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Inherited Thrombophilia*: Part 1

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Introduction

Although familial clustering of venous thromboembolic events was observed at the start of this century, it was not until the late nineteen-seventies before our insight into the organization of the haemostatic and fibrinolytic systems was sufficiently complete to enable a start to be made on a systematic search for genetic defects associated with familial thrombosis The discovery that heterozygosity of antithrombin deficiency co-segregated with the thrombotic tendency in affected families seemed to make a strong case for the association between single gene defects and thrombosis The results of these systematic studies had been in part very fruitful and could be considered in the context of two principle endogenous anticoagulant pathways (Fig 1), the antithrombin-heparan sulphate pathway and the protein C/protein S (PC/PS) pathway However only three single gene disorders had been identified that were associated with a significant increase in the risk for venous thrombembolism in families identified through a symptomatic deficient patient, with one of antithrombin, PC or PS deficiency

The awareness that in 85% of families predisposed to thrombosis no explanation could be found for the clustering of this disorder stimulated the search for alternative approaches A major breakthrough in the study of familial thrombosis has been achieved during the past two years Firstly, the concept and investigation of activated protein C resistance (APC-R) was introduced and, secondly, a mutation in the factor V gene (1691 G → A in exon 10, leading to 506Arg to Gln) was identified as the molecular basis for the phenotype of APC-R in the large majority of affected individuals (2, 3) This mutation, which is associated with a significant increase in thrombotic risk (3-5), has been found in about 50% of selected families with thrombophilia and in 20% of consecutive patients with thrombosis A consequence of this advance has been a conceptual change in how thrombophilia is viewed, which has implications for diagnosis and treatment of the disorder This review [published in two parts, see also (6)] attempts to summarise recent progress and to present recommendations for diagnosis, treatment and research in developed and developing countries

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Pathogenesis of Thrombophilia and Definition of Inherited Thrombophilia

Thrombophilia is a *tendency* to thrombosis The predisposing defects do not necessarily cause continuous clinical impairment they need only weaken the ability to cope with fluctuations induced by interactions with the environment Clinicians usually apply the term thrombophilia only to a subset of patients with atypical thrombosis Frequently cited features include (1) early age of onset (2) frequent recurrence, (3) strong family history (4) unusual, migratory or widespread locations, and (5) severity out of proportion to any recognized stimulus There are patients (see below) with fulminant thrombophilia who, without therapy, thrombose continuously But in most patients thrombosis is episodic, separated by often prolonged asymptomatic periods The discontinuity suggests that there is some trigger for each event, perhaps a direct stimulus a temporary deterioration of intrinsic resistance, or some combination of these factors

The term inherited thrombophilia acknowledges the presence of an inherited factor that by itself predisposes towards thrombosis but due to the episodic nature of thrombosis, requires interaction with other components (inherited or acquired) before onset of the clinical disorder, see Fig 2 Undoubtedly the concept of inherited thrombophilia is an operational one, the definition of which has undergone continuous refinement It was originally based upon early presentation of thrombosis, usually coupled with inherited phenotypic abnormality of one of the inhibitory proteins, antithrombin, PC or PS Progress in the molecular basis of thrombosis has enabled a more genetically based definition to be formulated *Inherited thrombophilia is a genetically determined tendency to venous thromboembolism Dominant abnormalities or combinations of less severe defects may be clinically apparent from early age of onset frequent recurrence or family history Milder traits may be discovered only by laboratory investigation All genetic influences and their interaction are not yet understood*

The identified and potential genetic factors predisposing for thrombophilia discussed fully below are summarised in Table 1 In its footnote are listed some of the many potential interacting acquired risk factors

Molecular Genetic Basis of Inherited Thrombophilia

In what follows there will be a summary of the available information on the nature and heterogeneity of the molecular defects associated with the established genetic risk factors for venous thrombosis antithrombin deficiency, PC deficiency PS deficiency and factor V gene mutation In the subsequent paragraphs those genetic defects will be

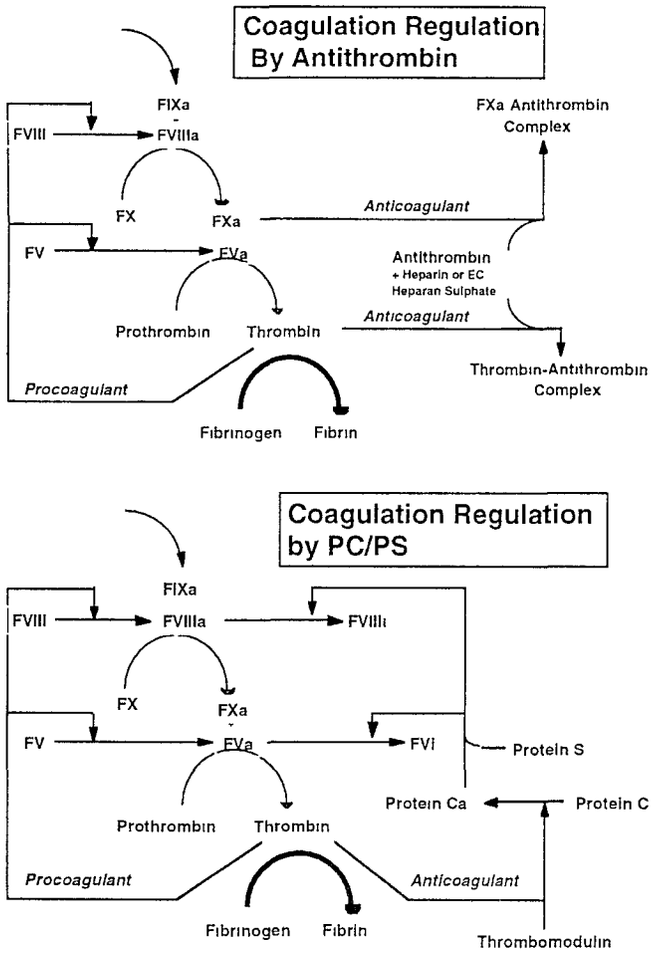

Fig 1 Representation of the two principle anticoagulant pathways known to be important in the regulation of coagulation proteinase activity To the left of each diagram is a simplified view of the coagulation cascade illustrating the positive 'procoagulant' feedback loops by which thrombin activates factors V and VIII To the right are the 'anticoagulant" pathways by which excessive activation of coagulation is prevented These pathways involve antithrombin (which directly inhibits the coagulation proteinases such as factor Xa and thrombin), and PC/PS (which inactivate factor Va and factor VIIIa) PS normally forms a complex with C4bBP and it is only the free form of PS that acts as a cofactor for PC

reviewed for which the available studies still do not permit definitive statements to be made on their association with a thrombotic risk

