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Characterization of an immunologic polymorphism (D79H) in the heavy chain of factor V

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Summary. *Background*: During the study of a family with hereditary factor (F)V deficiency (FV Amersfoort, 1102 A > T in exon 7) we identified an individual with 5% FV heavy chain antigen (FV_{HC}) and 50% FV light chain antigen (FV_{LC}). Further testing revealed that apart from the FV Amersfoort allele a second variant FV allele was segregating in this family, which encodes for a FV molecule with a reduced affinity for mAb V-23 used in the FV heavy chain ELISA ($ELISA_{HC}$). *Objective*: Identification and characterization of the molecular basis responsible for the reduced affinity of the variant FV for mAb V-23. *Methods*: Family members of the proband were screened for mutations in the exons coding for the heavy chain of FV, after which the recombinant variant FV could be generated and characterized. Next, the cases and controls of the Leiden Thrombophilia Study (LETS) were genotyped for carriership of the variant FV. *Results*: In the variant FV allele a polymorphism in exon 3 (409G > C) was identified, which predicts the replacement of aspartic acid 79 by histidin (D79H). Introduction of this mutation in recombinant FV confirmed that it reduces the affinity for binding to mAb V-23. The substitution has no effect on FV(a) stability and Xa-cofactor activity. In Caucasians the frequency of the FV-79H allele is ~5%. Analysis of the LETS revealed that the FV-79H allele is not associated with FV levels (FV_{LC}), activated protein C sensitivity (using an activated partial thromboplastin time-based test) or risk of venous thrombosis (OR 1.07, CI 95: 0.7–1.7). *Conclusion*: The D79H substitution in FV should be considered as a neutral polymorphism. The monoclonal antibody V-23, which has a strongly reduced affinity for FV-79H, is not suitable for application in diagnostic tests.

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Introduction

Human coagulation factor (F)V is a large plasma glycoprotein that plays an important role in blood coagulation. In plasma, FV circulates as a single-chain procofactor and is activated by thrombin via limited proteolysis [1–3]. Activated FV (FVa) consists of two polypeptide chains (designated as heavy and light chain), which are non-covalently linked via a Ca^{2+} ion [4]. FVa serves as the non-enzymatic cofactor of the serine protease Xa, which is responsible for the proteolytic activation of prothrombin in the prothrombinase complex, consisting of FVa, FXa, and negatively charged phospholipids. Down-regulation of FVa is mediated by activated protein C (APC) through proteolytic cleavage of the heavy chain of FVa at Arg306, Arg506 and Arg679 [5]. Procofactor FV contains also APC-cofactor activity in the inactivation of FVIIIa [6,7].

FV plays an important role both in the up and down-regulation of thrombin generation (see also reviews [8] and [9]). This dual role of FV is reflected in the disorders associated with molecular defects in FV, which can be either hemorrhagic or thrombotic. A mild to severe bleeding tendency has been reported in patients with nonsense or missense mutations in the FV gene (*F5*) which prevent FV synthesis or result in dysfunctional FV (a large selection of FV mutations is summarized in references [8] and [10]). On the other hand the common R506Q mutation (FV Leiden), which impairs degradation of FVa by APC, is associated with an increased risk of venous thrombosis [11–13].

Measurement of FV antigen levels is an important tool in the laboratory diagnosis of FV deficiency (parahemophilia), which is a rare autosomal recessive bleeding disorder with an estimated prevalence of 1–1000 000 [14]. Also, in the laboratory screening of thrombophilia FV antigen measurements are important, as compound heterozygosity for FV Leiden and a FV null allele results in a pseudo-homozygous phenotype (severe APC-resistance) [15–20].

Several immunologic assays for the measurement of FV in plasma are available. In our laboratory we have developed two enzyme-linked immunosorbent assays (ELISA) for measuring FV antigen, one for the light chain and one for the heavy chain. The light chain ELISA (ELISA$_{LC}$) has first been described by Guasch *et al.* [21] and uses two murine monoclonal antibodies (mAbs), V6 and V9, the epitopes of which have been mapped on the light chain of FVa. The heavy chain ELISA (ELISA$_{HC}$) uses two murine mAbs (V-23 and V39) directed against the heavy chain of FVa.

During the study of the family of an asymptomatic heterozygous carrier of the FV Amersfoort allele (1102 A > T in exon 7 [22]), we observed a striking discrepancy between FV light chain and heavy chain antigen levels in some of the family members. Further analysis showed that this abnormality cosegregated with a 409G > C transversion in exon 3 of *F5* predicting an amino acid substitution (D79H) in the heavy chain of FV (first reported by Cargill *et al.* [23]). So far, the effect of the D79H substitution on the function of FV is not clear. Recently, however, it has been reported that carriership of FV-79H is associated with moderately decreased FV levels and may serve as a trans-acting gene mutation in heterozygous carriers of FV Leiden by lowering the sensitivity of plasma to APC [24]. The latter observations prompted us to further investigate the effects of the D79H substitution on FV function, FV levels, APC-sensitivity ratios and risk of venous thrombosis.

Materials and methods

Materials

Plasmid isolation kits were from Qiagen (Chatsworth, CA, USA). DNA restriction fragments were purified from agarose gel using the Cleanmix kit (Talent, Trieste, Italy). Bovine Serum Albumin (BSA), benzamidine, phosphatidyl-choline (PC), N-hydroxy-succinimidobiotin was from Sigma (St. Louis, MO, USA). Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) were obtained from Avanti Polar Lipids (Alabaster, Al, USA). dNTP were from Serva (Heidelberg, Germany). Chromogenic substrate S-2238 was obtained from Chromogenix (Uppsala, Sweden).

