Association of Loss-of-Function Mutations in the *ABCA1* Gene With High-Density Lipoprotein Cholesterol Levels and Risk of Ischemic Heart Disease

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E PIDEMIOLOGICAL STUDIES CONSIS-tently demonstrate that a low plasma level of high-density lipoprotein (HDL) cholesterol is associated with increased risk of ischemic heart disease (IHD).[1] However, whether HDL cholesterol is a primary causal factor in the pathogenesis of IHD is unclear. Data from observational studies are potentially confounded by other factors related to low HDL cholesterol levels that may contribute independently to increases in cardiovascular events. One such factor is plasma triglycerides,[2] a marker for the presence of atherogenic remnant lipoproteins.[3-5]

Mendelian randomization, which is the random assortment of genes from parents to offspring that occurs during gamete formation, provides a method of assessing whether modifiable exposures are causally related to increased risk of IHD.[6] Thus, studies of genetic disorders that lower HDL cholesterol without increases in plasma triglycerides and remnant lipoproteins provide an ideal system in which to assess the consequences of isolated, lifelong low HDL

Context Low levels of high-density lipoprotein (HDL) cholesterol are inversely related to cardiovascular risk. Whether this is a causal effect is unclear.

Objective To determine whether genetically reduced HDL cholesterol due to heterozygosity for 4 loss-of-function mutations in *ABCA1* cause increased risk of ischemic heart disease (IHD).

Design, Setting, and Participants Three studies of white individuals from Copenhagen, Denmark, were used: the Copenhagen City Heart Study (CCHS), a 31-year prospective general population study (n=9022; 28 heterozygotes); the Copenhagen General Population Study (CGPS), a cross-sectional general population study (n=31 241; 76 heterozygotes); and the Copenhagen Ischemic Heart Disease Study (CIHDS), a case-control study (n=16 623; 44 heterozygotes). End points in all 3 studies were recorded during the period of January 1, 1976, through July 9, 2007.

Main Outcome Measures Levels of HDL cholesterol in the general population, cellular cholesterol efflux, and the association between IHD and HDL cholesterol and genotype.

Results Heterozygotes vs noncarriers for 4 *ABCA1* mutations (P1065S, G1216V, N1800H, R2144X) had HDL cholesterol levels of 41 mg/dL (interquartile range, 31-50 mg/dL) vs 58 mg/dL (interquartile range, 46-73 mg/dL), corresponding to a reduction in HDL cholesterol of 17 mg/dL (P < .001). A 17-mg/dL lower HDL cholesterol level in the CCHS was associated with a multifactorially adjusted hazard ratio for IHD of 1.70 (95% confidence interval [CI], 1.57-1.85). However, for IHD in heterozygotes vs noncarriers, the multifactorially adjusted hazard ratio was 0.67 (95% CI, 0.28-1.61; 1741 IHD events) in the CCHS, the multifactorially adjusted odds ratio was 0.82 (95% CI, 0.34-1.96; 2427 IHD events) in the CGPS, and the multifactorially adjusted odds ratio was 0.86 (95% CI, 0.32-2.32; 2498 IHD cases) in the CIHDS. The corresponding odds ratio for IHD in heterozygotes vs noncarriers for the combined studies (n=41 961; 6666 cases; 109 heterozygotes) was 0.93 (95% CI, 0.53-1.62).

Conclusion Lower plasma levels of HDL cholesterol due to heterozygosity for loss-of-function mutations in *ABCA1* were not associated with an increased risk of IHD.

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cholesterol levels. A genetic disorder that fulfills such a criterion is Tangier disease, which is due to loss-of-function mutations in the adenosine triphosphate–binding cassette transporter A1 (*ABCA1*; GenBank No. AF275948) gene and results in unmeasurable HDL cholesterol levels in homozygotes and half-normal

HDL cholesterol levels in heterozygotes. Family studies of homozygotes or heterozygotes for these severe HDL de-

ficiencies report modest risk of IHD[7-10]; however, IHD risk estimates were based on few individuals, were not compared with those in the background populations, and were not adjusted for age and other cardiovascular risk factors. The risk of IHD in heterozygotes for *ABCA1* mutations in the general population has never been determined.

To test the hypothesis that genetically reduced HDL cholesterol levels due to heterozygosity for 4 loss-of-function mutations in *ABCA1* associate with increased risk of IHD, we determined whether (1) HDL cholesterol levels associate inversely with risk of IHD, (2) *ABCA1* mutations associate with reduced HDL cholesterol levels in the general population, (3) the examined *ABCA1* mutations reduce cellular cholesterol efflux, and (4) *ABCA1* mutations associate with increased risk of IHD.

