

Remnant Cholesterol as a Causal Risk Factor for Ischemic Heart Disease

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Objectives	The aim of this study was to test the hypothesis that elevated nonfasting remnant cholesterol is a causal risk factor for ischemic heart disease independent of reduced high-density lipoprotein (HDL) cholesterol.
Background	Elevated remnant cholesterol is associated with elevated levels of triglyceride-rich lipoproteins and with reduced HDL cholesterol, and all are associated with ischemic heart disease.
Methods	A total of 73,513 subjects from Copenhagen were genotyped, of whom 11,984 had ischemic heart disease diagnosed between 1976 and 2010. Fifteen genetic variants were selected, affecting: 1) nonfasting remnant cholesterol alone; 2) nonfasting remnant cholesterol and HDL cholesterol combined; 3) HDL cholesterol alone; or 4) low-density lipoprotein (LDL) cholesterol alone as a positive control. The variants were used in a Mendelian randomization design.
Results	The causal odds ratio for a 1 mmol/l (39 mg/dl) genetic increase of nonfasting remnant cholesterol was 2.8 (95% confidence interval [CI]: 1.9 to 4.2), with a corresponding observational hazard ratio of 1.4 (95% CI: 1.3 to 1.5). For the ratio of nonfasting remnant cholesterol to HDL cholesterol, corresponding values were 2.9 (95% CI: 1.9 to 4.6) causal and 1.2 (95% CI 1.2 to 1.3) observational for a 1-U increase. However, for HDL cholesterol, corresponding values were 0.7 (95% CI: 0.4 to 1.4) causal and 1.6 (95% CI: 1.4 to 1.7) observational for a 1 mmol/l (39 mg/dl) decrease. Finally, for LDL cholesterol, corresponding values were 1.5 (95% CI: 1.3 to 1.6) causal and 1.1 (95% CI: 1.1 to 1.2) observational for a 1 mmol/l (39 mg/dl) increase.
Conclusions	A nonfasting remnant cholesterol increase of 1 mmol/l (39 mg/dl) is associated with a 2.8-fold causal risk for ischemic heart disease, independent of reduced HDL cholesterol. This implies that elevated cholesterol content of triglyceride-rich lipoprotein particles causes ischemic heart disease. However, because pleiotropic effects of the genetic variants studied cannot be totally excluded, these findings need to be confirmed using additional genetic variants and/or randomized intervention trials. (J Am Coll Cardiol 2013;61:427-36) © 2013 by the American College of Cardiology Foundation

Remnant cholesterol is the cholesterol content of triglyceride-rich lipoproteins, composed of very low-density lipoproteins and intermediate-density lipoproteins in the fasting state and of

these 2 lipoproteins together with chylomicron remnants in the nonfasting state (1,2). Elevated nonfasting plasma triglyceride is a marker of elevated nonfasting remnant cholesterol (1,3,4) and is associated with increased risk for cardiovascular disease (1-7). Because triglycerides per se are unlikely directly to cause cardiovascular disease (2,8), remnant cholesterol is more likely to be the causal factor.

See page 437

Elevated remnant cholesterol is also associated with reduced high-density lipoprotein (HDL) cholesterol (2,9,10); however, because HDL cholesterol increases have failed to reduce cardiovascular disease (11,12), elevated remnant cholesterol may be a more likely causal factor than reduced HDL cholesterol.

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Abbreviations and Acronyms

CI = confidence interval

HDL = high-density lipoprotein

LDL = low-density lipoprotein

We tested the hypothesis that elevated nonfasting remnant cholesterol is a causal risk factor for ischemic heart disease independent of reduced HDL cholesterol, using the Mendelian randomization approach (10,13,14): genetic variants robustly associated with a single lipoprotein, without effects on

other lipoproteins, were used to test the causal effect of lifelong exposure on risk for ischemic heart disease. Following a pre-specified plan and on the basis of current evidence from published research, we therefore carefully selected 15 genetic variants affecting levels of: 1) nonfasting remnant cholesterol alone; 2) nonfasting remnant cholesterol and HDL cholesterol combined; 3) HDL cholesterol alone; or 4) low-density lipoprotein (LDL) cholesterol alone as a positive control. Thus, several other genotypes were considered for inclusion but were a priori not included, exemplified by apolipoprotein E genotypes that affect levels of all the lipoproteins studied here and therefore would be difficult to use for the present studies.

We genotyped 73,513 white subjects of Danish descent from Copenhagen, of whom 11,984 had ischemic heart disease. Causal estimates for each lipoprotein on risk for ischemic heart disease were calculated in the same subjects and were thus directly comparable.

Methods

Studies were approved by institutional review boards and Danish ethical committees (H-KF-01-144/01, KF-100.2039/91, KF-01-144/01, KA-93125, and KA-99039) and conducted according to the Declaration of Helsinki. Informed consent was obtained from participants. All participants were white and of Danish descent. No subject was included in more than 1 study.

The CGPS (Copenhagen General Population Study). The CGPS is a prospective study of the general population initiated in 2003 with ongoing enrollment (1). Subjects were selected on the basis of the national Danish Civil Registration System to reflect the adult Danish population age 20 to ≥ 80 years. Data were obtained from a questionnaire, a physical examination, and blood samples including deoxyribonucleic acid extraction. Follow-up was 100% complete; that is, no subject was lost to follow-up. A total of 57,719 participants were included at the time of analyses; of these, 10,368 were used as controls in the CIHDS (Copenhagen Ischemic Heart Disease Study), leaving 47,351 participants.

The CCHS (Copenhagen City Heart Study). The CCHS is a prospective study of the general population initiated from 1976 to 1978, with follow-up examinations from 1981 to 1983, 1991 to 1994, and 2001 to 2003. Participants were recruited and examined exactly as in the CGPS. Blood samples for deoxyribonucleic acid extraction were drawn at the 1991 to 1994 and 2001 to 2003

examinations. A total of 10,609 participants were eligible for analyses.

The CIHDS. This study comprises 5,185 patients with ischemic heart disease from Copenhagen University Hospital during the period from 1991 to 2009 and 10,368 controls without ischemic heart disease matched by age and sex from the CGPS. Besides a diagnosis of ischemic heart disease, these cases also had stenosis or atherosclerosis on coronary angiography and/or positive results on exercise electrocardiography.

