Cardiovascular Biology and Cell Signalling

Functional promoter polymorphism in the VKORC1 gene is no major genetic determinant for coronary heart disease in Northern Germans

Matthias Watzka1,2, Almut Nebel17, Nour Eddine El Mokhtari4, Boris Ivandic5, Jens Müller1, Stefan Schreiber3, Johannes Oldenburg1
1Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Bonn, Germany; 2Institute of Transfusion Medicine and Immunohaematology, DRK Blood Donor Service Baden-Württemberg-Hessen, Frankfurt/Main, Germany; 3Institute of Clinical Molecular Biology, Christian-Albrechts-University, Kiel, Germany; 4Department of Cardiology, University Hospital Schleswig-Holstein, Kiel, Germany; 5Department of Internal Medicine III, University Hospital Heidelberg, Heidelberg, Germany

Summary
Recently, the C-allele of polymorphism rs2359612 (VKORC1: c.283+837C>T) in the VKORC1 gene has been reported to represent a major risk factor for coronary heart disease (CHD), stroke, and aortic dissection in Chinese patients. VKOR activity itself is the rate-limiting step in gamma-carboxylation of vitamin K-dependent coagulation factors (factors II, VII, IX, X, protein C, S, and Z) and proteins of calcium metabolism (matrix Gla protein and osteocalcin). Gamma-carboxylation is essential for the biological activity of these proteins that have been previously hypothesised to play a role in the pathogenesis of atherosclerosis. It was the objective of this study to analyse the VKORC1 genotype frequency in patients with CHD and controls from Northern Germany and to investigate the association of VKORC1 and CHD risk in patients with an European background. CHD patients (n = 901) and healthy controls (n = 521) were part of the PopGen biobank. Case and control samples were matched for ethnic and geographic origin, age and gender. After typing German CHD patients and control individuals, no evidence for a statistically significant association was detected between VKORC1 genotype and CHD phenotype. Also stratification for gender and myocardial infarction yielded no significant results. In conclusion, the discrepant association findings in Chinese and German populations may be explained by ethnic differences in genetic and perhaps environmental predisposition, modifying the polygenic CHD phenotype by interacting with VKORC1 variants and thus conferring disease susceptibility in some populations, but not in others.

Keywords
Coronary heart disease, haplotype, rs9923231, vitamin K, VKORC1

Introduction
Coronary heart disease (CHD) is one of the leading causes of morbidity and mortality worldwide (1–3). Recently, VKORC1 haplotypes have been associated with CHD, stroke, and aortic dissection in large collectives of Chinese patients (4). Since its discovery as the molecular target of coumarins (5–7), VKORC1 has been intensively investigated, in particular by addressing its role as a major dose determinant in oral anticoagulation (8–10). Up to now, only one functional single nucleotide polymorphism (SNP) has been identified in the promoter region of VKORC1 (rs9923231) that reduces mRNA expression and subsequent enzymatic activity of the protein to 30–50% (11, 12). This functional SNP is part of the wide-spread haplotype VKORC1*2 (10). Because of its population-specific distribution with low frequency in Africans, intermediate frequency in Europeans and high frequency in Chinese, VKORC1*2 was found to explain the clinically observed differences in coumarin dosage in these ethnic groups (10, 12). As VKOR activity is the rate limiting step in gamma-carboxylation of vitamin K-dependent proteins (13, 14), VKORC1 and its naturally occurring allelic variants were hypothesised to influence, apart from coagulation, other downstream physiological functions of vitamin K-dependent proteins e.g. in atherogenesis (15).

Specifically matrix Gla protein (MGP) and bone Gla protein (osteocalcin), two vitamin K-dependent proteins with regulating
effects on calcium homeostasis, have been suggested to play a role in the pathogenesis of atherosclerosis, myocardial infarction, and stroke (16–19). Calcification of arterial plaques (intimal calcification) and/or vessels (medial calcification) may be affected at least by MGP (15, 17). This protein, initially discovered in bone, but also highly expressed in heart, kidney, and lung, is a potent inhibitor of calcification of the extracellular matrix. Knock-out mice lacking MGP show premature bone mineralization and severe vascular calcification, leading to blood vessel rupture (17).

