Chapter 9

Early death of factor V Leiden hemizygous mice: a fatal absence of wild-type factor V anticoagulant function

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Summary

Background: Factor V (FV) has a dual role in coagulation by expressing both a procoagulant and an anticoagulant function. In factor V Leiden (FVL) hemizygosity, i.e. the co-inheritance of FVL and a quantitative FV deficiency, defects of both FV functions are combined. Surprisingly, this combination confers a thrombotic risk, suggesting a complex role for FV in (anti)coagulation.

Methods and results: To gain further insight in FV function, FVL ($FV^{Q/Q}$) and $FV^{+/-}$ mice were crossed to generate FVL hemizygous mice. An underrepresentation of $FV^{Q/-}$ mice as compared to $FV^{Q/+}$ mice was observed at the time of weaning. Timed matings showed that $FV^{Q/-}$ animals progressed normally to term, but half of the $FV^{Q/-}$ mice died in the immediate postnatal period coinciding with features of thrombosis. On a tissue factor pathway inhibitor heterozygosity background, the $FV^{Q/-}$ genotype was fully fatal confirming that FVL hemizygosity is associated with a thrombotic and not a bleeding phenotype. Analyses of the plasma coagulation profiles of $FV^{Q/+}$, $FV^{Q/Q}$ and viable $FV^{Q/-}$ mice showed a reduced overall procoagulability in $FV^{Q/-}$ as compared to $FV^{Q/+}$ and $FV^{Q/Q}$ mice with decreased thrombin generation and longer prothrombin times. Activated protein C resistance and FV-mediated $FVIII_a$ inactivation was decreased in $FV^{Q/-}$ mice as compared to $FV^{Q/+}$ animals, but comparable to that of $FV^{Q/Q}$ mice.

Conclusion: This mouse study demonstrates the physiological importance of the anticoagulant function of normal FV, of which a critical fraction is retained in factor V Leiden.

Introduction

Coagulation factor V (FV) is a central regulator of the coagulation cascade. It serves as a non-enzymatic cofactor for activated FX (FX_a) in the prothrombinase complex, enabling rapid thrombin formation. Thrombin bound to endothelial thrombomodulin can activate protein C, which subsequently inactivates FV_a and $FVIII_a$, thereby providing a natural feedback loop that down-regulates thrombin formation. To inactivate FV_a, activated protein C (APC) together with its cofactor protein S, cleaves FV_a at arginine 506, 306 and 679.[1,2] Although the Arg^{506} is the kinetically favored cleavage site, approximately 40% activity of FV_a remains and an additional cleavage at Arg^{306} is necessary for complete inactivation.[1]

Factor VIII is highly homologous to FV, not only in domain structure, but also in function as it acts as the non-enzymatic cofactor for FIX_a in the tenase complex. Activated FVIII is also inactivated by APC, which cleaves $FVIII_a$ at arginine 336 and 562. Although the cleavage at Arg^{336} is the fastest, this results in only partial inactivation, whereas the slower Arg^{562} cleavage completely inactivates $FVIII_a$.[2] To perform this function, APC needs protein S and factor V as cofactors,[3-5] indicating an additional anticoagulant function for FV.

The factor V Leiden mutation (FVL) leads to an arginine to glutamine substitution at position 506 of factor V and therefore the FVL protein confers a partial resistance to the inactivation by activated protein C. Additional to this reduced susceptibility to inactivation, it also possesses little cofactor activity in the inactivation of activated FVIII, as FV first has to be cleaved at Arg^{506} to express this cofactor function.[6,7] These two functional defects result in an increased risk for venous thrombosis, which is 7-fold increased in FVL heterozygotes and 80-fold increased in homozygotes.[8]