Antithrombin Deficiency

Antithrombin is a single chain plasma glycoprotein (58 kDa) which belongs to the superfamily of the Serine Protease Inhibitors (serpins) It is synthesized in the liver and its concentration in plasma is 2 5 μM Antithrombin is the primary inhibitor of thrombin and also inhibits most of the other activated serine proteinases involved in blood coagulation (factor Xa, factor IXa, factor XIa, factor XIIa, kallikrein) It is therefore one of the most important physiological regulators of fibrin formation

Inactivation of proteinases by antithrombin occurs via the formation of an irreversible 1 1 molar complex, in which Arg393 forms a stabilised bond with the active site of the proteinase The stable bond forms as the proteinase attempts to cleave the inhibitor Arg 393-Ser 394 bond (this bond is at the reactive centre of antithrombin and is

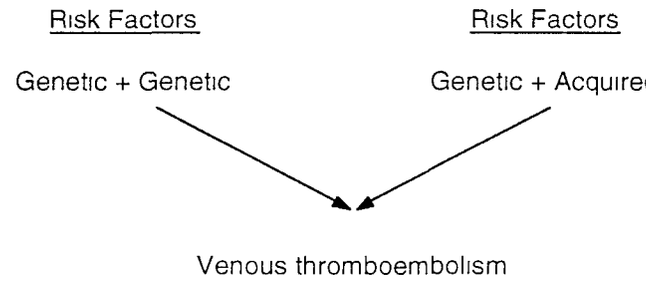

Fig 2 Venous thrombosis can be caused by interacting genetic and acquired risk factors

commonly referred to as the P1-P1' bond) Inhibition of most of the blood coagulation proteinases is relatively slow, but can be accelerated at least 1000-fold by the binding of heparin (and heparin-like com pounds, such as endothelial cell heparin sulphate) to antithrombin The interaction between heparin and heparin-binding domains in anti-thrombin results in a conformational change of the molecule which facilitates its interaction with the proteinase Inactive antithrombin serine proteinases complexes are rapidly cleared from the circulation More information on the structure, biochemistry and mechanism of action of antithrombin can be found in a number of recent reviews (7-9)

Human antithrombin cDNA clones have been isolated and se quenced (10 12) The gene coding for antithrombin is localized on chromosome 1 between 1q23 and 1q25 (13), it is 13 480 bp long and contains seven exons (1, 2 3A, 3B 4, 5, 6) (14), its nucleotide sequence has been recently completed (15) Several sequence variations or poly-morphisms have been described within the human gene [for reviews see (7-9)], including a highly polymorphic trinucleotide repeat sequence in intron 4 The latter, particularly, seems useful for haplotype analysis in the study of recurrent mutations or linkage analysis (16)

Antithrombin deficiency is a heterogenous disorder The subclassifi-cation of antithrombin deficiency was originally based mainly on the results of functional and immunologic assays in plasma Later after more information had become available on the actual mutations in the antithrombin gene, the nomenclature was modified (17, 18) Presently we recognize type I antithrombin deficiency (identified by a concordant

Table 1 Possible causes of inherited thrombophilia

(I) Inherited		
Antithrombin deficiency	PC deficiency	PS deficiency
APC R/factor V 506Arg to Gln	Dysfibrinogenaemia	Thrombomodulin
(ii) Acquired/Inherited (precise relative contribution as yet uncertain)		
Hyperhomocysteinemia	Elevated factor VIII	Elevated fibrinogen?
(iii) Potentially Inherited (but firm evidence lacking)		
Plasminogen deficiency	Heparin cofactor II deficiency	Elevated histidine rich glycoprotein
Plasminogen activator deficiency?	Elevated plasminogen activator inhibitor?	

As discussed in the text there is yet no firm evidence that conditions listed un der (iii) are linked to inherited thrombophilia The development of thrombosis is thought often to be caused by interaction between genetic and acquired factors the best recognised of the latter being advancing age immobilisation major surgery orthopaedic surgery and neurosurgery pregnancy puerperium use of oestrogen-containing hormones malignancies and the antiphospholipid syndrome

reduction of both functional and immunological antithrombin) and type II antithrombin deficiency [also identified by a variant antithrombin molecule, which has a defect in the Reactive Site (II RS), a defect affecting the Heparin Binding Site (II HBS) or multiple functional defects (Pleiotropic Effect) (II PE)] From a clinical point of view antithrombin deficiency is heterogenous, see below with mutations causing type II HBS deficiency being of much less risk than those causing the other subtypes (18, 19)

In 1991 the first database of antithrombin gene mutations was published (20) A revision of this database became available in 1993 as a report of the Thrombin and its Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (SSC ISTH) (18) In the 1993 database 39 distinct mutations and nine whole or partial gene deletions (>30 bp) are listed that have been found in type I antithrombin deficiency These mutations will introduce a frameshift (plus premature termination codon), a direct termination codon, a change in mRNA processing or unstable translation products In the type II deficiencies 11 different mutations have been reported in the type II RS group, 11 in the type II HBS group and 9 in the type II PE group Most of the mutations found in type II RS patients concern the reactive site of antithrombin (P12, P10, P2, P1 and P1') Type II HBS mutations are located mainly on the A, C and D α helices, sites that have been proposed to be involved in heparin binding (21) Mutations producing pleiotropic effects (type II PE) interestingly all are located in strand 1C close to the C-terminal end of the protein, this region has been shown to be essential both for the stability of the protein and for the transmission of conformational changes that endow the protein with its antithrombin activity (both in the presence and absence of heparin) (22) Since the publication of the first revision of the mutation database several reports on novel mutations in the antithrombin gene have been published (23-27)