Proteins

Restriction enzymes were from New England Biolabs (Beverly, MA, USA). Human FXa was from Hematologic Technologies Inc. (Essex, VT, USA). Amplitaq DNA polymerase was from Applied Biosystems (Foster City, CA, USA). Activated protein C, prothrombin, thrombin and sheep anti-FV polyclonal antibody were purchased from Enzyme Research Laboratories (South Bend, IN, USA). Neutralite^{TM} Avidin-HRP conjugate was from Southern Biotechnology Associated Inc. (Birmingham, AL, USA).

FV deficient family

We studied the family of an asymptomatic FV deficient individual who was heterozygous for an 1102-A > T substitution in exon 7 (FV Amersfoort, see van Wijk *et al.* [22]).

FV antigen assays

FV light chain antigen was detected in an ELISA using two different monoclonal antibodies (mAbs) directed against the light chain of FV as described before [21,25]. In this ELISA, mAb V-6 was used as a coating antibody and biotinylated mAb V-9 as tagging antibody. FV heavy chain antigen was detected in an ELISA recently developed in our laboratory. This ELISA shows identical dose–response curves for plasma and serum FV, indicating that it identifies a fragment of the heavy chain of FVa, which is not cleaved during activation or inactivation of FV(a). Monoclonal antibody mAb V-23 was used as coating antibody and biotinylated mAb V-39 as tagging antibody. For both light chain and heavy chain ELISA, samples were diluted in 50 mM triethanolamine (TEA), 100 mM NaCl, 10 mM EDTA and 0.1% Tween, pH 7.5. Dilutions of pooled normal plasma (1 : 25–1 : 1600 dilution) were used to calibrate the assays. Results are expressed in percentage, where 100% refers to the concentration of FV in pooled normal plasma.

DNA amplification and sequence analysis

The exons coding for the heavy chain of FV were amplified according to standard polymerase chain reaction (PCR) procedures using 0.1–1 µg genomic DNA. Details about primers and conditions are available on request (rjdirven@lumc.nl). Sequence reactions were performed using the CEQ2000^{TM} Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., Fullerton, CA, USA) according to the instructions of the manufacturer and subsequently analyzed on a SEQ^{TM}8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA). Nucleotides of the cDNA were numbered according to Jenny *et al.* [26].

Mutagenesis

Expression vector pMT2FV [27] was used as template to introduce the D79H (409G > C) variation in the heavy chain of FV. Mutations were introduced using the Quikchange^{TM} Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The forward mutagenic oligonucleotide used for construction of rFV-79H was:

5′-GTTCACTTTAA**G**AATAAGGCA**C**ATAAGCCCTT-GAGC-3′

The nucleotide responsible for the missense mutation is presented in bold and underlined. In this oligo an additional silent mutation (in bold) was introduced, which removes a *Dra*I restriction site, to serve as a marker. After mutagenesis, the presence of the mutations was confirmed by direct DNA

sequencing. Finally, the mutated fragment was inserted into a non-mutagenized pMT2FV by exchange of the appropriate restriction enzyme fragments. Details about this procedure are available on request.

Transient expression of recombinant FV

Recombinant FV was transiently expressed in COS-1 cells (75 cm^2 culture flasks) using Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Hague Road, IN, USA). Twenty-four hours after transfection cells were washed with phosphate-buffered saline (PBS) and incubated with serum-free medium (Optimem Glutamax, Life Technologies Ltd, Paisley, UK). Conditioned medium was harvested after 72 h, centrifuged for 20 min at 1000 r.p.m. (200 g) (4 °C) and frozen at −20°C.

FV activity assay

Total FV activity was measured in a two-step procedure as described before [28,29]. Briefly, FV was completely activated by 5 nM thrombin in HBS-Ca (25 mM Hepes, 175 mM NaCl, 3 mM CaCl$_2$, 5 mg mL^{-1} BSA, pH 7.5) during a 20-min incubation at 37 °C in a final volume of 150 μL. Subsequently, 10 μL was transferred to a 96 wells plate, 100 μL of prothrombinase mix (HBS-Ca containing FXa and DOPS/PC 10 : 90 molar ratio) was added and after 5 min the prothrombinase reaction was started by addition of 25 μL prothrombin (1.47 μM) in HBS-Ca. Final concentrations were: FXa (125 pM), prothrombin (280 nM) and DOPS/PC (50 μM). After a 4-min incubation at room temperature the reaction was stopped with 50 μL TN-EDTA (50 mM TRIS, 175 mM NaCl, 50 mM EDTA, 5 mg mL^{-1} BSA, pH 7.9). After the addition of 25 μL chromogenic substrate S-2238 (2.35 mM), the amount of thrombin formed was measured kinetically on a Spectra III Thermo microtiter plate reader from Tecan (Salzburg, Austria). The assay was calibrated using dilutions of pooled normal plasma (PNP) corresponding with 0–3 pM of FV.

FV(a) stability

FVa stability was assessed by following the spontaneous loss of Xa-cofactor activity as a function of time in the presence and absence of phospholipids as described before [30]. Spontaneous inactivation rates were obtained by fitting the curves in a single-exponential model using non-linear least-squares analysis.

Synthetic peptides overlapping region of amino acid 79

Two synthetic peptides were constructed homologous to the amino acid sequence of human FV with either an aspartic acid or histidine at position 79:

PEP79D: 71-KVHFKNKA**D**KPLSIH-85 (D at residue 79)
PEP79H: 71-KVHFKNKA**H**KPLSIH-85 (H at residue 79)

The affinity of these peptides for mAb V-23 was assessed from experiments in which plasma FV was used as competitor.

