Heterozygosity for R1141X in ABCC6 and Risk of Ischemic Vascular Disease

Running title: Hornstrup et al.; R1141X in ABCC6 and ischemic vascular disease

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Abstract:

**Background** - Pseudoxanthoma elasticum (PXE) is an autosomal recessive disease caused by loss-of-function mutations in *ABCC6*, and characterized by elastic calcification leading to dermal, ocular, and ischemic vascular disease (IVD). We tested the hypothesis that heterozygosity for R1141X, the most frequent PXE-causing mutation in Caucasians, associated with risk of IVD, as previous studies suggested 4-11 fold risk of ischemic heart disease (IHD) in heterozygotes.

**Methods and Results** - We studied 10,276 persons from the general population, including 1,985 with IHD and 989 with ischemic cerebrovascular disease (ICVD). We examined 45,603 individuals from a cross-sectional general population study, of whom 3,738 had IHD and 2,335 had ICVD. Finally, we compared 4,851 patients with IHD and 625 patients with ICVD with, respectively, 4,851 and 625 matched controls. We genotyped participants in all studies for *ABCC6* R1141X. The frequency of R1141X was 0.6% in all populations studied. *ABCC6* R1141X genotype was not associated with an increased risk of IHD, myocardial infarction, ICVD, or ischemic stroke. Furthermore, R1141X genotype did not interact with age on risk of the largest endpoint, IHD. Finally, R1141X genotype did not associate with variation in plasma levels of high-sensitivity C-reactive protein, fibrinogen, blood pressure, or lipid and lipoproteins in the general population.

**Conclusions** - In four studies including 66,831 participants and 13,642 cases with ischemic vascular events, heterozygosity for *ABCC6* R1141X did not associate with risk of IHD, myocardial infarction, ICVD, or ischemic stroke.

**Key words**: Genetics, Epidemiology, Ischemia; Cardiovascular diseases, Hypertension.
Introduction

Pseudoxathoma elasticum (PXE) is a rare recessive Mendelian disease associated with a substantially increased risk of ischemic heart disease (IHD)\(^1\). Patients are either homozygous or compound heterozygous for loss-of-function mutations in the causative gene, \textit{ATP-Binding Cassette Transporter C6 (ABCC6)}\(^2\). The exact biological function of the ABCC6 protein is unknown, however widespread calcification of elastic fibres particularly affecting the skin, retina and arteries are hallmarks of PXE\(^1\). The cardiovascular manifestations include hypertension, cardiac failure, angina pectoris, intermittent claudication and myocardial infarction (MI), mimicking more common heart disease\(^5\). The most common recurrent PXE mutation, R1141X (rs72653706), accounts for more than 30\% of all PXE mutations in the homozygous or compound heterozygous state in Caucasians\(^1\). Case-control studies of this mutation have detected 4-11 fold increased risk of IHD in heterozygotes\(^7\). Because these studies suggest a risk of IHD comparable or larger than the risk associated with low density lipoprotein receptor (\textit{LDLR}) or apolipoprotein B (\textit{APOB}) mutations, leading to familial hypercholesterolemia, and because R1141X is three times more common than \textit{LDLR} and \textit{APOB} mutations, this specific mutation is biologically well-suited to test in the general population\(^9\). We tested the hypothesis that heterozygosity for \textit{ABCC6} R1141X associated with ischemic vascular disease (IVD).

First, we tested whether heterozygosity for R1141X associated with increased risk of IVD in the general population. Second, we tested whether R1141X heterozygotes were more likely to develop premature ischemic heart disease compared to non-carriers. Finally, we tested whether R1141X genotype associated with levels of inflammatory markers (high-sensitivity C-reactive protein (hsCRP) and fibrinogen), with systolic or diastolic blood pressure or with lipids and lipoproteins, all risk factors for IVD. For these purposes, we genotyped the R1141X mutation in 10,276 participants from the Copenhagen City Heart Study, in 45,603 participants from the
Copenhagen General Population Study, and in, respectively, 9,702 and 1,250 individuals in two case-control studies, the Copenhagen Ischemic Heart Disease Study and the Copenhagen Carotid Stroke Study.