Factor V Leiden hemizygosity, also known as pseudo-homozygosity for APC resistance, is the co-inheritance of the FVL mutation and a quantitative FV deficiency on different alleles,[9] and thus combines the factor V Leiden-related increased venous thrombotic risk and the deficiency-related bleeding tendency. With the prevalence of parahemophilia (FV deficiency with levels <1%) being $1:10^6$ and the prevalence of factor V Leiden being 1:20 in the Caucasian population, it is estimated that 1:1000 FVL carriers is actually hemizygous and with approximately 26 million FVL carriers in Europe alone, this would make up for a substantial number of hemizygotes.[10] However, most knowledge on this condition comes from case reports and small patient studies. Thus far, it has been shown that FVL hemizygosity results in an increased venous thrombotic risk comparable to, or even

higher than, the risk in FVL homozygotes.[11-13] This unexpected finding suggests a more complex role and contribution for FV in coagulation. To gain further insight into the complex role of factor V in (anti)coagulation, we used an *in vivo* approach in which we crossed mice carrying the factor V Leiden knock-in allele with factor V heterozygous mice to generate FVL hemizygous animals.

Materials and Methods

Mice

Generation of mice carrying the R504Q knock-in mutation, which is orthologous to the human FVL mutation, was previously described as was the generation of $FV^{+/-}$ mice.[14,15] Tissue factor pathway inhibitor (TFPI) deficient mice were a kind gift of Dr. George Broze.[16] All mice used in this study were backcrossed to C57BL/6J mice for at least 20 generations before intercrossing. Genotyping for FV, FVL and TFPI was performed by PCR analysis of tail DNA using primers as previously reported.[14-16] Timed matings were performed to analyze in utero progeny from gestational day 18.5.[17] Mice were housed and cared for at the University of Michigan, under compliance with the University of Michigan Committee on the Use and Care of Animals.

Histology

Newborn pups were zinc formalin fixed and sagittally sectioned. Whole-mount paraffin sections were routinely stained with hematoxylin and eosin. Fibrin(ogen) staining was performed as previously described.[16]

Plasma analyses

Mice were anesthetized and blood samples on sodium citrate (final concentration 0.32%) were drawn directly from the inferior caval vein. Blood was centrifuged twice to obtain platelet free plasma which was immediately frozen at -80°C until analysis.

Plasma activity levels of factors II, VIII, IX, X, XI and XII were determined as described elsewhere.[18] The activity of FV was assessed in a 2-step procedure, in which FV is activated with thrombin and subsequently the FV_a activity is quantified by determining the rate of FX_a-catalyzed prothrombin action. Plasma FVII, antithrombin and TFPI activity levels were analyzed by means of commercially available kits from respectively Hyphen Biomed, Chromogenix and American Diagnostica. Fibrinogen antigen levels were measured with a commercial murine

ELISA kit (Affinity Biologicals), and protein C levels were determined with an in-house ELISA using antibodies from Haematologic Inc. Pooled mouse plasma from wild-type mice was used to generate standard curves from which the plasma activity and antigen levels of individual mouse samples could be calculated.
To evaluate overall coagulability of mouse plasmas, the activated partial thromboplastin time (aPTT-SP reagent, Instrumentation Laboratories) and the prothrombin time (STA neoplastin plus reagent, Roche) were measured on the STart 4 analyzer (Diagnostica Stago). Thrombin generation was assessed by means of the Calibrated Automated Thrombogram, using 1 pM tissue factor (PPP-reagent Low, Thrombinoscope) to trigger 1:6 diluted mouse plasma. Thrombin generation was measured on the Fluoroskan Ascent reader (Thermo Scientific) and the curves and area under the curve (endogenous thrombin potential; ETP) were calculated using the Thrombinoscope software. APC resistance was determined by quantifying the effect of added APC on the ETP.[19] The cofactor activity of FV on $FVIII_a$ inactivation was evaluated with the Immunochrom APC Response assay (Progen Biotechnik), according to the protocol described by Govers-Riemslag et al.[20] with the last incubation step being reduced to 2 minutes.

Statistical analyses
Plasma data is presented as mean ± standard error of the mean (SEM) and were analyzed with the Graphpad Instat software package. Survival data was evaluated using a Chi-square test and statistical differences between genotypes were evaluated using a one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test to compare FVL hetero- and homozygous mice with FVL hemizygous mice. A p-value <0.05 was considered to be statistically significant.

