Haplotypes encoding the factor VIII 1241Glu variation, factor VIII levels and the risk of venous thrombosis

A. Yaël Nossent[1], Jeroen C. J. Eikenboom[1], Hans L. Vos[1], Egbert Bakker[3], Bea C. Tanis[1, 2], Carine J. M. Doggen[2], Rogier M. Bertina[1], Frits R. Rosendaal[1, 2]

[1]Department of Haematology, Haemostasis and Thrombosis Research Center, [2] Department of Clinical Epidemiology and [3]Center for Human and Clinical Genetics; Leiden University Medical Center, Leiden, The Netherlands

Summary

Levels of factor VIII (FVIII) are associated with the risk of venous thrombosis. The FVIII variation D1241E has been reported to be associated with decreased levels of FVIII. Our objective was to study whether D1241E is associated with levels of FVIII and the risk of venous thrombosis and whether this association is caused by D1241E or another linked variation. We analyzed the association of three FVIII gene haplotypes encoding 1241E (further denoted as HT1, HT3, and HT5) with FVIII levels and thrombosis risk. This analysis was performed in the Leiden Thrombophilia Study (LETS). The control populations of two case-controls studies on arterial thrombosis in men and women, respectively, were used to confirm the effects observed on FVIII:C in the LETS. In men, HT1 was associated with a 6% reduction in FVIII:C and with a reduced risk of venous thrombosis [odds ratio 0.4 (CI95 0.2–0.8)]. Logistic regression showed that the risk reduction was only partially dependent of the reduction in FVIII levels. HT1 showed no effects in women on either FVIII:C or risk of thrombosis. The number of carriers of HT3 and HT5 was too low to make an accurate estimate of the risk of venous thrombosis. Neither HT3 nor HT5 showed effects on levels of FVIII:C. When we consider that all three haplotypes encoding 1241E show different effects on FVIII:C and thrombosis risk, it is possible that D1241E is not the functional variation. However, FVIII gene variations do contribute to both levels of FVIII and the risk of thrombosis.

Keywords

FVIII, haplotypes, levels, venous thrombosis, risk

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Introduction

Several studies have shown that elevated levels of coagulation factor VIII (FVIII) are an important risk factor of venous thrombosis. This risk remains after adjusting for the main determinants of FVIII levels, ABO blood group and von Willebrand factor (VWF) (1–8). FVIII activity (FVIII:C) ≥ 150 IU/dl increases the risk of a first venous thrombosis five-fold compared to levels below 100 IU/dl. The frequency of FVIII:C ≥ 150 IU/dl among thrombosis patients is 25%. Since these levels are found in 10% of the population, the contribution of elevated FVIII levels to all thrombotic events in the population is considerable (1).

Several lines of evidence support the idea that high FVIII levels are indeed causative to thrombosis and not a consequence of the thrombotic event, such as a dose-dependent relationship with risk (1), persistence of elevated levels over time (4), no indications for an acute phase response (9), the induction of thrombosis by high FVIII in mouse models (10), dependency on ABO blood group and the familial clustering of FVIII levels (11). The latter, familial clustering, supports the hypothesis that FVIII levels are, at least in part, determined genetically (12). So far, no genetic variations have been found in the gene encoding FVIII that are associated with an increase in levels and thrombotic risk (6, 13). Recently however, a single nucleotide polymorphism (SNP), c94901g (14), causing an amino acid change in the B-domain of FVIII, Asp1241Glu (D1241E), has been reported to be associated with a decrease in FVIII:C. Machiah et al. (15) found that this polymorphism accounts for approximately 5% of the total variation in FVIII:C in the families of the GAIT study. More recently, Scanavini et al. (16) reported that 1241E is as-

Correspondence to:
A. Y. Nossent
Department of Haematology, C2-R
Leiden University Medical Center
P.O. Box 9600
2300 RC Leiden, The Netherlands
Tel.: +31 71 526 6689/1648, Fax: +3 71 5266755
E-mail: A.Y.Nossent@LUMC.nl

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sociated with an 11% reduction in FVIII:C in 145 healthy women and 150 thrombotic women. Since high FVIII:C increases the risk of thrombosis, conditions associated with reduced FVIII:C levels are expected to be associated with a decrease in risk. However, Machiah et al. did not report estimations on risk, and Scanavini et al. did not find significant differences in genotype distribution between cases and controls (15, 16).