**Methods**

**Study cohorts**

We studied four independent cohorts of white people of Danish descent, two general population studies and two case-control studies. These studies were defined so that no person appears in more than one of the four analyses groups, thus permitting independent confirmation of the findings in each study. Studies were approved by institutional review boards and Danish ethical committees (KF V.100.2039/91, KF 01-144/01, KF 01-062/94, KA 99039, Copenhagen and Frederiksberg committee; and KA 93125 and KA 99039, Copenhagen County committee), and conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants.

**The Copenhagen City Heart Study (CCHS)**

This is a prospective study of the general population initiated in 1976-1978 with follow-up examinations in 1981-1983, 1991-1994, and 2001-2003. Individuals were randomly selected based on the national Danish Civil Registration System to reflect the adult Danish population aged 20-80+ years. Data were obtained from a questionnaire, a physical examination, and from blood samples. R1141X genotype was determined in 10,385 participants attending the 1991-94 and/or 2001-03 examinations. We excluded 109 individuals with IVD before study entry, leaving 10,276 for all further analyses. Of these, 1,985 developed IHD and 989 ischemic cerebrovascular disease (ICVD). Follow-up started at study entry and ended at occurrence of event or May 9th, 2009,
whichever came first. Median follow-up time was 25 years and was 100% complete, i.e. none were lost to follow-up.

The Copenhagen General Population Study (CGPS)

This is a cross-sectional study of the general population initiated in 2003 with ongoing enrolment\textsuperscript{11,14}. Participants were recruited from the Danish general population and examined as in the CCHS. We included 45,603 participants in the present study. Of these, 3,738 had IHD and 2,335 had ICVD. 4,851 additional participants were used as controls for the Copenhagen Ischemic Heart Disease Study, and 625 additional participants were used as controls for the Copenhagen Carotid Stroke Study.

The Copenhagen Ischemic Heart Disease Study (CIHDS)

This case-control study comprises 4,851 patients from the greater Copenhagen area referred for coronary angiography to Copenhagen University Hospital initiated in 1991 and still recruiting. Experienced cardiologists diagnosed IHD based on characteristic symptoms of stable angina pectoris\textsuperscript{15}, together with at least one of the following: >50% diameter stenosis or diffuse atherosclerosis on coronary angiography, a previous MI, or a positive bicycle exercise electrocardiography test. The 4,851 IHD cases were matched on sex and 1-year age strata with 4,851 controls free of IVD from the CGPS.

The Copenhagen Carotid Stroke Study (CCSS)

This case-control study comprises 625 patients from the greater Copenhagen area referred for carotid artery ultrasonography to Copenhagen University Hospital initiated in 1994 and still recruiting. Experienced neurologists and vascular surgeons diagnosed ICVD together with at least 50% stenosis of a carotid artery. Hemorrhage was excluded on computed tomography scan. The 625 ICVD cases were matched on sex and 1-year age strata with 625 controls free of IVD from the CGPS.
Endpoints

In all studies, information on diagnoses of IHD (World Health Organization; International Classification of Disease, 8th edition, 410-14; 10th edition, I20-I25) was collected and verified by reviewing all hospital admissions and diagnoses entered in the national Danish Patient Registry and all causes of death entered in the national Danish Causes of Death Registry. IHD was fatal or non-fatal MI or characteristic symptoms of stable angina pectoris, including revascularization procedures. Diagnosis of MI was based on characteristic chest pain, elevated cardiac enzymes, and/or electrocardiographic changes indicative of MI.

