Modulators of bleeding tendency in severe factor V deficiency

7

Summary and general discussion

Coagulation FV was first described by Paul Owren and was in fact discovered through the study of a patient with congenital FV deficiency.[1,2] Dating back to 1943, FV deficiency was (one of) the first of the rare bleeding disorders to be discovered and the description of that single patient revived research in the haemostasis field. The rare occurrence of FV deficiency, however, has hampered research into this condition, leaving many issues unsolved. In particular, the variable severity of bleeding symptoms presented by FV-deficient patients with similar plasma FV levels remains unexplained and makes it challenging to predict bleeding risk in individual patients.[3]

While several lines of evidence support the concept that FV is essential to blood clotting (*e.g.* the lethal phenotype of *Fv* knock-out mice[4] and the absence of gross genetic defects (large deletions, duplications or inversions) in the *F5* gene in humans),[5] the plasma level of FV actually needed for viability is very low (<1%).[6-9] Bleeding complications only occur when FV levels drop below 5%,[10] making symptomatic FV deficiency a recessive trait. When studying the bleeding tendency associated with congenital FV deficiency, several problems emerge. First, many patients with undetectable (<1%) plasma FV levels experience only mild-to-moderate bleeding.[3] Second, the severity of bleeding symptoms observed in the low FV range is very variable (even patients with the same genetic abnormality show phenotypic heterogeneity).[11] And third, *in vitro* coagulation tests do not reflect the *in vivo* situation; no thrombin generation or clotting is observed in plasma from many patients that do not experience life-threatening bleeding in real life.

In the present thesis we provide two complementary explanations for the overall moderate bleeding diathesis associated with FV deficiency, which are explained below. However, it must be kept in mind that the bleeding episodes an individual patient experiences in his life also reflect the exposure to risk situations. In fact, the same bleeding tendency might never result in life-threatening bleeding in the absence of traumatic situations, but turn out fatal in a car accident. Especially females face more risk situations as a result of menses and childbirth. Additionally, since FV levels increase with age,[12,13] the phenotype might improve during the course of a patient's life.

Plasma TFPI levels in FV deficiency

Many examples in literature illustrate that the co-inheritance of a prothrombotic mutation can improve haemostasis in patients with bleeding disorders such as haemophilia,[14-16] vWD,[17] and FVII deficiency.[18,19] Because no reports existed of procoagulant alterations in severe FV deficiency, while the comparatively mild phenotype of this disorder suggests the existence of a common compensation mechanism, we have explored the possibility that patients with se-

vere FV deficiency are protected from life-threatening bleeding by a concomitant procoagulant defect (Chapter 3). Using a test that reflects the overall coagulability of plasma (the thrombin generation test), we noticed that FV-deficient plasma supplemented with purified FV was more procoagulant than normal plasma in many respects. More specifically, reconstituted FV-deficient plasma was exceptionally APC resistant and showed increased thrombin generation when using a low amount of TF to trigger coagulation. Both findings suggested a malfunctioning of the TFPI pathway, as TFPI levels are a major determinant of APC resistance and of thrombin generation triggered with a low amount of TF.[20,21] As a matter of fact, (free) full-length TFPI levels were found to be markedly decreased in all FV-deficient plasmas under study.

Although plasma TFPI represents only a small fraction of all intravascular TFPI, several publications indicate that low plasma levels of (full-length) TFPI are associated with a hypercoagulable state; they increase the risk of venous thrombosis,[22-25] and are thought to compensate for the low levels of coagulation factors in neonates.[26] Additionally, TFPI inhibitors are known to improve haemostasis in animal models of haemophilia and might be used as therapeutic agents in haemophilic patients in the future.[27,28] In the light of these findings and of our own observation that low plasma TFPI levels decrease the FV requirement for minimal thrombin generation *in vitro*, we propose that the partial TFPI deficiency improves the bleeding diathesis associated with severe FV deficiency. Whether variation in the plasma TFPI level explains the phenotypic heterogeneity of patients with similar FV levels needs further investigation. Our study population (n=11) was too small to draw any conclusions.

While probably all patients with severe FV deficiency (at least those with a type I deficiency, *i.e.* low FV antigen and activity levels) present with low plasma TFPI levels, additional compensating mechanisms may be present in individual patients. A recent case-report, for example, describes a patient with severe FV deficiency and very mild bleeding symptoms, whose plasma showed a shorter aPTT than FV-depleted plasma when both plasmas were reconstituted with FV.[29] The causative factor, however, was not identified. Also in our own cohort, three FV-deficient patients carried additional thrombophilic defects (Chapter 3, Table 1).