Prompted by the recent observation of a disease association between the CHD phenotype and VKORC1 haplotypes in Chinese (4), we examined in the present study the potential association between the functional SNP rs9923231, which tags the VKORC1*2 haplotype, and early-onset CHD in a large, ethnically homogeneous sample from the northern-most province in Germany.

Subjects and methods

Patients
Unrelated German study participants were drawn anonymously from population-based samples. Samples from both patients with early-onset CHD (n = 901) and healthy controls (n = 521) were part of the PopGen biobank (20). Case and control samples were matched for ethnic ancestry (all Germans), geographic origin (all from the county of Schleswig in Northern Germany), age range (33–64 years), mean age (53.5 years) and gender ratio (84% males vs. 16% females). Significant CHD was confirmed by coronary catheterization demonstrating at least a 70% stenosis in one major epicardial coronary vessel before the age of 55 years. The average age of disease onset was 48 years (Table 1). The majority of subjects (90.3%) had undergone a coronary revascularization procedure (percutaneous coronary intervention or coronary artery bypass grafting), and a subset of individuals (~66%) had suffered a myocardial infarction. A detailed description of the samples and the recruitment procedure is given elsewhere (21). All subjects gave informed, written consent prior to participation. The study was approved by the Ethics Committee of the University Hospital Schleswig-Holstein in Kiel and by the data protection authorities.

Methods
The DNA samples were analysed for the SNP rs9923231 (VKORC1: c.-1639 G>A) in the VKORC1 gene using a TaqMan® SNP Assay based on fluorescence-labelled probes (primer and probe details are given in Table 2). Amplifications were carried out in a total volume of 5 µl using the Absolute™ QPCR Master-mixture (Abgene, Epsom, UK). Cycling conditions were: initial denaturation at 95°C for 15 minutes followed by 45 cycles of 95°C for 15 seconds and 62°C for 60 seconds. The typing was performed in the Institute of Clinical Molecular Biology in Kiel on a platform with an integrated laboratory information management system, as described earlier (22). The accuracy of the Taq-Man typing was demonstrated by testing 192 DNA samples that had previously been examined for the rs9923231 polymorphism by direct sequencing (10). For each test sample, the two methods yielded consistent results. The polymorphism was analysed for Hardy-Weinberg equilibrium using the software HaploView, version 3.2 (23; http://www.broad.mit.edu/mpg/haploview/). Single marker case-control analysis of allele and genotype frequency data was performed with Chi²-statistics using the web-based Simple Interactive Statistical Analysis (SISA) tool, available at http://home.clara.net/sisa. Power calculation was carried out by means of a program at http://www.openepi.com/Menu/OpenEpiMenu.htm.

Results
The polymorphism rs9923231 in the VKORC1 gene as a tag SNP for haplotype VKORC1*2 (10) was analysed in samples of 901 German CHD patients and 521 gender- and age-matched control individuals. The marker was found to be in Hardy-Weinberg equilibrium in both cases and controls (p-value >0.2). No evidence for a statistically significant association was detected between the tested polymorphism and the CHD phenotype, at the allele or at the genotype level (Table 3). Similarly, case-control analysis with gender-stratified samples showed no association, nor did stratification for myocard infarction (data not shown).

Given the sample size and carrier frequency of the rs9923231 G allele in the German population examined herein (Table 2), our study had a power >80% to detect the same effect that Wang

---

**Table 1: Characteristics of the German CHD patients.**

<table>
<thead>
<tr>
<th></th>
<th>Males (n = 759)</th>
<th>Females (n = 142)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at recruitment (years)</td>
<td>53.6</td>
<td>53.2</td>
</tr>
<tr>
<td>Mean age at disease onset (years)</td>
<td>48.2</td>
<td>48.2</td>
</tr>
<tr>
<td>Myocardial infarction (%)</td>
<td>65.9</td>
<td>65.0</td>
</tr>
<tr>
<td>Mean BMI with SD (kg/m²)</td>
<td>28.3 [4.3]</td>
<td>28.5 [5.2]</td>
</tr>
<tr>
<td>Past or current smoker (%)</td>
<td>80.9</td>
<td>72.7</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>70.0</td>
<td>76.2</td>
</tr>
<tr>
<td>Hypercholesterinemia (%)</td>
<td>80.0</td>
<td>75.5</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>17.3</td>
<td>20.3</td>
</tr>
</tbody>
</table>

CHD: coronary heart disease; BMI: body mass index; SD, standard deviation.