Results

Effect of FVL hemizygosity on survival
To generate FVL hemizygous ($FV^{Q/-}$) mice, $FV^{+/-}$ mice were crossed with $FV^{Q/Q}$ mice. Although equal numbers of $FV^{Q/+}$ and $FV^{Q/-}$ progeny were expected, only 23 of 91 mice carried the $FV^{Q/-}$ genotype at the time of weaning ($p<0.001$; table 1). To address the effect of FVL dosage on viability, $FV^{Q/Q}$ and $FV^{Q/-}$ mice were mated and again less $FV^{Q/-}$ mice were present at the time of weaning (53 $FV^{Q/-}$ vs. 131 $FV^{Q/Q}$, $p<0.001$), indicating that decreasing the dose of FVL in the absence of wild-type FV has adverse effects on viability. $FV^{Q/+}$ and $FV^{Q/-}$ mice were crossed to provide a direct comparison of $FV^{Q/+}$, $FV^{Q/Q}$ and $FV^{Q/-}$ in an identical genetic

context, which resulted in a significant reduction of $FV^{Q/-}$ progeny as compared to both $FV^{Q/+}$ and $FV^{Q/Q}$ ($p<0.001$ and $p<0.01$ respectively; table 1).
Timed matings were performed to analyze progeny from gestational day 18.5, just prior to birth. Genotypes for 171 mice from a $FV^{Q/+}$ x $FV^{+/-}$ cross showed that the observed distribution did not differ from the expected distribution (table 1), indicating that the $FV^{Q/-}$ mice progress normally to term but succumb between birth and weaning. A second series of timed matings between $FV^{+/-}$ and $FV^{Q/Q}$ mice were performed, resulting in 52 mice of which 29 carried the $FV^{Q/+}$ and 23 the $FV^{Q/-}$ genotype. However, 20 mice died within 24 hours after birth, of which 15 were $FV^{Q/-}$ ($p=0.02$). These data show that the distribution of genotypes at birth is as expected, but that a substantial fraction of the FVL hemizygotes dies in the immediate postnatal period.

Table 1: Genotype distribution of weaning pups and embryos obtained from experiments designed to assess the lethality of the $FV^{Q/-}$ genotype compared to $FV^{Q/+}$ and $FV^{Q/Q}$.

Number of offspring/genotype		$FV^{+/+}$	$FV^{+/-}$	$FV^{Q/+}$	$FV^{Q/Q}$	$FV^{Q/-}$	Total number of pups analyzed
Parental genotypes							
$FV^{+/-}$	$FV^{Q/Q}$	-	-	68	-	23**	91
$FV^{Q/Q}$	$FV^{Q/-}$	-	-	-	131	53**	184
$FV^{Q/-}$	$FV^{Q/+}$	-	18	23	14	5*	60
Embryonic mating							
$FV^{Q/+}$	$FV^{+/-}$	36	49	50	-	36	171

* $p<0.01$, ** $p<0.001$ (Chi square analysis)

To establish the cause of death, newborn pups that died shortly after birth were collected, of which some showed macroscopic features of coagulopathy (figure 1A). Additional histological analyses of $FV^{Q/-}$ pups revealed signs of macrovascular thrombosis and in 2 out of 6 $FV^{Q/-}$ pups large thrombi within vessels were found which demonstrated the typical layering of fibrin as a hallmark of thrombosis, discriminating these from postmortem blood clots (figure 1). Furthermore, livers of 2 $FV^{Q/-}$ animals featured infarcted areas with necrosis and the presence of multicellular infiltrates. Analysis of serial sections did not show thrombi located outside blood vessels or the presence of bleeding. No histological abnormalities were found in the $FV^{Q/+}$ pups (n=5).

