Coagulation factor V and thrombophilia: Background and mechanisms

Kenneth Segers 1, Björn Dahlbäck 2, Gerry A. F. Nicolaes 1

1Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands; 2Department of Laboratory Medicine, Clinical Chemistry, Lund University, Wallenberg Laboratory, University Hospital, Malmö, Sweden

Summary

Human coagulation factor V (FV) is an essential coagulation protein with functions in both the pro- and anticoagulant pathways. Failure to express and control FV functions can either lead to bleeding, or to thromboembolic disease. Both events may develop into a life-threatening condition. Since the first description of APC resistance, and in particular the description of the so-called factor V Leiden mutation, in which a prominent activated protein C cleavage site in FV has been abolished through a mutation in the FV gene, FV has been in the center of attention of thrombosis research. In this review we describe how the functions of FV are expressed and regulated and provide an extensive description of the role that FV plays in the etiology of thromboembolic disease.

Keywords

Factor V, coagulation, thrombosis, thrombophilia, protein C pathway

Introduction

In the Western world, thrombotic disease is a major cause of mortality. The clinical definition of thrombosis is that of the pathological presence of a clot (thrombus) in a blood vessel or in the heart that causes the obstruction of blood flow through the circulatory system. Depending on the location where thrombus formation takes place (i.e. in the venous or arterial part of the vessel tree), thrombotic disease can be classified into venous and arterial thrombosis. Both types of thrombosis are considered as distinct disease states that are characterized by different pathogenic mechanisms and underlying risk factors (1, 2). However, central to the pathogenesis of both venous and arterial thrombosis is the perturbation of the normal haemostatic balance. In healthy individuals, haemostasis is carefully controlled by several anticoagulant mechanisms that balance the procoagulant forces and thus prevent inappropriate vascular blood clotting. Several observations are even in favor of a slight dominance of the anticoagulant forces during homeostasis (3). This dynamic equilibrium between pro- and anticoagulant factors can be rapidly shifted in favor of coagulation in case of a physiological need for cessation of blood loss. However, this proneness towards clot formation implies a risk to develop thrombosis. As early as in the mid 1800s, the German pioneer in the field of haemostasis, Rudolf Virchow, postulated that three major causes contribute to thrombin formation (Virchow’s triad): changes in the blood composition, in the vessel wall or in the blood flow (4). Whereas arterial thrombosis is dominated by vessel wall changes (atherosclerosis), the known risk factors for venous thrombosis can be divided into those that are attributable to changes in the blood flow (stasis) or to changed blood composition. Although Virchow’s broad classification is still valid overall, the view of thrombogenicity in present day medicine is more refined as a result of progress that has been made in this field of research. A more common view is to divide those factors that contribute to thrombosis into two groups: the inherited and acquired (environmental) thrombosis risk factors. Acquired risk factors for thrombosis include changes induced by various influences such as pregnancy, surgery, immobilisation, hypobaric travel, autoimmune disease, diet, smoking and the use of oral contraceptives, or by intercurrent disorders such as diabetes mellitus, dyslipidemia, hypertension and hyperhomocysteinemia (1, 2, 5). Perturbation of the haemostatic balance towards coagulation may also be influenced by genetic changes, most of them affecting the protein C (PC) anticoagulant system, such as activated protein C (APC) resistance caused by the factor V Leiden mutation, or other polymorphic forms of FV, deficiencies of protein S (PS), PC or antithrombin or by increased levels of procoagulant factors such as prothrombin gene mutation, factor V Leiden, factor II or V Leiden, factor VIII or factor IX, or by an enhanced fibrinolytic activity.
as FVIII or prothrombin (3, 6–8). Other distinct factors are age and sex, each affecting an individuals’ cumulative thrombosis risk.

For a long time, thrombosis was commonly regarded as a single gene disorder. This view was conditioned largely by a seemingly obvious thrombosis etiology that was concluded from studies with small numbers of patients or families with inherited deficiencies in the anticoagulant proteins antithrombin (9), PC (10) and PS (11) and the associated high prevalence or severity of thrombosis. Over the last decade, however, additional risk factors for thrombosis have been described, and our understanding of the underlying mechanisms that contribute to the actual development of thrombosis has increased. It has now become widely accepted that thrombosis is a multifactorial disease that may occur as the result of the interplay between two or more genetic, environmental or behavioral risk factors (1, 5). These factors together are capable of synergistically passing a certain anticoagulant threshold, thereby tipping the natural haemostatic balance between pro- and anticoagulant forces. When the threshold is passed, the natural anticoagulant systems are insufficient to balance the procoagulant factors, resulting in the development of a thrombotic event (1).

Recently, it has become evident that hypercoagulability not only results in an increased tendency to thrombosis, but also triggers intracellular signaling for inflammation, given that the pathways of coagulation and inflammation are intertwined at several points. For many years it has been known that C4 binding protein (C4BP), a regulator from the complement pathway, is also a major regulator of the in-vivo activity of protein S (12). Furthermore, the procoagulant clotting factors (F) VIIa, Xa and thrombin can activate members of the protease-activated receptor (PAR) superfamily, which in turn initiate intracellular signaling pathways (13). The elicited inflammatory response includes cytokines, adhesion molecules and growth factors, which up-regulate the expression of the coagulation initiator tissue factor (TF), through their corresponding receptors. This feedback mechanism sustains the coagulation-inflammation cycle and thus contributes to an increased thrombotic risk. Therefore, increasing attention has been recently devoted to the development of PAR antagonists that may be useful in anti-thrombotic therapy (13–15). Lastly, in the process of inflammation the protein C pathway likely is of less importance, as is evidenced by the reduction in mortality after intravenous infusion of APC in patients that have developed life-threatening sepsis. The exact mechanisms have not been clarified, but the contribution of APC and EPCR (the endothelial cell protein C receptor molecule) appear evident (16).