This was tested in vitro (third question on cellular cholesterol efflux) and in 3 independent studies. To answer the first, second, and fourth questions, the Copenhagen City Heart Study (CCHS), a prospective study of 9022 white individuals from the Danish general population, was used. To reanswer the second and fourth questions, the Copenhagen General Population Study (CGPS), a cross-sectional study of 31 241 white individuals from the Danish general population, was used. To re-reanswer the fourth question, the Copenhagen Ischemic Heart Disease Study (CIHDS), a case-control study of 2498 white Danish IHD cases and 14 125 IHD-free controls from the CGPS, was used. Finally, for the fourth question, all 3 studies were pooled to achieve the maximal statistical power.

METHODS

Studies were approved by institutional review boards and Danish ethical committees (KF V.100.2039/91 and KF 01-144/01, Copenhagen and Frederiksberg committee; and KA 93125 and KA 99039, Copenhagen County committee), and conducted according to the Declaration of Helsinki. Written informed consent was obtained from participants. All participants were white and of Danish descent.

Copenhagen City Heart Study

The CCHS is a prospective cardiovascular study of the Danish general population initiated in 1976-1978 with follow-up examinations in 1981-1983 and 1991-1994.[11,12] Individuals were randomly selected based on the national Danish Civil Registration System to reflect the adult Danish general population aged 20 years or older. The 9022 individuals were genotyped for all non-synonymous mutations (S364C, T774P, K776N, P1065S, G1216V, N1800H, R2144X [http://www.hgmd.cf.ac.uk/ac/index.php; http://www.mutdb.org]), which were previously identified by re-sequencing the promoter, coding region, and consensus splice sites of *ABCA1* in 190 individuals of Danish ancestry with high and low HDL cholesterol levels.[13] All end points and data collection were recorded in the follow-up period of January 1, 1976, through July 9, 2007. Follow-up time was up to 31 years (214 750 person-years) and was 100% complete.

Information on diagnoses of IHD (*International Classification of Diseases, Eighth Revision,* codes 410-414; *International Classification of Diseases, 10th Revision*, codes I20-I25) was collected and verified by reviewing all hospital admissions and diagnoses entered in the national Danish Patient Registry, all causes of death entered in the national Danish Causes of Death Registry, and medical records from hospitals and general practitioners. Ischemic heart disease was defined as myocardial infarction or characteristic symptoms of stable angina pectoris.[14] A diagnosis of myocardial infarction required the presence of at least 2 of the following criteria: characteristic chest pain, elevated cardiac enzymes, and electrocardiographic changes indicative of myocardial infarction.

Copenhagen General Population Study

The CGPS is a cross-sectional study of the Danish general population initiated in 2003 and still recruiting[5]; the total aim is 100 000 participants ascertained exactly as in the CCHS, but with

a focus on all multifactorial diseases including IHD. At the time of genotyping for the present study, 31 241 individuals had been included. Information on diagnoses of IHD was ascertained as in the CCHS. End points were recorded in the period January 1, 1976, through July 9, 2007.

Copenhagen Ischemic Heart Disease Study

The CIHDS comprises 2498 patients from the greater Copenhagen area referred for coronary angiography to Copenhagen University Hospital during the period 1991 through 2004. These patients had documented IHD based on characteristic symptoms of stable angina pectoris,[14] plus at least 1 of the following: stenosis or atherosclerosis on coronary angiography, a previous myocardial infarction, or a positive bicycle exercise electrocardiography test. The diagnosis of myocardial infarction was established with the same criteria as described above. These 2498 cases were matched (6:1 when possible) by sex and 1-year age strata with 14 125 controls free of IHD from the CGPS. End points were recorded from January 1, 1976, through July 9, 2007. Participants in the CIHDS were genotyped for the 4 mutations (P1065S, G1216V, N1800H, R2144X) associated with reduced HDL cholesterol levels in the CCHS and the CGPS.

Laboratory Analyses

Genotyping. The ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City, California) was used for genotyping. All mutations identified in all studies were verified by sequencing.

Biochemical Analyses. Colorimetric and turbidimetric assays were used to measure plasma levels of total cholesterol, triglycerides, HDL cholesterol after precipitation of apolipoprotein B–containing lipoproteins, and apolipoprotein B and apolipoprotein A-I (Boehringer Mannheim GmbH, Mannheim, Germany, for all assays). Low-density lipoprotein (LDL) cholesterol was calculated according to the equation by Friedewald et al[15] if tri-

glycerides were less than 352 mg/dL (to convert to mmol/L, multiply by 0.0113), but measured directly at higher triglyceride levels (Konelab, Helsinki, Finland). Remnant lipoprotein cholesterol was total cholesterol minus HDL cholesterol and LDL cholesterol; because lipid profiles were measured in the nonfasting state, remnant lipoproteins constitute both chylomicron remnants and very low-density lipoprotein remnants.