Ischemic heart disease. Information on diagnoses of ischemic heart disease (World Health Organization International Classification of Diseases–Eighth Revision, codes 410 to 414; International Classification of Diseases–Tenth Revision, codes I20 to I25) were collected from 1976 through 2010 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, as previously described (1).

Laboratory analyses. Nonfasting total cholesterol, triglycerides, and HDL cholesterol were measured using colorimetric assays (Boehringer Mannheim, Mannheim, Germany; Konelab, Thermo Fisher Scientific, Waltham, Massachusetts). LDL cholesterol was calculated using the Friedewald equation when plasma triglycerides were ≤ 4.0 mmol/l and otherwise measured directly (Konelab). Nonfasting remnant cholesterol was calculated as nonfasting total cholesterol minus HDL cholesterol minus LDL cholesterol.

Genotypes. Genotyping was performed using TaqMan (Applied Biosystems, Carlsbad, California) or by restriction enzyme assays. Genotypes were verified by genotyping of randomly selected samples of each variant using 2 different methods (TaqMan plus sequencing or restriction enzyme assay). Call rates for genotypes were $>99.9\%$ for all assays.

Other covariates. Smokers were defined as current smokers. Hypertension was defined as systolic blood pressure ≥ 140 mm Hg (≥ 135 mm Hg for patients with diabetes), diastolic blood pressure ≥ 90 mm Hg (≥ 85 mm Hg for patients with diabetes), and/or the use of antihypertensive medications prescribed specifically for hypertension. Lipid-lowering therapy was self-reported.

Statistical analysis. Data were analyzed using Stata/SE version 11.2 (StataCorp LP, College Station, Texas). Two-sided p values <0.05 were considered statistically significant. Non-normally distributed variables were log transformed to approach a normal distribution. Chi-square tests were used to evaluate Hardy-Weinberg equilibrium.

In the prospective CGPS, CCHS, and CIHDS controls combined, Cox proportional hazards regression models with age as the time scale were used to estimate hazard ratios for ischemic heart disease by lipoproteins in quintiles; patients diagnosed with ischemic heart disease before study entry were excluded, and those dying or emigrating during follow-up were censored at their deaths or emigration dates, respectively. Multivariate adjustment was performed for age,

Table 1 Characteristics of the Participants in the 3 Studies

Variable	CGPS	CCHS	CIHDS
Number of participants	47,351	10,609	15,553
Women	28,806 (61%)	5,886 (55%)	4,418 (29%)
Age (yrs)	55 (46-65)	59 (46-69)	63 (56-71)
Triglycerides (mmol/l)	1.4 (1.0-2.1)	1.5 (1.1-2.2)	1.6 (1.1-2.3)
Remnant cholesterol (mmol/l)	0.6 (0.4-0.9)	0.7 (0.5-1.0)	0.7 (0.5-1.0)
HDL cholesterol (mmol/l)	1.6 (1.3-1.9)	1.5 (1.2-1.9)	1.4 (1.1-1.8)
LDL cholesterol (mmol/l)	3.2 (2.6-3.8)	3.7 (3.0-4.5)	3.2 (2.6-3.9)
Hypertension	32,081 (68%)	5,075 (48%)	7,741 (78%)*
Smoking, current	9,919 (21%)	4,589 (49%)	2,193 (23%)*
Statin use	4,620 (10%)	87 (1%)	1,008 (10%)*
Ischemic heart disease	4,582 (10%)	2,217 (21%)	5,185 (33%)*

Data are from study enrolment from 2003 to 2009 for the CGPS, from the 1991 to 1994 or 2001 to 2003 examinations of the CCHS when deoxyribonucleic acid was collected, and from study enrollment from 1991 to 2009 in the CIHDS. Data are expressed as median (interquartile range) or as number of participants (percent). Number of participants varies slightly according to availability of variables. To convert triglyceride values to milligrams per deciliter, multiply values in millimoles per liter by 88. To convert cholesterol values to milligrams per deciliter, multiply values in millimoles per liter by 38.6. *Values available only for controls.

CCHS = Copenhagen City Heart Study; CGPS = Copenhagen General Population Study; CIHDS = Copenhagen Ischemic Heart Disease Study; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

sex, smoking, hypertension, time since last meal, time of day for blood sampling, and lipid-lowering therapy. Analyses were purposely not adjusted for variables that in themselves through biological pathways affect lipoprotein levels, such as body mass index, alcohol consumption, and diabetes. Conventional hazard ratios and confidence intervals (CIs) were corrected for regression dilution bias using a nonparametric method (15), using lipoprotein values from 4,253 individuals without lipid-lowering therapy participating in both the 1991 to 1994 and 2001 to 2003 examinations of the CCHS; this correction helps avoid underestimation of risk esti-

mates, but it does not affect whether results are statistically significant. Regression dilution ratios of 0.48, 0.63, 0.73, and 0.60 were computed for nonfasting remnant cholesterol, the ratio of nonfasting remnant cholesterol to HDL cholesterol, HDL cholesterol, and LDL cholesterol, respectively.

One-way analysis of variance was used to compare lipoprotein levels as a function of genotypes in the CGPS, CCHS, and CIHDS controls combined. Genotypes were combined in 4 groups: 1) nonfasting remnant cholesterol-increasing alleles (*TRIB1* rs2954029, *GCKR* rs1260326, and *APOA5* rs651821); 2) nonfasting remnant cholesterol-increasing and HDL cholesterol-decreasing alleles (*LPL* rs1801177, *LPL* G188E, *LPL* rs268, and *LPL* rs328); 3) HDL cholesterol-decreasing alleles (*LIPC* -480C>T, *ABCA1* N1800H, and *ABCA1* R2144X), and, as a positive control; 4) LDL cholesterol-increasing alleles (*APOB* rs5742904, *LDLR* W23X, *LDLR* W66G, *LDLR* W556S, and *PCSK9* rs11591147). The p values for trends were estimated using Cuzick's extension of the Wilcoxon rank sum test.