Since thrombin is the dominant enzyme in so many humoral and cellular processes and since for the formation of thrombin in plasma there is an absolute requirement for the presence of FV, regulation of FV activities is a major regulatory mechanism for the control of thrombin formation. FV not only plays a role in procoagulant processes but, as will be detailed below, also plays an important role in the protein C pathway, and thus a dual role with its involvement in both pro- and anticoagulant processes.

This review will focus on inherited defects in blood coagulation FV that are considered as risk factors for thrombosis. Particular attention will be paid to the molecular mechanism by which FV polymorphisms contribute to APC resistance. Interactions with acquired risk factors that are known to synergistically increase the thrombotic risk that is related to FV genetic defects will also be discussed.

Haemostasis

Haemostasis is a physiological mechanism that controls blood fluidity and has the potential to rapidly induce haemostatic plug formation at sites of injury in order to stop or limit bleeding. The three distinct phases of the haemostatic process (primary haemostasis, coagulation and fibrinolysis) are closely linked to each other and are strictly regulated in order to efficiently close vessel wounds and promote vascular healing.

Primary haemostasis is initiated by the adhesion of platelets to collagen fibers underlying the vascular endothelium, which have become exposed following vascular injury. This adhesion is mediated by a specific platelet collagen receptor (glycoprotein IIb/IIIa) and von Willebrand factor (vWF), which forms links between the platelet and collagen fibers (17). As a result of the interaction with collagen, the platelets are activated and release a number of different coagulation factors and platelet activating factors, which in turn activate other platelets and white blood cells. The platelets adhere to each other via adhesion receptors (integrins) that bind to the same receptor on adjacent platelets via a central fibrinogen molecular bridge, which results in the formation of a haemostatic platelet plug. Platelet activation further results in the activation of several phospholipid transporter proteins, among which the protein scramblase, which causes the transport of negatively charged phospholipids from the inner to the outer leaflet of the platelet membrane. These negatively charged phospholipids provide a catalytic surface that is crucially important for efficient propagation of the coagulation through binding of several blood coagulation factors.

Initiation of blood coagulation (also known as secondary haemostasis) in vivo is triggered by the exposure of the transmembrane glycoprotein tissue factor (TF) to blood (18). Exposed TF from subendothelial layers binds with high affinity and specificity to both the zymogen and activated forms of coagulation FVII (FVII and FVIIa, respectively). A small fraction of FVII circulates in blood as an active protease (FVIIa) and activates FIX and FX when it is bound to TF. Activated FIX (FIXa) subsequently interacts with its nonenzymatic cofactor FVIIIa on the activated platelet surface to form the tenase complex, which efficiently activates FX. After its formation by the tenase complex, FXa assembles on the platelet membrane with its cofactor FVa to form the prothrombinase complex. The prothrombinase complex efficiently activates prothrombin to thrombin, whose feedback amplifies its own formation by activating FV, FVIII and FXI (19–25). Activation of FXI results in the generation of additional FIXa, which in turn activates FX, thus enforcing thrombin formation (26). Most importantly, thrombin converts soluble fibrinogen into insoluble fibrin fibers which aggregate to a soft fibrin clot.

The third phase of haemostasis involves fibrinolysis. The role of fibrinolysis is to remove fibrin deposits, after the damaged vessel wall has been restored. To this end, circulating plasminogen is activated by interactions between fibrin, plas-
minogen and the activator tPA. Plasmin, the product of plasminogen activation, is able to proteolytically degrade fibrin, such that the fibrin network is dissolved.

Anticoagulant mechanisms that regulate haemostasis

The formation of a stable plug by platelet aggregation and fibrin polymerization following vascular injury is a very rapid and important process that prevents extensive blood loss. However, intrinsic to the capacity of the blood clotting system to respond quickly to vascular injury is the danger of excessive clotting that may contribute to thrombosis. Therefore, nature has provided us with several anticoagulant mechanisms that control the coagulation pathway at different levels and that prevail over procoagulant mechanisms under normal conditions. Two major systems can be discriminated that provide efficient control of the activity of pro- and anticoagulant pathways. A first system comprises circulating inhibitors (e.g. TFPI [18, 27], antithrombin [28], α2 macroglobulin [29, 30], antitrypsin [31]) which can directly neutralise activated coagulation factors. A second important negative regulatory pathway of the coagulation cascade is the PC pathway (32–34). A key event in the initiation of the PC pathway is the formation of the thrombin-thrombomodulin complex (35). Thrombomodulin (TM) is a transmembrane protein present on intact endothelium, primarily that of the smaller vessels, that acts as a thrombin receptor by binding to thrombin exosite I and provides a binding platform for the substrate protein C. This thrombin exosite I plays an important role in the thrombomodulin-mediated recognition and activation of FV, FVIII and fibrinogen (28). As a consequence, the procoagulant properties of thrombin molecules, that escape a site of ongoing coagulation and migrate into the microvasculature, are lost upon binding to TM. In addition, TM-binding causes conformational changes near the active site of thrombin which alter the substrate specificity of thrombin (35, 36) such that thrombin becomes an anticoagulant protein that can efficiently activate PC in a process that is enhanced by the endothelial cell PC receptor (EPCR) (35).

In contrast to inhibitors that directly target protease activity, the PC pathway regulates the formation of the activated procoagulant enzymes FXa and thrombin by proteolytic inactivation of FVIIIa and FVa, respectively, both of which are the essential cofactors of the tenase and prothrombinase complex, respectively (37). Proteolytic inactivation of FVIIIa and FVa (as opposed to the spontaneous inactivation through cofactor dissociation which may be particularly important in the case of FVIIIa) is accomplished by APC and is stimulated by the non-enzymatic cofactor PS (38–40). During the degradation of FVIIIa, FVa acts as an additional cofactor to APC and the stimulating effect of FV and PS on FVIIIa inactivation was found to be synergistic rather than additive (41, 42).

