared).

and protein S drives the equilibrium towards complex formation, which results in low levels of free protein S. The higher concentrations of protein S and C4BPβ+ in type III than in type I may partly be an age-related phenomenon because individuals with type III deficiency tended to be older than those with type I. It may also be the result of an ongoing reactive process, because protein S and C4BPβ+ are both weak acute-phase reactants.[39]

The now reported results suggest that the concentration of free protein S is better than that of total protein S as a marker of protein S deficiency. Indeed, 47 patients deficient in free protein S had normal total protein S and would have

**Table 2. Correlation Between Protein S and C4BP Concentrations**

| | Protein S | | | |
|---|---|---|---|---|
| | Total | Free | Bound | C4BPβ+ |
| Normal members | | | | |
| C4BPt | 0.39* | 0.05 | 0.45* | 0.67* |
| C4BPβ+ | 0.63* | 0.19† | 0.68* | |
| Bound protein S | 0.94* | 0.30* | | |
| Free protein S | 0.61* | | | |
| Protein S-deficient members | | | | |
| C4BPt | 0.34* | 0.08 | 0.38* | 0.67* |
| C4BPβ+ | 0.46* | 0.04 | 0.49* | |
| Bound protein S | 0.98* | 0.04 | | |
| Free protein S | 0.24‡ | | | |
| Anticoagulated protein S-deficient members | | | | |
| C4BPt | 0.13 | 0.38 | 0.07 | 0.60‡ |
| C4BPβ+ | 0.07 | 0.15 | 0.10 | |
| Bound protein S | 0.99* | 0.05 | | |
| Free protein S | 0.18 | | | |
| Anticoagulated controls | | | | |
| C4BPt | 0.56* | 0.22 | 0.71* | 0.74* |
| C4BPβ+ | 0.57* | 0.12 | 0.80* | |
| Bound protein S | 0.92* | 0.02 | | |
| Free protein S | 0.42‡ | | | |
| Normal controls | | | | |
| C4BPt | 0.66* | 0.16 | 0.74* | 0.83* |
| C4BPβ+ | 0.72* | 0.12 | 0.86* | |
| Bound protein S | 0.86* | 0.16 | | |
| Free protein S | 0.64* | | | |

Relationship between the listed variables in citrated plasma was examined by calculating Pearson's correlation coefficients.

* $P < .001$.

† $P < .05$.

‡ $P < .01$.

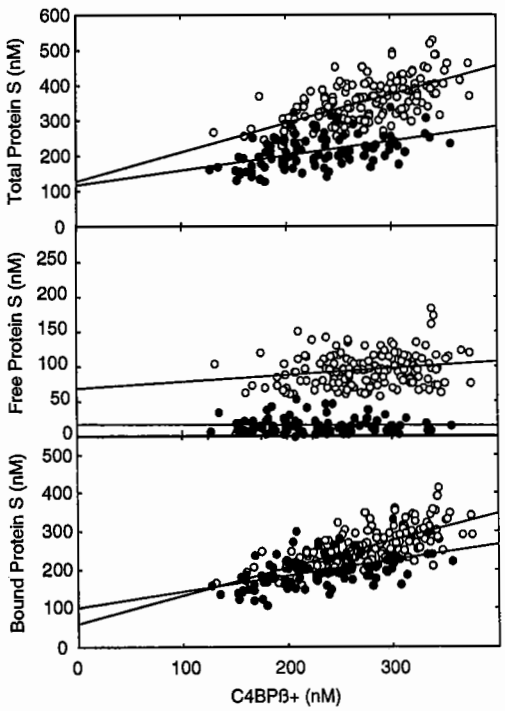


**Fig 3. Correlation between C4BPβ+ and protein S (total, free, and bound) in normal family members (○) and in nonanticoagulated protein S-deficient family members (●).**

