

# Genetic Approach to Thrombophilia

Rogier M. Bertina

Hemostasis and Thrombosis Research Center, Department of Haematology, Leiden University Medical Center, Leiden, The Netherlands

## Key words

Thrombophilia, venous thrombosis, genetic risk factors, polymorphisms

## Summary

Venous thrombosis is a multifactorial disease. Multiple interactions between genetic and environmental factors contribute to the development of the disease. Presently, we know of six or seven genetic risk factors for venous thrombosis. However, together these defects can explain the clustering of thrombotic events in only a small subset of families with thrombophilia. As to the identification of new genetic risk factors for thrombosis, we seem to have arrived at the end of a practicable road with the classical approach of thrombophilia, which usually starts with the study of the association of hemostatic phenotypes and thrombotic risk. At the same time we have undertaken various genetic approaches aiming at identifying polymorphisms/ mutations causing thrombotic risk. This review summarizes what we have learnt so far, what to do and what not to do. The odds for finding remaining common genetic risk factors for venous thrombosis during the next ten years may be predicted to be fairly high.

## Introduction

Thrombophilia is defined as a tendency to develop clots in veins or arteries. Both venous and arterial thrombosis are examples of a complex disease, in which multiple biological pathways contribute to the risk of developing the disease (e.g. blood pressure, blood flow, coagulation, inflammation, atherogenesis). The formation of an occlusive thrombus is the critical event in the acute phase of both diseases; however, the pathogenesis of venous and arterial thrombosis is sufficiently different to consider them as separate diseases. In Western countries thrombophilia is used most frequently in the context of venous thrombosis, to describe a subset of patients with early age of onset, recurrent events, a strong family history of thrombosis, an unusual clinical presentation or the absence of a recognized stimulus (1).

Venous thrombosis is an episodic disease. Present models of venous thrombotic risk hypothesize that a clinical event will occur only when the "thrombosis potential" – which is a function of age, genetic and environmental factors and their interactions (additive or synergistic) – has passed a certain threshold (2). Acquired or environmental risk factors for venous thrombosis include immobilisation, surgery, trauma, use of oral contraceptives and hormone replacement therapy, pregnancy, puerperium and malignancies. One or more of these risk factors are present in 33% of consecutive patients with a first deep-vein thrombosis

(Leiden Thrombophilia Study, unpublished observations). The impact of genetic factors on thrombotic risk is also substantial as illustrated by the many reports of familial clustering of thrombophilia (1, 3) and the finding that 23% of consecutive patients with a first venous thrombotic event report at least one first-degree relative with venous thrombosis (4).

During the past 35 years several genetic risk factors for venous thrombosis have been identified by studying families of thrombophilia patients (antithrombin deficiency, protein C deficiency, protein S deficiency, dysfibrinogenemia, APC-resistance associated with factor V Leiden) (5-10). On the other hand, non-O bloodgroup and the prothrombin 20210A mutation were found to be associated with increased risk of venous thrombosis in population based case-control studies (11, 12). Fig. 1 illustrates the progress in our search for genetic risk factors for venous thrombosis. At the end of the year 2000, we can find at least two genetic risk factors in 13% of the thrombophilia families, one genetic risk factor in 60% of the families and no genetic risk factor at all in 27% of the families. Considering the strong support for familial thrombophilia being an oligogenetic disease (13-19), we must conclude that we still lack information on several genetic factors contributing to the risk of venous thrombosis. Many of us expect that a genetic approach to thrombophilia will help us to identify these gene defects.

## The Classical Approach

During the past decades thrombophilia research has focused on the study of the association of thrombosis with functional phenotypes (isolated, persistent and inherited deficiencies or defects) (3, 20). These phenotypes were selected on the basis of their hypothetical effect on fibrin formation and/or degradation in the context of the thrombohaemorrhagic balance theory. Most of these studies used families recruited through symptomatic probands. Once an association was established between thrombosis and phenotype, the latter served as starting point for the search of the genes involved in its expression. This was a relatively easy task for simple phenotypes such as inherited reduced amounts and/or activity of antithrombin (21, 22), protein C (23-25) and protein S (26, 27). It was more difficult to find the gene responsible for the phenotype of APC-resistance (reduced sensitivity to APC). Careful selection of the candidate gene followed by linkage analysis in a large family with APC-resistance, demonstrated that APC-resistance was completely linked to a marker in the gene coding for factor V and was caused by a mutation in exon 10 of the factor V gene which predicts the replacement of Arg506 by Gln (Factor V Leiden) (10). However, later studies indicated that the sensitivity of a plasma to APC is also possibly influenced by other factor V alleles (R2-allele, R485K) or by non-O blood group alleles (via their effect on plasma factor VIII levels) (28-30). Unfortunately the degree to which these alleles will influence the APC sensitivity ratio is largely dependent on the precise formula of the laboratory test used to measure this phenotype.

