Genetic variation and susceptibility to venous thrombosis : Etiology and risk assessment

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Predictive Genetic Variants For Venous Thrombosis: What's New?

Chapter 3

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ABSTRACT

Various pathways lead to the development of venous thrombosis. Risk factors are common and can be genetic or acquired. Since the identification of factor V Leiden and prothrombin G20210A, the field of genetic epidemiology has developed rapidly and many new genetic variants have been described in the past decade. However, the association with venous thrombosis is often unclear and conflicting results have been reported in various studies. The aim of this review is to describe these candidate predictors of venous thrombosis and to put these in perspective.

Venous thrombosis is a complex condition in which genes and environment both contribute to the risk of disease. Many risk factors for venous thrombosis are common, and often, if not always, the coincidence of two or more risk factors is required to develop thrombosis. Heritable defects in factors that control the hemostatic balance have been identified since 1965 (for a comprehensive review, see Mannucci [45]). In that year, Egeberg described a family in which the incidence of venous thrombosis was higher and at a younger age than expected. It appeared that the affected family members had about 50% lower antithrombin levels than the non-affected family members. Deficiencies of protein C and protein S were identified in a similar manner during the 1980s. Families with a history of recurring venous thrombosis but no known hereditary abnormalities were studied to determine the role of plasma protein deficiencies. It appeared that affected family members had severely reduced protein levels of protein C or S. Today, numerous loss-of function mutations have been described in the genes encoding antithrombin, protein C, and protein S that lead to reduced plasma levels. In the heterozygous state these mutations lead to about halfnormal plasma levels. Homozygous mutations, especially in the antithrombin gene, are assumed to be incompatible with life.

In addition to these relatively rare protein deficiencies, two much more common genetic defects were described during the 1990s. Activated protein C (APC) resistance was identified as a risk factor for venous thrombosis in 1993. In 1994, Bertina et al found that factor V was involved in APC resistance. Subsequently, the factor V Leiden mutation was found by searching the factor V gene of APC resistant patients for mutations in APC binding- and cleavage sites. The second common genetic factor, prothrombin 20210 G>A, was identified in 1996 through screening the prothrombin gene for abnormalities among patients with a personal and family history of venous thrombosis. At present, the three plasma protein deficiencies and the two mutations are the main genetic risk factors for venous thrombosis. However, they still explain only part of venous thrombotic events [45]. The failure to identify a risk factor in many patients and the belief that genetic factors play

an important role in the development of venous thrombosis stimulate the search for novel predictive genetic variants.

Since the identification of prothrombin 20210 G>A in 1996, the field of genetic epidemiology has evolved rapidly and many genetic variants have been described that might influence the risk of venous thrombosis. In this review we will give an overview of the main genetic factors described in the past decade and put the findings in perspective. The main features are presented in Table 1.

NOVEL GENETIC VARIANTS

Fibrinogen

Fibrinogen is the precursor of fibrin, the fundamental constituent of the thrombus. The fibrinogen molecule consists of three chains: alpha (FGA), beta (FGB), and gamma (FGG), each encoded by a separate gene. High levels of fibrinogen increase the risk of venous thrombosis, mainly in elderly people [7]. The most frequently studied polymorphism is a G>A substitution at nucleotide -455 in the *FGB* gene. This genetic variant is associated with slightly increased fibrinogen levels but appears not to increase the risk of venous thrombosis [46,47]. Another, less frequently studied, polymorphism in the *FGA* gene, 4266 A>G (Thr312Ala), is associated with the risk of pulmonary embolism (PE) and is postulated to influence fibrin cross-linking [48]. The 312Ala genotype leads to reduced clot strength and a higher risk of embolization. Ko et al found an FGA haplotype, covering the Thr312Ala variant, to be related to both PE and deep vein thrombosis (DVT) in a Taiwanese population [49]. In another study that analyzed the association of haplotypes of the alpha, beta, and gamma genes and the risk of DVT, the only haplotype associated with DVT was a haplotype of the *FGG* gene, tagged by 10034 C>T (*FGG*-H2) [50] This polymorphism is located in a consensus sequence that is involved in cleavage and splicing of the *FGG* pre-mRNA. The *FGG*-H2 haplotype is associated with decreased levels of fibrinogen gamma' (FGG') and decreased FGG'/

total fibrinogen ratios. Interestingly, the *FGA* Thr312Ala polymorphism is strongly linked to this haplotype. Whether FGA Thr312Ala is a functional variant itself or only reflects the effect of the 10034C>T variation in *FGG*-H2 remains to be elucidated.