Under physiological conditions, the presence of the circulating inhibitors and the PC pathway together form the thrombosis threshold mentioned earlier that prevents the initiation and propagation of thrombin formation. When the coagulation trigger is such that sufficient amounts of proteolytically active enzymes are formed that cannot be controlled by the inhibitors or the PC pathway, the formation of large amounts of thrombin can no longer be prevented. At this stage the threshold is surpassed. The physiological importance of the PC pathway is illustrated by the observation that deficiencies of PC or PS result in an increased risk for venous thrombosis (6, 43).

Blood coagulation FV

Blood coagulation FV is a pivotal protein in haemostasis, playing a crucial role in both the procoagulant and anticoagulant pathways. In its activated form, FV serves as a cofactor of FXa in the prothrombinase complex that catalyzes the conversion of prothrombin into thrombin. In its unactivated form, however, FV serves as a cofactor of APC in the regulation of FVIIIa activity. Given the dual role of FV in the coagulation cascade, hence its description as a ‘Janus faced protein’ (44), genetic and acquired defects that affect the activity or expression level of the FV molecule may result in the manifestation of either thrombotic or haemorrhagic events (45, 46). In this paragraph, the structure and function of FV in the pro- and anticoagulant pathways will be detailed.

FV synthesis and primary structure

The gene encoding human FV (F5) is approximately 80 kilobases (kb) in size, is located on the long arm of chromosome 1 (1q23) and consists of 25 exons and 24 introns (Fig. 1A) (47, 48). Transcription of the F5 gene gives rise to a 6.8 kb mature mRNA, and the encoded protein consists of 2,224 amino acid residues including a 28 amino acid long signal peptide, which is removed after translocation to the endoplasmatic reticulum. The resulting FV molecule is a single-chain glycoprotein of 330 kDa that circulates in blood at a concentration of approximately 21 nM (49). In addition, 20–25% of total human FV is found in the α-granules of platelets, where it is stored in a partially proteolyzed form in association with multimerin (49). Plasma-circulating FV is synthesized in the liver, whereas the platelet fraction of FV is partly synthesized in the megakaryocytes and partly absorbed from plasma via endocytosis (50). Given the observation that liver transplantation can alter the platelet FV phenotype, the platelet FV pool is mainly derived from endocytosed molecules of plasma FV (51). During the initial phase of haemostasis, platelet FV is released from activated platelets, which causes a local increase in the FV concentration at sites of vascular injury (44).

FV possesses an A1-A2-B-A3-C1-C2 domain architecture that is similar to that of FVIII (Fig. 1B) (52). The FV and FVIII A-domains are highly homologous and share about 40% sequence identity with the copper-binding protein ceruloplasmin (48, 53, 54). The C-type domains share 46% sequence identity with their FVIII counterparts and belong to the discoidin domain of phospholipid-binding lectins (48, 55). The FV B-domain is poorly conserved among the various species of FV that have been studied, and shares low sequence identity (~15%) with the FVIII B-domain (48).

Maturation of FV includes extensive post-ribosomal modifications. Among these, glycosylation of FV has been best studied (56–59). A total of 37 potential N-glycosylation sites have been proposed on the surface of FV, and it is known that these influence the cofactor functions of this protein. Differential glyco-
sylation of the C2 domain in FV results in the formation of two different variants of FV, named FV₁ and FV₂, that both circulate in blood and in platelets at a 33:67 molar ratio (FV₁:FV₂) (57–59). The two FV isoforms possess different functional properties in both pro- and anticoagulant pathways (see below) and in model systems mimicking physiological conditions, FV₁ appears to be the more thrombogenic (60, 61). Tyrosine sulfation in regions preceding the thrombin activatable peptide bonds is another modification that has been shown to be of influence for efficient activation of FV by thrombin (62).

**Procoagulant function of FVa**

FV has little or no intrinsic procoagulant activity prior to its activation through limited proteolysis by thrombin or FXa at Arg709, Arg1018 and Arg1545. The resulting FVa molecule is a heterodimer that consists of a 105 kDa heavy chain (A₁-A₂ domains) and a 71/74 kDa light chain (A₃-C₁-C₂ domains) that are non-covalently associated in a calcium-dependent manner (Fig. 2) (63, 64). Unlike FV, FVa enhances the rate of FXa-catalyzed prothrombin activation of the prothrombinase complex by several orders of magnitude (65, 66). It has been proposed that the large B-domain keeps the FV molecule in the procofactor state by sterically and electrostatically inhibiting the interaction with FXa. Upon activation, however, the B-domain is released which results in the exposure of regions in the FV molecule that are important for its interaction with FXa in the prothrombinase complex (67, 68).

In recent years, detailed structural data have become available for the A- and C-domains of FV (69–72). Study of the structure of FV and of the complexes it is engaged in is of utmost importance for the understanding of its functions in both pro- and anticoagulant pathways and provides a rationale for potential future treatment. The available structural data are the result of several in-silico (homology modelling) studies (69–71) of the human FV A and C domains, which have allowed the construction of a number of models for the structure of membrane-bound FVAs. Also, X-ray crystallography has been applied to the study of FV structure and has yielded detailed information on the structure of the human C2 domain (72), as well as of the structure of bovine FVα, the APC-inactivated form of FV that lacks the A2 domain (73). In this latter structure, the position of the FVa C-domains relative to each other is totally different from that in other structures generated before, in which the C1-domain was predicted to be stacked above the membrane-bound C2 domain.

