Modulators of bleeding tendency in severe factor V deficiency

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Abbreviations

3'-UTR	3'-untranslated region
ACD	acid citrate dextrose
APC	activated protein C
aPTT	activated partial prothrombin time
AT	antithrombin
AUC	area under the curve
CAT	calibrated automated thrombography
CFT	clot formation time
CT	clotting time
CTI	corn trypsin inhibitor
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DOPS	dioleoylphosphatidylserine
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ETP	endogenous thrombin potential
F2	prothrombin gene
F5	FV gene
FII	prothrombin
FIIa	thrombin
FIX(a)	(activated) factor IX
FV:C	FV clotting activity
FV(a)	(activated) factor V
FVII(a)	(activated) factor VII
FVIII(a)	(activated) factor VIII
FVL	FV Leiden mutation (*F5* R506Q)
FX(a)	(activated) factor X
FXI(a)	(activated) factor XI
FXII(a)	(activated) factor XII
FXIII(a)	(activated) factor XIII
Gla	γ-carboxylated glutamate
HBS	Hepes-buffered saline
HRP	horseradish peroxidase
IVS	intervening sequence (intron)
MAXV	maximal velocity
MCF	maximum clot firmness

MLPA	multiplex ligation-dependent probe amplification
MMRN1	multimerin 1
nAPCsr	normalised APC sensitivity ratio
NMD	nonsense mediated decay
NPP	normal pooled plasma
OMIM	Online Mendelian Inheritance in Man
PC	protein C
PCR	polymerase chain reaction
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PS	protein S
PT	prothrombin (time)
QPD	Quebec platelet disorder
rFVIIa	recombinant FVIIa
RT	room temperature
RU	resonance units
RVV-x	Russel's viper venom - FX
SD	standard deviation
SDS	sodium dodecylsulfate
SPR	surface plasmon resonance
TAP	tick anticoagulant protein
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
u-PA	urokinase-type plasminogen activator
VTE	venous thromboembolism
vWD	von Willebrand disease
vWF	von Willebrand factor

Table of contents

1

General introduction

Introduction

Blood is a specialized fluid that serves the transport of oxygen, nutrients and waste products between tissues and organs. Additionally, blood takes a central position in the body's immune response and has a role in many other vital processes such as pH and temperature regulation. Blood is composed of a cellular compartment (erythrocytes, leukocytes and platelets) and a fluid compartment (plasma). Blood is enclosed in vessels of the circulatory system, which is divided in a low-pressure (venous) and a high-pressure (arterial) side. In case of blood vessel damage, a complex process called haemostasis limits extravasation of blood from the circulation. Haemostasis comprises three concurrent mechanisms: 1) constriction of the damaged vessel, 2) aggregation of platelets to form a platelet plug (primary haemostasis), and 3) the formation of a dense protein fibre network that reinforces the platelet plug (coagulation).

Primary haemostasis

Vessel wall injury results in the exposure of blood to proteins from the subendothelial layer (such as collagen) that can activate platelets that normally circulate in a quiescent state. Activated platelets release components (*e.g.* thromboxane A2, ADP and platelet activating factor) that promote attraction and activation of other platelets. As a result, platelets aggregate at the site of injury, thereby forming a physical barrier to blood loss. Additionally, activated platelets secrete coagulation factors and expose negatively charged phospholipids, *i.e.* phosphatidyl serine, at the outer layer of their plasma membrane, which provides a surface for the coagulation process.

Coagulation

In the absence of trauma, haemostasis is balanced towards processes that prevent blood coagulation, thereby maintaining blood fluidity. *In vivo*, coagulation is initiated when the endothelial cell layer that lines the vessel wall is damaged and blood becomes exposed to the transmembrane protein tissue factor (TF) that is abundantly expressed on the cells surrounding the endothelium. After initiation, coagulation proceeds via the sequential activation of inactive enzyme precursors (zymogens) present in plasma, eventually leading to the formation of thrombin, the key-enzyme that mediates the conversion of fibrinogen to fibrin polymers.[1,2] A simplified scheme of the coagulation cascade is presented in Figure 1.

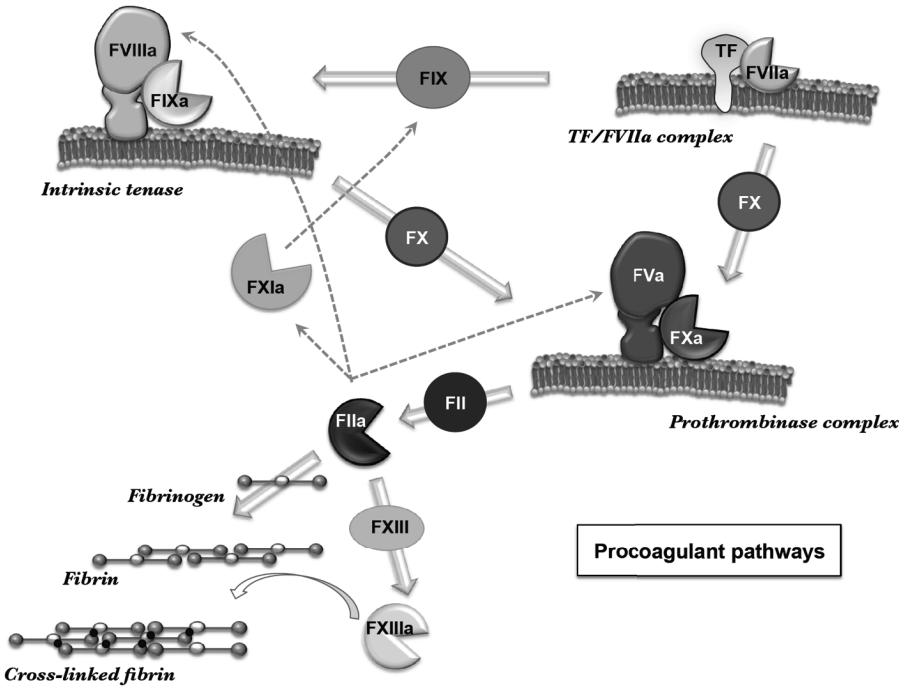

Figure 1. Simplified scheme of the coagulation cascade. Coagulation proceeds via the sequential activation of zymogens, culminating in the activation of prothrombin (FII) to thrombin (FIIa). Thrombin converts soluble fibrinogen to insoluble fibrin. Coagulation factors (F) are indicated with roman numbers. Positive feedback loops are represented by dashed lines.

Two potential ways to start coagulation exist, termed the intrinsic and extrinsic pathways. *In vivo*, coagulation is usually initiated *via* the extrinsic coagulation pathway, which starts when TF binds to trace amounts of activated factor VII (FVIIa) that circulate in plasma. The TF-FVIIa complex, also called the extrinsic tenase complex, activates two precursors of serine proteases: factor X (FX) and factor IX (FIX). Activated factor X (FXa) forms together with its cofactor activated factor V (FVa)* the prothrombinase complex on negatively charged phospholipids (*in vivo* provided by activated platelets), which converts prothrombin to thrombin. In the absence of FVa, the catalytic activity of FXa is reduced more than 1000-fold, making FV(a) essential for the generation of thrombin. Activated factor IX (FIXa), together with its cofactor activated factor VIII (FVIIIa), forms the intrinsic tenase complex on negatively charged phospholipids.[3] This complex provides an alternative way to activate FX when the extrinsic tenase complex is inhibited.

Positive feedback of coagulation occurs via thrombin, which activates the cofactors of the prothrombinase (factor V, FV) and intrinsic tenase (factor VIII, FVIII) complexes, as well

as factor XI (FXI). Activated FXI (FXIa) activates FIX, which in turn forms a complex with FVIIIa and activates FX. Thrombin also facilitates clot stabilization by activating factor XIII (FXIII). Activated FXIII (FXIIIa) is a transglutaminase that cross-links fibrin polymers, thereby strengthening the clot.[3] Moreover, thrombin promotes primary haemostasis by activating platelets via protease-activated receptors 1 and 4.[4]

Intrinsic initiation of coagulation occurs spontaneously after binding of factor XII (FXII) to negatively charged surfaces such as glass, resulting in autoactivation of FXII and ultimately in the activation of FXI. While the absence of bleeding in individuals with a deficiency of FXII is inconsistent with a role for FXII in normal haemostasis, recent investigations point to a function of this coagulation factor in atherothrombosis.[5]

Several coagulation factors (prothrombin, FVII, FIX, and FX) as well as anticoagulant proteins (protein C and S, which are discussed below) are characterised by an N-terminal Gla-domain, *i.e.* a cluster of γ-carboxylated glutamate (Gla) residues. The Gla-domain mediates the binding of these proteins to negatively charged phospholipid membranes in the presence of Ca^{2+} ions. These coagulation factors are termed "vitamin K-dependent" because vitamin K is required for the γ-carboxylation of Gla-residues.[3]

Regulation of coagulation

To prevent excessive thrombus growth as well as to avoid coagulation in the absence of vascular damage, blood coagulation is strictly regulated. Confinement of coagulation reactions to the site of injury is ensured by the fact that activated platelets become available only when the endothelial lining is disrupted, whereas temporal regulation is mediated by the onset of anticoagulant pathways as soon as coagulation begins. Anticoagulant proteins operate at all levels of coagulation: the initiation of coagulation is inhibited by tissue factor pathway inhibitor (TFPI);[6] activated protein C (APC) limits propagation of coagulation by inactivating FVa and FVIIIa;[7] antithrombin directly inhibits coagulation factors such as thrombin and FXa.[8]

TFPI is a slow tight-binding Kunitz-type inhibitor that inhibits both FXa and the TF-FVIIa complex.[6] TFPI can only inhibit TF-FVIIa after binding to FXa and therefore is only active after coagulation has started. TFPI contains three Kunitz-domains of which Kunitz-1 inhibits TF-FVIIa, Kunitz-2 binds to and inhibits FXa,[9] and Kunitz-3 binds to the TFPI-cofactor protein S (PS).[10] PS, a vitamin K-dependent protein, promotes the inhibition of FXa by TFPI.[11] Most TFPI is bound to the endothelium, whereas only ~10% circulates in plasma, either bound to lipoproteins or as free protein (5-20% of plasma TFPI). Plasma TFPI exists as many

isoforms, having a variable degree of truncation, but only the free full-length form of TFPI is physiologically relevant.[12]

The protein C (PC) anticoagulant pathway is initiated by the activation of PC to APC by thrombin bound to thrombomodulin (TM) on the surface of endothelial cells. APC is a serine-protease that proteolytically inactivates the membrane-bound cofactors FVa and FVIIIa. Both inactivation reactions require PS as a cofactor, while the inactivation of FVIIIa additionally requires APC-cleaved FV. Since thrombin is needed for PC activation, the PC pathway only comes in action when coagulation is ongoing.[7]

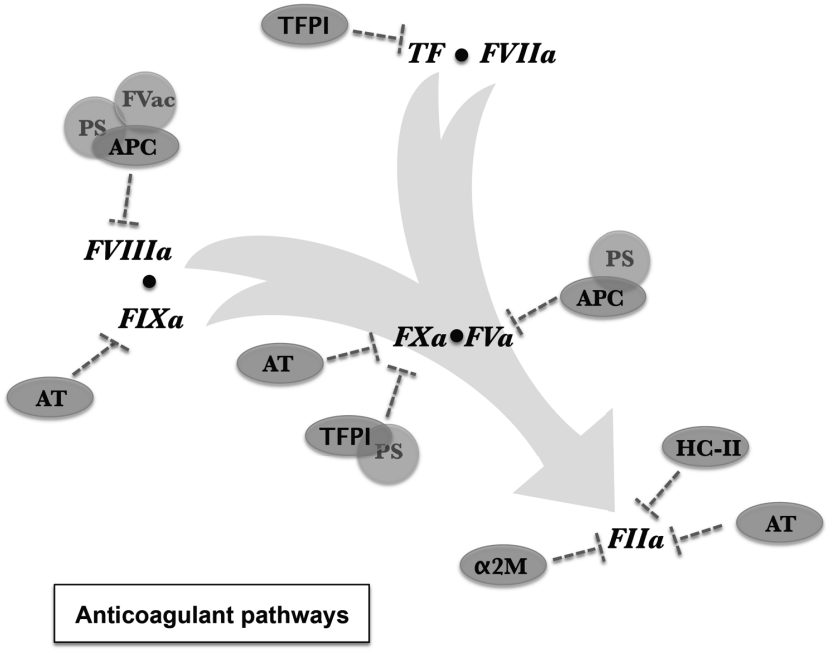

Figure 2. Regulation of coagulation. Coagulation is regulated at all levels by protease inhibitors (represented in black) and their cofactors (gray). α2M, α2-macroglobulin; APC, activated protein C; AT, antithrombin; FVac, anticoagulant form of FV (*i.e.* Arg506 cleaved FV); HC-II, heparin cofactor II; PS, protein S; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

Antithrombin is a serine-protease inhibitor (serpin) that directly inhibits activated coagulation factors and its inhibitory activity is greatly stimulated by heparin. Antithrombin inhibits several activated coagulation factors, but its most importantly targets are thrombin, FIXa and FXa.[8] Two additional thrombin inhibitors present in plasma are heparin cofactor II (HC-II) and α2-macroglobulin.[13,14]

Disturbances of the haemostatic balance

When the equilibrium between procoagulant and anticoagulant forces is disturbed, as a result of genetic defects or acquired factors, bleeding or thrombosis may occur.

The most prevalent hereditary bleeding disorder is von Willebrand disease (vWD), which results from a deficiency of von Willebrand factor (vWF) and segregates as an autosomal dominant or recessive trait. Patients with vWD suffer mainly from mucosal bleeding. Because vWF has a role in platelet adhesion and stabilizes FVIII, vWD affects both primary haemostasis and coagulation. Other common bleeding disorders are haemophilia A (1 in 5.000 males) and haemophilia B (1 in 30.000 males), which result from the deficiency of FVIII and FIX, respectively. The high frequency of these disorders in males originates from the localisation of the *F8* and *F9* genes on the X-chromosome and consequent hemizygosity in males. Both haemophilia subtypes are phenotypically indistinguishable and present as frequent joint and muscle bleeds as well as post-traumatic bleeding.[15]

Deficiencies of fibrinogen, prothrombin, FV, FVII, FX, FXI, and FXIII, and combined FV-FVIII deficiency are rare bleeding disorders (prevalence varying from 1 in 500.000 to 1 in 2 million persons) and are inherited as autosomal recessive traits.[16] The sporadic occurrence of these diseases has prevented adequate research regarding disease pathogenesis and management as opposed to haemophilia A and B. For most of the rare bleeding disorders fresh-frozen plasma or prothrombin complex concentrates (containing all the vitamin K dependent clotting factors) are still the treatment of choice. Both treatments are non-specific and carry the risk of infection with blood-borne pathogens.[17]

Venous thrombosis is a common disorder (annual incidence of 1 in 1000) with both genetic and environmental risk factors. Genetic risk factors include loss-of-function defects in anticoagulant proteins (*e.g.* AT,[18] PC,[19] or PS deficiency[20,21]) or gain-of-function mutations in coagulation factors (FV Leiden [FVL][22] and prothrombin G20210A[23]). Most important environmental risk factors are aging, immobilisation, surgery, cancer, and in women also pregnancy and hormonal contraceptive use.[24]

While severe deficiency of FV results in a bleeding tendency by interfering with prothrombin activation, the FVL mutation interferes with the inactivation of FVa and with FV's anticoagulant role. The FVL mutation is present in ~5% of the Caucasian population[25] and causes plasma APC resistance (*i.e.* a poor response of plasma to the anticoagulant action of APC)[26] by interfering both with the APC-catalysed inactivation of FVa[27,28] and with the cofactor activity of FV in FVIIIa inactivation.[29,30] The FVL mutation increases the risk of venous thrombosis 7-fold in heterozygous carriers and ~80-fold in homozygous carriers,[31] making it the most common cause of venous thrombosis in the Caucasian population.

The thrombin generation assay

The thrombin generation test, also known as the Calibrated Automated Thrombogram (CAT) method,[32] is an *in vitro* assay that reflects the overall tendency of a plasma sample to clot and has been frequently used in the studies presented in this thesis. In this test, coagulation is triggered *via* the extrinsic or intrinsic pathway and thrombin activity in plasma is monitored continuously *via* the conversion of a low-affinity fluorogenic thrombin substrate added to the plasma. The thrombin generation test has been shown to reflect prothrombotic and haemorrhagic tendencies and can be used to monitor anticoagulant treatment or factor replacement therapy.[33] The assay determinants are a function of the reaction conditions, which allows the study of specific aspects of coagulation by measuring thrombin generation under different conditions. The sensitivity of plasma to the anticoagulant action of APC is tested by measuring thrombin generation in the absence and presence of APC.[34] The TFPI/PS pathway can be studied by measuring thrombin generation after triggering coagulation with a low amount of TF.[35] Additionally, the contribution of platelets can be determined by measuring thrombin generation in platelet-rich plasma.

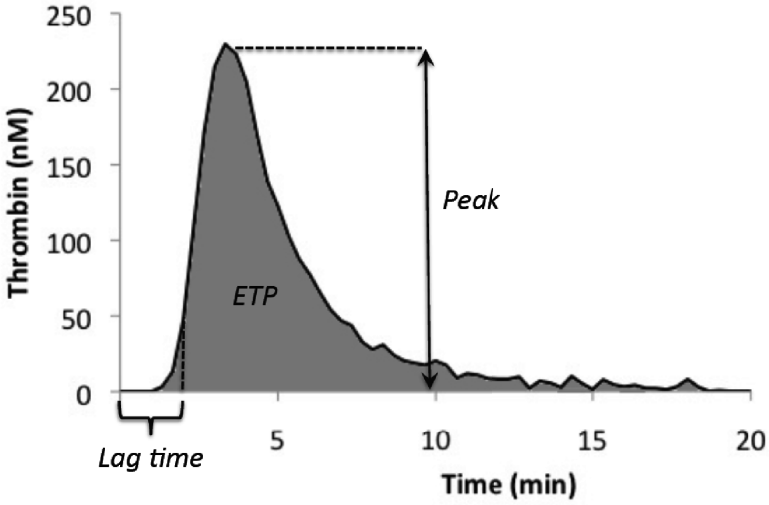

Figure 3. Thrombin generation in plasma. Normal plasma was triggered with TF and the formation and inhibition of thrombin was followed in time using a fluorogenic substrate. Commonly used thrombogram parameters are indicated. ETP, endogenous thrombin potential.

A typical thrombin generation curve obtained in normal plasma after triggering coagulation with TF is shown in Figure 3. The time until the onset of thrombin generation reflects the initiation of coagulation, while the increase and subsequent fall in thrombin concentration represent the propagation and termination phases of coagulation. From the thrombin generation

curve, several parameters can be obtained, but most commonly used are: 1) the lag time (minutes, the time until 1/8 of the maximal peak height is reached) which reflects the time until the explosive burst of thrombin formation and approximates the clotting time, as clotting occurs already at a few nM thrombin, 2) the peak height (nM thrombin) which is the maximum concentration of thrombin reached in plasma, and 3) the area under the curve which is named the endogenous thrombin potential (ETP, nM*min) and which reflects the total amount of "enzymatic work" than can be performed by thrombin.

Outline of this thesis

As a cofactor of FXa, FV(a) takes an essential position in coagulation. The absence of FVa from the prothrombinase complex reduces the rate of FXa-catalysed prothrombin activation by four orders of magnitude.[36] Not surprisingly, the complete absence of FV is assumed to be incompatible with life, supported by the fact that *Fv* knock-out mice have a lethal phenotype.[37] In humans, severe deficiency of FV is associated with a variable bleeding diathesis that poorly correlates with the residual amount of FV present in plasma.[38] Considering the pivotal role of FV in coagulation, many patients with unmeasurably low plasma FV levels bleed less than expected. With the research presented in this thesis we aimed to get a better understanding of the bleeding risk of patients with severe FV deficiency.

Chapter 2 provides an extensive overview of the current knowledge on the biology of FV and the clinical features of FV deficiency. Additionally, possible factors that could ameliorate the clinical presentation of FV deficiency are discussed.

Facing the observation that many patients with severe FV deficiency bleed less than anticipated, we sought for possible protective mechanisms in ***Chapter 3***. As a method we used the thrombin generation assay in FV-deficient plasma reconstituted with normal amounts of purified FV. The rationale behind this approach was that any other abnormalities would surface when FV levels were normalized. FV deficiency turned out to be associated with a deficiency of the natural anticoagulant TFPI, a procoagulant condition that partially compensates for the bleeding diathesis associated with FV deficiency.

While patients with severe FV deficiency intuitively benefit from low plasma TFPI levels, traces of FV are needed to support thrombin formation. Because many patients with severe FV deficiency are devoid of FV in their plasma and because FV is also known to be present in platelets, in ***Chapter 4*** we investigated the contribution of platelet FV to haemostasis in patients with severe FV deficiency. Remarkably, three patients whose platelet-poor plasma showed no thrombin generation appeared to have considerable thrombin generation in plate-

let-rich plasma. This, in conjunction with low plasma TFPI levels, provides an explanation for the mild-to-moderate bleeding phenotype of these patients.

In Chapter 4, we proposed that patients with an overall more severe clinical presentation are likely to have less residual platelet FV. This hypothesis was tested when we had the opportunity to obtain plasma from a patient with frequent and severe bleeding symptoms. The genetic and functional characterisation of the FV deficiency in this patient is described in ***Chapter 5***. In contrast to all previously studied patients, this patient showed no thrombin generation in platelet-rich plasma as a result of the virtual absence of functional FV in plasma and platelets. He appeared to have an intronic mutation predicting a gross anomaly in the *F5* mRNA.

While homozygous or compound heterozygous loss-of-function mutations in the FV gene result in a bleeding tendency, the *F5* R506Q (FVL) mutation interferes with FV's anticoagulant role and increases the risk for venous thrombosis. When the prothrombotic FVL mutation and a *F5* null mutation are co-inherited on different alleles (FVL pseudohomozygosity), only the FVL allele is expressed and the plasma FV level is reduced to ~50%. As a result of their partial FV deficiency, FVL pseudohomozygotes have reduced plasma TFPI levels. Whether this exacerbates the prothrombotic tendency of pseudohomozygous FV-deficient patients is explained in ***Chapter 6***.

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2

Advances in understanding the bleeding diathesis in factor V deficiency

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Summary

Coagulation factor V (FV), present in plasma and platelets, is an indispensable clotting factor, as demonstrated by the uniform lethality of FV knock-out mice. Surprisingly, however, severe FV deficiency is rarely fatal in humans. In fact, although several cases of life-threatening intracranial haemorrhage have been reported in FV-deficient newborns, many patients with undetectable FV levels experience only mild to moderate bleeding and do not require routine prophylaxis. While the reasons for this variable phenotypic expression are largely unknown, several observations from different laboratories indicate platelets as crucial players in FV deficiency. Moreover, as discussed in chapter 3, plasma levels of tissue factor pathway inhibitor are considerably reduced in FV-deficient plasma, which results in enhanced thrombin generation especially at very low FV levels (<2%). The present review discusses and integrates these findings in the context of the biology of FV and the clinical features of FV deficiency.

Introduction

Coagulation factor V (FV), which is present in plasma and platelets, is a versatile protein with both pro- and anticoagulant functions. Its essential role in the activation of prothrombin to thrombin and its interactions with several coagulation factors and inhibitors make it a central regulator of the coagulation process.[1]

The fact that FV is indispensable to life is demonstrated by the generalised embryonic/perinatal lethality of FV knock-out mice,[2] which can be rescued, at least partially, by the transgenic expression of tiny (<0.1%) amounts of FV.[3] In humans, FV deficiency states are associated with a bleeding tendency of variable severity, depending on the residual FV level. Among patients with undetectable FV levels, some present at birth with life-threatening intracranial haemorrhages, whereas others are born without complications and experience only mild or moderate bleeding throughout their lives.[4] The comparatively mild bleeding diathesis observed in many patients with severe FV deficiency may be explained by the fact that <1% FV is sufficient to guarantee minimal thrombin generation, as suggested by *in vivo*,[5] *in vitro*,[6] and *in silico* evidence.[7] However, there is no ready explanation for the differences in bleeding phenotype between patients with equally undetectable FV levels.

