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Mutation in *ABCA1* Predicted Risk of Ischemic Heart Disease in the Copenhagen City Heart Study Population

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OBJECTIVES We tested whether heterozygosity for the K776N mutation (frequency: 0.4%) in *ATP-binding cassette transporter A1* (*ABCA1*) predicted ischemic heart disease (IHD) in the Copenhagen City Heart Study population.

BACKGROUND In a complex trait like IHD, genetic variation is considered to be conferred by common DNA polymorphisms, although rare mutations may have a larger impact. Tangier disease, a rare high-density lipoprotein cholesterol (HDL-C) deficiency syndrome with IHD, is caused by homozygous *ABCA1* mutations.

METHODS We analyzed blood samples from a large cohort study of 9,076 Danish individuals followed for 24 years (167,287 person-years), during which 1,033 incident IHD events occurred. The hypothesis was retested in an independent case-control study comparing 562 IHD patients with 3,103 controls.

RESULTS The cumulative incidence of IHD as a function of age was increased in K776N heterozygotes compared with non-carriers (log-rank test: $p = 0.005$). At the age of 80 years, 48% of heterozygotes and 23% of non-carriers had IHD. Incidence rates in non-carriers and K776N heterozygotes were 61 and 157 per 10,000 person-years. The age-adjusted hazard ratio for IHD in K776N heterozygotes versus non-carriers was 2.4 (95% confidence interval 1.3 to 4.5). Adjusting for HDL-C, or for smoking, diabetes, and hypertension did not change the result, suggesting that genotype predicted risk of IHD beyond that offered by HDL-C, and by other conventional risk factors. Similar trends were obtained in an independent case-control study.

CONCLUSIONS Heterozygosity for an *ABCA1* mutation (K776N) conferred two- to three-fold risk of IHD in 37 participants in the Copenhagen City Heart study. (J Am Coll Cardiol 2005;46: 1516–20) © 2005 by the American College of Cardiology Foundation

The understanding of the genetic contribution to ischemic heart disease (IHD), the most common cause of death in developed countries (1), is very limited. Rare mutations in the heterozygous or homozygous state are known to cause familial hypercholesterolemia (2); however, the major genetic contribution to IHD is generally considered to be conferred by common DNA polymorphisms. Alternatively, rare mutations independent of plasma cholesterol levels may have a considerable impact on IHD risk (3).

The *ATP-binding cassette transporter AI* (*ABCA1*) is crucial in the initial step of high-density lipoprotein (HDL) formation and in reverse cholesterol transport. Tangier disease, a rare HDL deficiency syndrome (4–6) associated with an increased risk of IHD, is caused by homozygous *ABCA1* mutations. Genetic variation in several other genes such as *apolipoprotein AI* (*apoAI*), *apolipoprotein E*, *lecithin cholesteryl acyltransferase*, *lipoprotein lipase*, *hepatic lipase*, and *cholesteryl ester transfer protein* are known to influence HDL cholesterol levels (7,8). We and others have recently shown that rare *ABCA1* variants contribute to HDL-C levels in the general population (9,10). Thus, heterozygosity for mutations in *ABCA1* may influence risk of IHD in individuals in the general population.

Functional defects of mutations in Tangier disease and hypoalphalipoproteinemia have been extensively tested and verified in studies of apoAI cross-linking, cholesterol efflux, and intracellular signal trafficking (10–12). The K776 residue is localized in the middle of the *ABCA1* protein in a domain that is predicted to be either transmembrane or very close to the extracellular surface (13); very little is known about the functionality of this exact area. The K776N mutation is of particular interest because: 1) K776 is completely conserved between species; 2) the K > N amino acid substitution results in a change in side chain charge (basic to uncharged polar); 3) K776N is reported to be relatively frequent in Caucasians (3 per 1,000) (14); 4) disease-causing mutations have been identified in the corresponding region of a closely related gene, the *cystic fibrosis transmembrane conductance regulator* (*CFTR* or *ABCC7*) (15).