Recently, a low-resolution structure of membrane-bound (complete) FVAs was determined by transmission electron microscopy (EM) studies. The proposed membrane-bound FVα EM structure is in agreement with the APC-inactivated bovine FVα crystal structure and suggests a deeper insertion of both the C1 and C2 domains into the membrane than has been suggested before (Stoilova-McPhiee et al., manuscript submitted). The observation that both C domains participate in membrane binding is in agreement with mutagenesis studies showing that combined mutations in the hydrophobic solvent-exposed loops of these domains impair FV membrane binding (74, 75). Based on the crystal structure of APC-inactivated bovine FVα, a model of full-length human FVAs was constructed, enabling the generation of a structural model of the prothrombinase complex using protein-protein docking methods (76). The three A-domains, which are arranged in a triangular fashion, rest upon the platform that is formed by the C domains. In this way, the A-domains are present at a height above the phospholipid membrane surface that is ap-

![Figure 1: FV gene and molecule. A) Schematic representation of the exon-intron structure of the FV gene. Exons are represented by grey boxes, introns are drawn as lines. The scale is indicated at the bottom of the figure. B) Diagram of the domain organization of the human FV molecule. The N-terminal 28-aa residues signal peptide is represented in black, the homologous A-domains in light grey, the B-domain in white and the two homologous C-domains in dark grey. Grey rhombs represent the position of N-glycosylation sites. Free cysteines and cysteines involved in disulfide bridges are also indicated. The three thrombin activation cleavage sites are indicated by arrows.](https://www.thrombosis-online.com/533.png)
propriate for interaction with FXa and other physiologic partners like prothrombin or APC (77, 78) (Fig. 3A).

The mechanism by which FVa exerts its cofactor activity in the prothrombinase complex is still not fully understood, but it has been proposed that FVa acts as a receptor of FXa, because the presence of FVa on the phospholipid surface increases the affinity of the FXa-phospholipid interaction approximately 100-fold (79, 80). In addition, FVa also increases the catalytic activity of FXa more than 1,000-fold (66). A likely explanation for this rate enhancement has been provided by Walker et al. who have shown that, in contrast to what had previously been speculated, inclusion of FVa into the prothrombinase complex does not result in major changes in the FXa active site (81). Instead, a major role of FVa in the prothrombinase complex may be to provide extensive binding (exo)sites for prothrombin binding (82–84) and/or to increase the affinity of FXa for prothrombin (85).

FVa cofactor activity is efficiently downregulated by APC upon limited proteolysis at three cleavage sites that are located in the FVa heavy chain at Arg306, Arg506 and Arg679 (37). Of these cleavages, inactivation at Arg679 is slowest and does not appear important for the physiological inactivation of FVa (86, 87). A dual pathway model for the inactivation of FVa by APC has been proposed (8). A first option for FVa inactivation is via initial cleavage at Arg506, the preferred cleavage in FVa, that is subsequently followed by cleavage at Arg306. Alternatively, direct inactivation of the FVa molecule can occur by initial cleavage of Arg306. Cleavage at Arg506 results in a 40-fold reduction in the affinity of FVa for FXa, whereas the subsequent cleavage at Arg306 results in complete FVa inactivation. Complete loss of FVa cofactor activity is associated with the dissociation of the A2 domain, which is also likely to occur after initial cleavage of FVa at Arg306 (37, 88, 89). A 3D-model of the FVa-APC complex (Fig. 3B) suggests that a positively charged region in the serine protease domain of APC (comprising the 37, 60, 70 and 148 loops) interacts with a negatively charged region adjacent to the Arg506 cleavage site in FVa (90, 91). The cleavage at Arg306 is mediated by different interactions, as the binding between APC and the region around the Arg306 in FVa appears to be conferred by far less intermolecular contacts, than that around Arg506 (91). In line with this, it has been observed that the elimination of the positive cluster in the serine protease domain of APC had no effect on the rate of the Arg306 cleavage, but reduced the rate of cleavage at Arg506 (92, 93).

Anticoagulant function of FVa

Besides its procoagulant function in prothrombin activation, FV also accomplishes an anticoagulant function by stimulating the APC-mediated inactivation of FVIIIa (41, 94, 95). The import-
The observation that FV loses its anticoagulant activity after activation by thrombin suggested the involvement of the B-domain in this function (41, 97). This was confirmed by studies using recombinant FV variants which demonstrated that the anticoagulant activity of FV resides in the C-terminal part (last 70 amino acids) of the B-domain of FV (98). Additional evidence for the involvement of the B-domain of FV in the APC-catalyzed inactivation of FVIIIa was provided by the observation that a monoclonal antibody specific for the C-terminal part of the FV B-domain inhibited the anticoagulant function of FV (41). Such an inhibitory antibody has meanwhile also been observed in a patient with serious thrombotic complications (99).

In order to express its anticoagulant function, FV needs to be cleaved by APC at Arg506 (Fig. 2) (42, 100). In contrast, the APC-mediated cleavage at positions Arg306 or Arg679 does not result in the expression of FV anticoagulant activity (100).

**FV and thrombophilia**

Given the key role of FV in haemostasis, regulation of its pro- and anticoagulant cofactor activities is of prime importance for maintaining the haemostatic balance. As a consequence, perturbed FV functions can result in both thrombotic and haemophilic clotting disorders (45, 46). In the following paragraphs, we will discuss genetic and acquired defects that affect the activity and/or expression level of the FV molecule and therefore are considered as risk factors for thrombosis.