In brief, a 96-wells plate was coated with mAb V-23, after which the wells were incubated with pooled normal plasma (1/300 diluted in a buffer containing 50 mM TEA, 100 mM NaCl, 10 mM EDTA and 0.1% Tween, pH 7.5), mixed 1 : 1 with increasing concentrations of peptides in 50 mM TEA, 100 mM NaCl, pH 7.5 (final concentration 1 mM). After washing with ELISA buffer, the plate was incubated with biotinylated sheep antihuman FV polyclonal antibody (1 : 500 dilution), which was detected with Neutralite Avidin-HRP. In the final step, the plate was colored with 3,3′,5,5′-tetramethylbenzidine.

Study population

Patients and control subjects included in the present study were from a large population-based case-control study on risk factors for a first venous thrombosis, the Leiden Thrombophilia Study (LETS), which has been described in detail before [31,32].

Detection of 409G > C transversion in genomic DNA

Carriership of the 409G > C transversion in exon 3 of the *F5* (corresponding to the D79H mutation) was detected by PCR followed by restriction enzyme digestion. The PCR was performed using 0.1–1 μg genomic DNA, 200 ng primers, 200 μM dNTP and 1 University of *Taq* DNA polymerase in PCR buffer containing 67 mM TRIS-HCl (pH 8.8), 6.7 mM MgCl$_2$, 6.7 μM EDTA, 16.6 mM (NH$_4$)$_2$SO$_4$, 0.1 mg mL^{-1} BSA, 10% DMSO and 10 mM,-mercapto-ethanol. A 208-bp fragment was amplified with the following primers:

Primer A: (5′-GGAGGAATGGTAGCAATCACTCTT-GG-3′)
Primer B (5′-TCCTTGAGGATGGATGCTCA<u>GAT</u>GCT-TAT-3′)

In primer B a mismatch sequence (underlined) was introduced to generate a *BsaBI* restriction site after amplification of the FV-79D allele. Digestion of this fragment by *BsaBI* resulted in cleavage products of 25 bp and 183 bp, whereas the amplified fragment of the FV-79H allele was not digested by *BsaBI*.

APC-resistance test

The activated partial thromboplastin time (APTT)-based APC-sensitivity ratio (APC-SR) was measured as described by Koster *et al.* [31]. APC-sensitivity ratios were normalized by dividing them by the APC-SR of pooled normal plasma.

Statistical analysis

Odds ratios (ORs) and 95 per cent confidence intervals (95% CI) were calculated in the standard unmatched fashion. The OR estimates the risk of venous thrombosis in the presence of a risk factor (e.g. carriership of the FV-79H allele) relative to the absence of the particular risk factor, the reference category. An OR of 1 indicates no effect on risk, while an OR above 1 indicates an increase in risk.

Results

FV activity and antigen in FV deficient family

FV Amersfoort is characterized by the 1102 A > T substitution in exon 7 of *F5*, resulting in a premature stop at codon 310 [22]. To investigate whether the truncated FV protein (amino acids 1–310) is expressed in plasma, we measured FV heavy chain antigen (FV_{HC}) and FV light chain antigen (FV_{LC}) in plasma of an asymptomatic heterozygous carrier of FV Amersfoort and his first-degree relatives. The results are summarized in Table 1. In none of the FV Amersfoort carriers (father, child 1 and child 2) an excess of FV_{HC} was observed, because in none of the plasma samples the FV_{HC}/FV_{LC} ratio exceeded 1. Remarkably, in some individuals the FV_{HC} level was much lower than the FV_{LC} level, resulting in strongly reduced FV_{HC}/FV_{LC} ratios. The mother, child 1, 2 and 4 all seemed to be heterozygous for a variant FV molecule, which is poorly recognized by one or both mAbs used in the $ELISA_{HC}$. To test this, three additional FV ELISAs were designed using either mAb V-23, V-39 or V-6 as catching antibody and a polyclonal anti FV conjugate as tagging antibody. Subsequently, plasma of child 1 (FV_{HC}/FV_{LC} ratio 0.07) was analyzed with these ELISAs. The FV antigen levels were 0%, 41% and 61% as measured with $ELISA_{V-23}$, $ELISA_{V-39}$ and $ELISA_{V-6}$, respectively, which indicates that mAb V-23 poorly recognizes FV in this individual.

Identification of an immunologic polymorphism in the heavy chain of FV

The 'absence' of an epitope for mAb V-23 in the FV of some members of this FV deficient family most likely is the result of an amino acid substitution in the heavy chain of FV. Therefore, we sequenced the exons coding for the heavy chain of FV of the father and the mother. In total five nucleotide changes with respect to the sequence of Jenny *et al.* were identified (summarized in Table 2) including the FV Amersfoort mutation in exon 7. From the data in Table 1 and Table 2 haplotypes were constructed (Fig. 1). Carriership of the haplotype 327 A, 409C, 495 A, 642T, 1102 A cosegregated with reduced FV_{HC}/FV_{LC} ratios. Within this haplotype only the 409G > C polymorphism in exon 3 predicts an amino acid substitution (D79H). This

Table 1 FV activity and antigen levels in members of FV deficient family

Family	FV activity[2] (%)	FV_{LC} (%)	FV_{HC} (%)	FV_{HC}/FV_{LC}
Father [1]	34	51	41	0.80
Mother	125	136	89	0.65
Child 1[1]	62	69	5	0.07
Child 2[1]	60	64	65	1.02
Child 3	109	142	69	0.49
Child 4	93	136	57	0.42

[1]Heterozygous carrier of the FV Amersfoort allele. [2]FV activity was measured using a one stage clotting assay (data from van Wijk *et al.* [22]).