It is unclear through which mechanism the 1241E variant could influence levels of FVIII. Scanavini et al. hypothesized that the variation might be involved in APC resistance, but could not demonstrate this (16). It is very well possible that D1241E is not causal to the decrease in levels, but that c94901g encoding D1241E is in linkage disequilibrium with the causal variation. On the website of SeattleSNPs (14), SNP and haplotype data are presented for a wide variety of genes, including the gene encoding FVIII. These data are based on the re-sequencing of these genes in 23 subjects of European-American descent. In this population the SNP c94901g encoding 1241E is present in at least three different haplotypes of which possibly only one could be responsible for the reported effects on FVIII levels.

To investigate the influence on FVIII:C and on the risk of venous thrombosis, we studied the effect of these three common haplotypes encoding the 1241E variant in the Leiden Thrombophilia Study (LETS), a large population based case-control study on venous thrombosis. Associations found between these haplotypes and FVIII:C in the LETS were also evaluated in the control subjects of two large population-based case-control studies on arterial thrombosis, the Study of Myocardial Infarctions Leiden (SMILE), consisting of men under the age of 70 years, and the Risk of Arterial Thrombosis In Relation to Oral Contraceptives Study (RATIO), consisting of women under the age of 50 years.

Patients and methods

Study populations

Leiden Thrombophilia Study (LETS)

The LETS consists of 474 consecutive patients and 474 controls. All patients were referred for anticoagulant treatment after a first objectively confirmed episode of deep vein thrombosis without an underlying malignancy. Controls were matched for sex and age and were acquaintances or partners of the patients. Mean age for both patients and control subjects was 45 years, ranging from 15 to 69 years for patients and 15 to 72 years for controls. Both groups consisted of 272 women (57.4%) and 202 men (42.6%). All patients and controls were of Dutch origin. DNA samples were available of 471 cases and 471 controls. FVIII:C was measured in the plasma of all participants by a one-stage clotting assay. Levels are expressed as IU/dl (1). VWF:Ag was measured by ELISA in the plasma of the first 301 patients and 301 controls. Levels are expressed as IU/ml (1). Pooled normal plasma, calibrated directly against the WHO standard for VWF and FVIII (91/666) was used as a reference. In each assay, two control plasmas were included for inter-assay quality assurance. Plasma samples had not been thawed and refrozen before these measurements. The design of this study has previously been described in more detail (17).

Study of Myocardial Infarctions Leiden (SMILE)

The SMILE consists of 560 patients and 646 control subjects of which only the controls were included here. Cases were men below the age of 70, diagnosed with a first myocardial infarction. The control group consisted of men who were referred to the Leiden Anticoagulation Clinic for prophylactic anticoagulation therapy for a short period following minor orthopaedic interventions. Controls did not have a history of myocardial infarction and had not used anticoagulants for a period of at least six months prior to participating in this study. Controls were frequency matched to cases on 10-year age groups. All patients and controls were of Dutch origin. Plasma and DNA samples were available of all participants. Plasma FVIII:C was measured in two dilutions by a one-stage clotting assay with FVIII deficient plasma and automated activated partial thromboplastin time (APTT, Organon Teknika, Boxtel, The Netherlands) on a STA (Diagnostic Stago, Boehringer Mannheim, Mannheim, Germany). Levels are expressed as IU/dl. Pooled normal plasma, calibrated against an in-house standard, which was calibrated against the WHO standard for VWF and FVIII (91/666), was used as a reference. In each assay, two control plasmas were included for inter-assay quality assurance. Plasma samples had not been thawed and refrozen before these measurements. Exclusion criteria for this study were renal disease, severe (neuro-)psychiatric problems or a life expectancy of less than one year. The design of this study has previously been described in more detail (18).