Cellular Cholesterol Efflux Assays. HeLa cells were transfected (ExGen 500 in vitro transfection reagent, Fermentas Inc, Hanover, Maryland) with plasmids expressing the *ABCA1* mutations (P1065S, G1216V, N1800H) created by site-directed mutagenesis (Quick-Change II XL Site-Directed Mutagenesis Kit, Stratagene Inc, La Jolla, California); R2144X has previously been shown to cause reduced cholesterol efflux.[7] Sequences of all plasmids were confirmed by direct sequencing (Applied Biosystems Inc), and transfection efficiency was examined by flow cyto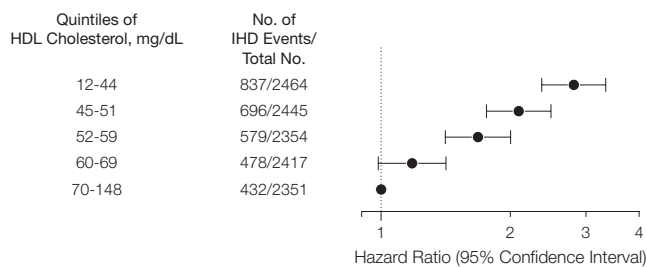metry of transfected HeLa cells. Cholesterol efflux was measured as described previously[16] (ie, HeLa cells were stimulated with increasing amounts of apolipoprotein A-I creating a dose-response curve for each plasmid). Michaelis-Menten kinetics was applied to each curve and V_{max} was then calculated. Data shown are change in V_{max} relative to the wild-type plasmid expressing the normal *ABCA1* gene.

Statistical Analyses

The statistical software package Stata special edition version 8.0 (StataCorp, College Station, Texas) was used. Two-sided probability values less than .05 were considered significant. The Mann-Whitney U test was used for continuous variables and the Pearson χ^2 test was used for categorical values. To examine the effect of mutations on intermediate phenotype in *ABCA1* heterozygotes identified in the general population, values for continuous variables for heterozygotes were converted to their respective percentiles and compared with values for the general population as a whole, using z scores, as previously described.[17-19] In the pro-spective CCHS (with the use of left truncation or delayed entry), Cox proportional hazards regression models with age as the time scale and adjusted for sex (or multifactorially) were used to estimate hazard ratios (HRs) for IHD as a function of HDL cholesterol levels and genotypes.[20] In the cross-sectional CGPS, logistic regression analysis adjusted for age and sex (or multifactorially) was used to estimate odds ratios (ORs) for IHD as a function of genotype. In the CIHDS, conditional logistic regression analysis using cases matched on age and sex with IHD-free controls from the CGPS was used to estimate ORs for IHD as a function of genotype. For the combined studies, logistic regression adjusted for age and sex (or multifactorial adjustment) was used. Hazard ratios as a function of plasma HDL cholesterol levels were corrected for regression dilution bias using a nonparametric method.[21] Power calculations assuming 1-sided probability values of less than .05 were performed using NCSS 2001 and PASS 2000 software (NCSS, Kaysville, Utah).

RESULTS
Plasma HDL Cholesterol and Risk of IHD

The HR for IHD as a function of HDL cholesterol in quintiles in the CCHS is shown in **FIGURE 1**, with the highest HDL quintile as the reference group. As expected, the risk of IHD increased with decreasing levels of HDL cholesterol; the HR adjusted for age, sex, total cholesterol, hypertension, diabetes, and smoking was 2.81 (95% confidence interval [CI], 2.37-3.33) for the lowest vs highest quintile. On a continuous scale, a 17-mg/dL (to convert to mmol/L, multiply by 0.0259) lower HDL cholesterol level associated with a multifactorially adjusted HR for IHD of 1.70 (95% CI, 1.57-1.85), similar to that reported in other studies.[1]

ABCA1 Mutation Heterozygotes and Plasma HDL Cholesterol

Four of 7 mutations (P1065S, G1216V, N1800H, R2144X) were associated with

Figure 1. Hazard Ratio for Ischemic Heart Disease (IHD) as a Function of High-Density Lipoprotein (HDL) Cholesterol in Quintiles in the Copenhagen City Heart Study

Quintiles of HDL Cholesterol, mg/dL	No. of IHD Events/ Total No.
12-44	837/2464
45-51	696/2445
52-59	579/2354
60-69	478/2417
70-148	432/2351

Hazard Ratio (95% Confidence Interval)

The HDL cholesterol values were from the second examination (1981-1983), and included a total number of 12 031 individuals. Hazard ratios were adjusted for age, sex, total cholesterol, hypertension, diabetes, and smoking. The highest quintile of HDL cholesterol (70-148 mg/dL) served as the reference group. To convert HDL cholesterol to mmol/L, multiply by 0.0259. Error bars indicate 95% confidence intervals.