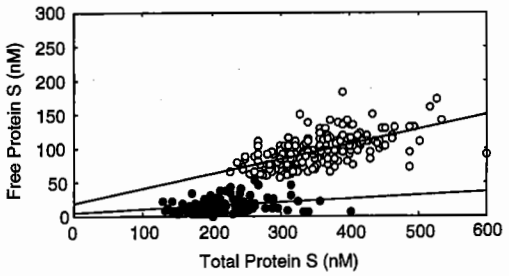


**Fig 4. Correlation between total protein S and free protein S in normal family members (○) and nonanticoagulated protein S-deficient members (●).**

been overlooked if only total protein S had been measured. In protein S-deficient family members, the mean total protein S concentration was 61% of that of normal relatives. This finding is not inconsistent with the conclusion that protein S deficiency is caused by a defect in the protein S gene. For instance, it has been shown that a proportion of patients with heterozygous deficiency of protein C have a normal plasma concentration.[50] Several genetic defects have been found and characterized in protein S-deficient patients, but so far mutations have only been found in approximately 50% of the investigated cases.[51-56] In the remaining 50%, it is still possible that the protein S deficiency is caused by a defect in the protein S gene. Thus, mutations may affect the poorly characterized 5′ end of the protein S gene.[55] Moreover, there may be gene conversion events between the protein S gene and the homologous protein S pseudogene. However, the possibility that protein S deficiency in some families is not caused by a defect in the protein S gene cannot be ruled out. For this reason, it will be important to perform genetic linkage studies in families in whom a mutation in the protein S gene is not found.

Anticoagulant therapy with vitamin K-antagonists such as warfarin inhibits the γ-carboxylation of glutamic acid residues, a process that is dependent on vitamin K.[57] Like the other vitamin K-dependent plasma proteins, the concentration of protein S decreases during warfarin therapy.[7,19,24,49] C4BP is not a vitamin K-dependent protein, but we find its plasma concentration to decrease during warfarin therapy, an observation that is in agreement with previous reports in which the concentration of C4BP in plasma of patients under oral anticoagulation was found to be 86% to 94% of normal.[19,24,49] The somewhat larger decrease now observed in both C4BPt and C4BPβ+ (approximately 25% reduction in plasma level) may be explained by differences in analytical methods, selection criteria, or intensity of anticoagulant treatment. The high correlation between protein S and C4BPβ+ in normal patients and in anticoagulated controls shows the plasma concentrations of these two proteins to be closely regulated. The decrease in plasma concentrations of C4BPβ+ during warfarin therapy, in both controls and in protein S deficiency, suggests that the C4BP concentration is affected by that of protein S rather than the reverse (Table 1 and Fig 2). However, a direct effect of warfarin on the synthesis of C4BP cannot be ruled out. The hypothesis that protein S partly regulates the C4BPβ+ concentration is in accordance with the maintained balance between protein S and C4BPβ+ during the acute-phase reaction.[39]

In conclusion, the present results show type I and type III protein S deficiency to be phenotypic variants of one genetic disease and support the concept that the concentration of free protein S is determined by the molar balance between C4BPβ+ and total protein S.[38,39] In protein S deficiency, the low level of free protein S is the result of an equimolar relationship between C4BPβ+ and protein S, which is the consequence of a reduced concentration of total protein S. These findings stress the importance of measuring free protein S in the elucidation of hereditary protein S deficiency and demonstrate that there are only two types of protein S deficiency, type I being characterized by a reduced protein S level and type II by a functional protein S defect.

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# Elevated levels of prothrombin activation fragment 1+2 in plasma from patients with heterozygous $Arg^{506}$ to Gln mutation in the factor V gene (APC-resistance) and/or inherited protein S deficiency.