Recurrent mutations are found in all types of antithrombin deficiency, but especially in the type II HBS From the 21 distinct repeat mutations 9 involved a CpG dinucleotide (hotspot for mutation) Only preliminary information is available (16) that addresses the issue of whether these recurrent mutations are the result of independent mutations or of a founder effect (identity by descent)

PC Deficiency

PC is a vitamin K dependent plasma glycoprotein which is the precursor of the serine proteinase Activated Protein C (APC) PC is synthesized in the liver as a single chain molecule (62 kDa) Single chain PC is converted into a two chain molecule by removal of a dipeptide (Arg157 - Thr158) probably in the Golgi In plasma most of the PC is in the two chain form (41 kDa heavy chain and 21 kDa light chain), the concentration of PC in plasma is 65 nM and is reduced during treatment with oral anticoagulants

PC is a multimodular protein the amino terminal light chain contains a γ-carboxyglutamic acid rich domain (Gla-domain) and two Epidermal Growth Factor like domains (EGF domains) These domains have been found to be involved in the formation of Ca^{2+}-induced conformational changes, binding to phospholipid surfaces, the activation of the molecule and its interaction with other proteins (such as its cofactor PS), while the carboxyterminal heavy chain contains the serine proteinase moiety of the molecule

During coagulation PC can be activated by thrombin via cleavage of the Arg169-Leu170 bond This reaction (which is normally very slow) can be greatly accelerated by the binding of thrombin to thrombomodulin, a transmembrane protein receptor present on the membrane of endothelial cells The APC, thus formed, inactivates the cofactors, factor Va and VIIIa, by selective proteolytic cleavages To do this efficiently APC needs to form a complex with PS on a suitable membrane surface Apart from these anticoagulant properties, APC also has antifibrinolytic properties and anti-inflammatory effects More detailed information on structural, biochemical and functional aspects of PC can be found in recent review articles (28-30)

Human cDNA clones have been isolated and sequenced (31, 32) Also the structure of the gene (PROC) has been resolved (33, 34) it contains 9 exons and 8 intrions on 11 kb of genomic DNA The gene transcript is 1795 bp It contains a 5' untranslated region of 74 bp a protein coding region (exons 2-9) and a 3' untranslated region of 294 bp The gene has been mapped to the chromosome 2q13-q14 region (35)

A number of DNA sequence polymorphisms are known to occur in the PC gene, both in the promotor region and in the coding region (36, 37) None of these variations leads to an amino acid dimorphism All these polymorphisms have been formatted for detection by PCR This is also true for the two restriction fragment length polymorphisms (RFLPs), reported by te Lintel-Hekkert et al that are located ~7 kb 5' to the PROC gene (38)

PC deficiency is a heterogenous disorder (37, 39) A phenotypic subclassification has been proposed that is based on the results of functional and immunologic PC assays In type I PC deficiency there is a concordant reduction in PC activity and PC antigen, while in type II PC deficiency there is evidence for the presence of an abnormal PC molecule (reduced PC activity normal PC antigen) A further classification of the type II PC deficiency can be made by comparing the results of different functional tests (clotting test versus chromogenic test)

In May 1995, the first update of the data base of PROC gene mutations was published on behalf of the Subcommittee on Plasma Coagulation Inhibitors of the SSC ISTH (37) The database contains 331 entries derived from 315 unrelated probands In total 160 different mutations were reported that had resulted in type I or type II PC deficiency Surprisingly ~60% (n = 132) of the mutations (n = 28) causing type I PC deficiency are missense mutations Probably these amino acid substitutions lead to changes in the interactions with other residues and thus interfere with protein folding, a condition associated with rapid intracellular degradation of the protein Missense mutations leading to type II PC deficiency predominantly are located in surface and solvent accessible areas of the protein that are relevant for the function of the protein (like propeptide cleavage, calcium binding, activation, interaction with thrombomodulin, active site, substrate binding) (40, 41) Further, there are mutations in the promoter and 5' untranslated region, splice site abnormalities, small deletions and insertions (eventually leading to a premature termination codon) and nonsense mutations that also are responsible for type I deficiency

About 30% of the unique events occur in CpG dinucleotides and are C → T and G → A transitions Interestingly, 18 transitions in 16 different CpG dinucleotides form about 40% of all the entries in the 1995 database At present there is insufficient information to distinguish recurrent events from events that are identical by descent

PROC gene mutations have been reported now for 18 true homozygotes (9 of these had severe clinical symptoms) and 17 compound heterozygotes (8 with severe clinical symptoms) PROC gene mutation analysis has not however solved an important clinical and epidemiological issue that of clinically recessive and dominant forms of PC deficiency (see below) The 1995 database already lists 14 different mutations that have been found in both these types of PC deficiency