This review discusses the possible mechanisms that can ameliorate the haemorrhagic diathesis associated with severe FV deficiency. After a general introduction to the biology of FV and an overview of FV deficiency states, we focus on classical FV deficiency (Owren parahaemophilia) and discuss the possible role of platelet FV. Moreover, we present some recent findings obtained in our laboratory concerning plasma tissue factor pathway inhibitor (TFPI) levels in FV deficiency.[8]

Biology of human FV

Biosynthesis

Whole blood FV is distributed between two pools: *c.* 80% circulates in plasma (at a concentration of 20–25 nM), whereas *c.* 20% is stored in platelet α-granules.[9] While plasma FV is synthesised in the liver, the origin of platelet FV has been debated for a long time, as cultured megakaryocytes (though not platelets) can both synthesise[10,11] and internalise[12,13] FV. Characterisation of platelet FV from patients who received bone marrow or liver transplants from donors with a different FV genotype has conclusively shown that endogenous synthesis hardly contributes to the platelet FV pool and that the vast majority of platelet FV is actually

of plasma (hepatic) origin.[14-16] The mechanism of plasma FV uptake by bone-marrow megakaryocytes has been recently shown to involve two distinct receptors and clathrin-dependent endocytosis.[12,17]

Structure

The human FV gene (*F5*) spans *c.* 80 kb on the long arm of chromosome 1 (1q23)[18] and comprises 25 exons and 24 introns.[19] The *c.* 7-Kb mRNA[20] encodes a precursor protein of 2224 amino acids, which undergoes several post-translational modifications (removal of the 28-amino acid leader peptide, multiple N- and O-linked glycosylation, sulphation and phosphorylation). Mature FV is eventually released into the bloodstream as a 330 kDa single-chain inactive protein of 2196 amino acids.

Factor V is highly homologous to factor VIII (FVIII) and comprises three A domains, a long B domain and two C domains, organised in an A1-A2-B-A3-C1-C2 structure.[20] When the coagulation cascade is activated, FV is cleaved by activated factor X (FXa) or thrombin at Arg709, Arg1018 and Arg1545.[21] Limited proteolysis at these sites removes the B domain and releases the activated form of FV (FVa), which consists of a heavy chain (A1-A2, 105 kDa) and a light chain (A3-C1-C2, 71–74 kDa) non-covalently associated *via* a Ca^{2+}-ion.

Procoagulant function

The activated form of FV acts as an essential non-enzymatic cofactor of FXa in the activation of prothrombin to thrombin. By promoting the assembly of the prothrombinase complex on the surface of activated platelets and by enhancing the catalytic activity of FXa, FVa accelerates the activation of prothrombin to thrombin by several orders of magnitude.[22] Given that FXa alone is a very inefficient prothrombin activator, and as there are no alternative pathways for prothrombin activation, FV(a) is absolutely required for thrombin generation.

The activity of FVa is down-regulated by activated protein C (APC), which cleaves the FVa heavy chain at Arg306, Arg506 and Arg679.[23,24] As a consequence, FVa loses its affinity for FXa and becomes inactive as a FXa-cofactor. Although Arg506 is the kinetically favoured cleavage site and is usually proteolysed first, cleavage at Arg306 is needed for complete FVa inactivation. Binding of FXa to FVa protects the Arg506 cleavage site, whereas the APC-cofactor protein S stimulates cleavage at Arg306 *c.* 20-fold.[25] The naturally occurring Arg506Gln (FV Leiden; *F5* R506Q) mutation, which abolishes the Arg506 APC-cleavage site, is associated with activated protein C (APC) resistance and increased thrombosis risk.[26]

Anticoagulant function

In addition to its procoagulant activity, FV also expresses an, as yet poorly characterised, anticoagulant activity by stimulating the inactivation of FVIII(a) by the APC/protein S complex.[27] The APC-cofactor activity of FV is elicited by APC-mediated cleavage of single-chain FV at Arg506[28] and is lost upon thrombin-mediated cleavage at Arg1545.[29] The physiological relevance of the anticoagulant function of FV is illustrated by the prothrombotic diathesis associated with FV Leiden,[30] which lacks the Arg506 APC-cleavage site and is therefore devoid of APC-cofactor activity.[28]

Special features of platelet FV

Although platelet FV originates from the plasma FV pool *via* endocytosis by bone marrow megakaryocytes (see above), it has several structural and functional peculiarities that distinguish it from plasma FV. These properties are acquired by 'post-translational re-tailoring' of the endocytosed plasma FV during its trafficking within the megakaryocyte.[31]

While plasma FV is a single-chain inactive procofactor, platelet FV is stored in a partially proteolysed form which already expresses considerable FXa-cofactor activity prior to exposure to FXa or thrombin.[31,32] In addition, platelet FV(a) is O-glycosylated at Thr402 and is resistant to phosphorylation of the heavy chain at Ser692.[16,31]

Platelet FV is activated by FXa 50-100 times more efficiently than by thrombin, whereas plasma FV is activated equally well by both enzymes.[32] Activation of platelet FV yields heavy (105 kDa) and light (72/74 kDa) chain fragments indistinguishable from those of plasma FVa,[32] except for the fact that the N-terminal residue of the light chain of platelet FVa is Tyr1543 instead of Arg1545.[31] Although APC cleaves platelet and plasma FVa at the same recognition sites, platelet FVa is proteolysed more slowly and cannot be completely inactivated by APC.[33,34]

Platelet FV resides in the α-granules, where it is bound to the soluble protein multimerin 1 (MMRN1).[35] Upon platelet activation, platelet FV dissociates from MMRN1 and is exposed on the platelet membrane as a fully activated cofactor, which promotes the assembly and activity of the prothrombinase complex at the platelet surface. Due to the localised release of platelet FV at the site of injury, it has been estimated that the concentration of platelet FV within a platelet-rich thrombus can exceed the plasma FV concentration >100 times.[36]

Storage in a partially activated form, targeted release, rapid activation by FXa and resistance to APC-mediated inactivation make platelet FVa a very effective FXa-cofactor, which can initiate prothrombinase activity before plasma FV is activated and sustain this activity long after plasma FVa has been inactivated.

Classification of FV deficiency states

Genetic FV deficiencies

Congenital FV deficiency (Owren parahaemophilia)

This form of FV deficiency [On-line Mendelian Inheritance in Man (OMIM) database #227400], which is caused by loss-of-function mutations in the *F5* gene, is the main subject of the present review and is described in detail below.

Combined FV and FVIII deficiency (F5F8D)

Combined FV and FVIII deficiency (OMIM #227300) is a rare ($1:10^6$) autosomal recessive bleeding disorder characterised by reduced levels (5-30%) of both FV and FVIII. In contrast to isolated FV or FVIII deficiency, it is due to mutations in the *LMAN1* or *MCFD2* genes,[37,38] which encode two proteins involved in the trafficking of FV and FVIII from the endoplasmic reticulum to the Golgi apparatus. Affected individuals present with a mild-to-moderate bleeding diathesis, the most common symptoms being mucous membrane bleeding (epistaxis, gum bleeding, menorrhagia) and post-traumatic or post-partum haemorrhages. Treatment requires supplementation of both FV (in the form of fresh frozen plasma) and FVIII. The molecular and clinical features of combined FV and FVIII deficiency have been recently reviewed elsewhere.[39,40]

Acquired FV deficiencies

Although pathological conditions such as severe liver disease or disseminated intravascular coagulation (DIC) can cause a (transient) decrease in FV levels, the most common form of acquired FV deficiency is associated with the development of FV inhibitors, *i.e.* antibodies that bind to FV and promote its degradation and/or block its activity.

FV inhibitor-mediated deficiency

Acquired FV inhibitors (reviewed elsewhere)[41-43] may cause various degrees of FV deficiency. The clinical presentation ranges from complete absence of symptoms to life-threatening haemorrhages. Acute bleeding episodes are treated with fresh frozen plasma and/or platelet concentrates, whereas the follow-up therapy (immunosuppression, injection of intravenous immunoglobulins, plasmapheresis or plasma adsorption) is aimed at lowering the antibody titre. In most cases, the FV inhibitor is transient and disappears within a few months.

Factor V inhibitors only rarely develop spontaneously. Most often, they are triggered by the exposure to topical bovine thrombin preparations (containing traces of bovine FVa) during surgical procedures, or by the use of certain antibiotics. On the whole, spontaneous FV inhibitors tend to cause a more severe bleeding diathesis than iatrogenic inhibitors.[41] However, the most important determinants of clinical outcome are the specific characteristics of the antibody, such as (i) the antibody titre, (ii) whether or not the antibody has access to platelet FV,[36,44] (iii) the FV epitope recognised by the antibody. In particular, inhibitors directed against the C2 domain of FV [which mediates the binding of FV(a) to phospholipid membranes] often result in clinical bleeding.[45]

In some cases, FV inhibitors have been reported to be associated with thrombotic rather than haemorrhagic manifestations. Such antibodies may act by selectively impairing FVa inactivation or the anticoagulant function of FV.[46]

Deficiencies of platelet FV

Québec platelet disorder (QPD)

The Québec platelet disorder [OMIM #601709, reviewed elsewhere][47] is an inherited bleeding disorder segregating as an autosomal dominant trait. All presently known cases are distantly related and belong to a single extended pedigree based in Québec (Canada), where the trait has an estimated prevalence of 1:300.000 in the general population. Affected individuals present with a variety of bleeding symptoms, ranging from easy bruising to joint bleeds, their most typical feature being delayed-onset bleeding after haemostatic challenges (*e.g.* surgery). As the laboratory findings include mild thrombocytopenia, abnormal response in some platelet aggregation tests and markedly reduced platelet FV (despite normal or low-normal plasma FV levels), the bleeding diathesis associated with QPD was originally attributed to the selective deficiency of platelet FV, hence the initial name of 'FV Québec'.[48]

However, later studies revealed that not only FV, but most α-granule proteins are decreased/degraded in platelets from QPD patients.[49,50] This is now known to be due to >100-fold increased expression of the urokinase-type plasminogen activator (u-PA) in QPD megakaryocytes, leading to plasmin generation within the platelets and proteolysis of α-granular proteins.[51,52] In line with these findings, accelerated fibrinolysis due to massive release of platelet-derived u-PA at the site of injury is currently considered the primary cause of the characteristic delayed-onset bleeding observed in QPD patients.[53] This view is also supported by the superior efficacy of anti-fibrinolytic drugs, as compared to fresh frozen plasma or platelet concentrates, in treating QPD-related haemorrhages. Linkage analysis has recent-

ly shown that QPD is linked to the u-PA structural gene (*PLAU*) on chromosome 10, although the causative mutation has not been identified yet.[54]

FV New York

Factor V New York is a bleeding disorder characterised by a mild deficiency (*c.* 50%) of platelet FV antigen and activity in the presence of normal levels of plasma FV. In contrast to QPD, FV New York is not a storage pool deficiency, as other α-granular proteins are normal. The only patient with this disorder described to date presented with a moderate bleeding diathesis, especially after surgical challenge. The molecular defect underlying FV New York is presently unknown.[55,56]

Congenital FV deficiency (Owren parahaemophilia)

Epidemiology

Congenital FV deficiency (OMIM #227400), first described by Owren (1947),[57] is an autosomal recessive bleeding disorder that reportedly affects 1:10^6 individuals in the general population. However, the prevalence is likely to be underestimated because mild cases often go undetected. Being a recessive disorder, FV deficiency is more prevalent in countries and cultures where consanguineous marriages are common. More than 200 cases have been reported to date. Registries of FV-deficient patients have been set up in Iran,[4] Italy,[58] as well as the United States and Canada.[59]

Most FV-deficient individuals have a parallel reduction of FV antigen and activity levels (type I deficiency). The only example of a qualitative (type II) FV deficiency known to date is FV New Brunswick,[60] which is characterised by decreased stability of FVa.[61]

Molecular basis

Congenital FV deficiency is caused by loss-of-function mutations in the *F5* gene, which result in reduced FV levels in both plasma and platelets. Currently, more than 100 *F5* mutations associated with reduced FV levels are listed in the online *F5* mutation database (January 2009 release) compiled by Dr H.L. Vos from the Leiden University Medical Centre, Leiden, the Netherlands.[62] Although most mutations are private, *i.e.* they have been described in only one patient or family, a few are common or recurrent (*cf.* the Tyr1702Cys, which was found in several individuals of both European and Asiatic descent).[63-66]

Causative mutations include missense, nonsense and splicing mutations as well as small insertions/deletions (frame-shift mutations) covering the whole *F5* gene, except for the pro-

moter region which has not been adequately investigated yet.[67] While nonsense and frameshift mutations are uniformly distributed throughout the gene, missense mutations, which usually impair folding and/or secretion, tend to cluster in the A and C domains and are characteristically absent from the B domain. This is due to the fact that amino acid changes in the B domain are unlikely to cause FV deficiency, because the B domain is subject to looser structural constraints than the A and C domains. In fact, even a naturally occurring 108-bp deletion causing the in-frame deletion of 36 amino acids within the B domain did not affect FV expression or activity.[68]

Interestingly, it has been observed that mutations predicting a premature stop codon account for as much as two thirds of all *F5* mutations and are significantly overrepresented in the *F5* gene as compared to other genes.[67,69] These molecular defects have been traditionally considered 'null' mutations, as mRNA containing a premature stop codon is normally degraded by nonsense-mediated decay (NMD). However, since point mutations and small insertions/ deletions can be overcome by an occasional somatic reversion or rare mistakes during translation (*e.g.* ribosome slippage), these defects may be actually compatible with the expression of traces of FV sufficient for minimal haemostasis.[3,70] In contrast, truly null FV defects, such as large *F5* deletions or chromosomal rearrangements involving the *F5* gene, would be incompatible with life if present in the homozygous or doubly heterozygous state, as suggested by the fact that gross *F5* gene deletions have never been found in FV-deficient patients. A complete deletion of one *F5* allele has been recently reported in a patient who also carried a missense mutation (Ser234Trp) on the other *F5* allele, resulting in 9% residual FV activity and (remarkably) no history of bleeding.[71]

Clinical presentation, diagnosis and treatment

Most cases of FV deficiency manifest themselves at birth or in early childhood, but some remain virtually asymptomatic until later in life and may be discovered by chance *via* routine coagulation screenings [*cf.* Patient A's brother in Castoldi *et al*.,[63] Patient 3 in Shinozawa *et al*.[72] and the proposita in Caudill *et al*.[71]. Heterozygotes are usually asymptomatic or experience only mild bleeding, whereas homozygotes and compound heterozygotes show a mild-to-severe bleeding diathesis, depending on the residual FV level. The correlation between FV level and bleeding phenotype is lost in the low FV range (<5%), where patients with equal FV levels may show very different clinical phenotypes.

According to the Iranian and North American registries,[4,59] the most common symptoms associated with FV deficiency are bleeding from mucous membranes (*e.g.* epistaxis, menorrhagia in females) and post-traumatic bleeding following surgery or delivery, which occur in approximately half of all FV-deficient individuals. Haemarthroses and muscle haematomas

are present in only one quarter of FV-deficient patients, and severe bleeding manifestations (*e.g.* intracranial or gastro-intestinal haemorrhages) are rare and confined to patients with undetectable FV levels. Despite this overall benign phenotype, several cases of severe FV deficiency presenting with life-threatening neonatal/perinatal intracranial haematomas have also been reported.[73-77]

The circumstances that most often lead to the diagnosis of congenital FV deficiency are bleeding episodes, positive family history or routine coagulation tests.[59] FV deficiency is suspected whenever the PT and aPTT are both prolonged, but conclusive diagnosis requires the measurement of plasma FV antigen and/or activity levels. If FV levels are reduced, additional testing is needed to exclude combined FV/ FVIII deficiency and acquired causes of FV deficiency. Due to the genetic heterogeneity of FV deficiency, identification of the molecular defect(s) in individual patients is still confined to specialised research laboratories, and prenatal diagnosis is not common practice.

In the absence of a FV concentrate, (virus-inactivated) fresh frozen plasma is the treatment of choice in symptomatic FV deficiency. However, since FV has a plasma half-life of only *c.* 13 h,[78] repeated infusion may lead to volume overload. Inhibitor development as a reaction to treatment is rare.[75] Mild bleeding episodes usually respond to anti-fibrinolytic agents, like aminocaproic acid.[59] In contrast to severe haemophiliacs, patients with severe FV deficiency do not need routine prophylaxis. Fresh frozen plasma is only administered on demand to stop acute bleeding or in view of risk situations. The clinical aspects of FV deficiency have been recently reviewed elsewhere.[79]

Open questions

Considering the pivotal role of FV in prothrombin activation, complete FV deficiency is expected to be incompatible with life. Accordingly, it is currently accepted that all FV-deficient patients actually have some residual FV.[3] Still, it remains puzzling that many patients with undetectable FV experience only mild-to-moderate bleeding (Fig. 1) and even have a more favourable prognosis than severe haemophiliacs. Moreover, it is not clear why some severe FV-deficient patients experience life-threatening haemorrhages, while others are only mild bleeders despite their equally low FV levels. The variable phenotype associated with low or undetectable FV levels strongly suggests the existence of additional factors modulating clinical bleeding in FV-deficiency.

Modulation of the bleeding diathesis in FV deficiency

Possible role of platelet FV[*]

Miletich et al. (1978)[80] reported that the bleeding tendency of FV-deficient patients was better predicted by the FXa-binding capacity of their platelets, i.e. the amount of FVa exposed on activated platelets, than by their plasma FV concentration. Despite this important preliminary observation, the possible role of platelet FV in congenital FV deficiency has not received much attention. In fact, platelet FV is not routinely determined in FV-deficient patients and most studies report only plasma FV levels, implicitly assuming that platelet FV is equally reduced. To our knowledge, platelet FV has been evaluated in only four patients with severe congenital FV deficiency.[5,72,76] No FV was found in platelets from two patients with undetectable plasma FV, one with mild[5] and one with severe[76] bleeding manifestations. However, platelet FV was measurable in two patients with 4% and 5% FV antigen in their plasma, respectively.[72] One of these two patients, who was homozygous for the Arg2174Leu mutation, even had a normal platelet FV level as determined by ELISA and Western blotting and no bleeding tendency whatsoever, despite plasma FV antigen and activity levels of 5% and 1%, respectively.[72] A patient with similar characteristics, *i.e.* undetectable plasma FV, 15% platelet FV ('FXa-binding capacity') and a mild haemorrhagic diathesis, was also present in Miletich's cohort of FV-deficient patients.[80] Such cases indicate that discrepancies between plasma and platelet FV levels do exist, although the underlying mechanism remains unclear. Moreover, they suggest the possibility that the observed differences in bleeding phenotype among FV-deficient patients with equally low plasma FV levels may be due to differences in platelet FV. Residual platelet FV may be particularly important for patients with undetectable plasma FV, where it might explain the discrepancy between the absence of *in vitro* thrombin generation in plasma[6,8] and the *in vivo* phenotype of mild/moderate bleeding (Fig. 1). To test this hypothesis, an effort is needed to determine platelet FV in a large cohort of patients with severe FV deficiency.

Several observations indicate that platelet FV is crucial in maintaining adequate haemostasis, especially in FV deficiency states. For example, the bleeding diathesis associated with FV New York, where platelet FV is decreased but plasma FV is normal, suggests that platelet FV is required for efficient haemostasis.[56] However, the importance of platelet FV is best illustrated by the comparison of two patients with acquired FV inhibitor reported in the literature. One patient had an antibody titre exceeding the plasma FV concentration 100-fold and still underwent surgery without any bleeding complication.[36] As it turned out, although

[*] At the time of writing this review, the research described in Chapter 5 regarding the role of platelet

the patient's plasma FV was fully neutralised, his platelet FV was relatively inaccessible to the antibody, ensuring normal haemostasis. In contrast, another patient with a relatively low antibody titre experienced repeated bleeding episodes, because the FV neutralising antibody was present both in her plasma and her platelets.[44] These observations also provide a rationale for the superior efficacy of platelet transfusions over fresh frozen plasma in treating patients with FV inhibitors, as the release of platelet FV at the site of injury results in a high local FV concentration that is sufficient to exceed the binding capacity of the inhibitor.[36]

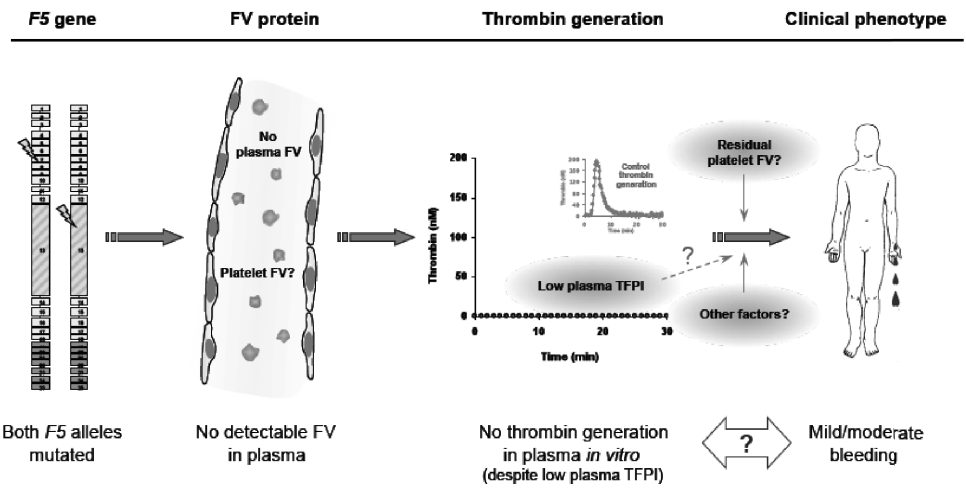

Figure 1. Genotype-to-phenotype flowchart in severe FV deficiency. Patients with severe FV deficiency are homozygous or compound heterozygous for loss-of-function mutations in the *F5* gene. Some mutations may impair gene expression to the point that FV antigen and/or activity are undetectable in the patient's plasma (and presumably platelets, although platelet FV is not routinely evaluated). Accordingly, no thrombin is formed when the patient's plasma is triggered with tissue factor *in vitro*, even if plasma free TFPI is low (inset: thrombin generation in control plasma is shown for comparison). Still, the patient may experience only mild or moderate bleeding. As suggested in this review, *in vivo* haemostasis may rely on possible traces of residual platelet FV and/or on other unknown factors synergizing with the low free TFPI levels to guarantee minimal thrombin generation.

Plasma levels of tissue factor pathway inhibitor

Thrombophilic mutations, such as *F5* R506Q (FV Leiden) and *F2* (prothrombin) G20210A have been reported to ameliorate the bleeding phenotype in several bleeding disorders, including haemophilia,[81] von Willebrand disease,[82] and FVII deficiency.[83] In an attempt to explain the relatively mild bleeding phenotype associated with FV deficiency, we recently tested 11 patients with severe congenital FV deficiency (nine with FV < 1%) for concomitant pro-

coagulant defects.[8] A complete thrombophilia screening, including the levels of antithrombin, protein C, protein S, prothrombin and FVIII, as well as the *F5* R506Q and *F2* G20210A mutations, was inconclusive, revealing only partial protein C deficiency in one patient and elevated FVIII levels in another patient. However, using thrombin generation assays as a functional screening tool, we discovered that FV-deficient plasma invariably contained low levels of the natural anticoagulant tissue factor pathway inhibitor (TFPI).[8] While total TFPI levels were only slightly reduced, free TFPI, *i.e.* the TFPI fraction not bound to lipoproteins, and TFPI activity were reduced to *c.* 30% of the normal plasma levels. FV and (free) TFPI were found to form a complex in plasma and their levels were therefore highly correlated, progressively decreasing from normal individuals to partial (heterozygous) and severe (homozygous) FV deficiency.[8]

Tissue factor pathway inhibitor [reviewed in Crawley & Lane, 2008][84] is a Kunitz-type protease inhibitor which down-regulates the initiation of coagulation by inhibiting both the tissue factor (TF)/FVIIa complex and FXa. Although most TFPI is associated with the vascular endothelium, *c.* 10% is present in plasma where it is largely truncated and bound to lipoproteins. Only *c.* 10% of plasma TFPI is in the free full-length form, which is considered the only active fraction. Since low plasma levels of TFPI have been shown to ameliorate the bleeding phenotype of haemophiliacs,[85] we reasoned that they might be beneficial to FV-deficient patients as well. To test this hypothesis, we supplemented plasma from a patient with undetectable FV with increasing amounts of purified FV (0-10% of the normal plasma concentration) and determined *in vitro* thrombin generation before and after normalising the plasma TFPI level (Fig. 2). In the low FV range (0-2% FV), thrombin generation was several-fold higher in the absence than in the presence of added TFPI, but this difference gradually disappeared at higher FV levels. At 0.5% FV, thrombin generation was distinctly measurable in the absence of added TFPI, but was completely abolished by the normalisation of plasma TFPI level. These findings suggest that patients with severe FV deficiency may benefit from their partial TFPI deficiency.[8] Whether interindividual differences in plasma TFPI levels contribute to the differences in clinical presentation among FV-deficient patients with similar residual FV levels remains to be elucidated (Fig. 1).

