Venous and arterial thrombosis : associations and risk factors

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Part I

High levels of protein C are determined by *PROCR* haplotype 3

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Abstract

Background
Genetic determinants of plasma levels of protein C (PC) are poorly understood. Recently, we identified a locus on chromosome 20 determining high PC levels in a large Dutch pedigree with unexplained thrombophilia. Candidate genes in the LOD-1 support interval included *FOXA2*, *THBD* and *PROCR*.
Objectives
To examine these candidate genes and their influence on plasma levels of PC.
Patients/Methods
Exons, promoter and 3'UTR of the candidate genes were sequenced in twelve family members with normal to high PC levels. Four haplotypes of *PROCR*, common in the European population, were examined in the family, and critical SNPs encountered during resequencing were genotyped in the family and in a large group of healthy individuals (the Leiden Thrombophilia Study (LETS) controls). Soluble endothelial protein C receptor (sEPCR) and soluble thrombomodulin (sTM) plasma levels were measured in the family.
Results
PROCR haplotype 3 (H3) and *FOXA2* rs1055080 were associated with PC levels in the family but only *PROCR H3* was also associated with plasma levels in the healthy individuals. Carriers of both variants had higher PC levels than carriers of only *PROCR H3* in the family but not in healthy individuals, suggesting that a second determinant is present between *FOXA2* and *PROCR*. Plasma levels of PC and sEPCR were associated in both studies, contrasting to sTM which was not associated with variations of *THBD* or with PC levels.
Conclusion
Chromosome 20 harbors a locus influencing PC and sEPCR plasma levels and a detailed analysis of candidate genes suggests that *PROCR H3* is responsible.

Introduction

Protein C (PC) is a vitamin K-dependent plasma glycoprotein that circulates at a concentration of ~40-80 nM and plays a major role in the control of the coagulation cascade. Upon activation by the thrombin-thrombomodulin complex, activated PC (APC) inactivates FVa and FVIIIa, consequently reducing thrombin formation[1]. Binding of PC to the endothelial protein C receptor (EPCR) increases the activation rate of PC 20-fold [2]. Individuals with abnormalities in components of the PC pathway (such as PC or protein S deficiency) have an increased risk of venous thrombosis[3].

Determinants of variation in PC levels are poorly known but, given the high heritability of about 50%, genetic determinants are likely to be important [4,5]. The PC gene (*PROC*) is located on chromosome 2 and around 6% of the variability in PC levels has been attributed to polymorphisms in the promoter region of the gene [6,7]. The GAIT (Genetic Analysis of Idiopathic Thrombosis) study estimated that additive effects of genes outside of the *PROC* structural locus cause approximately half of the phenotypic variation in PC levels [4]. The authors identified a major quantitative trait locus (QTL) for PC levels on chromosome 16, where *NQO1*, a gene encoding a quinone reductase involved in the metabolism of vitamin K, is located. Subsequently they found that variations in this gene were associated with PC levels [5].

Variations in the *PROCR* gene (encoding the EPCR) were associated with a moderate increase in levels of PC in the Leiden Thrombophilia Study (LETS) [8] and in the Cardiovascular Health Study (CHS) [9]. While this work was in progress, a genome-wide association scan (GWAS) in the Atherosclerosis Risk in Communities (ARIC) study claimed four loci associated with PC levels, that included *PROC*, *PROCR* and three other genes (*EDEM2*, *GCKR* and *BAZ1B*) [10].

The GENES study was designed to search for novel hereditary risk factors for venous thrombosis in families with unexplained thrombophilia [11]. Previously, a genome-wide linkage analysis was performed. In one particular family, a QTL influencing PC levels was found on chromosome 20 (chr 20), with a log-odds (LOD) score of 4.8 at 51cM [12]. In the 1-LOD support interval (38-64cM), three candidate genes encoding components potentially influencing PC levels are

present, namely forkhead box A2 (*FOXA2,* previously known as hepatic nuclear factor 3ß), thrombomodulin (*THBD*), and the endothelial protein C receptor (*PROCR*).

FOXA2 is part of the forkhead box family and encodes a transcription factor (FOXA2) for a large number of genes, including *PROC*. Two binding sites for FOXA2 in the promoter region of *PROC* have been described and mutations in this region were associated with type I PC deficiency [13].