**APC resistance**

APC resistance is an *in vitro* phenomenon that is characterized by a poor anticoagulant response to APC in plasma. This reduced susceptibility to APC of a plasma sample results in inefficient regulation of thrombin formation *in vitro* making this plasma APC resistant. APC resistance *in vitro* is as such associated with an increased risk for thrombosis (101). The phenomenon of APC resistance was first reported by Dahlbäck et al., who found that the plasma of a group of patients with a family history of venous thrombosis showed a reduced anticoagulant response to the addition of APC (94). Later it was found by several groups simultaneously that a single point mutation in the FV gene, which results in the substitution of arginine at position 506 by glutamine (FV*Leiden* mutation), was responsible for the observed APC-resistant phenotype of these patients (102–105). The R506Q mutation is the hallmark of the FV*Leiden* phenotype, and is a missense mutation at a dominant APC cleavage site in the heavy chain domain of FV (see Table 1). APC resistance represents the most frequent independent risk factor for (venous) thrombosis known to date (106). In most cases, it is associated with the FV*Leiden* mutation, but several other genetic and acquired risk factors such as the prothrombin G20210A mutation, pregnancy or the use of oral contraceptives are known to affect the anticoagulant response to APC. It should be noted that APC resistance as such is an independent risk factor for the development of thrombosis, even in the absence of the FV*Leiden* mutation (101).
The FV<sub>Leiden</sub> mutation as a major cause of APC resistance

Since its discovery in 1994, the FV<sub>Leiden</sub> mutation has been demonstrated to represent the most frequent cause of APC resistance (107). In healthy individuals of Caucasian origin, the prevalence of FV<sub>Leiden</sub> is between 2 and 10%, making the factor V<sub>Leiden</sub> mutation as an important factor of APC resistance (108). The highest prevalence of the mutation is found in Europe, most notably in Cyprus, Southern Sweden and Germany, but the mutation is also very common in Saudi Arabia and in Arab and Jewish populations of Israel (109). Although widely present in Caucasians, the FV<sub>Leiden</sub> mutation is not found in other ethnic groups such as in individuals of African, Chinese or Japanese origin. Haplotyping of FV<sub>Leiden</sub> homozygotes suggested a founder effect and that a single mutational event occurred approximately 21,000 years ago (110).

Among patients with venous thromboembolism, FV<sub>Leiden</sub> occurs in 20% of all cases and in up to 50% of selected patients with thrombophilia. The FV<sub>Leiden</sub> mutation seems to be a risk factor of much the same strength as the deficiencies of coagulation inhibitors, increasing the risk about five-fold in heterozygous carriers (111). Homozygous carriers of the mutation are considered to have a 50- to 100-fold increased risk of thrombosis.

The most common clinical manifestations of APC resistance caused by the FV<sub>Leiden</sub> mutation are venous thrombosis and pulmonary embolism. It is generally accepted that there is a much less clear link between the mutation and arterial thrombosis. Recent studies, however, have identified factor V<sub>Leiden</sub> as an important risk factor for arterial thrombosis (112, 113). Yet other studies have linked the FV<sub>Leiden</sub> mutation to atherosclerosis (114) and myocardial infarction (115). At the same time, however, several other studies have been unable to directly associate the FV<sub>Leiden</sub> mutation to arterial thrombotic disease (116, 117).

Molecular basis of FV<sub>Leiden</sub>-related APC resistance

The FV<sub>Leiden</sub> mutation, i.e. the substitution of a glutamine for an arginine at position 506, has important consequences for the function of FV in the pro- and anticoagulant pathways of the blood coagulation process. First, the loss of one of the three APC cleavage sites in FVa impairs efficient downregulation of procoagulant FVa activity (88). It was long believed that the reduced APC-mediated inactivation of FVa<sub>Leiden</sub> provides a rational molecular mechanism to explain the increased thrombogenicity in carriers of this mutation. However, in a purified system it was demonstrated that PS and FXa can almost completely eliminate the differences between the APC-mediated inactivation of normal FVa and FVa<sub>Leiden</sub>. This may be explained by the observed protective effect of FXa against FVa cleavage at Arg506 by APC (118), together with a stimulating effect of PS on mainly the APC-mediated cleavage at Arg306 (119, 120).

The discovery that FV serves as a cofactor during the APC-catalyzed inactivation of FVIIIa provided an important clue to possible mechanisms by which the FV<sub>Leiden</sub> mutation contributes to the APC resistance phenotype in vivo. It was found that FXa isolated from patients carrying the FV<sub>Leiden</sub> mutation was a much weaker APC cofactor in the inactivation of FVIIIa than normal FX (61, 97). Later, it was demonstrated using recombinant FX that cleavage at Arg506 is required for FV in order for FV to ex-

Table 1: List of missense variations in FV discussed in the text.