Table 2 Polymorphisms in exons coding for FV heavy chain in members of FV deficient family

Exon (Amino acid change)	Nucleotide change	Father	Mother	Child 1	Child 2	Child 3	Child 4
Exon 2	327G > A (Q51Q)	GA	AA	AA	AA	GA	GA
Exon 3	409G > C (D79H)	GC	GC	CC	GC	GC	GC
Exon 4	495G > A (A107A)	GA	GA	AA	GA	GA	GA
Exon 4	642G > T (S156S)	GT	GT	TT	GT	GT	GT
Exon 7	1102 A > T[1] (K310Term)	AT	AA	AT	AT	AA	AA

[1]Mutation resulting in premature stop (FV Amersfoort) [22].

made the D79H substitution a good candidate for the mutation that causes the reduced affinity for mAb V-23. It should be noted that the 409G > C transversion is also present in the FV Amersfoort allele (1102T), which is not expressed in plasma. Therefore, some members of this family are heterozygous for the D79H substitution, whereas they have a normal FV_{HC}/FV_{LC} ratio (i.e. child 2).

Analysis of the epitope of mAb V-23

To confirm that the molecular basis of the reduced affinity of FV for mAb V-23 resides in the D79H mutation, we introduced this mutation in an expression vector containing the cDNA of human FV (pMT2-FV). After transfection of COS-1 cells, conditioned medium containing recombinant FV was collected and analyzed (Table 3). The expression levels of both rFV-79D and rFV-79H were similar as measured by functional FV assay and $ELISA_{LC}$ (∼7% of normal plasma level), which indicates that rFV-79H has a normal specific activity (FVa/FV_{LC}). However, using the $ELISA_{HC}$ very low FV levels were detected in medium containing rFV-79H, while the FV_{HC} in the medium of rFV79D was similar to the FV_{LC}. This confirmed that the $ELISA_{HC}$ was affected by the D79H substitution, which most likely disrupts the epitope of mAb V-23 (see also first section of Results).

We tried to obtain more information about the epitope of mAb V-23 using synthetic peptides. Two peptides (PEP-79D and PEP-79H) homologous to the region surrounding amino acid 79 were synthesized and tested for their ability to compete with plasma FV for binding to mAb V-23. However, peptide concentrations up to 1 mg mL^{-1} did not affect the binding of plasma FV to mAb V-23 (data not shown).

The region surrounding residue 79 was also analyzed in a 3-dimensional model for the heavy chain of FVa [33]. This model revealed that this region is surface exposed, indicating that it might contain an epitope for an antibody. Replacement of 79D by 79H did not result in conformational changes in the experimental FVa model (data not shown), which suggests that
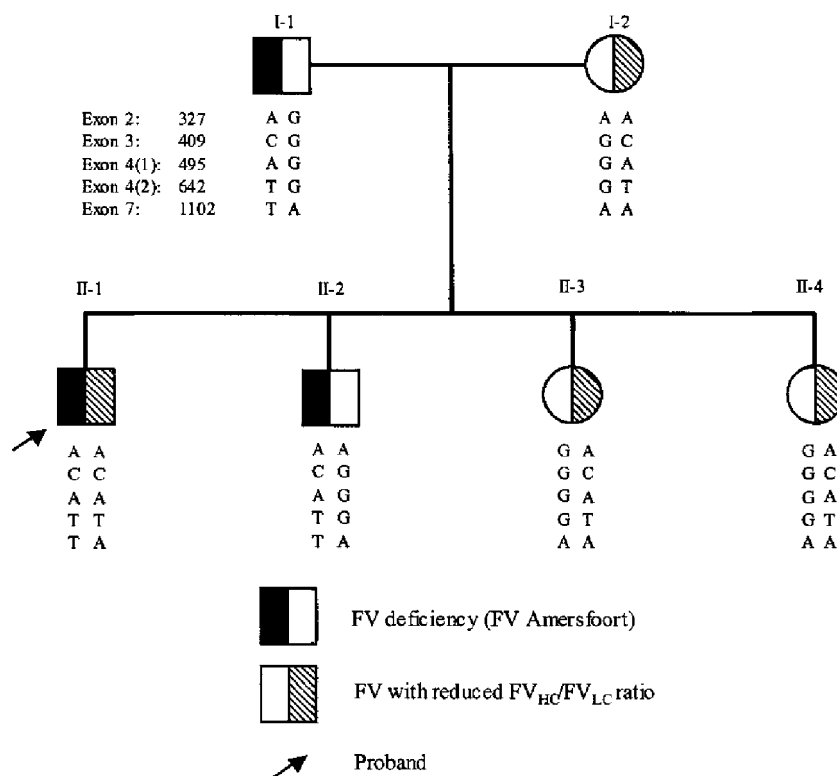
Fig. 1. Pedigree of FV deficient family. In this pedigree FV deficiency (FV Amersfoort) and reduced FV(HC)/FV(LC) antigen ratios (Table 1) are indicated together with the genotypes for the polymorphisms found in the exons coding for the heavy chain (Table 2). The proband of this family is child 1.

Table 3 Specific activity and FV_{HC}/FV_{LC} ratio of recombinant FV-79D and FV-79H. FV activity and FV antigen (FV_{HC} and FV_{LC}) were measured in conditioned medium containing either rFV-79D or rFV-79H

rFV	FVa/FV_{LC}[1]	FV_{HC}/FV_{LC}
rFV-79D	0.94	0.83
rFV-79H	1.04	0.06

[1]FV activity was measured as described in Materials and methods.

the D79H substitution affects electrostatic interactions between the epitope and mAb V-23.