THBD encodes thrombomodulin (TM), a transmembrane protein that, in complex with thrombin, enhances thrombin-mediated PC activation by more than 1000-fold [1].

PROCR encodes the already mentioned EPCR. Besides the membrane-anchored form, a soluble form of EPCR (sEPCR) lacking the transmembrane and cytoplasmic domain is present in human plasma. Like EPCR, sEPCR binds PC and APC with similar affinity [14]. Binding of sEPCR to APC inhibits its anticoagulant activity by impairing the inactivation of FVa, and binding to PC prevents PC activation by thrombin-TM complexes [15]. Four haplotypes of *PROCR* are present in the European population [8]. Haplotype 3 (H3) is tagged by a missense variation that leads to the Ser219Gly variant (rs867186). The Gly219 variant is associated with increased levels of sEPCR which can be explained by an increased sensitivity of the protein to sheddases such as metalloprotease ADAM17 16 and by the expression of an alternatively spliced mRNA that lacks the sequence encoding the transmembrane domain [17].

In this study, we investigated whether genetic variations in the three candidate genes on chr 20 influence the plasma level of PC.

Patients and methods

Subjects

GENES. The pedigree analyzed in the present study is one of the 22 families originally included in GENES, a study of Dutch families with unexplained thrombophilia [11]. Probands with personal and/or family history of venous thrombosis (defined as at least one first degree or two second degree relatives with venous thrombosis) but with none of the known inherited thrombophilic defects (i.e. PC-, protein S-, antithrombin deficiency, factor V Leiden or prothrombin

G20210A variation) were recruited together with their extended pedigree, including spouses. A standardized history was taken and for most individuals, plasma and DNA samples were obtained for coagulation tests and genotyping [12]. This analysis focuses on the largest pedigree in GENES, consisting of 185 individuals distributed over five generations. In this family, four individuals had a history of thrombosis of whom two had experienced more than one event. Detailed information about these individuals is given in table 1.

Leiden Thrombophilia Study (LETS). For replication of the results obtained in the family, relevant DNA variations were genotyped in healthy (i.e., non-thrombotic) individuals, who were the controls in a population-based case-control study for venous thrombosis (LETS). The design of this study has been described before [18]. Briefly, 474 consecutive patients with a first episode of deep vein thrombosis and 474 sex- and age-matched healthy controls were included. All patients were younger than 70 years and had no overt malignancy. Controls were healthy acquaintances and partners brought by the patients, accordingly to pre-established criteria. Mean age for patients and controls was 45 years (range 15-69 for patients and 15-72 for controls). Standardized questionnaire, DNA and plasma samples were obtained from all the participants. GENES and LETS were approved by the Central Committee on Research Involving Human Subjects (CCMO) and all participants have provided informed consent.

Table 1. Characteristics of family members with venous thrombosis.

	First episode of VT			Second episode of VT			PC	*PROCR*	*FOXA2*
Sex	Type	Age (y)	Risk factor	Type	Age (y)	Risk factor	(%)	genotype	rs1055080
Female	PE	32	Pregnancy	-	-	-	-	H1H2	CC
Male	DVT	41	Trauma	-	-	-	82	H2H2	CC
Male	DVT	4	Trauma	DVT	28	Idiopathic	114	H2H3	CT
Female	DVT	40	Idiopathic	DVT	44	Idiopathic	178	H3H3	CT

Abbreviations - VT: venous thrombosis; PE: pulmonary embolism; DVT: deep vein thrombosis. PC levels are presented as percentage of reference pooled plasma. *FOXA2* rs1055080: NM_021784.4:c.*50C>T or NM_153675.2:c.*50C>T.

Resequencing of candidate genes

Three genes in the linkage region (LOD-1 support interval) on chr 20 were selected for further analysis.

FOXA2 has two known mRNA splicing variants. The first variant (NM_021784) contains 2 exons and covers 2422 bases. Alternative splicing at the 5'-end results in a second mRNA variant (NM_153675) in which translation initiation starts 6 amino acids later than in variant 1. Variant 2 also has an additional (untranslated) 5'-exon, leading to a total mRNA length of 2410 bases. *THBD* is transcribed from an intron-less gene as a 4109 bases long mRNA (NM_000361). *PROCR* has four exons with transcription length of 1449 bases (NM_006404).