Potential cases with ICVD including ischemic stroke (IS) were gathered from the national Danish Patient Registry and the national Danish Causes of Death Registry (World Health Organization; International Classification of Diseases, 8th edition, 431-438; 10th edition, I60-I69, G45). In the CCHS, for each person registered with ICVD, hospital records were requested. Experienced neurologists reviewed all potential cases. Possible stroke events (hospitalized as well as non-hospitalized) were validated using the World Health Organization definition of stroke: an acute disturbance of focal or global cerebral function with symptoms lasting longer than 24 hours or leading to death with presumably no other reasons than of vascular origin. To distinguish between infarction (= IS), intracerebral hemorrhage, and subarachnoid hemorrhage, either a CT scan or an MRI scan, autopsy, spinal fluid examination, or surgical description was necessary. The event was diagnosed as ischemic stroke, if the scan did not visualize an infarction or hemorrhage, but the person had symptoms that met the criteria of the stroke definiton. The diagnosis of stroke was not applied in cases where a scan revealed signs of prior cerebrovascular disease, but without history of any symptoms. The diagnostic criteria for ICVD were IS, transient ischemic attack (focal
neurological symptoms lasting less than 24 hours), or amaurosis fugax (transient blindness on one eye only). Cardioembolic strokes were included in the IS diagnosis.

Information on a diagnosis of atrial fibrillation (World Health Organization International Classification of Diseases, 8th edition, 427.93 and 427.94; 10th edition, I48.9) was collected by reviewing all hospital admissions and diagnoses entered in the national Danish Patient Registry.

Genotyping

The ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, California) was used to genotype the R1141X mutation. TaqMan-based assays were used. Each run included a positive control. Due to two rounds of reruns, call rates for genotypes where above 99.9%. Heterozygosity for the R1141X mutation was verified by DNA sequencing in CCHS, CIHDS and CCSS. In CGPS, heterozygosity was verified by running the TaqMan-based assay twice. Concordance was 100%.

Biochemical analyses

Total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides and fibrinogen were measured using standard hospital assays (Boehringer Mannheim GmbH, Mannheim, Germany or Konelab, Helsinki, Finland). LDL cholesterol was calculated using the Friedewald equation if triglycerides were ≤4 mmol/L, but measured directly at higher triglyceride levels (Thermo Fisher Scientific, Waltham, Massachusetts, USA). High-sensitivity CRP (hsCRP) was measured by turbidimetry or nephelometry (DAKO, Glostrup, Denmark, or Dade Behring, Deerfield, Illinois, USA).
Other covariates

Diabetes mellitus, smoking, antihypertensive therapy, physical inactivity, alcohol and lipid lowering therapy were dichotomized and defined as diabetes (self-reported disease, use of insulin, use of oral hypoglycemic drugs and/or non-fasting plasma glucose >11mmol/L), smoking (current), antihypertensive medication (daily use of antihypertensive drugs), physical inactivity (fraction of individuals with less than 2-4 hours per week of light physical activity at leisure time), alcohol consumption (individuals with a consumption of beer, wine or spirits at least twice weekly), and lipid lowering medication (daily use of lipid lowering drugs).

Statistical analysis

Data were analyzed using STATA/S.E. version 10.0 (Stata Corp., College Station, Texas). Two-sided probability values <0.05 were considered significant. The Mann-Whitney U test and Pearson’s $\chi^2$-test were used in two-group comparisons.

