Genetic variation and susceptibility to venous thrombosis : Etiology and risk assessment

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Association Between F9 Malmö, Factor IX And Deep Vein Thrombosis

Chapter 6

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ABSTRACT

Background: We recently reported the association between the Malmö polymorphism in *F9* (rs6048) and deep vein thrombosis.

Objectives: To study whether the association between *F9* Malmö and deep vein thrombosis is explained by linkage with nearby SNPs, and whether the association is explained biologically by *F9* Malmö affecting factor IX levels or activation of factor IX.

Methods: We investigated the association of *F9* Malmö and 28 nearby SNPs with deep vein thrombosis in men from two case-control studies, LETS (n=380) and MEGA (n=1469). We assessed the association between *F9* Malmö and factor IX level in male control subjects from LETS (n=191) and two subsets of MEGA (n=823 and n= 484). We studied the association between *F9* Malmö and factor IX activation peptide in 1199 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 (0.71-0.92). One SNP in *F9*, rs422187, was strongly linked to *F9* Malmö (r^2=0.94) and was similarly associated with venous thrombosis. No other SNP or haplotype tested was more strongly associated. Factor IX level or factor IX activation peptide levels did not differ between *F9* Malmö genotypes.

Conclusions: The *F9* Malmö polymorphism appears to be causally related to deep vein thrombosis, as the association was not explained by linkage with other SNPs in the region. However, the biological mechanism by which *F9* Malmö affects risk remains unknown.

INTRODUCTION

The incidence rate of deep vein thrombosis in the general population is estimated to be about 1 per 1000 person-years leading to 500,000 new cases per year in Europe and 400,000 in the US [1,184]. 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 [185], IX [180], X [130], and XI [171]) have been shown to be important risk factors for deep vein thrombosis. 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 [180]. We recently tested 19,682 potentially functional SNPs for association with deep vein thrombosis [186]. One of the SNPs identified was an A>G polymorphism in the gene encoding factor IX (rs6048, "*F9* Malmö") and the G allele was associated with a 15 to 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 [187]. 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 men and women from 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 levels or activation of factor IX.

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 has been described previously [17]. 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 has been described previously [19,20]. Briefly, MEGA enrolled consecutive patients aged 18 to 70 years who presented with a first diagnosis of deep vein thrombosis in leg or arm or pulmonary embolism at any of 6 anticoagulation clinics in the Netherlands (Amsterdam, Amersfoort, The Hague, Leiden, Rotterdam, and Utrecht) between March 1, 1999 and May 31, 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 2 case-control studies, based on recruitment date and sample availability (blood or buccal swab). The first subset of MEGA, "MEGA-1", included 1398 patients and 1757 controls who donated a blood sample. The remaining 1314 patients and 2877 controls, who donated either a blood sample or a buccal swab sample, were included in "MEGA-2".

We excluded women heterozygous for *F9* Malmö from our combined analysis of *F9* Malmö and deep vein thrombosis in LETS, MEGA-1 and MEGA-2 because in women, one of the two X chromosomes in every cell is randomly inactivated early in embryonic development. Therefore, the expressed genotypes may be different from the determined genotypes. The analysis of gene variants in *F9* and deep vein thrombosis risk, and of *F9* Malmö and factor IX levels, was restricted to men. For the factor IX level analysis, vitamin K antagonist users were excluded. The LETS study included 188 male patients and 192 control men (191 with factor IX level measurements), the MEGA-1 study included 637 male patients and 832 control men (823 with factor IX level measurements) and the MEGA-2 study included 620 male patients and 1327 control men (484 with factor IX level measurements).

The Northwick Park Heart Study-II (NPHS-II) has been described previously [21]. Briefly, a cohort of 4600 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 [170]). 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 [183,188]. 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 level

The levels of factor IX were determined in LETS and MEGA by enzyme-linked immunosorbent assay (ELISA) as previously described [180]. 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 [189] 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.

Allele Frequency and Genotype Determination

DNA concentrations were standardized to 10 ng/µL using PicoGreen (Molecular Probes, Invitrogen Corp, Carlsbad, CA) fluorescent dye. Genotyping of individual DNA samples was performed as previously described [186] using 0.3ng of DNA in kPCR assays [165] or using multiplexed oligo ligation assays (OLA) [190]. 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 [166-168]. 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 [191].

