A common *VLDLR* polymorphism interacts with *APOE* genotype in the prediction of carotid artery disease risk[S]

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Abstract The genetic factors associated with carotid artery disease (CAAD) are not fully known. Because of its role in lipid metabolism, we hypothesized that common genetic variation in the very low density lipoprotein receptor (*VLDLR*) gene is associated with severe CAAD (>80% stenosis), body mass index (BMI), and lipid traits in humans. *VLDLR* was resequenced for variation discovery in 92 subjects, and single nucleotide polymorphisms (tagSNPs) were chosen for genotyping in a larger cohort (n = 1,027). Of the 17 tagSNPs genotyped, one tagSNP (SNP 1226; rs1454626) located in the 5′ flanking region of *VLDLR* was associated with CAAD, BMI, and LDL-associated apolipoprotein B (apoB). We also identified receptor-ligand genetic interactions between *VLDLR* 1226 and *APOE* genotype for predicting CAAD case status. These findings may further our understanding of VLDLR function, its ligand APOE, and ultimately the pathogenesis of CAAD in the general population.—Crawford, D. C., A. S. Nord, M. D. Badzioch, J. Ranchalis, L. A. McKinstry, M. Ahearn, C. Bertucci, C. Shephard, M. Wong, M. J. Rieder, G. D. Schellenberg, D. A. Nickerson, P. J. Heagerty, E. M. Wijsman, and G. P. Jarvik. **A common *VLDLR* polymorphism interacts with *APOE* genotype in the prediction of carotid artery disease risk. *J. Lipid Res.* 2008.** 49: **588–596.**

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Very low density lipoprotein receptor (*VLDLR*), located at chromosome 9p24, is a member of the low density lipoprotein receptor family and is highly expressed in heart, muscle, and adipose tissue, but it is barely detectable in liver (1–3). VLDLR is known to bind apolipoprotein E (apoE)-rich lipoproteins such as VLDL (3, 4). The binding of these ligands is stimulated by LPL, and it is thought that this process is important in the uptake and degradation of fatty acids and triglyceride-rich particles by monocytes, which may accelerate foam cell formation in atherosclerotic lesions (5). VLDLR also binds reelin, which triggers the signaling cascade required for the proper migration of neuroblasts in the central nervous system (6).

A role for VLDLR in atherosclerosis and vascular disease is logical based on the available functional data. Initial phenotypic observations of VLDLR-deficient mice on a hybrid background of C57BL/6J and 129/Sv strains fed various diets suggested that these mice were leaner but had normal lipid profiles compared with their control littermates (7). However, Yagyu et al. (8) observed an increase in triglycerides with fasted VLDLR-deficient mice compared with normal littermates, and this increase was associated with a decrease in LPL activity. Other reports suggested that abnormal lipid profiles are not observed unless VLDLR-deficient mice fed a high-fat diet are also LDLR-deficient (9). Subsequent studies of VLDLR-deficient mice fed a high-fat, high-calorie diet demonstrate that these mice have less obesity and insulin resistance compared with their normal littermates (10). No significant differences in food uptake or fat absorption were observed between VLDLR-deficient mice and their littermates; a decrease in the uptake of fatty acids is the likely mechanism that leads to protection against obesity in VLDLR-deficient mice (10).

In humans, VLDLR deficiency was reported in Hutterite families with autosomal recessive cerebellar hypoplasia with cerebral gyral simplification (11). Although lipids were not reported in these VLDLR-deficient families, it

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two tables.

is interesting that 50% of affected patients were underweight, with a body mass index (BMI) of <18.5 (11). Thus, there is evidence that VLDLR deficiency protects against obesity in both humans and mice, establishing phenotypic parallels between the human and mouse VLDLR-deficient state.

Given the phenotypic and possible functional parallels between the human and mouse deficient state and that *VLDLR* was in a linkage region for LDL particle size (12), we postulated that common genetic variation in *VLDLR* (as opposed to VLDLR deficiency) is also associated with lipid traits and BMI in humans. Furthermore, we hypothesized that common *VLDLR* variants are associated with carotid artery disease (CAAD), a common condition (13, 14) that is a major risk factor for stroke (15, 16) and whose risk factors overlap extensively with those of coronary artery disease (17, 18). The etiological pathways that lead to the development of CAAD remain to be fully elucidated, but it is suspected that high levels of LDL lead to the oxidation of LDL and the subsequent recruitment of monocytes from the peripheral blood to the vessel wall (19). Lesions are formed as monocytes differentiate into lipid-containing macrophages, the presence of which triggers various inflammatory responses (19) and the eventual development of disease over time.

To test our hypotheses, we resequenced a subsample of patients from the Carotid Lesion Epidemiology and Risk (CLEAR) study and a set of reference DNAs from presumably healthy Centre d'Etude du Polymorphisme Humain (CEPH) and Yoruban individuals for *VLDLR* SNP discovery, and we selected single nucleotide polymorphisms (tagSNPs) from common genetic variation for *VLDLR*. We then genotyped the larger CLEAR study cohort for 17 *VLDLR* tagSNPs. Additionally, we genotyped the two *APOE* polymorphisms (SNPs rs7412 and rs429358) that determine APOE ε2, ε3, and ε4 alleles to test for gene-gene interactions between the receptor and its APOE ligand.

