INHERITED THROMBOPHILIA

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1. 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. Understandably, most studies focused on the identification of isolated hereditary deficiencies or defects, because the majority of hereditary bleeding disorders had been explained by single factor deficiencies. In addition, the discovery that heterozygosity of antithrombin deficiency co-segregated with the thrombotic tendency in affected families (1) 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-heparin 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 thromboembolism in families identified through a symptomatic deficient patient, with one of antithrombin, PC or PS deficiency. Furthermore, these deficiencies were found in about 15% of families with familial thrombosis and only in a few percent of all patients with venous thrombosis (2).

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, based on genetics and on epidemiology. 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 → C6 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 (3, 4). This mutation, which is associated with a significant increase in thrombotic risk (4-6), 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 attempts to summarise recent progress and to present recommendations for diagnosis, treatment and research in developed and developing countries.

2. 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.
A list of conditions that have been associated with thrombosis is shown in Table 1. Very few are specifically associated with thrombophilia if the term is restricted to include only patients with some of the five atypical features of thrombosis listed above. In fact, nearly every condition or defect in Table 1 is more common than thrombophilia itself.

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.

3. MOLECULAR AND 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 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 mM. Antithrombin is the primary inhibitor of most of the activated serine proteinases involved in blood coagulation (thrombin, factor Xa, factor IXa, factor Xla, factor XIIa, kallikrein) and therefore is 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 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 compounds, such as endothelial cell heparin sulphate) to antithrombin. The interaction between heparin and heparin-binding domains in antithrombin 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 sequenced (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 (1-6, 14); its nucleotide sequence has been recently completed (15). Several sequence variations or polymorphisms 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 subclassification 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 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=A2). Type II HBS mutations are locate = d mainly on the A, C and D a 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 Ca2+-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 endothelialal 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 introns 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 promoter 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=3D132=) of the mutations (n=3D28) 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 → C6 T and G → C6 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.

**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 mg/ml and is reduced during treatment with oral anticoagulants.

PS is a multimodular protein: it contains a g-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 a chains (70 kDa) and one single b chain (45 kDa) which are linked to each other in the carboxyterminal region by disulphide bonds. The b 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 Ile 614 and Gly420 to His 434). 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/Tyro3 (45) and to a specific receptor on vascular smooth muscle cells (46). 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 PSA 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 PSb 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 PSb 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 CompOs 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 CompOs 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 =C6 C transition in codon 460, resulting in the replacement of Ser 460 by Pro in the consensus sequence for the N-linked glycosylation of Asn 458 (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. 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 mg/109 platelets. During blood coagulation factor V is converted into factor Va by (meizoenzyme) 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 Ca2+ ion. Factor Va serves as a nonenzymatic cofactor in prothrombinase (factor Xa, phospholipids, Ca2+) 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 Ca2+ 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 (A1-A2-B-A3-C1-C2) 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 cDNA's 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 (4).

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 (4, 6, 81). It involves a G=C6A transition of nucleotide 1691 in exon 10, which predicts the synthesis of a variant factor V molecule (factor V 506 Arg 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 (upto 6%) but a much lower frequency in the Japanese and other Eastern populations (down to 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 (4).

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 Aa chain, two mutations in the B8 chain and one mutation in the g 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).

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 (87). Two large patient-control studies have also found hyperhomocysteinemia to be a risk factor for recurrent thrombosis (88, 88a). Severe hyperhomocysteinemia has a population prevalence of \( \sim 1:300,000 \) and is most frequently caused by homozygous cystathionine-\( \beta \)-synthase deficiency. Homocysteine is a sulphhydril amino acid derived from metabolic conversion of methionine. Its intracellular metabolism occurs through remethylation to methionine or trans sulphuration to cysteine. Cystathionine-\( \beta \)-synthase is involved in the trans sulphuration 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 (89-90). A recently described mutation with thermolability of methylenetetrahydrofolate reductase may in the homozygous state be a significant and frequent cause for mild homocysteinemia (90a).

*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 thrombomodulin 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, 91). To date there is still very limited information of the co-segregation of these mutations with thrombophilia in the families of the probands.

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 (92). 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 (93). 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%) (94-95). Dysplasminogenemia or type II plasminogen deficiency (reduced plasminogen activity, normal antigen) associated with substitution of Ala 601 by threonine is a common variant in the Japanese population and seems not to be associated with thrombosis (96). Studies of plasminogen deficiency at the DNA level are still very rare.

In a model where 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 lysin binding sites) and thus reduces the free plasminogen concentration to around 50% (97). Complex formation with HRG interferes with the binding of plasminogen to fibrin. Although several families with thrombophilia and high HRG levels have been reported (98, 99), 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 (100-101).

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 82 glycoprotein 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) (102-103). Nevertheless, a few families have been reported in which an isolated heparin cofactor II deficiency seems to segregate with the thrombophilia (104-105). In two families with a type II heparin II cofactor deficiency the lesion in the gene was identified (replacement of Arg 189 by His) (106). This mutation is thought to effect the dermatan sulphate binding site and is analogous 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 (107). Subsequent studies have not supported these findings (108).

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 (109). The hereditability of this phenotype and the eventual underlying molecular defects have not been reported so far.
4. 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 (110). 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 (111). 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 (112-115), 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 (116). 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 x 1:700 = 4.9 x 10^6. Families in which intermarriage is still practised will of course have much higher risk of severe deficiency. The prevalence of severe antithrombin 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 (5, 117-118), and for several studies are based on genetic investigation (5, 118). 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 of homozygous factor V 506 Gln mutation has been estimated at ~1:4600 (5).

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% of consecutive patients with deep-vein thrombosis (5, 119).

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 (lifetime) 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 (120-121). Antithrombin deficiency especially has a much higher risk of thrombosis in pregnancy than deficiencies of PC or PS (122). The fifty-fold difference in the prevalence among patients with a first event of deep venous thrombosis and the prevalence in a healthy population (111-112, 123) 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 (123). There is some debate whether the more severe form of thrombophilia caused by antithrombin deficiency might also have a deleterious effect on mortality (124-125): in some pedigrees fatal thromboses have been observed (126), but in historic studies of Dutch pedigrees no excess mortality was evident (24, 127).

PC deficiency

Since 1981 (128), 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 (129, 130). In a population-based study (123), 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%) (111, 123) and healthy individuals from the general population (0.2%) (115), 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% 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 (131-132), 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 2) (133). 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 (123). 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 (117). At age 50, about 25% of individuals with APC-R had experienced at least one thrombotic event (117). This is lower than the figures reported for families with PC deficiency (130); 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% of patients with a first episode of deep-vein thrombosis and in 5% of controls, which led to an estimate of the relative risk associated with APC-R of 7 (119). 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 (5, 134). Still, the risk of thrombosis in those homozygous for the mutation is higher than for those who are heterozygous (10-fold higher), estimated at 90-fold increased compared to individuals without the mutation (5).

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) (116). 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 (130): many of them had APC-R (135). 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 (136-138). 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 (137).

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 (139), and is greatly increased among APC-R women who use oral contraceptives (140).
5. LABORATORY INVESTIGATION OF INHERITED THROMBOPHILIA

What laboratory assays should be included?