Risk of Arterial Thrombosis in Relation to Oral Contraceptives Study (RATIO)

The RATIO is a case-control study with patients with different vascular events (ischemic stroke, peripheral vascular disease and myocardial infarction) and healthy controls, of which only the controls were included here. The control group consisted of 925 women between the ages of 18 to 49 who had no history of coronary, cerebral, or peripheral arterial disease. Controls were selected via Random Digit Dialling. Only controls of which plasma samples were available (639 women) were included for the current study. Ninety-three percent of the controls were Caucasian.

Plasma FVIII:C was measured in two dilutions in duplicate by a one-stage clotting assay with FVIII deficient plasma and automated activated partial thromboplastin time (APTT, Organon Teknika) on a STA (Diagnostic Stago, Boehringer Mannheim). Levels are expressed as IU/dl. VWF:Ag was measured by ELISA, using polyclonal antibodies (Dako, High Wycombe, UK). Levels are expressed as IU/dl. Pooled normal plasma, calibrated against an in-house standard which was calibrated against the WHO standard for VWF and FVIII (91/666) was used as a reference. In each assay, two control plasmas were included for inter-assay quality assurance. Plasma samples had not been thawed and refrozen before these measurements. The design of this study has previously been described in more detail (19).

Combined control populations

Because the FVIII gene is located on the X-chromosome, effects were studied in detail in men and women separately. Combining the different control populations, two large control groups were

Table 1: Haplotypes on the FVIII gene. SNP numbering according to SeattleSNPs (April 2004). SNPs indicated with „*" are used to tag haplotypes (94901 encodes D1241E). SNP 94901 distinguishes HT1, HT3 and HT5 from the all-common form and 24052 and 27882 tag HT3 and HT5, respectively.

SNP Haplotype	*24052	*27882	54884	56667	58065	64384	69685	70105	71259	*94901	113927	115794	117638	118374	142242	142737
All-common	G	G	C	C	G	A	G	G	A	C	T	T	I	G	A	A
HT1	G	G	G	T	A	A	A	A	A	**G**	A	C	I	G	G	G
HT3	**A**	G	G	T	G	C	G	G	G	**G**	A	C	D	A	G	G
HT5	G	**C**	G	T	A	A	A	A	A	**G**	A	C	I	G	G	G

formed, all male and all female, respectively. The first control group consisted of all male control subjects from the LETS and all control subjects from the SMILE. The second control group consisted of all female control subjects from the LETS and all control subjects from the RATIO. Because controls were selected stratified for age and because age is known to influence FVIII levels, all analyses on levels were repeated after adjusting for age using linear regression. VWF levels and blood group were not determined in all individuals, so overall correction for these factors was not possible. Analyses were also performed after normalization of levels per study.

Genetic analysis

According to SeattleSNPs (April 2004), the SNP encoding the 1241E variant is present in at least three FVIII haplotypes, indicated throughout this paper as HT1, HT3 and HT5. Haplotypes are depicted in Table 1. In order to identify carriers of these haplotypes, c94901g [numbering according to SeattleSNPs, April 2004 (14); rs1800291], encoding D1241E was determined in all individuals. Subsequently, two haplotype tagging SNPs specific for HT3 and HT5, g24052a (rs6655259) and g27882c (rs-number pending), respectively, were determined in both hetero- and homozygous carriers of the 1241E encoding allele. Genotyping was performed using either polymerase chain reaction – restriction fragment length polymorphisms analyses (PCR-RFLP) or 5'nuclease/Taqman assays.