Association of genotypes with observed risk for ischemic heart disease was by logistic regression estimating age-adjusted and sex-adjusted odds ratios in the CGPS, CCHS, and CIHDS combined. Theoretically predicted risk was estimated from changes in lipoprotein values and known conventional associations of lipoproteins with ischemic heart disease in the prospective study.

Potential causal relationships between genetically elevated or reduced levels of the different lipoproteins and risk for ischemic heart disease were assessed by instrumental variable analyses using 2-stage least squares regression in an additive model. Genotypes associated with specific lipoprotein traits (i.e., nonfasting remnant cholesterol) were in-

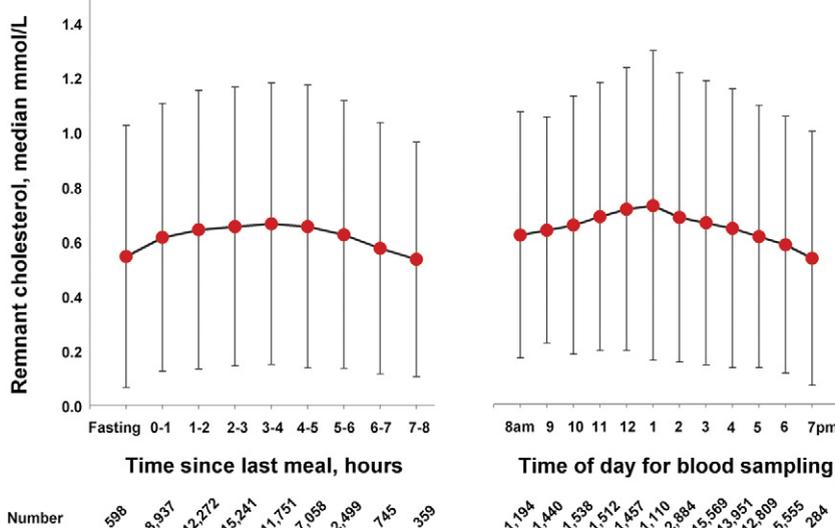


Figure 1 Remnant Cholesterol, Time Since Last Meal, and Time of Day for Blood Sampling

Remnant cholesterol as a function of time since last meal and time of day for blood sampling. Values are medians with interquartile ranges (error bars).

cluded as individual instruments in both first-stage and second-stage regressions; thereby, each variant was weighted by frequency and effect size. The strength of the separate and combined instruments was evaluated by F statistics from the first-stage regression, where an F statistic >10 indicates sufficient strength to ensure the validity of the instrumental variable analysis (13). R² values as percentages were used as a measure of the contribution of genotypes to the variation in the different lipoprotein levels. Lipoprotein values were not available for all cases in the CIHDS (7.6% missing) and were therefore imputed from age, sex, genotypes, and the known distribution of lipoproteins in the CGPS and the CCHS. The same was done for participants from the CGPS (7.4%) and the CCHS (0.6%) using lipid-lowering therapy; however, if subjects missing lipoprotein values or on lipid-lowering therapy were excluded instead, results were similar to those reported. Causal estimates from instrumental variable analysis were compared with observed risk for ischemic heart disease from conventional epidemiology using the Bland-Altman method.

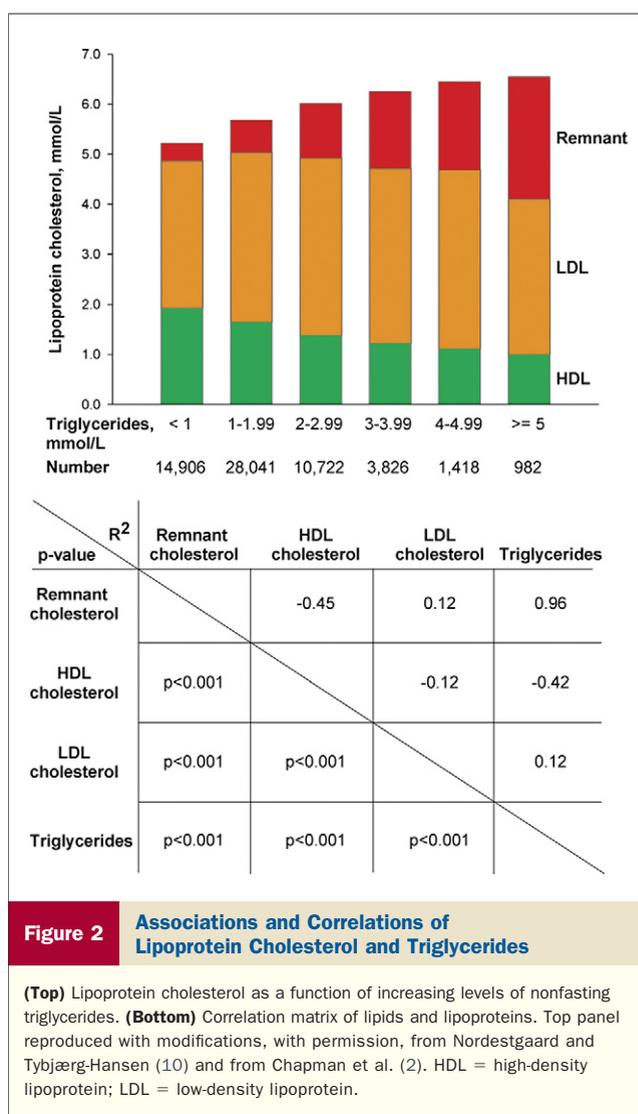
Results

Table 1 shows baseline characteristics of participants in the 3 studies. Participants in the CIHDS were older and more often men than in the CGPS and the CCHS. Genotype distributions for all studies were in Hardy-Weinberg equilibrium ($p > 0.10$).

Remnant cholesterol. Remnant cholesterol levels changed slightly as a function of time since last meal and time of day for blood sampling and had median levels of 0.55 mmol/l during fasting and 0.67 mmol/l at 3 to 4 h after the last meal (Fig. 1); the highest and lowest levels were seen at 1 and 7 PM, respectively. Increased levels of plasma triglycerides were associated with increased levels of remnant cholesterol and with reduced levels of HDL cholesterol, while the association with LDL cholesterol was less pronounced (Fig. 2, top). Remnant cholesterol levels were highly correlated with nonfasting triglyceride levels ($R^2 = 0.96$, $p < 0.001$), inversely correlated with HDL cholesterol levels ($R^2 = -0.45$, $p < 0.001$), and less correlated with LDL cholesterol levels ($R^2 = 0.12$, $p < 0.001$) (Fig. 2, bottom).