More recently, other complex phenotypes have been reported which in population-based case-control studies were found to be associated with increased risk of venous thrombosis: elevated plasma levels of

Correspondence to: Rogier M. Bertina, Hemostasis and Thrombosis Research Center, Leiden University Medical Center, C2-R, P.O. Box 9600, 2300 RC Leiden, The Netherlands – Tel.: +31 71 5261893; Fax: +31 71 5266755; E-mail: R.M.Bertina@lumc.nl

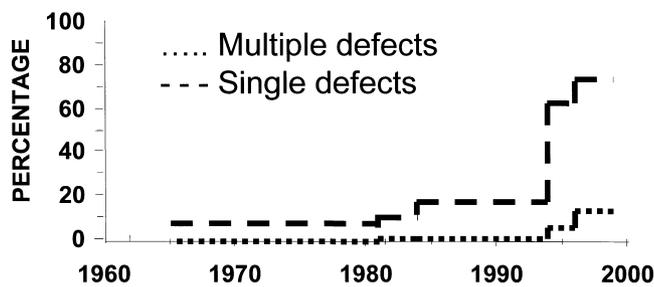


Fig. 1 Genetic defects in familial thrombophilia. The percentage of thrombophilic families known to have single and multiple genetic defects plotted as function of time. Hallmarks are the years that a new genetic defect was first reported: 1965 (antithrombin deficiency), 1981 (protein C deficiency), 1984 (protein S deficiency), 1994 (factor V G1691A), 1996 (prothrombin 20210A)

homocysteine (31), factor VIII (32), factor XI (33), factor IX (34) and possibly TAFI (35). Finding the genes that contribute to these functional phenotypes will be a major effort and is hardly feasible without a genetic approach. Retrospectively the classical approach for finding genetic risk factors for venous thrombosis has been relatively successful by limiting genetic analysis to only those functional phenotypes that were associated with increased thrombotic risk. As mentioned before, candidate risk-phenotypes have been and still are selected on the basis of our understanding of the regulation of coagulation and fibrinolytic processes. However, the number of candidate phenotypes left is very limited and we have to accept that pathways and proteins outside the coagulation system may be involved in the pathogenesis of venous thrombosis. For the identification of these proteins a genetic approach seems to be more promising.

### The Genetic Approach

Although we seem to have arrived at the end of a practicable road with the classical approach of thrombophilia, we hope that the genetic approach will help us to identify the remaining mutations and polymorphisms contributing to the interindividual variation in thrombotic risk (both risk factors and protecting factors). The general perspective is to generate a list of all the genetic factors that contribute to the devel-

opment of thrombotic events. Ideally, such a list will help to improve our understanding of the mechanism of thrombus formation in a variety of different environments and to design strategies for treatment and prevention tailored to the genetic profile of the individual (36). Whether the latter objective is meaningful in the context of venous thrombosis is still a matter of debate (37, 38). More discussion and especially more data are needed before we can define the role of genetic testing in the management of thrombophilia (39). Hopefully this debate will end before genetic screening of thrombophilia has become an established routine in our laboratories.

Interestingly, there is an increasing interest in finding genetic factors which influence the response of a patient to oral anticoagulant therapy (40-44). In this context, genetic testing might contribute to a reduction in bleeding risk, and indirectly to the possibility of developing more personalized treatment protocols (duration, intensity and type of treatment).

During the past decade thrombophilia researchers have explored the prospects and feasibilities of a genetic approach and I will try to summarize their achievements below. In the genetic approach, the study of the relationship between particular genotypes and the clinical phenotype precedes the study of the relationships between genotype and functional phenotype and between functional phenotype and clinical phenotype. Of course there are often hybrid approaches in which functional polymorphisms are selected as candidate risk markers.

