

ORIGINAL ARTICLE

Loss-of-Function Mutations in *APOC3* and Risk of Ischemic Vascular Disease

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ABSTRACT

BACKGROUND

High plasma levels of nonfasting triglycerides are associated with an increased risk of ischemic cardiovascular disease. Whether lifelong low levels of nonfasting triglycerides owing to mutations in the gene encoding apolipoprotein C3 (*APOC3*) are associated with a reduced risk of ischemic cardiovascular disease in the general population is unknown.

METHODS

Using data from 75,725 participants in two general-population studies, we first tested whether low levels of nonfasting triglycerides were associated with reduced risks of ischemic vascular disease and ischemic heart disease. Second, we tested whether loss-of-function mutations in *APOC3*, which were associated with reduced levels of nonfasting triglycerides, were also associated with reduced risks of ischemic vascular disease and ischemic heart disease. During follow-up, ischemic vascular disease developed in 10,797 participants, and ischemic heart disease developed in 7557 of these 10,797 participants.

RESULTS

Participants with nonfasting triglyceride levels of less than 1.00 mmol per liter (90 mg per deciliter) had a significantly lower incidence of cardiovascular disease than those with levels of 4.00 mmol per liter (350 mg per deciliter) or more (hazard ratio for ischemic vascular disease, 0.43; 95% confidence interval [CI], 0.35 to 0.54; hazard ratio for ischemic heart disease, 0.40; 95% CI, 0.31 to 0.52). Heterozygosity for loss-of-function mutations in *APOC3*, as compared with no *APOC3* mutations, was associated with a mean reduction in nonfasting triglyceride levels of 44% ($P < 0.001$). The cumulative incidences of ischemic vascular disease and ischemic heart disease were reduced in heterozygotes as compared with noncarriers of *APOC3* mutations ($P = 0.009$ and $P = 0.05$, respectively), with corresponding risk reductions of 41% (hazard ratio, 0.59; 95% CI, 0.41 to 0.86; $P = 0.007$) and 36% (hazard ratio, 0.64; 95% CI, 0.41 to 0.99; $P = 0.04$).

CONCLUSIONS

Loss-of-function mutations in *APOC3* were associated with low levels of triglycerides and a reduced risk of ischemic cardiovascular disease. (Funded by the European Union and others.)

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LOW-DENSITY LIPOPROTEIN (LDL) CHOLESTEROL is the principal target of lipid drugs that have been developed for the prevention of cardiovascular disease. However, even among patients with substantial reductions in LDL cholesterol levels, residual cardiovascular risk persists.¹ Spurred by the strong association between high levels of both fasting and nonfasting triglycerides and the risk of cardiovascular disease,²⁻⁶ recent genetic studies involving mendelian randomization have suggested that high levels of nonfasting triglycerides are causally associated with an increased risk of ischemic cardiovascular disease, independent of high-density lipoprotein (HDL) cholesterol levels.^{7,8} Plasma triglycerides are markers of so-called remnant particles, which include very-low-density lipoproteins (VLDLs), intermediate-density lipoproteins, and, in the nonfasting state, chylomicron remnants.

Apolipoprotein C3 is a component of remnant particles that is associated with high levels of triglycerides and thus remnant cholesterol. Apolipoprotein C3 increases plasma triglyceride levels by inhibiting hydrolysis of triglyceride-rich lipoproteins by lipoprotein lipase⁹ and by attenuating the uptake of triglyceride-rich remnant lipoproteins by the liver.¹⁰⁻¹³ A loss-of-function mutation in the gene encoding apolipoprotein C3 (*APOC3*) has been associated with markedly reduced levels of triglycerides and remnant cholesterol and with a decrease in coronary-artery calcification, a surrogate marker for atherosclerosis.¹⁴ Therefore, apolipoprotein C3 is a potential new target for reducing residual cardiovascular risk.

In the current study, we first confirmed that low levels of nonfasting triglycerides were associated with reduced risks of ischemic vascular disease and ischemic heart disease in two general-population studies. Then, in genetic analyses of participants in the same studies, we tested whether loss-of-function mutations in *APOC3*, causing lifelong low levels of nonfasting triglycerides, were associated with reduced risks of ischemic vascular disease and ischemic heart disease, to the extent predicted by the associations observed in the first part of the study.

METHODS

STUDY OVERSIGHT

The study was approved by the appropriate institutional review boards and Danish ethics com-

mittees and was conducted according to the principles of the Declaration of Helsinki. The funding bodies had no role in the conduct of the study, in the collection, management, analysis, or interpretation of data, or in the preparation, review, or approval of the manuscript.