Risk for ischemic heart disease: observational estimates. Associations of lipoproteins in quintiles with risk for ischemic heart disease in the CGPS, CCHS, and CIHDS controls combined in a prospective design are shown in Figure 3. Observational hazard ratios for the fifth versus first quintiles were 2.3 (95% CI: 1.7 to 3.1) for increasing nonfasting remnant cholesterol, 2.6 (95% CI: 2.1 to 3.2) for increasing ratio of nonfasting remnant cholesterol to HDL cholesterol, 2.5 (95% CI: 2.1 to 3.0) for decreasing HDL cholesterol, and 1.8 (95% CI: 1.4 to 2.2) for increasing LDL cholesterol.

Genotypes and lipoprotein levels. Lipoprotein levels as a function of increasing number of lipoprotein-increasing and lipoprotein-decreasing alleles for the CGPS, CCHS, and



CIHDS controls combined are shown in Figure 4 for groups of genotypes with similar effects. Corresponding effects of the individual genotypes are shown in Online Figure 1.

For nonfasting remnant cholesterol alone, 3 to 6 versus 0 or 1 alleles were associated with 15% (0.10 mmol/l [4 mg/dl]) elevated nonfasting remnant cholesterol and 19% elevated ratio of nonfasting remnant cholesterol to HDL cholesterol. HDL and LDL cholesterol differed only slightly by genotype.

For nonfasting remnant cholesterol and HDL cholesterol combined, 3 or 4 versus 0 or 1 alleles were associated with 22% (0.15 mmol/l [6 mg/dl]) elevated nonfasting remnant cholesterol, 37% elevated ratio of nonfasting remnant cholesterol to HDL cholesterol, and 11% (0.18 mmol/l [7 mg/dl]) reduced HDL cholesterol. LDL cholesterol differed only slightly by genotype.

For HDL cholesterol alone, 2 or 3 versus 0 alleles were associated with 8% (0.13 mmol/l [5 mg/dl]) reduced HDL cholesterol; the ratio of nonfasting remnant cholesterol to HDL cholesterol changed accordingly. Although nonfast-

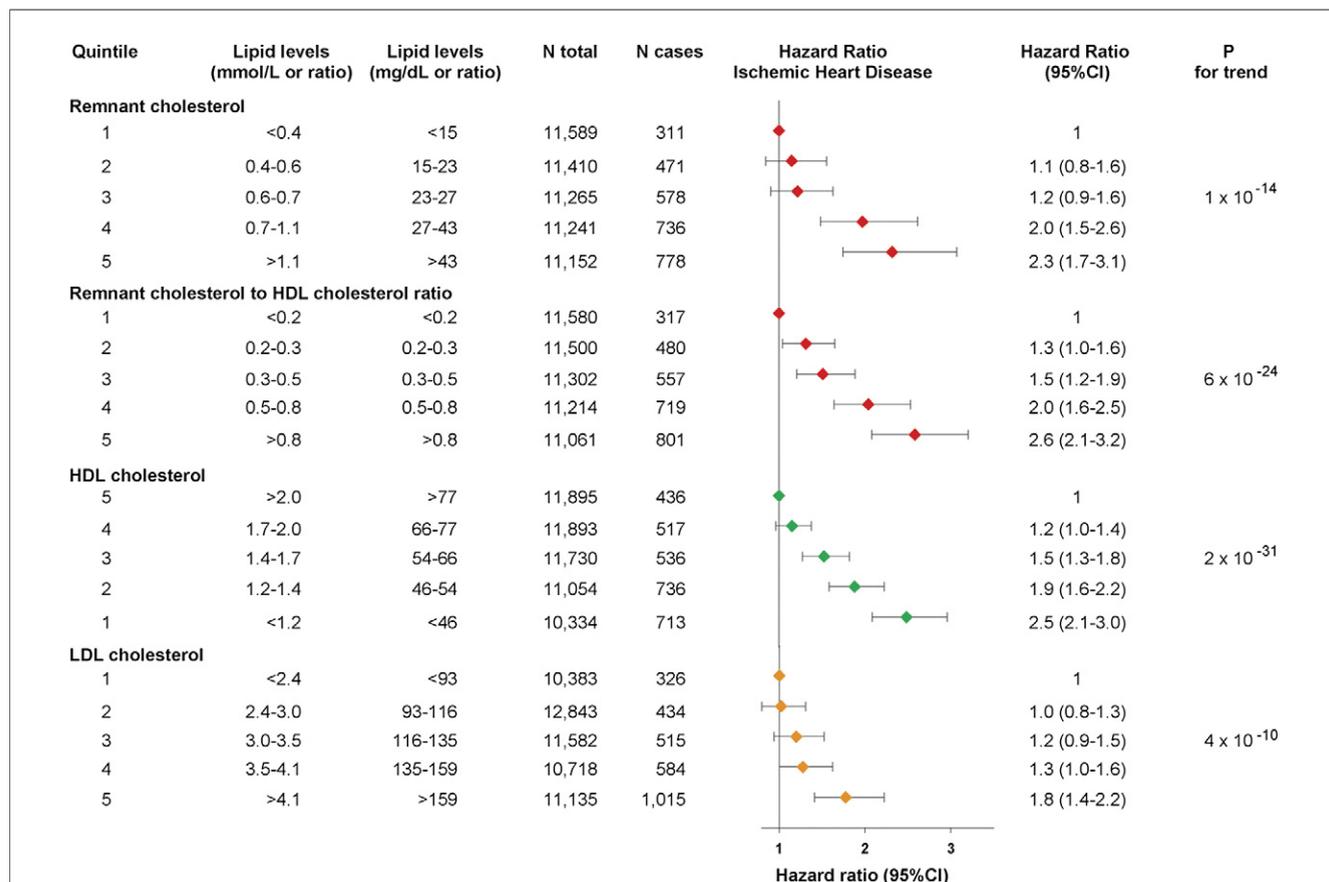


Figure 3 Risk for Ischemic Heart Disease: Observational Estimates

Risk for ischemic heart disease as a function of lipoprotein levels in quintiles in the prospective Copenhagen General Population Study, Copenhagen City Heart Study, and Copenhagen Ischemic Heart Disease Study controls combined. The p values are for trends across quintiles. CI = confidence interval; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

ing remnant cholesterol decreased slightly as a function of genotype, this was in the opposite direction of the usual inverse association between reduced HDL cholesterol and elevated nonfasting remnant cholesterol (2-4). LDL cholesterol did not differ by genotype.