<table>
<thead>
<tr>
<th>Nucleotide variation</th>
<th>Amino acid substitution</th>
<th>Alternative name</th>
<th>Domain</th>
<th>Exon</th>
<th>dbSNP number</th>
<th>MAF African</th>
<th>MAF Caucasian</th>
<th>MAF Oriental</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1691A</td>
<td>Arg506Gln</td>
<td>FV Leiden</td>
<td>A2</td>
<td>10</td>
<td>rs6025</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G1091C</td>
<td>Arg306Thr</td>
<td>FV Cambridge</td>
<td>A2</td>
<td>7</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>A1090G</td>
<td>Arg306Gly</td>
<td>FV Hong Kong</td>
<td>A2</td>
<td>7</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>T1250C</td>
<td>Ile359Thr</td>
<td>FV Liverpool</td>
<td>A2</td>
<td>8</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>G1628A</td>
<td>Arg485Lys</td>
<td></td>
<td></td>
<td>2</td>
<td>rs6020</td>
<td>30</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>T1328C</td>
<td>Met385Thr</td>
<td></td>
<td></td>
<td></td>
<td>rs6033</td>
<td>0</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>A4070G</td>
<td>His1299Arg</td>
<td>R2 haplotype</td>
<td>A2</td>
<td>8</td>
<td>rs6033</td>
<td>0</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>A5380G</td>
<td>Meth1736Val</td>
<td></td>
<td>B</td>
<td>13</td>
<td>rs1800595</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A6755G</td>
<td>Asp2194Gly</td>
<td></td>
<td>A3</td>
<td>16</td>
<td>rs6030</td>
<td>19</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>A2391G</td>
<td>Ser739Ser</td>
<td></td>
<td>C2</td>
<td>25</td>
<td>rs6027</td>
<td>0</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>A2663G</td>
<td>Lys830Arg</td>
<td></td>
<td>B</td>
<td>13</td>
<td>rs6021</td>
<td>7*</td>
<td>0*</td>
<td>?</td>
</tr>
<tr>
<td>A2664G</td>
<td>His837Arg</td>
<td></td>
<td>B</td>
<td>13</td>
<td>rs4524</td>
<td>17</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>A2863G</td>
<td>Lys897Asp</td>
<td></td>
<td>B</td>
<td>13</td>
<td>rs6032</td>
<td>17</td>
<td>26</td>
<td>17</td>
</tr>
</tbody>
</table>

The common allele is given before the coordinate, the rare allele after the coordinate. Numbering is according to the cDNA sequence of F5 (Jenny et al. [48]; GenBank accession number M16967). Population frequency data are obtained from an online database of mutations and polymorphisms in the FV gene, accessible at http://www.lumc.nl/4010/research/factor_V_gene.html [141]. Data from the HapMap project (http://www.hapmap.org/index.html.en), in which Africans, Caucasians, Han Chinese and Japanese were typed, have been used. The Oriental MAF (Minor Allele Frequency) represents the average from the Chinese and Japanese data. If the HapMap data are not available, data were obtained from Seattle SNPs (http://pga.mbt.washington.edu/), in which Afro-Americans and Caucasians were typed. Question marks have been entered when data were not available from the HapMap or Seattle project. Because of its presence in a repeated region, the H1299R-variant has not been typed in any of the projects.
press its anticoagulant cofactor activity (100). Thus, because FV
Leiden cannot be cleaved at Arg506, it cannot be transformed into
an anticoagulant FV molecule and therefore it is a poor cofactor of APC in the proteolytic inactivation of FVIIa.

The pathophysiologic relevance of the FVLeiden mutation was
further illustrated by thrombin generation experiments from our
laboratory, in which FV-deficient plasma was reconstituted with
increasing concentrations of normal FV or FVLeiden. In the
presence of APC, thrombin generation was progressively decreased
when increasing the concentration of normal FV, whereas the ad-
tion of FVLeiden had no effect on thrombin generation (96).
Moreover, addition of purified normal FV, was able to reduce
thrombin generation in pseudohomozygous carriers of the
FVLeiden mutation (who express 50% FVLeiden only since they also
carry a null-allele). These individuals have an associated throm-
bohris risk that is in the same range as that of homozygous carriers of
the FVLeiden mutation (50– to 80-fold increased). Addition of
purified FV to pseudohomozygous plasma, brings the response
to APC in the same range as that of heterozygous carriers of the
FVLeiden mutation, who have an associated risk of thrombosis that
is ~five-fold increased. It therefore appears as if the increased
risk of thrombosis in homozygous and pseudohomozygous car-
riers of the FVLeiden mutation is not so much a defect in the FV
that is present, more likely it illustrates the anticoagulant poten-
cy of normal FV, that is absent in these individuals.

Other FV mutations associated with APC resistance

Studies validated by FV gene analysis revealed that up to 20% of
randomly selected individuals exhibiting APC resistance do not
carry the FVLeiden mutation (121). Since the discovery of the
FVLeiden mutation, several other genetic defects in the FV gene
have been identified as possible risk factors for thrombosis.

Some of these mutations affect the APC cleavage site at posi-
tion Arg306. A first variant, FV Cambridge, contains a missense
mutation in the FV gene resulting in the replacement of Arg306 by
threonine. The FV Cambridge mutation was first discovered
in a British patient and his mother who where found to be APC
resistant (7). A second mutation at Arg306 is caused by a mis-
sense mutation in the FV gene resulting in an Arg to Gly substi-
tution at position 306. This variant was first described in two
Chinese thrombotic patients from Hong Kong and has been
named FV Hong Kong. Although phenotypically very similar to
FV Cambridge, the FV Hong Kong mutation has not been associ-
ated with APC resistance (8). However, in-vitro experiments em-
ploying recombinant FV Cambridge and FV Hong Kong mol-
ecules have shown that both variants cause mild APC resistance
due to a moderately impaired APC cofactor activity (122). It is
not known what has caused the different previous observations in
plasma, but other plasma factors that contribute to APC resist-
ance are likely candidates to explain the apparent difference be-
tween the plasma derived proteins and their recombinant
counterparts. Why FV Cambridge and FV Hong Kong are less
resistant to APC than FVLeiden remains unclear in particular since
it is evident from studies with purified proteins that both cleav-
ages at Arg506 and Arg306 are important for the loss of FVα ac-
tivity (88). It has been proposed that in the absence of the
normal Arg306 cleavage site, alternative APC cleavage sites
close to this region become relevant (123). Likewise, it is possi-
ble that reduced Arg306 cleavage in plasma can remain masked
as a result of low levels of FXa generated during the coagulation
assay: at low [FXa] the activity of FVα cleaved at only Arg506 is
virtually indiscriminable from completely inactivated FVα (88).
Due to the rare occurrence of both FV variants (124), extensive
epidemiological studies are not available, and as a result, it re-
 mains unanswered whether or not both mutations are risk factors
for venous thrombosis.