How low TFPI levels enhance thrombin generation in FV-deficient plasma is not obvious, as FV cannot be by-passed. Given that low plasma TFPI levels result in less down-regulation of the TF/FVIIa complex, more FX is likely to be activated in FV-deficient plasma. Although FXa alone is a very inefficient prothrombin activator,[22] increased FXa might act by protecting any available traces of FVa from APC-mediated inactivation.[25] In this respect, it is interesting to note that recombinant FVIIa (rFVIIa), which similarly stimulates the initiation of coagulation and the generation of FXa, has proved effective in the treatment of a severely affected FV-deficient patient who had become allergic to fresh frozen plasma.[86] The partial

TFPI deficiency that accompanies FV deficiency may also explain some rare cases of venous thrombosis reported in patients with severe FV deficiency.[87-89]

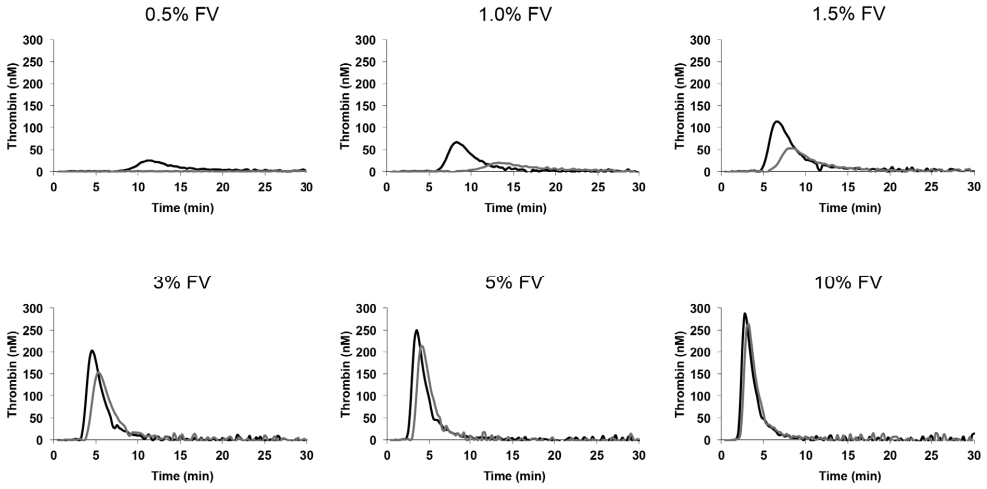

Figure 2. Beneficial effect of low TFPI level on thrombin generation in FV-deficient plasma. Plasma from a FV-deficient patient with undetectable FV (and decreased TFPI level) was reconstituted with increasing amounts of FV (0.5-10% of the normal plasma concentration). Thrombin generation in the reconstituted plasmas was triggered with 13.6 pmol/l tissue factor before (black) and after (grey) normalisation of the plasma TFPI level.

Pseudo-homozygous activated protein C resistance

A very special case of interaction between FV deficiency and a thrombophilic defect is represented by pseudo-homozygous APC resistance.[90] This rare condition is characterised by the co-inheritance of a loss-of-function mutation on one *F5* allele (predicting partial FV deficiency) and the *F5* R506Q mutation on the other *F5* allele. Although such patients have plasma FV levels (*c.* 50%) compatible with heterozygous FV deficiency and thus suggestive of a mild haemorrhagic diathesis, they actually carry a prothrombotic state comparable to that of FV Leiden homozygotes.[91] This is due to the fact that the non-Leiden *F5* allele is not expressed, leading to the exclusive presence of FV Leiden in plasma. Moreover, due to the reduced FV levels, plasma TFPI levels are also likely to be decreased,[8] further aggravating the hypercoagulable state. Therefore, caution is warranted in the management of heterozygous FV-deficient patients. In particular, FV Leiden genotype should always be tested in order to exclude pseudo-homozygous APC resistance, as prophylactic treatment in view of a risk situation might trigger life-threatening venous thromboembolism.

Conclusions

The complex pathophysiology of congenital FV deficiency is just starting to be unravelled. Given that the FV level required for minimal haemostasis is extremely low, subtle differences in plasma and/or platelet FV levels may be crucial to clinical outcome. In particular, residual platelet FV might be responsible for the vast differences in bleeding phenotype observed among patients with equally undetectable plasma FV levels. However, while several lines of evidence support the role of platelet FV in maintaining adequate haemostasis, available data on platelet FV in patients with severe congenital FV deficiency are too scanty to allow definite conclusions.

Although the residual FV level is the major determinant of clinical bleeding, the poor correlation between FV levels and bleeding manifestations in the low FV range suggests the existence of additional phenotype modulators. One of these might be the plasma TFPI level, which is markedly reduced in FV deficiency. While *in vitro* experiments show that low TFPI enhances thrombin generation in FV-deficient plasma, more research is needed to find out if (and to what extent) inter-individual differences in plasma TFPI levels contribute to clinical presentation differences among FV-deficient patients.

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3

Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency

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Summary

Severe factor V (FV) deficiency is associated with mild to severe bleeding diathesis, but many patients with FV levels lower than 1% bleed less than anticipated. We used calibrated automated thrombography to screen patients with severe FV deficiency for protective procoagulant defects. Thrombin generation in FV-deficient plasma was only measurable at high tissue factor concentrations. Upon reconstitution of FV-deficient plasma with purified FV, thrombin generation increased steeply with FV concentration, reaching a plateau at approximately 10% FV. FV-deficient plasma reconstituted with 100% FV generated several fold more thrombin than normal plasma, especially at low tissue factor concentrations (1.36 pM) or in the presence of activated protein C, suggesting reduced tissue factor pathway inhibitor (TFPI) levels in FV-deficient plasma. Plasma TFPI antigen and activity levels were indeed lower ($P < .001$) in FV-deficient patients (n = 11; 4.0 ± 1.0 ng/mL free TFPI) than in controls (n = 20; 11.5 ± 4.8 ng/mL), while persons with partial FV deficiency had intermediate levels (n = 16; 7.9 ± 2.5 ng/mL). FV immunodepletion experiments in normal plasma and surface plasmon resonance analysis provided evidence for the existence of a FV/TFPI complex, possibly affecting TFPI stability/clearance *in vivo*. Low TFPI levels decreased the FV requirement for minimal thrombin generation in FV-deficient plasma to less than 1% and might therefore protect FV-deficient patients from severe bleeding.

Introduction

Coagulation factor V (FV) is a large multidomain glycoprotein structurally and functionally homologous to factor VIII (FVIII).[1] After biosynthesis in the liver, FV is released in the bloodstream, where it is found in both plasma (80%; concentration of 21-25 nM) and platelets (20%). The activated form of FV (FVa) acts as an essential cofactor of activated factor X (FXa) in prothrombin (PT) activation, thereby enhancing thrombin formation by several orders of magnitude.[2]

The generation of thrombin is physiologically down-regulated by several anticoagulant mechanisms, including the protein C pathway[3] and the tissue factor pathway inhibitor (TFPI) system.[4] Activated protein C (APC) is a vitamin K–dependent serine protease that, in concert with its nonenzymatic cofactor protein S, inactivates FVa and FVIIIa by limited proteolysis. A poor anticoagulant response of plasma to exogenous APC (APC resistance[5]) is the most common risk factor for venous thrombosis. Conversely, TFPI is a Kunitz-type protease inhibitor that binds and inhibits both FXa and the tissue factor (TF)/FVIIa complex in a 2-step reaction,[6] the first step being stimulated by protein S.[7,8] TFPI is synthesized primarily by the vascular endothelium, and most of it (approximately 80%) is associated with the endothelial surface as a full-length protein, the form that most effectively inhibits FXa.[9] Another 2% of all TFPI is stored in platelets.[10,11] The remainder circulates in plasma at a concentration of 2.0 to 2.5 nM, of which approximately 80% is C-terminally truncated and bound to lipoproteins, while 5% to 20% is present as a free protein (both full-length and truncated forms).[12] Low levels of plasma TFPI, particularly free TFPI, have been associated with an increased risk of venous thrombosis.[13-16]

Severe FV deficiency (Owren parahemophilia; OMIM 227400) is a rare bleeding disorder with an estimated prevalence of $1:10^6$. It is inherited as an autosomal recessive trait, and several intragenic mutations impairing FV gene (*F5*) expression have been described (reviewed by Asselta *et al*[17]). Whereas most heterozygous carriers are asymptomatic, homozygotes and compound heterozygotes present with a wide spectrum of symptoms,[18,19] ranging from mucosal bleeding and postoperative/postpartum hemorrhages to life-threatening intracranial hematomas. Although severe bleeding manifestations are usually confined to people with FV levels less than 1%, there seems to be no clear-cut relationship between plasma FV levels and bleeding phenotype, and some FV-deficient patients have apparently remained completely asymptomatic for several years despite their undetectable plasma FV levels[20,21]

The overall moderate bleeding diathesis associated with severe FV deficiency in humans contrasts sharply with the invariable embryonic/perinatal lethality of the FV knockout

mouse model.[22] In addition, compared with patients with severe hemophilia, who frequently develop joint and muscle bleedings,[23] many patients with severe FV deficiency show a milder clinical course.[18] Even admitting that the FV level required for minimal hemostasis is 1% or less,[24] the general viability of FV deficiency in humans suggests the existence of widespread compensating mechanisms that protect FV-deficient patients from excessive bleeding. In contrast to severe hemophilia[25] and other bleeding disorders,[26-28] where coinherited thrombophilic mutations (*F5* Leiden, *F2* G20210A mutation) or low levels of the natural anticoagulants have been shown to mitigate the bleeding manifestations, no similar protective mechanisms have ever been reported for severe FV deficiency.

In this study, we have used *in vitro* thrombin generation assays to investigate the overall coagulation function in 11 patients with severe FV deficiency, and to screen for possible procoagulant defects that may contribute to improve their clinical phenotype.

Methods

Study population

Experiments were conducted in plasma from 11 subjects (10 unrelated) with congenital severe FV deficiency: 8 were patients referred to Padua Academic Hospital from district hospitals in northeastern Italy and 3 were blood donors from George King Bio-Medical (Overland Park, KS). Patient characteristics are reported in Table 1. No DNA and only limited information could be obtained for the George King donors.

Patients with severe FV deficiency were compared with 16 people with partial FV deficiency (9 men and 7 women; FV level 42.9%±9.9%) and to 20 healthy controls (8 men and 12 women; FV level 87.0%±17.8%) recruited at Padua Academic Hospital among relatives of FV-deficient and FV Leiden pseudohomozygous[29] patients and among healthy hospital personnel. Subjects with partial FV deficiency were all asymptomatic, except one who had experienced epistaxis and gum bleeding during childhood. None of the subjects under study was on oral contraceptives or hormone replacement therapy at the time of blood sampling.

As an additional control group, 15 unrelated (male) patients with hemophilia A (FVIII levels, 1%-23%; mean, 3.9%) and bleeding symptoms ranging from mild to severe were included in the study. Of these, 13 were patients at Padua Academic Hospital and 2 were blood donors from George King Biomedical.

The study was carried out in accordance with the Declaration of Helsinki, and all subjects gave informed consent to participation. George King Bio-Medical donors routinely sign an

informed consent statement at the moment of blood collection. The study was approved by the ethical commission of Padua Academic Hospital.

Blood collection and plasma preparation

Venous blood was drawn by venipuncture in 3.8% sodium citrate (wt/vol) and centrifuged at 2000g for 15 minutes. Platelet-poor plasma was aliquoted, snap-frozen, and stored at 80°C until use; buffy coats were stored at 20°C for later DNA isolation.

Plasma purchased from George King Bio-Medical was collected from individual donors by plasmapheresis in 3.8% sodium citrate, divided into aliquots, and immediately frozen at 80°C.

Commercial factor-depleted plasmas

FV-depleted plasmas were purchased from Affinity Biologicals (Ancaster, ON), Dade-Behring (Marburg, Germany), Diagen (Thame, United Kingdom), Diagnostica Stago (Asnières sur Seine, France) and Organon-Teknika (Durham, NC). PT-, FVII-, FX-, protein C- and antithrombin-depleted plasmas were from Affinity Biologicals.

Measurement of coagulation factor levels in plasma

A complete thrombophilia screening, including plasma levels of antithrombin, protein C, and protein S as well as PT and FVIII, was performed in all patients with severe FV deficiency. Antithrombin levels were measured with the Coamatic Antithrombin kit (Chromogenix, Mölndal, Sweden). For the quantification of protein C levels, protein C was activated in 1:20 diluted plasma with 0.05 U/mL Protac (Kordia Life Sciences, Leiden, The Netherlands) for 1 hour at 37°C, and the amidolytic activity of APC toward the chromogenic substrate S2366 was measured spectrophotometrically. Total and free protein S were determined by enzyme-linked immunosorbent assay (ELISA) as described.[30] PT levels were measured with a chromogenic assay after complete activation of PT with Ecarin (Kordia Life Sciences).[30] FVIII levels were measured by a one-stage activated partial thromboplastin time (aPTT)–based clotting assay in FVIII-deficient plasma.

FV and FX activity levels were determined with prothrombinase-based assays using in-house purified proteins, essentially as described.[31] Briefly, FV or FX was activated in highly diluted plasma with 2 nM thrombin (Innovative Research, Southfield, MI) or 0.27 μg/ml RVV-X (Kabi Diagnostica / Chromogenix), respectively, for 10 minutes at 37°C. The FVa or FXa concentration was subsequently quantified *via* a prothrombinase-based assay under the following conditions: limiting amounts of FVa (<25 pM), 5 nM FXa, 1 μM PT, 40 μM

DOPS/DOPC (10/90 mol/mol) phospholipid vesicles, and 2.6 mM $CaCl_2$ for the FVa assay; and limiting amounts of FXa (<25 pM), 5 nM FVa, 40 μM DOPS/DOPC (10/90 mol/mol) phospholipid vesicles, 1 μM PT and 3 mM $CaCl_2$ for the FXa assay (final concentrations). The minimum FV concentration that could be reliably detected with this assay in plasma was 0.5%. To attain maximal precision, the FV levels of the different FV-deficient patients were measured 2 times in duplicate using independent plasma dilutions.

Plasma levels of total and free TFPI antigen were measured with commercial ELISA kits (Asserachrom; Diagnostica Stago). Due to the poor sensitivity of the free TFPI ELISA in the low-level range, a more sensitive homemade full-length TFPI ELISA (C. F. A. Maurissen, J.R., and T. M. Hackeng, manuscript in preparation) was also performed in some cases. TFPI activity was determined with a thrombin generation–based assay (see "Thrombin generation assays").

A normal plasma pool prepared with plasma from 85 healthy donors not using any medication was used as a reference in all measurements. All factor levels were expressed as percentage of normal plasma, except total and free TFPI antigen levels, which were compared with a standard provided with the respective ELISA kit and expressed in nanograms per milliliter.

Thrombin generation assays

Thrombin generation was measured in platelet-poor plasma with the calibrated automated thrombogram (CAT) method.[32] Briefly, coagulation was initiated with varying concentrations of recombinant TF (Innovin; Dade-Behring), 30 μM DOPS/DOPC/DOPE (20/60/20 mol/mol/mol) phospholipid vesicles and 16 mM added $CaCl_2$, and thrombin activity was monitored continuously via a low-affinity fluorogenic substrate (I-1140; BACHEM, Bubendorf, Switzerland). A thrombin calibrator (Thrombinoscope, Maastricht, The Netherlands) was used to correct each curve for inner-filter effects and substrate consumption. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems, Helsinki, Finland), and thrombin generation curves were calculated with the Thrombinoscope software or as previously described[26] (Figure 1). Apart from the experiments presented in Figure 1, all thrombin generation curves were run in duplicate. The height of the thrombin peak or the area under the curve (endogenous thrombin potential [ETP]) was taken as a measure of the amount of thrombin formed. The lag time of the thrombin generation curve, which is a measure of the plasma clotting time, was also analyzed in some experiments.

To obtain reliable thrombin generation curves, FV-deficient plasma was supplemented with purified FV to various final concentrations (see "Results"), and FVIII-deficient plasma was spiked with 1% normal pooled plasma. FV was purified from normal pooled plasma as

described.[31] For reconstitution purposes, 23 nM FV was considered equal to 100% of the normal plasma concentration. In some experiments, FV-deficient plasma was supplemented with recombinant full-length TFPI (kind gift from Dr Lindhout, Maastricht University, Maastricht, The Netherlands). The amount of added TFPI was chosen to normalize thrombin generation in FV-deficient plasma reconstituted with 100% FV. To prevent contact activation, all thrombin generation curves were measured in the presence of 30 μg/mL corn trypsin inhibitor (CTI; Hematologic Technologies, Essex Junction, VT).

To determine plasma APC resistance, thrombin generation was triggered with 13.6 pM TF in the absence and presence of 12 nM purified human APC (Innovative Research). The APC concentration was chosen to reduce the ETP in normal plasma to approximately 10% of the ETP without APC.

By analogy with the diluted prothrombin time assay, which measures the ability of TFPI to prolong the plasma clotting time at a low TF concentration,[33] TFPI activity was quantified as the ability of TFPI to reduce the peak height of thrombin generation. Briefly, plasma was incubated with (and without) 16 μg/ml of a monoclonal antibody against human TFPI (TFPI-6; Sanquin Reagents, Amsterdam, The Netherlands) for 15 minutes at 37°C, after which thrombin generation was initiated with 1.36 pM TF. The outcome of the test was expressed as an anti-TFPI ratio, defined as the ratio of the peak heights of the thrombin generation curves determined in the presence and absence of antibody. The higher the anti-TFPI ratio, the more functional TFPI is present in plasma. Normal plasma yields an anti-TFPI ratio of approximately 4.0.

DNA analysis

Genomic DNA was isolated from peripheral blood leukocytes by “salting out.” Carriership of the *F5* Leiden and *F2* G20210A mutations was determined by polymerase chain reaction (PCR)–mediated amplification and restriction analysis, as described.[30] TFPI gene (*TFPI*) mutation screening was performed by PCR-mediated amplification and direct sequencing of all exons and splicing junctions, including approximately 500bp of the promoter region. Primer sequences and amplification/ sequencing conditions are available on request.

Plasma FV immunodepletion

For immunodepletion experiments, blood was drawn from healthy donors both in 3.2% sodium citrate (wt/vol) and in a mixture of recombinant hirudin (3.5 μM; Kordia Life Sciences), recombinant tick anticoagulant protein (TAP; 0.5 nM; Corvas International, San Diego, CA), and CTI (50 μg/ml). Platelet-poor plasma was prepared by centrifugation at 2000*g* for 15

minutes. Pooled citrated or hirudin/TAP/CTI-anticoagulated plasma (625 μl) was pretreated with 250 μL (drained volume) protein A sepharose beads (rProtein A Sepharose Fast Flow; GE Healthcare, Uppsala, Sweden) for 1 hour at room temperature to bind all endogenous immunoglobulins (preclearance). After removing the beads by centrifugation at 3000*g* for 2 minutes, precleared plasma was incubated with protein A sepharose beads bearing the anti-human FV monoclonal antibody 3B1 (kind gift from Prof B. N. Bouma, Utrecht University Hospital, Utrecht, The Netherlands) or no antibody (negative control) at room temperature under rotation. From these mixtures, 50-μl aliquots were taken at regular intervals and assayed for FV[34] and full-length TFPI antigen by ELISA.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) measurements were performed on a Biacore T100 (GE Healthcare). Three flow cells of a CM5 chip were coated with approximately 1500, 3400, and 7000 resonance units (RUs) of recombinant TFPI, respectively. The fourth cell was not coated and served as a reference cell. The chip was perfused with running buffer (25 mM HEPES, 175 mM NaCl, 0.005% Tween, and 5 mM $CaCl_2$ [pH 7.4]) until a stable baseline was obtained. Then, plasma-purified FV (2.6-100 nM in running buffer) was injected for 240 seconds and binding to immobilized TFPI was recorded. Since spontaneous dissociation was rather slow, the chip was stripped with regeneration buffer (25 mM HEPES, 1 M NaCl, and 0.005% Tween [pH 7.4]) for 150 seconds. All experiments were carried out at 25°C with a flow rate of 20 μl/min. Binding was expressed in RUs, after correction for the signal obtained in the reference cell.

Statistical analysis

All data are expressed as means plus or minus standard deviation. Due to the small number of people per group, factor levels and thrombin generation parameters were compared among groups using the nonparametric Mann-Whitney-Wilcoxon 2-sample test (*U*). The effect of age and sex on coagulation factor levels and thrombin generation parameters was assessed by (multiple) linear regression analysis. Statistical analyses were performed with SPSS 14.0 (SPSS, Chicago, IL).

Results

Characteristics of FV-deficient patients

The demographic and clinical characteristics of the 11 patients with congenital severe FV deficiency included in this study are presented in Table 1. Patients PD VII and PD VII-A were sisters, whereas all others were unrelated. Most patients had undetectable FV levels, except for PD III (0.6%), PD VII (4.8%), and PD VII-A (6.2%). Although all patients were bleeders, only patient PD V had experienced life-threatening events (hemorrhagic shock after tonsillectomy and severe hemoperitoneum following rupture of an ovarian cyst).

FV gene mutation screening was performed in all patients whose DNA was available for study. All were found to be homozygous or compound heterozygous for *F5* mutations that severely impair gene expression[36,37] (P.S. *et al*, manuscript in preparation). None of the patients was a carrier of the *F5* Leiden or *PT* G20210A mutations, and the levels of antithrombin, protein C and protein S, as well as PT, FVIII and FX, were in the normal range, unless otherwise stated in Table 1.

Table 1. Demographic and clinical characteristics of patients with severe FV deficiency.

Patient	Sex	Age (years)	FV level (%)	Co-inherited thrombophilic defects	Clinical phenotype[§]
PD I	F	64	<0.5	-	Moderate/severe (5)
PD II	F	44	<0.5	170% FVIII	Severe (7)
PD III	F	35	0.6	131% PT (no PT G20210A)	Asymptomatic (0*)
PD IV	F	27	<0.5	-	Mild (2)
PD V	F	52	<0.5	-	Severe (10*)
PD VI	M	28	<0.5	-	Mild (1*)
PD VII	F	62	4.8	-	Mild (1)
PD VII-A	F	46	6.2	-	Mild (1)
GK 502	F	57	<0.5	-	Mild
GK 505	F	56	<0.5	53% PC	Unknown
GK 506	M	65	<0.5	-	Unknown

PT, prothrombin; PC, protein C.

[§]Numbers in brackets represent the bleeding score calculated according to Rodeghiero *et al.*[35]

*Prophylaxis (with plasma and/or antifibrinolytic agents) often given during risk situations after the diagnosis of severe FV deficiency was made.

Thrombin generation in FV-deficient plasma

The coagulation phenotype of the different FV-deficient plasmas was characterized by thrombin generation measurements. When coagulation was initiated with 13.6 pM TF, a concentration that elicits maximal thrombin generation in normal plasma, very little thrombin, if any, was formed in most FV-deficient plasmas (data not shown), except for that of patient PD VII (patient PD VII-A was not tested). The thrombin generation curve obtained in this plasma was comparable with that of normal plasma (although slightly later in time), indicating that approximately 5% FV is sufficient for maximal thrombin generation at 13.6 pM TF.

When the TF concentration was increased, thrombin formation became apparent also in the plasmas with undetectable FV levels and progressively increased with the TF concentration (Figure 1A). At 544 pM TF, all FV-deficient patients showed some degree of thrombin generation, with peak heights ranging between 13.9 nM (PD IV) and 340 nM (PD VII) compared with approximately 500 nM in normal plasma (Figure 1B).

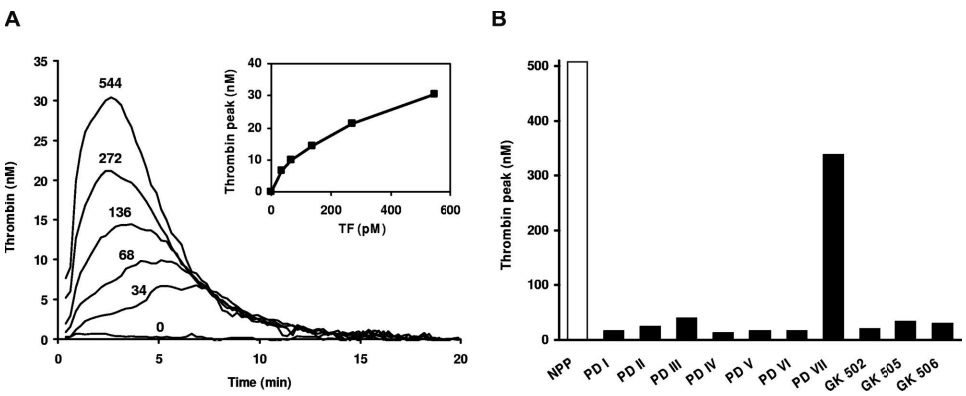

Figure 1. Thrombin generation in FV-deficient plasma. A) Tissue factor (TF) titration of thrombin generation in FV-deficient plasma. Thrombin generation was determined in plasma from patient GK 506 at 0, 34, 68, 136, 272 and 544 pM TF. Inset: Peak height of thrombin generation as a function of TF concentration. B) Peak height of thrombin generation evoked by 544 pM TF in normal pooled plasma (NPP) and in plasma from patients with severe FV deficiency

To determine the effect of FV level on thrombin generation, FV-deficient plasma was reconstituted with increasing amounts of purified FV ranging from 0% to 100% of the normal plasma concentration, and thrombin generation was measured at 13.6 pM TF (Figure 2). Although at this TF concentration no thrombin was formed in the absence of exogenous FV, thrombin generation increased steeply (and showed gradually shorter lag times) at increasing FV concentrations, reaching a plateau at approximately 10% FV. Thrombin generation

was already detectable at 0.5% FV and attained half-maximal ETP and peak height at 1% and 2% FV, respectively.