From the very beginning it was realized that venous thrombosis is a multifactorial disease and that this would add many complications to the genetic analysis of the disease, especially for the identification of thrombosis susceptibility genes in affected families with linkage or allele sharing methods. Fortunately we were well prepared by previous findings. We already knew that mutations in different genes (often coding for proteins in the anticoagulant pathways) can contribute to the same clinical phenotype and that carriers of the same gene mutation can have different clinical phenotypes (deep vein thrombosis, pulmonary embolism, superficial thrombophlebitis, cerebral vein thrombosis, or thrombosis in mesentery or retina) (21, 23, 26, 45). From the study of large collections of families with protein C -, protein S- or antithrombin deficiency, it was also learnt that the penetrance of the disease in mutation carriers is incomplete and largely dependent on the presence of other factors like age, environment or other mutations (21, 25, 26, 45). In general, thrombosis pedigrees will contain individuals who carry a disease allele but are still asymptomatic. On the other hand, we know

Table 1 Genetic risk factors for venous thrombosis

risk factor	prevalence (%)		
	healthy* controls	consecutive* patients	selected** patients
factor V 1691A	3	20	45
prothrombin 20210A	2.3	6.2	18
protein C deficiency	0.8	3.1	5.7
protein S deficiency	1.3	1.1	5.7
antithrombin deficiency	0.2	1.1	4.3
non-O blood group	57	73	-

\*Data are derived from the Leiden Thrombophilia Study on 474 consecutive patients with a first deep vein thrombosis and 474 healthy controls (12, 32, 95, 128). In this study, protein S deficiency was defined by a protein S level below the 5<sup>th</sup> percentile of the distribution in the controls.

\*\* Probands of families with thrombophilia (129).

that in such families there will also be individuals who have thrombosis but are not carriers of the private mutation(s) of that family (phenocopies). In these individuals, the thrombotic event might be triggered by an accumulation of (strong) environmental risk factors.

Reviewing the known genetic risk factors for thrombosis and their frequency in the general population, we recognize the existence of “loss of function” mutations and “gain of function” mutations (Table 1). “Loss of function” mutations result in deficiencies of protein C, protein S and antithrombin; many different mutations have been identified in these genes (46-48), all resulting in reduced activity or mass of the encoded protein in plasma. The frequency of these disease alleles in the general population is low ( $\leq 0.3\%$  for each of the three forementioned defects). On the other hand, “gain of function” mutations associated with thrombosis risk concern single point mutations which are quite common in the general population. Risk alleles which are common may confound the inheritance pattern in families studied by linkage analysis by introducing independent copies of the disease allele. An example of this comes from the study of Zöller and Dahlbäck in which the authors tried to link the phenotype of APC resistance to the factor V gene (49). Subsequent genotyping of all family members for the factor V Leiden mutation revealed that three independent copies of this allele were segregating in this family, probably as a result of the high frequency of this allele in the Swedish population.

As to the still unknown genetic risk factors for thrombosis we must be prepared to expect both (extremely) rare and rather common risk alleles. Recognition of this heterogeneity should guide us in the selection of suitable experimental approaches; family studies are useful for the identification of rare but relatively strong risk alleles, while case-control studies can be used to identify common (and generally also weaker) risk factors.

### Oligogenic Inheritance of Venous Thrombosis

As to the inheritance of thrombophilia, it has been recognised from the beginning that the disease is found in both men and women, and in successive generations (including male to male transitions), suggesting autosomal dominant inheritance. This was later confirmed by the identification of families with antithrombin-, protein C-, or protein S deficiency. It appeared that familial thrombophilia was an autosomal dominant trait with incomplete penetrance and that it concerned a collection of monogenetic diseases. Only recently has this view changed and now we accept the view that familial thrombophilia is an oligogenic disease.

There is strong support for the statement that, in thrombophilia families, individuals with two genetic defects will have thrombotic events more frequently and earlier in life than their relatives with a single defect (14-17, 50-52). In addition, Koeleman et al. reported that two locus linkage analysis supported the assumption that the factor V gene and the protein C gene were the two trait loci responsible for the thrombophilia in six families in which both the factor V Leiden allele and a defective protein C allele were segregating (14). This finding explained previous observations that the frequency of the factor V Leiden allele among symptomatic probands of protein C deficient families is much higher than expected on the basis of its population frequency. It also explained why the penetrance of thrombosis is low in protein C deficient family members of an asymptomatic protein C deficient healthy individual (absence of other genetic defects) (53, 54), but will increase after introduction of the factor V Leiden allele in the pedigree (55).