In the CCHS Cox proportional hazards regression models, with age as time scale and with the use of delayed entry (left truncation), estimated hazard ratios (HRs) for IHD, MI, ICVD, and IS as a function of R1141X genotypes. When age is used as time scale, age is automatically adjusted for in the best possible way, because the risk sets consist of all subjects still under follow-up with exact similar age as that of the subject who experience the event. Because participants were not followed since birth, the choice of age as time scale requires data to be analyzed using delayed entry$^{17}$. In the two case-control studies (CIHDS and CCSS) conditional logistic regression with sex and 1-year age strata as matching variables was used to estimate odds ratios. In the CGPS, unconditional logistic regression was used with age in 10-year age groups. Multifactorial adjustment in addition to age included sex, diabetes, smoking, and antihypertensive medication. Estimates on ICVD and IS in the general population cohorts (CCHS and CGPS) were furthermore
adjusted for atrial fibrillation to account for cases with cardioembolic stroke. Bivariate interaction terms between R1141X genotype and age (<55 years and ≥55 years) in predicting IHD were included in the models for CCHS and CGPS, and were tested statistically by likelihood ratio tests. These age groups and the specific endpoint were chosen in order to obtain large statistically valid groups for interaction tests. The proportional hazards assumption for Cox regression was tested graphically by plotting –ln(-ln[survival probability]) versus ln(analysis time); no violations were observed.

Levels of hsCRP, fibrinogen, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, systolic and diastolic blood pressure were adjusted for age, sex, body mass index and smoking by linear regression. All analyses were performed on individuals without IVD. In the analysis of blood pressure, individuals on antihypertensive medication were also excluded and in the analysis of hsCRP, fibrinogen and lipids and lipoproteins, individuals on lipid-lowering medication were excluded. Plasma hsCRP and triglyceride levels were ln transformed before analysis to obtain normal distribution.

To assess potential between study heterogeneity and summarize results from the present and previous studies of R1141X genotype and risk, meta-analyses were performed. $I^2$-statistics evaluated study heterogeneity. Results from both random and fixed effects models are presented and the meta-analyses are based on variance weighting.

**Results**

**Characteristics**

Clinical characteristics of the participants in each study cohort are shown in Tables 1 and 2.
We identified 60 R1141X heterozygotes in the CCHS (carrier frequency 0.6%), 265 in the CGPS (0.6%), 27 in the CIHDS (0.6%) and 4 in the CCSS (0.6%); none were homozygous. Genotype distributions did not deviate from Hardy-Weinberg equilibrium (P=0.53-0.94).

**R1141X heterozygosity and risk of ischemic vascular disease**

The multifactorially adjusted hazard ratios for IHD, MI, ICVD or IS as a function of R1141X genotype in the CCHS did not differ from 1.0 at a P-level <0.05 (Figure 1, left panel). This lack of association between genotype and IVD was confirmed in the CGPS (Figure 1, middle panel), the CIHDS (Figure 1, upper right panel), and the CCSS (Figure 1, lower right panel). When adjusted for age and gender only, or when unconditional logistic regression was performed in case-control studies instead of conditional models, results were similar.

To examine whether R1141X genotype interacted with age on risk, we stratified participants in those <55 and ≥55 years of age in order to obtain statistically valid groups. These analyses were performed in the two general population samples, CCHS and CGPS, for the largest endpoint, IHD. In these two analyses, R1141X genotype did not interact with age on risk of IHD (P-value for interaction: CCHS 0.76, CGPS 0.47).

**Inflammatory markers, blood pressure and lipid levels**

To examine whether R1141X genotype was associated with an exaggerated inflammatory response, increased blood pressure or an altered lipid profile, we determined the association of R1141X genotype with plasma levels of two inflammatory markers (hsCRP and fibrinogen), systolic and diastolic blood pressure, and plasma lipid and lipoprotein levels (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides) in participants in the CCHS and the CGPS (Figure 2).

Association tests were performed only in participants without ischemic vascular events, since
elevated plasma levels of hsCRP and fibrinogen, and increased blood pressure as well as altered lipid and lipoprotein levels are associated with increased risk of ischemic vascular events per se\textsuperscript{14,19,20}. R1141X genotype did not associate with plasma levels of hsCRP or fibrinogen, systolic or diastolic blood pressure or plasma levels of total cholesterol, LDL cholesterol, HDL cholesterol, or triglycerides (Figure 2, P-values, 0.34-1.00).