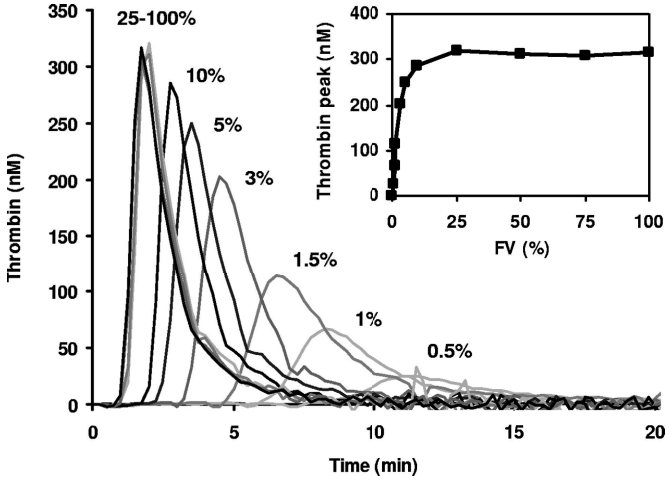

Figure 2. FV-titration of thrombin generation in FV-deficient plasma. Plasma from patient GK 502 was reconstituted with 0-23 nM purified FV (0%, 0.5%, 1%, 1.5%, 3%, 5%, 10%, 25%, 50%, 75% and 100% of the normal plasma concentration) and thrombin generation was determined at 13.6 pM tissue factor. Thrombin generation curves obtained at 25%, 50%, 75% and 100% FV are perfectly superimposable. Inset: peak height of thrombin generation as a function of FV concentration.

Comparison between FV-deficient plasma reconstituted with 100% FV and normal plasma

To screen FV-deficient plasma for the presence of procoagulant defects, thrombin generation determined under different experimental conditions was compared between FV-deficient plasma reconstituted with 100% purified FV (to eliminate the effect of low FV levels) and normal plasma. Since all reconstituted FV-deficient plasmas behaved essentially the same in these experiments, only a representative example is shown in Figure 3.

When the TF concentration was varied between 0.34 and 13.6 pM, much more thrombin was formed in reconstituted FV-deficient plasma than in normal plasma at the lowest TF concentrations, but this difference gradually disappeared as the TF concentration was increased. Thus, while the thrombin peak obtained at 1.36 pM was 4 times higher in reconstituted FV-deficient plasma than in normal plasma (183.6 nM *vs.* 44.6 nM; Figure 3A), the thrombin generation curves obtained at 13.6 pM TF were virtually superimposable (Figure 3B). However, when APC (12 nM) was included in the reaction mixture at 13.6 pM TF, thrombin generation was again much higher in reconstituted FV-deficient plasma (peak

height 166.3 nM; ETP 515.8 nM.min) than in normal plasma (peak height 21.3 nM; ETP 62.3 nM.min), revealing a pronounced APC resistance in (reconstituted) FV-deficient plasma (Figure 3C). Similarly, the lag time of thrombin generation was shorter in reconstituted FV-deficient plasma than in normal plasma, both at 1.36 pM TF (2.79 minutes *vs.* 3.67 minutes; Figure 3A) and at 13.6 pM TF in the presence of APC (1.75 minutes *vs.* 2.25 minutes; Figure 3C). Since TFPI is the major determinant of the lag time and peak height of thrombin generation at low TF concentrations and of APC resistance measured with the ETP-based test,[38,39] these findings suggested that TFPI levels might be decreased in FV-deficient plasma.

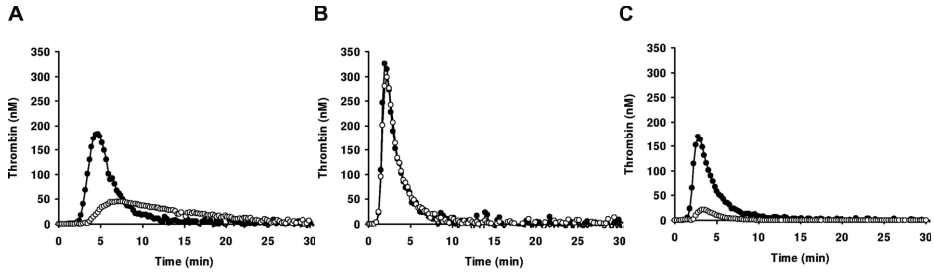

Figure 3. **Thrombin generation in FV-deficient plasma reconstituted with 100% FV and in normal plasma.** Thrombin generation was measured in FV-deficient plasma (pooled plasma from patients GK 502, GK 505 and GK 506) reconstituted with 100% FV (closed circles) and in normal plasma (open circles) after triggering coagulation with A) 1.36 pM tissue factor (TF); B) 13.6 pM TF; and C) 13.6 pM TF in the presence of 12 nM activated protein C.

Plasma TFPI levels in FV-deficient patients

Plasma levels of total and free TFPI antigen were determined in all FV-deficient patients and in 20 healthy controls. As an additional control, 16 people with partial FV deficiency (mean FV levels 42.9%) were also included. Total TFPI levels (Figure 4A) decreased slightly from normal controls (66.0±16.5 ng/ml) to individuals with partial FV deficiency (61.4±15.3 ng/ml, *P* = nonsignificant) to patients with severe FV deficiency (53.8±17.9 ng/ml, *P* = .036 *vs.* healthy controls). A similar trend, but more pronounced, was observed for free TFPI levels (Figure 4B), which were higher in healthy controls (11.5±4.8 ng/ml) than in those with partial FV deficiency (7.9±2.5 ng/ml, *P* = .023) than in patients with severe FV deficiency (4.0±1.0 ng/ml, *P*< .001 *vs.* healthy controls). Correction for age and sex, which are well-known determinants of TFPI levels,[15,40] did not alter these results. Full-length TFPI levels, as determined with an in-house ELISA, were also markedly reduced in FV-deficient plasma (23.9±17.4% of normal pooled plasma).

TFPI activity was determined by a thrombin generation–based assay in which coagulation was initiated with 1.36 pM TF in the absence and presence of a neutralizing anti-TFPI

antibody. To correct for a possible effect of FV on thrombin generation, the FV level was normalized in all FV-deficient plasmas by adding purified FV up to 100%. TFPI activity, expressed as anti-TFPI ratio (thrombin peak with antibody/thrombin peak without antibody), was 3.70 plus or minus 2.41 in healthy controls, 2.29 plus or minus 0.48 in those with partial FV deficiency (P = .046), and 1.18 plus or minus 0.08 in patients with severe FV deficiency (P = .001 *vs.* healthy controls), indicating that also the activity of TFPI is markedly reduced in FV-deficient plasma (Figure 4C).

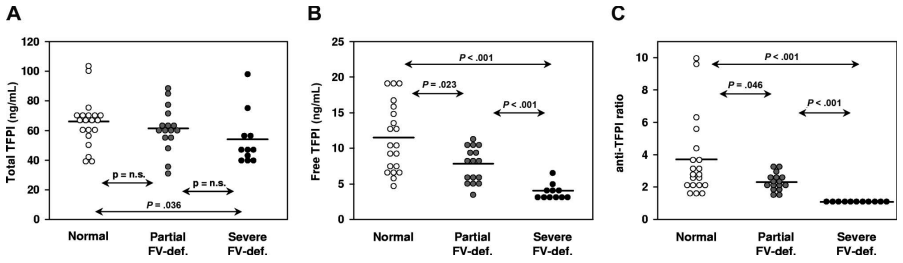

Figure 4. Plasma TFPI antigen and activity levels in groups of individuals with different FV levels. Total and free TFPI antigen levels were measured with commercial ELISAs, whereas TFPI activity levels were determined by a thrombin generation-based assay and expressed as anti-TFPI ratio, as described under Methods. A) Total TFPI antigen levels; B) free TFPI antigen levels; C) TFPI activity levels. The horizontal bars represent the means of the respective groups. Probabilities of the Mann-Whitney-Wilcoxon U are indicated.

Thrombin generation curves obtained in the absence and presence of anti-TFPI antibody were also analyzed separately. In the absence of antibody, plasma free TFPI levels showed a strong direct correlation with the lag time of thrombin generation (r = 0.582; P < .001) and an inverse correlation with the peak height (r = -0.725, P < .001), in line with the notion that free TFPI is a major determinant of these parameters at low TF concentration.[39] The lag time was 3.91 plus or minus 1.73 minutes in healthy controls, 2.90 plus or minus 0.44 minutes in those with partial FV deficiency (P = .020) and 2.47 plus or minus 0.29 minutes in patients with severe FV deficiency (P = .001 *vs.* healthy controls). The peak height was 61.4 plus or minus 34.6 nM in healthy controls, 81.2 plus or minus 27.1 nM in those with partial FV deficiency (P = .043) and 156.5 plus or minus 20.7 nM in patients with severe FV deficiency (P < .001 *vs.* healthy controls). Conversely, the lag time and peak height of thrombin generation obtained in the presence of antibody were virtually independent of the free TFPI level, confirming complete neutralization of plasma TFPI by the added antibody.

In the population as a whole, FV levels were strongly correlated with the plasma levels of total TFPI (r = 0.372, P = .010), free TFPI (r = 0.739, P < .001, Figure 5) and TFPI activity (r = 0.666, P < .001). Even in the small group of healthy controls, and after correction for age

and sex, the FV level correlated with the levels of total TFPI (P = .057) and free TFPI (P = .008), as well as with the anti-TFPI ratio (P = .015).

Effect of TFPI levels on thrombin generation in FV-deficient plasma

Since low plasma levels of TFPI are associated with a hypercoagulable state, they might be beneficial in (severe) FV deficiency. To quantify this possible protective effect, thrombin generation in FV-deficient plasma reconstituted with increasing amounts of FV (0.5%-10% of normal plasma) was determined in the absence and presence of 3.84 ng/ml exogenous TFPI (Figure 6). At the lowest FV concentrations, considerably more thrombin was formed in the absence than in the presence of added TFPI, but this difference gradually decreased at higher FV concentrations and eventually disappeared at 10% FV. In particular, in plasma containing 0.5% FV there was appreciable thrombin generation in the absence of TFPI, but no thrombin generation at all in the presence of TFPI.

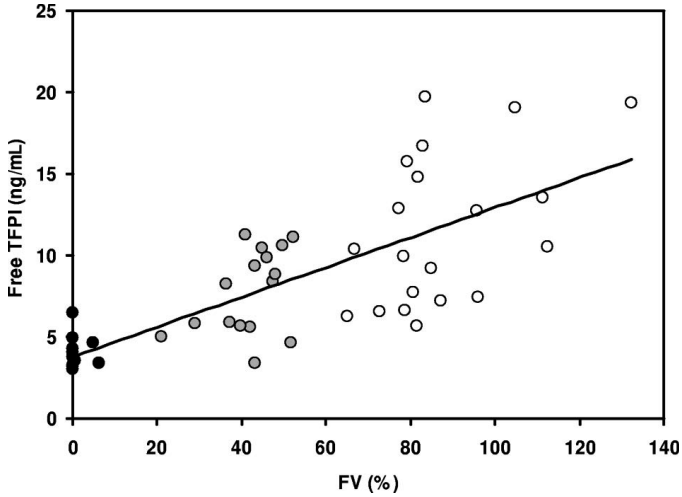

Figure 5. Correlation between FV and free TFPI levels in plasma. Plasma FV and free TFPI levels were determined with a prothrombinase-based assay and a commercial ELISA, respectively, in 11 patients with severe FV deficiency (closed circles), 16 individuals with partial FV deficiency (grey circles) and 20 normal controls (open circles).

Analysis of the TFPI gene in FV-deficient patients

To account for the observed low plasma levels of TFPI, the whole coding region and the proximal promoter of the TFPI gene were sequenced in 6 patients with severe FV deficiency (all unrelated). Apart from known polymorphisms, no mutations were found.

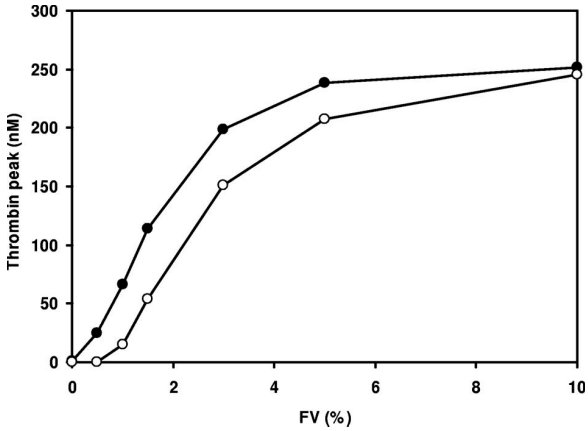

Figure 6. FV-titration of thrombin generation in FV-deficient plasma without and with added TFPI. Thrombin generation was triggered with 13.6 pM tissue factor in FV-deficient plasma from patient GK 502 reconstituted with increasing amounts of purified FV in the absence (closed circles) and presence (open circles) of 3.84 ng/ml added TFPI.

TFPI levels in factor-depleted plasmas

TFPI antigen levels were also measured in commercial plasmas depleted of FV or other factors (PT, FVII, FX, protein C and antithrombin, respectively). All FV-depleted plasmas (n=5) showed markedly reduced levels of both free (4.3±0.6 ng/ml) and full-length (26.3±12.5%) TFPI, whereas other depleted plasmas had normal TFPI levels (10.4±2.2 ng/ml free TFPI and 121.8±33.2 % full-length TFPI).

Interaction between FV and TFPI

To account for the low TFPI levels in FV-depleted plasma, we investigated the effect of FV immunodepletion on TFPI levels in normal plasma. For this purpose, we used both citrated plasma, in which free calcium is low, and hirudin/TAP/CTI-anticoagulated plasma, which retains normal levels of ionized calcium. Upon the addition of protein A sepharose–coupled anti-FV antibodies, FV disappeared with the same kinetics from both plasmas and became unmeasurable after approximately 20 minutes (Figure 7A). Interestingly, full-length TFPI also disappeared from plasma at a similar rate, suggesting that it binds to FV in plasma. Within 20 minutes from anti-FV addition, the full-length TFPI concentration had dropped to 43% and 20% of its initial value in citrated and hirudin/TAP/CTI-anticoagulated plasma, respectively, and remained constant afterward (Figure 7B). In the absence of anti-FV antibodies, no appreciable decrease of FV (Figure 7A) or TFPI (Figure 7B) levels was observed for 1 hour

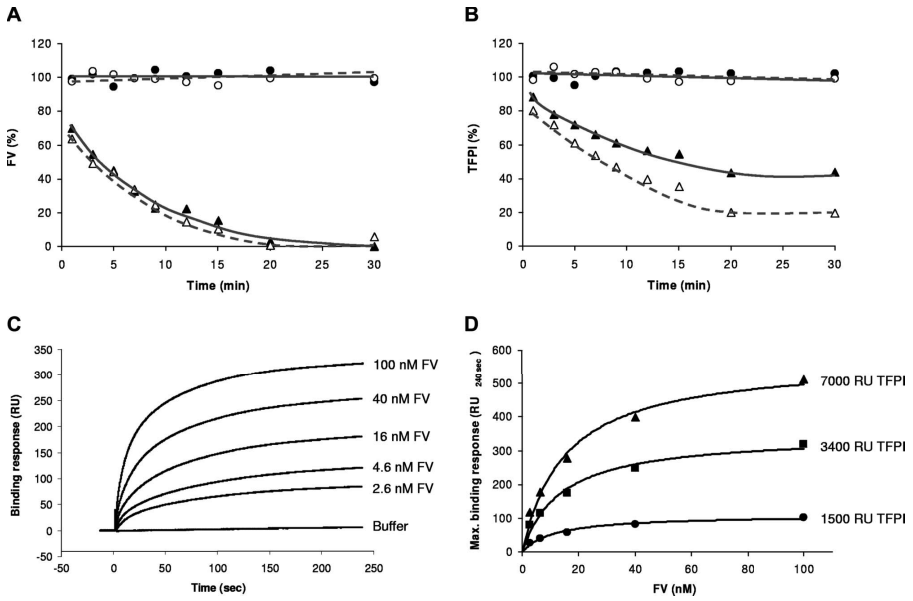

Figure 7. FV/TFPI interaction. Top: Plasma FV-immunodepletion. Pooled plasma from two healthy donors was incubated with protein A sepharose beads bearing an anti-FV monoclonal antibody (treated plasma, triangles) or no antibody (control plasma, circles). At regular intervals subsamples were taken and assayed for FV and full-length TFPI antigen, as described under Methods. FV levels (A) and TFPI levels (B) were expressed as percentage of the average control plasma level. The experiment was conducted at low (citrated plasma: closed symbols, solid lines) and normal (hirudin/tick anticoagulant protein/corn trypsin inhibitor-anticoagulated plasma: open symbols, dashed lines) levels of free calcium ions. Bottom: Measurement of FV/TFPI interaction by surface plasmon resonance. A CM5 chip with immobilised TFPI was perfused with increasing concentrations of FV as described under Methods. (C) Sensograms recorded at 3400 resonance units (RU) of immobilised TFPI with the indicated FV concentrations. (D) Dose-response curves showing the equilibrium binding response ($RU_{240\ sec}$) as a function of the FV concentration at 1500, 3400 and 7000 RU of immobilised TFPI.

Physical interaction between FV and TFPI was further verified by SPR measurements. FV bound to immobilized TFPI in a specific and concentration-dependent manner (Figure 7C). For all 3 TFPI-containing flow cells, the equilibrium binding response versus FV concentration plot could be fitted with a hyperbolic function (Figure 7D). The FV concentration at which half-maximal binding to TFPI was observed was 13.5 plus or minus 1.7 nM.

Plasma TFPI levels in patients with haemophilia A

To verify whether the reduction of TFPI levels is specific for FV deficiency, 15 patients with hemophilia A were also investigated and compared with the male healthy controls (Table 2).

Although both total and free TFPI antigen levels as well as TFPI activity levels determined via the thrombin generation–based assay were all lower in patients with hemophilia A than in healthy controls, only the difference in free TFPI levels reached statistical significance

Table 2. TFPI levels in patients with haemophilia A and normal controls.

	N	Age (years)	FVIII:C (%)	Total TFPI (ng/ml)	Free TFPI (ng/ml)	TFPI activity (anti-TFPI ratio)
Haemophiliacs	15	27.8±19.2	3.9 (range <1-23)	65.4±13.8	9.8±2.4*	3.51±1.40
Male controls	8	45.9±18.9	79.0±32.0	72.6±14.7	15.3±3.3*	5.22±2.92

(*P* = .001). Correction for the age difference between patients with haemophilia and controls did not alter this result.

*p=0.001

Discussion

Despite the pivotal role of FV in PT activation and the absence of alternative pathways to generate thrombin, many patients with FV levels lower than 1% experience only moderate bleeding and have a milder clinical course than patients with severe hemophilia.[18] Also in comparison with FV knockout mice, which die *in utero* or shortly after birth of massive hemorrhage,[22] most patients with severe FV deficiency show a relatively mild bleeding diathesis. This is generally attributed to the fact that FV gene mutations found in patients are compatible with some residual (though often undetectable) FV expression, which may be sufficient to prevent serious bleeding.[18,19] As a matter of fact, several *in vivo*, *in vitro* and *in silico* data,[20,24,41,42] as well as the thrombin generation experiments presented in Figures 1 and 2, support the concept that tiny amounts of FV are sufficient for minimal haemostasis.

In the present study, we have explored the additional possibility that patients with severe FV deficiency may be protected from life-threatening bleeding by a concomitant procoagulant defect. By performing a standard thrombophilic screening, low levels of protein C or high levels of PT or FVIII were found in 3 patients (Table 1). However, upon reconstitution with purified FV up to the normal plasma concentration, *all* FV-deficient plasmas generated considerably more thrombin than normal plasma, both at low TF without APC (Figure 3A) and at higher TF in the presence of APC (Figure 3C). This turned out to be due to markedly reduced TFPI levels in FV-deficient plasma. All measured TFPI-related parameters, including total and free antigen levels as well as activity levels determined with a thrombin generation–based functional assay, were significantly reduced in patients with severe FV deficiency

compared with controls, while those with partial FV deficiency showed intermediate levels (Figure 4). The value of 4.0 ng/mL for the mean free TFPI levels of patients with severe FV deficiency (corresponding to approximately 35% of the levels of healthy controls) is even likely to be an overestimate, because (1) in our hands this was approximately the lower limit of the commercial ELISA used for free TFPI quantification; (2) full-length TFPI levels as determined with a homemade ELISA were even lower; and (3) in the thrombin generation–based TFPI activity assay (which reflects free TFPI levels), addition of an anti-TFPI antibody hardly affected the peak height of thrombin generation in FV-deficient patients (mean anti-TFPI ratio, 1.18).

Since plasma levels of free TFPI are a major determinant of ETP-based and (to a lesser extent) aPTT-based APC resistance,[38,39] the low free TFPI levels may contribute to the APC resistance phenotype observed in FV-deficient plasma,[29,43] which had been previously entirely attributed to the missing/reduced APC-cofactor activity of FV.

Since plasma TFPI represents only a small fraction of all intravascular TFPI and is structurally and functionally heterogeneous,[12] its pathophysiological significance is uncertain. However, low plasma levels of (free) TFPI have been shown to increase the risk of venous thrombosis[13-16] and to functionally compensate for the low levels of the procoagulant factors in newborns.[44] Therefore, they might also enhance thrombin generation and mitigate bleeding symptoms in patients with severe FV deficiency. As a matter of fact, when FV-deficient plasma was reconstituted with increasing amounts of purified FV, considerably more thrombin was generated in the absence than in the presence of added TFPI (Figure 6), especially at the lowest FV concentrations (<2%). These results indicate that plasma TFPI deficiency lowers the FV level required for minimal thrombin generation *in vitro*. Whether this mechanism is also physiologically relevant remains to be established. In this respect, it should be emphasized that our thrombin generation experiments performed in platelet-poor plasma may not be entirely representative of the *in vivo* situation, where platelet FV also contributes to thrombin generation. Although platelets from patients with congenital severe FV deficiency contain hardly any FV,[20,45] preliminary thrombin generation experiments in platelet-rich plasma from FV-deficient patients suggest that platelets may play a pivotal role in maintaining the hemostatic balance in patients with severe FV deficiency.

The origin of the (partial) TFPI deficiency in plasma from FV-deficient patients is still under investigation. A genetic cause appears unlikely, as no mutation was identified in the coding region and proximal promoter of the *TFPI* gene in any of 6 FV-deficient patients. On the other hand, our experiments indicate that FV and TFPI can bind to each other and that a large fraction of full-length TFPI is actually complexed with FV in normal plasma (Figure 7). Although the physiologic significance of this complex is presently unknown, FV-bound TFPI might be protected from plasma proteases and/or from receptor-mediated clearance, and

therefore be more stable *in vivo*. This would account for the low levels of free/full-length TFPI in FV-deficient plasma and for the strong correlation between the levels of FV and TFPI observed in this (Figure 5) and other studies.[15,46] Moreover, the finding of a FV/TFPI complex in plasma provides a rationale for the low TFPI levels in FV-immunodepleted plasma.

Finally, since FVIII is structurally and functionally homologous to FV, we reasoned that patients with hemophilia A might also have low plasma levels of TFPI as compared with sex-matched healthy controls. This indeed appeared to be the case, at least for free TFPI, although the reduction of TFPI levels was less consistent and far less pronounced than in severe FV deficiency (Table 2). While the evidence that naturally occurring low TFPI levels could ameliorate the clinical course of patients with severe hemophilia is rather scanty,[47] newborns with hemophilia tend to be protected from bleeding by their low TFPI levels.[48] Moreover, TFPI inhibitors known as nonanticoagulant sulfated polysaccharides (NASPs) have been reported to effectively improve hemostasis in animal models of hemophilia A and B.[49,50]

In conclusion, we have shown that congenital FV deficiency is associated with reduced plasma levels of free/full-length TFPI. Partial TFPI deficiency might be a common feature of congenital FV deficiency and possibly help to counteract the severe bleeding tendency associated with this disorder. However, further research is required to fully appreciate the causes and physiologic significance of low plasma levels of TFPI in FV-deficient patients.