There is also strong support for epistatic interactions between the factor V Leiden allele and protein S gene defects (15, 17). In these studies, almost 40% of the symptomatic probands of protein S deficient thrombophilia families also carry the factor V Leiden mutation. However, Castaman et al. reported the complete absence of factor V Leiden in 16 Italian protein S deficient families (126). In 30% of these families the prothrombin 20210A allele was also segregating (and contributing to the thrombotic risk). Interaction between factor V Leiden and the prothrombin 20210A mutation is highly likely given the 10% prevalence of the latter mutation among symptomatic probands of thrombophilic factor V Leiden families (58, 127). However a formal study of thrombosis families in which both gene defects segregate is still lacking. Interaction between factor V Leiden and antithrombin gene defects has also been reported (16). Notable is the special situation where both gene defects are located on the same chromosome, and cosegregate in the family (16). Interestingly, not all combinations of genetic risk factors show interaction. Especially, there seems to be no interaction between the prothrombin mutation and protein C gene defects (56-58). There are also unexpected interactions such as those between the factor V Leiden allele and the factor V R2 allele (59) or a factor V null-allele (pseudohomozygous APC-resistance) (60-63). Together these recent studies have provided a genetic model for familial thrombophilia that can be used in the design of future genetic studies.

### Candidate Genes

Usually, several biological cascades can be identified that may contribute to the pathogenesis of a disease. This is also true for venous thrombosis where we can recognize the pro- and anticoagulant cascades and the pro- and antifibrinolytic cascades. Of course, each of these cascades has multiple interactions with other cascades as to the regulation of the concentration and activities of its individual components. Genes coding for proteins in these cascades form a first target (candidate genes) in the search for mutations/polymorphisms that may cause a functional phenotype associated with venous thrombosis. This explains the interest of researchers in finding single nucleotide polymorphisms (SNPs) in candidate genes for cardiovascular disease that subsequently can be tested in association or family studies (64, 65).

The main focus of these efforts is directed on the coding regions and promoters of these genes, because these regions are expected to contain most of the SNPs/polymorphisms that contribute to interindividual variation in (plasma) concentration and/or activity of these proteins. Because genotyping by use of the polymerase chain reaction has become a standard technique in most of the clinical laboratories, testing of the effect of SNPs on thrombotic risk in case-control studies has become very popular. As a result, the literature is full of conflicting and/or inconclusive results that subsequently can be used in meta-analyses (for discussion see ref. 66). It is clear that much more attention should be given to the design and interpretation of these association studies (67-69). Finding a higher frequency of an SNP in the patient group than in the control group can be a falsely positive finding, but there are also falsely negative findings. In the case of a true positive finding, it can be that the tested SNP itself causes the increased risk or that it is in linkage disequilibrium (LD) with another SNP which causes the risk. On the other hand, a negative result for an SNP in a particular gene does not mean that this gene is not involved in the pathogenesis of the disease. It may be that the SNP is not sufficiently linked to the disease-causing SNP or that the selected phenotype is too broad (relative risk too low).

Many of the SNPs that have been tested for their effect on venous thrombosis are common in the population (>10%). For these SNPs, it is possible to obtain reliable results from relatively small but properly designed case-control studies. However, to test the effect of less common SNPs (<5%), very large case-control studies may be needed to obtain a significant result (69). To study the effect of SNPs that are rare in the population but good candidate mutations, family studies might be more appropriate.

### Identification of Prothrombotic Mutations by Sequencing of Candidate Genes

At the end of the 1980s, researchers realised that the classical approach of finding genetic risk factors for thrombosis would not work for some candidate genes because it was not possible to measure their expression levels in the blood compartment (e. g. tissue factor, thrombomodulin, TFPI). Therefore, the genes coding for these proteins were sequenced in panels of selected thrombosis patients. We have used this approach to study the presence of prothrombotic mutations in the genes coding for TFPI, tissue factor, thrombomodulin and prothrombin. No candidate mutations were found in the first three genes in a panel of 28 symptomatic probands from families with unexplained thrombophilia. In 1985, Ohlin and Marlar reported the results of their analysis of a similar panel of 28 patients for mutations in the thrombomodulin gene (70). They found one patient heterozygous for a candidate mutation (Asp468Tyr). Since then, these authors have extended their analysis of the thrombomodulin gene to more patients and controls. Four additional candidate mutations have been observed, only one of which (Glu163Glu!) seems to segregate with thrombosis in the family (71). The previously reported Asp468Tyr polymorphism was also observed in one American control (71), but was not found in an Italian population (72).

Unfortunately, there is no information on the effects of these mutations on the biosynthesis, bioavailability or structure/function of the protein. Overall, we may conclude that defects in the thrombomodulin gene are rare and possibly as heterogeneous as in other deficiency states. Whether they are related to venous thrombosis is still an unanswered question. Interestingly, a common SNP in the thrombomodulin locus was found to increase the risk of myocardial infarction (especially when combined with classical risk factors as smoking) (73, 74). Whether this points to a causative role of this thrombomodulin allele in arterial thrombosis is not clear. Because of the small size of the TM gene, the SNP could easily be linked to a functional SNP in another gene.