Prothrombin

Prothrombin is the inactive precursor of thrombin. A principal role of thrombin is to cleave fibrinogen to form fibrin. In addition, thrombin gives positive feedback to the coagulation cascade by activating other coagulation factors and negative feedback by activating protein C in a complex with thrombomodulin. It influences fibrinolysis also, through thrombin activatable fibrinolysis inhibitor (TAFI). The 20210 G>A mutation in the prothrombin gene increases the risk of venous thrombosis by increasing plasma prothrombin levels (first described by Poort et al [51]. Around the 20210 position several additional variants were described, but there is no clear evidence that these rare variants contribute to the risk of venous thrombosis [52]. Another common variant in the prothrombin gene, 19911 A>G, is also associated with slightly higher prothrombin levels [3,53,54]. Initially, two studies reported that 19911 A>G modulates the risk in 20210A carriers [53,55]. Recently, two larger studies reported an increased risk associated with 19911 A>G, independently of other genetic risk factors [3,54].

Factor V

Activated factor V (FVa) is a cofactor for factor Xa in the conversion of prothrombin to thrombin. In addition, FV is a cofactor of APC-mediated FVIII degradation. Factor V Leiden (1691 G>A, Arg506Gln) is a mutation in the major cleavage site of FVa by APC, which makes FVa more resistant to inactivation. Recently, all known *FV* missense mutations that are relatively common were reviewed by Vos [68]. The most important variant, apart from factor V Leiden, is a common haplotype including several polymorphisms throughout the *FV* gene, first described by Lunghi et al [69]. This haplotype, *FV* HR2, is associated with decreased cofactor activity in FVIII inactivation and a more procoagulant isoform of FV. The variant responsible for the *FV*

Table 1 Candidate Predictors of Venous Thrombosis

Gene	Nucleotide	Amino Acid	Allele Frequency*	Phenotype	Odds Ratio†
Procoagulant proteins					
FGA	4266 A>G	Thr312 Ala	0,26 (G)	reduced clot strength	1,8 [49]
FGG	10034 C>T (FGG-H2)		0,26 (T)	alternatively spliced protein	2,4 [50]
Prothrombin	19911 A>G		0,49 (G) [3]	higher protein level	1,4 [3]
FV	6755 A>G (HR2)	Asp2194Gly	0,07 (G)	decreased FV cofactor activity	1,2 [56] (carriers)
FVIII	94901 C>G	Asp1241Glu	0,15 (G)	lower protein level	0,6 [57] (carriers)
FVIII	HT1		0,14 (HT1) [58]	lower protein level	0,4 [58] (men)
FXII	46 C>T		0,15 (T)	lower protein level	not replicated
FXIII	G>T	Val34Leu	0,24 (T) [59]	higher protein activity	0,6 [59]
FXIII	8259 A>G	His95Arg	0,08 (T) [60]	higher protein activity	1,5 [60]
TF	1208 I/D		0,52 (D) [61]	lower protein level	0,7 [61]
FSAP	1601 G>A (Gly511Gly)		-	impaired fibrinolysis inhibition	not replicated
ACE	I/D		0,51 (D) [62]	higher protein level	not clear
Anticoagulant proteins					
TFPI	536 C>T	Pro151Leu	<0,01 (D) [63]		not replicated
TFPI	-33 T>C		0,36 (C)	higher protein level	0,6 [64]
EPCR	4600 A>G	Ser219Gly	0,08 (G)	higher sEPCR level	not clear
EPCR	4678 G>C		0,34 (C)	higher APC level	not clear
Antifibrinolytic proteins					
PAI-1	4G/5G (D/I)		0,48 (5G) [65]	lower protein level	not clear
TAFI	505 G>A	Ala147Thr	0,30 (T)	higher protein level	0,7
Other					
Blood group	O		0,43 (O) [66]	lower FVIII level	0,6 [66]
ZPI	728 C>T	Arg67Stop	<0,01(T)	lower protein level	3,3 [67] (carriers)
MTHFR	677 C>T	Ala222Val	0,24 (T)	lower protein activity	not clear

* Allele frequencies as calculated in the study population referred to. When no reference is given, the allele frequency for Caucasian populations (applies to almost all studies) was obtained from dbSNP.