We tested the hypothesis that *ABCA1* K776N genotype is associated with risk of IHD in the general population. This was studied using blood samples from 9,076 Danish individuals followed for 24 years (167,287 person-years), during which 1,033 incident IHD events occurred. Further, the hypothesis was retested in an independent case-control

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Abbreviations and Acronyms

apoAI = apolipoprotein AI
CFTR = cystic fibrosis transmembrane conductance regulator
HDL-C = high-density lipoprotein cholesterol
IHD = ischemic heart disease

study comparing 562 IHD patients with 3,103 healthy controls.

METHODS

Participants. The Copenhagen City Heart Study is a large cohort study of the Danish general population initiated in 1976 to 1978 with follow-up examinations in 1981 to 1983 and 1991 to 1994 (16,17). At the third examination (1991 to 1994), 16,563 individuals were invited, 10,135 participated (response rate 61%), and clinical and laboratory data, DNA, and genotype information was available on 9,140. Informed consent was obtained from all participants, of which more than 99% were white and of Danish descent. The study was approved by the Danish Ethics Committee for the City of Copenhagen and Frederiksberg (No. 100.2039/91). To begin to verify the findings in the cohort study, 562 patients (45 to 64 years old) with IHD verified by coronary angiography at Department of Cardiology, Copenhagen University Hospital, Rigshospitalet, were compared with 3,103 healthy controls within the same age range.

Study designs. The 9,140 participants were followed from entry at the first (1976 to 1978), second (1981 to 1983), or third (1991 to 1994) examinations, and until end of follow-up, December 31, 1999. Information on diagnoses of IHD (World Health Organization International Classification of Diseases, 8th edition, codes 410 to 414; 10th edition, I20 to I25) was gathered until 1999 from the Danish National Hospital Discharge Register, from the Danish National Register of Causes of Death, and from medical records of general practitioners and hospitals. Of the 1,097 participants recorded with IHD, 64 were diagnosed before entry into the Copenhagen City Heart Study and were excluded, leaving 1,033 incident IHD cases, and a total of 9,076 individuals for all further analyses. Median follow-up time was 22 years (range, 0.04 to 24 years), representing 167,287 person-years. Follow-up was >99.9% complete.

Risk factors for IHD (i.e., diabetes mellitus, smoking, and hypertension) were dichotomized and defined as ever-diabetics (self-reported disease, use of insulin, use of oral hypoglycemic drugs, and/or non-fasting plasma glucose ≥11.1 mmol/l at any of the three examinations), ever-smokers (ex-smoker or current smoker at any of the three examinations), ever-hypertensive (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg or use of antihypertensive drugs at any of the three examinations).

DNA analyses. An ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, California) was used to genotype the K776N (nucleotide 2327G > C) mutation. TaqMan Universal PCR Master Mix (Applied Biosystems), wild-type and mutation-specific TaqMan probes (wild-type reporter probe: VIC-CACTCAAGATCTTCGC, mutation reporter probe: FAM-ACTCAACATCTTCGC), and one pair of polymerase chain reaction primers were used (forward: 5′TGTGGCATGGCAGGACTAC, reverse: 5′AGAAAGGCCAGAGGTACTCACA). A perfectly hybridized probe is cleaved by the 5′nuclease activity of Taq polymerase, releasing the 3′quencher linked to the probe, and resulting in a probe-specific increase in fluorescence. The assay was obtained from the Assay-by-Design Service using the Assay-by-Design File Builder software from Applied Biosystems.

Other analyses. Colorimetric and turbidimetric assays were used to measure plasma levels of total cholesterol, HDL-C, triglycerides, and apolipoproteins B and AI (all Boehringer Mannheim, Mannheim, Germany).