---

**Table 2: Oligonucleotide primer / probe sequences for genotyping of rs9923231.**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’ – 3’)</th>
<th>Positionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VKORC1-for</td>
<td>GGTAGGTGCAACATGTAAGGG</td>
<td>3603 – 3623</td>
</tr>
<tr>
<td>VKORC1-rev</td>
<td>AAATGTCTAGTTGATAGGCTGGTGA</td>
<td>3707 – 3685</td>
</tr>
<tr>
<td>Hybridization probesa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VKORC1-wt</td>
<td>ACCGGCACCCGGGCAATGTGAA</td>
<td>3681 – 3665</td>
</tr>
<tr>
<td>VKORC1-SNP</td>
<td>ACCGGCACCTGCGGCAATGGG</td>
<td>3681 – 3664</td>
</tr>
</tbody>
</table>

aThe wt probe was labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5’ end and the quencher 6-carboxy-6-carboxytetramethylrhodamine (TAMRA) at the 3’ end. To enable multiplex reactions, a different reporter dye (tetrachloro-6-carboxyfluorescein [TET]) was labeled at the 5’ end of the SNP probe, but TAMRA was still used as quencher at the 3’ end. b Nucleotide positions based on the GenBank accession no. AY587020.
Table 3: Summary association statistics for rs9923231 in the VKORC1 gene in German CHD and control samples.

<table>
<thead>
<tr>
<th></th>
<th>CHD samples^</th>
<th>Control samples^</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of individuals</td>
<td>901</td>
<td>521</td>
<td></td>
</tr>
<tr>
<td>Minor allele frequency</td>
<td>0.377</td>
<td>0.398</td>
<td>0.270</td>
</tr>
<tr>
<td>Genotype frequencies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>0.379</td>
<td>0.369</td>
<td>0.255</td>
</tr>
<tr>
<td>G/A</td>
<td>0.488</td>
<td>0.466</td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>0.133</td>
<td>0.165</td>
<td></td>
</tr>
</tbody>
</table>

^Analysis was performed with gender- and age-balanced case and control samples. Allele A, corresponding to haplotype VKORC1*2, is the minor allele.

Discussion

CHD is a complex phenotype with multiple influences affecting onset, formation, and progression. There are several congenital factors that increase an individual's risk of CHD. These include age, sex, and genetic background (24–26). However, other strong risk factors for CHD like smoking, overweight, diet low in fruits and vegetables, lack of physical activity, high blood pressure, and high cholesterol levels are modulated through individual nutrition habits and lifestyle (27–29).

Recently, the ancestral C allele of the polymorphism rs2359612 in the VKORC1 gene (VKORC1: c.283+837C>T) has been identified as a strong genetic risk factor for vascular diseases in Chinese (4). The derived T allele of this SNP is in strong linkage disequilibrium (LD) with haplotype VKORC1*2, while the C allele tags all the other known haplotypes VKORC1*1 (until now detected in African populations only), VKORC1*3, and VKORC1*4 (both haplotypes present in all populations examined so far). VKORC1*2 was found to comprise a functional promoter polymorphism (VKORC1: c.-1639G>A, rs9923231), whose A allele is in LD with haplotype VKORC1*2 and is associated with a notable reduced promoter activity, explaining interindividual and interethnical differences in e.g. coumarin dosage in oral anticoagulation (10, 11). The G allele of rs9923231 (as the C allele of rs2359612) also tags haplotypes VKORC1*1, VKORC1*3, and VKORC1*4 (i.e. all non-VKORC1*2 haplotypes).

