Venous and arterial thrombosis : associations and risk factors

Roshani, S.

Haplotypes of VKORC1, NQO1 and GGCX, their effect on activity levels of vitamin K-dependent coagulation factors, and the risk of venous thrombosis

Marieke C.H. de Visser, Sara Roshani, Julie W. Rutten, Astrid van Hylckama Vlieg, Hans L. Vos, Frits R. Rosendaal and Pieter H. Reitsma

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Dear Sirs,

Vitamin K antagonists, e.g. warfarin, acenocoumarol, and phenprocoumon, are widely used as treatment for individuals with increased thrombosis risk. The target of these drugs is the vitamin K epoxide reductase complex subunit 1 (VKORC1), a key enzyme in the vitamin K cycle. A reduced form of vitamin K (vitamin K hydroquinone) serves as a cofactor for gamma-carboxylase (GGCX) in the posttranslational carboxylation of vitamin K-dependent proteins, such as the coagulation proteins factor II, VII, IX and X, protein C, protein S, and protein Z. This carboxylation of glutamate residues is essential for full activation of these proteins. During gamma-carboxylation vitamin K epoxide is generated, which must be rapidly reduced again because of limited availability of reduced vitamin K. Via VKORC1 and NAD(P)H dehydrogenase [quinone] 1 (NQO1), vitamin K epoxide is recycled to its active form vitamin K hydroquinone. Binding of vitamin K antagonists to VKORC1 inhibits recycling of vitamin K, resulting in the formation of inactive, non-carboxylated proteins.

Whereas data on NQO1 and GGCX are scarce, genetic variation (single nucleotide polymorphisms, SNPs) in the *VKORC1* gene was repeatedly reported to influence the individual response of patients to vitamin K antagonists. Carriers of a specific *VKORC1* haplotype (A), consisting of several SNPs in complete linkage disequilibrium, were found to require a lower maintenance dose of vitamin K antagonists compared with haplotype B carriers [1].

Elevated plasma levels of vitamin K-dependent coagulation factors II, VII, IX, and X were previously found to be associated with an increased risk of venous thrombosis and they seem to have a significant genetic component [2]. However, only a few genetic determinants of these plasma levels have been identified. Furthermore, clustering of the levels of vitamin K-dependent proteins has been reported [3], suggesting that a common modifier gene exists. Genetic variation in *VKORC1*, *NQO1,* and *GGCX* might affect plasma activity levels of vitamin K-dependent proteins, and thereby thrombosis risk.

Several studies investigated the association between *VKORC1* variation and venous thrombosis risk [4-7], mainly by genotyping a SNP distinguishing haplotypes A and B. Lacut et al. [5] reported that haplotype A protected against thrombosis, whereas the other studies did not show any association. In a recent German study no association between SNPs in *VKORC1*, *NQO1,* and *GGCX* and activity levels

of vitamin K-dependent coagulation factors was found [8], whereas a Spanish study reported an association between an *NQO1* SNP with protein C levels [9].

Until now the association between genetic variation in *NQO1* and *GGCX* and venous thrombosis risk has not been studied. Furthermore, most previous studies only studied one SNP per gene, thereby not taking into account all common haplotypic variation. In the present study we investigated *VKORC1*, *NQO1,* and *GGCX* haplotypes and their association with activity levels of vitamin K-dependent coagulation proteins and venous thrombosis risk.

For our investigation we used the Leiden Thrombophilia Study (LETS), a population-based case-control study on venous thrombosis, including 474 consecutive patients aged 18-70 years with a first deep-vein thrombosis and 474 age- and sex-matched healthy controls [10]. Venous blood was collected into tubes containing 0.1 volume 0.106 mol/L trisodium citrate. Plasma was prepared by centrifugation for 10 minutes at 2000 g at room temperature and stored at -70°C. High molecular weight DNA was isolated from leukocytes and stored at 4°C. Measurements of factor II [11], factor VII [12], and protein C activity [13] have been described before.

Haplotype tagging SNPs in the *VKORC1* (n=4), *NQO1* (n=4), and *GGCX* (n=6) genes were identified in either the Caucasian Seattle PGA panel (*NQO1*, *GGCX*) or the Caucasian (CEU) Hapmap panel (*VKORC1*) using the Genome Variation Server (GVS, http://gvs.gs.washington.edu/GVS). Minor allele frequencies (MAF) were above 3%. All SNPs were genotyped using a 5'-nuclease/TaqMan assay (Applied Biosystems, Foster City, CA, USA). For all fourteen SNPs, the distribution of genotypes among control subjects was in Hardy-Weinberg equilibrium (tested using the χ^2-statistic). Tagging SNPs and haplotypes are shown in Table 1A. For *VKORC1* haplotypes, Geisen's nomenclature was used [14]. Analyses were performed with PLINK v1.06 software (http://pngu.mgh.harvard.edu/purcell/plink/) [15]. Haplotypes for the three genes were inferred in subjects without missing genotypes and haplotype allele frequencies were compared between patients and controls (Table 1A).

None of the haplotypes of the three genes affected venous thrombosis risk. Our results on *VKORC1* are in agreement with most previous findings. We could not confirm the finding that homozygous carriers of *VKORC1* haplotype A (tagged by rs2359612) are protected against thrombosis [5]. Individual SNPs were also not associated with the risk of venous thrombosis.

