F9 Malmö, factor IX and deep vein thrombosis

Irene D. Bezemer,[1] Andre R. Arellano,[2] Carmen H. Tong,[2] Charles M. Rowland,[2] Helen A. Ireland,[3] Kenneth A. Bauer,[4] Joseph Catanese,[2] Pieter H. Reitsma,[5,6] Carine J.M. Doggen,[1] James J. Devlin,[2] Frits R. Rosendaal,[1,5,3] and Lance A. Bare[2]

[1]Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands; [2]Celera, Alameda, CA, USA; [3]Department of Cardiovascular Genetics, Division of Medicine, University College London, London, UK; [4]Beth Israel Deaconess Medical Center, Molecular Medicine Unit, Boston, MA, USA; [5]Department of Thrombosis and Haemostasis, Leiden University Medical Center, Leiden, The Netherlands, and [6]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, the Netherlands

Acknowledgments: the authors acknowledge the contributions of the Celera High Throughput Laboratory and Computational Biology group and thank J. Sninsky and T. White for helpful comments on this manuscript. We thank R. van Eck, I.K. van der Linden, J. van der Meijden and P.J. Noordijk who performed laboratory measurements.

Funding: the Leiden Thrombophilia study was supported by the Netherlands Heart Foundation (grant 89.063). The Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis was supported by the Netherlands Heart Foundation (grant NHS 98.113), the Dutch Cancer Foundation (grant RUL 99/1992) and the Netherlands Organisation for Scientific Research (grant 912-03-033 | 2003). Ms. Bezemer was supported by a grant from the Leducq Foundation, Paris, France for the development of Transatlantic Networks of Excellence in Cardiovascular Research (grant 04 CVD 02).

Manuscript received November 7, 2008. Revised version arrived December 5, 2008. Manuscript accepted December 15, 2008.

Correspondence:
Frits R. Rosendaal, Department of Clinical Epidemiology, C7-P Leiden University Medical Center P.O. Box 9600 2300 RC Leiden, The Netherlands.
E-mail: f.r.rosendaal@lumc.nl

ABSTRACT

Background

We recently reported the association between the Malmö sequence variant in *F9* (rs6048) and deep vein thrombosis.

Design and Methods

We aimed to study whether the association between *F9* Malmö and deep vein thrombosis is explained by linkage disequilibrium with nearby single-nucleotide polymorphisms, and whether the association is explained biologically by *F9* Malmö affecting factor IX antigen levels or activation of factor IX. We investigated the association of *F9* Malmö and 28 nearby single-nucleotide polymorphisms with deep vein thrombosis in men from two case-control studies, LETS (n=380) and MEGA (n=1,469). We assessed the association of *F9* Malmö with factor IX antigen level in male control subjects from LETS (n=191) and two subsets of MEGA (n=823 and n=484) and the association with endogenous thrombin potential in LETS control men. We studied the association between *F9* Malmö and factor IX activation peptide in 1,199 healthy middle-aged men from the NPHS-II cohort.

Results

In the combined LETS and MEGA studies, the odds ratio (95% confidence interval) for the G allele of *F9* Malmö, compared with the A allele, was 0.80 (0.69-0.93). One single-nucleotide polymorphism in *F9*, rs422187, was strongly linked to *F9* Malmö (r^2=0.94) and was similarly associated with deep vein thrombosis. No other single-nucleotide polymorphism or haplotype tested was more strongly associated. Factor IX antigen level, factor IX activation peptide levels and endogenous thrombin potential did not differ between *F9* Malmö genotypes.

Conclusions

The *F9* Malmö sequence variant was the most strongly associated with deep vein thrombosis among common single-nucleotide polymorphisms in the region. However, the biological mechanism by which *F9* Malmö affects risk remains unknown.

Key words: factor IX, single nucleotide polymorphism, venous thrombosis.

Citation: Bezemer ID, Arellano AR, Tong C, Rowland CM, Ireland HA, Bauer KA, Catanese J, Reitsma PH, Doggen CJM, Devlin JJ, Rosendaal FR, and Bare LA. F9 Malmö, factor IX and deep vein thrombosis. Haematologica 2009; 94:693-699. doi:10.3324/haematol.2008.003020