To investigate genetic variations associated with PC levels in the family, we selected twelve family members based on their PC levels: (a) three with normal levels (72, 75 and 82%); (b) three with intermediate high levels (114, 116 and 128%); and (c) six with the highest levels (range: 166 – 212%). Three out of four patients with thrombosis were included in this panel (table 2). PC plasma level was not available for the fourth individual.

Exons and their flanking regions, 5' and 3' UTRs, and 1000 bp upstream to the initiation codon were resequenced. For *FOXA2*, the DNA sequence covering both splicing variants and 1000 bp upstream to exon 1 of both isoforms was analyzed. Primers and PCR conditions are available on request.

After amplification, the PCR product was sequenced using an ABI Prism® 3730 DNA Analyzer (Applied Biosystems, Carlsbad, California, USA). The results were analyzed using vector NTI® software version 10 (Invitrogen, Paisle, UK). Potentially interesting variations found by sequencing were investigated in all family members and in the healthy individuals using single nucleotide polymorphism (SNP) genotyping assays.

SNP genotyping assays

All SNPs were determined using TaqMan SNP genotyping assays (Applied Biosystems, California, USA). PCR reactions were performed in 384-well plates using the GeneAmp PCR System 9700 (Applied Biosystems, California, USA) and fluorescent endpoints were read on a 7900 HT Real-Time PCR System (Applied Biosystems, California, USA).

Common haplotypes of *PROCR* were tested in all family members with DNA available. Three haplotype tagging SNPs were chosen: rs2069952 (H1, pre-designed assay), rs867186 (H3, pre-designed assay) and rs2069951 (H4, custom

assay). The presence of the rare allele determines the mentioned haplotype and the presence of three common genotypes determines H2 [8].
Two common variations in *FOXA2* (rs1055080 and rs2277764) that were identified during sequencing analysis in individuals with high levels of PC were investigated in all family members and/or in healthy individuals using a custom TaqMan genotyping SNP assay.

Plasma assays
Protein C. In both the family and healthy individuals, blood was collected in tubes containing 0.106 mol L^{-1} trisodium citrate. Plasma was prepared by centrifugation at 2000 g for 10 min at room temperature and stored at -70°C [12,18]. PC levels were determined using a chromogenic assay (Chromogenix, Mölndal, Sweden). Levels were expressed as percentage of the level in a reference pooled plasma. Measurements in GENES and LETS were performed in different laboratories, several years apart, using different reference pooled plasmas.
Plasma soluble EPCR (sEPCR) levels were determined in the family using the Asserachrom sEPCR ELISA kit (Diagnostica Stago, Asnières, France) according to the manufacturer's instructions. Samples were tested in duplicate and plasmas were diluted 1/26 prior to the assay.
Plasma soluble TM (sTM) levels were measured in the family using the CD141 ELISA kit (Diaclone, Besançon, France) according to the manufacturer's instructions. Samples were tested in triplicate in non-diluted plasmas.

Linkage analysis
To assess the influence of the investigated genetic variations and plasma measurements of sEPCR and sTM on the QTL on chr 20 for PC levels, linkage analyses were performed using SOLAR [12]. The effects of the genetic variations were assessed by adding them to the marker set or by adding them as covariate to the linkage model (conditional analyses). Effects of sEPCR and sTM levels were assessed by adding them as covariates to the linkage model. In addition, LOD scores for sEPCR and sTM levels were determined on chromosome 20. Following Lander and Kruglyak [19], and correcting for two phenotypes, we used thresholds of 3.6 for genome wide significance and 2.2 for suggestive linkage.

Statistical analysis

Mean and 95% confidence interval (CI95) were used to compare continuous variables with normal distribution (i.e., PC and sEPCR). Patients using vitamin K antagonists at the time of venapuncture were excluded. Median and range were used to describe sTM because of the skewed distribution in the family. Linear regression analysis was used to analyze the correlation between sEPCR and PC levels. All calculations were performed using PASW Statistics 17.0 (IBM Corporation, Somers, USA).