Table 2 PROS gene sequence variations in patients with phenotypic PS deficiency

Exon	Codon, sequ mutat	Predicted mutation	Ref
1	-25, Ins T	-25, Leu → Leu frameshift, stop	(64)
2	- 2, G→T	-2, Arg → Leu	(68)
2	- 1, G→A	-1, Arg → His	(68)
2	22, C→A	22, Cys → stop	(68)
2	26, A→C	26, Glu → Ala	(68)
2	31, T→G	31, Phe → Cys	(68)
2	37, C→T	37, Thr → Met	(68)
3	43, del A	43, Lys → Asn frameshift, stop	(144)
4	49, G→A	49, Arg → His	(68)
5	82, del T	82, Pro → Pro, frameshift, stop	(145)
5	103, C→A	103, Thr → Asn	(68)
6	155, A→G	155, Lys → Glu	(69)
8	204, A→G	204, Asp → Gly	(68)
8	208, G→A	208, Glu → Lys	(68)
8	217, A→G	217, Asn → Ser	(146)
8	220, del G	220, Gly→ Glu frameshift, stop	(146)
8	224, C→G	224, Cys → Trp	(68)
8	224, T→C	224, Cys → Arg	(68)
9	261, del T	261, Leu → Trp frameshift, stop	(65)
9	267, del G	267, Gly → Gly frameshift, stop	(65)
10	335, G→A	335, Asp → Asn	(68)
10	340, G→T	340, Gly → Val	(65)
12	448, G→T	448, Gly → stop	(146)
13	467, T→G	467 Val → Gly	(65)
14	547-548, del AC	frameshift, stop	(146)
14	565, ins T	565, Val → Val frameshift, stop	
14	570, T→C	570, Met → Thr	(146)
14	578, ins C	578 Pro → Leu frameshift, stop	(65)
15	636, A→T	636 stop → Tyr new stop at 649	(64)
intron d	G → A exon 4+1		(146)
intron e	G → A exon 5+5		(68)
intron j	G → A exon 10+1		(146)
intron j	G → A exon 10+5		(64)

PS Deficiency

PS is a vitamin K dependent plasma glycoprotein (70 kDa) It is synthesized in the liver, but also in endothelial cells, megakaryocytes and Leydig cells in the testis The concentration of PS in plasma is 25 μg/ml and is reduced during treatment with oral anticoagulants

PS is a multimodular protein, it contains a γ-carboxyglutamic rich domain, a thrombin sensitive region, four epidermal growth factor like domains and a carboxyterminal region which is highly homologous to the sex hormone binding globulin (SHBG) PS serves as a non-enzymatic cofactor of activated protein C (APC) in the inactivation of factors Va and VIIIa probably by facilitating the formation of enzyme-substrate complexes on the surface of phospholipid/platelet membranes This APC-cofactor activity is destroyed by one of three proteolytic cleavages in the so-called "thrombin-sensitive region" (Arg49 Arg60, Arg70) In plasma PS circulates both free (40%) and in a 1 1 stoichiometric complex with C4b-binding protein (60%) The latter comprises 7 identical α chains (70 kDa) and one single β chain (45 kDa) which are linked to each other in the carboxyterminal region by disulphide bonds The β chain contains the PS binding site Two regions in PS have been reported to be involved in binding of the C4b binding protein (Gly605 to Ile614 and Gly420 to His434) Only the free form of PS has APC cofactor activity More recently it has been reported that PS itself also has anticoagulant activity under well-defined conditions it may inhibit (in an APC-independent way) the activity of both the tenase (IXa-VIIIa) and prothrombinase (Xa-Va) complexes, some reactions being independent of the presence of C4b-binding protein (42-44)

A novel functional aspect of PS has been revealed recently from studies reporting on its binding to the receptor tyrosine kinase known as Rse/Tyro 3 (45) and to a specific receptor on vascular smooth muscle cells This might indicate that PS also is involved in the regulation of cell proliferation More detailed information on structural, biochemical and functional aspects of PS can be found in a recent review article (47)

Human PS cDNAs have been isolated and sequenced (48-50) From the cDNA nucleotide sequence the amino acid sequence of human PS has been derived Two highly homologous PS genes have been identified and sequenced (51-53) The PROS or PSα gene is the active gene it consists of 15 exons which are spread over 80 kb of genomic DNA and has been mapped to the chromosome 3 p11 1-3 p11 2 region (54) The PSβ gene shows 96 5% homology with PROS in exon sequences and the positions of the introns are virtually identical to those in the PROS gene However the PSβ gene is a pseudogene, it contains only exons 2-15 and contains a large variety of detrimental aberrations (a splice site mutation, a frame shift mutation and three stop codons) With the use of a rare RFLP the pseudogene has been located within 4 cM of the PROS gene (55) Several DNA sequence polymorphisms have been reported in the PROS gene (48 49, 56) Some of these have been very useful for tracking PS deficiency through families, for prenatal diagnosis and for evaluating the possibility of allelic exclusion in the case of the study of reverse transcripts of platelet PROS mRNA (57, 58)

Discussions on the subclassification of PS deficiency have yet to be finalized Currently, two of the three proposed subclassification systems are still in use, the system proposed by Comp in 1990 (59) and the proposal recommended by the SSC ISTH in Munich, July 1992 Consistent with the subclassifications used for other hereditary deficiencies, type I deficiencies/defects result in a reduction of total PS antigen (and of free PS antigen and PS activity) Type II, or in Comp's notation type IIb, defines the presence of a functionally abnormal PS molecule (total PS antigen normal, free PS antigen normal but PS activity reduced) Type III PS deficiency (or in Comp's notation type IIa) is defined by normal total PS antigen but reduced free PS antigen and activity Although this phenotype seems to be rather prevalent, it is not yet clear whether it is caused by a hereditary defect and if so whether it is linked to the PS locus Recently Zoller et al reported that both type I and type III phenotypes are reflections of the same genotype (60) Furthermore, Duchemin et al reported among type III PS deficiencies an unusual high frequency (22%) of a mutation previously described as a rare polymorphism (61) It concerns a T → C transition in codon 460 resulting in the replacement of Ser460 by Pro in the

consensus sequence for the N-linked glycosylation of Asn458 (PS Heerlen) (62) The frequency of the PS Heerlen allele in the general population is 0 5% and not different from that in thrombophilic patients (0 7%) At present it is not clear why this genotype is so frequent among type III PS deficiencies