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VI

**Summary**

Inherited resistance to activated protein C (APC-resistance), caused by a point mutation in the factor V gene leading to replacement of $Arg(R)^{506}$ with a Gln (Q), and inherited protein S deficiency are associated with functional impairment of the protein C anticoagulant system, yielding lifelong hypercoagulability and increased risk of thrombosis. APC-resistance is often an additional genetic risk factor in thrombosis-prone protein S deficient families. The plasma concentration of prothrombin fragment 1+2 ($F_{1+2}$), which is a marker of hypercoagulable states, was measured in 205 members of 34 thrombosis-prone families harbouring the $Arg^{506}$ to Gln mutation (APC-resistance) and/or inherited protein S deficiency. The plasma concentration of $F_{1+2}$ was significantly higher both in 38 individuals carrying the $FV:Q^{506}$ mutation in heterozygous state (1.7±0.7 nmol/L; mean±SD) and in 48 protein S deficient cases (1.9±0.9 nmol/L), than in 100 unaffected relatives (1.3±0.5 nmol/L). Warfarin therapy decreased the $F_{1+2}$ levels, even in those four patients who had combined defects (0.5±0.3 nmol/L). Our results agree with the hypothesis that individuals with APC-resistance or protein S deficiency have an imbalance between pro- and anti-coagulant forces leading to increased thrombin generation and a hypercoagulable state.

**Introduction**

Blood coagulation is regulated by the protein C anticoagulant system (reviewed in 1). Protein C, which is a zymogen to a serine protease, is activated by thrombin bound to the endothelial cell membrane protein thrombomodulin. Activated protein C (APC) inhibits coagulation by degrading the activated forms of coagulation cofactors VIII and V (VIIIa and Va), whereas the intact procofactors are poor substrates for APC (1). The anticoagulant function of APC is stimulated by two cofactors, protein S and the intact form of factor V (1,2). The physiological importance of the protein C anticoagulant system is illustrated by the massive thrombo-embolic disease which affects individuals with homozygous protein C deficiency already in their neonatal life (3). Heterozygous deficiencies of protein C or protein S are associated with increased risk for thrombosis in adult life (1,4-7). Resistance to activated protein C (APC-resistance) is the most recently discovered defect in the protein C anticoagulant system (8). It is the most frequent inherited cause of thrombophilia yet described and it is associated with a lifelong 5-10-fold increased risk for thrombosis (9-15). In a majority of patients, APC-resistance is due to a single point mutation (a G to A transition at nucleotide position 1691) in the factor V gene, predicting replacement of $Arg(R)^{506}$ with a Gln (Q) (16-20). $Arg^{506}$ constitutes one of three APC-cleavage sites in the heavy chain of FVa and mutated FVa ($FVa:Q^{506}$) is more slowly degraded by APC than normal FVa ($FVa:R^{506}$) (16-26). This leads to stabilization of the prothrombin complex, a higher than normal rate of thrombin generation, and a

hypercoagulable state.

The prothrombin fragment 1+2 ($F_{1+2}$), which constitutes the N-terminal part of prothrombin, is formed upon activation of prothrombin by factor Xa (27) (reviewed in 28,29). The concentration of $F_{1+2}$ in plasma reflects the degree of activation of the common coagulation pathway. Several studies have found elevated levels of $F_{1+2}$ in clinical conditions which are associated with hypercoagulable states, i.e. in elderly individuals, in young asymptomatic individuals with inherited thrombophilia (deficiencies of protein S and protein C) and in patients with stroke, unstable angina pectoris and myocardial infarction (28-36), while patients with stable angina had normal $F_{1+2}$ levels (35). In contrast, the elevated levels of $F_{1+2}$ initially reported in patients with antithrombin III deficiency were in a subsequent study demonstrated to be caused by influence of anticoagulants used for blood collection (36,37). In two other studies no or only a small increase in $F_{1+2}$ levels was observed in antithrombin III deficient patients (32,38). Oral anticoagulant therapy has been reported to suppress the degree of activation of the haemostatic system, as monitored by decreased levels of $F_{1+2}$, even in patients with antithrombin III and protein C deficiency (39,40). High levels of $F_{1+2}$ have been reported in a few APC-resistant individuals, who all belonged to the same family (41). In contrast, $F_{1+2}$ was equally increased in stroke patients with APC-resistance (n=6) and in patients not harbouring the mutation (n=185) (33). Another group stated in an abstract that approximately one third of a mixed group of patients with APC-resistance (FV:$Q^{506}$ carriers), protein S, protein C or antithrombin III deficiency had increased levels of $F_{1+2}$ (42). To our knowledge, plasma levels of $F_{1+2}$ in a larger number of APC-resistant individuals or in warfarin treated protein S deficient patients have not been reported.