Finally, conservation of region surrounding amino acid 79 was analyzed between different species. In Table 4 the amino acid sequences surrounding residue 79 (residues 71–85) of 7 different species are shown. Homology ranges from 60% for the zebrafish to 93% for some mammals. The aspartic acid at position 79 was conserved between several species, but not in cow and pipid frog.

Further characterization of the D79H substitution

Recently, it has been reported that carriership of the FV-79H allele is associated with moderately reduced FV activity in plasma [24], as measured in a one stage clotting assay. Also, indications have been obtained that carriership of the FV-79H allele may lower the APC sensitivity in subjects carrying the FV Leiden allele [24]. Therefore, it may act as a *trans*-acting gene in

Table 4 Alignment of amino acid sequence surrounding residue 79 in the heavy chain of FV. Conservation of the region surrounding position 79 (residue 71–85) in the heavy chain of FV was assessed by comparing the human amino acid sequence with the amino acid sequence of 6 different species. Position 79 is bold and underlined

Species	FV amino acid sequence	Homology
Homo sapiens	KVHFKNKA**D**KPLSIH	–
Bos taurus	KVHFKNKA**H**KPLSIH	93%
Mus musculus	KVHFRNKA**D**KPLSIH	93%
Sus scrofa	KVHFRNKA**D**KPLSIH	93%
Gallus gallus	VVHFKNMA**D**KPISIH	87%
Xenopus tropicalis	QVHFKNMA**N**KPLTIH	80%
Danio rerio	IVTFRNMA**D**HPCSLH	60%

carriers of FV Leiden in a similar way as has been demonstrated for the FV-R2 allele [34–37]. To further investigate this, we genotyped the cases and controls from a large population-based case-control study on risk factors for venous thrombosis (LETS) and determined the effect on FV levels, n-APC-SR and risk of thrombosis.

To investigate the effect of the D79H substitution on plasma FV levels, we used the data from the 433 healthy controls from the LETS. Carriers of the FV-R2 allele were excluded from this analysis (no compound heterozygous carriers of the FV-79H allele and FV-R2 were found in this group). Table 5 shows that the mean FV_{LC} level in carriers of the FV-79H allele (138 U dL^{-1}) is similar to that in non-carriers (133 U dL^{-1}).

Table 5 FV antigen levels (U dL^{-1}) and APC sensitivity ratios according to carriership of FV-79H. Carriership of FV-79H allele, FV light chain (FV$_{LC}$) levels and n-APC-SR were determined in control subjects of the LETS

D79H	FVLC[1]		n-APC-SR[2]	
	n	Mean (95% CI) (U dL^{-1})	*n*	Mean (95% CI)
D/D	390	133 (130–139)	374	1.02 (1.01–1.03)
D/H	42	138 (129–147)	42	1.05 (1.02–1.08)
H/H	1	115	1	0.98

[1]FV-R2 carriers (*n* = 38) were excluded. [2]FV Leiden carriers and individuals using oral anticoagulants were excluded (*n* = 54).

Table 6 Stability of recombinant FV-79D and FV-79H in the absence or presence of phospholipids. Spontaneous inactivation rates (*k*s) were obtained by fitting time courses (30 h) of rFV(a) inactivation with a single exponential. Incubation conditions are described in Methods (∼1 nM FVa in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl$_2$, 5 mg mL^{-1} BSA ± 24.5 μM phospholipids (10 : 90 DOPS:DOPC) at 37 °C)

FV	+ PL	– PL
	Spontaneous inactivation rates (s^{-1})	
rFV-79D	N.D.	3.0×10^{-6}
rFV-79H	N.D.	2.6×10^{-6}
Activated		
rFV-79D	5.1×10^{-5}	2.6×10^{-5}
Activated		
rFV-79H	6.2×10^{-5}	3.6×10^{-5}

Theoretically, the reduced procoagulant FV activity reported by Bossone *et al.* [24] for carriers of FV-79H may be caused by increased instability of the activity of FV-79H. To assess this possibility, the loss of (activated) rFV activity was followed as a function of time. The first-order rate constant for spontaneous inactivation (k_s) of rFV-79D was similar to that for rFV-79H, being around 3.0×10^{-6} s^{-1} (Table 6). Also the k_s for activated rFV-79D was similar to that for activated rFV-79H, being around 3.0×10^{-5} s^{-1}. The presence of phospholipids did not influence the stability of the activated rFV molecules.

Next, we investigated the effect of the D79H substitution on the APTT-based normalized-APC sensitivity ratio [31]. For this analysis carriers of FV Leiden were excluded. The n-APC-SRs in the absence of the FV Leiden mutation are summarized in Table 5 and demonstrate that carriership of the FV-79H allele is not associated with a lower n-APC-SR. Exclusion of FV-R2 carriers did not alter this result (data not shown). In this analysis, it was not possible to investigate whether carriership of FV-Leiden in *trans* with the FV-79H allele is associated with a further decrease of the n-APC-SR, because none of the healthy controls were compound heterzozygous for FV-Leiden and FV-79H. Therefore, we screened all patients in the LETS for compound heterozygosity for FV-Leiden and FV-79H. In total 8 patients were found compound heterozygous for FV-Leiden and FV-79H with a mean n-APC-SR of 0.57 (range 0.53–0.64).