In order to evaluate the contribution of genetic defects in the pathogenesis of thrombosis, the laboratory investigation should include a functional APC-R test, determination of the factor V genotype and measurements of plasma concentrations of PC, PS (total and free PS) and antithrombin. In addition, a thrombin time will ensure that rare cases of dysfibrinogenemia are not overlooked. At present, available results suggest fibrinolytic parameters not to be of value in the evaluation of individual patients (141).

Before selecting the analytical procedure in the laboratory a number of questions should be addressed such as what are the sensitivity and specificity of the laboratory assay for the genetic defect to be detected? Moreover, the assay performance such as inter- and intra-day variation are important factors to take into account. Quality control should be of high priority. In the choice of assay it is crucial to consider possible influence by other defects, e.g., APC-R influence on coagulation-based functional assays for PC and PS (see below). An important parameter to be taken into consideration is the predictive value of a positive and a negative test. This is determined not only by the specificity and sensitivity of the assay but also by the prevalence in the population to be studied for the particular defect. Thus, unless the sensitivity and specificity are close to 100%, the predictive value of a positive test is usually quite low for defects which are rare in the population. Examples of this will be given below.

Anticoagulant protein deficiencies

A detailed evaluation of the possible contribution of anticoagulant protein deficiencies in the pathogenesis of thrombosis should include analysis of both protein levels with immunological assays and of the functional activity of each of these proteins. However, this is not always possible and practical compromises have to be made. In general, deficiencies of type I (low protein concentration) of any of the anticoagulant proteins are much more common than type II deficiencies (low functional activity but normal protein concentration). An optimal assay for each of these proteins is an easy and cheap functional assay which detects both type I and type II deficiencies. In the case of antithrombin, there are several commercially available functional assays (based on inhibition of either thrombin or factor Xa) which fulfill these criteria. An International Standard for antithrombin is available.

The situation for PC and PS is more complicated because the available coagulation based functional assays are complicated to perform and several of them have low sensitivity and specificity. Assays devised to measure the protein concentrations are often cheaper and easier to perform than several of the commercially available functional assays, and consequently such assays are used in most laboratories in the primary evaluation of the patients. Both sensitivity and specificity of immunological as well as of functional assays are lower when used to test individuals on oral anticoagulation than when used to test patients without anticoagulant therapy.

In the case of PC, depending on the site of the mutation there are many different possible types of functional defects. The interaction with the thrombin-thrombomodulin complex may for instance be impaired with a resulting poor activation; mutations in the vitamin K-dependent region may lead to poor phospholipid binding ability; mutations in other parts of PC may lead to functional defects due to poor interactions with either substrates or cofactors. There is at present no easily available
functional assay which monitors all functional aspects of PC and consequently, the laboratory evaluation is a practical compromise. A functional, easy, assay is available for PC and it is used in many laboratories as an alternative to immunological assays in the primary screening of the thrombosis patients. The assay includes activation of PC in plasma with a PC activator (Protac) isolated from a snake venom and measurement of the proteolytic activity of the activated PC against a synthetic substrate. The assay, which is readily automated, is available from several commercial sources. It should be borne in mind that the assay does not detect several of the type II defects. As there is an overlap in the PC concentrations between normals and those with the genetic defects (130), sensitivity and specificity of all available functional or immunological assays for the genetic defects causing PC deficiency are below 100%. The Protac based assays perform quite well and have coefficients of variation below 2%. The sensitivity has been estimated to be 85%, whereas the specificity is estimated to be 95% (130). When this assay is used to study a population in which the prevalence is around 0.3%, the predictive value of a positive test is 5% whereas the predictive value of a negative test 99.9%. Thus, in screening of the general population this assay is excellent to exclude PC deficiency, whereas a positive test is caused by a genetic defect in only 5% of the cases. When the assay is used to test patients with thrombosis (prevalence of genetic defect approximately 3%), the predictive value of a negative test is 34% whereas the predictive value to a positive test is 99.6%. The predictive value of a positive test is negatively related to the PC level, i.e., the lower the PC level the higher the predictive value. There is an International Standard for PC available.

At present, there are no functional PS assays which fulfill the requirements that can be put on an assay which is to be used in the primary evaluation of thrombosis patients. The low specificity of the available functional assays is a major problem as it has recently been shown that functional assays for PS are influenced by the APC-R associated with the presence of the factor V 506 Arg to Gln mutation (133). As PS circulates in plasma both as free protein (approximately 30%) and in complex with the C4b-binding protein (142), a relevant question is which fraction of PS should be measured with the immunological assays, the free form, the bound form or total PS. The current provisional conclusion is that the free PS assays have higher sensitivity and specificity for the genetic defects causing PS deficiencies than assays for total PS. This is because there is a great overlap in the total PS levels between normals and those with the genetic defect (60). Although the prevalence of PS deficiency in the general population is not well established it is reasonable to suspect that it is similar to that of PC and the discussion about the predictive value for a negative and positive test (see above) is therefore relevant also for PS. There are several commercially available assays for both free and total PS and an International Standard will become available.

The APC-R test

So far, practical clinical experience has mainly been obtained with the APTT-based test for APC-R (3). In an APTT reaction, the addition of APC results in degradation of both factor VIIIa and factor Va, which delays thrombin generation and hence the formation of the fibrin clot. The factor V 506 Arg to Gln mutation is associated with partial resistance to degradation of mutated factor Va by APC, but the mutated factor Va expresses normal procoagulant properties (83, 143-144). Consequently, thrombin generation is not properly impaired in the presence of APC, which results in less prolongation of the clotting time. =09

As the APC-R test is based on a standard APTT-reaction it is easy to perform. The APTT-reaction is run in the presence and absence of a carefully standardised amount of APC, which is included in the calcium chloride solution used to initiate the clotting reaction (3). The clotting time is
measured in the presence (a) and absence (b) of APC and a ratio (the APC-ratio) between the two clotting times (a/b) is calculated. If the assay is always done on the same instrument and also in other respects performed under strictly standardised conditions, the resulting APC-ratios can be used as they are (117, 119). However, it has been observed that different instruments give different clotting times and APC-ratios obtained on one type of instrument cannot be directly compared with those from another type (145). If results from different laboratories are to be compared, it is beneficial to normalise the APC-ratios against the APC-ratio of a normal plasma pool (146). There are some important considerations to take into account in relation to such a normalisation procedure. Due to the high prevalence of APC-R in the general population a pool of normal plasmas is fairly likely to contain plasma from individuals with APC-R and in accordance with this, it has indeed been found that APC-ratios of normal plasma pools are always lower than the mean of the individual APC-ratios. For laboratories not having the possibility to perform factor V genotyping, a practical solution to the problem may be to exclude plasmas with extreme APC-ratios from the pool, e.g., the 5 or 10% with the highest and the 5 or 10% with the lowest APC-ratios. In this context, it is obvious that an International Standard plasma would be useful.

The handling of the plasma samples is probably of importance and the quality of the results depend on strict standardisation. The centrifugation of blood should ensure that the resulting plasma is platelet poor (< 1% of normal platelet count), because even a small contamination of platelets affects the APC-ratio, in particular after freezing and thawing of the plasma; platelet contamination will lower the APC-ratio (147). For routine purposes it is recommended to centrifuge the plasma at 2000 g for 20 minutes at room temperature. When the plasma is pipetted off, only the middle portion should preferably be utilised to avoid platelet contamination by native or disrupted platelets.