Apart from the mutations affecting the APC cleavage sites
described so far, other mutations in the heavy chain of FV have
been reported that are associated with mild APC resistance. The
FV Liverpool mutation, a missense mutation that results in the
substitution of Ile359 by threonine (Fig. 4), introduces a novel
glycosylation consensus sequence at Asn357 (125). The pres-
ence of a carbohydrate side chain at this position hampers APC
cleavage at Arg306 and Arg506 and also impairs the APC cofac-
tor function of FV in the degradation of FVIIIa presumably by
steric hindrance (126).

The R485K mutation (see Fig. 4) has been found in several
Oriental populations and has been associated with venous
thrombosis (127), coronary artery disease (128) and pre-eclamps-
ia (129). Unfortunately, no data are available that might explain
the molecular mechanism that explains the observed APC resis-
tance in carriers of the R485K mutation.

A further common genetic variation in the FV gene is the R2
polymorphism, which is hallmarkized by a His to Arg substitution
at position 1299 in the FV B domain (130). A number of other
mutations in the FV gene are tightly linked to the R2 polymor-
phism. These mutations are collectively referred to as the R2 ha-

Figure 4: FVα missense mutations associated with APC resis-
tance. FVα is shown as a ribbon, using the same color code as in Figure
3. Polymorphisms in the FVα molecule that were discussed in the text
are displayed in CPK representation (red). The H1299R mutation is
present in the B domain of FV for which no structure is available at pres-
tent. The figure was created with ICM Pro (Molsoft LLC).
plotype and predict amino acid substitutions in both the heavy and light chain as well as the B-domain (131, 132). Based on its worldwide distribution and high prevalence in both the Caucasian and non-Caucasian population, it was proposed that the origin of the R2 haplotype dates back further than the FV_{Leiden} mutation (132). The Asp2194Gly mutation, which is tightly linked to the R2 haplotype, has been associated with reduced FV levels and a shifted FV1:FV2 ratio in favour of the more thrombogenic FV1 isoform (131). It has earlier (59) been shown that differential glycosylation of Asn2181 in the C2 domain of FV is the molecular cause of the coexistence of two forms of FV: FV1 and FV2. Yamazaki et al. (133) later proved that the Asp2194Gly mutation and not Met385Thr, His1299Arg or Met1736Val, plays a key role in the partial FV deficiency of the R2 haplotype, due to impaired secretion of recombinant molecules that carry this missense mutation. In-vitro experiments using recombinant FV molecules further indicate that the presence of a glycosylation site at position 2194 stimulates glycosylation at Asn2181 (Yamazaki and Nicolas, unpublished data).

Molecular dynamics simulations have demonstrated that the Asp2194Gly mutation results in a less stable FV C2 domain as a result of the loss of a salt bridge network between Asp2194 and surrounding Lys residues (134). Possibly, the non-glycosylated 2194Gly variant is cleared differently from the circulation than its glycosylated counterpart, which could explain the observed lower FV levels and increased FV1:FV2 ratio in FV R2 carriers. Likewise, the glycosylation at Asn2181 in the C2 domain may be an epiphenomenon that is not directly linked to the levels of circulating FV, but merely is an illustration of the overall intracellular processing of FV.

The molecular mechanism that causes the observed (mild) decrease in APC resistance in R2 carriers has still not been completely elucidated. The reduced level of expression of R2-FV is the most likely explanation for the enhanced thrombophilic phenotype in R2 carriers, in particular in FV_{Leiden} carriers. It is now indeed widely accepted that compound heterozygosity of the R2 and the FV_{Leiden} mutations is associated with a higher risk of venous thrombosis than heterozygous FV_{Leiden} alone (135–137). Until now, however, there are no conclusive data available that associate the R2 haplotype per se with a relevant increased risk of thrombosis (138). Recently, it was observed that male carriers of the FV R2 polymorphism have increased circulating levels of FVIII (139). No biochemical explanation has been given for this observation, but it is tempting to speculate that altered FV anticoagulant activity of R2 FV results in diminished proteolytic control of FVIII levels by the protein C pathway. Since increased levels of FVIII are an independent risk factor for the occurrence of venous thrombosis (140), this may explain a potential association between R2 FV and thrombosis.

Given the poor conservation of the FV B domain among different mammalian species and thus that mutations are obviously tolerated within this part of the FV gene, it is to be expected that more missense mutations are present in this domain. Besides the H1299R mutation, a number of other missense mutations in the B domain have been reported (141). However, most of these mutations are not associated with functional defects and, together with the overall poor conservation of the B domain, this may indicate that the amino acid sequence of the B domain is not an important determinant for FV function. Nevertheless, a number of polymorphisms in the FV B domain have been described that are always co-inherited in Caucasians and that contribute to the APC resistance phenotype (142). Because these mutations result from the A>G transitions at nucleotide positions 2391, 2663, 2684 and 2863, the terms A-allele and G-allele were introduced for the wild-type and variant allele, respectively. Of these four mutations, only the last three changes result in amino acid substitutions (K830R, H837R, K897E). The molecular mechanism involved in the APC resistance phenotype that is associated with these polymorphisms is still unknown. It may be anticipated that the G-allele mutations affect the anticoagulant function of FV rather than its procoagulant function, because they are present in a region that is removed upon activation. In the absence of direct proof of the effect that these mutations have on FV function, caution is to be taken with these FV variants. The changed APC resistance phenotype that is observed in the plasma of individuals carrying this haplotype may merely be a reflection of yet another (unknown) genetic variation outside the FV gene that causes the APC resistance to vary.