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 [192],

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 level 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. Subjects using vitamin K antagonists were excluded from the factor IX level analysis. Differences in factor IX activation peptide between *F9* Malmö genotypes in NPHS-II were assessed using a T-test. T-tests were performed with SAS version 9. Power to detect a difference in mean factor IX activation peptide between *F9* Malmö genotypes was calculated using nQuery Advisor version 4.0 [193] for a two sample t-test at a 0.05 two-sided significance level and assuming equal variance among the groups.

RESULTS

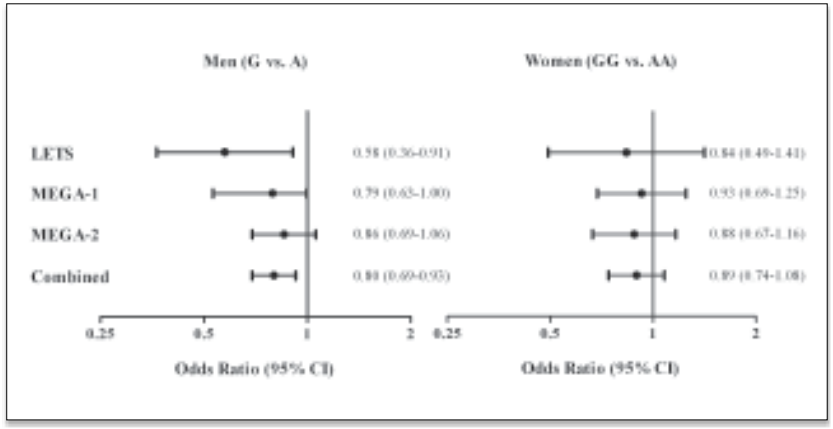

Figure 1. Combined analysis of F9 Malmö with deep vein thrombosis in men and women.

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, 0.71-0.92) for the G (n=1108) compared with the A genotype (n=2688). The pooled odds ratio in women was 0.86 (95% CI, 0.70-1.05) for the GG (n=405) compared with the AA genotypes (n=2190) (Fig. 1).

Factor IX level and risk of deep vein thrombosis

Factor IX 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 venous thrombosis. In women, the effect estimates depended on the use of oral contraceptives, with higher risk observed in non-users [180]. In the current analysis of MEGA, we found that individuals with factor IX 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) and similar to the risk estimate in LETS (OR 2.0, 95% CI, 1.3-3.0). Among men, the unadjusted odds ratio in MEGA (OR 1.8, 95%CI 1.3-2.4) was also similar to that in LETS (OR 1.9, 95%CI 1.0-3.6).

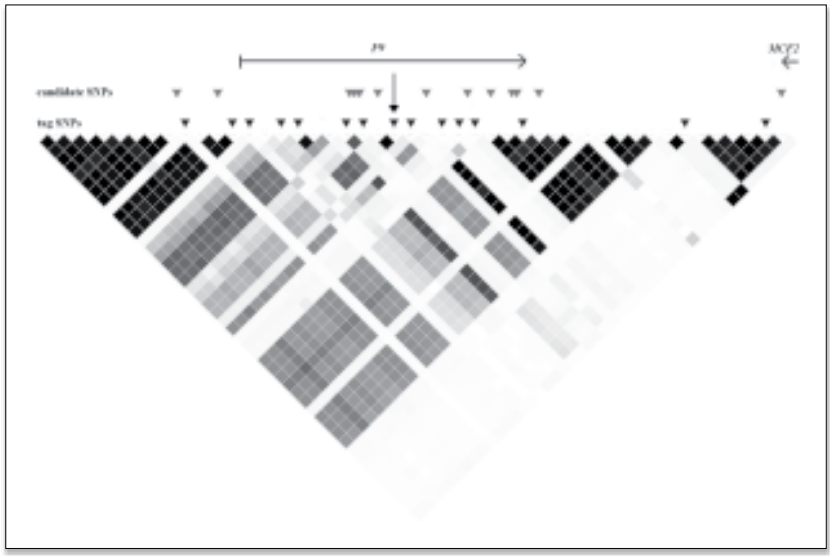

Figure 2 In the selected region on the X chromosome (positions 138,404,951 to 138,494,063), 48 SNPs with MAF>0.02 were genotyped in the HapMap CEPH population. The figure represent pairwise linkage disequilibrium (r2) between the 48 SNPs. High r2 values are represented as black squares, fading to gray and white as linkage becomes weaker. Based on these r2 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).