† By default, the odds ratio for homozygous carriers. For FV HR2, FVIII Asp1241Glu, FXIII His95Arg, and ZPI Arg69Stop, the odds ratio for all carriers (heterozygous and homozygous) is given.

HR2 phenotype is probably 6755 A>G (Asp2194Gly) [68]. Whether the *FV* HR2 haplotype affects the risk of venous thrombosis is not clear. Individual studies reported conflicting results and in a meta-analysis a pooled odds ratio of 1.15 (95% confidence interval [CI], 0.98 to 1.36) was calculated [56].

Factor VIII

The factor VIII (*FVIII*) gene has been studied extensively because there is a clear association between FVIII levels and the risk of venous thrombosis. Several studies screened cleavage sites, promoter and polyadenylation regions of the *FVIII* gene but found no mutation that corresponded with either FVIII levels or thrombosis [70-72]. However, two other studies reported lower FVIII levels associated with a 94901 C>G (Asp1241Glu) polymorphism that was therefore possibly protective for venous thrombosis [57,73]. In a recent study of the haplotypes carrying the 1241Glu variant (HT1, HT3, HT5), the protective effect of 1241Glu and lower levels of FVIII were confirmed, but seemed limited to male carriers of HT1 [58]. This indicates that Asp1241Glu is not likely to be functional but is probably linked to a functional variant. In addition, the risk reduction was only partially dependent on the lower levels of FVIII, which suggests that not only FVIII levels influence risk but also protein function may contribute.

Factor XII

Factor XII (FXII) is involved in both the intrinsic coagulation pathway and the fibrinolytic pathway. Although it has been suggested that deficiency of FXII may lead to an increased risk of thrombosis, there is not enough evidence to confirm an association. In 1998, Kanaji et al [74] described the *FXII* 46 C>T polymorphism that was associated with decreased plasma FXII levels. After finding *FXII* 46 C>T associated with FXII levels and venous thrombosis in a linkage study, Tirado et al reported an odds ratio of 4.8 (95% CI, 1.5 to 15.6) for the TT genotype [75]. However, other studies could not confirm this finding [76-78].

Factor XIII

Factor XIII (FXIII) stabilizes the fibrin clot by cross-linking fibrin monomers. A genetic variant that interferes with clot stabilization is the *FXIII* Val34Leu polymorphism. The *FXIII* 34Leu variant is more rapidly activated and thus more rapidly cross-links fibrin fibers. However, these fibers are thinner and the fibrin clots are more solid [79]. The 34Leu variant was found to be protective when first studied in relation to venous thrombosis [80]. Subsequent studies mostly failed to confirm this finding, perhaps due to small study sizes, but in a meta-analysis a pooled odds ratio of 0.63 (95% CI, 0.46 to 0.86) was computed [81]. Recently, this protective effect was shown to depend on fibrinogen levels [82]. High levels of fibrinogen also lead to less porous fibrin clots, and seem to act synergistically with *FXIII* 34Leu, being protective against venous thrombosis. Another polymorphism was reported in the FXIII B-subunit. This is a carrier protein for FXIII and dissociates upon activation of FXIII. The 8259 A>G (His95Arg) variant leads to increased dissociation and moderately increases the risk of venous thrombosis [60].