PARTICIPANTS

We included participants in two similar prospective studies involving persons from the general population in Denmark, the Copenhagen City Heart Study (CCHS) and the Copenhagen General Population Study (CGPS). All the participants were white and of Danish descent.

In the CCHS, baseline examinations were performed between 1976 and 1978, and follow-up examinations were performed between 1981 and 1983, between 1991 and 1994, and between 2001 and 2003.^{7,8} Participants were selected with the use of the Danish Civil Registration System to reflect the adult Danish population 20 to 100 years of age or older. Data were obtained by means of a questionnaire, a physical examination, and blood-sample collection. We included 10,333 consecutive participants in the current analyses. During follow-up (which ended in May 2011), 2817 participants had incident ischemic vascular disease (as defined below), of whom 2198 had ischemic heart disease (as defined below).

The CGPS was initiated in 2003, and enrollment is ongoing.^{7,8} Participants were recruited and examined exactly as in the CCHS. We included 65,392 consecutive participants in the current analyses. During follow-up (which ended in May 2011), 7980 participants had incident ischemic vascular disease, of whom 5359 had ischemic heart disease.

Combining the participants in the CCHS and the CGPS yielded a total of 75,725 participants; during a median follow-up of 34 years, ischemic vascular disease developed in 10,797 participants, with ischemic heart disease in 7557 of these participants. DNA was available for all participants, and lipid values were available for more than 98%. Written informed consent, including consent for genetic analysis, was obtained from all the participants.

LABORATORY ANALYSES AND ASSESSMENT OF OTHER COVARIATES

Plasma levels of triglycerides, total cholesterol, HDL cholesterol, apolipoprotein A1, apolipoprotein B,

alanine aminotransferase, and aspartate aminotransferase were measured in the nonfasting state,¹⁵ with the use of standard hospital assays. LDL cholesterol levels were calculated with the use of the Friedewald equation¹⁶ when plasma triglyceride levels were 4.00 mmol per liter (350 mg per deciliter) or less and were otherwise measured directly (Konelab). Lipoprotein(a) was measured as described previously.¹⁷ Levels of high-sensitivity C-reactive protein were measured by means of turbidimetry (Dako) or nephelometry (Dade Behring). Assessment of other covariates is described in the Supplementary Appendix, available with the full text of this article at NEJM.org.

CLINICAL END POINTS

Ischemic vascular disease was defined as either ischemic heart disease or ischemic cerebrovascular disease. Information on diagnoses of ischemic heart disease (*International Classification of Diseases*, 8th revision [ICD-8], codes 410 through 414; 10th revision [ICD-10], codes I20 through I25) and ischemic cerebrovascular disease (ICD-8, codes 431 through 438; ICD-10, codes I60 through I69 and G45) was collected and verified through a review of all hospital admissions and diagnoses entered in the Danish National Patient Registry, all causes of death entered in the National Danish Causes of Death Registry, and medical records from hospitals and general practitioners. Details of these and other end points are provided in the Supplementary Appendix.

RESEQUENCING OF APOC3 AND GENOTYPING

We resequenced the coding regions and consensus splice sites of *APOC3* in all 10,333 participants in the CCHS using three polymerase-chain-reaction (PCR) fragments covering the three coding exons and the exon–intron boundaries (*APOC3* consensus sequence NC_000011.9 GRCh37.p5) (Table S1 in the Supplementary Appendix). Mutational analysis was performed with the use of LightScanner (Idaho Technology), followed by sequencing on the ABI 3730 DNA Analyzer (Applied Biosystems). One splice variant (IVS2+1G→A, rs138326449) and three nonsynonymous variants (R19X, rs76353203; A43T, rs147210663; and V50M, rs201803883), all of which were identified in more than one participant in the CCHS, were subsequently genotyped in the CGPS participants with the use of the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and TaqMan-

based assays or with the use of an allele-specific PCR system (KASPer, LGC Genomics). Genotypes of all 302 heterozygotes identified were verified by direct sequencing of new PCR products.

STATISTICAL ANALYSIS

Data were analyzed with the use of Stata/SE software, version 12.0 (Stata). The Mann–Whitney U test and Pearson’s chi-square test were used for two-group comparisons of continuous and categorical variables, respectively. Cuzick’s test was used to test covariates as a function of triglyceride levels.