The genetic analysis of the PROS genes of symptomatic PS deficient probands has been hampered and delayed by the structural complexity of the PROS gene and the existence of the highly homologous pseudogene A further complication is the unexpectedly low yield of successful genetic analyses In three separate studies mutations were only found in 50 60% of the patients, although all coding and flanking regions had been amplified and sequenced (63-65)

Only two large deletions in the PROS gene have been reported as a cause of a type I PS deficiency (66, 67) Again, the majority of genetic lesions causing a type I deficiency are single nucleotide substitutions insertions and deletions So far 33 unique events have been reported see Table 2, but this number will increase rapidly in the near future At present these numbers are too low to make any further analysis Four different mutations have been reported that cause a type II PS deficiency (68, 69), two in the propeptide, one in the first EGF domain and one in the second EGF domain Table 2 gives a summary of all the PROS gene mutations reported to date in symptomatic PS deficient patients In 1995, in Jerusalem, the Subcommittee on Plasma Coagulation Inhibitors of the SSC ISTH decided to publish the first database of PROS gene mutations in 1996/1997

Factor V Arg506 to Gln

Factor V is a single chain plasma glycoprotein (300.000 kDa) It is synthesized in the liver and in megakaryocytes Human plasma has a concentration of 20 nM of this procofactor, while its concentration in platelets is 4 $\mu g/10^9$ platelets During blood coagulation factor V is converted into factor Va by (meizo) thrombin and/or factor Xa Thrombin-activated factor V is formed after cleavages of the Arg709-Ser710, Arg1018-Thr1019 and Arg1545-Ser1546 bonds It is composed of an amino terminal fragment (heavy chain 105 kDa) and a carboxy terminal fragment (light chain 74 kDa) non-covalently linked via a tightly bound Ca^{2+} ion Factor Va serves as a nonenzymatic cofactor in prothrombinase (factor Xa, phospholipids, Ca^{2+}) by increasing the catalytic efficiency approximately 2000-fold Factor Va light chain is reported to contain the phospholipid binding site, while the heavy chain site is mainly responsible for the cofactor activity

Factor Va is inactivated by proteolytic degradation of its heavy chain by APC, this inactivation is more efficient in the presence of phospholipids and Ca^{2+} and is according to some an ordered and sequential event with a first cleavage at Arg506 and subsequent inactivating cleavages at Arg306 and Arg679 (70) Others demonstrated that two random cleavages (mainly at Arg506 and Arg306) are involved and that both contribute to the inactivation of factor Va (71)

More recently it was reported that factor V is not only a procofactor in the prothrombinase reaction but also a cofactor in the inactivation of factor VIIIa by APC (72) More information on the structure and function of human factor V can be found in a recent review (73)

Partial and overlapping human factor V cDNAs have been isolated from HepG2 and human (fetal) liver cDNA libraries (74-76) From the nucleotide sequence, the complete amino acid sequence of factor V was derived It consists of 2196 amino acids and shows a characteristic domain structure (A_1-A_2-B-A_3-C_1-C_2) that is also found in factor VIII The characterization of the human factor V gene was reported in 1992 by Cripe et al (77) Twenty five exons and 24 introns span approximately 80 kb genomic DNA The nucleotide sequence of all coding and flanking regions has been determined The factor V gene has been mapped to chromosome 1 (1q21-25) and is closely linked to the antithrombin gene (78) A number of nucleotide sequence variations in human factor V cDNAs have been identified (74-76 79) but with some exceptions (79 80) the allele frequencies have not been reported The microsatellite marker D1S61 has been mapped within 4 cM of the factor V gene locus and has been used successfully for tracking the factor V gene in a family with hereditary APC-R (3)

In 1994 the single point mutation in the factor V gene was identified as the genetic defect causing the phenotype of APC-R in the vast majority of affected individuals (3 5 81) It involves a G → A transition of nucleotide 1691 in exon 10, which predicts the synthesis of a variant factor V molecule (factor V 506Arg to Gln or factor V Leiden)

The mechanism by which the mutation leads to the phenotype of APC-R is still subject of detailed biochemical studies However it is clear that the replacement of Arg506 by Gln will prevent cleavage of factor V(a) at this site by APC and by that delay the inactivation of factor Va (82, 83) either by preventing the conformational change necessary for the inhibitory cleavage at Arg306 or by preventing the kinetically more favourable inhibitory cleavage at Arg506

So far the factor V 506Arg to Gln mutation is the only genetic defect identified in APC-R families It has a relatively high frequency in Caucasian populations (up to ~6%) but a much lower frequency in the Japanese and other Eastern populations (~0%) (84) Preliminary evidence for a founder effect in the spread of this disorder was obtained from the results of haplotype analysis of 53 Dutch carriers of the mutation (3)

Other Candidates?

There are a number of other genetic defects or isolated deficiencies that have been implicated in contributing to the risk of thrombosis in families with thrombophilia In most cases these have been based on observations in case-families Sometimes genetic defects have been identified but no data on genotype-phenotype relationships are available as yet (85) In the next paragraphs the various candidates will be briefly discussed

Hereditary dysfibrinogenemia is detected by a prolonged plasma thrombin time Clinical symptoms vary from none mild bleeding to venous or arterial thrombosis The phenotype may follow recessive or dominant inheritance Recently the evidence for a causal relationship between an isolated dysfibrinogenemia and venous thrombosis has been critically reviewed and discussed by a working party of the SSC Subcommittee on Fibrinogen of the ISTH (86) In that investigation 5 families (from 5 different countries) were identified in which apart of the proband, two or more family members had both the defect and thrombosis In all five cases the genetic defect had been identified (two mutations in the Aα chain two mutations in the Bβ chain and one mutation in the γ chain of fibrinogen) In one family only homozygotes for the mutation (fibrinogen Naples) were clinically affected The relationship between the defect in the fibrinogen molecule and the phenotype of thrombophilia is still poorly understood Further studies are hampered by the low frequency of dysfibrinogenemia in cohorts of patients with thrombosis (0 8%) (86)