We have now measured the concentrations of $F_{1+2}$ in plasma from patients with heterozygosity for the FV:$Q^{506}$ mutation to investigate whether these individuals have a hypercoagulable state due to increased thrombin generation. APC-resistance is often an additional genetic risk factor in thrombosis-prone protein S deficient families (6,19,20,43). In addition, we therefore have measured the plasma levels of $F_{1+2}$ in relatives with inherited protein S deficiency, both those who were untreated and those who received warfarin. The results suggest individuals with inherited APC-resistance, like those with protein S deficiency, to suffer from an imbalance between pro- and anti-coagulant forces with an associated higher than normal rate of thrombin generation.

**Patients and Methods.**

*Patients.* The study population, selected from the files at the Department for Coagulation Disorders, at the University Hospital in Malmö, comprised 205 individuals (115 females and 90 males, mean age 40±19 years, range 6-87 years) from 34 previously described thrombosis-prone families with inherited protein S deficiency and/or APC resistance

(6,7,9,20). The project was approved by the ethics committee at Lund University, and written informed consent was obtained from all individuals. The family members were grouped into the following categories (table 1): 48 non-anticoagulated individuals with inherited protein S deficiency, 38 non-anticoagulated individuals with heterozygosity for the FV:$Q^{506}$ allele, 6 non-anticoagulated patients with a combination of the two defects, 13 warfarin treated patients with deficiency of protein S, 4 of which also had the FV:$Q^{506}$ allele and finally, 100 normal family members.

*Methods.* Blood was collected in 1/10 volume of 0.12M sodium-citrate (Vacutainer). Samples were centrifuged at 2000 x g for 20 minutes to obtain platelet-poor plasma. Plasma was frozen and stored at -70°C until analysis. Free and total protein S concentrations were determined with a RIA described previously (44). The plasma level of free protein S was determined after precipitation of plasma with 5% PEG 6000 (44). The diagnosis of protein S deficiency was based on free protein S below the normal reference range (56 to 182 nmol/L) combined with either low or normal total protein S (normal range, 219 to 407 nmol/L), as previously described (7). In patients on stable warfarin treatment, diagnosis was based on free protein S antigen level below the lower range observed in anticoagulated controls (16 to 91 nmol/L) in combination with low or normal total protein S (range in anticoagulated controls was 156 to 322 nmol/L), as described (7). Preparation of genomic DNA and determination of the FV genotype; FV:$Q^{506}$ or FV:$Q^{506}$ allele, was performed as described (19,20). The plasma concentration of $F_{1+2}$ was analysed with an ELISA (Enzygnost® $F_{1+2}$; Behring), as described by the manufacturer.

*Statistical Methods.* All data were expressed as mean±standard deviation (SD). N is the number of samples in each group. Calculations of mean, standard deviation, Pearsson's correlation coefficient (r), probability (*P*) and comparison by Mann-Whitney ($F_{1+2}$) were performed with Statview® SE+Graphics$^{TM}$ software (Abacus Concepts Inc, Berkeley CA). A $P < 0.05$ was considered significant.

**Results**

The main demographic and diagnostic features of the 205 members of the 34 thrombosis-prone families harbouring the $Arg^{506}$ to Gln mutation in the factor V gene (APC-resistance) and/or inherited protein S deficiency are presented in table I. Among protein S deficient patients, all had plasma levels of free protein S below the lower normal range, whereas the concentrations of total protein S were either subnormal or within the lower normal range. This is in accordance with a recent report demonstrating protein S deficiency of type I (low free and low total protein S) and type III (low free but normal total protein S) to be phenotypic variants of the same genetic disease (7). Only individuals

shown to be heterozygous for the FV:$Q^{506}$ allele were considered to be APC-resistant.