The APC-R test can be performed on both fresh and frozen plasma, but "like should be compared with like", i.e., when fresh plasma is analyzed, the normal range should have been determined with fresh plasma; when samples are frozen at -70 = BOC or at -20 = BOC they should be compared with normal controls handled in the same way. In support for this concept, it was recently reported that freezing of plasma results in decreased APC-R (Coatest=AE APC=81 Resistance from Chromogenix, M=F6indal, Sweden, was used = in the study) but of practical importance, the authors found no differences between freezing temperatures (-70 = BOC and -30 = BOC tested) (146). When all variables are strictly controlled, the APC-R test is reliable and gives good discrimination between normal and APC-R individuals. The reported sensitivity and specificity of the APC-R test for factor V 506 Arg to Gln are 85-90% or better. Variation in endogenous levels of PC has, of course, no influence on the APC-R because a standardised amount of APC is added. In addition, variation of the endogenous free PS level within the range expected for heterozygous PS deficiency and normals has no or only a very minor influence on the APC response in the APTT-based assay (3, 146). The original APC-R test has a sensitivity of 94% for detection of factor V 506 Gln allele, whereas the specificity is approximately 85% (similar results have been found for the commercial assay from Chromogenix). If this test is used for screening of a population in which the prevalence is around 10%, the negative predictive value is 99.7% whereas the positive predictive value is only 16%. In screening a population with thrombosis, the negative predictive value is 97% whereas the positive predictive value is around 73%. Thus, a positive test should always be confirmed by factor V genotyping. The sensitivity and specificity of the APC-R test appears to be related to the quality of the APTT reagent.

The original APC-R test is not reliable for analysis of plasmas from individuals receiving oral anticoagulants, or heparin, or if they are derived from individuals with other coagulation defects,
such as lupus anticoagulants or coagulation factor deficiencies (117, 146). In plasma from patients on oral vitamin K antagonists, the clotting times in the presence of APC are usually quite long (clotting may not even occur in such plasmas), which results in high ratios. In order to allow analysis of such plasmas, a modified APC-R test, in which sample plasma is prediluted with factor V deficient plasma before assay, was suggested (148-150). A predilution of 1 in 4 of sample plasma with factor V deficient plasma seems to provide sufficient amounts of vitamin K-dependent coagulation factors in the assay to yield normal basal APT times. This modification gives valid results not only for patients on oral anticoagulation but has been found also to provide an improved discrimination for the factor V 506Arg to Gln mutation. For some time this modification has been evaluated, using plasmas from individuals with or without oral anticoagulant therapy and a 100% sensitivity and specificity for the presence of the factor V 506Gln allele was obtained, irrespective of the plasma origin. Thus, the modified test is an excellent screening test for the presence of the factor V 506 Gln allele.

Analysis for the factor V 506 Arg to Gln mutation

The codon for Arg506 is positioned close to the exon-intron boundary in exon 10 of the factor V gene (77). Determination of the G to A mutation involves amplification of this nucleotide region either from genomic DNA or from mRNA. The detection of the point mutation can be made in many ways, e.g., by nucleotide sequencing, by different hybridisation techniques, by restriction enzyme cleavage or by allele specific amplification (4, 6, 80-81, 151-152). The methodology can be optimised to allow analysis of a large number of samples every day. The rate limiting step is usually the preparation of patient DNA, even though rather simple extraction procedures can be used. It is important to recognise the risk of contamination of PCR-based assays and hence it is of utmost importance to organise the work carefully and include both positive and negative controls.

The APC-R test versus the factor V gene mutation test

Optimal evaluation of a single patient requires both the functional APC-R test and the factor V gene mutation analysis to be used, because the two methods provide complementary information. However, for economical and practical reasons it will not always be possible to do both tests on all individuals. Until more experience is obtained by individual investigators, it seems that a rational approach would be to perform parallel APC-R tests in the absence and presence of factor V deficient plasma until confidence is obtained with the latter. Apart from allowing analysis of plasma from individuals on oral anticoagulant therapy, this approach will also decrease the need for confirmatory genetic testing.

When is it appropriate to perform the laboratory evaluation?

At present most testing is performed after a thrombotic episode and also after discontinuation of the oral anticoagulation. Most of the assays have their highest sensitivity and specificity for the genetic defects if this is strictly followed. Testing for PC and PS is not reliable during oral anticoagulation and it is preferable that the patient has been off this kind of therapy for at least 10 days. In rare cases it is possible to discontinue the therapy for 10 days during which the patient receives heparin (standard or low molecular weight heparin). A sample can be drawn on the morning of the tenth day before the heparin administration. It will be interesting to evaluate whether prophylactic testing of APC-R before surgery, oral contraception, hospitalisation will be beneficial. Highly sensitive and specific assays for APC-R with high predictive value are available.
and inexpensive. The main reasons why the other genetic defects are less interesting to evaluate for prophylactic screening are the relatively low levels of sensitivity and specificity, which gives low predictive value of a positive test. Cost-benefit analysis are required in the evaluation of prophylactic testing.

To date it has not been recommended to perform the laboratory investigation in association with the acute thrombotic event mainly because most assays are difficult to interpret and the therapeutic regimes may influence results of the assays. This recommendation may need to be re-evaluated with the availability of the modified highly specific and sensitive APC-R test (with factor V deficient plasma) and factor V genotyping.

Where should the laboratory investigation be performed?

The specialised assays for anticoagulant protein deficiencies are preferably performed in specialised coagulation units whereas it is a matter of debate where the APC-R testing should be done. Time will give the APC-R testing its right role in practical medicine and it will at this stage become obvious whether the testing should be performed not only in specialised laboratories but also in major or smaller hospitals.

Who should be tested?

This question is related to the questions what to test and why. It is recommendable that testing is performed on patients with thrombosis and possibly also on their first degree family members. It is valid to conclude that there are at present no data to support general screening of the population for APC-R. Whether prophylactic APC-R testing is beneficial in association with risk situations is yet to be evaluated.

Why should we test for genetic defects predisposing for thrombosis?

For each category of individuals it is important to ask the question why the testing is performed. The most pertinent question is whether the results of the assays will affect the handling of the patients. This is true for both general screening and for investigation of thrombosis patients. The rapid expansion of knowledge in this field will bring new therapeutic recommendations for individuals with genetic predisposition for thrombosis. There are two major reasons for testing of family members of patients with thrombosis, the prophylactic and diagnostic purposes. The prophylactic aspects relate to the possibilities to give adequate advice to family members and the diagnostic purposes to make reliable diagnosis of inherited defects.

6. CLINICAL MANIFESTATIONS OF INHERITED THROMBOSIS

The most common clinical problem is deep vein thrombosis of the lower limbs, with or without pulmonary embolism, accounting for approximately 90% of all the thrombotic episodes, Table 4. Unusual sites of venous thrombosis, such as those in the mesenteric or cerebral veins, account for less than 5% of the total episodes in patients with antithrombin, PC or PS deficiencies; in patients with APC-R, thrombosis seems to occur less frequently at these sites. Superficial thrombophlebitis is more frequent in patients with PC or PS deficiency and APC-R than in antithrombin deficient patients (153-156). Even though a role for PS deficiency as a risk factor for arterial thrombosis
has been postulated, there is little current evidence that this or other heterozygous defects of the anticoagulant systems increase the risk of arterial thrombosis (155-156).