**Meta-analyses**

A meta-analysis including five independent studies on risk of IHD as a function of R1141X genotype with a total of 11,376 cases and 54,992 controls resulted in overall random and fixed effects OR of 1.54 (95% CI: 0.87-2.72) and 1.30 (95% CI: 1.00-1.69), respectively, with an I\textsuperscript{2} of 70% (P for heterogeneity=0.01) (Figure 3, upper panel: the CCHS, the CGPS, the CIHDS, and the studies conducted by Trip MD et al\textsuperscript{7} and Köblös G et al\textsuperscript{8}). A meta-analysis comprising the studies from the present paper with a total of 10,574 cases and 53,186 controls resulted in overall random and fixed effects OR of 1.08 (0.81-1.44) and 1.07 (95% CI: 0.80-1.43), respectively, with an I\textsuperscript{2} of 0% (P for heterogeneity=0.63) (Figure 3, lower panel: the CCHS, the CGPS and the CIHDS).

**Discussion**

The principal finding of this study is that heterozygosity for the R1141X mutation in \textit{ABCC6} is not associated with increased risk of ischemic heart disease (IHD), myocardial infarction (MI), ischemic cerebrovascular disease (ICVD), or ischemic stroke (IS). The absence of an association with IVD was consistently observed in four large studies, including two studies of the general population, and two case-control studies, comprising more than 13,600 cases with IVD and more than 53,000 controls.
This is the largest study to date of heterozygous carriers of the most common PXE-causing mutation in Caucasians, accounting for more than 30% of all recurrent mutations. The risk of IHD in heterozygotes for the R1141X mutation has been reported in two previous case-control studies including, respectively, 441 and 361 cases\textsuperscript{7,8}, both reporting a marked increase in risk of IHD among carriers. Trip et al. reported in a Dutch population that 14 of 441 cases with premature coronary artery disease versus 8 of 1057 age-and sex matched controls were R1141X heterozygous carriers. Köblös et al. reported in a Hungarian population that 5 of 361 cases with coronary artery disease versus 1 of 749 healthy blood donor controls were R1141X heterozygous carriers. From these data, ORs for risk of coronary artery disease was 4.2 (95% CI: 1.76-10.2) and 10.5 (1.2-90.3), respectively. The frequency of R1141X heterozygosity in the control populations of these two case-control studies differed markedly, 0.8%\textsuperscript{7} and 0.1%\textsuperscript{8}, most likely contributing to the very different ORs estimates. In our study with a total of 66,831 participants, including 13,642 cases, the prevalence of R1141X heterozygosity was 0.8%, regardless of disease status. Meta-analyses of IHD risk revealed that large study heterogeneity was present when including the studies by Trip et al. and Köblös et al., whereas there were no signs of between study heterogeneity for the CCHS, CGPS, and CIHDS for IHD. The 4-fold increased risk observed by Trip et al.\textsuperscript{7} was obtained in patients with premature coronary artery disease. In our two general population samples, 775 individuals had premature IHD (<55 years), making this a robust setting in which to conclude that heterozygosity for R1141X is not associated with premature IHD in the Danish population.

It has been shown that mild, chronic oxidative stress is present in fibroblasts from PXE-patients\textsuperscript{21}, and genetic variation in antioxidant genes have been shown to affect the age of disease onset\textsuperscript{22}. Currently, it is not known whether this oxidative stress is a part of the disease mechanism and contributes to the increased risk of IHD, or if this rather reflects reverse causation. R1141X heterozygosity can hypothetically cause an intermediate PXE-phenotype and may
therefore affect levels of hsCRP and fibrinogen, as a proxy for increased oxidative stress.

Furthermore, PXE-patients have a higher prevalence of hypertension, hence heterozygotes may show a trend towards elevated blood pressure as well, which may indicate an increased stiffness of the arterial wall due to mineral deposits either locally or in the kidney. However, we could not detect a moderate intermediate PXE-phenotype as reflected in increased hsCRP or fibrinogen levels or increased blood pressure. In addition, genotype did not associate with variation in lipid and lipoprotein levels.