More successful was the candidate gene approach in the case of the prothrombin gene. Sequencing of the coding and flanking regions of the prothrombin genes of 28 unrelated probands of thrombophilic families revealed a new polymorphism in the 3'UT region of the gene (20210 G/A) (12). Heterozygosity for the 20210A allele was found in 18% of the selected thrombosis patients and only in 1% of healthy subjects. Subsequent genotyping of a population-based case-control study on venous thrombosis (the Leiden Thrombophilia Study) revealed that the 20210A allele was present in 6.2% of the patients and 2.3% of the controls (12). This study nicely demonstrates that narrowing the clinical phenotype from consecutive first deep-vein thrombosis to familial thrombophilia results in an increased frequency of the risk allele (and relative risk). Variations in patient selection may therefore explain the range of Odds ratios for venous thrombosis (OR 2.0-6.6) reported from other centers for the 20210A allele (reviews [56, 75, 76]). So far, there is only one report which could not confirm that the 20210A allele

is associated with venous thrombotic risk (77). The initial observation by Poort et al. that the 20210A allele is associated with a 30% increase in plasma prothrombin levels has also been confirmed in other centers. The observation made in the Leiden Thrombophilia Study that elevated plasma prothrombin levels (>115 U/dl) were associated with a 2.1 fold increased risk of thrombosis demonstrated that the 20210A allele acts on thrombotic risk via increased plasma prothrombin levels (gene → risk, gene → functional phenotype, functional phenotype → risk). Whether the 20210 G→A mutation causes the elevation in plasma prothrombin is not yet clear. In vitro studies need to answer the question of whether the G→A transition in nt 20210 causes increased transcription, polyadenylation or translation efficiency. Haplotype analysis of 20210 A carriers indicates strong linkage disequilibrium and suggests a founder haplotype (78). In our laboratory, we have used six prothrombin gene polymorphisms for haplotyping 22 homozygous 20210A carriers from different parts of the world. All 20210A alleles were found to have the same haplotype (3728T-4125C-8845G-9832T-19911A-20210A) (79). Like the Factor V Leiden allele, the prothrombin 20210A allele is extremely rare in non-Caucasians (80).

A more recent example of the candidate gene approach, is the work of Merati et al. (81) on the gene coding for the endothelial protein C receptor (EPCR). This is a transmembrane protein expressed in endothelial cells of large vessels that together with thrombomodulin is involved in the activation of protein C (for a review see ref 82). Systematic analysis of exonic regions in the EPCR genes of thrombosis patients revealed a rare sequence variation in exon 3. It concerns an insertion of 23 nucleotides preceding the insertion point (nt4031), which introduces a frameshift and premature stop (81). Because it is expected that the truncated protein encoded by this allele will lack the cytoplasmic tail, transmembrane domain and part of the extracellular domain, this mutation is probably a good model for an EPCR null-allele. Unfortunately, its allele frequency is low both in patients and controls (≤1%), so that it will be difficult to investigate its association with thrombotic risk in case-control studies. In our laboratory we have identified one heterozygote for this insertion among 50 patients with thrombophilia. Family studies might help to reveal the clinical phenotype of this mutation.

### Polymorphisms and Thrombotic Risk

As mentioned above, testing SNPs for their effects on risk for a particular disease phenotype is also very popular in thrombophilia research. Most of these SNPs seem to be functional in the sense that at least an association has been found with levels or activity of the affected protein. However it is often difficult to assess whether the SNP itself is the cause of the functional phenotype or whether it is in linkage disequilibrium with the functional mutation (64, 66, 83). Also, there are examples where several polymorphisms in the same gene contribute to the functional phenotype. For instance, polymorphisms in the promoter (84-86), coding region (87, 88) and intronic regions (89) of the factor VII gene all independently seem to influence the plasma factor VII levels (90). In such a situation it seems better to use dense haplotypes in the association studies. This is also the best approach when there is no functional SNP known in the gene under investigation. Using a single or even a few SNPs to study the effect of a gene on the risk of disease is not recommended, given that in the general population useful linkage disequilibrium might not extend beyond an average distance of 3 kb (83). However, the actual distance at which disequilibrium can be observed is largely dependent on the age of the SNP and may be as large as 100 kb (91).