The polymerase chain reactions with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA, USA) and for PCR-RFLP were performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany). Fluorescence endpoint reading for allelic discrimination of the 5'nuclease/Taqman assay was done on an ABI 7900 HT (Applied Biosystems). PCR conditions, restriction endonucleases and the sequences of probes and primers used for genotyping are available on request. Genotyping was successful in all but eight samples for g24052a (three from the LETS, four from the SMILE and one from the RATIO) and two samples for g27882c (1 from the SMILE and 1 from the RATIO). There was no evidence for the existence of other haplotypes than the three proposed by SeattleSNPs.

Sequencing

All exons and exon-intron boundaries of the FVIII gene were sequenced in DNA samples of nine individuals. Selection criteria for these individuals are described under „Results".

PCR Primers for all 26 exons of the FVIII gene were designed from the FVIIII reference sequence (Accession number AH002692.1). The primers are extended either with an M13-forward or with an M13-reverse sequence. All primers were designed to include complete exon-intron transitions. Fragments for exon 1 and exon 14 were split into two and 10 overlapping fragments, respectively. PCRs were performed in a 25-µl reaction volume, with a final $MgCl_2$ concentration of 1.5 mM except for exon 13. All PCR programs included an initial denaturation of 5 min by 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at either 55°C, 53°C, or 50°C and a final extension at 72°C was performed for 5 min. The PCR products were sequenced in both forward and reverse direction on an ABI Prism 3730 DNA Analyzer (Applied Biosystems) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The raw sequence data were analyzed using SeqScape Software for comparative sequencing analysis (Applied Biosystems). All primers and PCR-conditions are available on request.

Statistical analyses

FVIII:C levels are presented as means with their standard deviation (SD). To evaluate the difference in levels between groups Student's t-test was used. The differences, together with the 95% confidence intervals (CI95) of the differences, are given. The differences in FVIII levels between groups were also studied after adjustment for age using linear regression. To evaluate the influence of the different geno- and haplotypes on the risk of thrombosis, odds ratios (OR) and their corresponding CI95 according to Woolf (20) were calculated. ORs were corrected for levels of FVIII using logistic regression.

Results

c94901g, encoding D1241E, was in Hardy-Weinberg equilibrium for the female controls of the LETS and RATIO. The haplotypes present in the LETS, SMILE and RATIO were in accordance with those reported by SeattleSNPs. Haplotype frequencies were slightly lower than previously reported (14). They were approximately 14% for HT1 and 2% for both HT3 and HT5 in all three studies.

FVIII haplotypes and levels of FVIII

Effects of the FVIII gene variations on levels of FVIII in controls of the separate studies are presented in Tables 2A and B. Results and analyses of the combined control groups are presented in Tables 3A and B.

For D1241E in all the controls combined, lower levels of FVIII:C were observed in the absence of the 1241D encoding allele. Heterozygous carriers and homozygous wildtype carriers had similar levels of FVIII.

In both the separate as well as the combined male control groups, hemizygous carriers of the 1241E encoding alleles had lower levels of FVIII (Tables 2A and 3A). In the combined control group, FVIII:C was reduced by 6%. Looking at the three sep-

arate haplotypes, HT1, HT3 and HT5, the reduction disappeared for carriers of HT3. For carriers of HT5, a reduction in FVIII:C was observed; however, with wide confidence intervals. Also, correction for age made the reduction much smaller while confidence intervals remained wide. For HT1 however, the 6% reduction in FVIII:C remained (Table 3A).

In the female control groups, no clear effect of the 1241E encoding alleles on levels of FVIII was observed (Tables 2B and 3B). Also, the separate haplotypes showed no consistent effects.

In both the male and the female control group, VWF:Ag, which was measured in the RATIO and in part of the LETS only, was not influenced by either D1241E or by the three haplotypes. In those subgroups where VWF and ABO blood group were measured, correction for VWF and blood group did not influence the outcomes.