There is history of thrombosis at diagnosis in 50-60% of individuals with antithrombin, PC and PS deficiencies, with a 50% recurrence rate; the first thrombotic episode occurs as early as before 40 years in approximately 80% of patients. In antithrombin deficiency, the overall risk of venous thrombosis is considered greater than in PC or PS deficiency (157), but contradictory results have been obtained (156). On the other hand, individuals with APC-R appear to have a lesser tendency to thrombosis than those with the defects of the naturally occurring anticoagulants. There is history of thrombosis in only 23% to 31% of cases with APC-R (117, 151) and only 30% of them develop thrombosis before age 45 (158). The presence of APC-R however magnifies the risk of thrombosis in patients with antithrombin, PC and PS deficiencies (135-137), as discussed earlier.

In antithrombin, PC and PS deficiencies, 32% to 50% of the venous thrombotic episodes occur when other risk factors are concomitantly present (surgery, pregnancy, immobilization) (153-156). In individuals with APC-R, the need of the existence of such risk factors to trigger thrombotic episodes appears to be greater (62%) than for the other thrombophilic syndromes (151).

The risk factors that are more often associated with the occurrence of thrombosis are pregnancy, the puerperium and surgery (151, 153-154, 156). In women with antithrombin deficiency, the frequency of thrombosis during pregnancy and the puerperium is between 37% and 44%; in PC or PS deficiency, between 12% and 19% (122, 159); in APC-R, 28% (160). Thrombotic episodes occur most frequently during the puerperium, accounting for 60-75% of all the episodes complicating pregnancy (122, 159). A retrospective analysis of a large number of antithrombin, PC or PS deficient individuals gave an overall frequency of venous thrombosis complicating surgery of 22%, with no significant differences due to the type of deficiency or surgical procedure (159). Intake of oral contraceptives is associated with an increased thrombotic risk, particularly in women with antithrombin deficiency and APC-R (139-140). Patients with dysfunctional defects of antithrombin, PC and PS have thrombotic risks similar to those of the corresponding quantitative defects. A notable exception is the antithrombin HBS type II subtype, with a prevalence of thrombosis in these (heterozygous) cases of only 6%, contrasting with 52-68% in patients with other types of antithrombin deficiencies (19).

Homozygous antithrombin deficiency is extremely rare and almost exclusively reported in patients with HBS defects. These individuals have a severe thrombotic history of early onset, often affecting arteries (161). Homozygous type I antithrombin deficiency is, presumably, incompatible with life: in one report, two brothers with this defect died within three weeks of birth (162). Homozygous PC deficiency has peculiar phenotypic and clinical expressions (reviewed in (163)). In patients with unmeasurable PC, purpura fulminans, due to thrombosis of small vessels with cutaneous and subcutaneous ischemic necrosis, may occur soon after birth or in the first year of life (164, 165). In patients with very low but measurable PC (5-20%), clinical manifestations are milder and generally similar to those for heterozygous deficiency (166). Homozygous PS deficiency has been rarely reported, but is also associated with neonatal purpura fulminans (167). Due to the high frequency of the mutant factor V in the general population, homozygous APC-R is relatively frequent, ~1: 5,000 (5). Whether homozygotes have a risk of arterial thrombosis greater than that of the general population remains to be elucidated (118, 168).
MANAGEMENT OF INHERITED THROMBOPHILIA

Acute Events

The management of acute venous thrombosis or pulmonary embolism in patients with inherited thrombophilia is generally not different from other patients. Thrombolytic therapy can be used in patients with massive acute venous thrombosis or pulmonary embolism. Heparin should be initiated with an intravenous bolus of 5,000 units followed by an infusion of 1400 units per hour (169), or if a weight adjusted regimen is used, a bolus of 80 units per kg body weight followed by an infusion of 18 units per kg per hour (170). The APTT should be performed approximately 6 hours after therapy is initiated, and at least daily thereafter to maintain the clotting time in the therapeutic range. For many commercial APTT reagents, this corresponds to an APTT that is 1.8 to 3.0 times the mean of the normal range, or an anti factor Xa heparin level of 0.3 to 0.7 units per ml (171). For less sensitive APTT reagents, the therapeutic APPT ratio is 1.5 to 2.0 (172). Warfarin can be started within the first 24 hours. Heparin is continued for at least 5 days (173) or until the prothrombin time is in the therapeutic range, namely an International Normalized Ratio (INR) of 2.0 to 3.0.

Patients with antithrombin deficiency can usually be treated successfully with intravenous heparin (174), though in some situations unusually high doses of the drug are required to achieve adequate anticoagulation. In antithrombin deficient patients receiving heparin for the treatment of acute thrombosis, the adjunctive role of antithrombin concentrate purified from human plasma is not clearly defined, as controlled trials have not been performed (174). This product should probably be administered when difficulty is encountered in achieving adequate heparinization, or recurrent thrombosis is observed despite adequate anticoagulation. It is also reasonable to treat antithrombin deficient subjects with concentrate before major surgeries or in obstetrical situations where the risks of bleeding from anticoagulation are unacceptable. The manufacturing processes used to prepare antithrombin concentrate result in a product that is greater than 95% pure; they also inactivate the hepatitis B and C viruses and human immunodeficiency virus I (175-176). Hence, it is preferable to administer antithrombin concentrate rather than fresh frozen plasma.

The infusion of 50 units of antithrombin concentrate per kilogram of body weight (one unit is defined as the amount of antithrombin in one ml of pooled normal human plasma) will usually raise the plasma antithrombin level to approximately 120% in a congenitally deficient individual with a baseline level of 50% (176-180). Plasma levels should be monitored to ensure that they remain above 80%; the administration of 60% of the initial dose at 24 hour intervals is recommended to maintain inhibitor levels in the normal range (180).

Due to the infrequent occurrence of coumarin induced skin necrosis, it may be advisable to take special precautions when initiating oral anticoagulant treatment in a patient who is previously known or likely to have PC deficiency. Warfarin should be started only when the patient is fully heparinized and the dose of the drug should be increased gradually, starting from a relatively low level (e.g., 2 mg for the first 3 days and then in increasing amounts of 2 to 3 mg until therapeutic anticoagulation is achieved). Patients with heterozygous PC deficiency and a history of warfarin-induced skin necrosis have been successfully retreated with oral anticoagulants. Here PC administration either in the form of fresh frozen plasma or PC concentrate provides protection against the development of recurrent skin necrosis until a stable level of anticoagulation is achieved (181-182).
After an episode of venous thrombosis or pulmonary embolism, patients are usually continued on oral anticoagulants for 3 to 6 months. Recent data indicates that the risk of recurrence is greater in patients with permanent as opposed to temporary risk factors for thrombosis (183a, 184) and it is therefore appropriate that warfarin should be continued for at least 6 months at an INR of 2.0 to 3.0 in patients with inherited thrombophilia.

After 6 months of anticoagulant treatment for an acute thrombotic event, an assessment must be made as to the relative benefit conferred by long term anticoagulant therapy in preventing future thromboembolic complications versus the potential side-effects, cost, and inconvenience for the patient. Unfortunately there is a paucity of reliable data regarding the magnitude of the thrombotic risk or the benefit of anticoagulant treatment in patients with deficiencies of antithrombin, PC, or PS as these are relatively uncommon disorders. Due to the high frequency of APC-R in patients presenting with a first episode of venous thrombosis, reliable data is just emerging regarding the risk of recurrence (118). At this time however, only general guidelines for managing patients with the various hereditary defects predisposing to thrombosis will be proposed rather than to provide rigid recommendations.