Statistical analyses. We used the statistical software package Stata (STATA Corp., College Station, Texas). Two-sided probability values <0.05 were considered significant. Pearson chi-square test and Student *t* test were used for two-group comparisons. Cox proportional hazards regression models estimated hazard ratios for IHD as a function of K776N genotype, and Kaplan-Meier plots and log-rank tests evaluated the cumulative incidence of IHD as a function of age and K776N genotype. For all survival statistics, age was the time scale using left truncation (or delayed entry). When age is used with left truncation, it implies that age is automatically adjusted for, and therefore not included as a covariate in the model (18). The assumption of proportional hazard ratios was checked by visual inspection of graphs of the log of the cumulative hazard function in the exposed and unexposed groups. If these graphs for the exposed and unexposed groups are parallel, the assumption is not violated. Smoking, diabetes, and hypertension dichotomized, and HDL-C in tertiles were forced into the regression models. Hazard ratios are presented for heterozygotes versus non-carriers, because no homozygotes were identified. Logistic regression analysis adjusted for age in 10-year age groups estimated odds ratio for IHD in the case-control study.

RESULTS

Among the 9,076 participants in The Copenhagen City Heart Study, 37 (frequency: 0.4%) were heterozygous and none were homozygous for K776N. Genotype frequencies did not differ from those predicted by the Hardy-Weinberg equilibrium ($p = 0.85$). Risk factors for IHD did not differ between non-carriers and K776N heterozygotes, except for levels of HDL-C in men (Table 1). The cumulative incidence of IHD as a function of age was increased in K776N heterozygotes compared with non-carriers (log-

Table 1. Characteristics of Individuals in the General Population by *ABCA1* K776N Genotype

	Women		Men	
	Non-Carriers (n = 5,003)	K776N Heterozygotes (n = 19)	Non-Carriers (n = 4,036)	K776N Heterozygotes (n = 18)
Age at entry (yrs)	46 ± 0.2	45 ± 3.0	45 ± 0.2	48 ± 2.5
Cholesterol (mmol/l)	6.3 ± 0.02	6.2 ± 0.3	6.0 ± 0.02	5.6 ± 0.3
Apolipoprotein B (mg/dl)	86.4 ± 0.3	83.8 ± 5.3	86.2 ± 0.3	85.1 ± 6.2
HDL-C (mmol/l)	1.72 ± 0.01	1.82 ± 0.11	1.38 ± 0.01	1.18 ± 0.09*
Triglycerides (mmol/l)	1.7 ± 0.02	1.4 ± 0.2	2.1 ± 0.03	2.3 ± 0.3
Body mass index (kg/m^2)	25.2 ± 0.07	25.0 ± 1.0	26.1 ± 0.06	24.7 ± 1.1
Smoking (%)	73	89	84	88
Diabetes mellitus (%)	3	0	6	11
Hypertension (%)	51	37	59	56

Values are mean ± SE, or percentages. Non-normally distributed variables were transformed before comparisons; untransformed values are presented. Heterozygotes were compared with non-carriers by Pearson chi-square test or Student *t* test. *p = 0.05.
HDL-C = high-density lipoprotein cholesterol.

rank test: p = 0.005) (Fig. 1). At the age of 80 years, about 48% of heterozygotes and 23% of non-carriers had IHD. Incidence rates in non-carriers and K776N heterozygotes were 61 and 157 per 10,000 person-years (Table 2). The age-adjusted hazard ratio for IHD in K776N heterozygotes versus non-carriers was 2.4 (95% confidence interval 1.3 to 4.5) (Table 2). Adjusting for HDL-C, or for smoking, diabetes, and hypertension did not substantially change the hazard ratio, suggesting that genotype predicted risk of IHD beyond that offered by HDL-C, and by other conventional risk factors. Finally, in an independent case-control study comparing 562 patients with IHD with 3,103 healthy controls within the same age range, the odds ratio for IHD in K776N heterozygotes versus non-carriers was 2.8 (95% confidence interval 0.8 to 9.4) (Table 3).

Mean plasma HDL-C in non-carriers and K776N heterozygotes was 1.72 mmol/l and 1.82 mmol/l in women (p = 0.42), and 1.38 mmol/l and 1.18 mmol/l in men (p = 0.05). Mean plasma apoAI levels in non-carriers and K776N heterozygotes were 151 mg/dl and 150 mg/dl in women (p = 0.93), and 130 mg/dl and 117 mg/dl in men (p = 0.03) (data not shown); HDL-C levels in the 37 individuals heterozygous for K776N ranged from 1.0 to 2.7 mmol/l in women, and from 0.5 to 2.0 mmol/l in men. Three of 19 women (16%), and 6 of 18 men (33%) had non-fasting triglycerides >2.2 mmol/l. Five of 19 women (26%) and 5 of 18 men (28%) had IHD; of these, one woman and one man had premature IHD, and two men had ischemic stroke.