In an attempt to account for the low TFPI levels in FV-deficient plasma, we observed that in normal plasma a large proportion of the full-length TFPI circulates in a complex with FV (Chapter 3, Figure 7) and that there is a correlation between the plasma levels of FV and TFPI (Chapter 3, Figure 5 and references[24,30,31]). How FV regulates plasma TFPI level remains to be elucidated. Since FV did not influence TFPI stability *in vitro*, we consider a reduced *in vivo* stability or an increased clearance as the most likely explanation for the low plasma TFPI levels in patients with severe FV deficiency. Furthermore, because approximately half of all plasma full-length TFPI was found to be complexed to protein S in normal

plasma,[32] we can speculate that all plasma "free" TFPI (*i.e.* the fraction that is not bound to lipoproteins) circulates in complex with either FV or protein S and that non-bound TFPI is rapidly degraded or cleared from the circulation.

Role of platelet FV in severe FV deficiency

While low plasma TFPI levels are clearly beneficial to those severe FV-deficient patients with residual FV expression, nine patients described in Chapter 3 did not have any detectable FV or thrombin generation in plasma, even though many of them experienced only mild or moderate bleeding. Because trace amounts of FV are already sufficient for minimal haemostasis,[6,9] we wondered whether platelet FV plays a role in severe FV deficiency.

Already in 1978, the importance of platelet FV in severe FV deficiency was predicted following the observation that the bleeding tendency of FV-deficient patients better correlated with the "FXa binding capacity" of their platelets (*i.e.* the amount of FVa present on their platelets) than with their plasma FV level.[33] Later publications on platelet FV levels in patients with severe FV deficiency are scarce and provide inconclusive data. To date, platelet FV levels have been reported for five patients with severe FV deficiency (not counting the patients described in this thesis). Three of them had non-detectable platelet FV level but a variable bleeding diathesis,[7,29,34] and two patients had detectable platelet FV and mild/moderate bleeding symptoms.[35] We studied the role of platelet FV in severe FV deficiency more extensively using thrombin generation experiments in platelet-rich plasma (PRP) of FV-deficient patients and FV activity and antigen measurements (Chapters 4 and 5).

Four patients with relatively mild bleeding symptoms, among whom only one had detectable plasma FV (Chapter 4), showed FV-dependent thrombin generation in PRP already at a low trigger concentration (1 pM TF). All four appeared to have residual platelet FV. In contrast, a patient with frequent severe bleeding episodes (Chapter 5) did not have detectable thrombin generation even at a high trigger concentration (50 pM TF) and at maximal platelet activation. Not surprisingly, his platelets were devoid of FV activity. Our findings suggest that thrombin generation in PRP may discriminate between mild and severe bleeders. To illustrate this, thrombin generation curves obtained in platelet-poor and platelet-rich plasma from a healthy control (C) and from patients with severe FV deficiency with different amounts of plasma and platelet FV are presented in Figure 1. While patient PD VII has measurable FV both in plasma and platelets, patient PD III has only platelet FV. Both are described in Chapter 4 and have minimal bleeding problems. PB, the proband described in Chapter 5, has no detectable FV in plasma or platelets and experiences severe bleeding manifestations.