Inherited thrombotic disorders and recommendations pertaining to duration of anticoagulant treatment

When a heterozygous patient with one of the hereditary thrombotic disorders is identified, family studies should be conducted since approximately half of their first-degree relatives will be affected. Affected asymptomatic individuals should receive counselling regarding the implications of the diagnosis and advice regarding symptoms that require immediate medical attention. In women of child-bearing age, oral contraceptives are contraindicated in view of the increased thrombotic risk associated with the use of these medications. The replacement dose of estrogens that is administered to postmenopausal women is much lower than the contraceptive dose and has not been shown to increase the risk of venous thrombosis in the general population (185). As there are not currently data indicating that these medications increase the risk of thrombosis in patients with a hereditary thrombotic disorder, postmenopausal estrogen replacement is not absolutely contraindicated in women who have a strong indication for replacement therapy.

All biochemically affected individuals should be carefully evaluated prior to surgical, medical or obstetric procedures that carry an increased thrombotic risk. These subjects should then receive appropriate prophylactic anticoagulation regimens. If specific concentrates are available for the patient’s deficiency state, under some restricted circumstances these might also be administered to raise the plasma levels of the protein to the normal range during the peri-operative period.

In patients with an inherited thrombotic disorder, the occurrence of two or more spontaneously occurring thromboembolic episodes generally leads to the continuation of oral anticoagulants for life.

Given that future events in an asymptomatic patient or in an individual with only one prior thrombotic episode cannot currently be accurately predicted and that there is a finite risk of bleeding associated with warfarin therapy, recommendations relating to long-term anticoagulation are best individualized at the current time (186-187). The clinical features that should be considered in making this decision include:
1. Whether the thrombotic episodes were spontaneous or whether precipitating factors were present; e.g., if a precipitating event such as a major abdominal operation was present, it would be reasonable to manage the patient without long-term oral anticoagulation after the acute episode was adequately treated;

2. The sex and lifestyle of the individual; e.g., situations where these factors may influence the decision-making process include: women of child-bearing age planning to conceive, occupations that entail prolonged periods of immobilization and thereby might be associated with an increased risk of thromboembolism, jobs with higher than average chance of trauma that might lead to thrombotic or bleeding complications; and

3. A history of thromboembolism in other biochemically affected members of the family though marked intra- and inter-familial heterogeneity has been observed in the phenotypic expression of the inherited thrombotic disorders.

4. The number, sites, and severity of thrombosis; e.g., a patient who previously sustained a massive pulmonary embolus is more likely to receive long-term warfarin than a subject who developed deep venous thrombosis in a calf vein.

Management of pregnancy

The management of pregnancies in women with hereditary thrombotic disorders poses special problems (121). The incidence of thrombotic complications during pregnancy and the postpartum period appears to be greater in women with antithrombin deficiency than in those with deficiencies of PC or PS (122). Recent data also indicates that 60% of women who develop a first episode of venous thrombosis during pregnancy have a diagnosis of APC-R (144). During pregnancy, adjusted dose heparin administered by the subcutaneous route is the anticoagulant of choice in many Centres because its efficacy and safety for the fetus are established (188). Centres with increasing experience of low MW heparins, however, might find some advantage in the use of these agents as laboratory monitoring may not be required. Patients with a history of thrombotic episodes should receive treatment throughout pregnancy, while affected women with antithrombin deficiency who have not yet experienced such events should probably receive treatment. Treatment of asymptomatic women with other hereditary thrombotic disorders should be considered on an individual basis.

The dose and duration of heparin therapy in pregnancy is uncertain as appropriately designed clinical trials have not been performed in these patient populations. Patients considered to be at high risk should receive full-dose heparin by subcutaneous injection every 12 hours for the duration of pregnancy. The dose of heparin should be adjusted to maintain the 6 hour postinjection-APTT 1.5 times the control value. In women considered to be at intermediate risk, lower doses of heparin can be used (5,000 to 10,000 units subcutaneously every 12 hours) and therapy can be started during the second or third trimester and continued for approximately 6 weeks into the postpartum period. Low risk patients can be observed closely throughout the pregnancy with duplex ultrasound imaging of the leg veins at regular intervals.

In women who are planning pregnancy while chronically taking oral anticoagulants, several approaches can be taken to minimize the risk of both thrombotic complications and warfarin embryopathy. One is to stop warfarin and commence subcutaneous heparin therapy; this
potentially exposes the patient to many months of heparin therapy and the risk of osteoporosis while she is trying to conceive. An approach in women with antithrombin deficiency is to use antithrombin concentrates until conception. This product, however, is costly and needs to be administered intravenously at frequent intervals. Finally, warfarin therapy could be continued with the performance of pregnancy tests on a frequent basis. As soon as pregnancy is diagnosed, and prior to the sixth week of gestation, oral anticoagulants must be discontinued and heparin therapy initiated. Though the risk of warfarin embryopathy appears to be quite small during the first six weeks of pregnancy (189), even the small risk of this complication makes this the least preferable of the three approaches.

Coumarin-induced skin necrosis and neonatal purpura fulminans

A clear association has been established between the rare complication of coumarin induced skin necrosis and hereditary PC deficiency (181, 190). About a third of patients with coumarin induced skin necrosis will prove to have hereditary PC deficiency (191). This complication has also been described in a patient with heterozygous PS deficiency (192). As coumarin induced skin necrosis is a rare complication, therapy has been guided primarily by knowledge regarding its pathogenesis. The diagnosis should be suspected in patients with painful, red skin lesions developing within a few days after the initiation of the drug and immediate intervention is required to prevent rapid progression and reduce complications. Therapy should consist of immediate discontinuation of warfarin, administration of vitamin K, and infusion of heparin at therapeutic doses. Lesions, however, have been reported to progress despite adequate anticoagulation with heparin. In patients with hereditary PC deficiency, the administration of a source of PC should be seriously considered, and it may also be appropriate in other patients with warfarin-induced skin necrosis as they invariably have reduced plasma levels of functional PC when the skin lesions first appear. Fresh frozen plasma has been used, but improved results can be expected with the administration of a highly purified PC concentrate, which facilitates the rapid and complete normalization of plasma PC levels=20(193).