Table 2 Polymorphisms in candidate genes; effects on levels, function and thrombotic risk (I)

gene	polymorphism <sup>1)</sup>	association with		effect on thrombotic risk		comments
		levels	function	yes	no	
tissue factor	-1208 <b>D/I</b> <sup>130</sup>	yes (↓) <sup>130</sup>	-	reduction <sup>130</sup>	-	I: insertion 18nt
factor VII	Arg 353 <b>Gln</b> <sup>87</sup>	yes (↓) <sup>87,85,90,131</sup>	-	-	no <sup>132</sup>	-
factor X	-343 D/I -222 C/T -220 C/A	no <sup>133</sup>	-	-	no <sup>133</sup>	I: insertion TTGTGA
factor X	-40 C/T <sup>134</sup>	no <sup>133</sup>	-	-	no <sup>133</sup>	-
factor XII	46 C/T <sup>135</sup>	yes (↓) <sup>135</sup>	-	-	-	-
factor II	19911 <b>G/A</b> <sup>79</sup>	yes (↑) <sup>79</sup>	-	-	no <sup>79</sup>	intron 13
factor V	Arg 485 <b>Lys</b> <sup>136</sup>	-	yes? <sup>29</sup>	? <sup>137</sup>	-	APC-resistant? <sup>29</sup>
factor V	His1299 <b>Arg</b> <sup>28</sup>	yes (↓) <sup>28,198,140</sup>	yes? <sup>141</sup>	increase <sup>59,139</sup>	no <sup>198,138</sup>	marker for the R2-FV allele
VWF	-1234 C/T <sup>142</sup>	yes (↑) <sup>142</sup> , no <sup>143</sup>	-	-	no <sup>143</sup>	marker for VWF promoter allele
fibrinogen Aα	Thr 312 <b>Ala</b> <sup>145</sup>	-	? <sup>144</sup>	? <sup>144</sup>	-	only for pulmonary embolism?
fibrinogen Bβ	-455 G/A	yes (↑) <sup>146-148</sup>	-	-	no <sup>132</sup>	Hae III restriction site
factor XIII	Val34 <b>Leu</b> <sup>152</sup>	no <sup>156,160</sup>	yes <sup>156-159</sup>	reduction <sup>150,151</sup>	no <sup>149,153-156,160</sup>	-
TAFI	-438 G/A <sup>161</sup>	yes (↓) <sup>161</sup>	-	reduction? <sup>161</sup>	no <sup>161</sup>	marker for TAFI promoter allele
TAFI	-152 A/G	no <sup>161</sup>	-	-	no <sup>161</sup>	-

<sup>1)</sup> The allele in bold was tested for its effect on level, function or thrombotic risk. References are given as indices in each column

Tables 2 and 3 provide a summary of the SNPs/polymorphisms which have recently been used in the study of thrombophilia. Not included in these Tables are factor V G1691A (factor V Leiden), prothrombin 20210A and MTHFR C677T. The association or lack of association of these genotypes with venous thrombotic risk has been amply documented in the literature (for recent reviews see refs 92, 56, 93 and 197).

Presently, there is no firm support for an effect of the MTHFR 677A allele on the risk of venous thrombosis, either in the absence or presence

of factor V Leiden (93, 94); it is surprising therefore that this polymorphism is still frequently included in genetic studies of thrombophilia. Furthermore, it is interesting to see that SNPs in the genes coding for factor VII, fibrinogen, PAI-I, and tPA receive much less attention in the study of thrombophilia than they do in the study of arterial thrombosis (review [92]).

All studies have investigated the effect of a particular SNP on the risk of venous thrombosis. There are, however, considerable differences in the selection of patients; first events/recurrent events, consecu-

Table 3 Polymorphisms in candidate genes; effects on levels, function and thrombotic risk (II)