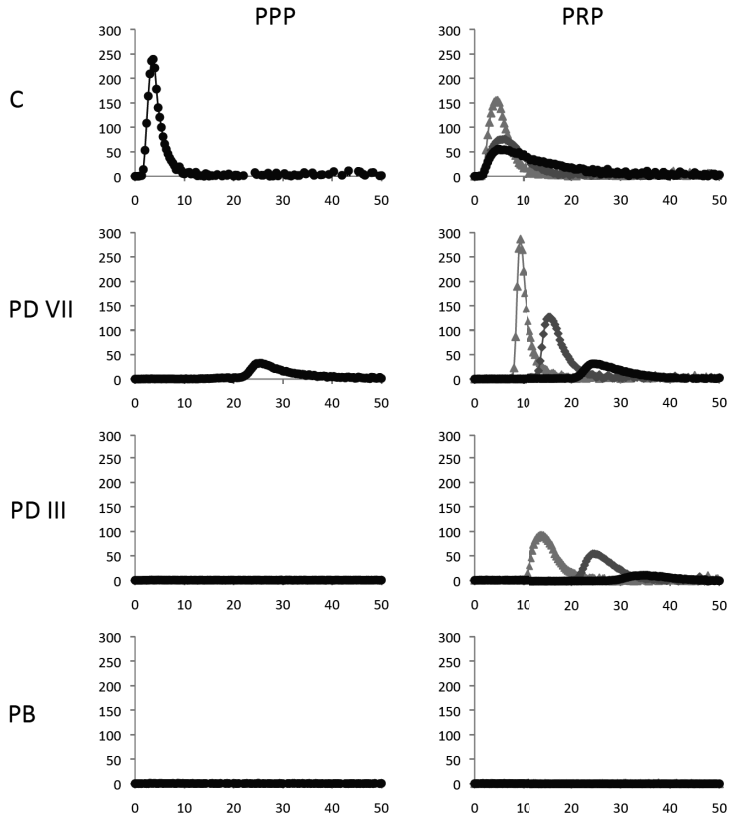

Figure 1. Thrombin generation in platelet-poor (PPP) and platelet-rich plasma (PRP) from a healthy control (C) and from patients with severe FV deficiency having both plasma and platelet FV (PD VII), only platelet FV (PD III) or no functional FV (PB). Coagulation was triggered with 5 pM TF. PPP was supplemented with synthetic phospholipid vesicles. Platelets were non-preactivated (black) or preactivated with collagen (middle gray) or Ca^{2+}-ionophore (light gray).

As a result of the low FV requirement for minimal haemostasis, residual platelet FV might explain the relatively mild bleeding diathesis presented by many FV-deficient patients. Additionally, the special features of platelet FV (storage as a partially activated form and resistance to APC catalyzed inactivation)[36,37] make platelet FV a better procoagulant than plasma FV and circumvent the necessity of plasma FV for haemostasis. As demonstrated in Chapter 4 (Figure 1), in the presence of normal platelets, plasma FV does not even contribute to thrombin generation. Also other lines of evidence illustrate the superior role of platelet FVa as FXa cofactor. A patient with an acquired FV inhibitor that only inhibited plasma FV underwent surgery without any complications,[38] whereas the selective deficiency of platelet FV (FV New York) is associated with a bleeding diathesis.[39] Besides the procoagulant char-

acteristics of platelet FV, also the partial TFPI deficiency of patients with severe FV deficiency contributes to thrombin generation in FV-deficient PRP as shown in Chapter 4, (Figure 5). Normalizing the TFPI level completely abolished thrombin generation in platelet-rich FV-deficient plasma.

The presence or absence of residual FV inside platelets of FV-deficient patients with undetectable plasma FV may be related to the molecular defect responsible for the FV deficiency. Sequencing of the *F5* gene revealed that all four patients with residual platelet FV carried missense mutations. Such mutations do not necessarily prevent protein synthesis[5] and their effects can be abolished by rare somatic reversion events or by occasional mistakes during mRNA translation.[9] In contrast, the patient with undetectable functional FV in plasma and platelets was homozygous for a deep-intronic splicing mutation that causes most of the *F5* mRNA to be degraded and prevents the synthesis of full-length FV. Interestingly, six out of seven previously described patients with homozygous splicing mutations in the *F5* gene (for whom phenotypic information was available) had a severe bleeding diathesis.[40-44] Most splicing mutations, however, do not completely prevent protein expression, as a tiny fraction of all primary transcripts may be spliced correctly.[44] Therefore traces of normal FV might still be present in our patient (even though they are not detectable by our assays) and allow minimal haemostasis *in vivo*. This is supported by the observation that clots of normal size and strength were formed in this patient's blood, albeit at a reduced rate, as shown by thromboelastrometry (Chapter 5, Table 2). This suggests the presence of traces of FV that allow the formation of the few nM of thrombin that are needed for clot formation, but that are barely detectable in the thrombin generation assay. This once more illustrates how low the FV requirement for minimal haemostasis really is.