Finally, we analyzed the association of the D79H substitution with risk of thrombosis. For this analysis all cases and

Table 7 Frequencies of D79H polymorphism in LETS

D79H	Patients	Controls
D/D	426	428
D/H	45	42
H/H	1	1

Odds ratio of FV-79H carriers: 1.07 (CI 95: 0.7–1.7).

controls of the LETS were genotyped for this polymorphism. The allele frequency of the FV-79H allele was 5.0% in patients and 4.7% in controls (Table 7), which is in agreement with previously reported allele frequencies (5–15%) in different healthy Caucasian populations [23,24]. The odds ratio (OR), calculated as a measure of the relative risk of venous thrombosis, for subjects carrying the FV-79H allele (both heterozygous and homozygous) was 1.07 (CI 95: 0.7–1.7) compared to homozygous FV-79D carriers. Exclusion of all FV Leiden carriers did not alter this OR.

Discussion

In this study, we have analyzed a variant FV that was not recognized by a monoclonal antibody (mAb V-23) directed against the heavy chain of FV. Haplotype analysis of the variant FV allele suggested that an amino acid substitution at residue 79 (D79H) was responsible for the absence of the epitope for mAb V-23. This was confirmed by introducing this substitution in recombinant FV (rFV-79H). Functional tests with rFV-79H showed that this substitution did not affect the Xa-cofactor function or the stability of FV(a). Furthermore, carriership of the FV-79H allele (allele frequency ∼5%) is not associated with FV levels (FV$_{LC}$), APC sensitivity (using an APTT-based test) or risk of venous thrombosis (OR 1.07, CI 95: 0.7–1.7). Together, these results indicate that the frequent D79H polymorphism in FV is neutral.

Initially, we were interested in the question whether truncated FV molecules were expressed in plasma from carriers of the FV Amersfoort allele, which refers to a 1102-A > T mutation in exon 7 of *F5* resulting in a premature stop in codon 310 [22]. For this purpose plasma samples of the relatives of a heterozygous carrier of FV Amersfoort were screened with ELISAs for FV heavy chain (FV$_{HC}$) and FV light chain (FV$_{LC}$). Recent data indicate that both antibodies used in the ELISA$_{HC}$ recognize an epitope at the amino-terminal end (1–306) of FV (data not shown). Because no FV$_{HC}$/FV$_{LC}$ ratios higher than 1 were observed (Table 1) it seems unlikely that substantial amounts of FV Amersfoort are expressed, although it can not excluded that the affinity of the truncated FV molecule for mAb V-23 or V-39 has been affected. This possibility could not be tested, because we have not generated recombinant FV Amersfoort. Instead, we have tested another truncated rFV molecule, labeled M-fragment, consisting of the first 909 amino acids of FV, which has been constructed before [29]. This showed that the affinity of the M-fragment for Mab V-23 and V-39 was not affected (data not shown). Taken together, it seems most likely that mRNA transcribed from the

FV Amersfoort allele is degraded in the nonsense-mediated mRNA decay pathway [38].

Unexpectedly, we observed strongly reduced FV_{HC}/FV_{LC} ratios in some family members (see Table 1). This observation suggested that in this family a variant FV was segregating, which was poorly recognized by one of the mAbs (V-23 or V-39) used in our $ELISA_{HC}$. Further testing revealed that mAb V-23 poorly recognized this variant FV and that the strongly reduced FV_{HC}/FV_{LC} ratios cosegregated with a FV haplotype containing one single missense mutation in the heavy chain (D79H). This made this mutation a strong candidate to affect the epitope for mAb V-23 (Fig. 1). Introduction of the D79H mutation in recombinant FV confirmed that it strongly reduced the affinity for mAb V-23 (FV_{HC}/FV_{LC} antigen ratio for rFV-79H is ∼0.06 compared to 0.83 for rFV-79D, Table 3). Further tests revealed that mAb V-23 did not bind synthetic peptides homologous to the region around residue 79 (71–85). This cannot easily be explained by the absence of post-translational modifications on these peptides, because in the region from 71 to 85 no modifications are known. This suggests that the epitope for mAb V-23 is not linear, which is in agreement with the observation that mAb V-23 did not detect FV in Western blots under reducing conditions (data not shown). Furthermore, replacement of 79D into 79H in the experimental model of FVa [33] did not result in conformational changes in the heavy chain of FVa, which suggests that the D79H mutation rather has an effect on the electrostatic interactions between the epitope and mAb V-23.

Little is known about the effect of the D79H substitution on the function of FV. So far, this polymorphism has only been reported twice. The first time was in 1999 in a study by Cargill et al. [23]. They included this polymorphism in an extensive catalog of single-nucleotide polymorphisms (SNPs) in the coding regions of human genes, which could be used in association studies. This report, however, gives no information on the functionality of this polymorphism. More recently, it has been reported that carriership of FV-79H is associated with slightly reduced procoagulant FV activity levels and possibly with a lower sensitivity to APC in heterozygous carriers of the FV Leiden allele [24]. This suggested that the D79H substitution, in analogy to the FV-R2 mutation, increases the APC-resistent phenotype of plasma from heterozygous carriers of the FV Leiden mutation via reduced FV expression of the FV-79H allele [34,39].