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 (Fig. 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.

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	0.33	0.32 (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	0.23	0.39 (A)	0.66 (0.43 - 1.01)	*0.058*
rs378815 (T)	138439863	LETS	0.23	0.39 (T)	0.67 (0.44 - 1.03)	*0.067*
rs3817939 (T)	138440752	LETS	0.01	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	0.03	0.08 (T)	1.07 (0.52 - 2.18)	*0.855*
rs398101	138451623	LETS	0.83	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)	*1000*
rs422187	138460525	LETS	0.95	0.33 (C)	0.61 (0.39 - 0.97)	*0.035*
		MEGA-1	0.94	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	0.09	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	0.02	0.01 (T)	0.00 (NA)	*0.999*
rs4149730 (T)	138467398	LETS	0.01	0.03 (T)	0.99 (0.31 - 3.14)	*0.993*
rs421766	138468258	LETS	0.44	0.24 (C)	0.56 (0.33 - 0.94)	*0.028*
		MEGA-1	0.48	0.22 (C)	0.85 (0.65 - 1.09)	*0.196*
rs4149762 (T)	138469863	LETS	0.00	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*

rs number	Position (chr X)	Study	r^2 with F9 Malmö	MAF (allele)	OR (95 % CI) *	*p*
rs4149749	138470891	LETS	0.00	0.00 (T)	NA	-
rs4149763	138472372	LETS	0.00	0.00 (A)	NA	-
rs440051 (T)	138472583	LETS	0.44	0.24 (A)	0.61(0.36 - 1.00)	*0.052*
rs434144	138474091	LETS	0.41	0.26 (G)	0.50 (0.30 - 0.84)	*0.009*
		MEGA-1	0.45	0.23 (G)	0.86 (0.67 - 1.10)	*0.233*
rs17342358 (T)	138482244	LETS	0.00	0.01 (A)	0.00 (NA)	*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	0.00	0.01 (A)	0.00 (NA)	*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.

F9 Malmö and factor IX 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 levels. Among the male control subjects from LETS, MEGA-1 and MEGA-2 for whom factor IX 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 1199 men of NPHS-II for whom factor IX activation peptide levels were available 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).

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

	F9 Malmö A		F9 Malmö G		Difference (95% CI)*
	N	Mean (sd)	N	Mean (sd)	
factor IX 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)

* T-test.

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 level assays had a standard deviation of 17 U/dl and the combined study had 90% power to detect mean factor IX 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.

DISCUSSION

We investigated whether the previously reported association between *F9* Malmö and deep vein thrombosis could be explained by linkage disequilibrium with SNPs that had a stronger association with deep vein thrombosis than *F9* Malmö. We also studied whether factor IX levels or factor IX activation peptide 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 similar in women and in men, but the removal of heterozygous women from the analysis resulted in broader confidence intervals 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 venous thrombosis in postmenopausal women [183]. 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 levels is consistent with the results of the GAIT study, where none of 27 SNPs including *F9* Malmö were associated with FIX:C [188]. Our investigation of factor IX activation did not yield an explanation for the observed association between *F9* Malmö and risk of deep vein thrombosis either. The power calculations suggest we can likely (90% power) exclude differences of 19pmol/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 [194]. Direct measurements of factor IX activation by TF:FVIIa or FXIa might reveal effects on the activation of factor IX caused by *F9* Malmö.

If the associations of *F9* Malmö with deep vein thrombosis observed in this study are replicated in future studies, a question that remains is what is the mechanism by which *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 support for the proposed mechanism for the association of *F9* Malmö with deep vein thrombosis. We found no evidence for an association of *F9* Malmö with either factor IX levels or activation of factor IX, indirectly measured by factor IX activation peptide plasma levels. Additional studies should focus on investigating direct measurements of factor IX activation by the extrinsic pathway (e.g. FVII:TF complex) and intrinsic pathway (e.g. Factor XIa), and on linkage disequilibrium between *F9* Malmö and a rare (minor allele frequency <0.02) polymorphism.

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