For LDL cholesterol alone, 3 versus 0 or 1 alleles were associated with 90% (2.66 mmol/l [103 mg/dl]) elevated LDL cholesterol. Other lipoprotein classes did not differ by genotype.

Genotypes and risk for ischemic heart disease. Assuming that each of the lipoproteins is causally associated with risk for ischemic heart disease, we would theoretically expect genetically elevated or reduced levels to be associated with risk for ischemic heart disease in the same direction as observed for conventional lipoprotein levels, as shown in Figure 3. For nonfasting remnant cholesterol alone, for nonfasting remnant cholesterol and HDL cholesterol combined, and for LDL cholesterol alone, the observed associations of the genotypes with risk for ischemic heart disease were in the same direction and more pronounced than the theoretically predicted risk for ischemic heart disease; however, this was not the case for HDL cholesterol alone (Fig. 5).

For nonfasting remnant cholesterol alone for 3 to 6 versus 0 or 1 alleles, the observed risk was increased by 15% (95% CI: 9% to 21%) and the theoretically predicted risk by 5% (95% CI: 4% to 6%) (Fig. 5). For nonfasting remnant cholesterol and HDL cholesterol combined for 3 or 4 versus 0 or 1 alleles, the corresponding risks were increased by 29% (95% CI: 18% to 42%) and 9% (95% CI: 7% to 11%). However, for HDL cholesterol alone for 2 or 3 versus 0 alleles, we observed no association with risk for ischemic heart disease by genotype, despite a theoretically predicted increased risk of 7% (95% CI: 5% to 8%). Finally, for LDL cholesterol alone for 3 versus 0 or 1 alleles, corresponding risks were increased by 525% (95% CI: 314% to 845%) and 45% (95% CI: 29% to 63%).

Other risk factors for ischemic heart disease than lipoproteins were generally distributed equally among genotypes, confirming that the genotypes are not confounded (Online Table 1).

Risk for ischemic heart disease: causal estimates. We also examined the potential causal association of the lipoproteins with risk for ischemic heart disease in instrumental variable analyses. For nonfasting remnant cholesterol alone, for the ratio of nonfasting remnant cholesterol to HDL

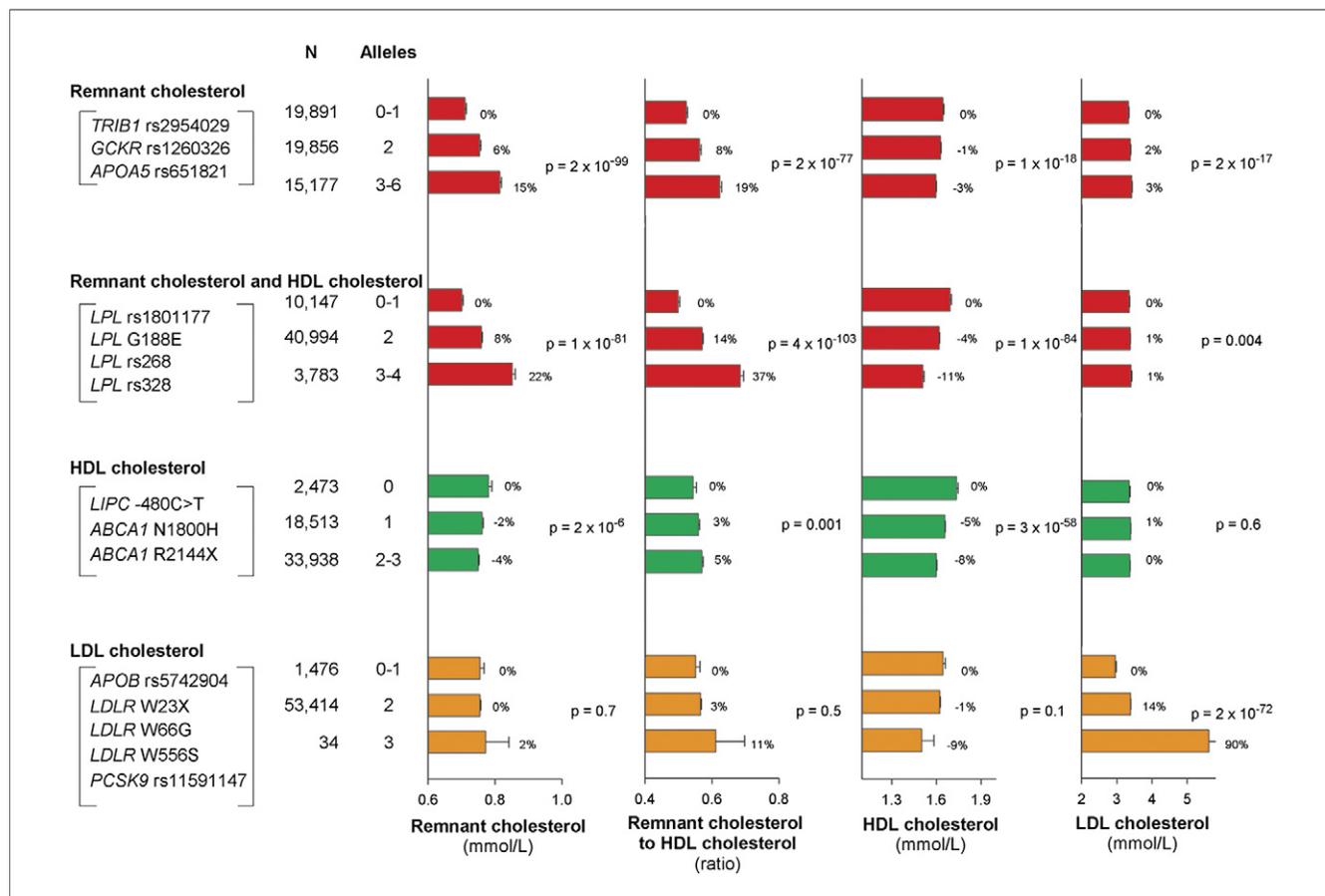


Figure 4 Genotypes and Lipoprotein Levels

Lipoprotein levels as a function of genotype in allele counts in the Copenhagen General Population Study, Copenhagen City Heart Study, and Copenhagen Ischemic Heart Disease Study controls combined. Genetic variants included in each lipoprotein group are shown in brackets. Columns show mean lipoprotein levels with standard error bars, percent change in lipoprotein levels, and p values for trends across allele counts. Participants using lipid-lowering therapy were excluded. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