The management of neonatal purpura fulminans in association with homozygous or doubly heterozygous PC deficiency is more complicated and heparin therapy as well as antiplatelet agents have not been shown to be effective (164, 194-196). The administration of a source of PC appears to be critical in the initial treatment of these patients. Fresh frozen plasma has been used with success to treat these infants. However, the half-life of PC in the circulation is only about 6 to 12 hours (197-198), and the administration of plasma on a frequent basis is limited by the development of hyperproteinemia, hypertension, loss of venous access, and the potential for exposure to infectious viral agents. A highly purified concentrate of PC has been developed and is efficacious in treating neonatal purpura fulminans (199). Warfarin has been administered to these infants without the redevelopment of skin necrosis during the phased withdrawal of fresh frozen plasma infusions (164, 196, 200-202), and this medication has been used chronically to control the thrombotic diathesis. A 20 month old child with liver failure and homozygous PC deficiency has undergone successful liver transplantation which normalized his PC levels and resolved the thrombotic diathesis (203). Neonatal purpura fulminans has been described in association with homozygous PS deficiency (204-205).
8. PRENATAL DIAGNOSIS IN INHERITED THROMBOPHILIA

Prenatal diagnosis can only be considered appropriate in possible cases of severe thrombophilia where the fetus is expected to be homozygous or compound heterozygous for inhibitor deficiency. Only in a few instances has prenatal diagnosis been performed, in attempts to avoid severe thrombophilia. The initial procedure used was fetal blood sampling by cordocentesis during the 19th week of gestation in a woman who had previously delivered two newborns who died of massive thrombosis and purpura fulminans due to homozygous PC deficiency (206). At this stage of gestation the mean level of PC antigen is about 10 u/dl (206-208), and thus, care should be taken in distinguishing between results consistent with heterozygosity with a PC level of about 5 u/dl (206) and homozygosity. In two reports the mean level of free PS antigen at 15-23 weeks of normal gestation was 27 and 38 u/dl, respectively (207, 208), and hence the diagnosis of homozygous PS deficiency by fetal blood sampling should be easier than the diagnosis of severe PC deficiency. Such a procedure has not so far been carried out in cases of suspected severe PS deficiency.

A more direct and precise approach to prenatal diagnosis of severe deficiencies of PC, PS and antithrombin depends primarily on the identification of the mutation or mutations responsible for the disease in the proband and on devising an easy method for its detection, i.e., polymerase chain reaction and restriction analysis or Southern analysis. Such work can be performed in highly specialized centers that have a particular interest in this issue. For identification of the mutation causing PC deficiency a scanning method was devised using denaturant gradient gel electrophoresis (DGGE) of 13 PCR amplified fragments that cover exon I and most of the PC coding regions (209). A similar approach was also used for detection of mutations causing PS deficiency (68). Once these prerequisites have been met, prenatal diagnosis can be provided during the 9-10th week of gestation by chorionic villus sampling and DNA analysis. Such an approach was recently used in a family affected by severe PC deficiency (210). Prenatal exclusion of a severe thrombophilia may also be carried out by indirect restriction fragment length polymorphism (RFLP) tracking. Such a procedure was used in a family in which a previous infant died of bilateral renal vein thrombosis and both parents were found to be heterozygotes for PS deficiency (57).

9. INHERITED THROMBOPHILIA IN THE DEVELOPING COUNTRIES

The prevalence and morbidity of thrombophilia in different geographic regions of the world may be different because of genetic differences in populations or because the phenotypic expression of the disease is altered by environmental factors. While several studies on inherited thrombophilia have been published, these should be interpreted with caution, due to many problems involved in the execution of the work. For example, there are concerns with quality control of laboratory assays, with objective diagnosis of thrombosis and with study design. Furthermore, no large epidemiological studies on groups such as blood donors have been performed in developing countries and there are therefore no reasonable estimates of the prevalence of the inhibitor deficiencies.

Despite these reservations, a number of published reports have attempted to address the issues of frequency of venous thrombosis following surgery (211-215) and of hereditary thrombophilia (216-218). The available data suggests that the prevalence of deep venous thrombosis may be less in the developing than in the developed countries. However, there are suggestions that patients who do get venous thrombosis are more likely to have an underlying inhibitor deficiency. Another
potentially interesting aspect is that early onset of stroke may be associated with inhibitor deficiency (219). Further work is clearly required to consolidate these various observations.

10. CONCLUSIONS

1. At the present time, mutations in four genes are clearly linked to increased risk for venous thromboembolism. Many discrete mutations cause deficiencies of antithrombin, PC and PS that diminish the capacity to balance procoagulant activity. One specific mutation in factor V 506Arg to Gln has a similar impact by rendering this procoagulant factor resistant to proteolytic degradation. With these four established risk factors for thrombosis, roughly 50% of the familial thrombophilias can be explained. Apparently a number of other genetic risk factors so far has escaped detection. It is however unlikely that these will be found among deficiencies of plasminogen, heparin cofactor II, tissue factor pathways inhibitor or b2 glycoprotein. Other candidates will need further evaluation (for instance, dysfibrinogenemia, thrombomodulin defects and inherited hyperhomocysteinemia). It is increasingly apparent that co-inheritance of more than one relatively mild thrombophilic risk factor causes more severe clinical expression.

2. Increasingly, attempts are being made to assign a magnitude to genetic and acquired risk factors. The risks estimates that have been found in various studies depend heavily on the way the subjects for those studies were selected and do not necessarily apply to individuals selected differently. In particular, results from studies among selected families with striking thrombophilia are probably overestimating the risk when applied to individuals who were found because of a single thrombotic event. The most stringent selection criteria have been used to ascertain families with the rare abnormalities (PC, PS or antithrombin), which more readily explains the differences found in family studies on these disorders as compared to APC-R than a true difference in severity. Finally, when gene-gene and gene-environment interactions are required to bring about thrombosis, there will be differences within families as much as between families.

3. Laboratory evaluation of thrombophilia should involve the use of assays with highest possible sensitivity and specificity for the genetic defect that is to be detected. Assays can be of immunological and functional nature, in the case of immunological assays it should be realized that cases with truly functional defective proteins may not be detected. A practically useful approach should be taken and the selection of analytical procedures should be governed by the aim of the investigation as well as of locally determined factors such as prevalence of the genetic defects to be detected and availability of technical support. Based on available scientific information, the laboratory evaluation should include measurements of PC, total and free PS, antithrombin and a functional APC-R test which is sensitive and specific for the presence of the factor V 506Gln allele.

In the case of PC, assays which are based on its activation with the PC activator Protac and measurements of the active enzyme with synthetic substrate, fulfill required quality criteria. At present no functional PS assays can be recommended for general screening of thrombophilic patients. Immunological assays of total as well as of free PS are recommended. Recently published results indicate free PS to be the best marker for genetically determined PS deficiency but further studies are required before a
recommendation only to measure free PS can be made. The functional assays for antithrombin which are based on heparin stimulated inhibition of factor Xa are recommended for screening of thrombophilic patients. For initial screening of APC-R, functional tests are recommended. The test can be improved by diluting the patient plasma in FV-deficient plasma. As assays for PC and PS have distinctly lower sensitivity and specificity for the presence of inherited deficiency during the acute thrombotic episode and oral anticoagulation, it is at present recommended to perform the laboratory investigation for these components after discontinuation of the therapy.

4. The clinical manifestations of the defects of naturally-occurring anticoagulant systems (AT, PS and PC deficiencies; APC-R) are similar. In heterozygotes, typical manifestations are those of venous thromboembolism, such as deep-vein thrombosis of the legs, pulmonary embolism and superficial thrombophlebitis. Visceral and cerebral vein thrombosis are rarer but quite typical for inherited thrombophilia. Patients with homozygous defects have usually more severe clinical manifestations with an earlier age of onset. Some manifestations are quite typical for homozygous defects, such as skin necrosis and widespread neonatal thrombosis in PC and PS deficiencies. There are preliminary data suggesting that some homozygous defects (antithrombin type II HBS deficiency) may be also associated with an increased risk for arterial thrombosis in the young, but more data on this and other homozygous deficiencies are warranted to establish any relationship with arterial disease.