Results

Genetic analysis of candidate genes PROCR, FOXA2 and THBD

Resequencing of candidate genes yielded five variations in *FOXA2*, four variations in *THBD* and five variations in *PROCR*. All non-synonymous variations are summarized in table 2. For *PROCR*, variations were within the expected for the haplotypes and only the haplotypes are shown. All SNPs have been previously reported. One variation in *PROCR* (rs867186, a tagging SNP for H3) and two variations in *FOXA2* (rs1055080, in the 3'- UTR and rs2277764, in the 5'-UTR) were associated with higher levels of PC. Other SNPs found in *FOXA2* were rs1800847, rs1203910 and rs1212275, all leading to synonymous amino acid substitutions (not shown). Relevant variations found in *THBD* were: rs1042579 (p.Val473Ala), rs1042580, rs3176123 and rs1962 (last three in the 3'-UTR) but none of these was associated with PC plasma levels. This excludes *THBD* as a likely determinant of PC levels.

The co-inheritance of the rare variations of *PROCR* (rs817186) and *FOXA2* (rs1055080 and rs2277764) in individuals with high PC plasma levels suggests that *PROCR* and *FOXA2* SNPs are inherited as a single haplotype in these individuals. In an attempt to distinguish which gene is actually responsible, we genotyped all members of the family for *FOXA2* rs1055080 and for the tagging SNPs of common *PROCR* haplotypes (table 3). Genotyping of *PROCR* showed 52 heterozygotes and two homozygotes for rs817186 minor allele, whereas for *FOXA2* rs1055080, thirty four individuals were heterozygotes and no homozygote for the minor allele was present. Except for two individuals, all

Table 2. Polymorphisms detected in twelve family members with normal, intermediate and high PC levels. Only non-synonymous variations are shown in the table.

Sample	PC (%)	PROCR haplotype	FOXA2 rs1055080	rs2277764	THBD rs1962	rs3176123	rs1042580	rs1042579
36049	72	H2H4	1	1	1	1	3	1
29640	75	H1H2	1	1	2	2	1	2
29448*	82	H2H2	1	1	1	1	2	1
29494*	114	H2H3	2	2	1	1	1	1
29599	116	H1H3	1	1	2	2	1	2
29495	128	H2H3	2	2	1	1	1	1
29600	166	H2H3	2	2	1	2	1	2
29680	166	H1H3	2	2	2	1	1	1
29552	169	H3H3	2	2	1	2	1	2
29678	170	H1H3	2	2	2	1	1	1
29529*	178	H3H3	2	2	1	2	1	2
29687	212	H2H3	2	2	2	1	1	1

*individuals with thrombosis; 1 homozygous for the common allele, 2 heterozygous and 3 homozygous for the rare allele. PC levels are presented as percentages of the reference pooled plasma.

carriers of the *FOXA2* rs1055080 minor allele were also carriers of *PROCR* H3, again suggesting co-inheritance in the family.

To answer the question whether *PROCR* H3 or FOXA2 rs1055080 is responsible for PC levels variation, we investigated these SNPs in LETS controls. *FOXA2* rs1055080 was determined in 465 healthy individuals, out of whom thirty seven carried the minor allele, two in homozygous state (table 3). Only eight carriers of the minor *FOXA2* rs1055080 allele also carried *PROCR* H3, which suggests that, in this population-based study, *PROCR* and *FOXA2* are not inherited together, reinforcing the idea that the co-inheritance of the rare variations is particular to this family.

FOXA2 rs2277764 was also determined in LETS controls but because of its tight linkage with *FOXA2* rs1055080 (r^2=0.98), this variation was not analyzed further.

PROCR H3 is associated with high levels of PC in the family and in healthy individuals