To examine the association between nonfasting triglyceride levels and the risk of ischemic vascular disease or ischemic heart disease, we used Cox proportional-hazards regression models, with age as the time scale and left truncation (delayed entry), to estimate hazard ratios. For this analysis, the median follow-up period from the time of baseline blood sampling was 4 years. We examined risk as a function of the triglyceride level in increments of 1.00 mmol per liter (90 mg per deciliter) as well as in quintiles, with adjustments for age (as the time scale), sex, current smoking status, presence or absence of hypertension, physical inactivity (yes vs. no), and alcohol consumption (yes vs. no) or for all of the above plus HDL cholesterol level in quintiles. Hypertension was defined by a systolic pressure of 140 mm Hg or more, a diastolic pressure of 90 mm Hg or more, or the use of antihypertensive medication; physical inactivity by less than 2 hours of light exercise per week; and alcohol consumption by consumption two or more times per week. Hazard ratios were corrected for regression dilution bias with the use of a nonparametric method.¹⁸

To examine the association between *APOC3* genotypes and levels of nonfasting triglycerides and other lipids and lipoproteins, we used the nonparametric Mann–Whitney U test. To examine the association between *APOC3* genotypes and the risks of ischemic vascular disease and ischemic heart disease, we used Kaplan–Meier plots and log-rank tests to compare cumulative incidences as a function of age. Cox proportional-hazards regression models were used to estimate hazard ratios for clinical end points as a function of *APOC3* genotypes. Models were adjusted for multiple factors as described above; for vascular disease end points, the models were additionally adjusted for triglyceride levels. The median follow-up

period (from birth or the establishment of the Danish National Patient Registry) was 34 years.

The observed hazard ratio for vascular risk associated with a 1-unit increment in log-transformed triglyceride levels was used to calculate theoretically predicted risks of ischemic vascular disease and ischemic heart disease corresponding to the magnitude of the changes in nonfasting triglyceride levels associated with *APOC3* genotype. Further details of the statistical analyses are provided in the Supplementary Appendix.

RESULTS

PARTICIPANTS

Baseline characteristics of the 75,725 study participants according to increments of nonfasting triglyceride levels are shown in Table 1. Charac-

teristics according to disease status are shown in Table S2 in the Supplementary Appendix.

NONFASTING TRIGLYCERIDE LEVELS AND ISCHEMIC VASCULAR DISEASE

The risks of ischemic vascular disease and ischemic heart disease decreased in a stepwise fashion as a function of decreasing levels of nonfasting triglycerides (Fig. 1). For participants with nonfasting triglyceride levels of less than 1.00 mmol per liter, as compared with participants with levels of 4.00 mmol per liter or more, the hazard ratio for ischemic vascular disease was 0.43 (95% confidence interval [CI], 0.35 to 0.54), and the hazard ratio for ischemic heart disease was 0.40 (95% CI, 0.31 to 0.52). Corresponding hazard ratios for participants with nonfasting triglyceride levels in the lowest quintile as compared with participants with levels in the highest quintile were 0.55 (95% CI,

Table 1. Characteristics of the 75,725 Study Participants According to Plasma Levels of Nonfasting Triglycerides.*

Characteristic	Triglyceride Level					P Value†
	<1.00 mmol/liter (N=19,924)	1.00–1.99 mmol/liter (N=35,122)	2.00–2.99 mmol/liter (N=12,699)	3.00–3.99 mmol/liter (N=4419)	≥4.00 mmol/liter (N=3561)	
Age — yr						
Median	54	59	60	60	57	<0.001
Interquartile range	44–64	48–68	50–69	50–68	48–65	
Female sex — no. (%)	14,010 (70)	19,808 (56)	5463 (43)	1541 (35)	1087 (31)	<0.001
Body-mass index‡						
Median	24	26	27	28	28	<0.001
Interquartile range	22–26	23–28	25–30	26–31	26–31	
Diabetes — no. (%)§	407 (2)	1,175 (3)	691 (5)	321 (7)	339 (10)	<0.001
Current smoker — no. (%)	3,852 (19)	8,425 (24)	3319 (26)	1284 (29)	1147 (32)	<0.001
Hypertension — no. (%)¶	8,702 (44)	19,914 (57)	8458 (67)	3101 (70)	2519 (71)	<0.001
Physical inactivity — no. (%)	1,030 (5)	2,459 (7)	1171 (9)	451 (10)	440 (12)	<0.001
Alcohol consumption — no. (%)**	14,451 (73)	25,173 (72)	9113 (72)	3208 (73)	2564 (72)	0.53
Lipid-lowering therapy — no. (%)††	1,270 (6)	3,325 (9) ^o	1473 (12)	564 (13)	430 (12)	<0.001

* To convert the values for triglycerides to milligrams per deciliter, divide by 0.01129.

† P values are for the comparison of trend across groups of triglyceride levels and were calculated with the use of Cuzick's extension of a Wilcoxon rank-sum test for trend.