MATERIALS AND METHODS

Patients and samples

Subjects were ascertained as part of the CLEAR study conducted by the University of Washington and the Veterans Affairs Puget Sound Health Care System in Seattle, Washington. This study was approved by the institutional review boards of those institutions, and informed consent was obtained from all human subjects. Details of ascertainment, subject characteristics, and lipid measurements on fasting whole plasma are given elsewhere (20). Briefly, total cholesterol, HDL, and triglycerides were measured using standard enzymatic methods on an Abbott Spectrum analyzer (21–23). LDL-cholesterol was calculated using the Friedewald equation (24). ApoA-I was measured using standardized methods as reported previously (25). LDL B (for apolipoprotein B associated with LDL-cholesterol) was measured after pooling the LDL-containing density gradient ultracentrifugation fractions (26). LDL density (LDL-Rf) was evaluated by nonequilibrium density gradient ultracentrifugation (27). For this study, 1,027 white male subjects were identified, and subject characteristics are given in **Table 1**.

For variation discovery, we resequenced 23 CEPH samples of European descent (NA11995, NA12892, NA11882, NA11994, NA12815, NA12891, NA06985, NA11840, NA11881, NA11993, NA12751, NA12814, NA06993, NA07056, NA11832, NA11839, NA11992, NA12057, NA12156, NA12239, NA12750, NA12813, and NA07055) and 24 Yoruban samples (NA18502, NA19153, NA19223, NA19201, NA18504, NA18870, NA19137, NA19238, NA19144, NA19203, NA19200, NA18855, NA18505, NA18501, NA18861, NA19193, NA19143, NA18517, NA18856, NA19239, NA18871, NA19209, NA19152, and NA19210) obtained from Coriell Cell Repositories. Both variation discovery sample sets overlap with the recently completed International HapMap Project (28).

Sequencing and genotyping

Overlapping primers for PCR were designed to span *VLDLR* based on the longest genomic transcript in EntrezGene. The entire gene was targeted for PCR-based sequencing, including ~2 kb upstream of the start of the gene, all introns, all exons, and ~2 kb downstream of the gene, resulting in a total target of

TABLE 1. CLEAR study subject characteristics for cases and controls

	Cases		Controls		
Characteristic	*mean or %*	*SD*	*mean or %*	*SD*	*P*
Male	100%	—	100%	—	—
White	100%	—	100%	—	—
Age	65.59	8.85	64.18	9.22	0.020
BMI	27.92	5.14	28.95	5.08	0.003
Diabetic	25.72%	—	11.23%	—	<0.0001
On lipid-lowering medication	65.27%	—	20.98%	—	<0.0001
Current smokers	31.37%	—	9.62%	—	<0.0001
Total cholesterol (mg/dl)	182.63	38.89	194.60	36.52	<0.0001
LDL-cholesterol (mg/dl)	103.51	30.97	116.45	31.38	<0.0001
LDL B (mg/dl)	60.24	17.39	68.11	18.34	<0.0001
HDL-cholesterol (mg/dl)	43.34	13.44	48.84	15.55	<0.0001
Triglycerides (mg/dl)	164.58	114.07	141.76	111.89	0.0035
APOAI	131.26	24.84	141.21	26.06	<0.0001
LDL Rf (mg/dl)	0.26	0.03	0.26	0.03	0.586

APOAI, apolipoprotein A-I; BMI, body mass index; CLEAR, Carotid Lesion Epidemiology and Risk; LDL B, apolipoprotein B associated with LDL-cholesterol. Cases are defined as having >80% stenosis (n = 310), and controls are defined as having <15% stenosis (n = 517). *P* values are from tests of association using *t*-tests for continuous data and Chi-square tests for categorical data. Diabetics are defined as individuals taking oral hypoglycemic agents with or without insulin.

36,686 bp. The PCR products were sequenced using standard dye primer and termination chemistry on an ABI 3730. Polymorphisms were identified using PolyPhred 5.0 (29). Analysts reviewed all polymorphisms identified by PolyPhred for false-positives associated with features of the surrounding sequence or biochemical artifacts. If the polymorphism identified was an insertion/deletion polymorphism, the analysts manually genotyped each sample and designed primers from the other strand to sequence past the polymorphism. A detailed sequencing protocol is available on the Program for Genomic Applications SeattleSNPs website (pga.gs.washington.edu).

All SNPs annotated in the SeattleSNPs DNA samples were submitted to GenBank (accession number DQ067198) and dbSNP. The location and flanking sequence for each SNP discovered in the reference and CLEAR samples are given in supplementary Table I. SIFT (for Sorting Tolerant from Intolerant) (30), a normalized probability of substitution at each position in a multiple alignment, and PolyPhen (31) were used to predict the effect of the alternate allele of a nonsynonymous cSNP on protein function (see supplementary Table II).