DISCUSSION

A total of 20% to 44% of Tangier disease patients have been reported to have cardiovascular disease compared with only 5% to 6% in control populations (19). Heterozygotes for Tangier disease do not have the classical Tangier symptoms caused by massive deposition of cholesteryl esters in various tissues, in particular tonsil anomalies and neuropathy are not characteristic. Biochemically heterozygotes are sometimes, but not always, characterized by half-normal serum concentrations of HDL-C, and by apoAI levels below the fifth percentile of sex-matched controls (20), but it is unclear at present whether heterozygotes for mutations in *ABCA1* have an increased risk of IHD, and whether this risk is correlated to HDL-C levels. The frequency of IHD in K776N heterozygotes in the present study was 26% to 28% (women: 5 of 19; men: 5 of 18), comparable to the frequency in Tangier disease. A heterozygous mutation in *ABCA1* in the Copenhagen City Heart Study population predicted risk of IHD independent of plasma HDL-C. The present study supports experimental evidence that *ABCA1* may have antiatherosclerotic or anti-ischemic properties independent of plasma HDL-C (21–24). Thus, risk of IHD in individuals homozygote or heterozygote for mutations in *ABCA1* may not only be related to levels of HDL-C in plasma, but may also depend on local effects of *ABCA1* mutations in the arterial wall (21–23) or in platelets (24), promoting atherosclerosis.

The K776 residue is localized in the middle part of the *ABCA1* protein in a fragment that is predicted to be either transmembrane, or very close to the extracellular surface (13). Very little is known about the functionality of this exact area. In contrast, the two major extracellular loops (11,12), the ATP-binding cassettes, a regulatory proline-glutamic acid-serine-threonine (PEST) sequence (residues 1283 to 1306) (25,26), and the C-terminal region have been extensively studied (27). A rare single nucleotide polymorphism (SNP) (V771M, allele frequency 0.03) and a muta-

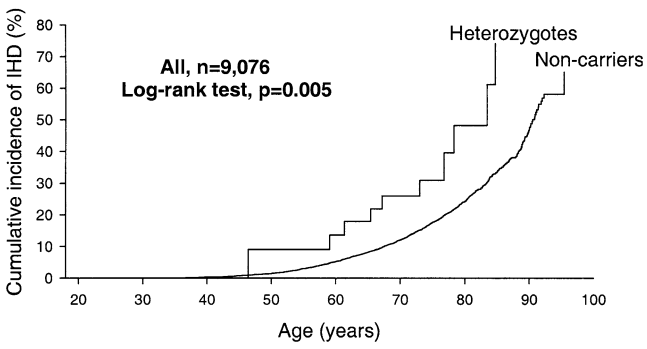

Figure 1. Cumulative incidence of ischemic heart disease (IHD) as a function of age and *ABCA1* K776N genotype.

Table 2. Risk of Ischemic Heart Disease as a Function of *ABCA1* K776N Genotype in the General Population

	Events		Incidence Rate/10,000 Person-Years (95% CI)	Hazard Ratio (95% CI)		
	Observed	Expected		Age-Adjusted	HDL-C–Adjusted	Multifactorial-Adjusted
Non-carriers	1,023	1,029	61 (58–65)	1	1	1
K776N heterozygotes	10	4	157 (76–290)	2.4 (1.3–4.5)	2.4 (1.3–4.5)	2.4 (1.3–4.5)

In the Cox regression model (age-adjusted, HDL-C–adjusted, and multifactorial-adjusted), age is adjusted for by incorporating age in the baseline hazard function (left truncation). HDL-C–adjusted: HDL-C in tertiles. Multifactorial adjusted: smoking, diabetes mellitus, and hypertension dichotomized.
CI = confidence interval; HDL-C = high-density lipoprotein cholesterol.