Introduction

The incidence rate of deep vein thrombosis in the general population is estimated to be about 1 per 1,000 person-years leading to 500,000 new cases per year in Europe and 400,000 in the US.[1,2] Deep vein thrombosis is a multicausal disease that results from acquired (e.g., age, oral contraceptive use, surgery) and genetic risk factors (e.g., factor V Leiden, prothrombin G20210A). Elevated plasma levels of coagulation factors (e.g., VIII,[3] IX,[4] X,[5] and XI[6]) have been shown to be important risk factors for deep vein thrombosis. Antigen levels of factor IX above the 90th percentile (129U/dL) have been associated with a 2 to 3-fold increased risk of deep vein thrombosis.[4] We recently tested 19,682 potentially functional single-nucleotide polymorphisms (SNPs) for association with deep vein thrombosis.[7] One of the SNPs identified was an A>G sequence variant in the gene encoding factor IX (rs6048, *F9 Malmö*) and the G allele was associated with a 15-43% decrease in deep vein thrombosis risk compared with the A allele, in three case-control studies of deep vein thrombosis. This common variant (minor allele frequency = 0.32) leads to the substitution of alanine for threonine at amino acid position 148 in the portion of the factor IX zymogen that is cleaved from the zymogen to activate factor IX.[8] This variant has not been reported to be associated with hemophilia B. The mechanism by which F9 Malmö affects risk of deep vein thrombosis is unclear.

In this study we calculated the pooled odds ratios in the case-control studies in which the association of *F9* Malmö with deep vein thrombosis was initially identified. We investigated whether the association between *F9* Malmö and deep vein thrombosis could be explained by linkage disequilibrium between *F9* Malmö and other *F9* variants. We assessed whether the association could be explained biologically by *F9* Malmö affecting factor IX antigen levels, activation of factor IX or endogenous thrombin potential.

Design and Methods

Study populations and data collection

The case-control populations (LETS, MEGA-1 and MEGA-2) used to analyze the association of genotypes with deep vein thrombosis are derived from two large population-based case-control studies; the Leiden Thrombophilia Study (LETS) and the Multiple Environmental and Genetic Assessment (MEGA) of risk factors for venous thrombosis. Samples from the Northwick Park Heart Study–II (NPHS-II) were used to evaluate the association of *F9* Malmö genotype with factor IX activation peptide.

Collection and ascertainment of events in LETS have been described previously.[9] Briefly, patients were recruited between January 1, 1988 and December 30, 1992 from three anticoagulation clinics in the western part of the Netherlands: Leiden, Amsterdam, and Rotterdam. A total of 474 consecutive patients, 70 years or younger, with a diagnosis of a first deep vein thrombosis of the leg and without a known malignancy were included. For each patient, an age- (±5 years) and sex-matched control subject who had no history of deep vein thrombosis was enrolled. In the present analysis of LETS, we excluded 52 participants due to inadequate quantity or quality of DNA. After these exclusions, 443 patients and 453 controls remained.

Collection and ascertainment of events in MEGA have been described previously.[10,11] Briefly, MEGA enrolled consecutive patients aged 18-70 years who presented with a first diagnosis of deep vein thrombosis in leg or arm or pulmonary embolism at any of six anticoagulation clinics in the Netherlands (Amsterdam, Amersfoort, The Hague, Leiden, Rotterdam, and Utrecht) between March 1st, 1999 and May 31st, 2004. Control subjects in MEGA included partners of patients and random population control subjects frequency-matched on age and sex to the patient group and were selected so that the distribution of age and sex matched that of the patient group. For the present analyses we excluded patients who had a pulmonary embolism without a documented deep vein thrombosis or a history of malignant disorders to obtain a study population similar to the LETS population. We split the MEGA study to form two case-control studies, based on recruitment date and sample availability (blood or buccal swab). The first subset of MEGA, *MEGA-1*, included 1,398 patients and 1,757 controls who donated a blood sample. The remaining 1,314 patients and 2,877 controls, who donated either a blood sample or a buccal swab sample, were included in *MEGA-2*.

We calculated the pooled odds ratio for deep vein thrombosis in men and women from the LETS and MEGA studies. The gene effect in men, who have one X-chromosome, was assumed to be equivalent to the effect in women homozygous for one allele, compared to women homozygous for the other allele. Heterozygous women were assumed to have an in-between gene effect. The analysis of gene variants in *F9* and deep vein thrombosis risk was restricted to men. The analysis of *F9* Malmö with factor IX antigen levels and endogenous thrombin potential, was restricted to men not using vitamin K antagonists. The LETS study included 188 male patients and 192 control men (191 with factor IX antigen level measurements and 157 with endogenous thrombin potential measurements), the MEGA-1 study included 637 male patients and 832 control men (823 with factor IX antigen level measurements) and the MEGA-2 study included 620 male patients and 1,327 control men (484 with factor IX antigen level measurements).

The Northwick Park Heart Study-II (NPHS-II) has been described previously.[12] Briefly, a cohort of 4,600 men aged 50-63 years registered with 9 general medical practices in England and Scotland were screened for eligibility in the NPHS-II. Exclusion criteria for the original study included: a history of unstable angina or acute myocardial infarction (AMI); a major Q wave on the electrocardiogram (ECG); regular aspirin or anticoagulant therapy; cerebrovascular disease; life-threatening malignancy; conditions exposing staff to risk or precluding informed consent. Factor IX activation peptide was measured in the first available sample from individuals who, at the time of sampling, had not had a coronary heart disease event.