In healthy individuals, virtually all FV present in platelets derives from plasma FV,[45,46] even though megakaryocytes (the platelet precursors) are capable of FV synthesis.[47] Furthermore, plasma and platelet FV levels correlate, implying that the plasma FV concentration regulates the amount of FV present in platelets. Why FV in patients with severe FV deficiency preferentially resides in platelets is still uncertain. One possibility is that the plasma FV pool is depleted when megakaryocytes take up all FV available in plasma. Alternatively, plasma FV may be cleared more rapidly than platelet FV. The latter hypothesis is supported by the observation that FV-deficient patients are longer protected from bleeding by administration of platelet concentrates than by fresh-frozen plasma, suggesting that platelet FV has a longer half-life than plasma FV.[48]

Plasma TFPI levels in FVL pseudohomozygotes

While the low plasma TFPI levels that accompany low FV levels are beneficial to patients with severe FV deficiency, they may also enhance the hypercoagulable state and thrombosis risk associated with prothrombotic conditions. This is well illustrated by the condition known as FVL pseudo-homozygosity, where a *F5* loss-of-function mutation is co-inherited with the prothrombotic FV Leiden (FVL) mutation on the counterpart allele.[49-51] Although pseudohomozygotes are genotypically heterozygous for the FVL mutation, their plasma contains only FVL and their APC resistance has been reported to approximate that of FVL homozygotes.[49-57] However, since FVL pseudohomozygotes are partially FV-deficient, their TFPI levels are reduced (Chapter 6), which may affect APC sensitivity, as plasma TFPI level is one of the major determinants of APC resistance.[20,21] As shown in Chapter 6, as a result of their low plasma TFPI levels, FVL pseudohomozygotes had increased thrombin generation at low TF and a higher APC resistance compared to FVL homozygotes. On the basis of these observations, FVL pseudohomozygotes might have a higher risk of venous thrombosis than FVL homozygotes.

Low FV levels might likewise increase the risk of venous thrombosis when co-inherited with other procoagulant mutations in the *F5* gene, *e.g.* the HR2 haplotype[58] or the FV Cambridge[59] and Liverpool[60,61] mutations. Moreover, also in the absence of such risk factors, low FV levels may increase the risk of venous thrombosis by lowering the plasma TFPI levels as well as by decreasing the APC-cofactor activity of FV in FVIII(a) inactivation.[62,63] This is supported by several reports on the occurrence of venous thrombosis in patients with moderate FV deficiency (*i.e.* ~ 10% plasma FV),[64-67] and by the association of low FV levels with venous thrombosis in the Japanese population.[68] However, in a large case-control study no association between FV levels and the risk of venous thrombosis was found.[12]

Conclusions and future perspectives

In conclusion, we have identified two important phenotypic modulators of the bleeding tendency associated with severe FV deficiency. First, patients with severe FV deficiency have low plasma levels of the natural anticoagulant TFPI, which increases thrombin generation and might improve haemostasis. Second, patients with undetectable plasma FV may have residual platelet FV, depending on the severity of the *F5* gene mutation. Among patients with non-detectable plasma FV levels, the amount of residual platelet FV might be responsible for the vast differences in bleeding phenotype.

Our findings suggest that measuring thrombin generation in PRP of patients with severe FV deficiency might be useful in clinical practice to estimate bleeding risk and to monitor treatment. In comparison to other tests that can provide useful information about plasma/platelet FV level in patients with severe FV deficiency (*e.g.* the prothrombinase-based activity assay and western blotting after immunoprecipitation), the thrombin generation test is relatively easy to perform.

While low plasma TFPI levels are beneficial to patients with severe FV deficiency, they enhance the hypercoagulable state (and probably contribute to the thrombotic risk) of FVL pseudohomozygotes. Also in other prothrombotic conditions, low plasma FV levels might further increase the risk for venous thrombosis by lowering plasma TFPI levels and decreasing the APC-cofactor activity of FV in FVIII(a) inactivation.

Due to the rare occurrence of FV deficiency, a large multicenter study would be necessary to fully appreciate the effect of platelet FV and plasma TFPI levels on bleeding symptoms in FV-deficient patients.

Finally, our studies may represent a precedent to investigate the role of platelets in the deficiencies of other coagulation factors that are present in platelets as well as in plasma (*e.g.* fibrinogen).

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Samenvatting

Hemostase is een complex proces dat overmatig bloedverlies bij een verwonding voorkomt. Een belangrijk onderdeel van de hemostase is de bloedstolling die zorgt voor de vorming van een netwerk van fibrinedraden in het bloed. Het stollingsproces begint wanneer bij beschadiging van het bloedvat het bloed in aanraking komt met een eiwit uit de vaatwand, de z.g. weefselfactor. Weefselfactor initieert de opeenvolgende activering van een aantal stolfactoren die als inactieve pro-enzymen in het bloed circuleren. Uiteindelijk resulteert dit in de vorming van het enzym trombine dat de omzetting van fibrinogeen in onoplosbare fibrinedraden katalyseert. Verschillende regulerende anticoagulante mechanismen voorkomen enerzijds dat een stolsel ongelimiteerd doorgroeit en anderzijds dat stolling optreedt zonder dat er schade aan de vaatwand is. Een nauwgezette balans tussen pro- en anticoagulante mechanismen zorgt ervoor dat het bloed normaal in vloeibare toestand blijft, maar stolt wanneer de vaatwand beschadigd wordt. Een verschuiving in deze balans kan ervoor zorgen dat er enerzijds trombose ontstaat of anderzijds een bloedingsneiging.