FVIII haplotypes and the risk of venous thrombosis

The risk of venous thrombosis in the LETS (Table 4A)

Overall, men and women combined, 1241E appeared to slightly decrease the risk of venous thrombosis. Homozygous carriers of the E encoding alleles had an OR of 0.7 (CI95 0.4–1.1). When stratified for haplotypes, it became clear that this risk reduction was caused by HT1. Homozygous carriers of HT1 had an OR of 0.5 (CI95 0.3–0.9). For HT3 and HT5 no reduction in risk was observed.

The risk of venous thrombosis in men (Table 4B)

In men alone we found a more pronounced protective effect of the 1241E encoding alleles against venous thrombosis. Carriers of 1241E had an OR of 0.5 (CI95 0.3–0.9). After stratification for haplotypes this reduction in risk remained mainly for HT1. It was even more pronounced for carriers of HT1 for which the OR was 0.4 (CI95 0.2–0.8).

Table 2: A) FVIII:C (IU/dl) in male control subjects from the LETS and SMILE separately. B) FVIII:C (IU/dl) in female control subjects from the LETS and RATIO separately.

A)

Male controls	Geno- / haplotype *(N)*	LETS		SMILE	
		N	Mean FVIII:C *(SD)*	N	Mean FVIII:C *(SD)*
D1241E	D *(701)*	163	110.6 *(28.8)*	538	124.3 *(33.2)*
-	E *(145)*	38	101.9 *(31.6)*	107	119.2 *(35.6)*
HT1	HTx *(726)*	170	110.2 *(29.4)*	556	124.2 *(33.1)*
-	HT1 *(118)*	30	102.5 *(29.8)*	88	119.2 *(36.4)*
HT3	HTx *(829)*	196	109.1 *(29.1)*	633	123.5 *(33.6)*
-	HT3 *(15)*	4	109.0 (50.4)	11	125.8 *(33.4)*
HT5	HTx *(835)*	198	109.2 *(29.3)*	637	123.6 *(33.6)*
-	HT5 *(10)*	3	90.7 *(36.5)*	7	114.9 *(30.2)*

B)

Female controls	Geno- / haplotype *(N)*	LETS		RATIO	
		N	Mean FVIII:C *(SD)*	N	Mean FVIII:C *(SD)*
D1241E	D / D *(619)*	184	111.0 *(28.2)*	435	114.5 *(47.3)*
-	D / E *(245)*	77	114.4 *(33.5)*	168	108.6 *(39.9)*
-	E / E *(31)*	9	117.4 *(32.1)*	22	125.4 *(56.0)*
HT1	HTx / HTx *(673)*	197	111.4 *(28.7)*	476	114.1 *(46.2)*
-	HTx / HT1 *(197)*	67	113.8 *(33.0)*	130	108.4 *(41.7)*
-	HT1 / HT1 *(24)*	6	118.5 *(35.4)*	18	127.6 *(61.4)*
HT3	HTx / HTx *(865)*	266	112.2 *(29.8)*	599	113.4 *(46.3)*
-	HTx / HT3 *(29)*	4	106.9 *(39.1)*	25	111.3 *(32.1)*
HT5	HTx / HTx *(862)*	258	111.8 *(29.7)*	604	113.5 *(46.2)*
-	HTx / HT5 *(32)*	12	120.7 *(32.7)*	20	109.5 *(32.2)*

Table 3: A) FVIII:C (IU/dl) in all male control subjects from the LETS and SMILE. Δ is the difference between the means given with the corresponding CI95. Δ* is the difference between the mean distances to the regression line after linear regression adjusting for age. HTx signifies all haplotypes but the one given. **B) FVIII:C (IU/dl) in all female control subjects from the LETS and RATIO.** Δ is the difference between the means given with the corresponding CI95 calculated vs. the wildtype (D / D or HTx / HTx). Δ* is the difference between the mean distances to the regression line after linear regression adjusting for age. HTx signifies all haplotypes but the one given.