Gene variants in the F9 region and risk of deep vein thrombosis

To investigate whether other SNPs in this region might be associated with deep vein thrombosis, we used results from the HapMap Project to identify a region defined by linkage disequilibrium with *F9* Malmö ($r^2 \geq 0.2$, chr X: 138,404,951 to 138,494,063). Apart from the *F9* gene, this region also contains part of the gene encoding MCF.2 cell line derived transforming sequence (*MCF2*). This region contained 48 SNPs with allele frequencies >0.02 (HapMap data release #221, phase II April07, on NCBI B36 assembly, dbSNP 126). Allele frequencies and linkage disequilibrium were calculated from the SNP genotypes in the HapMap CEPH population, which includes Utah residents with ancestry from Northern and Western Europe. Eighteen SNPs were selected for genotyping using pairwise tagging in Tagger (implemented in Haploview).[13] We were unable to construct assays for 3 (rs4149754, rs438601, rs17340148) of the 18 tag SNPs; the remaining 15 SNPs are reasonable surrogates for 45 of the 48 SNPs in this region because the 45 SNPs are either directly genotyped or in strong linkage disequilibrium ($r^2 > 0.8$) with at least one of the 15 genotyped SNPs. In addition, we genotyped 14 candidate SNPs.[14,15] Thus, in total 29 SNPs were initially investigated in the men of LETS. SNPs that were associated with deep vein thrombosis were subsequently investigated in the men of *MEGA-1*.

Factor IX antigen level

The levels of factor IX were determined in LETS and MEGA by enzyme-linked immunosorbent assay (ELISA) as previously described.[4] This ELISA is highly specific for factor IX and results are not affected by the levels of the other vitamin K–dependent proteins. The intra-assay and interassay coefficients of variation were 7% and 7%, respectively, in LETS and 4 % and 3% in MEGA. Results are expressed in units per deciliter (dL), where 100 U is the amount of factor IX present in 1 dL pooled normal plasma.

Factor IX activation peptide

Factor IX activation peptide was determined by double antibody radioimmunoassay[16] as a marker of turnover of factor IX in the NPHS-II samples. The intra-assay coefficient of variation was less than 5% and the interassay coefficient of variation was about 10% for factor IX activation peptide.

F9 Malmö and endogenous thrombin potential

Endogenous thrombin potential is an activate protein C (APC) sensitivity test that quantifies the time integral of thrombin generated in plasma in which coagulation is initiated via the extrinsic pathway.[17,18] The sensitivity of the plasma endogenous thrombin potential to APC was measured in LETS under conditions where the test is insensitive for small amounts of phospholipid present in plasma as previously described.[19,20]

Allele frequency and genotype determination

DNA concentrations were standardized to 10 ng/μL using PicoGreen (Molecular Probes, Invitrogen Corp, Carlsbad, CA, USA) fluorescent dye. Genotyping of individual DNA samples was performed as previously described[7] using 0.3ng of DNA in kPCR assays[21] or using multiplexed oligo ligation assays (OLA).[22] Genotyping accuracy of the multiplex methodology and kPCR has been assessed in three previous studies by comparing genotype calls from multiplex OLA assays with those from real time kPCR assays for the same SNPs, and the overall concordance of the genotype calls from these two methods was >99% in each of these studies.[23-25] Primer sequences are available upon request.

Statistical analysis

The combined analysis of *F9* Malmö in LETS, MEGA-1 and MEGA-2 was performed using the meta package version 0.8-2 available in R software language version 2.4.1 (*http://www.r-project.org*) by treating the individual studies as fixed effects and using the inverse variance method to estimate the pooled effect of the SNP.[26]

Deviations from Hardy–Weinberg expectations were assessed using an exact test in controls. Odds ratios (OR) and 95% confidence intervals (95% CI) were computed as an estimate of risk of deep vein thrombosis associated with each SNP using logistic regression.

The association between haplotype and deep vein thrombosis was assessed using the R language package (*http://cran.us.r-project.org/*) of haplo.stats,[27] which uses the expectation maximization algorithm to estimate haplotype frequencies followed by testing for association between haplotype and disease using a score test. A sliding window was used to select and test haplotypes consisting of 3, 5, and 7 adjacent SNPs from the set of SNPs including *F9* Malmö and the 28 other SNPs.

Differences in the factor IX antigen level and endogenous thrombin potential between *F9* Malmö genotypes were assessed in control subjects of LETS, MEGA-1 and MEGA-2 using a *t*-test assuming equal variance among the groups. Differences in factor IX activation peptide between *F9* Malmö genotypes in NPHS-II were also assessed using a *t*-test. *t*-tests were performed with SAS version 9. Power to detect a difference in mean factor IX antigen levels, activation peptide or thrombin potential between *F9* Malmö genotypes was calculated using nQuery Advisor version 4.0[28] for a two sample *t*-test at a 0.05 two-sided significance level and assuming equal variance among the groups.