gene	polymorphism <sup>1)</sup>	association with		effect on thrombotic risk		comments
		levels	function	yes	no	
protein C	-1641 <b>G/A</b> , -1654 <b>C/T</b> <sup>162</sup>	yes (↓) <sup>162,163,164</sup>	-	increased? <sup>162,163</sup>	-	GC homozygotes versus AT homozygotes
protein S	A 2148 <b>G</b> <sup>165</sup>	yes (↓) <sup>166,167</sup>	-	-	-	Pro 626 dimorphism
protein S	C 2698 <b>A</b> <sup>168</sup>	yes (↑) <sup>167</sup>	-	-	-	in 3'UT region
thrombomodulin	Ala 455 <b>Val</b> <sup>169</sup>	-	-	-	no <sup>169</sup>	-
EPCR	4031 I/D <sup>82</sup>	-	-	increased <sup>82</sup>	-	insertion 23 bp in exon 3
TFPI	-287 T/C <sup>174</sup>	yes (↓) <sup>174</sup>	-	-	-	-
TFPI	-399 C/T <sup>171</sup>	no <sup>171,174</sup>	-	-	no <sup>171</sup>	-
TFPI	Val264 <b>Met</b> <sup>175</sup>	yes (↓) <sup>175</sup>	-	-	no <sup>172</sup>	-
TFPI	<b>T</b> 384 C <sup>175</sup>	no <sup>175</sup>	-	-	-	Tyr 57 dimorphism
TFPI	Pro151 <b>Leu</b> <sup>170</sup>	-	-	increased <sup>170</sup>	no <sup>173,176,177</sup>	-
tissue plasminogen activator	I/D intron h <sup>178</sup>	yes <sup>178,180,181</sup>	-	-	-	-
plasminogen activator inhibitor	-675 <b>4G/5G</b> <sup>183</sup>	yes <sup>182,183,186-189</sup>	-	*increased <sup>185,194</sup>	no <sup>184,185,190,192,193</sup>	*in carriers FV Leiden or protein S gene defect
	-844 <b>A/G</b> <sup>184,191</sup>	no <sup>191</sup>	-	*increased <sup>184</sup>	-	*in carriers FV Leiden
protease activated receptor -1	-506 I/D <sup>195</sup>	-	-	decreased <sup>195</sup>	-	only in men
	-1426 C/T <sup>195</sup>	-	-	-	no <sup>195</sup>	-
	IVS -14 A/T <sup>195</sup>	-	-	-	no <sup>195</sup>	-
Angiotensin I converting enzyme	I/D(intron 16) <sup>196</sup>	yes (↑) <sup>104,196</sup>	-	increased <sup>104</sup>	-	insertion 287 bp

<sup>1)</sup> The allele in bold was tested for its effect on level, function or thrombotic risk. References are given as indices in each column

tive patients/selected patients, deep-vein thrombosis/all thrombotic events. Of course this may influence the outcome of the studies as well as the extent of agreement that can be obtained between studies of the same genotype in different centers. Also, a polymorphism might differ in its effect on different phenotypic expressions of venous thrombosis. For example, factor V Leiden is a risk factor for deep-vein thrombosis (95), cerebral vein thrombosis (96), superficial vein thrombosis (97) and portal vein thrombosis (98), but not for primary pulmonary embolism (99) and retinal vein thrombosis (100). Finally, differences in exposure to environmental factors may influence the results. The use of oral contraceptive enhances the risk of factor V Leiden and prothrombin 20210 A alleles (101, 102), while there is no interaction between these alleles and surgery (103). Thus, Factor V Leiden is not an important risk factor for postoperative thrombosis in patients undergoing hip arthroplasty, while the D allele of the polymorphism in intron 16 of the gene coding for the angiotensin-I converting enzyme increases the risk almost tenfold (104). The latter polymorphism had only a weak effect on thrombotic risk in a small case control study in African-Americans, but only in men (105). This result can possibly be explained by the observation that surgery is more frequently found in men than in women to be a precipitating factor for a thrombotic event.

A final observation that can be made from Table 2 is that there is a growing interest in polymorphisms that protect against venous thrombosis (e. g. factor XIII Val 34Leu, and TAFI -4386 G/A). This mirrors the growing interest in the effect of prothrombotic mutations on the clinical phenotype of congenital and acquired bleeding disorders (106, 107).

## Family Studies

To find susceptibility loci for complex diseases, investigators have studied twins, sib pairs, small nuclear families and large extended pedigrees. In the field of thrombophilia, there is still limited experience with these types of studies, but this situation will probably change rapidly now that the first genome-wide scan in thrombosis families has been recently completed (XVIII ISTH Congress, Paris, 2001).

In the context of this review, I will not discuss the various statistical genetic approaches. During the past decade there has been enormous progress in what these programs can do. Using more of the available information (e. g. multipoint analysis) in both affected and non-affected individuals leads to more statistical power and thus enhances the probability of finding significant effects. Genetic markers can be either chosen in specific candidate genes or be a selection that covers the whole genome at the required density (e. g. with an intermarker distance of 1 cM). Markers should have a high polymorphic information content (e. g. CA repeats) because this will prevent loss of information. Polymorphic markers in or very close to candidate susceptibility genes for thrombosis, have been identified by determining the location of the genes on the linkage map (108, 109).