‡ The body-mass index is the weight in kilograms divided by the square of the height in meters.

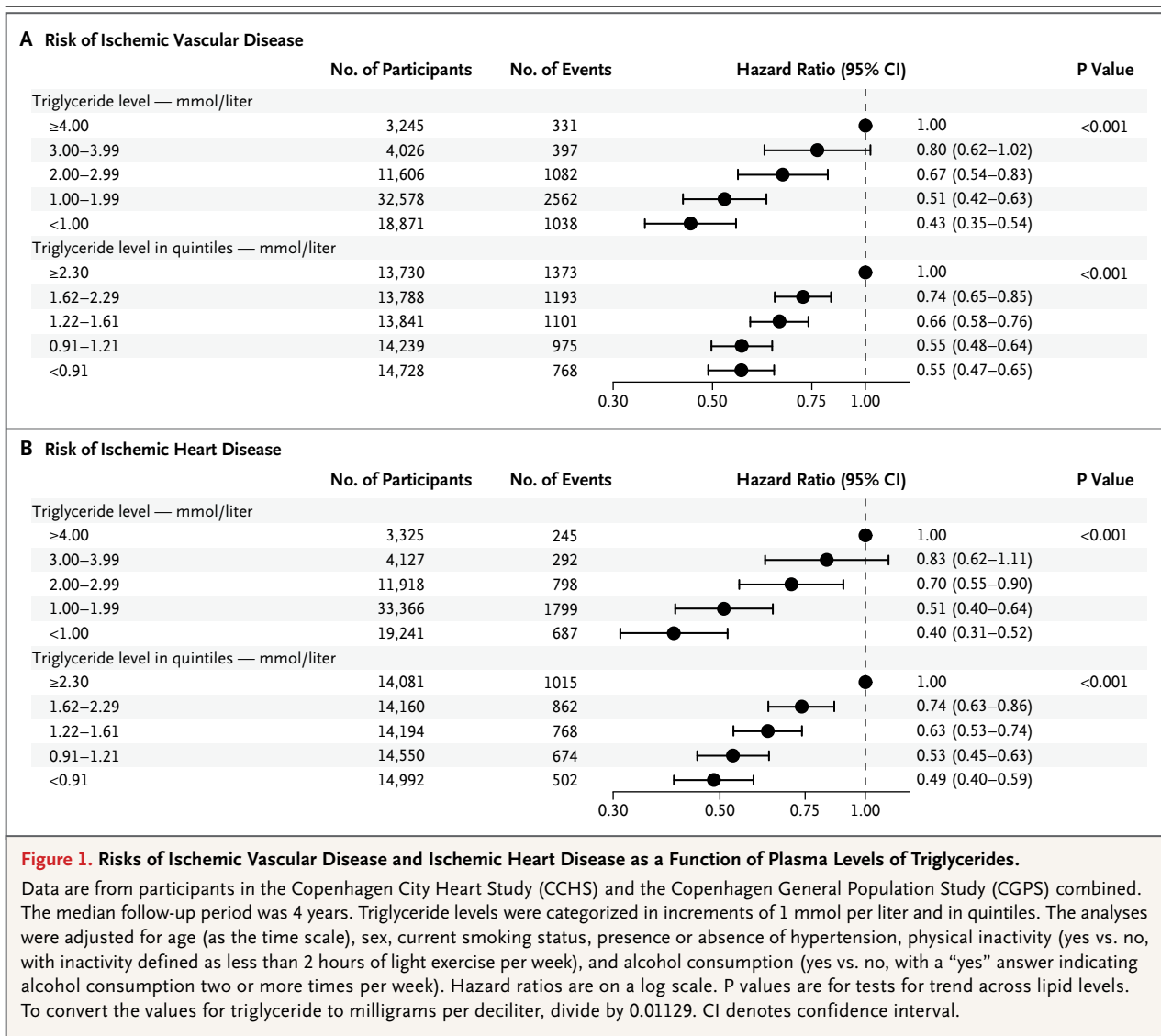
§ Participants were considered to have diabetes if they self-reported the disease, reported using antidiabetic medication, or had a nonfasting plasma glucose level of more than 11.0 mmol per liter (200 mg per deciliter).

¶ Participants were considered to have hypertension if they had a systolic pressure of 140 mm Hg or more or a diastolic pressure of 90 mm Hg or more or if they used antihypertensive medication.

|| Physical inactivity was defined as less than 2 hours of light exercise per week.

** Included in this category were participants who consumed alcohol two or more times per week.

†† Use of lipid-lowering therapy was self-reported. Among participants who reported using lipid-lowering therapy, more than 97% took statins.