Results

Combined analysis of F9 Malmö and deep vein thrombosis

In the combined analysis of LETS, MEGA-1 and MEGA-2, we found that *F9* Malmö was associated with deep vein thrombosis in men: the pooled odds ratio was 0.80 (95% CI, 0.69-0.93) for the G (n=1,108) compared with the A genotype (n=2,688). The pooled corresponding odds ratio in women was 0.89 (95% CI, 0.74-1.08) for the GG (n=405) compared with the AA genotypes (n=2,190), assuming an in-between effect in heterozygous women (n=1,750) (Figure 1).

Factor IX antigen level and risk of deep vein thrombosis

Factor IX antigen levels in LETS were previously reported to be positively associated with risk of deep vein thrombosis. Individuals with factor IX levels above the 90th percentile of the control group levels had a 2- to 3-fold higher risk of deep vein thrombosis.[4] In the MEGA study, we confirmed the results published for LETS. We found that individuals with factor IX antigen levels above the 90th percentile had a 1.7-fold (95% CI 1.4-2.1) increased risk of deep vein thrombosis, compared with those with levels below the 90th percentile. After adjustment for age, sex, oral contraceptive use and vitamin K-dependent coagulation factors II, VII and X (all coagulation factors dichotomized at the 90th percentile) the odds ratio was 1.8 (95% CI 1.4-2.2). Among men, the unadjusted odds ratio in the MEGA study was 1.8 (95%CI 1.3-2.4).

Gene variants in F9 region

To study the region surrounding *F9* Malmö we investigated 15 tag SNPs that served as surrogates for other SNPs in the region and 14 additional candidate SNPs in LETS men (Figure 2). We found that 7 of the 29 SNPs, including *F9* Malmö, were associated ($p \leq 0.05$) with deep vein thrombosis (Table 1). We then investigated the association between these 7 SNPs and deep vein thrombosis in MEGA-1 men and found that 2 were significantly associated with deep vein thrombosis in MEGA-1: *F9* Malmö and rs422187, a SNP that is located 421 bp from *F9* Malmö in intron 5 (Table 1). The minor allele frequency and risk estimate for the association between rs422187 and deep vein thrombosis was similar to that of *F9* Malmö as LD between rs422187 and the *F9* Malmö SNP was high (r^2=0.94). We did not find any haplotype of adjacent SNPs (*data not shown*) that was more strongly associated with deep vein thrombosis in LETS or MEGA-1 than *F9* Malmö or rs422187.

F9 Malmö and factor IX antigen level

We investigated whether the association between *F9* Malmö and deep vein thrombosis could be explained by an association between *F9* Malmö and factor IX antigen levels. Among the male control subjects from LETS, MEGA-1 and MEGA-2 for whom factor IX antigen levels were measured we did not find a difference in factor IX level between the A and the G genotype (Table 2).

F9 Malmö and factor IX activation

We investigated whether the association between *F9* Malmö and deep vein thrombosis could be explained by differential activation of factor IX. Among men of NPHS-II for whom factor IX activation peptide levels were avail-

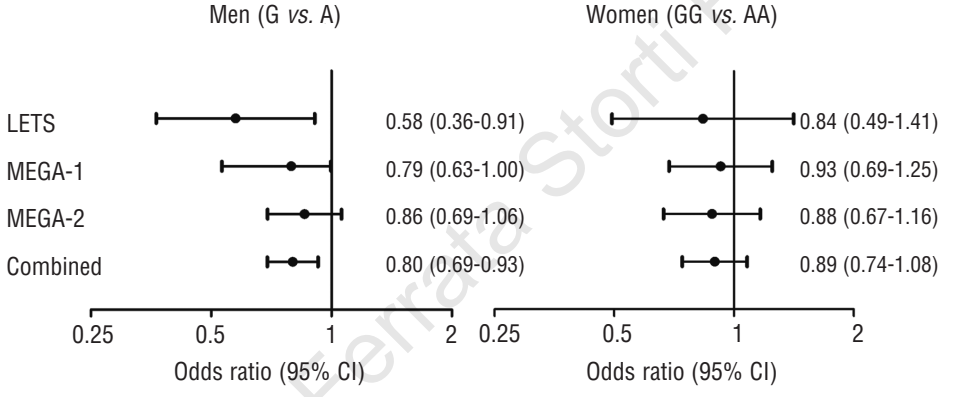

Figure 1. Combined analysis of *F9* Malmö with deep vein thrombosis in men and women.