Individuals with either APC-resistance or protein S deficiency had significantly higher mean levels of $F_{1+2}$ than their biochemically unaffected relatives (Table II and Fig.1). This was the case even when only asymptomatic individuals were included in the calculations. $F_{1+2}$ is known to increase with age (30), but the age distribution was similar among biochemically unaffected relatives and cases with either APC-resistance or protein S deficiency (Table 1). Moreover, there were no age differences between asymptomatic normal relatives (mean age 37 years [range 6-86]), asymptomatic APC-resistant relatives (mean age 38 years [range 7-74]) and asymptomatic protein S deficient relatives (mean age 32 years [range 7-66]), which could explain the results. Among protein S deficient family members, the concentrations of $F_{1+2}$ were higher in those with a thrombotic history than in those without thrombosis. However, this may be related to the higher age among thrombotic protein S deficient patients as compared to those without symptoms (mean age 51 years [range 28-82] vs. 32 years [range 7-66], p=0.003). Thrombotic relatives without any detectable genetic defect, as compared to asymptomatic normal relatives, tended to have higher mean level of $F_{1+2}$, though the number of thrombotic patients without APC-resistance/protein S deficiency was rather low (n=7). Moreover, the 7 thrombotic relatives tended to be older than the 93 asymptomatic relatives without any genetic defects (mean age 56 years [range 42-67] vs. mean age 37 years [range 6-86], p=0.006). In contrast, corresponding difference in $F_{1+2}$ levels between symptomatic and asymptomatic individuals with APC-resistance was not observed, though thrombotic APC-resistant individuals tended to be older than asymptomatic carriers of the FV:$Q^{506}$ allele (mean age 52 years [range21-87] vs. mean age 38 years [range 7-74], p=0.08).

No significant difference in mean $F_{1+2}$ levels between APC-resistant and protein S deficient patients was observed. The six family members who carried a combination of the two defects did not have higher plasma levels of $F_{1+2}$ than those with either of the two defects, but the number of family members with both defects was too small to allow firm conclusions to be drawn .

There was a large overlap in $F_{1+2}$ levels between the different groups (Fig.1) and the $F_{1+2}$ analysis is obviously not a useful parameter to distinguish the presence or absence of a prothrombotic genetic defect such as protein S deficiency or APC-resistance in an individual. Neither were $F_{1+2}$ levels useful to distinguish between asymptomatic and thrombotic patients with protein S and/or APC-resistance.

In warfarin treated patients, the levels of $F_{1+2}$ were decreased below the levels observed in family members without any known prothrombotic defect. Very low levels of $F_{1+2}$ were even observed in the 4 anticoagulated patients with combined genetic defects. Only in one individual with isolated protein S deficiency was the level of $F_{1+2}$ found not to be markedly reduced during anticoagulant treatment.

**Discussion**

Inherited defects affecting the function of the protein C natural anticoagulant pathways, such as deficiency of protein C and protein S, are associated with increased thrombin generation and a life-long increased risk of thrombosis (31,32). The hypercoagulable state associated with these genetic defects is reflected by elevated plasma levels of markers for activation of coagulation such as the prothrombin activation fragment $F_{1+2}$. The continuous increase in the levels of $F_{1+2}$ in individuals with congenital anticoagulant pathway defects suggests both the pro- and the anti-coagulant pathways to be activated during normal physiological situations and inherited defects to be associated with a procoagulant imbalance. The increased levels of $F_{1+2}$ in APC-resistant individuals, as compared to their normal relatives, demonstrate carrier ship of the FV:$Q^{506}$ allele to be associated with a hypercoagulable state. The increased levels of $F_{1+2}$ in individuals with APC-resistance and/or protein S deficiency agree with the clinically observed hypercoagulable state, as reflected by an increased incidence of thrombosis, in these genetically affected individuals (6,7,20,45,46).