Het stollingseiwit factor V (FV) bevindt zich zowel in het bloedplasma als in bloedplaatjes. FV is essentieel voor de vorming van trombine en heeft daarom in de eerste plaats een procoagulante rol in de bloedstolling. Daarnaast heeft FV ook een anticoagulante functie, het stimuleert de activiteit van het anticoagulante eiwit geactiveerd proteïne C. Een ernstig gebrek aan FV ten gevolge van mutaties in het *F5* gen (congenitale FV deficiëntie) zal tot overmatig bloedverlies leiden omdat er niet genoeg trombine gevormd kan worden. Aangezien slechts weinig FV nodig is voor de vorming van trombine, zullen bloedingen slechts optreden wanneer het plasma FV niveau lager is dan 5%. Anderzijds kunnen mutaties in het *F5* gen ook interfereren met de anticoagulante functie van FV en de procoagulante werking intact laten. Dit leidt tot een verhoogd risico op veneuze trombose.

Hoewel congenitale FV deficiëntie al in 1947 voor het eerst beschreven werd, heeft de lage prevalentie van deze aandoening (naar schatting $1:10^6$ individuen is homozygoot FV-deficiënt) wetenschappelijk onderzoek beperkt. Met name de grote variatie in fenotype bij patiënten met een gelijke mate van FV deficiëntie vraagt verdere opheldering. Daarnaast is het ziekteverloop van veel patiënten milder dan verwacht en weerspiegelen laboratoriumtesten niet het ziektebeeld; volgens deze testen is stolling volledig afwezig in plasma van veel patiënten, wat eigenlijk tot levensbedreigende bloedingen zou moeten leiden, terwijl dit in de praktijk veelal niet het geval is. In dit proefschrift hebben wij geprobeerd de bloedingsneiging van patiënten met congenitale FV deficiëntie beter te begrijpen.

In hoofdstuk 2 wordt een overzicht gegeven van de huidige kennis over FV en FV deficiëntie. Verder worden in dit hoofdstuk een aantal aspecten besproken die van invloed zouden kunnen zijn op het fenotype van patiënten met FV deficiëntie. Deze factoren komen uitvoeriger aan de orde in hoofdstuk 3, 4, 5 en 6.

In hoofdstuk 3 is onderzocht of er compenserende factoren in FV-deficiënt plasma aanwezig zijn die het bloedingsrisico van FV deficiënte patiënten kunnen verminderen. In vergelijking met andere bloedingsneigingen, zoals bijvoorbeeld hemofilie, heeft FV deficiëntie namelijk een milder verloop, ondanks het feit dat FV een essentiële rol speelt in de trombinevorming en in de bloedstolling. Wij hebben dit probleem benaderd met behulp van een test die de globale stolbaarheid van het plasma weerspiegelt (de trombine generatie test). Alvorens de stolbaarheid van het FV-deficiënte plasma te testen, is het plasma FV niveau genormaliseerd door toevoeging van gezuiverd FV. Hierdoor wordt het effect dat de FV deficiëntie op deze test heeft teniet gedaan, waardoor andere afwijkingen meetbaar worden. Uit deze experimenten bleek, na normalisatie van het FV niveau, het FV-deficiënte plasma onder bepaalde omstandigheden zeer procoagulant te zijn. Nader onderzoek wees uit dat dit veroorzaakt werd door een verlaagde hoeveelheid “tissue factor pathway inhibitor” (TFPI) in het plasma. TFPI is een belangrijk anticoagulant eiwit dat de initiatie van de bloedstolling remt. In totaliteit zijn elf patiënten met congenitale FV deficiëntie onderzocht, waaruit bleek dat partiële TFPI deficiëntie een gemeenschappelijk kenmerk van FV-deficiënte is. Omdat het verlaagde TFPI niveau de hoeveelheid FV die nodig is voor de trombine vorming verlaagt, stellen we voor dat dit het bloedingsrisico van patiënten vermindert.