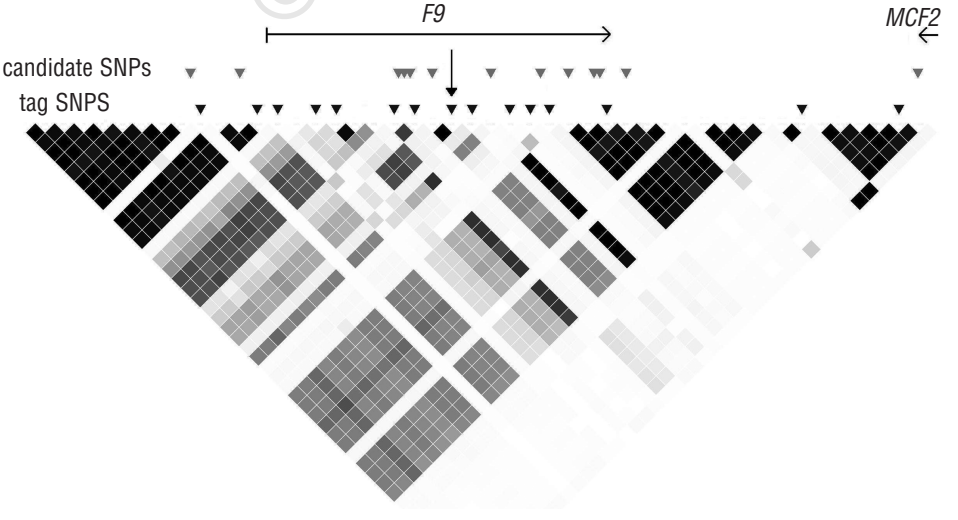

Figure 2. In the selected region on the X chromosome (positions 138,404,951 to 138,494,063), 48 SNPs with MAF>0.02 were genotyped in the HapMap CEPH population. The figure represents pairwise linkage disequilibrium (r^2) between the 48 SNPs. High r^2 values are represented as black squares, fading to gray and white as linkage disequilibrium becomes weaker. Based on these r^2 values, 18 tag SNPs were selected of which 15 were genotyped in LETS (black triangles). In addition, we selected 14 candidate SNPs (gray triangles), of which one (rs2235708) was located outside the selected region. The arrow indicates *F9* Malmö (rs6048).

able we compared the mean activation peptide levels between the A and G genotypes. There was no evidence of lower levels of factor IX activation peptide in men with the *F9* Malmö genotype (Table 2).

F9 Malmö and endogenous thrombin potential

We also investigated the association between *F9* Malmö and deep vein thrombosis through measuring the endogenous thrombin potential. In the test performed in LETS, coagulation is initiated via the extrinsic pathway. Among the male control subjects of LETS for whom endogenous thrombin potential was measured, we found no difference between *F9* Malmö genotypes (Table 2).

The LETS, MEGA and NPHS-II studies had sufficient power to detect small differences in factor IX or factor IX activation peptide levels between *F9* Malmö genotypes A and G. The combined LETS and MEGA factor IX antigen level assays had a standard deviation of 17 U/dL and the combined study had 90% power to detect mean factor IX antigen level differences of 0.18 standard deviations (3.1 U/dL) or greater. The factor IX activation peptide assay had a standard deviation of 75 pmol/L and the study had 90% power to detect mean factor IX activation peptide level differences of 0.25 standard deviations (19 pmol/L) or greater. The endogenous thrombin potential assay had a standard deviation of 348 Nm.min and the study had 90% power to detect mean thrombin potential differences of 0.55 standard deviations (190 Nm.min) or greater.

Discussion

We investigated whether the previously reported association between *F9* Malmö and deep vein thrombosis could be explained by linkage disequilibrium with SNPs

Table 1. Gene variants in *F9* region and risk of deep vein thrombosis in LETS and MEGA-1.