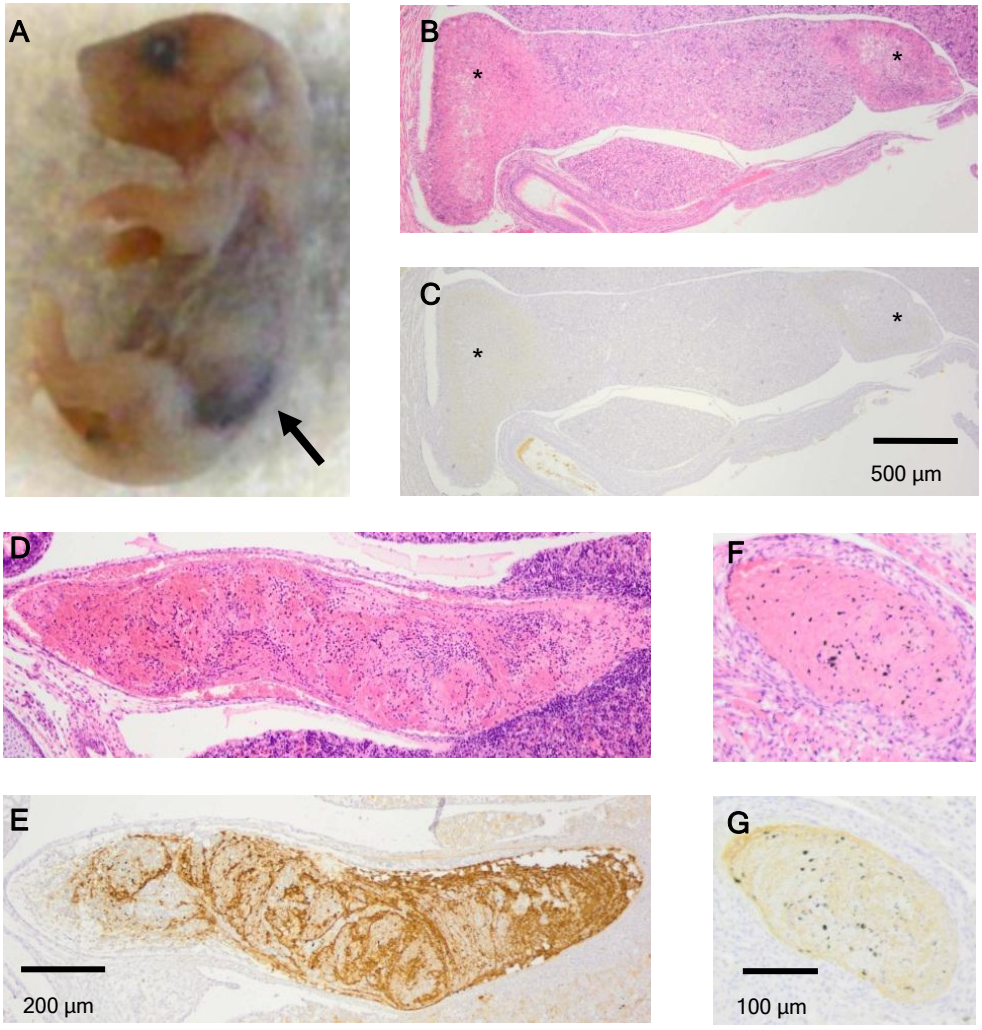

Figure 1: Macroscopic and microscopic examination of dead factor V Leiden hemizygous mice.
Macroscopic features of coagulopathy were observed in $FV^{Q/-}$ mice that succumbed within 24 hours after birth (A). Microscopic analyses of these $FV^{Q/-}$ animals (hematoxylin/eosin and fibrin(ogen) staining) showed infarcted areas in the liver (asterisks in panels B and C) and revealed macrovascular thrombi (panels D-G).

In order to confirm that the $FV^{Q/-}$ genotype predisposes to a thrombotic and not bleeding tendency, we combined these mice with mice lacking one allele of TFPI. Whereas either FVL homozygosity or TFPI heterozygosity does not result in early lethality, we previously demonstrated that a $TFPI^{+/-}$ background transforms homozygosity for factor V Leiden into nearly complete lethality in the immediate perinatal period due to widespread thrombosis.[21] Of the 203 progeny analyzed from a $FV^{Q/-}$,$TFPI^{+/+}$ x $FV^{Q/+}$,$TFPI^{+/-}$ cross, 27 mice were $FV^{Q/Q}$ $TFPI^{+/+}$ which corresponds with the expected 12.5% distribution. However, only 16 $FV^{Q/-}$ $TFPI^{+/+}$ mice were present at the time of weaning, confirming the decreased viability of the hemizygous mice on a wild-type background. On a $TFPI^{+/-}$ background, only 3 $FV^{Q/Q}$ mice and 1 $FV^{Q/-}$ mouse out of 203 were present. Thus, on a prothrombotic $TFPI^{+/-}$ background, the $FV^{Q/-}$ genotype was nearly fully fatal supporting the presence of a thrombotic and not a bleeding phenotype.