A)

Male controls	Geno- / haplotype *(N)*	Mean FVIII:C *(SD)*	Δ *(CI95)*	Δ* *(CI95)*
D1241E	D *(701)*	121.1 *(32.7)*	-	-
-	E *(145)*	114.7 *(35.3)*	6.4 *(0.4 to 12.3)*	6.0 *(0.2 to 11.7)*
HT1	HTx *(726)*	120.9 *(32.8)*	-	-
-	HT1 *(118)*	115.0 *(35.5)*	5.9 *(-0.5 to 12.4)*	6.3 *(0.1 to 12.5)*
HT3	HTx *(829)*	120.1 *(33.2)*	-	-
-	HT3 *(15)*	121.3 *(37.4)*	–1.3 *(-18.3 to 15.7)*	–2.6 *(-19.0 to 13.8)*
HT5	HTx *(835)*	120.2 *(33.2)*	-	-
-	HT5 *(10)*	107.6 *(32.3)*	12.6 *(-8.2 to 33.3)*	5.3 *(-14.7 to 25.4)*

B)

Female controls	Geno- / haplotype *(N)*	Mean FVIII:C *(SD)*	Δ *(CI95)*	Δ* *(CI95)*
D1241E	D / D *(619)*	113.5 *(42.5)*	-	-
-	D / E *(245)*	110.4 *(38.0)*	3.1 *(-3.0 to 9.2)*	3.4 *(-3.4 to 10.1)*
-	E / E *(31)*	123.1 *(49.9)*	–9.6 *(-25.1 to 5.9)*	–11.2 *(-28.4 to 5.9)*
HT1	HTx / HTx *(673)*	113.3 *(41.8)*	-	-
-	HTx / HT1 *(197)*	110.2 *(39.0)*	3.1 *(-3.5 to 9.6)*	3.3 *(-4.0 to 10.6)*
-	HT1 / HT1 *(24)*	125.3 *(55.5)*	–12.0 *(-29.2 to 5.3)*	–14.5 *(-33.6 to 4.7)*
HT3	HTx / HTx *(865)*	113.1 *(41.9)*	-	-
-	HTx / HT3 *(29)*	110.7 *(32.4)*	2.4 *(-13.1 to 17.8)*	3.0 *(-14.2 to 20.1)*
HT5	HTx / HTx *(862)*	113.0 *(42.0)*	-	-
-	HTx / HT5 *(32)*	113.7 *(32.3)*	–0.7 *(-15.4 to 14.0)*	–0.3 *(-16.6 to 16.1)*

Table 4: A) Distribution of FVIII geno- and haplotypes in all patients with venous thrombosis and controls from the LETS. † the homozygous genotypes also include hemizygotes. * reference group. **B) Distribution of FVIII genotypes and haplotypes in all male patients with venous thrombosis and all male controls from the LETS.** * reference group. **C) Distribution of FVIII geno- and haplotypes in all female patients with venous thrombosis and all female controls from the LETS.** * reference group.

A)

LETS	Geno- / haplotype†	Cases	Controls	OR *(CI95)*
D1241E	D / D	363	347	**1***
-	D / E	75	77	**0.9** *(0.7 to 1.3)*
-	E / E	33	47	**0.7** *(0.4 to 1.1)*
HT1	HTx / HTx	386	367	**1***
-	HTx / HT1	65	67	**0.9** *(0.6 to 1.3)*
-	HT1 / HT1	20	36	**0.5** *(0.3 to 0.9)*
HT3	HTx / HTx	460	462	**1***
-	HTx / HT3	9	4	**2.3** *(0.7 to 7.4)*
-	HT3 / HT3	2	4	**0.5** *(0.1 to 2.8)*
HT5	HTx / HTx	454	456	**1***
-	HTx / HT5	11	12	**0.9** *(0.4 to 2.1)*
-	HT5 / HT5	6	3	**2.0** *(0.5 to 8.1)*

B)