rs number	Position (chr X)	Study	r^2 with F9 Malmö	MAF (allele)	OR*	(95 % CI)	*p*
rs4829996	138418800	LETS	0.36	0.36 (A)	0.67	(0.43-1.05)	0.084
rs7055668 (T)	138427049	LETS	0.02	0.08 (G)	0.92	(0.43-1.97)	0.835
rs411017	138439768	LETS	0.22	0.39 (A)	0.66	(0.43-1.01)	0.058
rs378815 (T)	138439863	LETS	0.01	0.39 (T)	0.67	(0.44-1.03)	0.067
rs3817939 (T)	138440752	LETS	0.04	0.02 (G)	0.67	(0.11-4.04)	0.659
rs371000 (T)	138443187	LETS	0.04	0.44 (C)	1.14	(0.76-1.70)	0.536
rs4149674 (T)	138444467	LETS	0.62	0.38 (T)	0.74	(0.48-1.14)	0.177
rs392959 (T)	138449920	LETS	0.02	0.08 (T)	1.07	(0.52-2.18)	0.855
rs398101	138451623	LETS	0.82	0.34 (G)	0.61	(0.39-0.96)	0.031
		MEGA-1	0.85	0.33 (G)	0.83	(0.66-1.03)	0.094
rs4149755	138451778	LETS	0.03	0.06 (A)	1.42	(0.63-3.17)	0.395
rs4149758	138455143	LETS	0.19	0.08 (A)	0.73	(0.32-1.62)	0.434
rs374988 (T)	138458881	LETS	0.03	0.07 (G)	1.00	(0.45-2.22)	1.000
rs422187	138460525	LETS	0.95	0.33 (C)	0.61	(0.39-0.97)	0.035
		MEGA-1	0.93	0.34 (C)	0.77	(0.61-0.96)	0.022
rs6048 (Malmö) (T)	138460946	LETS	NA	0.33 (G)	0.58	(0.36-0.91)	0.017
		MEGA-1	NA	0.32 (G)	0.79	(0.63-1.00)	0.046
rs4149759 (T)	138462720	LETS	0.08	0.09 (C)	0.74	(0.35-1.58)	0.439
rs413957	138465182	LETS	0.45	0.24 (G)	0.58	(0.35-0.96)	0.035
		MEGA-1	0.45	0.23 (G)	0.83	(0.65-1.07)	0.159
rs4149761 (T)	138467129	LETS	0.01	0.01 (T)	0.00	–	0.999
rs4149730 (T)	138467398	LETS	0.01	0.03 (T)	0.99	(0.31-3.14)	0.993
rs421766	138468258	LETS	0.43	0.24 (C)	0.56	(0.33-0.94)	0.028
		MEGA-1	0.47	0.22 (C)	0.85	(0.65-1.09)	0.196
rs4149762 (T)	138469863	LETS	<0.01	0.05 (A)	1.76	(0.78-3.95)	0.171
rs370713	138470059	LETS	0.45	0.25 (C)	0.57	(0.34-0.95)	0.031
		MEGA-1	0.46	0.22 (C)	0.85	(0.66-1.10)	0.220
rs4149749	138470891	LETS	0.00	0.00 (T)	NA	–	–
rs4149763	138472372	LETS	0.00	0.00 (A)	NA	–	–
rs440051 (T)	138472583	LETS	0.43	0.24 (A)	0.61	(0.36-1.00)	0.052
rs434144	138474091	LETS	0.40	0.26 (G)	0.50	(0.30-0.84)	0.009
		MEGA-1	0.44	0.23 (G)	0.86	(0.67-1.10)	0.233
rs17342358 (T)	138482244	LETS	<0.01	0.01 (A)	0.00	–	0.999
rs5907573 (T)	138489652	LETS	0.01	0.45 (T)	1.21	(0.81-1.81)	0.355
rs3128282	138490821	LETS	0.01	0.44 (C)	1.26	(0.84-1.88)	0.262
rs2235708	138506410	LETS	<0.01	0.01 (A)	0.00	–	0.999

*(T): tag SNP; MAF: minor allele frequency; *OR is the odds ratio for the minor relative to the major allele; NA: not applicable, minor allele not observed.*

Table 2. Association of *F9* Malmö (rs6048) with factor IX antigen level in male control subjects of LETS and MEGA, with factor IX activation peptide in NPHS-II participants and with endogenous thrombin potential in male control subjects of LETS.

	F9 Malmö A		F9 Malmö G		Difference
	N	Mean (sd)	N	Mean (sd)	(95% CI)[1]
Factor IX antigen level (U/dL)					
LETS	127	104 (17)	64	109 (24)	5 (-1 to 11)
MEGA-1	562	104 (17)	261	102 (18)	-3 (-5 to 0)
MEGA-2	344	104 (15)	140	104 (18)	0 (-3 to 3)
Combined	1033	104 (17)	465	103 (19)	0 (-2 to 1)
Factor IX activation peptide (pmol/L)					
NPHS-II	840	217 (78)	359	226 (85)	8 (-1 to 18)
Endogenous thrombin potential (Nm.min)					
LETS	102	1657 (341)	55	1583 (361)	-73 (-189 to 41)

[1] *T-test.*

that had a stronger association with deep vein thrombosis than *F9* Malmö. We also studied whether factor IX antigen levels, factor IX activation peptide or endogenous thrombin potential differed by *F9* Malmö genotype, which would provide a biological explanation for the association with deep vein thrombosis. However, the present results did not support any of these hypotheses.

The OR for the association of *F9* Malmö with deep vein thrombosis was slightly lower in men than in women of all three studies, and broader confidence intervals were observed in women.