Table 1. Number of Participants Heterozygous for Missense or Nonsense Mutations in *ABCA1* in the Studied Populations

Mutation	CCHS (n = 9022)	CGPS (n = 31 241)	CIHDS (n = 2498)
P1065S	1	0	0
G1216V	3	3	1
N1800H	22	70	3
R2144X	2	3	1

Abbreviations: CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; CIHDS, Copenhagen Ischemic Heart Disease Study.

reductions in levels of HDL cholesterol in plasma in heterozygotes vs noncarriers in the CCHS as well as in the CGPS, while 3 were not (S364C, T774P, K776N). The distribution of heterozygotes per mutation in the 3 studies is shown in **TABLE 1**. The overall heterozygote frequency in the general population was approximately 3:1000 in both the CCHS and the CGPS, the majority carrying the N1800H mutation. All mutations were in Hardy-Weinberg equilibrium in all 3 studies (*P* value range, .84->.99)

Characteristics of the 28 heterozygotes for loss-of-function mutations in the CCHS are shown in **TABLE 2** compared with noncarriers. Unadjusted plasma levels of HDL cholesterol were reduced by, respectively, 17 mg/dL in heterozygotes overall and 16 mg/dL in N1800H heterozygotes alone (*P*<.001); the corresponding reductions for apo-lipoprotein A-I were 40 mg/dL and 34 mg/dL (to convert to g/L, multiply by 0.01) (*P*<.001). The age- and sex-adjusted mean percentile for HDL cholesterol in heterozygotes in the CCHS was at the 16th percentile (95% CI, 9th-23rd percentile; *P*<.001), and this reduction was reflected in the corresponding percentiles for total cholesterol (31st percentile [95% CI, 22nd-41st percentile]; *P*<.001) and apolipoprotein A-I (17th percentile [95% CI, 9th-24th percentile]; *P*<.001) (**FIGURE 2**). In contrast, percentiles for LDL cholesterol, apolipoprotein B, triglycerides, and remnant lipoprotein cholesterol did not differ between heterozygotes and noncarriers. As expected, not all heterozygotes had a low plasma level of HDL cholesterol, but 25 of 28 heterozygotes (90%) in the CCHS (**FIGURE 3**), and 69 of 76 heterozygotes (91%) in the CGPS (**FIGURE 4**) had levels of HDL cholesterol below the 50th percentile for age and sex.

ABCA1 Mutations and Cellular Cholesterol Efflux in Vitro

In agreement with the observed lower plasma HDL cholesterol levels associated with these mutations in vivo, 4 mutations were associated with impaired cholesterol efflux in vitro: 79% (95% CI, 56%-103%) for P1065S, 74% (95% CI, 54%-95%) for G1216V, 49% (95% CI, 37%-60%) for N1800H, and 48%[7] for R2144X compared with 100% in wild-type (*P*=.04 for all).

ABCA1 Heterozygotes and Risk of IHD

Characteristics of individuals in the 3 different studies are shown in **TABLE 3**. For IHD in heterozygotes vs noncarriers, the multifactorially adjusted HR was 0.67 (95% CI, 0.28-1.61) in the CCHS,

Table 2. Characteristics of Individuals Heterozygous for Missense or Nonsense Mutations in *ABCA1* and Cardiovascular Risk Factors Among 9022 Participants in the Copenhagen City Heart Study[a]

Characteristic	Noncarriers (n = 8994)	Heterozygotes		
		N1800H (n = 22)	Rare Mutations (n = 6)[b]	All Mutations (n = 28)[c]
Age, median (IQR), y	60 (48-70)	64 (58-78)[d]	61 (54-74)	64 (57-77)[d]
Sex, No. (%)				
Female	5006 (56)	12 (55)	2 (33)	14 (50)
Male	3988 (44)	10 (45)	4 (67)	14 (50)
Lipid level, median (IQR), mg/dL				
Total cholesterol	236 (205-266)	237 (181-247)	199 (162-228)[d]	230 (180-245)[e]
Triglycerides	135 (96-197)	147 (112-179)	162 (104-396)	147 (108-186)
LDL cholesterol[f]	142 (114-172)	161 (112-171)	106 (92-123)	154 (109-167)
HDL cholesterol	58 (46-73)	42 (31-50)[g]	35 (27-46)[g]	41 (31-50)[g]
Apolipoprotein A-I	139 (122-160)	105 (90-123)[g]	97 (94-123)[g]	99 (90-123)[g]
Body mass index, median (IQR)[h]	25 (23-28)	25 (22-30)	27 (24-31)	26 (23-30)
Alcohol, median (IQR), g/d[i]	10 (2-22)	10 (3-17)	9 (2-12)	10 (2-14)
Physical inactivity, No. (%)[j]	5819 (65)	14 (64)	2 (33)	16 (57)
Hypertension, No. (%)	4992 (55)	19 (86)[d]	4 (67)	23 (82)[d]
Diabetes, No. (%)	409 (5)	1 (5)	2 (33)[e]	3 (11)
Smoking, No. (%)	7014 (78)	18 (82)	6 (100)	24 (86)