In their original reports on Chinese, Wang et al. (4) showed a statistically significant association of CHD with the C allele of rs2359612 that represents all non-VKORC1*2 haplotypes. In their samples, the C allele reached a carrier frequency of 16.9% in CHD cases compared to 11.2% in matched controls with an odds ratio of 1.72, indicating non-VKORC1*2 haplotypes as a risk factor for CHD.

The authors further reported that levels of undercarboxylated vitamin K-dependent proteins (osteocalcin and PIVKA-II) were directly correlated with VKORC1 haplotypes (4). Highest levels of undercarboxylated proteins were found in subjects homozygous for the rs2359612 T allele (VKORC1*2 haplotype), lowest levels in individuals homozygous for the C allele (non-VKORC1*2 haplotypes). However, the finding of the C allele as a CHD risk factor is difficult to reconcile with the higher VKORC1 mRNA level and VKOR activity and a subsequent higher gamma-carboxylation level of e.g. MGP, which is thought to exhibit an effect on vessel calcification (16, 18, 19). Wang et al. acknowledged that the pathophysiology underlying their observation is unclear and still needs clarification.

In view of the disease association found in Chinese (4), we analysed the functional SNP rs9923231 in a well-characterized sample of German early-onset CHD patients. With a similar gender ratio (85 % vs. 84 % male cases), mean age (59 vs. 53.5 years), and proportion of patients with myocardial infarction (58% vs. 66%) and diabetes mellitus (15.3% vs. 17.8%), the collectives of Wang et al. and from our study were quite comparable (Table 1). Furthermore, in both studies the inclusion criterion of at least 70% stenosis in one of the major coronary arteries was identical. Nevertheless, we did not confirm the previous findings of Wang et al., and in our sample the shift from 86.7% carriers of the rs9923231 G allele (non-VKORC1*2 haplotypes) in cases compared to 83.5% in controls was not significant (Table 1). Similarly, case-control analysis with gender-stratified samples as well as stratification for survival of myocardial infarction did not show any significant differences.

The high frequency of the rs9923231 G carriers in the German samples is striking, but is in agreement with previous observations that VKORC1*2 is most frequent in Chinese and non-VKORC1*2 haplotypes are predominant in Europeans (10, 11). The remarkable differences in the geographic distribution of the VKORC1 haplotypes could be due to (neutral) random genetic drift. It may also reflect varying selective pressures acting on the VKORC1 gene in Asians and Caucasians during the last several thousand years. If the latter scenario indeed played a role, selection would not relate to the effect VKORC1 has on vascular diseases, but to other (unknown) functions of the gene. CHD is usually confined to aged populations and does not affect reproductive success. Any genetic variant contributing to CHD is therefore unlikely to have been subjected to direct selective pressure during evolution (30). However, the question remains as to which factors could have contributed to the discrepant association findings in Chinese and German CHD patients.

It has to be pointed out that our investigation is not a true replication study. Wang et al. analysed the VKORC1 haplotype distribution by investigating a SNP (rs2359612) that is in strong, but not perfect LD with VKORC1*2. As the authors stated, this SNP was chosen for analysis because its frequency is slightly higher than those of the other polymorphisms forming haplotype VKORC1*2. In our study, we analysed VKORC1*2 by using the tagging SNP rs9923231, which is known to reduce VKORC1 haplotype-dependent promoter activity (11). Sequence analysis in 200 German blood donors did not show any recombination event or deviating polymorphism frequency in any of the haplotypes of the complete VKORC1 gene region (previously published in Geisen et al. [10]). Therefore, rs9923231 is in perfect LD with the other SNPs of haplotype VKORC1*2 (including rs2359612) in Germans. The lower LD in VKORC1 in Chinese indicates a higher heterogeneity of this gene region in Asians. The SNP rs2359612 could therefore be a population-specific marker for...
an as yet unidentified causative variant that is in LD with VKORC1 in Chinese, but not in Germans. However, the deviations in the LD patterns in the two groups appear to be minor and given the functional evidence for an involvement of VKORC1 in angiogenesis and vascular diseases (15, 31) and the lack of appropriate candidate genes surrounding VKORC1 (4), this explanation seems unlikely.