We did not identify any SNP that was more strongly associated with deep vein thrombosis than *F9* Malmö in LETS and MEGA-1 among 29 SNPs we genotyped in the *F9* region. For 3 SNPs initially selected for genotyping in LETS we were unable to construct genotyping assays. These 3 SNPs are unlikely to be responsible for the observed association between *F9* Malmö and deep vein thrombosis since they were not in strong LD (r^2<0.2) with *F9* Malmö in the HapMap CEPH population. A recent study found that rs4149755 in the *F9* gene was associated with deep vein thrombosis in postmenopausal women.[15] We included rs4149755 in our analysis in LETS, but rs4149755 was not associated with deep vein thrombosis in men (Table 1) or in women (*data not shown*). We also did not find any haplotype that was more strongly associated with deep vein thrombosis in both LETS and MEGA-1 than *F9* Malmö.

The lack of an association between *F9* Malmö and factor IX antigen levels is consistent with the results of a previous study, where none of 27 SNPs including F9 Malmö were associated with factor IX levels.[14] In a recent analysis in LETS, no association was observed either;[29] the present study also included the LETS and confirmed the findings in the MEGA study, which has a much larger number of samples. The combined analysis of LETS and MEGA had sufficient power to detect small factor IX antigen level differences between genotypes; the observed levels were almost identical. In addition to factor IX antigen levels we studied factor IX activation. This analysis did not yield an explanation for the observed association between *F9* Malmö and risk of deep vein thrombosis either. The power calculations suggest that we can likely (90% power) exclude differences of 19 pmol/L or greater, but we cannot rule out smaller differences. We used an indirect assay to estimate activation of factor IX. The plasma level of the factor IX activation peptide is dependent on the release of activation peptide that occurs when factor IX is activated to factor IXa and the steady clearance rate of the activation peptide in the kidneys.[30] Direct measurements of factor IX activation by tissue factor (TF):factor VIIa or factor XIa might reveal effects on the activation of factor IX caused by *F9* Malmö.

The endogenous thrombin potential is the end product of the TF activation pathway and is therefore an alternative measure of the activity of factor IX or any other factor in the coagulation cascade. We found no evidence of an association between *F9* Malmö and thrombin potential, although we cannot rule out a small difference between the genotypes.

If the associations of *F9* Malmö with deep vein thrombosis observed in this study are replicated in future studies, the question remains by which mechanism *F9* Malmö reduces risk of deep vein thrombosis. Our study included SNPs with minor allele frequencies of at least <2% and was not designed to detect functional variants that are less frequent. It is also possible that one or more of the observed associations is the result of linkage disequilibrium with a low frequency variation.

In conclusion, the Malmö SNP in *F9*, rs6048, was associated with a 20% reduction in risk of deep vein thrombosis in a combined analysis of men from LETS, MEGA-1 and MEGA-2. The association was not explained by linkage disequilibrium with other SNPs in the *F9* region. We were not able to provide a biological explanation for the association of *F9* Malmö with deep vein thrombosis, as we found no evidence for an association of *F9* Malmö with either factor IX antigen levels or activation of factor IX, indirectly measured by factor IX activation peptide plasma levels and by the endogenous thrombin potential. Additional studies should focus on investigating direct measurements of factor IX activation and on linkage disequilibrium between *F9* Malmö and a rare (minor allele frequency 0.02) sequence variant.

Authorship and Disclosures

IDB, HAI, KAB, PHR, CJMD, FRR, LAB: study design; IDB, ARA, HAI, JC, CJMD, FRR: data acquisition; IDB, ARA, CT, CMR, HAI, KAB, PHR, CJMD, JJD, FRR, LAB: analysis and interpretation; IDB, LAB: drafting of the manuscript; critical revision of the manuscript: all authors. HAI, KAB, PHR, CJMD, JJD, FRR, LAB: study supervision.

The funding organizations did not play a role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; or the preparation, review, or approval of the manuscript. LAB, ARA, CHT, CMR, JC, and JJD have employment and ownership interests.

Preliminary results were presented at the International Society on Thrombosis and Haemostasis (ISTH) Congress; July 10, 2007; Geneva, Switzerland.

The remaining authors have no disclosures.