Linear regression analysis was used to test for an association between haplotypes and activity levels of vitamin K-dependent coagulation proteins in the LETS control population. Table 1B shows the regression coefficients β. Only two regression coefficients were significantly different from zero ($p<0.05$). *NQO1* H4 was associated with reduced factor II activity. Each copy of *NQO1* H4 was associated with a reduction in factor II activity of 2.68 % (i.e., linear regression coefficient $\beta= -2.68$; $p=0.04$). This reduction was consistent as also factor VII and protein C activity were reduced in *NQO1* H4 carriers. *NQO1* H4 is tagged by rs1800566 (p.Pro187Ser). The proline to serine substitution was found to be associated with loss of NQO1 protein and NQO1 activity [16], which may explain the observed reduction in levels. The rs1800566 SNP is in complete linkage disequilibrium with rs1437135 (http://www.hapmap.org) which was previously reported to be associated with protein C levels in the GAIT study [9]. Individual analysis of all fourteen SNPs also showed an association between rs1800566 and factor II activity. This finding was the only significant result in the single SNP analysis. The second significant result in the haplotype analysis was the association between *GGCX* H1 and reduced factor II activity ($\beta= -1.90$; $p=0.048$), but this result may be spurious. The reduction was not consistent as factor VII activity was not reduced. Rieder et al. previously showed that *VKORC1* haplotype A (combination of VKORC1*2A and VKORC1*2B) is associated with a reduced expression of VKORC1 in the liver [1]. In LETS controls a trend towards lower activity of factor II, factor VII and protein C was observed in haplotype A carriers, which is in accordance with Rieder's report.

In conclusion, we did not find an association between haplotypes of *VKORC1*, *NQO1* and *GGCX* and venous thrombosis risk. *NQO1* H4 does possibly have a small influence on activity levels of vitamin K-dependent proteins. However, these changes are too subtle to noticeably change thrombosis risk.

Table 1A. Haplotypes and tagging SNPs of *VKORC1*, *NQO1* and *GGCX*

	Tagging SNPs rs number (SeattleSNPs numbering[1])						Frequency LETS	
VKORC1 haplotypes[2] (clusters)[3]	rs2884737 (5808)	rs17708472 (6009)	rs2359612 (7566)	rs7294 (9041)			Patients n=469	Controls n=466
VKORC1*2A (A)	T	C	**T**	G			13.2	14.5
VKORC1*2B (A)	**G**	C	**T**	G			26.9	26.3
VKORC1*4 (B)	T	**T**	C	G			22.0	21.7
VKORC1*3 (B)	T	C	C	**A**			36.8	36.2
VKORC1*1 (B)	T	C	C	G			1.2	1.3
NQO1 haplotypes	rs689453 (1910)	rs2965753 (2898)	rs1800566 (9144)	rs10517 [4]			Patients n=461	Controls n=462
NQO1 H1	**A**	A	C	C			7.1	8.7
NQO1 H2	G	A	C	C			60.7	61.8
NQO1 H3	G	A	C	**T**			1.2	1.4
NQO1 H4	G	A	**T**	C			19.2	17.1
NQO1 H5	G	**G**	C	**T**			11.9	11.0
GGCX haplotypes	rs6738645 (7475) [5]	rs699664 (10067)	rs10179904 (10496)	rs11676382 (12970)	rs17026447 (13031)	rs2028898 (13333)	Patients n=465	Controls n=461
GGCX H1	A	G	C	G	T	C	43.6	41.6
GGCX H2	A	G	C	**C**	T	C	9.8	11.5
GGCX H3	C	A	C	G	**G**	C	2.9	2.7
GGCX H4	**C**	**A**	C	G	T	**T**	30.6	30.2
GGCX H5	**C**	G	**T**	G	T	C	10.9	10.5
GGCX H6	**C**	G	C	G	T	C	2.2	3.4

Minor alleles in bold and underlined [1] http://pga.mbt.washington.edu/, [2]According to Geisen et al (14), [3]According to Rieder et al (1), [4]Not determined in SeattleSNPs panels, [5]in Hapmap CEU population A is minor allele

Table 1B. Association of *VKORC1*, *NQO1* and *GGCX* haplotypes with activity of vitamin K-dependent coagulation proteins in controls

	Factor II (%)	Factor VII (%)	Protein C (%)
VKORC1 haplotypes			
VKORC1*2A	1.57	0.81	1.60
VKORC1*2B	-1.11	-1.46	-1.17
VKORC1*4	0.02	2.36	-0.96
VKORC1*3	0.07	-0.36	0.98
VKORC1*1	-1.63	-5.67	-4.12
VKORC1 haplotype A[1]	-0.07	-0.73	-0.16
NQO1 haplotypes			
NQO1 H1	1.18	1.21	-0.31
NQO1 H2	0.75	2.28	1.35
NQO1 H3	0.85	-4.31	-2.05
NQO1 H4	**-2.68***	-2.85	-1.03
NQO1 H5	1.47	-1.21	-0.92
GGCX haplotypes			
GGCX H1	**-1.90***	0.94	-2.02
GGCX H2	1.76	-2.71	-1.40
GGCX H3	2.32	-0.16	-0.18
GGCX H4	1.21	0.95	2.29
GGCX H5	-0.95	-2.09	-0.59
GGCX H6	-0.70	4.15	2.78

Regression coefficients β are shown. The direction of the regression coefficient represents the effect of each extra copy of the haplotype (i.e. a positive regression coefficient means that the haplotype increases phenotype mean). Activity in pooled normal plasma is 100%.

[1]According to Rieder et al (1)

* $p<0.05$

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