A more plausible alternative for the discrepant association findings in Chinese and Germans may be other population-specific differences in genetic and perhaps environmental predisposition to CHD. CHD is a polygenic phenotype influenced by polymorphisms in many genes. Variants that are unique to some ethnic groups may alone or in interaction with other genetic markers confer disease susceptibility or protection that is relevant only to these populations. In addition, varying strong environmental factors, such as diet, that modify the influence of a risk allele in different populations may have contributed to the inconsistency between the studies. This assumption might be reflected by a higher body mass index (BMI) in our collective with a value of 28.3 ± 4.5 in German cases (compared to Wang et al.: 25.2 ± 3.1) and 26.8 ± 4.5 in controls (Wang et al.: 24.5 ± 3.2), a higher proportion of individuals with hypertension (71% in German cases vs. 44.4% in Chinese CHD patients) as well as higher cigarette consumption in our samples with 79.6% smokers in cases compared to 56.9% in Chinese cases (Table 1).

Another factor influencing interethnic differences in development of cardiovascular phenotypes might be unequal dietary intake of phylloquinone (vitamin K1) and menaquinone (vitamin K2) in Chinese and Europeans. Phylloquinone is found in high quantities in dark green leafy vegetables, onions, and cabbage, whereas menaquinone is present in meat and fermented foods like cheese and curds. When comparing vitamin K intake in Chinese and Europeans, two main differences are obvious: first, total vitamin K intake in Chinese is reported to be significantly higher. In a study performed by Yan et al. (32), in elderly people from Shenyang (China) phylloquinone intake was 2.5 times higher compared to individuals from Cambridge (UK).

This difference was reflected by three times higher plasma phylloquinone concentrations (32, 33).

Second, although menaquinone intake and vitamin K2 plasma concentrations were not considered in the study of Yan et al. (32), consumption of meet and dairy products was considerably higher in the Cambridge group (32). With regard to Chinese nutrition habits avoiding fermented milk (curds / cheese), menaquinone intake in Europeans might be higher. Recent studies documented that high dietary intake of menaquinone, but not phylloquinone, was associated with a significantly lower risk of CHD (34). Additionally, only menaquinone, but not phylloquinone was found to prevent warfarin induced vascular media calcification in rats (35). However, so far studies related to menaquinone and CHD in Chinese are missing.

Considering all known variables, association between vitamin K type, VKORC1 genotype, and CHD becomes more and more complex, especially since CHD is controlled by multiple exogenous (mostly habitual and nutritive) and inherited (genetic) factors other than VKORC1. The variable composition of these factors is likely causing the unequal outcome in studies addressing CHD and VKORC1 in different ethnic groups. Therefore, further studies have to be undertaken to clarify the role of phylloquinone, menaquinone, VKORC1 genotype and vitamin K-dependent factors, particular MGP and osteocalcin, in development and progression of atherosclerosis and myocardial infarction.

Acknowledgements

The work of J.O. was supported by grants from the Deutsche Forschungsgemeinschaft (DFG – OL 100/3–1), the Bundesministerium für Bildung und Forschung – Forschungszentrum Jülich (BMBF/PTJ – 0312708E), the National Genome Research Net Cardiovascular Diseases (BMBF/DLR-01GS0424/NHK-S12T21) and Baxter Germany. S.S. was supported by grants from the National Genome Research Net Cardiovascular Diseases (BMBF/DLR-01GS0426), for the National Genotyping Platform (BMBF/DLR-01GR0412) and PopGen (BMBF/DLR-01GR0468).

References

5. Rost S, Fregin A, Ivaskovichuk V, et al. Mutations in VKORC1 cause warfarin resistance and multiple congenital anomalies in rats (35). However, so far studies related to menaquinone, VKORC1 genotype and vitamin K-dependent factors, particular MGP and osteocalcin, in development and progression of atherosclerosis and myocardial infarction.