5. When a symptomatic patient with inherited thrombophilia due to a known genetic defect is identified, family studies should be conducted since approximately half of their first-degree relatives will be affected. Asymptomatic individuals who carry the genetic defect should receive counselling regarding the implications of the diagnosis and symptoms that require medical attention. In general, the management of symptomatic individuals with the genetic defect is similar to that for symptomatic patients without an identifiable genetic defect. An exception is patients with neonatal purpura fulminans in association with homozygous or doubly heterozygous PC deficiency in whom the administration of a source of PC is critical in initial treatment. Given that future thrombotic events in patients with inherited thrombophilia cannot be accurately predicted and there is a finite risk of bleeding associated with anticoagulant therapy, recommendations relating to long-term treatment are best individualized at the current time.

6. Once an individual is defined as being affected by hereditary thrombophilia as many family members as possible are examined for the particular defect detected in the proband and a pedigree is constructed. Family members who are found to be affected are counselled about the risk of thrombosis. An evaluation of the potential risk of birth of severely affected newborns is usually carried out in families in which intermarriage is practised, and consequently counselling, extensive carrier detection and prenatal diagnosis is planned and executed.

The target families for prenatal diagnosis of hereditary thrombophilias are those families that had already been afflicted by infants with severe thrombosis due to homozygosity or compound heterozygosity for PC, PS or antithrombin deficiency, as well as those families mentioned above. In these target families an attempt is made to detect the responsible mutation(s), and to devise a simple method for their detection, e.g., PCR and restriction analysis or Southern analysis. This is followed by an extensive study of family members.
in the child-bearing age for carrihership and as a consequence counselling is provided. Prenatal diagnosis is then based on DNA obtained by chorionic villus sampling or amniocentesis.

In instances in which the mutation cannot be identified, specific RFLP tracking in family members and eventually in fetal DNA is an alternative approach. In still other instances of PC or PS deficiency fetal blood sampling at 18-22 weeks of gestation is an option since data on the levels of these components in normal fetuses are available. For antithrombin such values are unavailable and thus at present fetal blood sampling for affected families cannot be offered, unless the specific mutation has been identified.

7. Deep vein thrombosis and pulmonary embolism have a lower incidence in the developing world when compared to the West, and this may be due to a combination of racial and environmental factors. There are limited studies with complete laboratory evaluation on inherited thrombophilia from the developing countries. It appears that there is a higher chance of finding an underlying genetic defect (PC, PS and antithrombin deficiency) in patients with thrombosis in the developing world. Preliminary data on APC-R suggests that this defect is rare in Asians, Africans and Chinese.

11. RECOMMENDATIONS

1. Recent reports in the literature support the hypothesis that familial thrombophilia is a multiple gene disorder and that the penetrance of the disease is higher in carriers of multiple gene defects. In relation to this it is to be expected that prophylactic and therapeutic measures need to be adjusted to the number of independent risk factors present in an individual. Therefore efforts should be intensified to identify those genetic risk factors that so far have escaped detection, so that these can be included in diagnostic screening procedures.

2. Guidelines need to be developed for the use of specific laboratory tests in screening procedures aiming at the identification of individuals who carry a genetic risk factor for venous thrombosis.

3. Given the large number of patients that must be followed to detect new abnormalities, collaborative international investigations with standardized recruitment protocols should be encouraged.

4. More information linking genetic causes of hyperhomocysteinemia to venous thromboembolism is necessary.

5. An estimate of the prevalence of PS deficiency in the population would help evaluate its relative risk.

6. More specific recommendations need to be developed for the classification of hereditary PS deficiency. Within this context it needs to be established whether so called type III PS deficiency is an independent risk factor for venous thrombosis or a different phenotype of type I PS deficiency.
7. As recent data suggest that measurement of free PS may be more valuable than total PS assays for the diagnosis of PS deficiency, it is recommended that studies are performed which evaluate the performance of different methods for determination of free PS.

8. General screening of the population for genetic defects of PC, PS and antithrombin can at present not be recommended mainly based on the low prevalence of these defects in the population and on the low predictive value of a positive test. It is recommended that studies are performed which address the question whether general screening for APC-R (FV:506Gln allele), e.g., before oral contraception, surgery or hospitalization, is beneficial for the decision on therapeutic and prophylactic regimes.

9. Further clinical studies should be organized to evaluate whether defects causing inherited thrombophilia are a cause of or contribute to arterial thrombosis.

10. Heterozygous deficiencies of PC, PS or antithrombin should not be the target of prenatal diagnosis. In view of the very low expected frequency of the severe homozygous or compound heterozygous defects in PC, PS and antithrombin in the general population, it is not recommended to carry out population screening for carriership.

11. It would be preferable to establish reference centres where work up of the mutations and DNA based prenatal diagnosis can be performed in cases with severe defects.

12. It is necessary to obtain accurate data on the frequency and impact of thrombophilia in the developing world. In order to do this individual laboratories should be identified in different regions and these should develop the necessary expertise to screen for and document the genetic defect responsible, in association with WHO Collaborating Centres.

13. For the present screening should be done on all patients with venous thromboembolism in the context of a study to determine the percentage of patients with hereditary thrombophilia in the developing world where thrombotic disorders in general appear to have a low prevalence. Family studies should be done in all patients in whom a genetic defect is documented.

14. Since facilities for screening may be available only in reference centers treatment should be initiated without delay where appropriate and tests performed in the reference center after anticoagulants have been discontinued.

15. Data on the thrombotic risk in patients and asymptomatic family members with thrombophilia should be collected to determine whether the risk profile is different in the developing world.

16. Careful documentation of the risk of hemorrhage on anticoagulants is necessary in order to determine the risk benefit ratio of therapeutic intervention in patients with thrombophilia.

17. Recommendations for screening for hereditary thrombophilia at a national level in health care services can be made only after adequate data on the epidemiology, risk of thrombosis and result of therapeutic intervention is available.
18. Since there is a higher incidence of stroke in the young in the developing world this population needs to be studied in order to determine the number of patients who have thrombophilia as the underlying cause.

19. In order to increase awareness of inherited thrombophilia in developed and developing countries, it is proposed, in cooperation with the ISTH, to select a centre for designation as a WHO Collaborating Centre to improve diagnosis, clinical recognition and treatment of related thrombosis. This Centre will serve as a reference centre for an appropriate WHO Region and will improve education of both health professionals and the general public.

20. The next meeting of WHO/ISTH experts focusing on the clinical problems of inherited thrombophilia might be organized at the proposed centre for designation as a WHO Collaborating Centre. A training course on the diagnosis for thrombophilia for local doctors and invited participants from countries within the WHO Region should also be foreseen at the same time.
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14. REFERENCES


134. Greengard JS, Eichinger S, Griffin JH, Bauer KA. Variability of thrombosis among homozygous siblings with resistance to activated protein C due to an Arg-->Gln mutation