Thrombomodulin (TM) is another component of the PC anticoagulant pathway (47) It is a transmembrane protein synthesized by endothelial cells which acts as a receptor for thrombin and as cofactor of thrombin in the activation of PC By analogy with PC and PS deficiencies one would expect that deficiencies of or defects in thrombo

modulin may be associated with an increased risk of thrombosis Unfortunately plasma is not an abundant source of TM and therefore cannot be used for the laboratory diagnosis of TM defects Analysis of the TM genes in cohorts of symptomatic probands with a family history of thrombophilia did not reveal any alteration in the coding and flanking regions of the gene (0/30) in one study (Reitsma PH & Bertina RM unpublished observations) while four different mutations (4/87) were identified by a second group (85 87) To date there is still very limited information of the co-segregation of these mutations with thrombophilia in the families of the probands

Recently mild *hyperhomocysteinemia* was found in 19% of patients with juvenile venous thrombosis and family studies showed that in most cases this phenotype was inherited (88) Two large patient-control studies have shown that hyperhomocysteinemia is a risk factor for recurrent thrombosis (89) and for thrombosis in unselected patients, among whom elevated levels were present in 10% (90) Severe hyperhomocysteinemia has a population prevalence of ~1 300 000 and is most frequently caused by homozygous *cystathionine β synthase* deficiency Homocysteine is a sulphydryl amino acid derived from metabolic conversion of methionine Its intracellular metabolism occurs through remethylation to methionine or transulphuration to cysteine Cystathionine β-synthase is involved in the transulphuration pathway A small number of cases of severe hyperhomocysteinemia are caused by homozygous deficiency of *methylenetetrahydrofolate reductase* Gene defects in both of these metabolic enzymes are implicated in mild hyperhomocysteinemia (91, 92) A recently described mutation with thermolability of methylenetetrafolate reductase may in the homozygous state be a significant and frequent cause for mild hyperhomocysteinemia (93)

Plasminogen deficiency and dysplasminogenemia have been reported frequently to be associated with thrombophilia However, family studies reveal that in most families with a type I plasminogen deficiency (parallel reduction of plasminogen activity and antigen) only the proband suffers from thrombotic disease (94) In a recent retrospective analysis of 20 families, heterozygotes of a type I plasminogen deficiency were found to experience significantly more thrombotic events (although rather late in life) than their normal family members (95) The frequency of plasminogen deficiency in the general population seems to be slighter lower (0 4%) to that in cohorts of patients with thrombosis (1 3%) (96, 97) Dysplasminogenemia or type II plasminogen deficiency (reduced plasminogen activity, normal antigen) associated with substitution of Ala601 by threonine is a common variant in the Japanese population and seems not to be associated with thrombosis (98) Studies of plasminogen deficiency at the DNA level are still very rare

In a model in which it is expected that reduced plasminogen levels may cause thrombophilia it seems reasonable to propose that an inherited elevated *Histidine Rich Glycoprotein (HRG)* level in plasma is also a risk factor for thrombosis HRG (a non enzymatic protein) forms a 1 1 complex with plasminogen in plasma (via binding to its lysine binding sites) and thus reduces the free plasminogen concentration to around 50% (99) Complex formation with HRG interferes with the binding of plasminogen to fibrin Although several families with thrombophilia and high HRG levels have been reported (100 101) there is still no formal evidence for its association Surprisingly, recently two families have been reported where a partial deficiency of HRG seems to be associated with thrombophilia (102 103)

Another potential risk factor of thrombosis, deficiency of the *Tissue Factor Pathway Inhibitor (TFPI)* has been investigated but no mutation in TFPI genes of 30 symptomatic probands of families with thrombophilia (Reitsma PH & Bertina unpublished observations) could be found Other potential candidate risk factors for thrombosis are *heparin cofactor II deficiency* and *β2 glycoprotein I deficiency* Laboratory analysis of large groups of patients with thrombophilia and of controls revealed however, that the frequency of heterozygotes for these defects among patients and controls is very similar (0 6% and 6% respectively) (104 105) Nevertheless a few families have been reported in which an isolated heparin cofactor II deficiency seems to segregate with the thrombophilia (106 107) In two families with a type II heparin II cofactor deficiency the lesion in the gene was identified (replacement of Arg 189 by His) (108) This mutation is thought to effect the dermatan sulphate binding site and is analagous to certain defects in type II HBS antithrombin deficiency that effect the heparin binding

A last genetic defect that has been discussed during the last years with respect to its possible association with thrombophilia is a partial *factor XII* deficiency Original studies from Mannhalter et al suggested a high frequency of heterozygotes for factor XII deficiency in cohorts of thrombophilic patients (109) Subsequent studies have not supported these findings (110)

Finally there is a phenotype that recently has been identified as a risk factor for thrombosis in large patient-control studies elevated *factor VIII* levels (111) The hereditability of this phenotype and the eventual underlying molecular defects have not been reported so far

Epidemiology of Inherited Thrombophilia

Prevalence of Hereditary Thrombophilia

Venous thrombosis has an overall annual incidence of <1 in 1000 It is rare in the young, and becomes more frequent with advancing age The true prevalence of hereditary thrombophilia is not yet known It seems clear that we do not know all genetic abnormalities causing a tendency to venous thrombosis, since even in patients from families selected on the basis of a high number of unexplained thromboses in only about half an underlying defect will be found (112) This indicates that the prevalence of hereditary thrombophilia in the general population will be higher, possibly up to two fold than estimates from large prevalence studies on the known genetic defects A high prevalence of hereditary thrombophilia will make this an important factor in the overall incidence of thrombosis This may also be assessed by taking the presence of a positive family history into account Among unselected consecutive patients with deep vein thrombosis a family history was reported by one out of every four patients (113) Even though a positive family history may occur by chance as venous thrombosis is not a rare disorder, these figures indicate that genetic causes are prominent in the etiology of venous thrombosis