Table 3 shows the mean plasma levels of PC and 95% confidence intervals (CI95) for the different genotype groups. Levels of PC cannot be compared directly between the family and healthy individuals because different pooled plasmas were used as a reference. In the healthy individuals, levels of PC were systematically lower than the levels in the family. In the family, mean PC level was higher in *PROCR* H3 carriers (mean: 131%; CI95: 123-138), than in non-carriers (mean: 106%; CI95: 102-110). In two individuals from the family who were homozygous for H3, PC levels were 169% and 178%. In healthy individuals, mean PC level was higher in H3 heterozygotes (mean: 113%; CI95: 110-117) than in non-carriers of H3 (mean: 99%; CI95: 97-100). Mean PC level in homozygotes for H3 was 127% (CI95: 109-144) which is somewhat higher than the levels in heterozygote carriers, but the confidence intervals overlap.
Carriers of the *FOXA2* rs1055080 minor allele in the family had increased levels of PC (mean: 139%; CI95: 128-150) in comparison with non-carriers (mean: 109%; CI95: 105-113). This was not seen for heterozygous carriers and non-carriers in the healthy individuals (mean: 100% (CI95: 93-106) and 103% (CI95: 101-104), respectively). Two individuals were homozygous for the minor allele and PC levels were 92 and 123%. This indicates that the minor allele of *FOXA2* rs1055080 is not associated with plasma level of PC in the population.
We further examined whether family members carrying both rare variants in *PROCR* and *FOXA2* had increased levels of PC in comparison to carriers of only one rare variant (table 3). Mean level of PC was higher in carriers of both *PROCR* H3 and *FOXA2* rs1055080 rare variants (mean: 142%; CI95: 131-153) than in carriers of *PROCR* H3 only (mean: 120%; CI95: 111-129). This indicates that, in this family, a second gene variation (other than *PROCR* H3) may determine protein C levels. Since in the healthy individuals no effect of *FOXA2* rs1055080 on PC levels was found, this second gene variation is not *FOXA2* rs1055080.

PROCR H3 is associated with elevated sEPCR levels in the family and in the healthy individuals

In the family, sEPCR was increased in carriers of *PROCR* H3 (mean: 261 ng/ml; CI95: 240-281) in comparison to non-carriers (mean: 103 ng/ml; CI95: 97-108). Carriers of the *FOXA2* rs1055080 minor allele also had increased sEPCR in the family but not in healthy individuals (table 3). No difference in the level

of sEPCR was observed between carriers of the rare allele for both variations and carriers of *PROCR* H3 only, either in the family or in the health individuals. sEPCR and PC levels were correlated in both the family (r^2=0.18) and healthy individuals (r^2=0.15).

sTM concentration is not different between PROCR H3 carriers and non-carriers

Median sTM plasma level was 1.2 ng/ml (range: 0.1 to 4.0) in the family. Median levels were not different between *PROCR* H3 carriers (median: 1.3 ng/ml; range: 0.3 to 4.0) and non-carriers (median: 1.2 ng/ml; range: 0.1 to 4.0). sTM was not measured in LETS.

Linkage analysis

Finally, we (re)examined linkage between PC, sEPCR and sTM levels and genetic markers on chr 20, now including *PROCR* H3 and *FOXA2* rs1055080. For PC levels, the addition of new genetic markers did not change the LOD-score (Fig. 1a). When the analysis was performed conditional on *PROCR* H3 and *FOXA2*, the LOD score for PC went down to <2.0, suggesting that most of the variation of PC levels can be attributed to these genetic markers (Fig. 1b).

For sEPCR, the linkage analysis performed with the initial genetic markers yielded a LOD score of 6.2. Adding *FOXA2* rs1055080 to the analysis increased the LOD score to 7.7 and addition of *PROCR* H3 increased it further to 9.3, reinforcing the idea that this haplotype is largely responsible for sEPCR plasma level variation (Fig. 1c). For sTM, the LOD score remained <1.0 for any model (Fig. 1d).

Table 3. PC and sEPCR plasma levels for *PROCR* H3 and *FOXA2* rs1055080 carriers and non-carriers in the family and in the healthy individuals from LETS.