Plasma coagulation

The mating studies suggested that the reduced anticoagulant rather than the reduced procoagulant function of FV(L) contributed to the early lethality of the FVL hemizygous mice as compared to the FVL heterozygous mice. Therefore we studied the effect of hemizygosity on coagulation by comparing plasmas from viable $FV^{Q/-}$ mice and $FV^{Q/+}$ littermates. In addition, plasmas from $FV^{Q/Q}$ mice from a parallel cross were included in the comparison.

As expected, FV activity levels were decreased in $FV^{Q/-}$ mice as compared to both $FV^{Q/+}$ and $FV^{Q/Q}$ animals (table 2). In addition, FVIII levels were also lower whereas FII and FVII levels were higher in $FV^{Q/-}$ mice than in $FV^{Q/+}$ and $FV^{Q/Q}$ mice. To assess the impact of the differences in individual coagulation factors on the overall coagulability, thrombin generation assays were performed showing a significantly higher endogenous thrombin potential (ETP) in $FV^{Q/+}$ mice (272±5 nM*min) as compared to $FV^{Q/-}$ mice (213±13 nM*min, $p<0.01$), with an intermediate ETP value for $FV^{Q/Q}$ mice (232±10 nM*min, p=n.s.). Furthermore, the prothrombin time was longer in $FV^{Q/-}$ (11.9±0.1 sec) than in $FV^{Q/+}$ and $FV^{Q/Q}$ animals (both 11.3±0.1 sec, $p<0.05$). The activated partial thromboplastin time was also increased in $FV^{Q/-}$ mice, although this did not reach statistical significance ($FV^{Q/+}$ 29.3±0.6 sec, $FV^{Q/Q}$ 31.0±0.9 sec and $FV^{Q/-}$ 31.3±0.9 sec).

Table 2: Plasma levels of procoagulant factors from factor V Leiden heterozygous ($FV^{Q/+}$), homozygous ($FV^{Q/Q}$) and viable hemizygous mice ($FV^{Q/-}$).

	$FV^{Q/+}$	$FV^{Q/Q}$	$FV^{Q/-}$	p-value
Fibrinogen	121.8±5.0	112.5±6.0	106.7±8.5	n.s.
Factor II	126.2±3.9	121.4±2.5**	134.6±2.9	0.012
Factor V	101.8±4.7**	97.2±6.1**	56.5±3.8	<0.001
Factor VII	103.4±2.7*	103.6±3.0	115.9±5.1	0.034
Factor VIII	110.0±3.4*	101.9±6.8	88.5±6.3	0.020
Factor IX	96.3±1.8	98.7±3.7	100.6±3.9	n.s.
Factor X	99.6±3.1	101.6±2.9	109.9±4.1	n.s.
Factor XI	104.9±5.3	107.4±9.7	106.1±7.7	n.s.
Factor XII	105.3±2.0	98.8±2.3*	108.8±1.8	0.014

Data are expressed as mean percentage of pooled mouse plasma ± standard error of the mean of n=15 ($FV^{Q/+}$ and $FV^{Q/Q}$) or n=10 ($FV^{Q/-}$) mice. *p<0.05 and **p<0.01 as compared to $FV^{Q/-}$ mice by performing an analysis of variance (ANOVA) with an additional Bonferroni post-hoc test.