cholesterol, and for LDL cholesterol alone, causal risk estimates for genetically elevated levels were in the same direction and higher than corresponding risk estimates for corresponding increases in conventional plasma levels of the same lipoproteins; however, this was not the case for HDL cholesterol alone (Fig. 6).

The causal odds ratio for a 1 mmol/l (39 mg/dl) genetic increase of nonfasting remnant cholesterol was 2.8 (95% CI: 1.9 to 4.2), with a corresponding observational hazard ratio of 1.4 (95% CI: 1.3 to 1.5) (Fig. 6). For the ratio of nonfasting remnant cholesterol to HDL cholesterol, corresponding values were 2.9 (95% CI: 1.9 to 4.6) causal and 1.2 (95% CI: 1.2 to 1.3) observational for a 1-U increase. However for HDL cholesterol, corresponding values were 0.7 (95% CI: 0.4 to 1.4) causal and 1.6 (95% CI: 1.5 to 1.7) observational for a 1-mmol/l (39 mg/dl) decrease. Finally for LDL cholesterol, corresponding values were 1.5 (95% CI: 1.3 to 1.6) causal and 1.1 (95% CI: 1.1 to 1.2) observational for a 1 mmol/l (39 mg/dl) increase.

Results were similar for a 1 standard deviation increase or decrease in genetic and plasma levels of the lipoproteins

(Online Fig. 2). Online Figure 3 illustrates that all genetic variants separately and combined for each lipoprotein studied were valid instruments (F statistics >10). Figure 6 shows causal odds ratios for the CGPS, CCHS, and CIHDS combined; however, analyzing these studies separately gave similar results (Online Fig. 4).

Discussion

This study suggests that a 1 mmol/l (39 mg/dl) increase in nonfasting remnant cholesterol is associated with a 2.8-fold causal risk for ischemic heart disease, independent of reduced HDL cholesterol. These findings are novel.

That the causal risk for ischemic heart disease was higher than the observational risk for elevated nonfasting remnant cholesterol suggests that lifelong exposure through genetically elevated levels may have a larger effect on risk than suggested from observational data alone. This is in accordance with parallel results for LDL cholesterol in present and former studies (16,17).

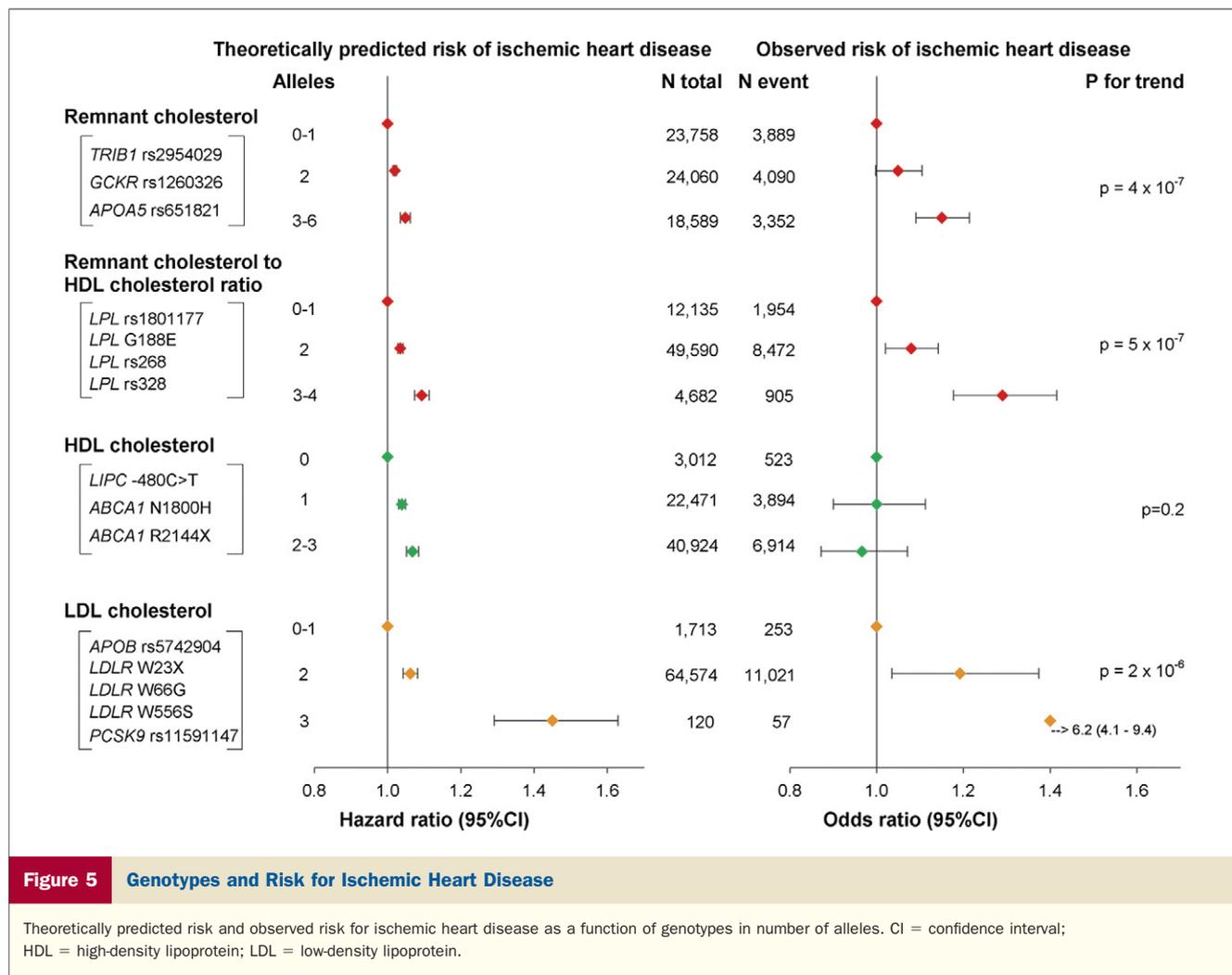


Figure 5 Genotypes and Risk for Ischemic Heart Disease

Theoretically predicted risk and observed risk for ischemic heart disease as a function of genotypes in number of alleles. CI = confidence interval; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