With regard to thrombophilia, linkage studies might be used to answer two types of questions. First, what are the genetic determinants of a functional phenotype, e. g. a phenotype that has previously been shown to be associated with venous thrombotic risk. It is also possible of course to select a phenotype for which it is unknown whether there is an associated risk. Finding the genotypes that influence such a phenotype may reveal new candidate genes, after which the effect of these genes on thrombotic risk can be explored in the usual association studies. Before actually performing the linkage analysis, it is important to have an estimate of the heritability of the phenotype. For many of the hemostasis and fibrinolysis parameters such heritabilities have been

published (110-114). It should however be recognized that observed heritability may be different in different environments or geographical areas.

The second obvious application is to use linkage in thrombosis families to identify thrombosis susceptibility loci. For such studies it is generally important to have an adequate genetic model for the disease. At the present time, we may have such a model for familial thrombophilia (see above). Of course it is also possible to combine both types of studies in the same (thrombosis) families, in which case it is possible to test for pleiotropic effects of loci on (a) quantitative trait(s) and susceptibility to thrombosis. An example of such a study was recently published by Soria et al. (115). In this study, the authors demonstrated that the prothrombin 20210A mutation jointly influences thrombosis and plasma prothrombin activity. This study was performed within the framework of the GAIT project, a project that focuses on the identification of susceptibility loci for hemostatic phenotypes and thrombosis using multipoint linkage analysis by variance components techniques (116, 117). The published results of this project are very promising and partly agree with views based on the interpretation of findings in association studies; such as the genetic linkage of the ABO locus to plasma VWF, factor VIII, and APTT levels (118). They also agree with the reported significant genetic correlations between thrombosis and VWF/factor VIII, Factor IX, factor XI, the APC sensitivity ratio and homocysteine levels (119). Two comments may be made. First, the authors used a rather broad definition of the clinical phenotype which includes arterial thrombosis. And secondly, many of the hemostatic phenotypes used in this study concern functional phenotypes. Although in general these phenotypes will give more information, results might be reagent specific (e. g. one stage clotting assays, APC sensitivity ratios measured with different methods). For instance high factor VIII levels might cause falsely high readings of other intrinsic factors in one stage assays. Confirmation of the results in different sets of families will therefore be important.

As mentioned above, there is strong support for the hypothesis that familial thrombophilia is an oligogenetic disease. This would mean that in general at least two different genetic defects will segregate in a thrombophilic family. Knowing one gene defect in such a family might offer a good opportunity for finding the second by linkage analysis. This is the approach followed by Bovill and coworkers in their study of the genetic causes of thrombosis in a very large pedigree with protein C deficiency and thrombosis. This family was first reported in 1989 (120), later the authors reported the presence of the private protein C gene mutation (His107 Pro) (121) and concluded that a still unknown genetic defect interacted in this family with the protein C gene defect to increase thrombotic risk (122). Subsequently, they found that the prothrombin 20210A allele was fairly frequent in this family but did not contribute significantly to the thrombotic risk (57). For this analysis they used a transmission/disequilibrium test (123) modified for calculating likelihoods in pedigrees. In their most recent paper, the authors reported the results of genetic screening of 34 candidate genes for a prothrombotic interaction with the protein C gene defect in an informative subset of the family (109). However, although almost all known hemostatic genes were included (blood group and factor VIII were not included) none of them was implicated as the second prothrombotic gene in this family.

A few years ago we obtained very similar results when we tried to find (a) second gene defect(s) in four protein C deficient families using markers for 24 thrombosis candidate genes (124). A total of 66 individuals were genotyped, 22 of which were affected by thrombosis. We used the TLINK program for the analysis, and assumed that the

protein C gene was one of the two disease genes. No significant lod scores were obtained for any of the tested markers. A marker for the protein C gene itself gave in single locus analysis a lodscore of 1.8 at theta = 0.05!

We appeared to have more success with a very similar approach in a set of thrombophilia families with one known genetic defect (either Factor V Leiden, antithrombin deficiency, or protein S deficiency) (125). In these families, we specifically investigated whether there was support for a second thrombosis susceptibility locus in the neighbourhood of the protein C locus (2q13-14), using nine highly polymorphic markers. This region was difficult to study in the protein C deficient families, while it could not be excluded that in some of the symptomatic protein C deficient families the apparent dominant inheritance of the disease was caused by two different but closely linked defects on chromosome 2. Results of parametric (TLINK) and non-parametric linkage analysis (SIMIBD, GENEHUNTER) provided weak evidence (lodscores 1.7-1.9) for a second thrombosis susceptibility locus in these families (apart from the FV gene, the protein S gene or the antithrombin gene). This locus is located close to the gene coding for interleukin 1. However, sequencing of a number of candidate genes in this region, did not reveal any sequence variation that could constitute the disease determinant. Confirmation of this result in different sets of families is needed before a more systematic analysis of this region is considered.

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