To evaluate the effects of FVL hemizygosity on anticoagulation, plasma levels of antithrombin, protein C and TFPI were measured. However, no differences between genotypes were present (data not shown). In addition, an ETP-based APC sensitivity ratio was determined. The APC resistance of $FV^{Q/-}$ mice was comparable to $FV^{Q/Q}$ (2.85±0.12 vs. 3.12± 0.31) but significantly higher than $FV^{Q/+}$ mice (1.78±0.11, p<0.01; figure 2). As the ETP-based APC sensitivity ratio does not distinguish between the 2 functional defects of factor V Leiden, i.e. the decreased susceptibility to be inactivated by APC and the reduced cofactor activity in $FVIII_a$ inactivation, we performed an APTT-based APC sensitivity test in which pooled mouse plasma from either wild-type or FVL homozygous mice were titrated in FV deficient plasma.(22) Figure 2b shows that in wild-type plasma, factor V has an obvious cofactor function, whereas this is less present in FVL plasma. Therefore, the specific FV-mediated $FVIII_a$ inactivation was measured, showing a significant decreased capability of $FVIII_a$ inactivation in $FV^{Q/-}$ mice (1.25±0.03) than in $FV^{Q/+}$ mice (1.59±0.07, p<0.01), with $FV^{Q/Q}$ having an intermediate phenotype (1.40±0.03, fig. 2c).

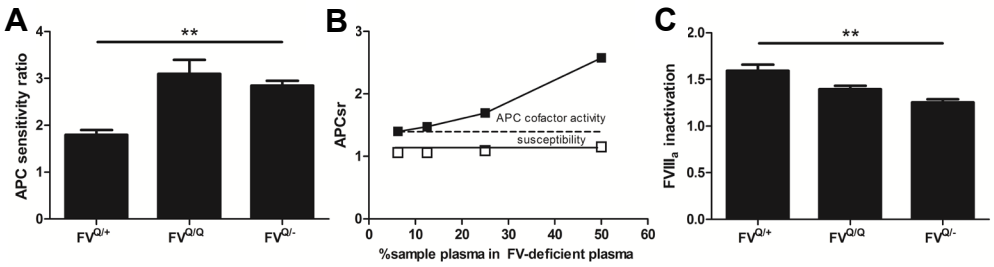

Figure 2: Overview of anticoagulant properties of factor V Leiden heterozygous ($FV^{Q/+}$), homozygous ($FV^{Q/Q}$) and viable hemizygous mice ($FV^{Q/-}$).

The activated protein C (APC) sensitivity ratio for individual mouse plasmas was based on the endogenous thrombin potential (panel A). The APC sensitivity ratio was also assessed in an APTT-based assay, in which pooled wild-type mouse plasma (black) or factor V Leiden mouse plasma (open) was titrated with human factor V deficient plasma, showing that murine FV acts as a cofactor in $FVIII_a$ inactivation (B). FV-mediated $FVIII_a$ inactivation per genotype is shown in panel C. Data are expressed as mean ± standard error of the mean of n=15 ($FV^{Q/+}$ and $FV^{Q/Q}$) or n=10 ($FV^{Q/-}$) mice. **p<0.01 as compared to $FV^{Q/-}$ mice by performing an analysis of variance (ANOVA) with an additional Bonferroni post-hoc test.

Discussion

In the present study we combined $FV^{Q/Q}$ and $FV^{+/-}$ mice to generate unique factor V Leiden hemizygous mice that allowed us to gain further insight into the complex role of factor V and factor V Leiden in (anti)coagulation. The data presented here demonstrate that the $FV^{Q/-}$ genotype induces an unexpected partial lethality in the immediate postnatal period that is related to macrovascular thrombosis; findings that were also confirmed on a $TFPI^{+/-}$ background. Compared to $FV^{Q/+}$ mice, viable $FV^{Q/-}$ mice had an expected overall reduced procoagulant plasma profile, with less thrombin generation and longer prothrombin times. Anticoagulability was also decreased with a more pronounced APC resistance and a diminished capability to inactivate activated FVIII in a FV-dependent manner. As $FV^{Q/-}$ mice die with features of thrombosis and not bleeding, we conclude that the impaired FV anticoagulant function in FVL hemizygosity underlies the fatal phenotype. In addition, comparison of plasma coagulation profiles of $FV^{Q/-}$ and $FV^{Q/Q}$ animals showed only subtle differences. However, as $FV^{Q/Q}$ mice do not display early lethality, this implies that factor V Leiden has maintained a vital and critical fraction of its anticoagulant activity. Overall, this mouse study demonstrates the physiological importance of the anticoagulant function of the factor V allele.