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4

Residual platelet factor V ensures thrombin generation in patients with severe congenital factor V deficiency and mild bleeding symptoms

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Summary

Coagulation factor V (FV), present in plasma and platelets, is indispensable to thrombin formation, yet patients with undetectable plasma FV seldom experience major bleeding. We used thrombin generation assays to explore the role of platelet FV in 4 patients with severe congenital FV deficiency (3 with plasma FV:C <1%). When triggered with tissue factor (TF) concentrations up to 50 pM, platelet-poor plasma (PPP) from the patients with undetectable plasma FV showed no thrombin generation, whereas platelet-rich plasma (PRP) formed thrombin already at 1 to 5 pM TF. Thrombin generation in PRP from the FV-deficient patients was enhanced to near-normal levels by platelet activators (collagen or Ca^{2+}-ionophore) and could be completely suppressed by specific FV inhibitors, suggesting FV-dependence. Accordingly, platelet FV antigen and activity were measurable in all FV-deficient patients and platelet FVa could be visualized by Western blotting. Normalization of the tissue factor pathway inhibitor (TFPI) level, which is physiologically low in FV-deficient plasma, almost completely abolished thrombin generation in PRP from the FV-deficient patients. In conclusion, patients with undetectable plasma FV may contain functional FV in their platelets. In combination with the low TFPI level, residual platelet FV allows sufficient thrombin generation to rescue these patients from fatal bleeding.

Introduction

Coagulation factor V (FV) is a 330-kDa glycoprotein which is present in plasma (80%) and platelets (20%).[1] Plasma FV (20-25nM) is synthesized in the liver and is released in the bloodstream as a single-chain inactive procofactor. After limited proteolysis by thrombin or activated factor X (FXa), FV is converted to its activated form (FVa), consisting of a heavy chain (105 kDa) and a light chain (71/74 kDa) that are noncovalently linked via a single Ca^{2+}-ion.[2] FVa acts as a nonenzymatic cofactor of FXa in the conversion of prothrombin to thrombin and increases the rate of prothrombin activation more than 1000-fold.[3,4] FVa activity is down-regulated by activated protein C (APC), which inactivates FVa by cleaving the heavy chain at Arg306, Arg506, and Arg679.[5,6]

Although megakaryocytes are capable of FV synthesis,[7,8] platelet FV originates from the plasma pool.[9-11] Bone marrow megakaryocytes internalize plasma FV via a specific receptor-mediated process[12] and store it in secretory α-granules.[13] After endocytosis, FV gains several unique modifications that make platelet FV structurally and functionally different from plasma FV. In particular, platelet FV is stored in a partially activated from that, after exposure on the platelet surface, is further activated by FXa or thrombin and is resistant to APC-catalysed inactivation.[14-16]

Congenital FV deficiency (Owren parahaemophilia),[17,18] caused by loss-of-function mutations in the *F5* gene,[19,20] is a rare ($1:10^6$ [1 person per million]) bleeding disorder inherited as an autosomal recessive trait. Homozygous and compound heterozygous individuals have FV levels lower than 10% and present with a life-long haemorrhagic diathesis whose severity is poorly correlated with the plasma FV level.[21] Although a few cases of neonatal intracranial haemorrhage have been reported,[22-24] many patients with undetectable FV experience only mild to moderate bleeding[21] and do not require routine prophylaxis. The reason for the relatively mild clinical presentation of many FV-deficient patients is presently unknown.

Given the uniform lethality of FV-null mice,[25] it has been argued that FV-deficient persons who survive to postnatal life always have some residual FV expression.[19,25,26] Since the FV requirement for minimal thrombin generation is well below 1%,[26-29] traces of FV would already be sufficient to guarantee thrombin formation and to rescue FV-deficient persons from fatal bleeding. However, *in vitro* experiments have failed to detect any thrombin generation in plasma from patients with undetectable FV,[29,30] although FV-deficient patients have low plasma levels of the anticoagulant protein tissue factor pathway inhibitor (TFPI),[29] which considerably reduces the FV requirement for thrombin generation.

Some 30 years ago, Miletich *et al*.[31] showed that, in patients with severe FV deficiency, the FXa-binding capacity of platelets (which is a measure of platelet FV) is a better predictor of bleeding tendency than the plasma FV level. Despite this important observation, platelet FV is not routinely evaluated in FV-deficient patients and only 3 studies report platelet FV levels in patients with severe FV deficiency.[32-34] No platelet FV antigen or activity could be demonstrated in 2 patients with undetectable plasma FV.[32,33] In another FV-deficient patient platelet FV could be visualised by Western blotting, but its activity was not determined.[34] To get more insight into the role of platelet FV in FV deficiency, we have measured thrombin generation and platelet FV levels in 4 patients with severe congenital FV deficiency.

Methods

Patients

Four unrelated female patients with severe congenital FV deficiency were enrolled. All 4 were referred to Padua Academic Hospital from district hospitals in northeastern Italy and their demographic and clinical characteristics have been previously described.[29] Briefly, patient PD I (age 64 years, FV clotting activity [FV:C] < 1%) was diagnosed at the age of 5 years because of recurrent epistaxis and gum bleeding. Immediately after the menarche at the age of 8 years, she presented with abundant menses. During the postpartum period of her only pregnancy, she developed 3 severe hemorrhages, which were treated by transfusion with fresh-frozen plasma, platelets, and red blood cell concentrates. However, she never experienced major spontaneous bleeding. Patient PD II (age 44 years, FV:C < 1%) suffered from recurrent epistaxis in childhood, which led to the diagnosis of severe FV deficiency at the age of 8 years. At the age of 15 years, she presented with excessive bleeding after a tooth extraction. She has always had abundant menses and in her thirties she was admitted to hospital twice for severe metrorrhagia. On both occasions, administration of fresh-frozen plasma was effective in controlling the hemorrhage. Her parents are first-degree cousins. Patient PD III (age 35 years, FV:C < 1%) experienced only very mild episodes of epistaxis and gum bleeding and was diagnosed at the age of 8 years. Her occasional abundant menses have worsened lately because of the presence of uterine fibromatosis. To reduce menstrual bleeding, she was prescribed oral contraceptives, but the treatment had to be stopped after a few cycles because of her intolerance to the drug. Fresh-frozen plasma and/or antifibrinolytic agents were given during all risk situations and bleeding complications never ensued. Patient PD VII (age 62 years, FV:C 6%) has experienced only mild menorrhagia. She was diagnosed at the age of 23 years, when she presented with a postpartum hemorrhage requiring transfu-

sion with fresh-frozen plasma. She also experienced one uncomplicated spontaneous abortion. None of the patients received substitutive therapy in the 3 months preceding blood sampling. All patients were homozygous or compound heterozygous for missense mutations in the *F5* gene (Table 1).[35-37]

The study was approved by the Ethics Committee of Padua Academic Hospital and conducted in accordance with the Declaration of Helsinki. All patients gave informed consent to participate. Eight volunteers with normal FV levels recruited among hospital employees served as healthy controls.

	***F5* gene mutations**	
	Nucleotide change	**Amino acid change**
PD I	1744G>C (homozygous)	524 Asp/His
PD II	1744G>C (homozygous)	524 Asp/His
PD III	853T>C; 4957G>C	227 Trp/Arg; 1595 Tyr/Asp
PD VII	6509G>A (homozygous)	2112 Gly/Asp

Table 1. *F5* gene mutations in FV-deficient patients

Blood collection and plasma preparation

Blood was collected from each FV-deficient patient on 2 separate occasions, with an interval of approximately 6 months. On each occasion, 40 mL of blood was drawn in 129mM sodium citrate (1:10 vol/vol) for thrombin generation experiments, and 20 mL of blood was drawn in 80mM sodium citrate, 52mM citric acid, 183mM glucose (1:7 vol/vol) for platelet isolation (see “Platelet isolation and preparation”). For each patient, blood from 2 healthy controls was also collected on the same day and handled in the same way.

Citrated blood was centrifuged at 250g for 15 minutes to obtain platelet-rich plasma (PRP). Part of the PRP was further centrifuged at 14000g for 5 minutes to yield platelet-poor plasma (PPP).

The FV-deficient plasma used in the experiment shown in Figure 1 was purchased from George King Biochemical Inc.

Thrombin generation assays

Thrombin generation in PPP and PRP was determined using the Calibrated Automated Thrombogram method.[38] Coagulation was triggered by recalcification (16mM added $CaCl_2$) in the presence of recombinant tissue factor (Innovin; Dade-Behring). The tissue factor (TF)

concentration in the Innovin stock solution (prepared according to the manufacturer's instructions) was measured with the Imubind TF-ELISA (American Diagnostica) and found to be 331 ng/mL (7.36nM). For the measurement in PPP, synthetic phospholipids composed of phosphatidylcholine, phosphatidylserine, and sphingomyelin (Phospholipid-TGT; Rossix) were added to a final concentration of 20 M. PRP was adjusted to $1.5*10^8$ platelets/mL. In some experiments, PRP was preincubated with collagen (10 g/mL; Dynamyte Medical) or Ca^{2+}-ionophore A23187 (20 μM; Sigma-Aldrich) for 10 minutes at 37°C to activate the platelets before triggering coagulation. To prevent thrombin formation via the intrinsic pathway, all thrombin generation experiments were performed in the presence of 32 μg/mL of corn trypsin inhibitor (Haematologic Technologies Inc). Thrombin generation curves were determined in duplicate and were calibrated against the fluorescence signal obtained in the same plasma with 100nM thrombin calibrator (Thrombinoscope BV). Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems) and thrombin generation curves were analyzed for lag time, peak height, and endogenous thrombin potential (ETP) using the Thrombinoscope software (Thrombinoscope BV).

In some thrombin generation experiments, an anti-FV polyclonal antibody (SAFV-IG; Affinity Biologicals Inc), plasma-purified human APC (Innovative Research), in-house plasma-purified FV, or recombinant human full-length TFPI (kind gift of Prof W. Buurman, Maastricht University) was added to the plasma.

Platelet isolation and preparation

Washed platelet suspensions were prepared as described previously[11] and divided in 2 aliquots: one, with a concentration of $0.7*10^9$ platelets/mL, was frozen for the preparation of platelet lysates; the other was diluted to $0.5*10^9$ platelets/mL in platelet buffer (PB, 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, pH 7.5, containing 2 mg/mL bovine serum albumin (BSA) and 2 mg/mL glucose) and used for the preparation of activated platelet suspensions. Platelet lysates were used for the measurement of FV and TFPI antigen levels, whereas activated platelet suspensions represented the starting material for platelet FVa activity determination and platelet FVa immunoprecipitation.

For the preparation of platelet lysates, 0.5% (vol/vol) Triton X100 (Fluka) was added to the frozen platelet suspension in the presence of the following protease inhibitors: 5 mM EDTA (Merck), 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Fluka), 5 mM N-ethylmaleimide (NEM, Merck), 10 μg/mL leupeptin (Sigma-Aldrich), 10 mM benzamidine (Sigma-Aldrich) and 25 μg/mL soybean trypsin inhibitor (Sigma-Aldrich). After 2-hour lysis on ice, platelet lysates were analyzed immediately for FV and TFPI antigen levels.

For the preparation of activated platelet suspensions, Arg-Gly-Asp-Ser-peptide (H-1155, BACHEM AG) was added to the washed platelet suspensions at a final concentration of 0.3 mg/mL to inhibit platelet aggregation, and platelets ($0.5*10^9$/mL in PB pH 7.5) were activated with 34 nM thrombin and 20 μM Ca^{2+}-ionophore (A23187) in the presence of 5 mM $CaCl_2$ for 30 minutes at 37 °C. Added thrombin was subsequently neutralized with hirudin (Kordia Life Sciences, Leiden, The Netherlands). Activated platelet suspensions were frozen at -80 °C for later determination of FV activity.

Measurement of FV antigen levels in plasma and platelets

Plasma and platelet FV antigen levels were quantified with an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated overnight at 4 °C with 2.2 μg/well of polyclonal anti-FV antibody (The Binding Site Ltd) in carbonate buffer. After rinsing 5 times with washing buffer (0.05 M tris(hydroxymethyl)-aminomethane/0.15 M NaCl/5 mM ethylenediaminetetraacetic acid, pH 7.5, 0.1% Tween 20), 100 μL 1:200-diluted plasma or platelet lysate ($2.5*10^7$ or $5*10^7$ platelets/mL in washing buffer) was added to the wells and incubated at room temperature (RT) for 2 hours. After washing, bound FV was detected by incubation with an HRP-conjugated polyclonal anti-FV antibody (The Binding Site Ltd, 0.5 μg/well) for 1.5 hours, and plates were developed with tetramethylbenzidine/ peroxide.

Measurement of FV activity levels in plasma and platelets

FV activity levels of plasma and washed platelets were determined with prothrombinase-based assays. Plasma FV was determined as previously described.[29] For platelet FV determination, activated platelet suspensions were thawed at 37 °C and diluted with Hepes-buffered saline (HBS, 25 mM Hepes, 175 mM NaCl, pH 7.7) containing 0.5 mg/mL ovalbumin and 2.7 mM $CaCl_2$ to obtain a final concentration of $12*10^6$ platelets/mL in the assay. Platelet FVa was quantified with 5 nM FXa, 1 μM prothrombin, 40 μM di-oleoyl phosphatidylserine/di-oleoyl phosphatidylcholine (10/90 mol/mol) phospholipid vesicles and 2.5 mM $CaCl_2$. Pooled normal plasma and an activated platelet pool prepared from platelets from 20 healthy individuals were used as a reference for plasma and platelet FV measurements, respectively.

Immunoprecipitation of FVa from plasma and platelets

Activated platelet suspensions (individual patient samples and a pool of activated platelets from 20 controls) were solubilized by the addition of 0.5% (final concentration) Triton X100 in the presence of protease inhibitors (2 mM AEBSF, 5 mM NEM, 10 μg/mL leupeptin, 10 mM benzamidine, 25 μg/mL soybean trypsin inhibitor) and 5 mM $CaCl_2$ for 30 minutes at RT.

FV-deficient or normal pooled plasma was defibrinated with 1 U/mL ancrod (National Institute for Biological Standards and Control) for 10 minutes at 37°C. Plasma FV was activated with 34nM thrombin and 16mM added $CaCl_2$ in the presence of a polyclonal sheep anti–protein C antibody (0.16 mg/mL; Innovative Research) for 10 minutes at 37°C, after which the same cocktail of protease inhibitors as used for the platelet preparation was added.

Platelet lysates and plasma were precleared of endogenous immunoglobulin G with protein A–Sepharose beads (rProtein A Sepharose Fast Flow; GE Healthcare) that were subsequently removed by centrifugation. Precleared platelet lysate (200 μL of $1.5*10^8$ platelets/mL) or precleared plasma was added to protein A-Sepharose beads (15 μL drained volume) bearing monoclonal anti-FV heavy chain antibodies (3B1, a kind gift from Prof B. N. Bouma, Utrecht University Hospital, Utrecht, The Netherlands) and FVa was immunoprecipitated for 30 minutes at RT under rotation. The beads were collected by centrifugation, washed 3 times with HBS containing 5mM $CaCl_2$ and 10mM benzamidine, and resuspended in HBS containing 1:2 volume sample buffer (40mM tris(hydroxymethyl)aminomethane, pH 6.7, 3.33% sodium dodecyl sulfate (SDS), 6.25% mercaptoethanol, 50% glycerol, 0.01% bromophenol blue). Plasma or platelet samples from FV-deficient patients were resuspended in 33.3 μL (to achieve a 6-fold concentration of the FVa), while control samples were resuspended in 200 μL (no concentration). After 5 minute incubation at 96 °C, samples were subjected to SDS-polyacrylamide gel electrophoresis on 6% polyacrylamide gels according to Laemmli under reducing conditions. Proteins were subsequently transferred to polyvinylidene fluoride membranes by semidry blotting. Membranes were blocked with 5% (wt/vol) skim milk (Merck) in HBS/0.5% Tween 20 for 1 hour at RT and the primary antibody (monoclonal anti-FV heavy chain AHV-5146 [Haematologic Technologies Inc], 5 μg/mL in blocking buffer) was incubated overnight at 4 °C under shaking. Membranes were washed with HBS/0.3% Tween 20 and incubated with a secondary antibody (HRP-conjugated rabbit anti-mouse immunoglobulins (DAKO), 1:2000 in blocking buffer) for 1 hour at RT. HRP activity was detected using chemiluminescence (Supersignal West Pico Chemiluminescent Substrate, Pierce) and the LAS-3000 image analyzer (Fujifilm).

TFPI ELISA

TFPI levels were determined by a full-length TFPI ELISA in plasma as well as in platelet lysates containing 10^8 platelets/mL. The ELISA was calibrated against normal pooled plasma or a pool of lysed platelets from 20 healthy persons.

Statistical analysis

Data are expressed as mean plus or minus SD. Correlations are expressed as Pearson coefficients (r). Statistical analysis was performed using SPSS 15.0 (SPSS).

Results

Contribution of plasma and platelet FV to thrombin generation

To explore the relative contributions of plasma and platelet FV to thrombin generation, FV-deficient plasma with undetectable FV was supplemented with normal platelets and with increasing concentrations (0%-100%) of purified plasma FV. After preactivating the platelets with collagen, coagulation was triggered with 5pM of TF. Thrombin generation was already optimal in the absence of plasma FV and did not increase further when the concentration of plasma FV was increased up to 100% (Figure 1). This experiment shows that *in vitro* thrombin generation in the presence of normal platelets does not require plasma FV, in line with the *in vivo* observation that platelet FV could maintain hemostasis in a patient with an acquired antibody that fully neutralized plasma FV.[39]

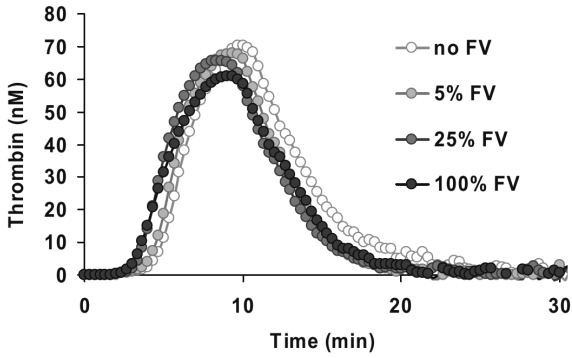

Figure 1. Contribution of plasma and platelet FV to thrombin generation. FV-deficient plasma was reconstituted with $1.5*10^8$ normal platelets/mL and increasing amounts of purified plasma FV (0%, 5%, 25%, and 100% = 23 nM). Platelets were activated with collagen and thrombin generation was triggered with 5 pM tissue factor.

Thrombin generation in PPP and PRP from FV-deficient patients

To investigate the role of platelet FV in congenital FV deficiency, 4 patients with severe congenital FV deficiency were enrolled. All were homozygous or compound heterozygous for missense mutations in the *F5* gene (Table 1).[35-37] Plasma FV clotting activity (FV:C) was undetectable in all patients, except PD VII (FV:C 6%).

Thrombin generation was determined in PPP and PRP from these patients after triggering coagulation with 1, 5, 10, or 50pM of TF. Thrombin generation curves obtained in plasma from the 4 patients and 1 representative control are shown in Figure 2. Thrombin generation parameters obtained at 5pM of TF are presented in Table 2.

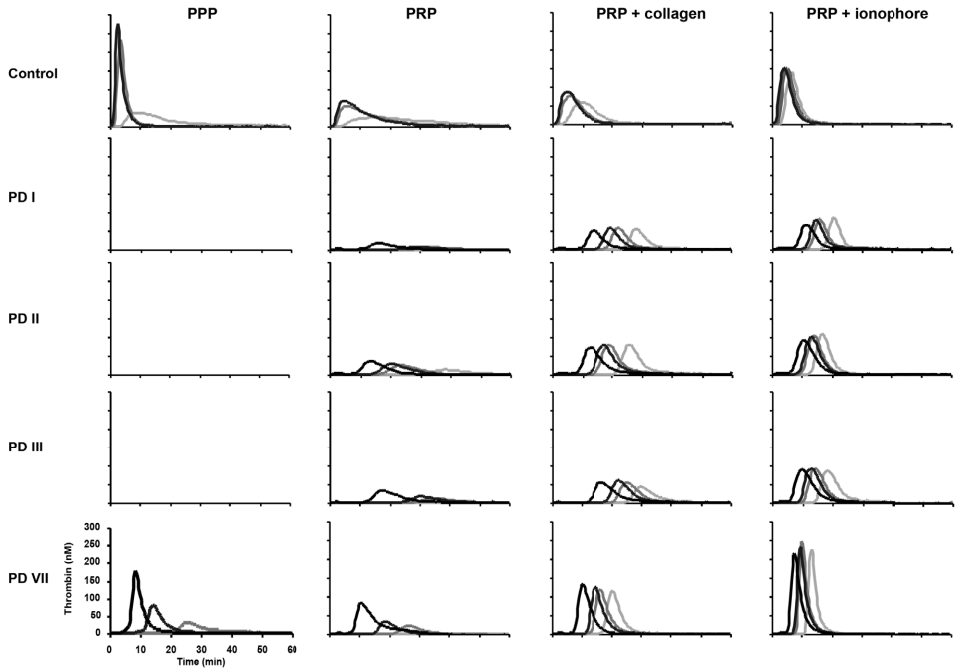

Figure 2. Thrombin generation in platelet-poor plasma (PPP) and platelet-rich plasma (PRP) from patients with severe congenital FV deficiency. PPP and PRP from patients PD I, PD II, PD III and PD VII were triggered with 1 (light gray), 5 (middle gray), 10 (dark gray), or 50 (black) pM tissue factor, and thrombin generation was determined as described under Methods. Thrombin generation in PRP was measured without the addition of any platelet agonist and after pre-activation of platelets with collagen (10 μg/mL) or Ca^{2+}-ionophore (20 μM). A representative control is shown for comparison.

In control PPP and PRP, thrombin generation was already appreciable at 1pM of TF and increased at increasing TF concentrations. The lag time of thrombin generation decreased at increasing TF concentrations and at 50pM of TF it became too short to be measurable. At

Table 2. Thrombin generation parameters in PRP triggered with 5 pM of TF.

	No platelet agonist			Collagen, 10 μg/mL			Ionophore, 20μM		
	Lag time min	Peak height nM	ETP nM · min	Lag time min	Peak height nM	ETP nM · min	Lag time min	Peak height nM	ETP nM · min
PD I	27.2	6	85	19.2	60	384	13.0	87	469
PD II	17.7	26	290	15.3	82	624	10.7	107	632
PD III	28.3	12	152	21.7	56	438	11.0	94	610
PD VII	21.5	31	283	13.7	127	661	8.3	271	808
Controls, n = 8	3.0 ± 0.5	48 ± 49	671 ± 155	3.0 ± 0.6	86 ± 55	681 ± 69	2.3 ± 0.2	191 ± 44	725 ± 94

Thrombin generation parameters were obtained from the thrombin generation curves shown in Figure 2. ETP indicates endogenous thrombin potential.

Table 3. Plasma and platelet FV antigen and activity levels in FV-deficient patients.

	Plasma FV		Platelet FV	
	Antigen, %	Activity, %*	Antigen, %	Activity, %*
PD I	1.8	< 0.5	4.7	1.7
PD II	5.7	< 0.5	1.7	3.7
PD III	4.2	< 0.5	0.4	6.4
PD VII	9.6	4.4	4.6	11.9
Controls, n = 8	98.7 ± 20.9	106.5 ± 26.8	93.9 ± 32.5	93.6 ± 38.6

* FVa activity was determined with a prothrombinase-based assay (see “Measurement of FV activity levels in plasma and platelets”).

each TF concentration, thrombin generation was lower in PRP than in PPP, most probably because activated platelets provide a less favorable phospholipid surface for coagulation reactions than the synthetic phospholipid vesicles added to PPP.

No thrombin generation was observed in PPP from patients PD I, PD II, and PD III at any of the TF concentrations tested. PPP from patient PD VII showed thrombin generation at TF concentrations of 5pM or higher, although this was lower and started later in time than in control plasma. In contrast to PPP, PRP from all 4 FV-deficient patients showed thrombin generation, suggesting the presence of some residual FV in the patients' platelets. Thrombin generation was already measurable at 1pM of TF in PRP from patients PD II and PD VII and at 5pM of TF in PRP from patients PD I and PD III.

FV dependence of thrombin generation in PRP from the FV-deficient patients

To investigate whether thrombin generation observed in PRP from the FV-deficient patients was FV dependent, thrombin generation was determined in the patients' PRP in the presence of specific inhibitors of FV(a) (*ie*, an anti-FV antibody or APC). When coagulation was triggered with 50 pM of TF in the presence of Ca^{2+}-ionophore, the addition of an anti-FV antibody completely inhibited thrombin generation in FV-deficient PRP (Figure 3A), whereas it had no effect on thrombin generation in control PRP (data not shown).