Tissue Factor

Tissue factor (TF) initiates coagulation by activating factor VII. TF is expressed by most cells and organs and a soluble form of TF might also circulate in plasma. The relationship between circulating TF levels and venous thrombosis is not clear. Arnaud et al [61] screened the promoter region of the *TF* gene in blood donors and identified a deletion/insertion polymorphism, 1208 D/I, of which the 1208 D variant was associated with lower circulating TF levels and a lower risk of venous thrombosis in a subsequent case-control study. The coding region of the *TF* gene was screened in a case-control setting by Zawadzki et al [83]. They found a C>T (Arg200Trp) variant that might lower monocyte TF concentrations, but the variant was too rare to study its effect on the risk of thrombosis.

Factor VII–Activating Protease

Factor VII-activating protease (FSAP) stimulates coagulation by activating FVII. It also stimulates fibrinolysis by activating urokinase precursor. The

Marburg I polymorphism (1601 G>A, Gly511Gly) impairs the profibrinolytic activity of FSAP but has no effect on its FVII-activating function [84]. Initially, an increased risk of venous thrombosis was reported for carriers of the Marburg I polymorphism, mainly for idiopathic cases [85]. However, this finding could not be confirmed by others [86,87].

Blood Group

ABO blood group is associated with both FVIII and von Willebrand factor (VWF) levels, and a reduced risk of venous thrombosis for phenotypic blood group O was already recognized by Jick et al [88] in 1969. The protective effect of blood group O is mainly explained by FVIII levels [66,89]. In 2005, several investigators reported on the association between blood group genotype and venous thrombosis [66,90-92]. All studies confirmed the lower risk for blood group O (genotypes O^1O^1 and O^1O^2), and the highest risk for carriers of the A^1 allele. Blood group O genotypes were associated with lower FVIII and VWF levels [91,92]. The protective effect is restricted to homozygous carriers of O alleles; hence individuals with phenotypic blood group O [90]. There is some evidence that the risk for non-OO genotypes is higher in carriers of factor V Leiden [66].

Angiotensin-Converting Enzyme

Angiotensin-converting enzyme (ACE) stimulates platelet activation and regulates fibrinolysis. The relationship between ACE levels and the risk of venous thrombosis is, however, not clear. Variation in ACE levels is largely explained by a 287-bp insertion/deletion (I/D) polymorphism in the *ACE* gene. Individuals with the DD genotype have higher plasma ACE than individuals with the II genotype [93]. Initially, the DD genotype was reported to increase the risk of venous thrombosis [94,95]. Subsequent studies reported varying associations between the I/D variant and venous thrombosis, as summarized by Okumus et al [96] and Ekim et al [97]. An important conclusion from both reviews was that studies were heterogeneous in patient selection, with regard to etiology and ethnicity. The most recent and largest study found a slightly protective effect of the DD genotype, which was restricted to women. However, the authors concluded that the I/D polymorphism itself is unlikely to be functional [62].

Tissue Factor Pathway Inhibitor

The first step in the extrinsic coagulation pathway is inhibited by tissue factor pathway inhibitor (TFPI). Low TFPI levels are associated with an increased risk of venous thrombosis [98]. Five polymorphisms have been described in the *TPFI* gene. The -399 C>T polymorphism, reported by Miyata et al [99] was not associated with TFPI concentrations or with venous thrombosis. Kleesiek et al [63] screened the coding region of the gene and found a 536 C>T (Pro151Leu) variant that was associated with an increased risk of venous thrombosis but not with TFPI plasma activity and concentration. The association with venous thrombosis was not confirmed by others [100-102]. Three other polymorphisms were reported by Moatti et al [103,104]. The 874 G>A (Val264Met) variant might influence TFPI levels, but it was not associated with venous thrombosis [98,105]. The intron variant -33 T>C and the promoter polymorphism -287 T>C were both associated with higher TFPI levels [64,104]. Accordingly, -33C was found to be protective for venous thrombosis [64]. The -287 T>C variant has not yet been studied in venous thrombosis patients.