Abbreviations: HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein.
SI conversion factors: To convert apolipoprotein A-I to g/L, multiply by 0.01; HDL, LDL, and total cholesterol to mmol/L, multiply by 0.0259; triglycerides to mmol/L, multiply by 0.0113.
[a]The risk factors of diabetes mellitus, smoking, and hypertension were dichotomized and defined as ever having diabetes (self-reported disease, use of insulin, use of oral hypoglycemic drugs, and/or nonfasting plasma glucose >198 mg/dL; to convert to mmol/L, multiply by 0.0555), ever smokers (ex-smoker or current smoker), or ever having hypertension (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg and/or use of antihypertensive drugs).
[b]Probands heterozygous for P1065S, G1216V, or R2144X.
[c]Probands heterozygous for P1065S, G1216V, R2144X, or N1800H.
[d]*P*<.01 vs noncarriers.
[e]*P*<.05 vs noncarriers.
[f]Calculated with the equation by Friedewald et al[15] if triglycerides were less than 352 mg/dL, but measured directly at higher triglyceride levels.
[g]*P*<.001 vs noncarriers.
[h]Calculated as weight in kilograms divided by height in meters squared.
[i]A total of 12 g of alcohol equals 1 glass of wine or 1 beer.
[j]Individuals with less than 2 to 4 hours per week of light physical activity.

the multifactorially adjusted OR was 0.82 (95% CI, 0.34-1.96) in the CGPS, and the multifactorially adjusted OR was 0.86 (95% CI, 0.32-2.32) in the CIHDS (**TABLE 4**). When restricting the analyses to N1800H heterozygotes, the corresponding values were an HR of 0.50 (95% CI, 0.16-1.56), an OR of 0.87 (95% CI, 0.36-2.10), and an OR of 0.51

(95% CI, 0.15-1.80). When the number of IHD events/cases and controls from all 3 studies were combined to achieve the maximal statistical power (n=41 961; n=6666 cases; 109 heterozygotes), the corresponding OR for IHD in heterozygotes vs noncarriers was 0.93 (95% CI, 0.53-1.62); with 80% statistical power to exclude an OR of 1.77

or more. When restricting the analyses to N1800H heterozygotes (n=95), the equivalent OR was 0.77 (95% CI, 0.41-1.45); with 80% statistical power to exclude an OR of 1.85 or more.

COMMENT

The principal finding of this study is that heterozygosity for loss-of-function mutations in *ABCA1* associated with substantial, lifelong lowering of plasma levels of HDL cholesterol, but not with corresponding higher levels of plasma triglycerides or atherogenic remnant lipoproteins, did not predict an increased risk of IHD.

The risk of IHD in heterozygotes for *ABCA1* mutations identified either in families with Tangier disease or with low HDL cholesterol is unclear. Without reporting IHD risk in the background populations and without adjustment for age and other cardiovascular risk factors, Schaefer et al[8] reported the presence of angina or evidence of other vascular disease in 7 of 22 obligate heterozygotes from 11 kindreds from the United States, Europe, and Australia. Similarly, without reporting IHD risk in the background populations but only in relatives and without adjustment for age and other cardiovascular risk factors, Clee et al[7] reported coronary artery disease in 8 of 62 heterozygotes for different *ABCA1* mutations vs 5 of 122 noncarrier relatives from 11 kindreds from Canada and the Netherlands. Hence, from these studies it is difficult to determine whether *ABCA1* heterozygosity increases the risk of IHD or not because (1) the number of IHD cases was limited, (2) heterozygotes were only ascertained from families and therefore prone to ascertainment bias, (3) both studies lacked population controls and did not adjust for cardiovascular risk factors, and (4) participants were ethnically heterogeneous.[7,8] In contrast, we identified *ABCA1* heterozygotes either from the general population or from consecutive IHD cases, and adjusted IHD risk estimates for known cardiovascular risk factors. We included a total of 109 heterozygotes of Danish de-