Mechanistically, the explanation for a causal effect of elevated nonfasting remnant cholesterol on ischemic heart disease risk could be that remnants (i.e., triglyceride-rich lipoproteins) enter and get trapped in the intima of the arterial wall (18,19). Like LDL trapping in the intima, this would lead to the accumulation of intimal cholesterol, atherosclerosis, and ultimately ischemic heart disease (2,9). Unlike LDL, remnants and triglyceride-rich lipoproteins may not need to be oxidized to be taken up by macrophages to cause foam cell formation and atherosclerosis (20).

Previous case-control (21-31) and prospective (32-36) studies have also found observationally that elevated remnant cholesterol is associated with increased risk for cardiovascular disease. However, these studies were relatively small and, unlike the present study, were unable to investigate the causality of remnant cholesterol, because of confounding by various cardiovascular risk factors and because of the inverse association with reduced HDL cholesterol (2,10). To circumvent these problems, on one hand, we used the Mendelian randomization approach to avoid confounding from other cardiovascular risk factors: genetically determined levels of lipoproteins are present from birth and are

therefore generally not confounded by other risk factors (13,14). On the other hand, by using carefully selected genetic variants, we were able to deduct the causality of lipoproteins by using genetic variants as instruments. Variants causing only lifelong elevated nonfasting remnant cholesterol were associated with increased risk for ischemic heart disease. The same was seen for variants causing both lifelong elevated nonfasting remnant cholesterol and reduced HDL cholesterol; however, variants causing only lifelong reduced HDL cholesterol were not associated with increased risk for ischemic heart disease. Taken together, this implies that it must be the elevated nonfasting remnant cholesterol and triglyceride-rich lipoproteins that are causally related to increased risk for ischemic heart disease.

In our study, remnant cholesterol was determined in the nonfasting state and thus included cholesterol in all triglyceride-rich lipoproteins, that is, very low-density lipoproteins, intermediate-density lipoproteins, and chylomicron remnants combined. By using nonfasting remnant cholesterol calculated as nonfasting total cholesterol minus HDL cholesterol minus LDL cholesterol, remnant cholesterol can be calculated directly from a standard lipid profile,