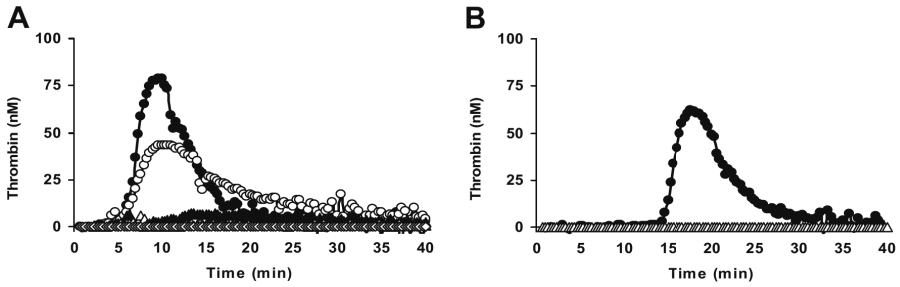

Figure 3. FV-dependence of thrombin generation in platelet-rich plasma (PRP) from a FV-deficient patient. (A) Effect of an anti-FV antibody on thrombin generation in FV-deficient PRP. PRP from patient PD III was triggered with 50 pM tissue factor (TF) in the presence of Ca^{2+}-ionophore and thrombin generation was measured in the absence of an anti-FV antibody (●) and in the presence of 8 μg/mL (○), 28 μg/mL (◆), 60 μg/mL (r) and 10^8 μg/mL (◇) anti-FV antibody. (B) Effect of APC on thrombin generation in FV-deficient PRP. PRP from patient PD III was triggered with 5 pM TF in the presence of calcium ionophore and thrombin generation was measured in the absence of activated protein C (APC) (●) and in the presence of 50 nM APC (○).

Since APC concentrations up to 200nM had no effect on thrombin generation in PRP triggered with 50pM of TF in the presence of Ca^{2+}-ionophore, the APC sensitivity of thrombin generation in PRP was tested at 5pM of TF in the presence of Ca2 -ionophore. Under these conditions, thrombin generation in FV-deficient PRP was completely inhibited by 50nM APC (Figure 3B). Thrombin generation in control PRP could also be fully inhibited by APC, but a considerably higher APC concentration (200nM) was required (data not shown).

FV antigen and activity levels in plasma and platelets

The fact that thrombin generation was observed in PRP, but not in PPP, from the FV-deficient patients suggested that their platelets contained FV. To verify this, FV antigen and activity levels were measured in washed platelets from the FV-deficient patients and 8 healthy controls, and compared with plasma FV levels (Table 3).

Although FV antigen was detectable in plasma from all 4 FV-deficient patients, plasma FV activity was below detection limit in all FV-deficient patients except PD VII (4.4%). In platelets, not only FV antigen but also FV activity was measurable in all 4 FV-deficient patients, confirming the presence of functional platelet FV even in the patients with undetectable plasma FV activity. Although platelet FV antigen and activity levels did not show a correlation in the FV-deficient patients, they were well correlated in the healthy controls ($r = 0.94$, $P = .002$). Moreover, despite the very small sample size, platelet FV activity levels in FV-deficient patients correlated ($r = 0.91$, $p = .09$) with the peak height of thrombin generation determined in the respective PRPs in the presence of Ca^{2+}-ionophore, a condition where all platelet FV(a) is exposed.

Immunoprecipitation of plasma and platelet FVa

To visualize any residual FV present in plasma and/or platelets from the FV-deficient patients, FV was concentrated from plasma and platelets by immunoprecipitation and detected by Western blotting (Figure 4). Since plasma FV is structurally different from platelet FV, whereas the corresponding activated forms are identical,[40] activated plasma and platelets (containing FVa) were used as a starting material for the immunoprecipitation. As shown in Figure 4A, no FVa was detectable in PPP from the FV-deficient patients, except for PD VII. In contrast, all patients showed FVa on the Western blot of activated platelet lysates (Figure 4B). Since an anti–heavy chain antibody was used for the detection of FVa, only the heavy chain is visible on the Western blot. This appears as a single band of 105 kDa in plasma FVa, and as multiple bands in platelet FVa. The additional bands of 75/80 kDa observed in platelet FVa represent degradation products of the FVa heavy chain that are probably generated during platelet activation and/or immunoprecipitation.[41,42]

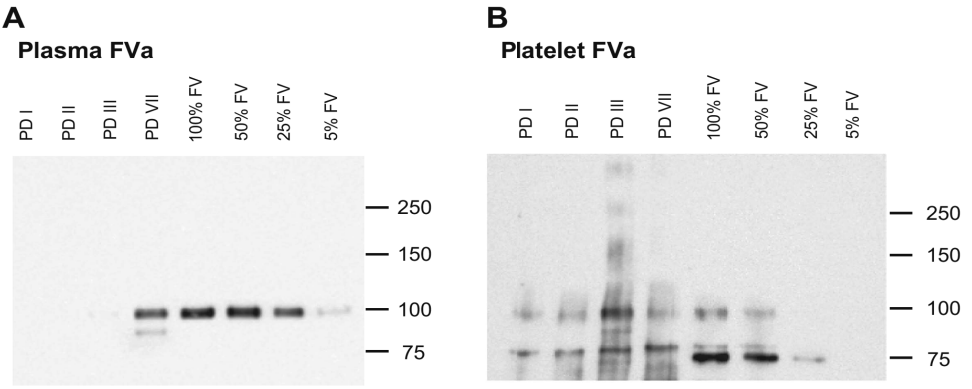

Figure 4. Immunoprecipitation of FVa from plasma and platelets. FVa was immunoprecipitated from 200 μL plasma (A) or 200 μL $1.5*10^8$ activated platelets/mL (B), concentrated 6 times (FV-deficient patients PD I, PD II, PD III, PD VII) or not concentrated (control), and subjected to SDS-PAGE and Western blotting. Control plasma and platelet FVa preparations were run at different dilutions (100%, 50%, 25% or 5%). FVa was detected with a monoclonal anti-FV heavy chain antibody and chemiluminescence. The FVa heavy chain has a molecular weight of 105 kDa, smaller fragments (only visible in platelet FVa) are degradation products of the FVa heavy chain.

Platelet TFPI level

We have recently shown that full-length TFPI is markedly reduced in FV-deficient plasma,[29] a finding that was confirmed in this study (Table 4). Since platelets also contain full-length TFPI,[43,44] we measured platelet TFPI antigen in the FV-deficient patients (Table 4). Platelet TFPI levels showed a large interindividual variation both in controls and in FV-deficient patients. On average, platelet TFPI levels were slightly but not significantly lower in FV-deficient patients (58.3±19.9%) than in healthy controls (77.4±31.7%).

Table 4. Plasma and platelets TFPI antigen levels.

	Full-length TFPI level	
	Plasma, %	**Platelet, %**
PD I	36.9	81.6
PD II	55.8	58.7
PD III	22.0	32.9
PD VII	51.0	60.1
Controls, n = 8	96.7 ± 22.0	77.4 ± 31.7

Effect of TFPI on thrombin generation in PRP

To verify whether the low plasma levels of full-length TFPI also improve thrombin generation in FV-deficient PRP (as they do in PPP),[29] increasing concentrations of TFPI (0.8 to 14.8 ng/mL) were added to PRP from patient PD II and to control PRP, and thrombin generation was initiated with 50pM of TF in the presence of collagen. In control plasma, addition of TFPI increased the lag time of thrombin generation from 4.2 minutes to 8.0 minutes, but did not affect peak height (Figure 5). In contrast, in FV-deficient PRP, increasing the TFPI concentration not only prolonged the lag time (from 14.3 minutes to 29.5 minutes), but it also profoundly reduced the amount of thrombin formed (Figure 5). The addition of 7.8 ng/mL of TFPI, a concentration that normalizes the plasma TFPI level in FV-deficient PRP, almost completely abolished thrombin generation in FV-deficient PRP, which confirms the beneficial effect of low plasma levels of TFPI for thrombin generation in FV-deficient patients.

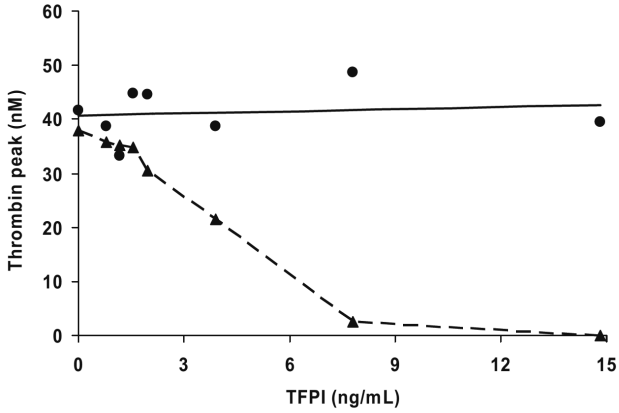

Figure 5. TFPI-titration of thrombin generation in platelet-rich plasma (PRP). Control PRP (●) and FV-deficient PRP (patient PD II, ▲) were supplemented with increasing amounts of recombinant full-length TFPI and coagulation was initiated with 50 pM tissue factor in the presence of collagen (10 μg/mL). The peak height of thrombin generation is plotted as a function of the concentration of added TFPI.

Discussion

Despite the essential role of FV in prothrombin activation, many persons with severe congenital FV deficiency experience only mild to moderate bleeding.[21] The present study provides an explanation for this paradox by showing that patients with undetectable plasma FV may

contain enough functional FV in their platelets to guarantee thrombin generation and protect them against major bleeding.

Four patients with severe congenital FV deficiency and relatively mild bleeding symptoms were investigated. Although no thrombin generation was observed in PPP from the 3 patients with undetectable plasma FV even when plasma was triggered with 50pM of TF, the corresponding PRP showed appreciable thrombin generation already at 5pM of TF. Thrombin generation in PRP from the FV-deficient patients was already observed in the absence of platelet agonists and was greatly stimulated by preactivating the platelets with collagen or Ca^{2+}-ionophore. The progressive increase in the amount of thrombin formed going from no platelet agonist to collagen and Ca^{2+}-ionophore probably reflects the extent of FV secretion and the parallel exposure of procoagulant phospholipids (phosphatidylserine) on the outer platelet membrane surface.[45,46] Remarkably, the total amount of thrombin formed in PRP from FV-deficient patients stimulated with collagen or Ca^{2+}-ionophore was similar to that of controls, although the lag time of thrombin generation was considerably prolonged. Moreover, when PRP from the FV-deficient patients was activated with collagen or Ca^{2+}-ionophore, the amount of thrombin formed was independent of the TF concentration, suggesting that a fixed prothrombinase activity was generated at all TF concentrations. A possible explanation for this observation is that the amount of platelet FV(a) that is released is the limiting factor for prothrombinase activity in FV-deficient PRP, although more factor X (FX) is activated at increasing TF concentrations.

To prove that thrombin generation in PRP from the FV-deficient patients was dependent on platelet FV and not caused by an unknown platelet protein capable of stimulating prothrombin activation, we showed that thrombin generation in PRP from FV-deficient patients could be completely abolished by 2 specific inhibitors of FV, an anti-FV antibody and APC. Thrombin generation in control plasma was much less sensitive to the FV antibody and to APC, which is likely caused by the excessive amount of FVa in combination with the reduced sensitivity of platelet FVa for APC.[14-16]

The presence of FV in platelets from the FV-deficient patients was confirmed in several ways. First, platelet preparations from all 4 patients showed FV activity in a FVa activity assay, that is, they were able to stimulate FXa-catalyzed prothrombin activation in a model system. Second, platelet FVa from the FV-deficient patients could be visualized on Western blot. In addition to the 105-kDa band, corresponding to the full-length heavy chain, platelet FVa showed several smaller fragments on the Western blot. These fragments represent degradation products of the FVa heavy chain that are probably generated during platelet activation[47] and/or FVa immunoprecipitation, as many platelet proteases, including calpain[41] and lysosomal enzymes,[42] can cleave FV(a). Finally, FV antigen was also detectable in the patients' platelets by an ELISA. Although FV antigen was also measurable in the patients' plasma, this

is most likely nonfunctional as the patients' PPP showed no FV activity in the prothrombinase-based and thrombin generation assays (except PD VII). The rather poor correlation between the amount of platelet FV antigen measured by ELISA and that visible on Western blot (eg, for PD III) might be due to interference of the *F5* mutations with the recognition of FV by the different antibodies used in the ELISA, immunoprecipitation, and Western blot.

The presence of functional FV in platelets from patients with congenital FV deficiency can be explained only if the underlying *F5* gene mutations are compatible with some residual FV expression. Remarkably, all patients in the study carried *F5* missense mutations as the underlying cause of FV deficiency. Although all amino acid substitutions involved charge changes at highly conserved residues, it should be noted that missense mutations do not per se prevent protein synthesis, but rather impair the folding, secretion, and/or stability of the mutant protein.[19] Moreover, being point mutations, their effects can be abolished by rare somatic reversion events or by ribosome slippage during mRNA translation.[26] Therefore, they can hardly be considered truly "null" mutations. Although it has been argued that *all* patients with severe FV deficiency actually retain some minimal FV expression,[19,25,26] as suggested by the uniform lethality of FV-knock out mice[25] and by the absence of gross deletions from the *F5* mutational spectrum,[19] further studies are needed to verify the presence of functional platelet FV in patients with *F5* molecular defects other than missense mutations. As a matter of fact, no functional FV could be demonstrated in plasma or platelets from 2 young patients with severe FV deficiency caused by short out-of-frame deletions/ insertions[32,33] and from 2 other patients with unknown *F5* mutations.[31] Patients with undetectable platelet FV tend to have more severe clinical manifestations[31,33] than the patients in our study and their PRP would be expected to generate less or no thrombin.

The mechanism underlying the preferential localization of FV in platelets rather than in plasma from patients with severe FV deficiency remains unclear. One possibility is that megakaryocytes take up all FV available in plasma, simultaneously depleting the plasma FV pool. This would imply a high-affinity receptor for FV endocytosis. Alternatively, minimal FV synthesis might occur in megakaryocytes as well as in the liver, but plasma FV might be more rapidly cleared than the FV stored in platelets. This hypothesis is supported by the observation that platelet concentrates confer FV-deficient patients a longer-lasting protection from bleeding than fresh-frozen plasma, suggesting that platelet FV has a longer half-life than plasma FV.[48]

Since the FV requirement for minimal hemostasis is less than 1%, the small amount of FV found in platelets may well be sufficient to explain the relatively mild clinical phenotype of our patients. In fact, the specific characteristics of platelet FV (rapid activation by FXa and resistance to APC-catalyzed inactivation)[14,40] and its targeted release at the site of injury, resulting in high local concentrations,[39] make platelet FV a very efficient procoagulant, able to

maintain hemostasis even in the absence of plasma FV. This concept is illustrated by the *in vitro* observation that, in the presence of normal activated platelets (and thus of platelet FVa), plasma FV does not contribute to thrombin generation (Figure 1). Moreover, in a patient with a circulating FV inhibitor and undetectable plasma FV, platelet FV proved sufficient to ensure adequate hemostasis even during surgery.[39] The pivotal role of platelet FV is further supported by the bleeding diathesis associated with FV New York,[49] a selective deficiency of platelet FV with normal levels of plasma FV.

As we have recently reported,[29] FV deficiency is associated with markedly reduced plasma levels of full-length TFPI, a condition that reduces the FV requirement for minimal thrombin generation. In the present study, we show that normalization of the plasma TFPI level almost completely abolishes thrombin generation in PRP from FV-deficient patients. This effect of TFPI is likely due to inhibition of the initiation phase of coagulation, since physiologic concentrations of TFPI do not inhibit prothrombin activation.[50] The low TFPI level contributes to enhance thrombin generation in patients with severe FV deficiency and may explain the near-normal thrombin generation observed in collagen- and Ca^{2+}-ionophore–stimulated PRP from these patients (Figure 2, Table 2).

In conclusion, this study demonstrates that patients with congenital FV deficiency and undetectable plasma FV may contain functional FV in their platelets. In combination with the low TFPI level, residual platelet FV supports enough thrombin generation to rescue patients with undetectable plasma FV from fatal hemorrhage. These findings further suggest that differences in residual platelet FV level, possibly determined by the nature of the underlying molecular defect, may be responsible for the variation in clinical phenotype observed among patients with equally undetectable plasma FV levels. Therefore, platelet FV should be routinely evaluated for a more accurate estimate of bleeding tendency in these patients.

Acknowledgments

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5

Homozygous *F5* deep-intronic splicing mutation resulting in severe factor V deficiency and undetectable thrombin generation in platelet-rich plasma

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Summary

Background: Coagulation factor V (FV) deficiency is associated with a bleeding tendency of variable severity, but phenotype determinants are largely unknown. Recently, we have shown that three patients with undetectable plasma FV and mild bleeding symptoms had sufficient residual platelet FV to support thrombin generation in platelet-rich plasma (PRP). Therefore, we hypothesized that FV-deficient patients with severe bleeding manifestations may lack platelet FV. ***Objectives:*** To characterise a FV-deficient patient with a severe bleeding diathesis. ***Patients/Methods:*** We performed FV mutation screening and functional studies in a 31-year-old male (FV:C <1%) with umbilical bleeding at birth, recurrent haemarthrosis and muscle haematomas, and a recent intracranial haemorrhage. ***Results:*** The proband was homozygous for a deep-intronic mutation (*F5* IVS8 +268A→G) causing the inclusion of a pseudo-exon with an in-frame stop codon in the mature *F5* mRNA. Although platelet FV antigen was detectable by immunoprecipitation followed by Western blotting, no FV activity could be demonstrated in the proband's plasma or platelets with a prothrombinase-based assay. Moreover, no thrombin generation was observed in PRP triggered with 1-50 pM TF (even in the presence of platelet agonists), whereas an acquired FV inhibitor was excluded. Clot formation in the proband's whole blood, as assessed by thromboelastometry, was markedly delayed but not abolished. ***Conclusions:*** This is the first report of a pathogenic deep-intronic mutation in the *F5* gene. Our findings indicate that the minimal FV requirement for viability is extremely low and suggest that thrombin generation in PRP may predict bleeding tendency in patients with undetectable plasma FV.

Introduction

Coagulation factor V (FV)[1] is an essential clotting factor whose active form (FVa) is required for the efficient conversion of prothrombin into thrombin. FV is synthesised in the liver and is subsequently secreted in the blood stream, where it circulates as an inactive single-chain precursor with an A1-A2-B-A3-C1-C2 domain structure. When coagulation is initiated, FV is activated through limited proteolysis by thrombin or factor Xa (FXa).[2] In this process the B domain is released, yielding a heavy chain (A1-A2, 105 kDa) and a light chain (A3-C1-C2, 71/74 kDa) linked *via* a Ca^{2+}-ion. FVa acts as a non-enzymatic cofactor of FXa and accelerates FXa-catalysed prothrombin activation more than 1000-fold.[3] FVa activity is down-regulated by activated protein C (APC)-mediated proteolysis of the heavy chain at Arg^{306}, Arg^{506} and Arg^{679}.[4,5]

Approximately 20% of the FV present in blood is stored in platelet α-granules, from which it is released upon platelet activation.[6] Platelet FV is not synthesised endogenously, but originates from endocytosis and intracellular processing of plasma FV by bone marrow megakaryocytes.[7-11] Platelet FV is stored in a partially activated form and is more resistant to APC-mediated inactivation than plasma FV(a).[9,12]

Congenital FV deficiency (Owren parahaemophilia, MIM #227400) is an autosomal recessive bleeding disorder with an estimated prevalence of $1:10^6$.[13,14] The clinical presentation of severe (homozygous) FV deficiency ranges from occasional mucosal bleeding to life-threatening haemorrhages,[15-17] without a clear-cut correlation with residual plasma FV levels. To date, >100 detrimental FV gene (*F5*) mutations (including missense, nonsense and splicing mutations as well as small insertions/deletions) have been described in FV-deficient patients.[18]

While the complete absence of FV is generally considered incompatible with life (based on the uniform embryonal/perinatal lethality of FV knock-out mice[19]), several lines of evidence indicate that the minimal FV requirement for viability is well below 1%.[20-22] In previous studies, we have identified residual platelet FV in combination with low plasma levels of the anticoagulant protein tissue factor pathway inhibitor (TFPI) as a possible rescue mechanism in patients with undetectable plasma FV.[23,24] In particular, we showed that three patients with undetectable plasma FV and relatively mild bleeding symptoms had sufficient residual FV in their platelets to support thrombin generation in platelet-rich plasma (PRP), which may protect them from major bleeding.[24] Therefore, FV-deficient patients with severe bleeding manifestations might have less or no platelet FV. In the present study we report on a FV-deficient patient with a severe bleeding tendency and undetectable thrombin generation in PRP.

Materials and methods

Blood collection and plasma preparation

Venous blood was obtained from the proband, his parents and four healthy controls on two different occasions with an interval of 8 months. From each donor, 40 mL blood were drawn in 129 mM sodium citrate (1/10 vol/vol) for thrombin generation and thromboelastometry experiments, and 20 mL blood were drawn in 80 mM sodium citrate, 52 mM citric acid and 183 mM glucose (ACD, 1/7 vol/vol) for platelet isolation (see "Platelet preparation") and platelet RNA extraction (see "Genetic analysis"). PRP and platelet-poor plasma (PPP) were prepared from citrated plasma as described before[24] and immediately tested in the thrombin generation assay. Buffy coats were stored at -20 °C for later DNA isolation. The study was conducted according to the Helsinki protocol and all subjects provided informed consent to participation.

Thrombin generation

Thrombin generation was measured using the Calibrated Automated Thrombogram method,[25] essentially as described.[24] Briefly, coagulation in PPP (with 20 μM added phospholipids, TGT-lipids, Rossix, Mölndal, Sweden) and PRP (platelet count standardised to $1.5*10^8$ platelets/mL) was triggered by recalcification (16 mM added $CaCl_2$) and 1-50 pM tissue factor (TF; Innovin, Dade-Behring, Marburg, Germany). In some experiments with PRP, platelets were pre-activated with 10 μg/mL collagen (Dynamyte Medical, München, Germany) or 20 μM Ca^{2+}-ionophore (A23187, Sigma, Buchs, Switzerland). All measurements were performed in the presence of 32 μg/mL corn trypsin inhibitor (Haematologic Technologies, Essex Junction VT, USA) to prevent contact activation.

To exclude the presence of inhibitory anti-FV antibodies in the proband's plasma, normal pooled plasma was mixed with the proband's plasma or with a commercial congenitally FV-deficient plasma (George King Biomedical, Overland Park KS, USA) to final concentrations of 0%, 0.5%, 1%, 2.5%, 5% and 10%. Plasma mixtures were incubated at room temperature for 2 hours and FV activity was subsequently assayed by measuring thrombin generation at 13.6 pM TF.

Platelet preparation

Platelets were isolated and washed as described previously.[8] Washed platelets were divided in two aliquots: one ($0.7*10^9$ platelets/mL) was frozen as such, whereas the other ($0.5*10^9$ platelets/mL) was activated with thrombin and Ca^{2+}-ionophore as in ref.[24] and then frozen.

Platelet lysates, prepared by thawing non-activated platelets in the presence of Triton X100 (Fluka, Buchs, Switzerland) and protease inhibitors,[24] were used for the determination of platelet FV antigen levels (ELISA). Activated platelet suspensions were the starting material for platelet FVa immunoprecipitation and activity measurement.

Measurement of FV antigen levels

Plasma and platelet FV antigen levels were measured using an ELISA assay, as described before.[24]

Measurement of FV activity levels

Plasma and platelet FV activity levels were determined with a prothrombinase-based assay, as described previously.[24] Assay conditions were as follows: 5 nM FXa, 1 μM prothrombin, 40 μM phospholipid vesicles (DOPS/DOPC, 10/90 mol/mol), 2.5 mM $CaCl_2$, and limiting amounts of FVa.

To investigate to what extent the platelet-dependent stimulation of prothrombin activation was attributable to platelet FV(a) rather than to other platelet components, the assay was performed in the absence and presence of specific FV inhibitors (anti-FV antibody, APC/protein S). However, since these inhibitors were unable to completely block platelet FVa activity in control platelets, EDTA was used as an alternative means to abolish FVa activity. Chelation of Ca^{2+} ions by EDTA results in the dissociation of the heavy and light chains of FVa, causing loss of cofactor activity. At low FVa concentrations this effect is largely irreversible, as only 10% of the original FV activity was recovered when the free Ca^{2+} concentration was restored. In detail, 11.4 mM EDTA was added to the activated platelet suspension (containing 4.3 mM $CaCl_2$) and incubated for 10 minutes at room temperature. Subsequently, the original Ca^{2+} concentration was restored, and platelet FVa activity was assayed immediately. The difference in prothrombinase activity before and after EDTA treatment was taken as a measure of FVa activity.