0.47 to 0.65) and 0.49 (95% CI, 0.40 to 0.59) (Fig. 1). Further adjustment for HDL cholesterol levels modestly attenuated these risk estimates (Fig. S1 in the Supplementary Appendix). Results were similar in separate analyses of data from the CCHS and the CGPS (Fig. S2 and S3 in the Supplementary Appendix).

APOC3 GENOTYPES AND LEVELS OF NONFASTING TRIGLYCERIDES

Resequencing the coding regions and exon–intron boundaries of *APOC3* in the entire CCHS population (10,333 participants) identified a total of 13 genetic variants (Table S3 in the Supplementary Appendix).

Three rare variants — R19X, IVS2+1G→A, and A43T — were identified in a total of 41 participants (approximately 1 in 250 participants) and were associated with substantially reduced levels of nonfasting triglycerides (Fig. S4 in the Supplementary Appendix). Two of these variants (IVS2+1G→A and A43T) were also associated with increased levels of HDL cholesterol and apolipoprotein A1 in the CCHS population. Genotyping these three variants in the CGPS participants identified an additional 219 heterozygotes, totaling 260 heterozygotes in the two studies combined (heterozygote frequency, 1 in 290 participants; allele frequency, 1 in 580 alleles).

As compared with noncarriers of *APOC3* mutations, heterozygotes for these mutations had mean reductions of 44% (0.77 mmol per liter [70 mg per deciliter]) in levels of nonfasting triglycerides ($P<0.001$) (Fig. 2). Results were similar for the individual mutations. Heterozygotes, as compared with noncarriers, also had mean reductions of 16% (17 mg per deciliter) in apolipoprotein B levels and increases in levels of HDL cholesterol and apolipoprotein A1 of 24% (0.38 mmol per liter [15 mg per deciliter]) and 9% (15 mg per deciliter), respectively (Fig. S5 in the Supplementary Appendix).

A fourth variant, V50M, was not associated with levels of triglycerides or other lipid measures in the CCHS, the CGPS, or the two studies combined and was therefore considered unlikely to be functional — an observation that was consistent with *in silico* predictions (Fig. S4 and S6 in the Supplementary Appendix). No homozygotes or compound heterozygotes for any of the four variants were identified (expected frequency, 1 in 251,000 participants for any mutation). Rates of other well-known cardiovascular risk factors were similar in heterozygotes and noncarriers of *APOC3* mutations (Table S4 in the Supplementary Appendix), confirming that associations of *APOC3* genotype with risks of ischemic vascular disease and ischemic heart disease were not confounded by conventional cardiovascular risk factors.

APOC3 GENOTYPES AND RISKS OF ISCHEMIC VASCULAR DISEASE

During follow-up, 10,797 participants had incident ischemic vascular disease, and of these 7557 had ischemic heart disease. The cumulative incidences of ischemic vascular disease and ischemic heart disease as a function of age were decreased in *APOC3* heterozygotes as compared with noncarriers of *APOC3* mutations ($P=0.009$ and $P=0.05$, respectively, by the log-rank test) (Fig. 3). Results were similar when the V50M variant was included (Fig. S7 in the Supplementary Appendix).

We estimated that low levels of nonfasting triglycerides due to mutations in *APOC3* would theoretically predict hazard ratios for ischemic vascular disease and ischemic heart disease of 0.77 (95% CI, 0.73 to 0.81) and 0.74 (95% CI, 0.69 to 0.78), respectively (Fig. 4). The observed hazard ratios were 0.59 (95% CI, 0.41 to 0.86)