			Protein C				Soluble EPCR			
Genotype	Family (n)	LETS (n)	Family (n)	Mean (CI95)	LETS (n)	Mean (CI95)	Family (n)	Mean (CI95)	LETS (n)	Mean (CI95)
PROCR H3										
HxHx	106	360	85	106 (102-110)	360	99 (97-100)	96	103 (97-108)	360	94 (91-96)
H3Hx	52	100	47	131 (123-138)	100	113 (110-117)	47	261 (240-281)	99	258 (248-269)
H3H3	2	10	2	169; 178*	10	127 (109-144)	1	337*	10	439 (399-478)
Total	160	470	134	-	470	-	144		469	-
FOXA2										
CC	126	428	104	109 (105-113)	428	103 (101-104)	114	131 (118-144)	427	138 (129-146)
CT	34	35	30	139 (128-150)	35	100 (93-106)	30	249 (216-282)	35	117 (96-139)
TT	-	2	-	-	2	92;123*	-	-	2	54;176*
Total	160	465	134	-	465	-	144	-	464	-
PROCR H3 + *FOXA2*										
HxHx +CC	104	326	83	106 (102-110)	326	99 (97-100)	94	103 (98-109)	326	94 (92-96)
HxHx +CT/TT	2	29	2	87;117*	29	97 (90-104)	2	66;81*	29	90 (81-98)
H3Hx/H3H3+CC	22	102	21	120 (111-129)	102	115 (111-119)	20	263 (235-291)	101	279 (265-294)
H3Hx/H3H3+CT/TT	32	8	28	142 (131-153)	8	111 (101-121)	28	262 (232-292)	8	217 (168-265)
Total	160	465	134	-	465	-	144	-	464	-

Abbreviations - (n): number of individuals per group; PC levels are presented as percentages of the reference pooled plasma and sEPCR as ng/ml. *only one or two measurements were available and therefore, mean and CI95 were not calculated. Genotype for *FOXA2* rs1055080 (NM_021784.4:c.*50C>T or NM_153675.2:c.*50C>T) was performed in 465 controls from LETS with DNA available. PC measurement was not available for 26 individuals from the family and sEPCR measurement was not available for 16 individuals from the family and for 1 control from LETS.

Discussion

In this study, we provide evidence for the association of a common haplotype (H3) of *PROCR* with high plasma levels of PC. Resequencing of *PROCR* did not provide proof for additional determinants of plasma PC levels. Furthermore, we excluded that two other genes involved in the PC anticoagulant pathway, i.e. *FOXA2* and *THBD*, both located on chr 20 close to the *PROCR* gene, determined the plasma level of PC, both at the level of common variations as well as on the level of rare sequence variations. Finally, variations in *THBD* did not influence the levels of sTM (data not shown) nor were these levels associated with the level of PC. This contrasted with the clear relationship between levels of PC and sEPCR.

Our results regarding the role of chr 20 in determining PC levels are in agreement with previous reports [8-10,20]. Most notably, a recent study by Tang et al. that was published while this work was in progress, came to very similar findings using a radically different approach [10]. In that study, two neighboring genes, i.e. *PROCR* and *EDEM2*, were found as determinants of variations in PC levels. In the family, carriers of *PROCR* H3 who also carried the rare allele of *FOXA2* rs1055080 had higher PC levels than carriers of H3 alone. The fact that this effect was absent in the healthy individuals from LETS indicates that a second determinant is present between the *FOXA2* and *PROCR* genes, which could well be *EDEM2*.

When we genotyped the family and the healthy individuals from LETS for *EDEM2* rs6120846 and rs3746429 and analyzed the association to PC plasma levels, PC levels were lower in carriers of one or two minor alleles in both the family and healthy controls (unpublished observations), which is in accordance with the data from Tang et al [10]. In addition, we observed that sEPCR levels were also lower in carriers of the minor alleles of *EDEM2* which suggests that, also in this case, PC levels are influenced through EPCR. This is supported by the fact that we could not see any relationship between the minor alleles and levels of other coagulation proteins (FII, FV, FVII, FVIII, FIX, FX or FXI).

The precise mechanisms underlying the association of *PROCR* and PC levels are not known, and there might be an important role for sEPCR. It is known that PC (and APC) has a comparable affinity (Kd≈30nM) for membrane bound EPCR and sEPCR [14]. Therefore, complex formation between PC and sEPCR in plasma

is to be expected and thus, high levels of sEPCR might drive high levels of PC. The problem with this explanation is that, in general, PC levels are much higher than sEPCR levels, even in the presence of *PROCR* H3. PC levels in carriers of H3 in the family is estimated to range from 63 to 163 nM whereas sEPCR levels in the same individuals is estimated to range from 4 to 18 nM. Based on these estimations it is difficult to simply explain the relationship between PC and sEPCR levels. Perhaps, higher sEPCR levels are associated with low levels of EPCR on the endothelial membrane, thus leading to a redistribution of PC between the membrane-bound compartment and a soluble compartment. There is indeed evidence that the H3 haplotype associated Gly219 involves increased EPCR shedding from the endothelium [16], but evidence that this leads to lower density on the endothelial membrane is not available. In preliminary studies we have analyzed blood-originated endothelial cells (BOECs) from carriers and non-carriers of *PROCR* H3 (three individuals of each) by flow cytometry, but the data suggests that the expression of EPCR on the membrane is not different between the groups (unpublished data). It has also been hypothesized that the local concentration of sEPCR at the endothelial surface is higher than the concentrations measured in the plasma, possibly exceeding the Kd of PC interaction [20], but this also does not readily explain the increased plasma PC levels. Future research might solve this problem.