During APC-mediated degradation of FVa, the initial proteolytic event occurs at $Arg^{506}$ (22). This cleavage facilitates subsequent proteolysis at $Arg^{306}$, which is associated with loss of FVa procoagulant activity. Compared to FVa:$R^{506}$, the rate of APC-mediated degradation of FVa:$Q^{506}$ is approximately 10-fold lower. The partial APC-resistance of mutated FVa leads to stabilization of the prothrombinase complex and concomitantly to higher rates of thrombin generation. This is the probable molecular mechanism which leads to the increased $F_{1+2}$ levels in APC-resistant individuals (23-25).

Protein S deficient patients with a history of thrombosis had significantly higher plasma levels of $F_{1+2}$ than those protein S deficient individuals who had not had thrombosis. A similar difference was also observed between asymptomatic and symptomatic individuals who had no identifiable congenital defect. As $F_{1+2}$ levels have been reported to increase with age (30), these observations may be related to the higher age among thrombotic patients as compared to those without thrombosis.

Warfarin treatment suppresses the hypercoagulable states affecting patients with APC-resistance, protein S deficiency, or a combination of the two, as reflected by a drop in the levels of $F_{1+2}$. This suggests warfarin therapy to be an efficient means to control the hypercoagulability which is associated with genetic defects of the protein C anticoagulant pathway. In this study, patients received full-dose warfarin therapy and it remains to be elucidated whether low dose warfarin is sufficient to normalize the hypercoagulability. That low-dose warfarin is potentially useful as therapy was suggested by the recent demonstration of normalization of $F_{1+2}$ levels without concomitant reduction in the plasma concentrations of protein C or protein S in patients with angina pectoris (39).

In conclusion, the presented data demonstrate the existence of a procoagulant

imbalance with increased levels of $F_{1+2}$ in individuals with either APC-resistance or protein S deficiency and that warfarin treatment is associated with normalization of this imbalance.

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**Table 1 Main features of the 205 individuals from 34 families with inherited APC-resistance and/or protein S deficiency.**

| Population | Number of Individuals | Number of families | Age (years) (mean±SD, range) | Male/female | History of thrombosis no/yes |
|---|---|---|---|---|---|
| Normal relatives | 100 | 19 | 38±19, 6-86 | 46/54 | 93/7 |
| FV:$Q^{506}$ heterozygosity | 38 | 15 | 42±21, 7-87 | 15/23 | 29/9 |
| Protein S deficiency | 48 | 15 | 38±19, 7-82 | 18/30 | 33/15 |
| Protein S deficiency + FV:$Q^{506}$ heterozygosity | 6 | 5 | 46±19, 23-74 | 4/2 | 3/3 |
| Anticoagulated Protein S deficiency and/or FV:$Q^{506}$* heterozygosity | 13 | 6 | 50±15, 21-69 | 7/6 | 0/13 |

**Table 2 Plasma concentrations of prothrombin fragment 1+2 ($F_{1+2}$) in the different patient groups and in normal relatives.**

| Patient group | All individuals (No. of subjects) | Asymptomatic (No. of subjects) | Thrombotic (No. of subjects) |
|---|---|---|---|
| Normal relatives | 1.3±0.5 (n = 100) | 1.3±0.5 (n = 93) | 1.8±0.5 c** (n = 7) |
| FV:$Q^{506}$ heterozygosity | 1.7±0.7 a*** (n = 38) | 1.7±0.7 b*** (n = 29) | 1.8±0.6 $c^{NS}$ (n = 9) |
| Protein S deficiency | 1.9±0.9 a*** (n = 48) | 1.7±0.7 b*** (n = 33) | 2.4±0.9 c** (n = 15) |
| Combined Protein S deficiency and FV:$Q^{506}$ heterozygosity | 1.9±0.3 a** (n = 6) | 1.8±0.2 b* (n = 3) | 2.0±0.5 $c^{NS}$ (n = 3) |
| Anticoagulated protein S deficiency and/or FV:$Q^{506}$ heterozygosity | 0.5±0.3 a*** (n = 13) | ------- (n = 0) | 0.5+0.3 (n = 13) |

Values of $F_{1+2}$ given as nanomoles per liter (mean ± SD). Abbreviations: a, significance vs. normal relatives in column one (n=100); b, significance vs. asymptomatic normal relatives (n=93); c, significance vs. asymptomatic relatives in the same row; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and NS > 0.05 (analyzed with Mann-Whitney U test).