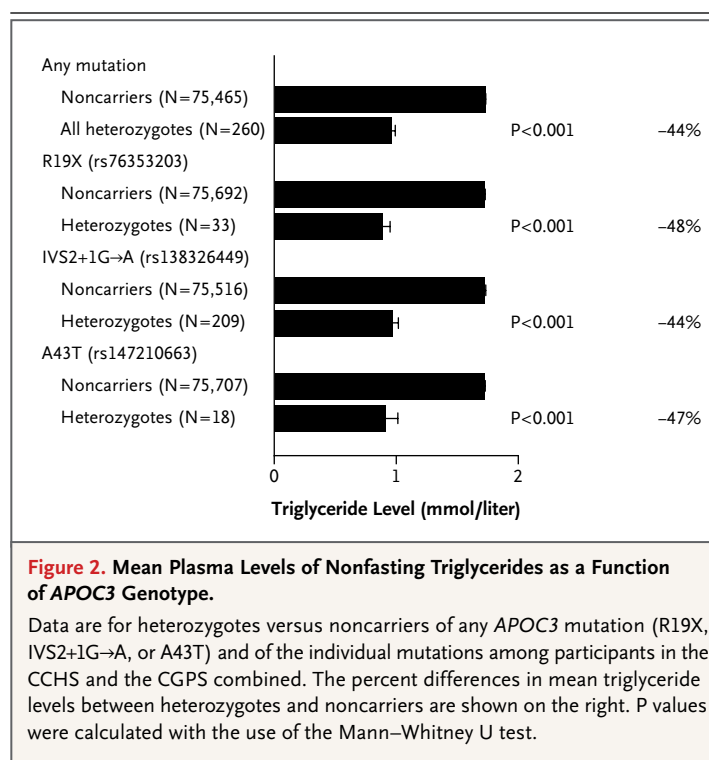


Figure 2. Mean Plasma Levels of Nonfasting Triglycerides as a Function of *APOC3* Genotype.

Data are for heterozygotes versus noncarriers of any *APOC3* mutation (R19X, IVS2+1G→A, or A43T) and of the individual mutations among participants in the CCHS and the CGPS combined. The percent differences in mean triglyceride levels between heterozygotes and noncarriers are shown on the right. P values were calculated with the use of the Mann–Whitney U test.

for ischemic vascular disease ($P=0.007$) and 0.64 (95% CI, 0.41 to 0.99) for ischemic heart disease ($P=0.04$) (Fig. 4, and Fig. S8 in the Supplementary Appendix). Risk estimates were similar whether participants were followed from the time of DNA blood sampling or from either the time of the establishment of the Danish National Patient Registry (January 1, 1977) or the participant's date of birth, whichever was later. When the association between any *APOC3* mutation and the risk of ischemic vascular disease or ischemic heart disease was adjusted for levels of nonfasting triglycerides, the risk estimates were attenuated and became nonsignificant (hazard ratios, 0.71 [95% CI, 0.49 to 1.04] and 0.80 [95% CI, 0.51 to 1.24], respectively) (Fig. S9 in the Supplementary Appendix).

SENSITIVITY ANALYSES

The reduced risks of ischemic vascular disease and ischemic heart disease in *APOC3* heterozygotes as compared with noncarriers of *APOC3* mutations were similar across subgroups defined according to individual ischemic vascular disease end points, individual mutations, and individual cardiovascular risk factors. However, not all risk

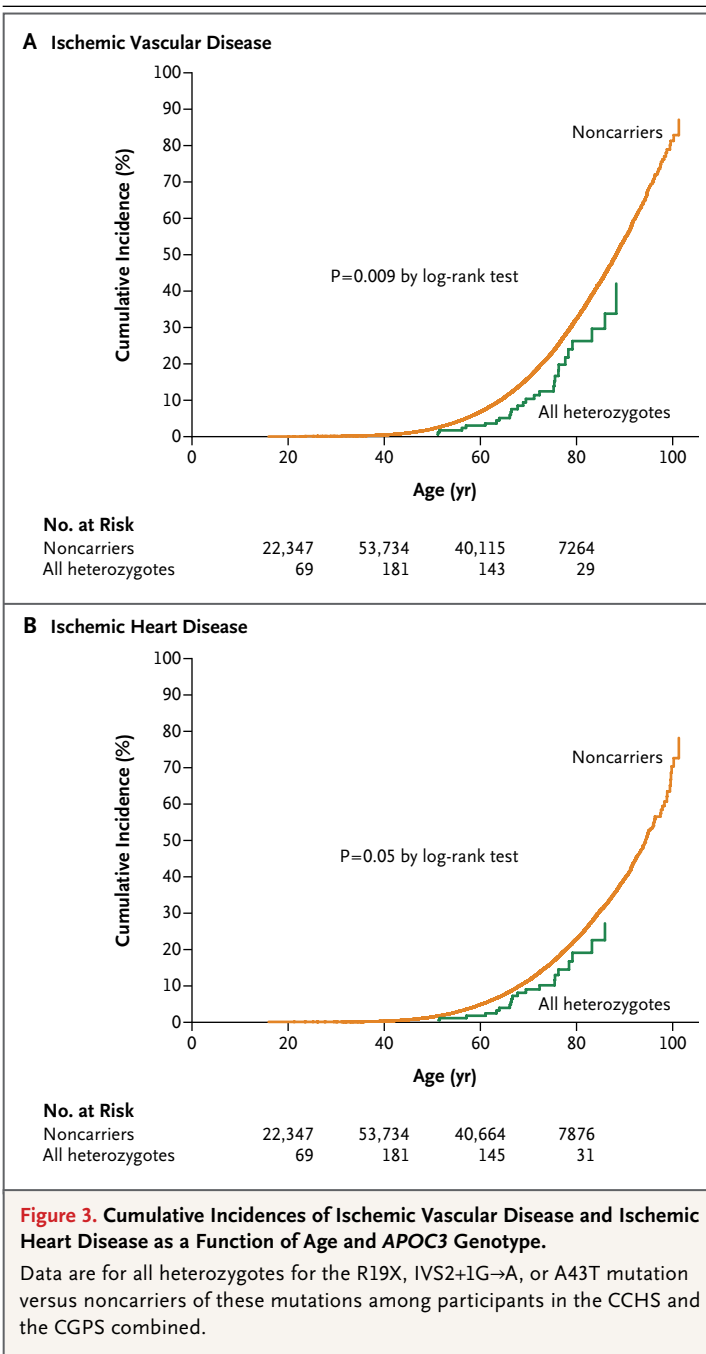
cant association between *APOC3* genotype and the risk of dementia, any cancer, or death from any cause (Fig. S14 in the Supplementary Appendix).