Because the chromosomal region spanning \textit{ABCC6} (chromosome 16: 16,243,422-16,317,328) is well tagged on Affymetrix 500K and 6.0 chips (17 SNPs on the Affymetrix 500K chip and 28 SNPs on the 6.0 chip), the associations between tagSNPs within or near the \textit{ABCC6} gene and ischemic vascular disease has already been thoroughly investigated in large genome-wide association studies (GWAS) totalling more than 78,000 participants\textsuperscript{23,25}. None of these large GWAS have identified common variants in \textit{ABCC6} or in any region on chromosome 16 to be associated with ischemic cardiovascular disease down to a \(P\)-value threshold of \(10^{-3}\)\textsuperscript{25}, thus, it is highly unlikely that common variants in \textit{ABCC6} in the present studies would add to risk prediction.

Importantly, the primary aim with the present study was not to detect specific disease associated SNPs or haplotypes in the \textit{ABCC6} gene, but to evaluate whether the most common disease causing mutation in PXE, in the heterozygous state conferred any PXE associated, attenuated phenotypes in the general population, a question that could not be answered by addressing common \textit{ABCC6} variants.

Even though this study is performed in large, well-characterized cohorts of the general population and in large patient cohorts, our study has limitations. Each of the individual four studies has limitations and potential biases that differ from study to study owing to their different designs. Despite this, the results of the four studies were similar. Further, the results for ICVD and IS were
not confounded by cardioembolic stroke caused by atrial fibrillation, as adjustment for atrial fibrillation did not alter the primary results. Finally, because we studied Caucasians only our results may not necessarily apply to other ethnic groups, although PXE has been identified among most ethnicities. R1141X is the major PXE-causing mutation in Europeans possibly representing a founder mutation\textsuperscript{26}, whereas other ABCC6 mutations may be more common in other ethnic groups.

In conclusion, our results show that in the Danish general population heterozygosity for R1141X, the most common PXE-causing mutation in Caucasians, does not associate with ischemic vascular disease, as previously suggested in moderately sized case-control studies in Dutch and Hungarian populations. This suggests that one functional ABCC6 allele is sufficient to retain normal vasculature in Caucasians in the Danish general population.

**Acknowledgements:** We thank Mette Refstrup, Karen Aagaard Hansen and Christina Dam for their persistent attention to the details of the large-scale genotyping. We are indebted to the staff and participants of the Copenhagen City Heart Study, the Copenhagen General Population Study and the participants in the case-control studies for their important contributions.

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**Conflict of Interest Disclosures:** None

**References:**


Table 1. Characteristics of subjects by study and ischemic heart disease status

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<th>Copenhagen City Heart Study</th>
<th>Copenhagen General Population Study</th>
<th>Copenhagen Ischemic Heart Disease Study</th>
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<td>No. of subjects</td>
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<td>41,865</td>
<td>4,851</td>
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<tr>
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<td>55 (45-65)</td>
<td>64 (56-71)</td>
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<td>Smoking (%)</td>
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<td>Systolic blood pressure (mmHg)*</td>
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<td>Alcohol consumption&gt; twice weekly (%)</td>
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<td>Body mass index (kg/m²)</td>
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<td>Total cholesterol (mmol/L)</td>
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<td>LDL cholesterol (mmol/L)</td>
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<td>Lipid lowering medication (%)</td>
<td>0.5</td>
<td>7</td>
<td>9</td>
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All continuous traits are given as median and interquartile range. All categorical variables are shown in numbers and percentages. Diabetes mellitus, smoking, antihypertensive medication, physical inactivity, alcohol and lipid lowering therapy were dichotomized and defined as diabetes (self-reported disease, use of insulin, use of oral hypoglycemic drugs and/or non-fasting plasma glucose >11mmol/L), smoking (current), antihypertensive medication (daily use of antihypertensive drugs), physical inactivity (fraction of individuals with less than 2-4 hours per week of light physical activity at leisure time), alcohol consumption (individuals with a consumption of beer, wine or spirits at least twice weekly) and lipid lowering medication (daily use of lipid lowering drugs). * Individuals on antihypertensive medication were excluded in the analysis. NA=not available.
Table 2. Characteristics of subjects by study and ischemic cerebrovascular disease status