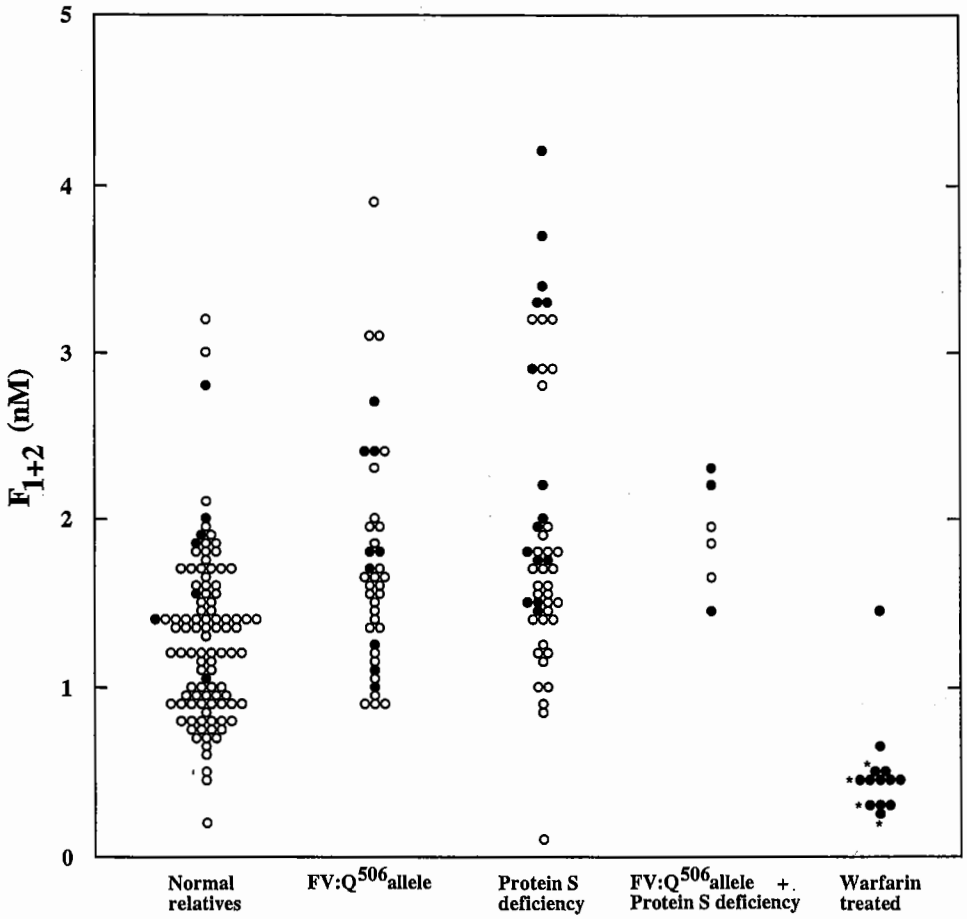


**Figure 1.** Hypercoagulable state in APC-resistance. The prothrombin activation fragment $F_{1+2}$ was measured using a commercial ELIZA (Enzygnost® $F_{1+2}$; Behring) in 205 individuals from 34 thrombosis-prone families with inherited protein S deficiency and/or APC-resistance (100 normal relatives, 38 individuals with heterozygosity for the FV:$Q^{506}$ allele, 48 protein S deficient, 6 with combined defects and 13 anticogulated protein S deficient patients out of which four also had the FV:$Q^{506}$ allele). Patients having thrombotic histories are marked with filled symbols. The four patients with combined defects in the anticoagulant group are indicated with asterisks.