Figure 2. Mean Percentiles for Heterozygous Carriers of *ABCA1* Mutations in the Copenhagen City Heart Study (CCHS)
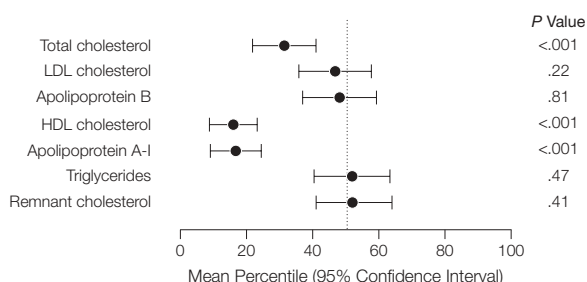
Values given are relative to age in 10-year age groups and sex-matched individuals in the CCHS as a whole (N=9022). Heterozygotes were compared with the CCHS as a whole using the *z* test. Error bars indicate 95% confidence intervals. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Figure 3. Plasma High-Density Lipoprotein (HDL) Cholesterol and Apolipoprotein A-I Levels for Heterozygous Carriers of *ABCA1* Mutations in the Copenhagen City Heart Study
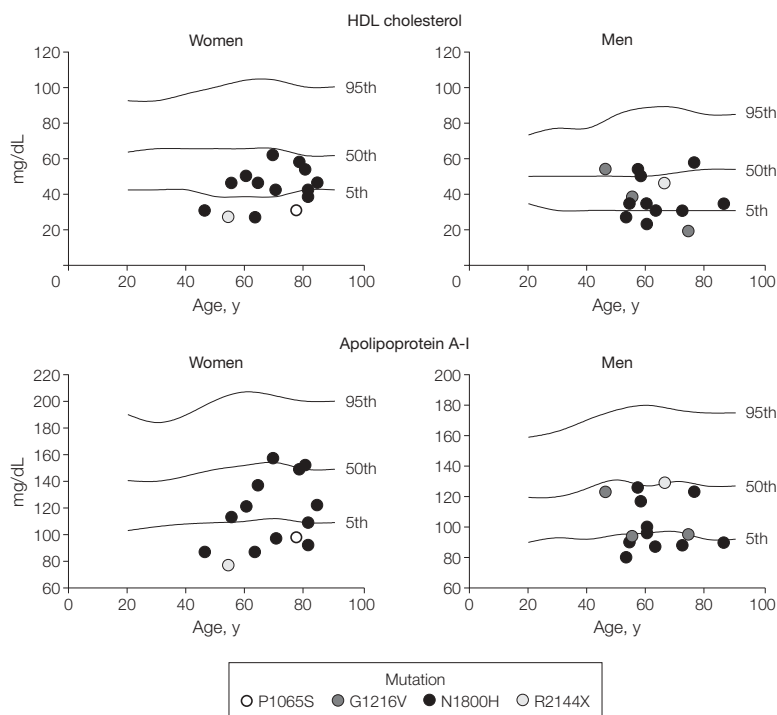
Exact values for each heterozygous mutation carrier are superimposed on the 5th, 50th, and 95th percentiles for age and sex as a whole (N=9022). To convert HDL cholesterol to mmol/L, multiply by 0.0259.

scent in studies with 6666 IHD cases and a total of 41 961 participants.

Because the extremely low or half-normal HDL cholesterol levels in Tangier disease and thus *ABCA1* homozygotes and heterozygotes are not reflected in a corresponding marked increase in risk of IHD, a simultaneous low LDL cholesterol level in some of these patients has been suggested to account for the lower than expected risk.[9,22] Our data do not favor this explanation because plasma LDL cholesterol levels were similar in *ABCA1* heterozygotes and noncarriers of the same age and sex. The present study suggests that low HDL cholesterol in *ABCA1* heterozygotes does not cause IHD. In support of this interpretation, functional mutations in apolipoprotein A-I (*APOAI*) and lecithin cholesterol acyltransferase (*LCAT*) associated with isolated low HDL cholesterol also does not consistently associate with increased risk of IHD.[23,24] Thus, taken together these data including 3 different genes suggest that low HDL cholesterol is associated with increased risk of IHD only in combination with a simultaneous increase in triglycerides and atherogenic remnant lipoproteins.[3,5,25,26] Remnant lipoproteins enter into the arterial intima like LDL,[27] and may even be trapped preferentially within the arterial intima.[28] In support of this idea, genetic variation in lipoprotein lipase associated with increases in plasma triglycerides as well as reductions in plasma HDL cholesterol are consistently associated with an increased risk of IHD.[18,29-34]

Genetically isolated high HDL cholesterol due to genetic variants in the cholesteryl ester transfer protein (*CETP*) gene likewise do not consistently translate into the expected reduced risk of IHD. Although, several studies suggest a protective effect of HDL increasing *CETP* alleles,[35-38] large studies have either shown no association to IHD,[39] or a paradoxical increase in IHD risk associated with common *CETP* variants.[40,41] These latter findings are consistent with the initial Japanese report that high HDL choles-

terol due to genetic *CETP* deficiency did not protect from IHD, but rather the contrary.[42] This is indirectly supported by recent reports[43,44] that torcetrapib, a *CETP* inhibitor that increases plasma levels of HDL cholesterol, failed

to protect against the progression of atherosclerosis and even increased the risk of cardiovascular disease, cardiovascular mortality, and overall mortality.[45] Finally, exactly as for genetic variants in *CETP*, functional variants in hepatic li-