Acquired risk factors that increase thrombotic risk by affecting FV function

Apart from genetic defects, other factors that influence FV function and impair the response to APC have been reported. Over the last decade, several individuals have been described who have developed auto-antibodies against FV (143–147). In most instances, these antibodies inhibit the expression of the normal functions of FV in haemostasis. The most common cause of FV inhibitors has been exposure to bovine thrombin. Bovine thrombin is commonly mixed with fibrinogen derived from cryoprecipitate to make fibrin sealant, a haemostatic preparation that is widely used in cardiovascular surgery (148). The presence of small amounts of contaminating proteins (among which FV) in the thrombin preparation that has been purified from bovine plasma may elicit a strong immune response in recipients. Although antibodies against FV generally have no clinical consequences or induce a bleeding tendency, a number of cases have been reported in which the development of FV auto-antibodies was associated with thrombosis. Sufficient data to establish a direct causal link between FV antibodies and thrombosis have only been obtained in a couple of instances (144–147). It is unknown what the underlying mechanisms are that result in the increased thrombotic risk in these patients. It is possible that the auto-antibodies block the anticoagulant properties of FV, similar to the confirmed effect that a monoclonal FV antibody, directed against an epitope in the B domain of FV, has on the in-vitro APC cofactor activity of FV (41). Since patients with FV antibodies had additional risk factors for thrombosis, it cannot be excluded that the thrombotic phenotype is to be attributed to the presence of these risk factors.

Risk factor interactions determine thrombotic risk

Although the pathogenesis of venous thrombosis is still far from being fully elucidated, substantial progress has been made over...
the last decade towards a better understanding of the pathophysiological mechanisms involved in this disease. The most significant finding was the confirmation of the concept that inherited hypercoagulable conditions are present in a large proportion of patients with venous thrombosis or pulmonary embolism. It has been estimated that inherited genetic risk factors may account for more than 60% of the total risk to develop thrombosis (149).

The high prevalence of the FV<sub>Leiden</sub> mutation and other frequently occurring prothrombotic mutations (e.g. the G20210A prothrombin mutation) have made it possible to study the cumulative effects of individual risk factors on the overall thrombotic risk. In individuals where multiple risk factors for thrombosis occur, the risk factors appear to have a synergistic effect. Among thrombophilic families with antithrombin, PC and PS deficiency, the HR2 haplotype or the prothrombin G20210A mutation, it was found that the prevalence of the FV<sub>Leiden</sub> mutation was much higher than in the general population. Moreover, the combined risks in these families are much higher than the risk conferred by the sum of the single thrombophilic defects (136, 150–153). It is now widely accepted that interactions between more than one genetic or acquired risk factor are a prerequisite for the development of thrombosis (1, 139). Given the ubiquitous presence of FV prothrombotic genetic variants, FV is one of the important factors that contribute to development of thrombosis worldwide.

The earlier mentioned FV<sub>Leiden</sub> pseudohomozygosity (a combination of the FV<sub>Leiden</sub> mutation and a null mutation on different alleles (in-trans)) is in fact a special type of gene-gene interaction, where the presence of a nonsense and a missense mutation on different alleles coincide within the same gene. Alternatively, the lack of expression of the FV null-allele can mask a detrimental effect of the FV<sub>Leiden</sub> mutation when both mutations are present on the same allele (in-cis). This may cause a discrepancy in the FV genotype/phenotype, which in this case appears to be in favor of the carrier: the FV<sub>Leiden</sub> mutation is silenced by another null-mutation of the same allele (154), and therefore only normal FV is expressed from the remaining normal FV allele. Notably, both these cases illustrate that APC resistance should preferably be evaluated with a functional assay since PCR-based mutation analysis of the FV<sub>Leiden</sub> mutation may result in test outcomes that do not represent the biologically relevant phenotype in case of in-trans or in-cis combinations of the FV<sub>Leiden</sub> allele and a null-mutation.

Besides gene-gene interactions, interactive effects between genetic and acquired risk factors also contribute significantly to a higher thrombotic risk. Such interaction is well illustrated when analyzing the risk for venous thrombosis in women carrying the FV<sub>Leiden</sub> mutation who use oral contraceptives (155, 156). It has been estimated that the relative risk for the development of thrombosis in FV<sub>Leiden</sub> carriers taking oral contraceptives is approximately five times higher than in FV<sub>Leiden</sub> carriers who do not use oral contraceptives (157). The factors that increase thrombosis risk in women taking oral contraceptives are clearly not independent of the mechanism that increases APC resistance in FV<sub>Leiden</sub> carriers, as is concluded from the overall multiplicative risk of thrombosis (as compared to the risk increases that are conveyed by each of the factors separately).

Other examples of synergistic interactions between environmental factors and FV genetic risk factors are those observed between FV<sub>Leiden</sub> and the antiphospholipid syndrome (158) and long-distance travel (159). Also in these cases there is an apparent interaction between the processes that are the cause of the thrombosis risk increase for each of the factors separately, and the combined risk increase outweighs the sum of the increases due to individual risk factors.

This review has concentrated on FV, its functions and inherited defects in the FV gene that may contribute to an increased thrombotic risk. It should be kept in mind, however, that thrombosis is a multifactorial disease in which genetic and acquired risk factors interact dynamically. Therefore, the role that FV plays in the etiology of thrombosis is largely dependent on other factors that influence the impact of any altered FV phenotype. Although an increasing number of genetic risk factors have been identified in recent years, little is known about the combined effect of multiple genetic and acquired risk factors on the overall thrombotic risk. Future studies will need to focus on gene-gene and gene-environment interactions to fully appreciate the impact of a given polymorphism in venous and arterial thrombosis. The central role that FV plays both in pro- and anticoagulant pathways suggests that any alterations in the FV gene or its expression may have more pronounced systemic functional effects than alterations in the genes of other coagulation factors.

References
Segerse et al. Coagulation factor V and thrombophilia