tion (T774P, allele frequency 0.004) situated, respectively, five and two amino acids N-terminal of the K776 residue, have been reported. Although we have recently shown that V771M is associated with increased HDL-C levels (9), effects on risk of IHD have not been documented for either of these variants (14,28). In contrast to the V771 and K776 residues, T774 is not conserved between species. The high degree of conservation of K776 between species and between human ABCAs with very different transport functions could indicate that this part of the protein is essential for normal function. To our knowledge, no homozygotes for K776N have been described so far, and we also did not identify any. Mean HDL-C levels in heterozygotes were 1.82 mmol/l (range: 1.0 to 2.7 mmol/l) in women, and 1.18 mmol/l (range: 0.5 to 2.0 mmol/l) in men. It is therefore unlikely that the majority of K776N homozygotes would express an HDL-C deficiency phenotype comparable to Tangier disease, where HDL-C levels are generally below 0.2 mmol/l. However, as is the case for K776N in the present study, in Tangier disease there also does not seem to be a clear correlation between the reduction in plasma HDL-C levels and risk of IHD. This is in agreement with previous studies that did not determine genetic variation in *ABCA1* as strong predictors of HDL-C (9,14,29). A likely reason for this is that *ABCA1* mainly affects pre-beta HDL, a type of HDL very poor in cholesterol content. However, this does not preclude an effect of genetic variants in *ABCA1* on risk of IHD.

Although K776N is a relatively common mutation, it is not a common cause of IHD. The population-attributable fraction of K776N to IHD is about 0.4% in the Copenhagen City Heart Study, or comparable to the risk of IHD attributed to low-density lipoprotein receptor mutations in the same study. However, at the individual level, K776N appears to have a marked impact on risk.

As this is a novel observation, it may represent a chance finding. However, several arguments favor a true observation: 1) the involved amino acid residue is completely conserved between species and relatively conserved between 12 ABCAs with very different transport functions; 2) the amino acid substitution changes the charge of the side-chain, potentially leading to structural alterations of the protein, and consequently to altered protein interactions or transport properties; 3) in the *CFTR* (or *ABCC7*), a disease-causing mutation (R347P) has been identified at a site that corresponds to residue 764 in *ABCA1* (15), and thus in close vicinity to K776N; 4) the present study is of a large cohort, and therefore includes only incident cases, avoiding the normal pitfalls of case reports and case-control studies (30); 5) we observed a similar trend on risk of IHD in a separate case-control study; 6) we have previously determined effects on lipids and lipoproteins of all non-synonymous SNPs identified in *ABCA1* (R219K, V771M, V825I, I883M, E1172D, R1587K). When taking multiple testing into account, the log-rank test for the cumulative incidence of ischemic heart disease as a function of age and K776N genotype fulfilled a Bonferroni-corrected p value <0.007 (0.05 of 7) on a two-sided test (seven different genetic variants tested including K776N).

The fact that DNA samples were not obtained before the 1991 to 1994 examination is a source of potential bias. If mortality rate from ischemic heart disease was higher among K776N heterozygotes and homozygotes than among non-carriers, our study would underestimate the association between *ABCA1* K776N and risk of ischemic heart disease. However, the fact that K776N was in Hardy-Weinberg equilibrium suggested that no serious selection bias had occurred in the cohort during follow-up.

This study demonstrated that heterozygosity for a common mutation in *ABCA1* increased risk of IHD in the Copenhagen City Heart Study population, and predicted risk of IHD beyond traditional cardiovascular risk factors. Additional large cohort studies should address whether the

Table 3. Risk of Ischemic Heart Disease as a Function of *ABCA1* K776N Genotype in the Case-Control Study

		Frequency		Odds Ratio (95% CI)
		IHD	Controls	Age-Adjusted
All	Non-carriers	558 (99.3)	3,095 (99.7)	1
	K776N heterozygotes	4 (0.7)	8 (0.3)	2.8 (0.8–9.4)

A total of 562 45-to 64-year-old patients with ischemic heart disease (IHD) verified by coronary angiography were compared with 3,103 healthy controls within the same age range.
CI = confidence interval.

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present findings can be generalized to populations other than the Danish.

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