<table>
<thead>
<tr>
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<th>Copenhagen City Heart Study</th>
<th>Copenhagen General Population Study</th>
<th>Copenhagen Carotid Stroke Study</th>
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<td>No event ICVD</td>
<td>Controls ICVD</td>
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<td>43,268 2,335</td>
<td>625 625</td>
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<tr>
<td>Age (years)</td>
<td>57 (43-68) 68 (62-74)</td>
<td>55 (46-65) 70 (61-78)</td>
<td>65 (59-71) 64 (58-70)</td>
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<td>Sex (F/M)</td>
<td>5,224/4,063 518/471</td>
<td>25,547/17,721 1,094/1,241</td>
<td>234/391 234/391</td>
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<tr>
<td>Diabetes (%)</td>
<td>4 (2)</td>
<td>2 (2)</td>
<td>5 (4)</td>
</tr>
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<td>Smoking (%)</td>
<td>47 (29)</td>
<td>21 (24)</td>
<td>21 (48)</td>
</tr>
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<td>Systolic blood pressure (mmHg)*</td>
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<td>Diastolic blood pressure (mmHg)*</td>
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<td>81 (75-90) 84 (76-90)</td>
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<td>Body mass index (kg/m²)</td>
<td>24.8 (22.4-27.8) 26.3 (23.5-28.8)</td>
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<td>26.4 (24.2-28.7) 25.3 (23.0-27.2)</td>
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<td>Alcohol consumption&gt; twice weekly (%)</td>
<td>63 (71)</td>
<td>51 (60)</td>
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<td>Antihypertensive medication (%)</td>
<td>10 (23)</td>
<td>17 (44)</td>
<td>25 (49)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.5 (1.0-2.1) 1.8 (1.3-2.5)</td>
<td>1.4 (1.0-2.4) 1.5 (1.1-2.2)</td>
<td>1.6 (1.1-2.4) 1.6 (1.1-2.3)</td>
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<td>LDL cholesterol (mmol/L)</td>
<td>3.6 (2.8-4.4) 4.0 (3.2-4.7)</td>
<td>3.2 (2.6-4.8) 2.9 (2.2-3.7)</td>
<td>3.4 (2.8-3.9) 3.6 (2.9-4.5)</td>
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<td>HDL cholesterol (mmol/L)</td>
<td>1.5 (1.2-1.8) 1.5 (1.2-1.9)</td>
<td>1.6 (1.3-2.0) 1.6 (1.2-1.9)</td>
<td>1.5 (1.2-1.9) 1.4 (1.1-1.7)</td>
</tr>
<tr>
<td>Lipid lowering medication (%)</td>
<td>1 (8)</td>
<td>8 (35)</td>
<td>8 (21)</td>
</tr>
</tbody>
</table>

All continuous traits are given as median and interquartile range. All categorical variables are shown in numbers and percentages. Diabetes mellitus, smoking, antihypertensive medication, physical inactivity, alcohol and lipid lowering therapy were dichotomized and defined as diabetes (self-reported disease, use of insulin, use of oral hypoglycemic drugs and/or non-fasting plasma glucose >11mmol/L), smoking (current), antihypertensive medication (daily use of antihypertensive drugs), physical inactivity (fraction of individuals with less than 2-4 hours per week of light physical activity at leisure time), alcohol consumption (individuals with a consumption of beer, wine or spirits at least twice weekly) and lipid lowering medication (daily use of lipid lowering drugs). * Individuals on antihypertensive medication were excluded in the analysis. NA=not available.
Figure Legends:

Figure 1. Risk of ischemic heart disease (IHD), myocardial infarction (MI), ischemic cerebrovascular disease (ICVD) and ischemic stroke (IS) as a function of R1141X genotype in The Copenhagen City Heart Study (CCHS), The Copenhagen General Population Study (CGPS), The Copenhagen Ischemic Heart Disease Study (CIHDS) and The Copenhagen Carotid Stroke Study (CCSS). In the right hand panel, CIHDS is shown above the break, and CCSS is shown below. CC = non-carriers, CT = R1141X heterozygotes. Hazard ratios and odds ratios were multifactorially adjusted for age, sex, diabetes, smoking and antihypertensive medication.

Figure 2. Plasma levels of high-sensitivity CRP (hsCRP), fibrinogen, systolic and diastolic blood pressure (BP), total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides as a function of R1141X genotype. Data are from the Copenhagen City Heart Study (CCHS) and the Copenhagen General Population Study (CGPS). We excluded participants with known ischemic heart or cerebrovascular disease, and individuals who were receiving antihypertensive (analyses of systolic and diastolic BP) or lipid-lowering therapy (analyses of hsCRP, fibrinogen and lipids and lipoproteins). All covariates were adjusted for age, sex, body mass index and smoking. CC = non-carriers, CT = R1141X heterozygotes. Boxes represent median (horizontal line) and interquartile ranges; whiskers represent 5th and 95th percentiles. P-values by Mann-Whitney U test.

Figure 3. Meta-analysis summarizing risk of IHD by R1141X genotype. Horizontal lines correspond to 95% confidence intervals by forest plots. Diamonds and broken vertical lines represent summary estimates. Confidence interval for the summary estimate corresponds to the width of the diamond. The grey shaded areas correspond to the weight of the study in the meta-analysis (far right); weights are from random effects model. GP= General population, CC=case-control.
Ischemic heart disease

<table>
<thead>
<tr>
<th>Study design</th>
<th>No. of cases</th>
<th>No. of controls</th>
<th>OR (95% CI)</th>
<th>% Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Copenhagen City Heart Study (CCHS)</td>
<td>GP</td>
<td>1,985</td>
<td>8,291</td>
<td>0.94 (0.49-1.81)</td>
</tr>
<tr>
<td>The Copenhagen General Population Study (CGPS)</td>
<td>GP</td>
<td>3,738</td>
<td>40,044</td>
<td>1.24 (0.82-1.86)</td>
</tr>
<tr>
<td>The Copenhagen Ischemic Heart Disease Study (CIHDS)</td>
<td>CC</td>
<td>4,851</td>
<td>4,851</td>
<td>0.93 (0.55-1.57)</td>
</tr>
<tr>
<td>Trip MD et al. (Circulation 2002, ref. 7)</td>
<td>CC</td>
<td>441</td>
<td>1,057</td>
<td>4.30 (1.79-10.32)</td>
</tr>
<tr>
<td>Köblös G et al. (Genet test Mol Biomarkers 2009, ref. 8)</td>
<td>CC</td>
<td>361</td>
<td>749</td>
<td>10.51 (1.22-90.26)</td>
</tr>
<tr>
<td>Random effects: Overall ($I^2 = 70%, P = 0.01$)</td>
<td>11,376</td>
<td>54,992</td>
<td>1.54 (0.87-2.72)</td>
<td>100</td>
</tr>
</tbody>
</table>

Fixed effects

1.30 (1.00-1.69)
Heterozygosity for R1141X in ABCC6 and Risk of Ischemic Vascular Disease
Louise S. Hornstrup, Anne Tybjærg-Hansen, Christiane L. Haase, Børge G. Nordestgaard, Henrik Sillesen, Peer Grande and Ruth Frikke-Schmidt

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