LETS men	Geno- / haplotype	Cases	Controls	OR *(CI95)*
D1241E	D	180	163	**1***
-	E	22	38	**0.5** *(0.3 to 0.9)*
HT1	HTx	188	170	**1***
-	HT1	14	30	**0.4** *(0.2 to 0.8)*
HT3	HTx	200	196	**1***
-	HT3	2	4	**0.5** *(0.1 to 2.7)*
HT5	HTx	196	198	**1***
-	HT5	6	3	**2.0** *(0.5 to 8.2)*

C)

LETS women	Geno- / haplotype	Cases	Controls	OR *(CI95)*
D1241E	D / D	183	184	**1***
-	D / E	75	77	**1.0** *(0.7 to 1.4)*
-	E / E	11	9	**1.2** *(0.5 to 3.0)*
HT1	HTx / HTx	198	197	**1***
-	HTx / HT1	65	67	**1.0** *(0.7 to 1.4)*
-	HT1 / HT1	6	6	**1.0** *(0.3 to 3.1)*
HT3	HTx / HTx	260	266	**1***
-	HTx / HT3	9	4	**2.3** *(0.7 to 7.6)*
HT5	HTx / HTx	258	258	**1***
-	HTx HT5	11	12	**0.9** *(0.4 to 2.1)*

After adjustment for FVIII:C with logistic regression, the OR for HT1 in men was 0.5 (CI95 0.2–1.0). The reduction in FVIII levels accounted for part of the reduction of the risk of venous thrombosis, but HT1 seems to influence risk in a manner independent of levels as well. HT3 and HT5 were too rare to allow meaningful risk estimates.

The risk of venous thrombosis in women (Table 4C)

In women, virtually no effect on the risk of venous thrombosis was observed. For both hetero- and homozygous carriers of 1241E, the OR was approximately 1. Similar ORs were observed for heterozygous carriers of HT1 and HT5 and homozygous carriers of HT1. In heterozygous carriers of HT3, the risk appeared increased (OR 2.3); however, the confidence interval was wide (0.7–7.6).

Sequencing of the FVIII gene

The effects observed on levels of FVIII and the risk of thrombosis appeared to depend mainly on HT1. To identify haplotype-specific variations that may explain this effect, all exons and exon-intron boundaries of the FVIII gene were sequenced in nine male individuals, three per haplotype. Individuals were selected on phenotype for HT1. Three male controls from the LETS, hemizygous for HT1, were chosen with FVIII:C < 100 IU/dl because of the protective effect on venous thrombosis in men and the reduction in FVIII:C associated with HT1. HT3 and HT5 appeared not to be associated with any particular phenotype. Therefore, for both HT3 and HT5 three hemizygous male carriers were selected without reference to FVIII levels.

Besides the SNPs already reported by SeattleSNPs, no haplotype-specific variations were found. Since none of these previously reported variations are likely to influence either protein levels or protein function, it is possible that functional variations lie in intronic regions of the FVIII gene.

Discussion

Elevated levels of FVIII are an important risk factor for venous thrombosis. There are strong indications that FVIII levels are, at least in part, determined genetically, beyond the well-known effect of ABO blood group. To assess whether the D1241E variation in the B-domain of FVIII, which in previous studies was linked to FVIII levels, is indeed causative to the previously reported effects on FVIII:C (15, 16), we genotyped two additional SNPs to tag three haplotypes that all carry the minor allele of this variation.

We studied these three haplotypes separately in the LETS and in the control populations of the SMILE and the RATIO studies. In general, FVIII:C was relatively high in all three studies. It was shown previously in the LETS that this is not a result of activation (6) since FVIII:C were strongly associated with the actual protein concentration (FVIII:Ag). Nor were these high FVIII levels the result of an acute phase response (9). The high mean FVIII levels were not the result of a shift in the distribution of ABO blood groups (LETS control group, 42.6% with blood group O). So there is no explanation for the overall slightly elevated FVIII levels in our study populations. However, this elevation does not affect analyses comparing effects of the different geno- and haplotypes within these populations.