DISCUSSION

The principal finding of this study is that lifelong low levels of nonfasting triglycerides due to loss-of-function mutations in *APOC3* are associated with reduced risks of ischemic vascular disease and ischemic heart disease in the general population. These findings are of potential clinical importance, because they suggest that *APOC3* is a relevant drug target for reducing residual cardiovascular risk. Inhibition of *APOC3* by antisense oligonucleotides has recently been shown to reduce plasma levels of apolipoprotein C3 and triglycerides in animal models and in a phase 1 clinical trial involving humans.¹⁹

The hypertriglyceridemic effects of apolipoprotein C3 are attributable to both extracellular and intracellular roles in triglyceride metabolism. Extracellularly, plasma apolipoprotein C3 inhibits hydrolysis of triglyceride-rich lipoproteins catalyzed by lipoprotein lipase⁹ and attenuates the uptake of triglyceride-rich remnant lipoproteins by the liver.^{11,13} Intracellularly, apolipoprotein C3 promotes triglyceride synthesis and VLDL assembly and secretion.²⁰⁻²² All these mechanisms lead to high levels of triglyceride-rich remnant lipoproteins in plasma and hence to atherosclerosis and an increased risk of ischemic cardiovascular disease.²⁻⁶ Indeed, like LDLs, triglyceride-rich remnant lipoproteins can penetrate the arterial intima and may be retained preferentially, thus causing atherosclerosis owing to their cholesterol content.^{23,24}

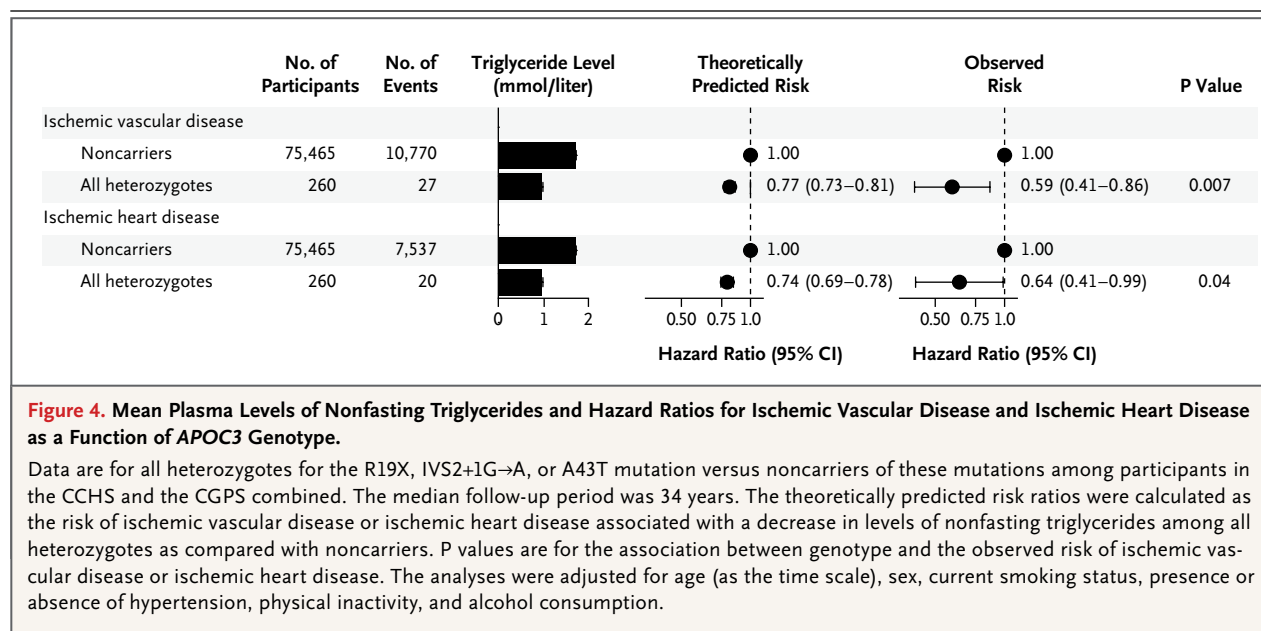
Similarly low levels of nonfasting triglycerides were observed in association with three of the mutations (R19X, IVS2+1G→A, and A43T) identified in the current study. In a prior study, the R19X loss-of-function mutation in *APOC3* was shown to result in a reduction of 46% in triglyceride levels and in a decrease of 60% in the risk of coronary-artery calcification, a surrogate marker for subclinical atherosclerosis.¹⁴ The A43T mutation has been identified previously in Yucatan Indians with low triglyceride levels²⁵ and has been shown to compromise assembly of VLDL particles in the liver and hence prevent VLDL maturation.²² The IVS2+1G→A mutation is located in the consensus splice site, most likely resulting in alternative splicing and