Figure 4. Plasma High-Density Lipoprotein (HDL) Cholesterol and Apolipoprotein A-I Levels for Heterozygous Carriers of *ABCA1* Mutations in the Copenhagen General Population Study
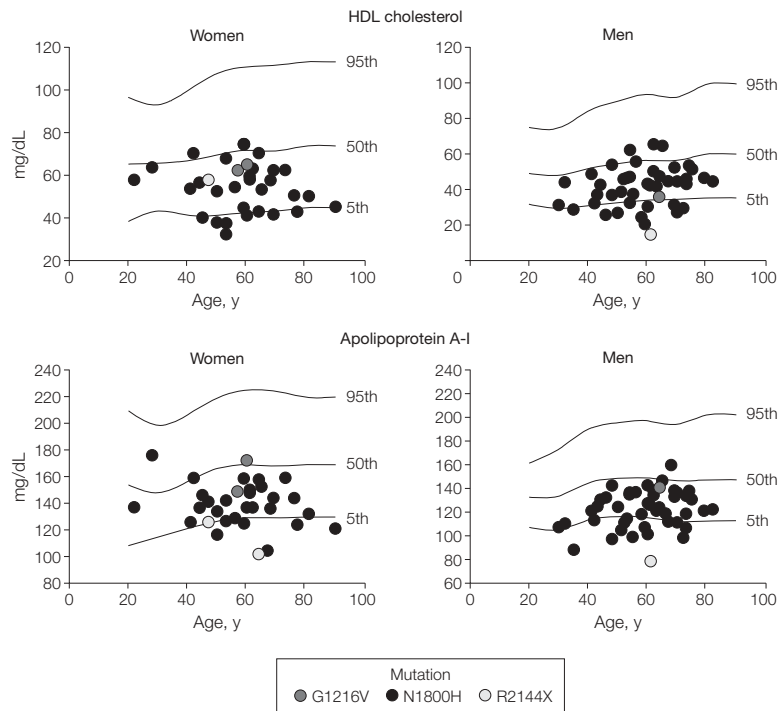
Exact values for each heterozygous mutation carrier are superimposed on the 5th, 50th, and 95th percentiles for age and sex as a whole (n=31 241). To convert HDL cholesterol to mmol/L, multiply by 0.0259. P1065S was not identified in the Copenhagen General Population Study.

Table 3. Characteristics of Participants of Danish Descent in the 3 Studies Conducted in Copenhagen, Denmark[a]

	CCHS (n = 9022)	CGPS (n = 31 241)	CIHDS (n = 16 623)
Age, median (IQR), y	60 (48-70)	60 (49-68)	61 (54-69)
Sex, No. (%)			
Female	5020 (56)	16 250 (52)	4865 (29)
Male	4002 (44)	14 991 (48)	11 758 (71)
Total cholesterol, median (IQR), mg/dL	236 (205-266)	220 (193-247)	220 (193-247)
Hypertension, No. (%)	5015 (56)	19 570 (63)	10 484 (63)
Diabetes, No. (%)	412 (5)	1297 (4)	845 (5)
Smoking, No. (%)	7038 (78)	19 509 (62)	11 160 (67)

Abbreviations: CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; CIHDS, Copenhagen Ischemic Heart Disease Study; IQR, interquartile range.
SI conversion factor: To convert total cholesterol to mmol/L, multiply by 0.0259.
[a] The risk factors of diabetes mellitus, smoking, and hypertension were dichotomized and defined as ever having diabetes (self-reported disease, use of insulin, use of oral hypoglycemic drugs, and/or nonfasting plasma glucose >198 mg/dL; to convert to mmol/L, multiply by 0.0555), ever smokers (ex-smoker or current smoker), or ever having hypertension (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg and/or use of antihypertensive drugs).

Table 4. Risk of Ischemic Heart Disease as a Function of *ABCA1* Mutations in the 3 Different Studies

	CCHS	CGPS	CIHDS	All Studies
Controls, No.	7281	28 014[a]	14 125[a]	35 295
Events/cases, No.	1741	2427	2498	6666
	HR (95% CI)	**OR (95% CI)**		
Age- and sex-adjusted				
All mutations	0.65 (0.27-1.56)	0.96 (0.41-2.26)	0.73 (0.29-1.86)	0.84 (0.48-1.45)
N1800H	0.47 (0.15-1.46)	1.04 (0.44-2.47)	0.46 (0.14-1.51)	0.69 (0.37-1.29)
Multifactorially adjusted[b]				
All mutations	0.67 (0.28-1.61)	0.82 (0.34-1.96)	0.86 (0.32-2.32)	0.93 (0.53-1.62)
N1800H	0.50 (0.16-1.56)	0.87 (0.36-2.10)	0.51 (0.15-1.80)	0.77 (0.41-1.45)