Our data regarding the reduced anticoagulability in surviving FVL hemizygous mice reproduce two important observations for their human counterpart, i.e. decreased FV-mediated $FVIII_a$ inactivation and pseudo-homozygosity for APC resistance.[11] However, the recently reported lower TFPI levels associated with FV deficiency and FVL hemizygosity that is thought to significantly contribute to increased coagulability[12] could not be reproduced in mice as we did not detect a lower plasma TFPI levels upon lack of one FV allele (9.4±0.3 U/mL vs. 10.0±0.2 U/mL for $FV^{Q/+}$ and $FV^{Q/-}$ mice, respectively). Both in mice and human, TFPI has 2 isoforms: TFPIα and TFPIß, which are different in protein structure. The predominant isoform in mouse plasma is TFPIß, whereas in human TFPIα is the main plasma isoform,[23] which may explain the absence of an association between FV and TFPI levels in mice. Besides the absence of a FV-TFPI association, we found an evident postnatal lethality associated with the FVL hemizygous state in mice. For humans, it is estimated that 1:1000 FVL carriers is actually hemizygous, and with approximately 26 million FVL carriers in Europe alone, this would make up for a considerable number of hemizygotes.[10] Whether reduced postnatal survival in humans contributes to the relatively limited reports on factor V Leiden hemizygotes is subject to speculation.

Viable $FV^{Q/-}$ animals had lower fibrinogen and FVIII plasma levels as compared to $FV^{Q/+}$ mice. Although we have no good explanation for these findings, they were confirmed in offspring from multiple matings with different parental genotypes. Low levels of FVIII and fibrinogen provide a reduced procoagulant state and may have enabled survival of those expressing the low levels through selection. On the other hand, the lower fibrinogen and FVIII levels may also be the result of consumptive coagulopathy and thus a secondary effect.[24] In this respect, it is interesting that $FV^{Q/Q}$ mice, which have increased tissue fibrin deposition as a marker of microvascular thrombosis, show intermediate levels of both fibrinogen and factor VIII. Whether $FV^{Q/-}$ mice have more microvascular thrombosis and therefore lower plasma levels of both factors as compared to $FV^{Q/Q}$ mice has not been determined. Since FV and FVIII are structurally related and share steps in posttranslational processing,[25] we considered that the lower FVIII levels result from the decreased FV levels as a significant correlation between these factors was found (Pearson $r=0.52$, $p<0.001$). However, in $FV^{+/-}$ mice, we did not observe lower FVIII levels, thus making a direct relation between FV and FVIII in these mice less likely.

Although the survival of $FV^{Q/Q}$ and viable $FV^{Q/-}$ mice differs markedly, we were unable to detect major differences in plasma coagulation profiles. This implies a

residual anticoagulant function for the factor V Leiden allele that reaches a functionally adequate level in the homozygous but not hemizygous state. Indeed, the level of $FVIII_a$ inactivation appeared to be somewhat higher in $FV^{Q/Q}$ mice as compared to $FV^{Q/-}$, which has also been observed in humans.[11] However, FV might also have an additional function besides it pro- and anticoagulant role in coagulation. For example, a role for FV in fibrinolysis has been revealed,[26-28] and a more impaired fibrinolysis in FVL hemizygosity as compared to FVL homozygotes may contribute to the observed thrombotic phenotype.

In conclusion, our data show partial lethality of factor V Leiden hemizygous mice in the immediate postnatal period due to macrovascular thrombosis. These observations indicate that the lack of the anticoagulant function rather than the lack of the procoagulant function of factor V is of vital importance. In addition, our data imply a residual anticoagulant function of the factor V Leiden allele which can rescue the lethal phenotype associated with factor V Leiden hemizygosity.

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