We did not observe an association between carriership of the FV-79H allele and lower FV_{LC} levels in 433 healthy individuals from the LETS (Table 5). This suggests that this allele is not associated with reduced FV antigen levels, which seems to be different from the results reported by Bossone et al. [24]. This may be explained by the relatively small size of the group studied by Bossone ($n = 150$ compared to 433 in our study). It also is possible that FV-79H has a slightly reduced specific procoagulant activity. However, we found no indications that the D79H substitution has an effect on the Xa-cofactor activity of FVa: the specific activity (FVa/FV_{LC}) of rFV-79H was similar to that of rFV-79D (Table 3). This is in agreement with the results from Zeibdawi et al. [40], who studied the effect of the D79A substitution on the cofactor function of FVa. Also, the D79H substitution did not affect the stability of the (activated) FV molecule (see Table 6), like the amino acid substitutions A221V and D111A, which have been reported to weaken the interaction between the heavy and light chain of activated FV [40,41].

Carriership of the FV-79H allele is not associated with a lower n-APC-SR after exclusion of FV Leiden carriers (Table 5), which is in line with the findings from Bossone et al. [24]. We did not find that heterozygous carriers of FV Leiden, who carry the FV-79H allele on the other chromosome have a more APC-resistant phenotype than subjects ($n = 8$) carrying the alleles for FV Leiden and FV-79D (mean n-APC-SR = 0.57). This ratio is identical to the ratio of 0.57 (range 0.5–0.67) previously reported for heterozygous carriers of FV Leiden [11,37]. Finally, carriership of the FV-79H has no effect on the risk of venous thrombosis (Table 7), which is reflected in an odds ratio of 1.07 (CI 95: 0.7–1.7).

Taken together, our data suggest that the D79H substitution has no effect on FV(a) function or thrombosis risk. This is in agreement with the fact that so far no functional domains have been identified in the region surrounding residue 79 in FV (see also the review by Mann and Kalafatis [8]). Also the fact that the aspartic acid at position 79 is not fully conserved between the species aligned in Table 4, suggests that residue 79 is not critical for the function of FV.

We conclude that the D79H substitution should be considered as a neutral polymorphism with an allele frequency of ∼5% in the Caucasian population. FV-79H is poorly recognized by mAb V-23. Therefore, it is recommended not to use mAb V-23 in diagnostic tests, because it would result in falsely reduced FV levels in 10% of the population.

References

1 Esmon CT. The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits, and reconstitution of biological activity. J Biol Chem 1979; **254**: 964–73.

2 Kane WH, Majerus PW. Purification and characterization of human coagulation factor V. J Biol Chem 1981; **256**: 1002–7.

3 Suzuki K, Dahlbäck B, Stenflo J. Thrombin-catalyzed activation of human coagulation factor V. J Biol Chem 1982; **257**: 6556–64.

4 Guinto ER, Esmon CT. Formation of a calcium-binding site on bovine activated factor V following recombination of the isolated subunits. J Biol Chem 1982; **257**: 10038–43.

5 Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem 1994; **269**: 31869–80.

6 Shen L, Dahlbäck B. Factor V, protein S, as synergistic cofactors to activated protein C, in degradation of factor VIIIa. J Biol Chem 1994; **269**: 18735–8.

7 Varadi K, Rosing J, Tans G, Schwarz HP. Influence of factor V and factor Va on APC-induced cleavage of human factor VIII. Thromb Haemost 1995; **73**: 730–1.

8 Mann KG, Kalafatis M. Factor V: a combination of Dr Jekyll and Mr Hyde. Blood 2003; **101**: 20–30.

9 Nicolaes GA, Dahlbäck B. Factor V and thrombotic disease: description of a janus-faced protein. Arterioscler Thromb Vasc Biol 2002; **22**: 530–8.

10 Montefusco MC, Duga S, Asselta R, Malcovati M, Peyvandi F, Santagostino E, Mannucci PM, Tenchini ML. Clinical and molecular characterization of 6 patients affected by severe deficiency of coagulation factor V. broadening of the mutational spectrum of factor V gene and in vitro analysis of the newly identified missense mutations. *Blood* 2003; **102**: 3210–6.

11 Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64–7.

12 Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Eisenberg PR, Miletich JP. Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke, and venous thrombosis in apparently healthy men. *N Engl J Med* 1995; **332**: 912–7.

13 Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood* 1995; **85**: 1504–8.

14 Owren PA. Parahaemophilia. haemorrhagic diathesis due to absence of a previously unknown clotting factor. *Lancet* 1947; **1**: 446–51.

15 Girolami A, Simioni P, Venturelli U, Girolami B, Zanon E. Factor V antigen levels in APC resistance, in factor V deficiency and in combined APC resistance and factor V deficiency (pseudohomozygosis for APC resistance). *Blood Coagul Fibrinolysis* 1997; **8**: 245–8.

16 Guasch JF, Lensen RPM, Bertina RM. Molecular characterization of a type I quantitative factor V deficiency in a thrombosis patient that is 'pseudo homozygous' for activated protein C resistance. *Thromb Haemost* 1997; **77**: 252–7.

17 Kalafatis M, Simioni P, Bernardi F. Phenotype and genotype expression in pseudohomozygous R2 factor V. *Blood* 2001; **98**: 1988–9.

18 Lunghi B, Castoldi E, Mingozzi F, Bernardi F, Castaman G. A novel factor V null mutation detected in a thrombophilic patient with pseudo-homozygous APC resistance and in an asymptomatic unrelated subject. *Blood* 1998; **92**: 1463–4.

19 Kalafatis M, Bernardi F, Simioni P, Lunghi B, Girolami A, Mann KG. Phenotype and genotype expression in pseudohomozygous factor VLEIDEN. the need for phenotype analysis. *Arterioscler Thromb Vasc Biol* 1999; **19**: 336–42.