The prevalence of deficiencies of PC and antithrombin has been investigated in a very large study of almost 10,000 blood donors (114-117), Table 3 Repeated testing of the levels of these proteins coupled with family studies and DNA analysis led to estimates of 1 in 500 for PC deficiency and 1 in 5000 for type I antithrombin deficiency This is in the same range of the findings of a previous study among over 5000 blood donors where 1 in 250 were considered PC deficient (118) Although some caution is needed in interpreting data based on studies among blood donors who are a self selected sample from the general population these seem to be fair estimates Accepting the approximate prevalence of PC deficiency to be 1 350 the prevalence of severe (homozygous or compound heterozygous) deficiency will be $1\ 700 \times 1\ 700 = 4.9 \times 10^{-7}$ Offspring from related parents will of course have much higher risk of severe deficiency The prevalence of severe anti-

Table 3 Prevalence of the major thrombophilic clotting abnormalities

	PC deficiency	PS deficiency	Antithrombin deficiency	APC R
Healthy individuals				
Tait et al (n = 9669) (114 117)	0.2%+		0.02%[1]	
Miletich et al (n = 5422) (118)	0.4%			
Svensson and Dahlbäck (n = 130) (119)				7%
Rosendaal et al (n = 474) (4)				3%+
Ridker et al (n = 704) (120)				6%+
Consecutive patients with first DVT				
Heijboer et al (n = 277) (113)	3%	2%	1%	
Koster et al (n = 474) (125)	3%+	1%	1%	
Rosendaal et al (n = 471) (4)				20%+
Thrombophilic patients				
Briet et al (n = 113) (147)	8%	13%	4%	
Scharrer et al (n = 158) (148)	9%	6%	5%	
Ben Tal et al (n = 107) (149)	6%	3%	7%	
Taberno et al (n = 204) (150)	1%	1%	0.5%	
Griffin et al (n = 25) (112)				52%

+ DNA confirmed [1]type I Antithrombin deficiency

thrombin deficiency is likely to be 100 1000 less than severe PC deficiency There are no studies of sufficient size on PS deficiency among healthy individuals on which a reliable estimate of its prevalence can be based

For APC-R, the groups that have been studied are not as large as in the blood donor studies However since the prevalence of this abnormality is an order of magnitude higher than the other inhibitor deficiencies the estimates are as reliable The estimates for Caucasians range from 3 to 7 percent (4 119 120), and for several studies are based on genetic investigation (4, 120) Since these estimates are also based on self selected individuals without a history of cardiovascular disease or venous thrombosis they are under rather than over-estimates The prevalence at birth of homozygous factor V 506Gln mutation has been estimated at ~1 5000 (4)

Among consecutive patients with objectively confirmed deep vein thrombosis, deficiencies of PC PS and antithrombin combined account for ~5% APC-R is present in 20 percent of consecutive patients with deep-vein thrombosis (4, 121)

Several studies have focused on selected patients with venous thrombosis and usually found a higher prevalence of deficiencies of PC, PS and antithrombin than has been reported among unselected consecutive patients Different criteria were used in selecting the patients for these series some authors included patients who experienced a first thrombosis at a young age others studied individuals with recurrent thrombotic events or thrombotic events that occurred in the absence of any of the classical risk factors for venous thrombosis, while some included individuals with a positive family history Because of these heterogeneous inclusion criteria the prevalences that have been reported in these studies should not be compared too closely The prevalences that have been reported among selected patient groups for deficiencies of PC PS and antithrombin are mostly between 5 and 10%, much higher than the prevalences found in the population studies, and also somewhat higher than the frequency among consecutive unselected patients These higher prevalences in patients with thrombosis than in healthy individuals and in thrombophilic individuals as compared to unselected patients also indicate that these deficiencies indeed lead to venous thrombosis and venous thrombophilia APC R appears to account for half of all cases of hereditary thrombophilia and clearly emerges from Table 3 as the most important cause of hereditary thrombosis and perhaps of venous thrombosis in general

Risk of Venous Thrombosis

The risk of venous thrombosis for individuals with clotting factor gene abnormalities has largely been investigated by two approaches first by studies in family members of probands with one of these abnormalities and second by population-based studies (case control studies) These different approaches do not necessarily yield the same information The former are based on families in which the hereditability of the abnormality has been shown by including only families with one or more individuals with the clotting abnormality apart from the proband who is both symptomatic for thrombosis and has the clotting defect In the analysis typically the occurrence of thrombosis is compared between the family members with and without the clotting factor abnormality while the proband is excluded from the analysis Since hereditability is a prerequisite in studies of this design they are most fitting to directly answer questions concerning risks of genetic disorders In population based studies patients with thrombosis are compared to healthy individuals with regard to the prevalence of clotting factor abnormalities These case control studies yield relative risk estimates which indicate how much higher the risk of thrombosis is for an individual with a particular risk factor compared to an individual without that factor Since only individuals are included and not families, no direct statements about hereditability result from these studies Furthermore case control studies can only yield estimates of relative risks not of absolute (life time) risks

When consecutive patients are included in a population-based study (with population-controls) the results associating a particular risk factor with thrombosis apply indiscriminately to all individuals with that abnormality in the population The results from family studies are based on families that stood out and were recognised because of a conspicuous high frequency of thrombosis strictly speaking these results only apply to families detected in a similar way In other words population-based studies yield an average risk for individuals with a particular abnormality whereas family studies are conducted among those with higher risks Since it is likely and has now been shown to be

true for APC-R that many families with thrombophilia display more than one genetic abnormality one should exercise extreme caution in applying results from family studies to unselected individuals or results from population-based studies to thrombophilic families If an individual is identified as carrying a thrombogenic abnormality by means of a study among the general population in all likelihood he only carries that one abnormality if he is found as proband of a family with thrombophilia he might well carry two or more and if he is an unselected patient with thrombosis he may well be one of both