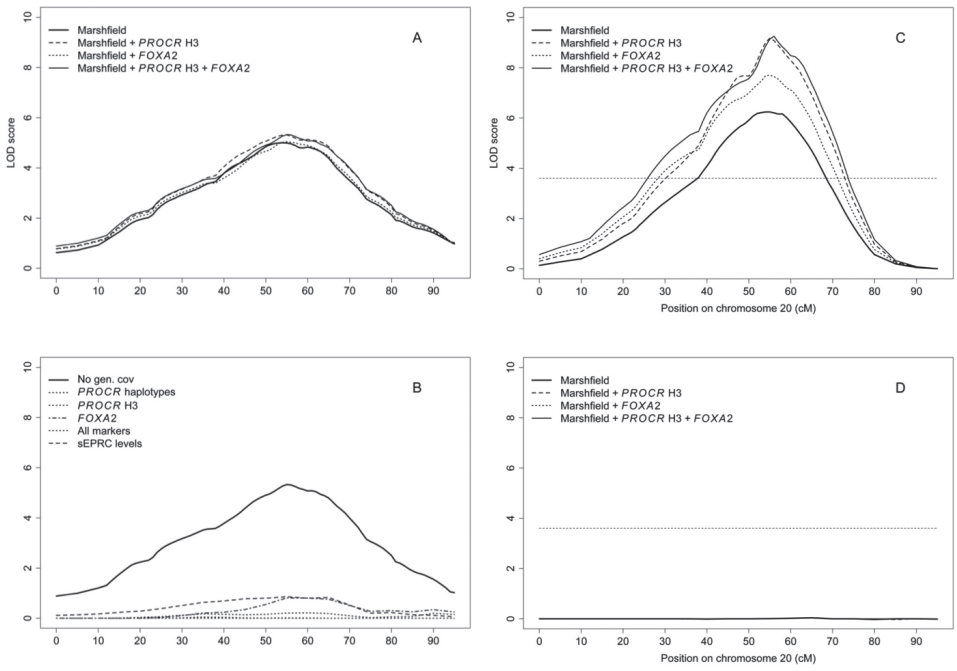
There are claims that H3 not only influences PC levels but also the level of other coagulation proteins, e.g. FVII [21,22]. In the present GENES study or in the healthy individuals from LETS, this association was not confirmed (data not shown), neither was H3 associated with levels of FII, FV, FVIII, FIX or FXI [8].

Since low levels of PC, as in individuals with inherited PC deficiency, are a risk factor for venous thrombosis, it is tempting to assume that high levels of PC are protective, but there is no evidence that the latter is indeed the case. Thus, it is also reasonable to assume that the H3 haplotype, from the perspective of PC levels, would protect against venous thrombosis. This does not seem to be the case: in the family, two of the H3 carriers who had high levels of PC experienced recurrent venous thrombosis, but this family study obviously does not have sufficient power to determine the relationship between H3 and venous thrombosis. Population-based case-control studies have not been conclusive. Some authors report increased risk of venous [20,23] and arterial thrombosis [24] in H3

carriers, and others claim no association [8,9,25,26]. It seems fair to conclude though, that the markedly elevated levels of PC associated with H3 do not protect against thrombotic disease.

In conclusion, our data provide new evidence for the association of *PROCR* H3 and sEPCR with plasma levels of PC and suggest that *FOXA2* and *THBD*, two other genes on chr 20, are not involved. Further studies are necessary to elucidate the mechanisms underlying this association.

Figure 1. LOD-scores for Protein C levels using additional markers (a) or additional covariates (b), and for sEPCR (c) and sTM (d) levels using additional markers. The horizontal line indicates the genome-wide significance threshold.



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