20 Castoldi E, Kalafatis M, Lunghi B, Simioni P, Ioannou PA, Petio M, Girolami A, Mann KG, Bernardi F. Molecular bases of pseudo-homozygous APC resistance: the compound heterozygosity for FV R506Q and a FV null mutation results in the exclusive presence of FV Leiden molecules in plasma. *Thromb Haemost* 1998; **80**: 403–6.

21 Guasch JF, Cannegieter S, Reitsma PH. Van't Veer-Korthof ET, Bertina RM. Severe coagulation factor V deficiency caused by a 4 bp deletion in the factor V gene. *Br J Haematol* 1998; **101**: 32–9.

22 van Wijk R, van den Nieuwenhuis KBM, Huizinga EG, van der Meijden BB, Kraaijenhagen RJ, van Solinge WW. Five novel mutations in the gene for human blood coagulation factor V associated with type I factor V deficiency. *Blood* 2001; **98**: 358–67.

23 Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Shaw N, Lane CR, Lim EP, Kalyanaraman N, Nemesh J, Ziaugra L, Friedland L, Rolfe A, Warrington J, Lipshutz R, Daley GQ, Lander ES. Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet* 1999; **22**: 231–8.

24 Bossone A, Cappucci F, D'Andrea G, Brancaccio V, Cibelli G, Iannaccone L, Grandone E, Margaglione M. The factor V (FV) gene ASP79HIS polymorphism modulates FV plasma levels and affects the activated protein c resistance phenotype in presence of the FV Leiden mutation. *Haematologica* 2003; **88**: 286–9.

25 Kamphuisen PW, Rosendaal FR, Eikenboom JC, Bos R, Bertina RM. Factor V antigen levels and venous thrombosis: risk profile, interaction with factor V leiden, and relation with factor VIII antigen levels. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1382–6.

26 Jenny RJ, Pittman DD, Toole JJ, Kriz RW, Aldape RA, Hewick RM, Kaufman RJ, Mann KG. Complete cDNA and derived amino acid sequence of human factor V. *Proc Natl Acad Sci USA* 1987; **84**: 4846–50.

27 Kaufman RJ. Vectors used for expression in mammalian cells. *Meth Enzymol* 1990; **185**: 487–511.

28 van der Neut Kolfschoten M, Dirven RJ, Vos HL, Bertina RM. The R2-haplotype associated Asp2194Gly mutation in the light chain of human factor V results in lower expression levels of FV, but has no influence on the glycosylation of Asn2181. *Thromb Haemost* 2003; **89**: 429–37.

29 van der Neut Kolfschoten M, Dirven RJ, Tans G, Rosing J, Vos HL, Bertina RM. The activated protein C (APC) -resistant phenotype of APC cleavage site mutants of recombinant factor V in a reconstituted plasma model. *Blood Coagul Fibrinolysis* 2002; **13**: 207–15.

30 van der Neut Kolfschoten M, Dirven RJ, Tans G, Rosing J, Vos HL, Bertina RM. Factor Va is inactivated by APC in the absence of cleavage sites at Arg306, Arg506 and Arg679. *J Biol Chem* 2004; **279**: 6567–75.

31 Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C. Leiden Thrombophilia Study. *Lancet* 1993; **342**: 1503–6.

32 van der Meer FJ, Koster T, Vandenbroucke JP, Briet E, Rosendaal FR. The Leiden Thrombophilia Study (LETS). *Thromb Haemost* 1997; **78**: 631–5.

33 Villoutreix BO, Dahlbäck B. Structural investigation of the A domains of human blood coagulation factor V by molecular modeling. *Protein Sci* 1998; **7**: 1317–25.

34 Bernardi F, Faioni EM, Castoldi E, Lunghi B, Castaman G, Sacchi E, Mannucci PM. A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. *Blood* 1997; **90**: 1552–7.

35 Alhenc-Gelas M, Nicaud V, Gandrille S, van Dreden P, Amiral J, Aubry ML, Fiessinger JN, Emmerich J, Aiach M. The factor V gene A4070G mutation and the risk of venous thrombosis. *Thromb Haemost* 1999; **81**: 193–7.

36 Faioni EM, Franchi F, Bucciarelli P, Margaglione M, De Stefano V, Castaman G, Finazzi G, Mannucci PM. Coinheritance of the HR2 haplotype in the factor V gene confers an increased risk of venous thromboembolism to carriers of factor V R506Q (factor V Leiden). *Blood* 1999; **94**: 3062–6.

37 de Visser MC, Guasch JF, Kamphuisen PW, Vos HL, Rosendaal FR, Bertina RM. The HR2 haplotype of factor V. effects on factor V levels, normalized activated protein C sensitivity ratios and the risk of venous thrombosis. *Thromb Haemost* 2000; **83**: 577–82.

38 Frischmeyer PA, Dietz HC. Nonsense-mediated mRNA decay in health and disease. *Hum Mol Genet* 1999; **8**: 1893–900.

39 Lunghi B, Iacoviello L, Gemmati D, Dilasio MG, Castoldi E, Pinotti M, Castaman G, Redaelli R, Mariani G, Marchetti G, Bernardi F. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. *Thromb Haemost* 1996; **75**: 45–8.

40 Zeibdawi AR, Grundy JE, Lasia B, Pryzdial EL. Coagulation factor Va Glu96-Asp111. A chelator-sensitive site involved in function and subunit association. *Biochem J* 2004; **377**: 141–8.

41 Steen M, Miteva M, Villoutreix BO, Yamazaki T, Dahlbäck B, Factor V New Brunswick. Ala221Val associated with FV deficiency reproduced in vitro and functionally characterized. *Blood* 2003; **102**: 1316–22.