FVa immunoprecipitation

The FV heavy chain was immunoprecipitated from plasma or activated platelet suspensions with a monoclonal anti-FV heavy chain antibody (3B1, a kind gift of Prof. B.N. Bouma) coupled to protein G Sepharose beads (Protein G Sepharose 4 Fast Flow, GE Healthcare, Uppsala, Sweden). After immunoprecipitation, the beads were recovered and boiled in SDS-containing gel sample buffer under reducing conditions to release the FV heavy chain. Samples were then subjected to gel electrophoresis and Western blotting. Bands were visualised

using a monoclonal anti-FV heavy chain antibody (AHV-5146, Haematologic Technologies) and chemiluminescence. During this procedure, samples from the proband (but not his parents' samples) were concentrated 6 times to increase the chances of detecting any residual FV(a).

Measurement of TFPI levels

Plasma free TFPI levels were determined using a commercial ELISA kit (Asserachrom, Diagnostica Stago, Asnières sur Seine, France).

Thromboelastometry

Rotation thromboelastometry in whole blood was carried out on a ROTEM® Analyzer (Tem International GmbH, Munich, Germany) according to the manufacturer's instructions. Coagulation was initiated with the extrinsic (EXTEM) or intrinsic (INTEM) trigger provided by the manufacturer. The following parameters were derived from the thromboelastogram: clotting time (CT), clot formation time (CFT), alpha-angle (α), maximum clot firmness (MCF), maximal velocity (MAXV) and area under the curve (AUC).

Genetic analysis

DNA mutation screening. Genomic DNA from the patient and his parents was isolated from peripheral blood leukocytes using the Wizard® Genomic DNA Purification kit (Promega, Madison WI, USA). All 25 exons, the proximal promoter (~1000 bp) and the 3'-UTR of the *F5* gene were amplified by polymerase chain reaction (PCR) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Bedford MA, USA), essentially as described.[8]

Multiplex ligation-dependent probe amplification (MLPA) analysis. To check for possible large deletions (or duplications) within the *F5* gene, MLPA analysis was performed. This technique[26] makes it possible to "count" the copies of specific target sequences in a person's genomic DNA by comparison with control sequences present in two copies in the same genomic DNA. In the absence of a commercial *F5* MLPA kit, two sets of 11 *F5*-specific probes with lengths between 88 and 148 nt were designed and ordered from Integrated DNA Technologies (Leuven, Belgium). The first set (probemix A) included probes for exons 1, 4, 7, 10, 13 (proximal portion), 16, 19, 22 and 24, as well as intron 3 and the 3'-UTR of the *F5* gene. The second set (probemix B) included probes for the promoter region as well as intron 2 and exons 6, 9, 11, 13 (distal portion), 14, 15, 17, 18 and 21 of the *F5* gene. Each *F5*-specific probemix was combined with the control probemix from the SALSA MLPA kit P200-A1 Hu-

man DNA Reference 1 (MRC Holland, Amsterdam, The Netherlands), which contains 14 probes (172-250 nt in length) recognising control genes on various chromosomes. Genomic DNA from the proband, his parents and four normal controls was standardised to 20 ng/μL and MLPA reactions were carried out according to the manufacturer's instructions. MLPA products were separated on an ABI 3730 DNA Analyzer (Applied Biosystems) and results were analysed using the Peak Scanner software.

cDNA analysis. Although FV is synthesised in the liver, *F5* cDNA analysis was conducted on RNA extracted from platelets, which are readily accessible and known to contain (ectopic) *F5* mRNA. Total platelet RNA from the patient, his parents and two normal controls was isolated using TRIzol® Reagent (Invitrogen, Breda, The Netherlands). Briefly, 20 mL ACD-anticoagulated blood were centrifuged at 100×*g* for 10 minutes. The resulting PRP was collected and centrifuged again at 1000×*g* for 10 minutes to precipitate the platelets. After discarding the supernatant, the platelet pellet was resuspended in 2 mL TRIzol® Reagent. Further steps were according to the manufacturer's instructions. The washed RNA pellet was eventually resuspended in RNase-free water and quantified spectrophotometrically. Total RNA was subsequently reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Ten overlapping fragments spanning the whole *F5* mRNA were amplified from the obtained cDNA and analysed by agarose gel electrophoresis and direct sequencing. Primers and conditions are available on request. Gel bands were evaluated by densitometric analysis using the UN-SCAN-IT gel Version 6.1 software (Silk Scientific, Orem UT, USA).

Bioinformatic analysis

Splice site consensus values for the sequences flanking the intron 8 pseudo-exon were evaluated with three different online bioinformatic tools: NNSPLICE version 0.9 from the Berkeley Drosophila Genome Project†, SpliceView from the Italian National Research Council# and Human Splicing Finder‡.[27] The latter programme was also used to identify and score potential branch-point sequences.

† http://www.fruitfly.org/seq_tools/splice.html

http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html

‡ http://www.umd.be/HSF/

Results

Case history

The proband is a 31-year-old Caucasian male from North-Eastern Italy. The diagnosis of severe FV deficiency (FV:C <1%) was made at birth, when he presented with numerous haematomas of the limbs and prolonged bleeding after resection of the umbilical cord. At the age of 1 month, he developed a hand haematoma that required administration of fresh frozen plasma. Throughout childhood he experienced recurrent epistaxis and ecchymoses of the limbs, which were treated with anti-fibrinolytic agents (tranexamic acid). From the age of approximately 10 years, he has been suffering from haemarthrosis (2-3 episodes/year, especially at the knees, shoulders and right elbow) and muscle haematomas following even minor traumas. The patient has undergone two minor surgical operations (excision of a cyst from the oral cavity and removal of a birthmark from the shoulder) which were successfully managed with prophylactic administration of fresh frozen plasma. However, he is not on routine prophylaxis; he is only treated on demand. No plasma or platelets were administered to him in the two months preceding each blood sampling for this study.

Recently, the patient developed a syncope associated with haemorrhagic shock (haemoglobin level of 4.5 g/dL; normal range, 14.6-17.7 g/dL) and he was admitted to the Intensive Care Unit, where a diagnosis of spontaneous right pneumothorax with haemothorax was made. Following administration of large amounts of plasma and red blood cells and the nsertion of a drainage tube into the right pleural cavity, he progressively recovered from the pneumothorax. Plasma was administered for several days, without bleeding recurrences, and the patient was eventually discharged one month after the admission in good clinical conditions. However, 15 days later he reported to the Emergency Room because of a sudden headache and blurred vision in the left eye. A brain CT-scan revealed a subdural frontal intracranial haemorrhage, which was treated conservatively with the administration of plasma on admission and during the following days. Partial re-absorption of the intracranial haemorrhage was observed at day 10, and almost complete resolution at day 30. The patient was discharged and has no relapses up to now.

The proband's parents are reportedly unrelated. Both have reduced FV levels (FV:C 42% in the mother and 79% in the father), but no history of bleeding.

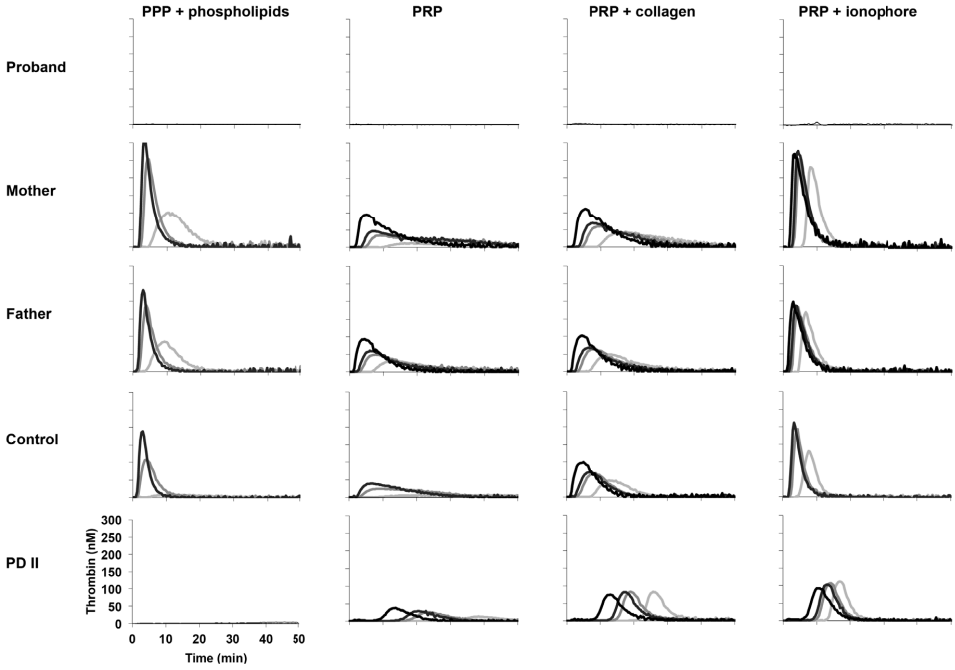

Figure 1. Thrombin generation in platelet-poor plasma (PPP) and platelet-rich plasma (PRP). Thrombin generation was measured in PPP and PRP from the proband, his parents, four normal controls and patient PD II[24] after activation of coagulation with 1 (light grey), 5 (middle grey), 10 (dark grey), or 50 (black) pM tissue factor. Thrombin generation in PRP was determined in the absence of platelet agonists and after pre-activation of platelets with collagen (10 μg/mL) or Ca^{2+}-ionophore (20 μM). Only one representative normal control is shown. In some panels, the thrombin generation curve evoked by 50 pM TF is not visible, because the lag time was too short to be measurable.

Thrombin generation

Thrombin generation was measured in PPP and PRP from the proband, his parents, one of the previously investigated FV-deficient patients (PD II)24 and a normal control after triggering coagulation with 1, 5, 10 or 50 pM TF (Figure 1). Thrombin generation in control PPP and PRP was already measurable at 1 pM TF and peak height increased (and lag time decreased) at higher TF concentrations. Moreover, platelet pre-activation with collagen or Ca^{2+}-ionophore greatly enhanced thrombin generation in PRP. Thrombin generation in the proband's parents was similar or even higher (especially in the mother) than in control plasma. In contrast, no thrombin generation could be detected in the proband's PPP or PRP at any of the TF concentrations used, not even after pre-activation of platelets with collagen or Ca^{2+}-ionophore. In this respect, the proband of the present study is different from patient PD II (Figure 1) and the other two previously investigated FV-deficient patients with undetectable

plasma FV (PD I and PD III), who all showed substantial thrombin generation in PRP.[24] The presence of an acquired antibody against FV in the proband's plasma was excluded using a thrombin generation-based Bethesda assay (data not shown).

Genetic analysis

To identify the genetic defect(s) responsible for FV deficiency, the coding sequence (including splicing junctions), the proximal promoter and the 3'-UTR of the *F5* gene were sequenced in the proband and his parents, but no mutation was found. Remarkably, however, the proband turned out to be homozygous for all polymorphisms covered by the sequencing (n=38), except one (327A/G in exon 2). This suggested that he might be hemizygous for a large portion of the *F5* gene. To check for the presence of large deletions within the gene, MLPA analysis was performed using 22 *F5*-specific probes, but no abnormalities were noticed in the MLPA profiles (data not shown), strongly arguing against the presence of large *F5* deletions in the family.

Alternatively, the proband might have inherited two identical alleles bearing the same mutation. Since the coding region had already been screened without success, we reasoned that the causative mutation might be located in an intron and possibly affect splicing. To test this hypothesis, total platelet RNA from the proband, his parents and a normal control was isolated and reverse-transcribed to cDNA. When *F5* cDNA fragments were amplified and analysed by agarose gel electrophoresis, a difference was noticed in the amplicon spanning exons 8-11 (Figure 2A). While the normal control showed the expected 470-bp PCR product, the proband showed a higher-molecular-weight product, suggesting the retention of >100 nt of intronic sequence in his mature *F5* mRNA. Although hardly any normally spliced *F5* cDNA was visible in the proband's lane, densitometric analysis of the gel disclosed the presence of a very faint band co-migrating with the control 470-bp band (Figure 2B, arrow). The proband's parents showed both the normal and the abnormal band.

To understand why part of intron 8 was retained in the patient's mature mRNA, three splice site prediction tools were employed to analyse the insert and the surrounding intronic sequence for the presence of splicing regulatory elements (Figure 3). All three programmes identified a rather strong acceptor splice site consensus sequence (score between 0.83 and 0.90) at the 5' end of the insert. Moreover, according to the Human Splicing Finder tool, which can also score branchpoint sequences, a sequence (TGCTCAT, branch-point adenine underlined) with a branch-point consensus value of 0.90 was present 67-60 nt upstream of the 5' end of the insert. In contrast, no donor splice site consensus sequence (two

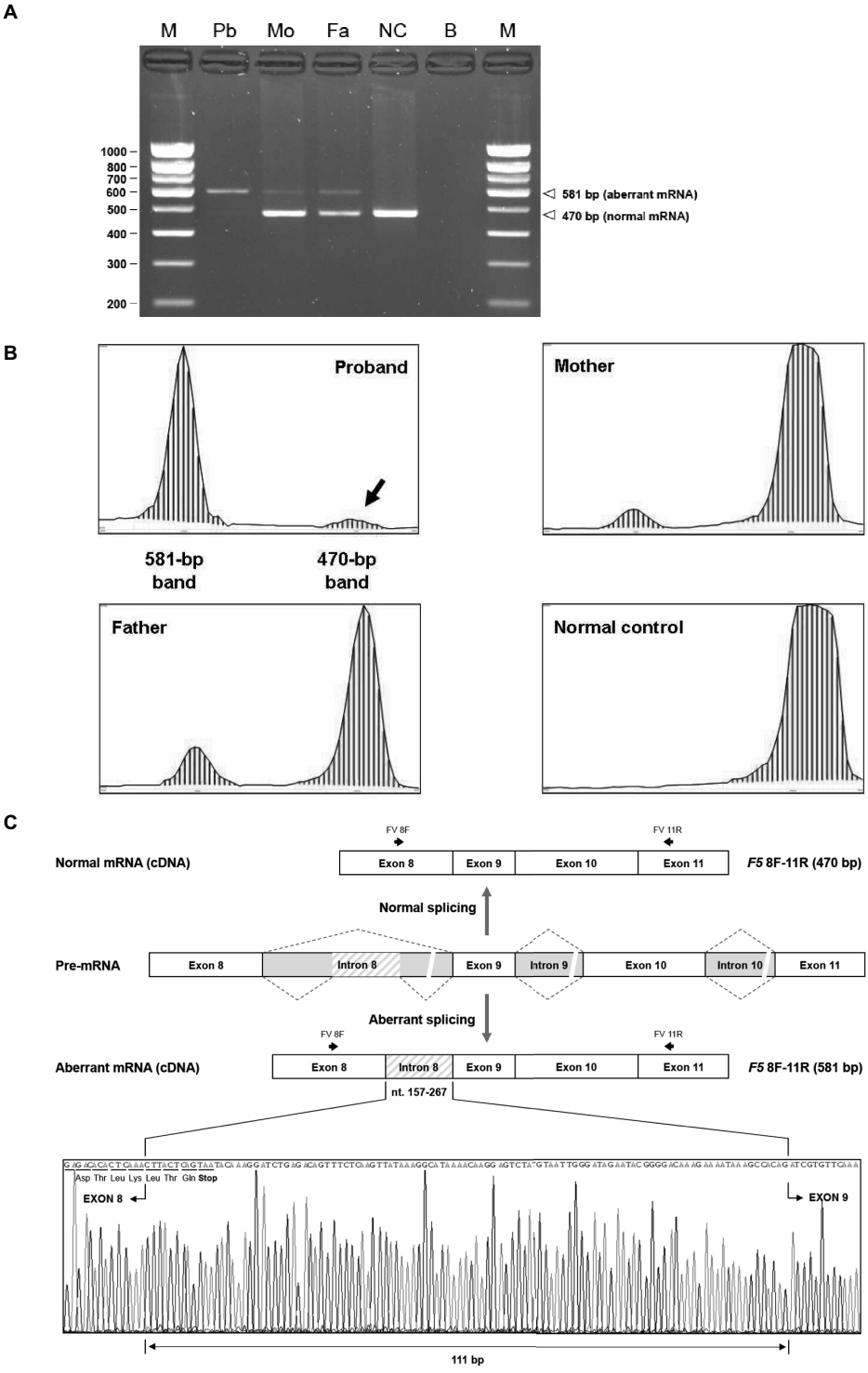
A
M
Pb
Mo
Fa
NC
B
M
1000
800
700
600
500
400
300
200
581 bp (aberrant mRNA)
470 bp (normal mRNA)
B
Proband
Mother
581-bp band
470-bp band
Father
Normal control
C
FV 8F
FV 11R
Normal mRNA (cDNA)
Exon 8
Exon 9
Exon 10
Exon 11
F5 8F-11R (470 bp)
Normal splicing
Pre-mRNA
Intron 8
Intron 9
Intron 10
Aberrant splicing
Aberrant mRNA (cDNA)
F5 8F-11R (581 bp)
nt. 157-267
Asp Thr Leu Lys Leu Thr Gln Stop
EXON 8
EXON 9
111 bp

Figure 2. *F5* cDNA analysis. A) Detection of the splicing abnormality. A *F5* cDNA fragment spanning exons 8-11 was amplified in the proband (Pb), his parents (Mo, Fa) and a normal control (NC), and run on a 2% agarose gel. B, no cDNA control (blank). M, molecular weight marker. The 470-bp product represents the expected normal fragment, the 581-bp product visible in the proband and his parents is aberrant. B) Densitometric scans of the proband's, mother's, father's and normal control's lanes of the gel shown in Figure 2A. The small peak in the proband's densitometric profile (arrow) indicates the presence of a very faint band co-migrating with the control's 470-bp band, which corresponds to the correctly splices *F5* cDNA. C) Characterisation of the aberrant mRNA (cDNA). The *F5* pre-mRNA is spliced differently in the normal control (top) and in the proband (bottom). The sequencing chromatogram of the *F5* 8F-11R fragment amplified from the proband's cDNA (primers are indicated by thick arrows) shows the insertion, between exons 8 and 9, of 111 bp derived from intron 8 (hatched). The insert contains an in-frame stop codon predicting premature termination of translation. Interestingly, the abnormal band was less intense than the normal band, suggesting that the aberrant mRNA is subject to partial degradation *in vivo*. Sequencing of the abnormal amplification product revealed that the proband's *F5* cDNA contained a large (111 bp) insert between exons 8 and 9, whose sequence was identical to nucleotides 157-267 of intron 8 (Figure 2C). Although the inserted sequence did not alter the mRNA reading frame, it contained an in-frame stop codon predicting premature termination of translation at codon 436 (within the A2 domain).

programmes) or only a weak one (score of 0.73, one programme) was identified at the 3' end of the insert, which may explain why this intronic sequence is not normally included in the mature *F5* mRNA. Following amplification (from genomic DNA) and sequencing of the relevant portion of intron 8, the proband was found to be homozygous for an A→G transition at nucleotide +268 of intron 8, whereas both parents were heterozygous (Figure 3). Interestingly, the IVS8 +268A→G mutation affects the first nucleotide following the insert in the genomic sequence and creates a perfect donor splice site consensus sequence (score between 0.92 and 1.00) at the 3' end of the insert, thereby causing exonization of this intronic region. These features make this portion of intron 8 a typical pseudo-exon[28] activated by the IVS8 +268A→G mutation.

Polymorphism analysis indicated that the proband's mother, who was heterozygous for the IVS8 +268A→G mutation, carried the R2 haplotype[29] on the "normal" *F5* allele.

FV levels in plasma and platelets

To verify whether the *F5* IVS8 +268 A→G mutation is compatible with the expression of any (functional) FV, plasma and platelet FV antigen and activity levels were measured in the proband and his parents. Normal pooled plasma and a pool of platelets from 20 healthy individuals were used as references. FV-deficient patient PD II from our previous study[24] was also included as an additional control.

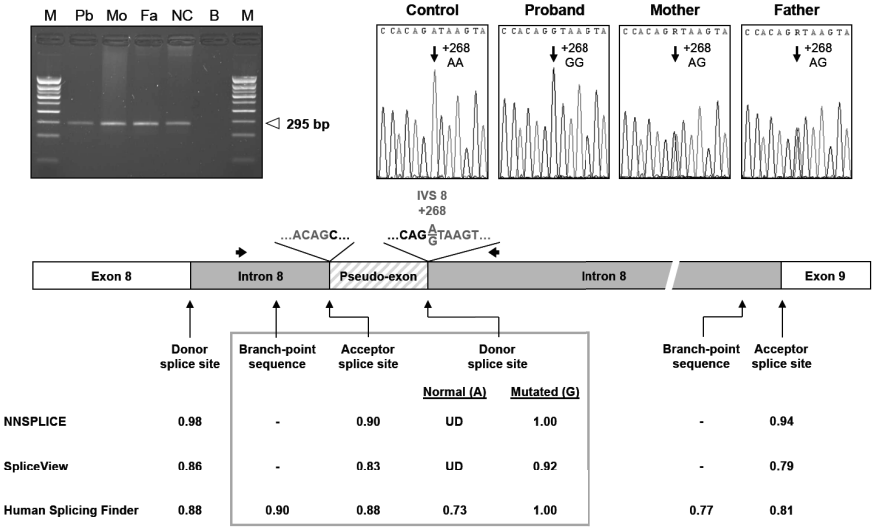

Figure 3. Identification and bioinformatic analysis of the *F5* IVS8 +268A→G mutation. Top left: Amplification from genomic DNA of a 295-bp fragment spanning the pseudo-exon in *F5* intron 8 (primers are indicated by thick arrows, gel lanes are labeled as in Figure 2A). Top right: sequencing chromatograms showing the IVS8 +268A→G substitution in the proband (homozygous) and his parents (both heterozygous) *vs*. a normal control. Bottom: Splice site and branch-point sequence consensus values for the intronic sequences flanking the pseudo-exon in intron 8. Splice site consensus values were determined with three different splice site prediction tools, whereas branch-point sequence consensus values could only be scored with Human Splicing Finder. The consensus values of the canonical splice sites at either end of intron 8 and of the canonical branch-point sequence at the 3' end of intron 8 are also shown for comparison. Consensus values range from 0 to 1.00. UD, undetectable.

Table 1. FV antigen and activity levels in plasma and platelets

	Plasma FV antigen (%)	Plasma FV activity (%)	Platelet FV antigen (%)	Platelet FV activity (%)
Proband	UD	<0.5	6.6	UD
Mother	43.0	37.2	33.8	26.5
Father	67.9	65.1	43.0	31.5
PDII	6.3	<0.5	1.7	3.3

UD, undetectable.

FV antigen, as determined by ELISA, was detectable both in plasma and platelets from patient PD II, but only in platelets from the proband. The proband's parents had plasma and platelet FV antigen levels compatible with their heterozygous FV deficiency (Table 1).

FV activity levels were measured using a prothrombinase-based assay. No FV activity was detectable in the proband's plasma or platelets, whereas the platelets of patient PD II expressed ~3.3% FV activity. The proband's parents had reduced FV activity levels in both plasma and platelets (Table 1).

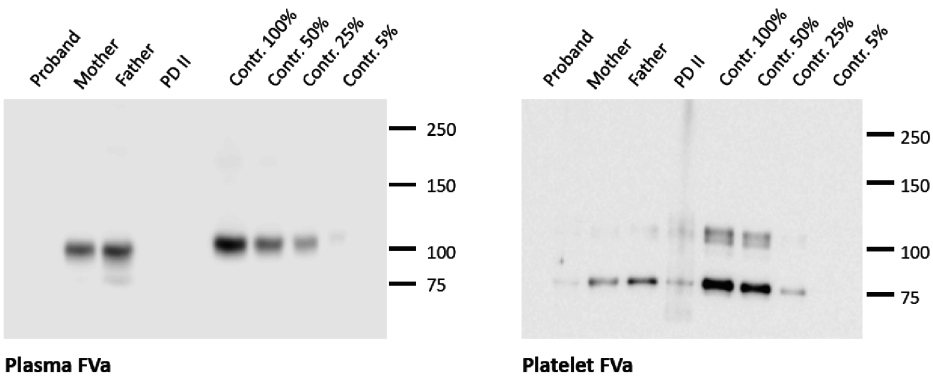

Figure 4. Western blot analysis of FV heavy chain after immunoprecipitation. The heavy chain of FV was immunoprecipitated from plasma (left) and activated platelet suspensions (right), concentrated 6 times (proband's and patient PD II's samples) or not concentrated (parents' samples), and subjected to gel electrophoresis and Western blotting. The control sample was run at different dilutions (100%, 50%, 25%, 5%). The FV heavy chain was detected using a monoclonal anti-FV heavy chain antibody and chemiluminescence. The molecular weight of the FV heavy chain is 105 kDa, the ~75-kDa band visible in platelet FVa from all individuals is a typical degradation product.[24]

The FV heavy chain was also visualized on Western blot after immunoprecipitation from plasma and activated platelet suspensions (Figure 4). No FV heavy chain was detectable in plasma from the proband or patient PD II (Figure 4, left panel). Differently, platelet FV heavy chain fragments were visible in both patients, although the signal was lower in the proband than in patient PD II (Figure 4, right panel). Interestingly, the proband showed only the expected FV heavy chain band, suggesting that the truncated protein, if synthesised at all, is rapidly degraded. Also in the parents' plasma and platelets, only the expected FV heavy chain band was present, which was less intense than in the normal control.