Antithrombin Deficiency

When the data from all the available family reports of antithrombin deficiency are combined it appears to confer a higher risk of thrombosis than deficiencies of PC and PS Thrombosis is not uncommon before age 16 and about half of the patients from these reported families experienced a first thrombotic event before age 25 (122 123) Antithrombin deficiency especially has a much higher risk of thrombosis in pregnancy than deficiencies of PC or PS (124) The fifty fold difference in the prevalence among patients with a first event of deep venous thrombosis and the prevalence in a healthy population (113 114 125) also suggests a higher thrombotic risk in antithrombin deficiency than in PC deficiency, although such a difference could not be substantiated in a population-based study (125) There is some debate whether the more severe form of thrombophilia caused by antithrombin deficiency might also result in increased mortality (126 127) in some pedigrees fatal thromboses have been observed (128) but in historic studies of Dutch pedigrees no excess mortality was evident (24 129)

PC Deficiency

Since 1981 (130) many families with hereditary protein C deficiency have been reported The risk of thrombosis appears not to be different for the different types of protein C deficiency (type I low plasma level and type II low activity) nor for the large number of mutations identified in PC deficiency (37) In family studies it has been shown that family members who are PC deficient have an increased risk of venous thrombosis (about 8 10 fold) and that by age 40 about half of them will have experienced at least one thrombotic event (131, 132) In a population-based study (125), a relative risk estimate of 7 has been reported which (surprisingly in view of the comments made above) is very similar to the relative risk derived from family studies The prevalences reported in unselected patients with first thrombotic event (3%) (113 125) and healthy individuals from the general population (0 2%) (117) also support a relative risk of this size or slightly higher

These data all apply for heterozygous PC deficiency in which PC activity on average is 50 percent of normal The rare homozygous patients with no PC activity in plasma has a much higher risk of thrombosis and develop purpura fulminans shortly after birth (see below)

PS Deficiency

Since 1984 (133 134) many families have been reported with venous thrombophilia and PS deficiency It is not clear whether the three different subtypes that have been described (types I II and III) confer similar risks of thrombosis It is very difficult to arrive at risk estimates for PS deficiency since the prevalence in the general population is unknown there are no reports from family studies formally assessing the relative risk the molecular basis in many cases remains unclear and several families with APC-R appear to have been misclassified as PS deficient (type II) (135) In one population based case control study the prevalence of individuals with repeatedly low levels of PS did not differ between cases and controls which does not support an association of PS deficiency and venous thrombosis (125) It is difficult to reconcile this finding with the reports from several families as well as the higher prevalence of PS deficiency in selected patients with thrombophilia than in unselected patients with a first event (Table 2) Although the available evidence generally leads to the conclusion that PS deficiency increases the risk of thrombosis this evidence is much less solid than for PC deficiency Homozygous PS deficiency has been reported and while extremely rare appears to be as severe as homozygous PC deficiency (see below)

APC R/Factor V 506Arg to Gln

In a study in 34 families with APC R the life time risk of thrombosis was clearly higher in family members with this phenotype than in those who did not (119) At age 50, about 25 percent of individuals with APC-R had experienced at least one thrombotic event (119) This is lower than the figures reported for families with PC deficiency (132) it should be borne in mind, however, that these families with APC R were not selected on the severity of thrombophilia as was the case in family studies of PC deficiency In a population based case control study APC-R was found in 21 percent of patients with a first episode of deep vein thrombosis and in five percent of controls which led to an estimate of the relative risk associated with APC-R of 7 (121) This risk estimate is very similar for that found for PC deficiency

Because of its high allele frequency homozygous carriers of the factor V Arg506 to Gln alleles are not uncommon The homozygous abnormality appears much less severe than homozygous PC deficiency since several of the homozygous patients have remained thrombosis free well into adult life (4 136) Still the risk of thrombosis for those homozygous for the mutation is higher than for those heterozygous (10-fold higher), estimated at 90 fold increased compared to individuals without the mutation (4)

Combined Abnormalities

Deficiencies of PC, PS and antithrombin are rare while APC R is very common Because they are so rare these former deficiencies have been studied mostly in referred and highly selected families In these selected families e g with PC deficiency, high risks of thrombosis have been observed (PC deficiency is then said to be clinically dominant), much higher than in relatives of patients homozygous for PC deficiency (PC deficiency is then said to be clinically recessive) (118) This discrepancy in clinical expression cannot be explained by different mutations underlying PC deficiency (37) The most plausible explanation for the difference in clinical expression in PC deficiency arises from the view that more than one abnormality may be required to cause thrombosis, see Fig 2 In patients with homozygous PC deficiency, two identical defects are present so that their heterozygous relatives will often have just one defect which carries a smaller risk Individuals with heterozygous PC deficiency from families with striking thrombophilia among heterozygous carriers might be expected to have additional abnormalities contributing to the risk It has been shown that APC-R may be such a second risk factor and this explains why in family studies among selected families with thrombophilia the risk of thrombosis among non deficient family members was higher

than in the general population (132) many of them had APC R (137) In this study it was shown that the combination of PC deficiency and APC-R conferred a higher risk than each of these abnormalities separately Similar conclusions have been drawn regarding the increased risk of thrombosis following interaction of factor V 506Arg to Gln mutation with the other inhibitor deficiencies PS and antithrombin deficiencies (138 140) The situation with combined factor V 506Arg to Gln and antithrombin mutation is further complicated by their close genetic proximity In some families co segregation of two genetic defects can occur resulting in high thrombotic risk to all affected individuals (139)

Acquired risk factors are important in the development of thrombosis Unfortunately there are few reported studies that contain quantitative information It has been shown that the risk of thrombosis is further increased among women with PC deficiency who use oral contraceptives (141), and is greatly increased among APC-R women who use oral contraceptives (142) The risk is reported to vary with the type of progestagen used (143)

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