Endothelial Protein C Receptor

The endothelial protein C receptor (EPCR) is expressed on the endothelium of large vessels. Binding of protein C stimulates protein C activation by the thrombin–thrombomodulin complex. A soluble form of EPCR (sEPCR) also binds protein C but inhibits protein C activity. Low levels of sEPCR have been found to reduce the risk of venous thrombosis [106]. Biguzzi et al screened the *EPCR* gene in venous thrombosis patients and found a 23-bp insertion (4031ins23) that impaired EPCR function, and four promoter polymorphisms that did not affect transcription in vitro [107,108]. These variants were rare in both patients and control subjects and therefore the effect on thrombosis risk could not be established [109-111]. España et al [112] reported more common polymorphisms in exon 4 (4600 A>G, Ser219Gly) and in the 3′UTR (4678 G>C). The 4600G genotype was associated with increased sEPCR levels and the 4678C genotype was associated with increased APC levels. Conflicting results were published about the association with venous thrombosis [106,113-115]. Two studies constructed haplotypes of the *EPCR* gene. Saposnik et al [116] found

three haplotypes of which the haplotype corresponding to the 4600G genotype (H3) was associated with increased sEPCR levels and an increased risk of venous thrombosis. Uitte de Willige et al [106] identified an additional haplotype (H4) that also contained 4600G and was part of H3 in the study of Saposnik et al. In this study, carriers of H4 (not H3) had a slightly increased risk of venous thrombosis, which suggests that 4600G itself is not a functional variant.

Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) indirectly inhibits the fibrinolytic activity of plasminogen. There is no clear relationship between PAI-levels and venous thrombosis [117]. Dawson et al [118] described a guanine deletion/insertion (4G/5G) upstream of the *PAI-1* gene that influences PAI-1 activity. A literature review regarding 4G/5G and venous thrombosis by Francis [117] showed that although most studies find higher plasma levels of PAI-1 in individuals with 4G/4G, the effect on the risk of venous thrombosis is not clear. There is some evidence that 4G/4G increases the risk of venous thrombosis in subgroups with additional genetic risk factors [65,119], but these findings need to be confirmed. The fact that the 4G/5G variant is associated with PAI-I levels but not with venous thrombosis suggests that PAI-I levels have no major effect on the risk of venous thrombosis.

Thrombin-Activatable Fibrinolysis Inhibitor

During fibrinolysis, partially degraded fibrin is a cofactor for plasminogen activation. Thrombin-activatable fibrinolysis inhibitor (TAFI) inhibits fibrinolysis by suppressing this cofactor activity. Increased TAFI levels were found to be associated with a slightly increased risk of venous thrombosis. Zhao et al [120] described the 505 G>A polymorphism (Ala147Thr) that was associated with higher TAFI concentrations. In addition, several promoter polymorphisms (-438 A>G, 1102 T>G, 1690 G>A) and an additional polymorphism in the coding region (1040 C>T, Thr325Ile) were described that influenced TAFI levels and possibly the risk of venous thrombosis, but these variants were strongly linked to the 505 polymorphism [121,122]. Martini et al [122] analyzed haplotypes constructed of -438 G>A, 505 G>A and 1040

C>T and found that only 505A was associated with a reduced risk of venous thrombosis. This was an unexpected finding because 505A is the variant associated with higher TAFI levels. Further research is needed to unravel the relationship between TAFI and venous thrombosis.

5,10-Methylenetetrahydrofolate Reductase

Increased homocysteine has been associated with venous thrombosis in many studies but the mechanism by which homocysteine affects coagulation is not clear. The common 677 C>T (Ala222Val) polymorphism in the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene, leading to a thermolabile variant of the enzyme, slightly increases plasma homocysteine levels and has been studied extensively. Odds ratios computed from a large meta-analysis indicate that *MTHFR* 677 C>T is a weak risk factor for venous thrombosis [123]. However, we have recently studied *MTHFR* 677 C>T in a large case-control study but found no evidence of an association with venous thrombosis [124].

Protein Z–Dependent Protease Inhibitor

Although in vitro studies suggest that protein Z–dependent protease inhibitor (ZPI) might influence coagulation by inhibiting FXa and FXIa [125], ZPI plasma levels do not seem to affect the risk of venous thrombosis [126]. Van de Water et al [125] screened the coding region of the *ZPI* gene for mutations and identified two premature stop codons at Arg67 and Trp303 that were associated with venous thrombosis. However, in subsequent studies, the Trp303 stop codon was not detected [127] or the presence of either Arg67Stop or Trp303Stop was not associated with venous thrombosis [67]. Recently, however, Corral et al [128] studied haplotypes of the *ZPI* gene and found the haplotype covering Arg67Stop to be associated with venous thrombosis with a 3.3-fold increased risk. Since Arg67Stop is a rare mutation, large studies are needed to confirm this finding.