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

Risk Factors
Genetic + Genetic

Risk Factors
Genetic + Acquired

Venous thromboembolism
<table>
<thead>
<tr>
<th>Acquired</th>
<th>Acquired/Inherited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Elevated lipoprotein (a)</td>
</tr>
<tr>
<td>Prior thrombus</td>
<td>Elevated factor VIII</td>
</tr>
<tr>
<td>Immobilization</td>
<td>Elevated fibrinogen</td>
</tr>
<tr>
<td>Foreign surfaces*</td>
<td>Elevated histidine rich glycoprotein*</td>
</tr>
<tr>
<td>Malignancy</td>
<td>Elevated factor VII*</td>
</tr>
<tr>
<td>Cancer chemotherapy</td>
<td>Elevated plasminogen activator inhibitor*</td>
</tr>
<tr>
<td>Obesity*</td>
<td>Hyperhomocysteinemia</td>
</tr>
<tr>
<td>Atherosclerosis*</td>
<td>Inherited</td>
</tr>
<tr>
<td>Use of oral contraceptives</td>
<td>Disfibrinogenemia</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
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<tr>
<td>Hyperlipidaemia*</td>
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<td>Myeloproliferative disorders</td>
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</tr>
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<td>Antithrombin deficiency</td>
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<tr>
<td>Diabetes*</td>
<td>PC deficiency</td>
</tr>
<tr>
<td>Antiphospholipid syndrome</td>
<td>PS deficiency</td>
</tr>
<tr>
<td>Paroxysmal nocturnal haemoglobinuria</td>
<td>APC-R (factor V 506 Arg to Gin)</td>
</tr>
<tr>
<td>Polycythemia vera and stress polycythemia</td>
<td></td>
</tr>
</tbody>
</table>

¹This table lists conditions often considered to be associated with thrombophilia, but should not be taken to imply that firm evidence for a causal relationship is available. Certainly, there is no clear evidence for the conditions marked with an asterisk.
### Table 2

"PROS" GENE SEQUENCE VARIATIONS IN PATIENTS WITH PHENOTYPIC PS DEFICIENCY

<table>
<thead>
<tr>
<th>exon</th>
<th>codon, sequence mutation</th>
<th>predicted mutation</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ins T</td>
<td>-25, Leu → Leu</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>frameshift, stop</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>G → T</td>
<td>-2, Arg → Leu</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>G → A</td>
<td>-1, Arg → His</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>C → A</td>
<td>22, Cys → stop</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>A → C</td>
<td>26, Glu → Ala</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>T → G</td>
<td>31, Phe → Cys</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>C → T</td>
<td>37, Thr → Met</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>del A</td>
<td>43, Lys → Asn</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td></td>
<td>frameshift, stop</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>G → A</td>
<td>49, Arg → His</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>del T</td>
<td>88, Pro → Pro,</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>frameshift, stop</td>
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<td>103, Thr → Asn</td>
<td>68</td>
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<td>68</td>
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<td>222</td>
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<td>del G</td>
<td>220, Gly → Glu</td>
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</tr>
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<td>T → C</td>
<td>224, Cys → Arg</td>
<td>68</td>
</tr>
<tr>
<td>9</td>
<td>del T</td>
<td>261, Leu → Trp</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>frameshift, stop</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>del G</td>
<td>267, Gly → Gly</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>frameshift, stop</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>G → A</td>
<td>335, Asp → Asn</td>
<td>68</td>
</tr>
<tr>
<td>10</td>
<td>G → T</td>
<td>340, Gly → Val</td>
<td>65</td>
</tr>
<tr>
<td>12</td>
<td>del G</td>
<td>420, frameshift</td>
<td>222</td>
</tr>
<tr>
<td>12</td>
<td>G → T</td>
<td>448, Gly → stop</td>
<td>222</td>
</tr>
<tr>
<td>13</td>
<td>T → G</td>
<td>467, Val → Gly</td>
<td>65</td>
</tr>
<tr>
<td>14</td>
<td>del AC</td>
<td>547-548, frameshift</td>
<td>222</td>
</tr>
<tr>
<td>14</td>
<td>ins T</td>
<td>565, Val → Val</td>
<td>222</td>
</tr>
<tr>
<td>14</td>
<td>T → C</td>
<td>570, Met → Thr</td>
<td>222</td>
</tr>
<tr>
<td>14</td>
<td>ins C</td>
<td>578, Pro → Leu</td>
<td>65</td>
</tr>
<tr>
<td>15</td>
<td>A → T</td>
<td>636 stop → Tyr</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>new stop at 649</td>
<td></td>
</tr>
<tr>
<td>intron 4</td>
<td>nt +1, G → A</td>
<td>donor splice intron 4</td>
<td>222</td>
</tr>
<tr>
<td>intron 5</td>
<td>nt +5, G → A</td>
<td>donor splice intron 5</td>
<td>68</td>
</tr>
<tr>
<td>intron 9</td>
<td>nt-1, G → A</td>
<td>acceptor splice intron 10</td>
<td>222</td>
</tr>
<tr>
<td>intron 10</td>
<td>nt +5, G → A</td>
<td>donor splice intron 10</td>
<td>64</td>
</tr>
</tbody>
</table>
### Table 3

**PREVALENCE OF THE MAJOR THROMBOPHILIC CLOTTING ABNORMALITIES**

<table>
<thead>
<tr>
<th></th>
<th>Protein C deficiency</th>
<th>Protein S deficiency</th>
<th>Antithrombin deficiency</th>
<th>APC-R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy individuals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tait et al (n=9669) [112,115]</td>
<td>0.2% +</td>
<td></td>
<td>0.02% + 1</td>
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</tr>
<tr>
<td>Miletich et al (n=5422) [116]</td>
<td>0.4%</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Svensson and Dahlbäck (n=130)[117]</td>
<td></td>
<td></td>
<td>7%</td>
<td></td>
</tr>
<tr>
<td>Rosendaal et al (n=474) [5]</td>
<td></td>
<td></td>
<td>3% +</td>
<td></td>
</tr>
<tr>
<td>Ridker et al (n=704) [118]</td>
<td></td>
<td></td>
<td>6% +</td>
<td></td>
</tr>
<tr>
<td><strong>Consecutive patients with first DVT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heijboer et al (n=277) [111]</td>
<td>3%</td>
<td>2%</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Koster et al (n=474) [123]</td>
<td>3% +</td>
<td>1%</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Rosendaal et al (n=471) [5]</td>
<td></td>
<td></td>
<td></td>
<td>20% +</td>
</tr>
<tr>
<td><strong>Thrombophilic patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Briët et al (n=113) [223]</td>
<td>8%</td>
<td>13%</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>Scharrer et al (n=158) [224]</td>
<td>9%</td>
<td>6%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>Ben Tal et al (n=107) [225]</td>
<td>6%</td>
<td>3%</td>
<td>7%</td>
<td></td>
</tr>
<tr>
<td>Taberno et al (n=204) [226]</td>
<td>1%</td>
<td>1%</td>
<td>0.5%</td>
<td></td>
</tr>
<tr>
<td>Griffin et al (n=25) [110]</td>
<td></td>
<td></td>
<td></td>
<td>52%</td>
</tr>
</tbody>
</table>

+ DNA confirmed
+ type I Antithrombin deficiency
Table 4

CLINICAL FEATURES OF PATIENTS WITH INHERITED THROMBOPHILIA DUE TO DEFECTS IN THE ANTICOAGULANT PATHWAYS

Venous thromboembolism (> 90% of cases)
- Deep vein thrombosis of lower limbs (common)
- Pulmonary embolism (common)
- Superficial thrombophlebitis
- Mesenteric vein thrombosis (rare but characteristic)
- Cerebral vein thrombosis (rare but characteristic)

Family history of thrombosis*

First thrombosis usually at young age (< 45 yrs.)*

Frequent recurrences*

Neonatal purpura fulminans (homozygous protein C and protein S deficiency)

* All these features are less evident in patients with APC-R, who appear to be less severely affected clinically.