References

1. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerström J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost 2007; 5:692-9.
2. Cohen AT, Agnelli G, Anderson FA, Arcelus JI, Bergqvist D, Brecht JG, et al. Venous thromboembolism (VTE) in Europe. The number of VTE events and associated morbidity and mortality. Thromb Haemost 2007; 98:756-64.
3. Kyrle PA, Minar E, Hirschl M, Bialonczyk C, Stain M, Schneider B, et al. High plasma levels of factor VIII and the risk of recurrent venous thromboembolism. N Engl J Med 2000;343:457-62.
4. van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. Blood 2000;95:3678-82.
5. de Visser MCH, Poort SR, Vos HL, Rosendaal FR, Bertina RM. Factor X levels, polymorphisms in the promoter region of factor X, and the risk of venous thrombosis. Thromb Haemost 2001;85:1011-7.
6. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000;342:696-701.
7. Bezemer ID, Bare LA, Doggen CJM, Arellano AR, Tong C, Rowland CM, et al. Gene variants associated with deep vein thrombosis. JAMA 2008; 299:1306-14.
8. Graham JB, Lubahn DB, Lord ST, Kirshtein J, Nilsson IM, Wallmark A, et al. The Malmö polymorphism of coagulation factor IX, an immunologic polymorphism due to dimorphism of residue 148 that is in linkage disequilibrium with two other F.IX polymorphisms. Am J Hum Genet 1988;42:573-80.
9. Koster T, Rosendaal FR, De Ronde H, Briët E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 1993; 342:1503-6.
10. Blom JW, Doggen CJM, Osanto S, Rosendaal FR. Malignancies, prothrombotic mutations, and the risk of venous thrombosis. JAMA 2005; 293:715-22.
11. van Stralen KJ, Rosendaal FR, Doggen CJM. Minor injuries as a risk factor for venous thrombosis. Arch Intern Med 2008;168:21-6.
12. Miller GJ, Bauer KA, Barzegar S, Cooper JA, Rosenberg RD. Increased activation of the haemostatic system in men at high risk of fatal coronary heart disease. Thromb Haemost 1996;75:767-71.
13. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263-65.
14. Khachidze M, Buil A, Viel KR, Porter S, Warren D, Machiah DK, et al. Genetic determinants of normal variation in coagulation factor (F) IX levels: genome-wide scan and examination of the FIX structural gene. J Thromb Haemost 2006; 4:1537-45.
15. Smith NL, Hindorff LA, Heckbert SR, Lemaitre RN, Marciante KD, Rice K, et al. Association of genetic variations with nonfatal venous thrombosis in postmenopausal women. JAMA 2007;297:489-98.
16. Bauer KA, Kass BL, ten Cate H, Hawiger JJ, Rosenberg RD. Factor IX is activated in vivo by the tissue factor mechanism. Blood 1990;76:731-6.
17. Hemker HC, Beguin S. Thrombin generation in plasma: its assessment via the endogenous thrombin potential. Thromb Haemost 1995;74:134-8.
18. Nicolaes GA, Thomassen MC, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. Blood Coagul Fibrinolysis 1997;8:28-38.
19. Rosing J, Tans G, Nicolaes GA, Thomassen MC, van Oerle R, van der Ploeg PM, et al. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. Br J Haematol 1997;97:233-8.
20. Tans G, van Hylckama Vlieg A, Thomassen MC, Curvers J, Bertina RM, Rosing J, et al. Activated protein C resistance determined with a thrombin generation-based test predicts for venous thrombosis in men and women. Br J Haematol 2003; 122:465-70.
21. Germer S, Holland MJ, Higuchi R. High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. Genome Res 2000;10:258-66.
22. Iannone MA, Taylor JD, Chen J, Li MS, Rivers P, Slentz-Kesler KA, et al. Multiplexed single nucleotide polymorphism genotyping by oligonucleotide ligation and flow cytometry. Cytometry 2000;39:131-40.
23. Iakoubova OA, Tong CH, Chokkalingam AP, Rowland CM, Kirchgessner TG, Louie JZ, et al. Asp92Asn polymorphism in the myeloid IgA Fc receptor is associated with myocardial infarction in two disparate populations: CARE and WOSCOPS. Arterioscler Thromb Vasc Biol 2006;26:2763-8.
24. Shiffman D, Rowland CM, Louie JZ, Luke MM, Bare LA, Bolonick JI, et al. Gene variants of VAMP8 and HNRPUL1 are associated with early-onset myocardial infarction. Arterioscler Thromb Vasc Biol 2006; 26:1613-8.
25. Shiffman D, O'Meara ES, Bare LA, Rowland CM, Louie JZ, Arellano AR, et al. Association of gene variants with incident myocardial infarction in the Cardiovascular Health Study. Arterioscler Thromb Vasc Biol 2008;28:173-9.
26. Cooper H, Hedges LV. The Handbook of Research Synthesis. Newbury Park, CA, USA. Russel Sage Foundation, 1994.
27. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. Am J Hum Genet 2002;70:425-34.
28. Elashoff JD. NQuery Advisor Version 4.0 User's Guide. Los Angeles, CA, USA. Statistical Solutions Limited. 2008.
29. van Minkelen R, de Visser MCH, van Hylckama Vlieg A, Vos HL, Bertina RM. Sequence variants and haplotypes of the factor IX gene and the risk of venous thrombosis. J Thromb Haemost 2008;6:1610-3.
30. Lowe GD. Factor IX and thrombosis. Br J Haematol 2001;115:507-13.