De oorzaak voor de verlaging van het plasma TFPI niveau bij patiënten met FV-deficiëntie is nog niet geheel duidelijk. Wel hebben wij aangetoond dat FV en TFPI in plasma een complex vormen en dat er een correlatie bestaat tussen de plasma FV en TFPI niveaus. Naar alle waarschijnlijkheid verhoogt FV de stabiliteit en / of vermindert het klaring van TFPI *in vivo*, wat betekent dat lage hoeveelheden FV in het plasma gepaard gaan met lage TFPI spiegels.

Een verlaagd TFPI niveau is alleen voordelig voor de patiënt als er spoortjes FV aanwezig zijn die de vorming van een kleine hoeveelheid trombine toelaten. Bij een lager TFPI niveau zal dan meer trombine gevormd worden. In plasma van veel FV-deficiënte patiënten is FV echter niet aantoonbaar, terwijl vele van hen slechts een matige bloedingsneiging hebben. Omdat een deel van het FV in bloed is opgeslagen in de α-granulae van bloedplaatjes, hebben we in hoofdstuk 4 en 5 de rol van bloedplaatjes FV bij congenitale FV deficiëntie onderzocht. In hoofdstuk 4 worden vier patiënten gepresenteerd die slechts een milde tot matige bloedingsneiging hebben hoewel plasma FV bij drie van de patiënten afwezig is. Het stolvermogen van deze patiënten werd *in vitro* getest door de vorming van trombine in plaatjesrijk plasma na initiatie van de stolling in de tijd te volgen met behulp van de trombine genera-

tie test. Hoewel bij geen van hen trombinevorming aanwezig was in plaatjes-arm plasma, kon in hun plaatjes-rijk plasma een bijna normale hoeveelheid trombine gevormd worden. Echter, in hoofdstuk 5 wordt een patiënt besproken die ernstige en frequente bloedingen heeft. In zijn plaatjes-rijk plasma was geen trombine vorming aantoonbaar. Het type mutatie in het *F5* gen ligt waarschijnlijk ten grondslag aan dit verschil. Terwijl de patiënten met trombinevorming in plaatjes-rijk plasma allen “missense” mutaties hadden die de productie van eiwit niet volledig voorkomen, had de patiënt zonder trombine-vorming geen functioneel FV in zijn plaatjes, t.g.v. een mutatie die interfereert met de normale splicing van het FV mRNA en die de productie van functioneel FV verhindert.

Wij stellen voor dat in patiënten bij wie geen plasma FV aangetoond kan worden, de kleine hoeveelheid FV in bloedplaatjes een belangrijke modulator van het bloedingsrisico is. Laboratoriumtesten die gebaseerd zijn op de stolling, trombinevorming of FV bepaling in plaatjes-rijk plasma zullen daarom het ziekteverloop beter weerspiegelen dan testen in plaatjes-arm plasma. Daarnaast vermindert de partiële TFPI deficiëntie de bloedingsneiging bij patiënten met congenitale FV-deficiëntie. Verder onderzoek is nodig om te bepalen of variatie in het plasma TFPI niveau de heterogeniteit van het fenotype van patiënten met een gelijke mate van FV deficiëntie kan verklaren.

In hoofdstuk 6 komt de anticoagulante functie van FV aan bod. Een puntmutatie in het *F5* gen, de FV Leiden (FVL) mutatie genoemd, die zowel interfereert met de inactivering van FVa door APC als met de anticoagulante werking van FV, verhoogt het risico op veneuze trombose ongeveer 7 maal in FVL heterozygoten en 80 maal in FVL homozygoten. Een zeldzaam voorkomende afwijking is de combinatie van FV deficiëntie en de FVL mutatie. Dit is het gevolg van een FV nul-mutatie op het ene *F5* allel en een FVL mutatie op het andere *F5* allel. Wij stellen voor dat deze z.g. FVL pseudohomozygoten blootgesteld zijn aan een hoger tromboserisico dan heterozygote FVL carriers en zelfs een hoger tromboserisco hebben dan FVL homozygoten. We postuleren dat ten gevolge van de partiële FV deficiëntie, FVL pseudohomozygoten lagere plasma TFPI waarden hebben waardoor hun tromboserisico hoger is dan dat van FVL homozygoten aangezien die normale FV en dus normale TFPI niveaus hebben. Deze hypothese wordt met behulp van trombine generatie experimenten ondersteund in hoofdstuk 6.