We observed an association between 1241E and lower FVIII:C, but in men only. In men, a reduction of approximately 6% in FVIII:C was observed in those individuals with 1241E. After stratifying for haplotypes, this effect appeared to be restricted to HT1. The data also imply a reductive trend of FVIII:C for HT5, which is genetically very similar to HT1. However if

HT1 and HT5 are looked at as one haplotype, the effects are weaker then for HT1 alone.

In contrast to Scanavini et al. (16), who described an 11% reduction in FVIII:C in female carriers of the 1241E encoding alleles, we found no effects in female control subjects. Stratifying for haplotypes made no difference. There are few differences between the female controls from the LETS and the RATIO and the controls used by Scanavini et al. (16) that could explain these contrasting results. Scanavini et al. (16) excluded users of oral contraceptives, whereas we did not. However, exclusion or inclusion of oral contraceptive users is highly unlikely to have influenced the results of D1241E in either study. Since prescription of oral contraceptives does not depend on genotype, there is no reason to assume that oral contraceptives users will be unequally distributed over the different geno- and haplotypes.

In summary, we showed that in our study populations a reduction in FVIII:C is restricted to male carriers of HT1, indicating that there is another variation on this haplotype that influences FVIII:C in men only. There is no satisfactory explanation for the discrepancy in effects between the two sexes.

As expected, we observed decreases in risk of venous thrombosis in the group where FVIII levels were decreased. In male carriers of 1241E and particularly carriers of HT1, who showed a 6% reduction in FVIII:C, a strong protection against venous thrombosis was observed. It is tempting to hypothesize that this reduction in risk is caused solely by the reduction in FVIII levels; however, logistic regression showed that only a small part of this effect is caused by the reduction in FVIII:C. Nonetheless, similar to the role of ABO blood group, this again is an indication for a causative association between FVIII:C and the risk of venous thrombosis. Besides the small effect HT1 has on the risk of venous thrombosis via a reduction in FVIII:C, adjustment with logistic regression showed us that HT1 influences the risk of venous thrombosis in a manner independent of FVIII levels as well.

The data presented here suggest that the effects associated with D1241E are limited to HT1. Thus, D1241E is not likely to be the functional variation responsible for the effect on FVIII levels and thrombosis risk as it is present in all three haplotypes. Indeed, so far, no mechanisms for the functionality of D1241E have been identified. The B-domain of the FVIII molecule, in which D1241E is located, is not necessary for procoagulant activity (21) but probably plays an important role in determining levels of FVIII in the circulation (22). The B-domain is heavily glycosylated. At least 19 of the 25 N-linked glycosylation sites on FVIII are located in the B-domain (23, 24). It has been shown that these sites play an important role in the secretion of FVIII (25) and that they may also influence FVIII clearance from the circulation (26). The nearest N-linked glycosylation site to D1241E, however, is 14 residues away. Although it cannot be excluded, the minor change in the side group from aspartic acid to glutamic acid makes it highly unlikely that D1241E would influence glycosylation in the B-domain.

After re-sequencing of the FVIII gene, we did not identify other possibly functional variations specific for HT1. However, we only sequenced exons and exon-intron boundaries, so additional variations undoubtedly exist within non-coding regions. So even though no functional variant was found in HT1, and numbers of carriers of the other two haplotypes, HT3 and HT5, remained very small, we should seriously consider the possibility of the existence of an as yet unidentified functional mutation in HT1 other than D1241E. At the same time, the possibility also exists that as yet unidentified variations in HT3 and HT5 exist that counteract the effects of D1241E, masking its functionality in these two haplotypes.

In conclusion, we observed a reduction in FVIII:C that was restricted to male carriers of HT1. HT1 was also associated with a decrease in risk of venous thrombosis. A small part of this decrease in risk could be explained by the reduction in FVIII levels. In addition, HT1 had a protective effect against venous thrombosis, which could not be explained by the reduction in FVIII:C as well. Although this should be investigated further, it is possible that D1241E is not the main player in these effects, since both carriers of HT3 and HT5 apparently show no reduction in either FVIII:C or in the risk of venous thrombosis.

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