Abbreviations: CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; CIHDS, Copenhagen Ischemic Heart Disease Study; HR, hazard ratio; OR, odds ratio.
[a] Individuals with cardiovascular events were excluded.
[b] Adjusted for age, sex, total cholesterol, hypertension, diabetes, and smoking.

pase associated with higher HDL cholesterol levels have been shown to associate with increased risk of IHD.[46,47]

Recent studies suggest that the levels of HDL cholesterol per se are not relevant for IHD risk, but rather the functionality or dysfunctionality of the lipoprotein, possibly influencing cellular cholesterol efflux.[48-50] Individuals with established cardiovascular disease are suggested to have more proinflammatory HDL than healthy controls[48]; however, whether this is the cause of increased atherosclerosis development, or whether it is a consequence of a chronic inflammatory state in established atherosclerosis remains to be determined in humans. Further, bone marrow transplantation studies in mice suggest that genetically reduced *ABCA1* expression might cause increased atherosclerosis risk independent of HDL cholesterol levels, most likely due to reduced efflux in *ABCA1*-deficient macrophages.[51,52] In contrast, whole-body *ABCA1* knockout mouse models, which resemble the human model most, display no increased atherosclerosis development despite dramatically reduced cholesterol efflux and HDL cholesterol levels.[52,53] Our results in humans of loss-of-function *ABCA1* mutations with low HDL cholesterol levels and low cellular cholesterol efflux, but no increased risk of IHD, is in complete agreement with these latter findings. In humans, it has only been assumed, but never shown convincingly that low choles-

terol efflux due to loss-of-function mutations in *ABCA1* directly causes atherosclerosis. Despite 30 years of work, the relationship of reverse cholesterol transport to atherosclerosis remains more of a hypothesis than an established fact.[54] The present study of human *ABCA1* mutations associated with low cellular cholesterol efflux, as well as with low levels of HDL cholesterol but without increased risk of IHD, questions the hypothesis of reverse cholesterol transport.

Limitations to our studies include that we might have missed other important mutations in *ABCA1* because the effects of *ABCA1* on HDL cholesterol do not necessarily have to be mediated only by variants in coding sequences.[13,55] However, we also screened *ABCA1* in consensus splice sites and known regulatory regions without detecting any mutations.[13] Also, although each of our individual 3 studies has limitations and potential biases that differ from study to study due to the different designs, the results of the 3 studies were similar. Furthermore, because we studied whites only our results may not necessarily apply to other ethnic groups. Also, we cannot completely exclude that other unmeasured metabolic or phenotypic changes associated with these mutations could be countering an actual increased risk conferred by lower HDL cholesterol. Finally, the limitations inherent in the Mendelian randomization study design also need to be considered.[56]

Mendelian randomization can be used for the study of causation between modifiable exposures and disease, provided that the following 6 criteria are fulfilled[56]: (1) the presence of suitable genetic variants for the study of the modifiable exposures of interest (heterozygotes for loss-of-function mutations in *ABCA1* as used in the present study are ideal for this purpose); (2) reliable genotype-intermediate-phenotype and genotype-disease associations can be established (we demonstrated lower HDL cholesterol levels in *ABCA1* mutation heterozygotes, and tested whether these mutations associated with increased risk of IHD in 3 independent studies); (3) there is no confounding of these relationships (an obvious confounder for HDL cholesterol could be triglycerides and remnant lipoproteins; for this reason we chose a gene in which mutations associated with low HDL cholesterol levels, but not with high levels of triglycerides and remnant lipoproteins, namely *ABCA1*); (4) there are no pleiotropic effects of the genetic variants of interest (we cannot exclude unknown pleiotropic effects of *ABCA1*; however, we can exclude pleiotropic effects due to effects on lipid and lipoprotein levels other than HDL cholesterol and apolipoprotein A-I); (5) there is no compensation by other genes during development (canalization)[56,57] (canalization or compensation by other genes during intra-

uterine development is generally difficult to assess, however, the fact that the heterozygotes as expected had half normal or lower HDL cholesterol levels suggests that any canalization would have affected factors other than HDL cholesterol); and (6) population admixture that differs between cases and controls may severely affect risk estimates (however, our participants were all white and of Danish descent). Hence classic limitations of Mendelian randomization do not appear to be of major importance in this study.

In conclusion, lower plasma levels of HDL cholesterol due to heterozygosity for loss-of-function mutations in *ABCA1* were not associated with an increased risk of IHD.

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