estimates remained significant (Fig. S10, S11, and S12 in the Supplementary Appendix).

APOC3 GENOTYPES AND OTHER END POINTS

APOC3 genotypes, individually or combined, were not significantly associated with plasma levels of alanine aminotransferase, aspartate aminotransferase, or C-reactive protein (Fig. S13 in the Supplementary Appendix). There was also no signifi-



loss of function, probably owing to nonsense-mediated decay of the messenger RNA transcript.^{26,27} The fourth mutation that we identified, V50M, has not been described previously but, as evidenced by the lack of effect on triglyceride levels in both the CCHS and the CGPS, is probably not functional — an observation that is also supported by *in silico* predictions. Nevertheless, the V50M mutation had a correlation with ischemic vascular disease, though not a significant one. Therefore, we cannot rule out an effect of this mutation on ischemic vascular disease, independent of triglyceride levels.

Because common, noncoding variants in *APOC3* have been associated with fatty liver disease and longevity in some,^{28,29} but not all,^{30,31} studies, we examined the association between genetic variants in *APOC3* and markers of liver disease; in addition, to test whether targeting *APOC3* would be safe, we examined the association between genetic variants in *APOC3* and inflammation, dementia, cancer, and total mortality. We found no significant associations.

In this study and in previous studies, plasma levels of nonfasting triglycerides and remnant cholesterol were strongly associated with the risk of ischemic cardiovascular disease.^{2–6} Recent genetic studies involving mendelian randomization suggest that this association may be causal.^{7,8} The reduced risks of ischemic vascular disease and ischemic heart disease in *APOC3* heterozygotes observed in our study are there-

fore probably mediated by the reduced levels of triglyceride-rich remnant lipoproteins that are associated with these mutations. The reductions in risk determined from genetic analyses tended to be nominally larger than the reductions in theoretically predicted risk estimated from observational analyses, most likely reflecting the beneficial effects of lifelong reductions in levels of triglycerides and remnant cholesterol.^{7,8}

Some limitations to our study must be considered. First, the inverse association between triglyceride levels and HDL cholesterol levels in plasma represents the most important pleiotropic effect of the genetic variants in *APOC3*. Although the risk of ischemic cardiovascular disease is consistently inversely related to plasma levels of HDL cholesterol in observational studies,⁴ clinical trials as well as genetic studies involving mendelian randomization have failed to establish a causal link between plasma levels of HDL cholesterol and the risk of ischemic cardiovascular disease.^{32–38} Second, we did not measure plasma levels of apolipoprotein C3. However, apolipoprotein C3 levels have consistently been shown to be highly correlated with plasma levels of triglycerides in humans as well as in mice.³⁹ Third, we did not measure triglyceride levels in the fasting state, and we are therefore unable to determine whether levels of nonfasting triglycerides are associated with ischemic vascular disease independent of levels of fasting triglycerides. There are, however, no definitive

data to suggest that one is superior to the other in the prediction of ischemic cardiovascular disease. Finally, the CCHS and the CGPS involved white participants only; however, we are not aware of data to suggest that our results would not apply to populations of other races or ethnic groups.

In conclusion, we identified three loss-of-function mutations in *APOC3* that were associated with markedly lower levels of nonfasting triglycerides. These mutations were also associated with corresponding reductions in the risk of ischemic vascular disease and ischemic heart disease in the general population.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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