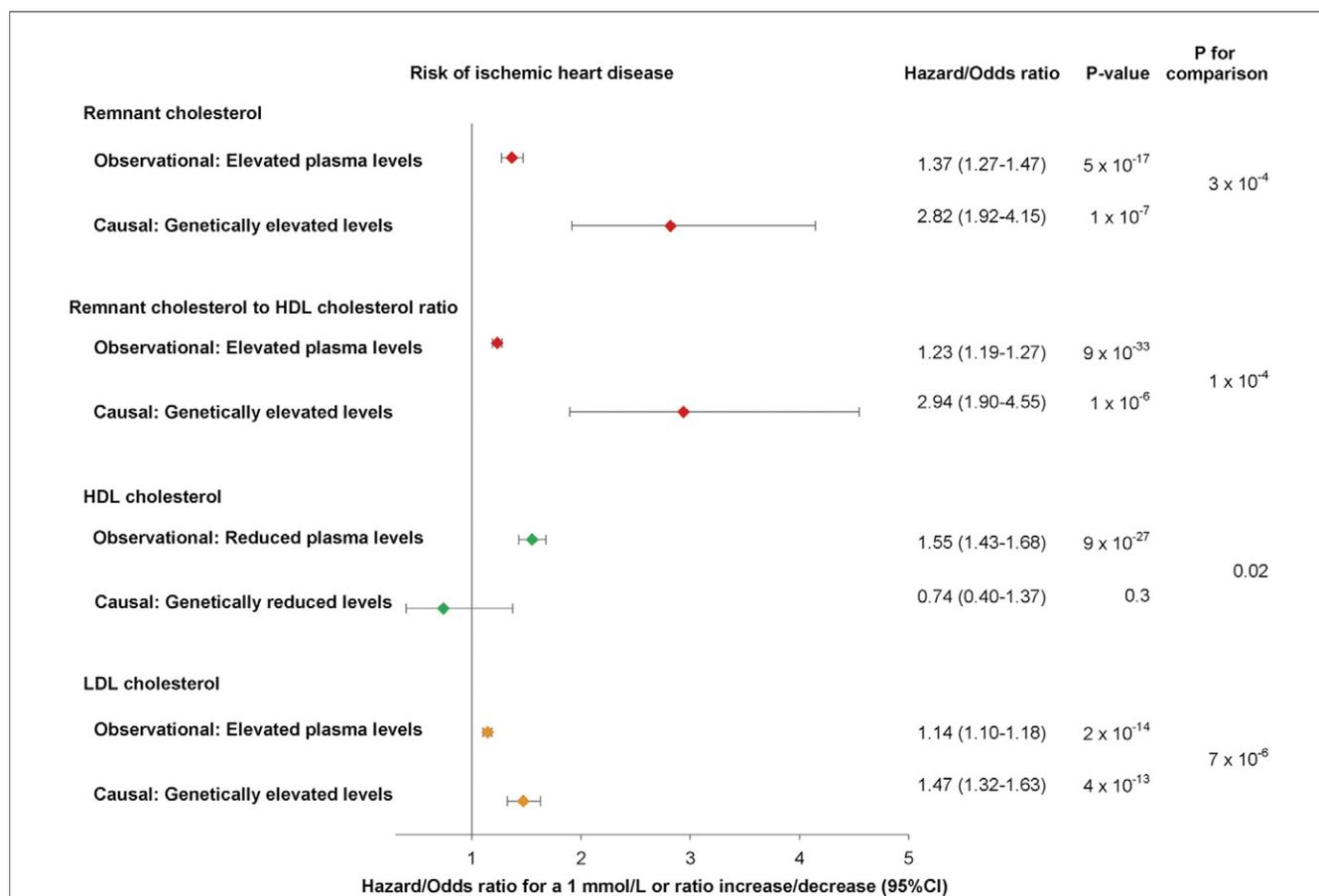


Figure 6 Causal and Observational Risk Estimates for Ischemic Heart Disease

Observational risk estimates in hazard ratios for a 1 mmol/l or a 1 ratio unit increase or decrease in plasma lipoprotein levels are from the prospective Copenhagen General Population Study, Copenhagen City Heart Study, and Copenhagen Ischemic Heart Disease Study controls combined, adjusted for age, sex, smoking, hypertension, time since last meal, time of day for blood sampling, and lipid-lowering therapy. Causal risk estimates for a 1 mmol/l or a 1 ratio unit increase or decrease in genetic lipoprotein levels in odds ratios are from the Copenhagen General Population Study, Copenhagen City Heart Study, and Copenhagen Ischemic Heart Disease Study combined, estimated by instrumental variable analyses. The p values are for significance of risk estimates, and p values for comparison are for differences between observational and causal estimates. CI = confidence interval; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

provided it is taken in the nonfasting state, as has been recommended in Denmark since 2009. Thus, the presently used calculated nonfasting remnant cholesterol comes at no extra cost and is easily available.

The Mendelian randomization approach has potential limitations, with the most important being pleiotropy of the genetic variants used; that is, the genetic variants may affect risk for ischemic heart disease through mechanisms other than their effects on lipoprotein levels. For a proper Mendelian randomization study, a variant that exclusively affects plasma remnant cholesterol is needed. Because remnant cholesterol is the result of multiple interrelated metabolic processes involving many genes, an ideal variant that affects only remnant cholesterol simply may not exist. Some pleiotropic effects are evident for the 3 genetic variants associated with remnant cholesterol and risk for ischemic heart disease. From previous studies, it is known that *TRIB1* and *APOA5* are also associated with HDL cholesterol and LDL cholesterol and *GCKR* with HDL choles-

terol, fasting glucose levels, and risk for diabetes (37,38). Most important, remnant cholesterol is directly related to plasma triglycerides, because remnant cholesterol is the cholesterol content of triglyceride-rich lipoproteins. However, because elevated triglycerides per se are unlikely to be the direct cause of ischemic heart disease (2,8), the cholesterol content of the triglyceride-rich lipoproteins is more likely to be the cause of the risk marked by high triglycerides. However, when we studied nonfasting plasma triglycerides instead of nonfasting remnant cholesterol, results and conclusions were largely similar (data not shown). Except for triglycerides and other components of triglyceride-rich lipoproteins, the presently used variants were not associated to any large extent with other known risk factors, and choosing more than 1 variant from different genes on different chromosomes makes it unlikely that the variants have the same pleiotropic effects (14). Nevertheless, rather than remnant cholesterol per se, it is possible that triglyceride-rich lipoprotein particles per se or other unmea-

sured covariates of triglycerides could also be implicated as being causal for ischemic heart disease on the basis of our findings.

Also, it could be argued that a relatively larger population variation in remnant cholesterol compared with HDL and LDL cholesterol would make it easier to detect associations of genetic variants with remnant cholesterol; however, because the variation over time was larger for remnant cholesterol than for HDL and LDL cholesterol, with regression dilution ratios of 0.48, 0.73, and 0.60, respectively, it is more likely that it is the opposite; that is, it is more difficult to detect genetic variation associated with remnant cholesterol than with HDL and LDL cholesterol. Furthermore, analyses are limited to a number of genetic variants and genes, for example, 4 variants in *LPL* for remnant cholesterol and HDL cholesterol, variants in 2 genes for HDL cholesterol, and variants in 3 genes for LDL cholesterol. Because different biological pathways lead to variability in these lipid fractions, the interpretation of the results is limited to the processes regulated by the genes included in the analysis. For HDL cholesterol, Mendelian randomization studies do not support a casual relationship between common genetic variants near *ABCA1*, *LCAT*, *LIPC*, or *LIPG* (39–42), but it is possible that genetic variation altering the expression or function of other genes in the HDL pathway, such as *CETP* and *APOA1*, might alter risk for ischemic heart disease. Finally, because we studied white subjects only, our results may not necessarily apply to other races.

Strengths of our study include sufficient statistical power and no bias from admixture, because of the large sample size of all white subjects of Danish descent. Another strength of the present study is the control for the validity of the study design by the inclusion of a positive LDL cholesterol control in all analyses; LDL cholesterol is well documented as a causal risk factor for ischemic heart disease (10,16,43). Finally, although remnant cholesterol varies slightly more than LDL cholesterol in the same subjects over 10 years (compare regression dilution ratios of 0.48 vs. 0.60), we corrected for this regression dilution bias for all lipoproteins.

Conclusions

A 1 mmol/l (39 mg/dl) increase in nonfasting remnant cholesterol is associated with a 2.8-fold causal risk for ischemic heart disease, independent of reduced HDL cholesterol. This implies that the elevated cholesterol content of triglyceride-rich lipoprotein particles causes ischemic heart disease. However, because we cannot totally exclude pleiotropic effects of the genetic variants studied, our findings need to be confirmed using additional genetic variants and/or randomized intervention trials. These findings may help direct future efforts to reduce cardiovascular disease beyond the reduction achieved using statins. The focus of future intervention studies should be not only on lowering LDL cholesterol levels but also on lowering nonfasting

remnant cholesterol levels and triglyceride-rich lipoproteins, or alternatively on reduction in nonfasting apolipoprotein B or non-HDL cholesterol, both of which include remnant or triglyceride-rich lipoproteins.

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Key Words: atherosclerosis ■ cardiovascular disease ■ lipoproteins ■ myocardial infarction.

 **APPENDIX**

For supplementary tables and figures and their legends, please see the online version of this article.