TFPI levels

Free TFPI levels in plasma were 22% in the proband, 43% in the mother and 46% in the father, in line with the respective FV levels.[23]

Thromboelastometry

The overall coagulation phenotype of the proband and his parents was evaluated by rotation thromboelastometry in whole blood activated with either TF (EXTEM) or an intrinsic trigger (INTEM). Five healthy individuals served as controls. As shown in Table 2, the proband had markedly prolonged clotting time and clot formation time. Nonetheless a clot was formed and maximal clot firmness was similar to that of normal controls, both after extrinsic and intrinsic activation of coagulation. The proband's parents had thromboelastogram parameters similar to the normal controls. The mother had somewhat prolonged clotting time and clot formation time after intrinsic activation (probably attributable to her reduced FXII level: 33%), but once started, clot formation proceeded even more efficiently than in the normal controls.

Table 2. Thromboelastogram parameters

	Controls (n=5)	**Proband**	**Mother**	**Father**
EXTEM				
CT (s)	58.8 ± 12.9	291	58	46
CFT (s)	80.8 ± 25.9	247	36	65
α (°)	74.2 ± 5.0	48	83	78
MCF (mm)	57.6 ± 4.5	52	75	56
MAXV (mm/s)	16.6 ± 3.6	10	35	20
AUC (mm)	5746 ± 454	5185	7414	5562
INTEM				
CT (s)	161.2 ± 11.8	798	385	156
CFT (s)	69.2 ± 16.0	85	143	53
α (°)	76.2 ± 2.7	73	70	79
MCF (mm)	59.8 ± 3.6	62	77	62
MAXV (mm/s)	18.8 ± 3.6	15	22	22
AUC (mm)	5956 ± 364	6122	7879	6144

CT, clotting time; CFT, clot formation time; α, alpha-angle; MCF, maximum clot firmness; MAXV, maximal velocity; AUC, area under the curve. Parameters determined in the normal controls are expressed as mean ± standard deviation.

Discussion

Patients with severe FV deficiency express a highly heterogeneous clinical phenotype, but the determinants of bleeding tendency other than FV levels are poorly understood.[14] In a previous study, we have shown that three patients with undetectable plasma FV and mild bleeding symptoms had residual FV in their platelets, which was sufficient to support throm-

bin generation in PRP and probably protected them from major bleeding [24]. We therefore speculated that patients with equally undetectable plasma FV and severe bleeding symptoms might have less or no platelet FV. In the present study we tested this hypothesis by characterising a FV-deficient patient with a severe bleeding tendency.

This patient was diagnosed with FV deficiency already at birth because of umbilical bleeding, which is a rare (3%) and potentially life-threatening manifestation of the disease.[15,16] Unlike the previously investigated FV-deficient patients, who experienced only mucosal or post-traumatic bleeding,[24] he suffers from recurrent spontaneous joint and muscle bleedings which, though not infrequent (20-25%) among FV-deficient patients,[15,17] represent a more serious bleeding manifestation[16] Moreover, he recently developed a spontaneous haemothorax and an intracranial haemorrhage. Overall, diagnosis at birth, the very young age (1 month) at first plasma transfusion, the nature and frequency of his bleeding symptoms and the frequent need of substitutive treatment all point towards a rather severe bleeding diathesis.

Sequencing of the *F5* proximal promoter, coding region, splicing junctions and 3'-UTR yielded no mutation, but showed the proband to be homozygous at virtually all polymorphic positions. After excluding carriership of a large *F5* deletion, this strongly suggested that the patient had inherited two alleles identical-by-descent and that his parents were (distantly) related. As a matter of fact, both parents turned out to carry the same splicing mutation in intron 8 (*F5* IVS8 +268A→G), whereas the proband was homozygous. Since this mutation is deep-intronic, it was not detected during the initial genetic screening and could only be identified because of its impact on mRNA splicing. The *F5* IVS8 +268A→G mutation activates a cryptic donor splice site in intron 8, causing a 111-nt long pseudo-exon to be retained in the mature *F5* mRNA. Since the inserted sequence contains an in-frame stop codon, no full-length FV can be synthesised from the aberrant mRNA, which also appears to be largely degraded by nonsense-mediated decay *in vivo*. However, a tiny fraction of the proband's *F5* pre-mRNA is spliced correctly (Figure 2B), allowing for the possibility that the patient may have traces of functional FV.

To our knowledge, only 11 splicing mutations have been reported in the *F5* gene so far.[22,30-37] All are located close to a splicing junction and disrupt an existing splice site consensus sequence, leading either to exon skipping or to the activation of a nearby cryptic splice site. Differently, the IVS8 +268A→G mutation is deep-intronic and results in the inclusion of a whole new exon (a pseudo-exon) in the mature mRNA. Although only a few of such mutations have been described to date in haemostasis-related genes, notably in the factor VIII gene[38] and in the fibrinogen gene cluster,[39,40] several examples have been reported in other genes and mutational activation of intronic pseudo-exons is emerging as a common mechanism of disease[28]

While no FV antigen or activity could be demonstrated in the proband's plasma, his platelets contained traces of FV antigen but no detectable FV activity. Accordingly, no thrombin generation was observed in his PPP or PRP on two different occasions. This is in striking contrast to the previously studied patients with undetectable plasma FV and relatively mild bleeding tendencies, who all showed thrombin generation in PRP already at 1 pM or 5 pM TF.[24] Since the proband of the present study had equally low or even lower free TFPI levels than the previously studied FV-deficient patients,[24] his undetectable thrombin generation in PRP is solely attributable to the virtual absence of functional FV in his plasma and platelets. This is likely a consequence of his more severe genetic defect (splicing mutation) as compared to the previously investigated patients, who all carried missense mutations. In fact, while the effects of missense mutations can be abolished by occasional translation mistakes, splicing mutations produce grossly abnormal mRNA molecules that are subject to nonsense-mediated decay. Remarkably, almost all FV-deficient patients with homozygous splicing mutations have experienced severe bleeding manifestations, like intracranial bleeding[22,31,33] or repeated haemarthrosis,[32] suggesting that the type of genetic defect might determine the amount of residual platelet FV and thus clinical outcome in patients with severe FV deficiency.

Despite undetectable thrombin generation in the proband's PRP, thromboelastometry showed that his blood could form clots of normal size and strength, although clot formation was markedly delayed. These findings are not conflicting, because the thrombin concentration (a few nM) needed for plasma to clot is too low to be detectable in the thrombin generation assay. Therefore, the patient may have traces of functional FV that, though below the detection limit, do afford minimal thrombin generation and clot formation *in vivo*. This once more illustrates the very low FV requirement for viability.[21,22]

The proband's parents had FV antigen and activity levels compatible with their heterozygous FV deficiency, and thrombin generation and thromboelastographic profiles similar to those of normal controls. The reduced plasma TFPI levels associated with their partial FV deficiency[23,24] may explain their comparatively high thrombin generation at low TF. As a carrier of a *F5*-null mutation (IVS8 +268A→G) and the *F5* R2 haplotype on different alleles, the proband's mother was "R2 pseudo-homozygous". Accordingly, she had consistently lower FV levels than the father and particularly high thrombin generation.

In conclusion, we have characterised a FV-deficient patient with a severe bleeding diathesis. In contrast to the previously studied patients with equally undetectable plasma FV but mild bleeding symptoms, this patient had undetectable platelet FV activity and showed no thrombin generation in PRP, due to homozygosity for a splicing mutation that virtually precludes FV synthesis. In combination with our previous observations,[24] these findings suggest that thrombin generation in PRP may help predict bleeding risk in patients with undetectable

plasma FV. However, this conclusion needs to be validated in a larger group of patients with severe FV deficiency.

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6

FV Leiden pseudo-homozygotes have a more pronounced hypercoagulable state than FV Leiden homozygotes

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Summary

The factor V Leiden (FVL) mutation causes activated protein C (APC) resistance and increases the risk of venous thrombosis. FVL pseudo-homozygotes are heterozygotes that carry a null mutation on the counterpart FV allele. Like homozygotes, they have only FVL in plasma, but their partial FV deficiency also predicts low plasma levels of tissue factor pathway inhibitor (TFPI), which may exacerbate their hypercoagulable state. We compared 9 FVL pseudo-homozygotes and 18 homozygotes. Pseudo-homozygotes had lower TFPI levels, higher thrombin generation and higher APC resistance than homozygotes, indicating a more pronounced hypercoagulable state and possibly a higher thrombosis risk than in homozygotes.

The factor V (FV) R506Q mutation (FV Leiden),[1] which is present in ~5% of Caucasians, is associated with activated protein C (APC) resistance[2] and increases the risk of venous thrombosis ~7-fold in heterozygotes and ~80-fold in homozygotes.[3] The rare FV Leiden heterozygotes who carry a loss-of-function mutation on the counterpart FV allele (FV Leiden pseudo-homozygotes), express only the FV Leiden allele and have plasma FV levels of ~50%.[4] Based on APC resistance measurements, FV Leiden pseudo-homozygotes are generally considered to have a hypercoagulable state similar to that of FV Leiden homozygotes.[4-6] Their risk of venous thrombosis is not well established and was found to be comparable to that of FV Leiden heterozygotes in one study[7] and to that of homozygotes in another study.[6] However, both studies may have been biased by the inclusion of probands in the analysis. Therefore, an intermediate phenotype such as thrombin generation may be more suitable to quantify the thrombotic tendency associated with FV Leiden pseudo-homozygosity.

Recently, we have shown that plasma levels of tissue factor pathway inhibitor (TFPI) are markedly reduced in FV deficiency.[8] Since FV Leiden pseudo-homozygotes have low FV levels, we hypothesized that they might have reduced TFPI levels as well. This may exacerbate their hypercoagulable state, as low TFPI levels are also associated with an increased risk of venous thrombosis.[9] To test this hypothesis, we compared plasma TFPI levels and thrombin generation in 9 FV Leiden pseudo-homozygotes and 18 FV Leiden homozygotes.

Nine FV Leiden pseudo-homozygotes (5 males and 4 females, mean age 48.4 years), of whom 5 (55.5%) had experienced venous thrombosis, were included. Eighteen FV Leiden homozygotes (9 males and 9 females, mean age 44.8 years), of whom 6 (33.3%) had experienced venous thrombosis, served as controls. Genotyping for the FV Leiden mutation was performed as described before.[8] All participants gave informed consent to the study, which was performed in accordance with the Declaration of Helsinki.

Platelet-poor plasma was prepared from venous blood as described previously.[8] Plasma levels of prothrombin, FV, total protein S and free TFPI was measured as described before.[8] Pooled normal plasma, prepared by pooling plasma from 15 healthy individuals without FV Leiden (7 males and 8 females, mean age 45.1 years), was used as a reference.

Thrombin generation was determined using the Calibrated Automated Thrombogram method[10] under conditions sensitive that are sensitive to the TFPI anticoagulant pathway, *i.e.* at low (1.7 pM) tissue factor (TF) and at high (6.8 pM) TF in the presence of APC. Measurements at low TF were performed in the absence and presence of inhibitory anti-TFPI antibodies (an equimolar mixture of anti-TFPI Kunitz-1, anti-TFPI Kunitz-2, anti-TFPI Kunitz-3 and anti-TFPI C-terminus monoclonal antibodies from Sanquin Reagents, Amsterdam, The Netherlands). Moreover, 32 μg/mL corn trypsin inhibitor (Haematologic Technologies, Essex Junction VT, USA) was added to inhibit contact activation. Measurements at high TF were

performed in the absence and presence of APC (Innovative Research, Novi MI, USA). Since the low APC concentration (~5 nM) normally used in the thrombin generation-based APC resistance test has relatively little effect on thrombin generation in FV Leiden homozygous plasma, precluding discrimination between homozygotes and pseudo-homozygotes,[5,6] a higher APC concentration (16 nM) was used in the present study. This APC concentration completely abolished thrombin generation in pooled normal plasma and reduced the endogenous thrombin potential (ETP) of a FV Leiden homozygous plasma pool (prepared by pooling plasma from 5 FV Leiden homozygotes: 1 male and 4 females, mean age 37.8 yearsto ~30% of the ETP measured in the absence of APC. This FV Leiden homozygous plasma pool was used instead of pooled normal plasma to normalise the APCsr. The normalised APC sensitivity ratio (nAPCsr) was therefore defined as the ration of the ETPs determined in the presence and absence of APC in sample plasma divided by the ETP ration in the FV Leiden homozygous plasma pool.

All data are presented as mean ± standard deviation. Differences in factor levels and thrombin generation parameters between FV Leiden pseudo-homozygotes and homozygotes were evaluated with Student's t-test and corrected for age and sex by multiple linear regression analysis. Statistical analyses were performed with IBM SPSS Statistics 18.0.

Table 1. Demographic characteristics of the study subjects and plasma levels of coagulation factors and inhibitors

	N	Sex (M/F)	Age (years)	VTE (%)	Prothrombin (%)	FV (%)	Free TFPI (ng/mL)	Total PS (%)
FVL homozygotes	18	9/9	44.8±13.6	33.3	104.3±13.0	104.1±19.3*	10.5±3.4**	109.0±22.7
FVL pseudo-homozygotes	9	5/4	48.4±18.3	55.5	102.0±23.4	62.9±8.5*	6.5±2.0**	110.1±18.1

VTE, venous thromboembolism; PT, prothrombin; PS, protein S; * p<0.001; ** p=0.004

The demographic characteristics and the plasma levels of coagulation factors and inhibitors in the 18 FV Leiden homozygotes and the 9 FV Leiden pseudo-homozygotes are presented in Table I. FV Leiden pseudo-homozygotes had reduced FV and TFPI levels as compared to homozygotes (FV 62.9±8.5% *vs*. 104.1±19.3%, p<0.001; TFPI 6.5±2.0 ng/mL *vs*. 10.5±3.4 ng/mL, p=0.004). These differences persisted after correction for age and sex. The levels of prothrombin and protein S, two other major determinants of thrombin generation and APC resistance,[11,12] did not differ between the two groups.

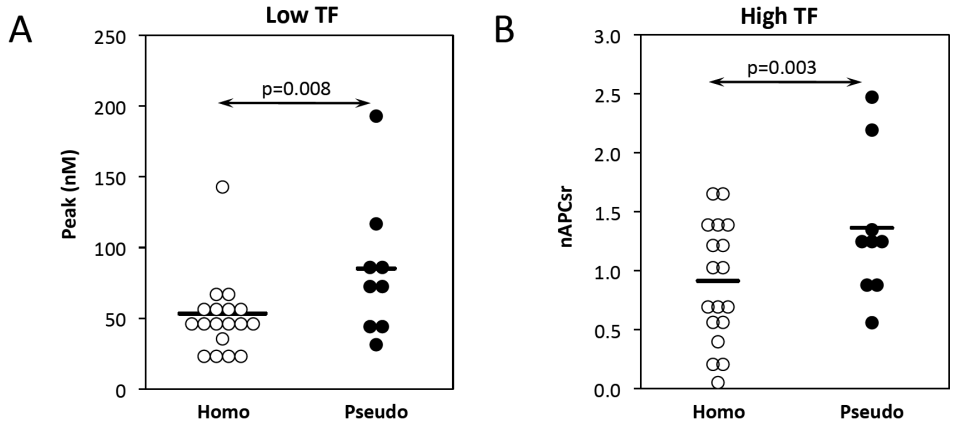

Figure 1. Thrombin generation in FV Leiden homozygotes and pseudo-homozygotes. Thrombin generation was measured in plasma from 18 FV Leiden homozygotes (open circles) and 9 FV Leiden pseudo-homozygotes (closed circles) after initiating coagulation with A) a low tissue factor (TF) concentration (1.7 pM) and B) a higher TF concentration (6.8 pM) in the absence and presence of 16 nM activated protein C (APC). Measurements initiated with low TF are expressed as the peak height of the thrombin generation curve. Measurements at higher TF are expressed as normalized APC sensitivity ratio (nAPCsr), normalised against a FV Leiden homozygous plasma pool. Horizontal lines represent the means of the respective distributions.

To investigate the effect of reduced plasma TFPI levels in FV Leiden pseudo-homozygotes, thrombin generation was measured under TFPI-sensitive conditions. At low TF in the absence of APC (Figure 1A), the peak height of thrombin generation was 1.6-fold higher in FV Leiden pseudo-homozygotes than in homozygotes (85.2±48.3 nM *vs*. 53.3±26.8 nM, p=0.008 after correction for age and sex). This difference disappeared in the presence of anti-TFPI antibodies (212.9±63.0 nM *vs*. 210.5±36.0 nM, p=n.s.), indicating that the low TFPI levels are responsible for the elevated thrombin generation in FV Leiden pseudo-homozygotes. At high TF in the absence of APC, the ETP was similar in FV Leiden pseudo-homozygotes and homozygotes (706±276 nM.min *vs*. 692±150 nM.min, p=n.s.). However, in the presence of APC the ETP was higher in FV Leiden pseudo-homozygotes than in homozygotes (ETP 312 nM.min *vs*. 213 nM.min, p=0.030 after correction for age and sex), making FV Leiden pseudo-homozygotes ~1.5 times more APC resistant than homozygotes (nAPCsr 1.36 *vs*. 0.91, p=0.003 after correction for age and sex; nAPCsr normalised against the FV Leiden homozygous plasma pool) (Figure 1B). Since TFPI is the major determinant of APC resistance measured with the thrombin generation-based assay,[11,12] the observed difference in APC resistance between FV Leiden pseudo-homozygotes and homozygotes is likely attributable to their difference in plasma TFPI levels. A control experiment performed with pooled plasma from FV Leiden homozygotes and pooled plasma from FV Leiden pseudo-

homozygotes indeed showed that the difference in APC resistance between the two pools could be abolished by the addition of anti-TFPI antibodies (data not shown).

The higher thrombin generation and APC resistance in FV Leiden pseudo-homozygotes indicate a more pronounced hypercoagulable state than in homozygotes, which may translate in a higher risk of venous thrombosis. The clinical implication of this finding is that protocols for VTE prophylaxis and treatment in FV Leiden pseudo-homozygotes should be similar to those used in FV Leiden homozygotes (or double heterozygotes for the FV Leiden and prothrombin G20210A mutations) according to the current antithrombotic guidelines.

In a previous study we have shown that the lack of normal FV expressing APC-cofactor activity in factor VIIIa inactivation makes FV Leiden pseudo-homozygotes more APC resistant than FV Leiden heterozygotes and similar to homozygote.[5] In the present study we show that FV Leiden pseudo-homozygotes also have reduced plasma levels of TFPI, which further enhances their hypercoagulable state, making them possibly more procoagulant than FV Leiden homozygotes. This difference has been previously overlooked because APC resistance was measured with assays that are rather insensitive to TFPI (*e.g.* the aPTT-based assay) and/or because the APC concentration was too low to discriminate between FV Leiden homozygotes and pseudo-homozygoytes.

Our data further suggests that differences in the expression of the normal FV allele in FV Leiden heterozygotes may modulate APC resistance and thrombosis risk not only by affecting the APC-cofactor activity of FV in factor VIIIa inactivation,[5] but also by modifying the plasma TFPI levels. The relative contributions of these two mechanisms will depend on the determinants of the assay used to measure APC resistance.[11]

Similar considerations apply to individuals who are pseudo-homozygous for other procoagulant FV gene polymorphisms/mutations, including the common R2 haplotype[13] as well as the Cambridge,[14] Hong-Kong,[15] and Liverpool[16] mutations. Moreover, partial FV deficiency might *per se* increase the risk of venous thrombosis *via* the associated decrease in both APC-cofactor activity of FV and in plasma TFPI levels. Evidence for this has recently been reported in the Japanese population,[17] whereas low FV levels were not associated with the risk of venous thrombosis in the Leiden Thrombophilia Study (LETS).[18] This discrepancy might be due to the different patient selection criteria (only idiopathic thromboses in the Japanese study) and/or the relatively high prevalence of partial FV deficiency[17] in the absence of other common thrombophilic defects (FV Leiden and the prothrombin G20210A mutation) in the Japanese population. In either case, further studies are needed to establish whether low FV levels are *per se* a risk factor for venous thrombosis.

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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.

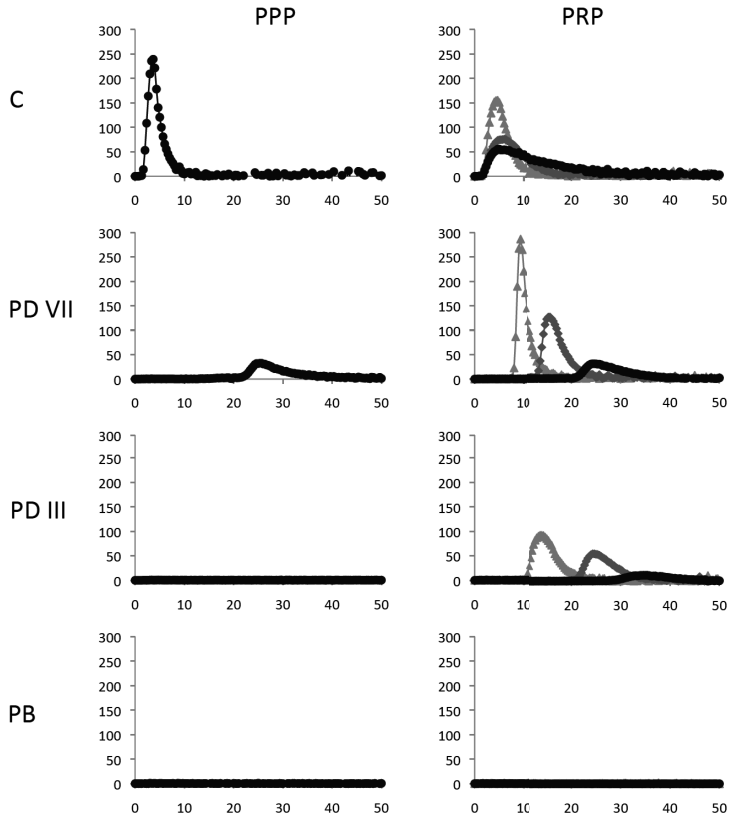

Figure 1. Thrombin generation in platelet-poor (PPP) and platelet-rich plasma (PRP) from a healthy control (C) and from patients with severe FV deficiency having both plasma and platelet FV (PD VII), only platelet FV (PD III) or no functional FV (PB). Coagulation was triggered with 5 pM TF. PPP was supplemented 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.

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Curriculum Vitae

Connie Duckers was born on the 27th of June 1984 in the hospital of Kerkrade, the Netherlands. After graduating secondary school (VWO Gymnasium) at College Rolduc in Kerkrade, she started to study Molecular Life Sciences at Maastricht University. During this bachelor study she followed internships at various departments at Maastricht University as well as at the Biomedical Research Institute (BIOMED, Diepenbeek, Belgium) under the supervision of dr. A. Hopman and dr. A. Duijvestijn. In 2005, she started with her master study in Biomedical Sciences at the University of Hasselt (Diepenbeek, Belgium), specialization Clinical Molecular Sciences. She followed her graduation internship at the Department of Molecular Cell Biology (Maastricht University) on nuclear intermediate filaments and laminopathies under supervision of dr. J. Broers. In 2006 she received her master's degree with great distinction. In the same year she started her PhD research described in this thesis under supervision of prof. dr. J. Rosing and dr. E. Castoldi at the Department of Biochemistry (Maastricht University). During her PhD period she visited the lab of prof. dr. P. Simioni at the University of Padua (Italy) to perform research and the Department of Clinical Epidemiology at Leiden University (The Netherlands) to analyse data. She presented her research at the XXIIth congress of the International Society on Thrombosis and Haemostasis in Boston (United States of America), which was awarded a "young investigator award". From April 2011 onward she will be working at the VieCuri medical centre in Venlo (The Netherlands) as a trainee clinical chemist under the supervision of dr. J. Swaanenburg.

List of publications

Papers

- **Duckers C**, Simioni P, Spiezia L, Radu C, Gavasso S, Rosing J, Castoldi E. Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency. Blood 2008; 112 (9); 3615-3623.
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- *Symposium van de Nederlandse Vereniging voor Trombose en Hemostase (NVTH), Koudekerken, NL, April 2008.*
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