Factors VII, IX, X, XI, von Willebrand Factor, and Thrombomodulin

Despite a clear association between plasma levels and the occurrence of

venous thrombosis, no predictive genetic variants are known in the genes encoding factor IX and factor XI. The promoter region of the *FXI* gene has been screened and two single-nucleotide polymorphisms (SNPs) were identified [129], but the association with plasma concentrations of FXI and venous thrombosis has not yet been studied. Plasma levels of factor X and von Willebrand factor are also related to the risk of venous thrombosis, but the association is dependent on levels of other coagulation factors [66,89,130]. For both factors, gene variants were studied in relation to plasma concentrations and the risk of venous thrombosis, but no association was found [72,130]. Plasma concentrations of factor VII and thrombomodulin are not related to the risk of venous thrombosis. Some genetic variants were identified that were related to levels but not to the risk of venous thrombosis [46,47,131-134].

FUTURE PERSPECTIVES

Research Goals

All variants that were initially found to be associated with venous thrombosis are listed in Table 1. For each factor, the magnitude of the association with venous thrombosis is given in the right column. However, most of these findings have not yet been replicated, and others have failed to replicate. Failure of replication may occur for several reasons.First, many genetic variants of modest effect are expected to contribute to the risk of venous thrombosis. To detect these modest effects large sample sizes are needed. All of the genetic variants discussed above are at most weak risk factors and were studied in samples of at most several hundreds of study subjects.

Another obstacle for replication is phenotype definition. Venous thrombosis is a multicausal disease and genetic variants that influence the risk of disease are expected to affect only one of the causal pathways. The intermediate phenotype of that causal pathway (for example, protein levels) will be more strongly correlated to the genetic variant. On the other hand, when the

intermediate phenotype is not associated with venous thrombosis, which is the case with FVII, FX, FXII, von Willebrand factor, and thrombomodulin, finding an association between venous thrombosis and the genetic variant is less likely.

Alternatively, the initial finding may have been a false-positive result. As the amount of genetic variants tested increases, the possibility of finding false-positive results also increases when multiple testing is not correctly accounted for. The problem of false-positive findings probably accounts for many of the non-replicated associations.

Once a finding is replicated, the knowledge from previous studies can be used to further explore the relationship between the replicated variant and disease. This can be done by constructing haplotypes in the genes where association was found, as was the case in relation to the fibrinogen [50], *FVIII* [58], and *EPCR* [116] genes. In these studies, haplotypes were identified that increased the risk of thrombosis and probably explained the association observed before with a single nucleotide polymorphism. Knowledge from previous research can further be used to study interaction of genetic variants that seem to affect the same causal pathway. The synergistic effect of the *FXIII* Val34Leu variant and fibrinogen levels observed by Vossen et al [82] is an example of this approach.

Clinical Implications

The ultimate goal of genetic epidemiologic research is to be able to predict each individual's risk of disease on the basis of genetic and acquired factors. With a genetic profile it would then be possible to give lifestyle recommendations or prophylaxis in high-risk situations to maximally reduce the risk of disease.

However, venous thrombosis is a complex disease and prediction requires an extensive model including many acquired and genetic factors. To be clinically useful, the genetic variants described above should be evaluated together with many other, as yet unknown, risk factors. In addition, the outcome of testing the genetic variant should influence treatment or preventive measures. The

various possible or weak genetic risk factors for venous thrombosis that have been identified in the past years are not of clinical importance.

CONCLUSION

In the past decade, many factors have been described that might influence the risk of venous thrombosis. Some of these, like ABO blood group, FXIII Val34Leu, or haplotypes of fibrinogen, *FV*, and *FVIII*, are possible novel predictive variants. Others have failed to replicate in subsequent studies. More well-designed, large studies are needed to unravel the various pathways that lead to the development of venous thrombosis.