Genotyping was performed using TaqMan™ Assays by Design™ and Assays on Demand™ with the Applied Biosystems 7900HT real-time PCR system under standard conditions (32). The average genotyping success rate was 97% (range, 88–100%), and all SNPs were in Hardy-Weinberg equilibrium.

Statistical methods

TagSNPs were chosen from the variation discovery *VLDLR* CLEAR data using LDSelect at default settings ($r^2 > 0.64$) from SNPs with a minor allele frequency (MAF) of >10% (33). The r^2 threshold used here represents a balance between the more stringent threshold of 0.80 and the more lenient threshold of 0.50 (33). For bins with more than one tagSNP available, SNPs in exons, untranslated regions, or regions conserved with mouse (determined using the ECR Browser at http://ecrbrowser.dcode.org) were preferentially selected for genotyping. Of the 20 tagSNPs targeted for genotyping, two (rs12057080 and rs7874933) could not be converted into genotyping assays, and one SNP genotyping assay failed to validate (rs12379259).

All statistical tests were performed using R (http://www.r-project.org/). Cases were defined as patients having >80% stenosis of one or both internal carotid arteries. Controls were defined as patients with <15% stenosis bilaterally on duplex ultrasound. Because the overwhelming majority of CLEAR study subjects are males of European descent, we excluded females and non-European-descent subjects from analysis to reduce heterogeneity. Logistic regression was performed to test for associations between case status and *VLDLR* tagSNPs using an additive genetic model unadjusted and adjusted for age, current smoking status, and the presence of diabetes, all three of which are associated with CAAD case status (Table 1). Diabetics were defined as individuals taking oral hypoglycemic agents with or without insulin. Kruskal-Wallis tests were performed for LDL density (LDL-Rf), and triglycerides were also included in adjusted models for this lipid trait. For BMI and the remaining lipid trait phenotypes, linear regression was performed for control subjects not on lipid-lowering medication using an additive genetic model unadjusted and adjusted for age, current smoking status, and the presence of diabetes. BMI, LDL-cholesterol, LDL B, and triglycerides were log-transformed before the regression analysis to reduce non-normality. False discovery rate analysis was performed to account for multiple testing (34).

Because of the small sample sizes of the *APOE* genotypes 2/2 and 4/4 (n = 3 and 13, respectively), we combined these genotypes with the larger *APOE* 2/3 and 3/4 genotype groups for all analyses and refer to them here as ε2 and ε4 carriers. *APOE* genotype 2/4 was omitted from the analysis because of its small sample size (n = 16).

To test for interactions between *VLDLR* and *APOE* in CAAD case status prediction and prediction of the quantitative lipid traits, we considered additive terms for both *VLDLR* genotype (AA = 0, AC = 1, and CC = 2) and *APOE* group (ε2 carrier = 0, 3/3 = 1, and ε4 carrier = 2; 1 degree of freedom) as well as a multiplicative interaction term (1 degree of freedom). To further explore any interactions, the single multiplicative term was replaced by four separate genotype combinations that considered *VLDLR* and *APOE* genotype groups as 0, 1 dummy variables, with *VLDLR* 1226 AA and *APOE* 3/3 used as the reference groups. For the lipid trait prediction, linear regression was performed both unadjusted and adjusted for age, current smoking status, and the presence of diabetes. Marginal terms for *VLDLR* genotype and *APOE* genotypes were also included in all interaction models.

Unadjusted odds ratios were calculated for CAAD case status for *VLDLR* SNPs predicting CAAD stratified by *APOE* (ε2 carriers, 3/3, and ε4 carriers). We also calculated odds ratios adjusted for other factors associated with CAAD case status, BMI, and lipid levels (Table 1: age, current smoking status, and the presence of diabetes). Unadjusted and adjusted linear regression was performed for BMI and each lipid trait using controls who were not on lipid-lowering medication stratified by *APOE* genotype.

RESULTS

VLDLR genetic variation discovery

Of the combined total of 271 SNPs identified by resequencing (see supplementary Table I), 157 SNPs were identified in the CLEAR study samples, 203 SNPs were identified in the Yoruban samples, and 135 SNPs were identified in the 23 European-American CEPH samples. Approximately 70% of the SNPs were shared between the CLEAR study and the European-American CEPH samples. Of the SNPs identified in the CLEAR study but not identified in the CEPH SeattleSNPs reference samples, the vast majority were either relatively rare (1–5% minor allele frequency) or had insufficient data in the SeattleSNPs. Only three common SNPs (>5% MAF) were identified in the CLEAR study but not the CEPH SeattleSNPs reference samples: site 2750 (rs7874933), site 9839 (rs7047850), and site 21008 (rs10967306). We also identified several SNPs in the coding region of *VLDLR*, most of which were rare (see supplementary Table II).

VLDLR tagSNP associations with case status, BMI, and lipid traits

Because the CLEAR study samples identified common SNPs not found among the European-American SeattleSNPs reference samples, we used the CLEAR study variation data to select tagSNPs to represent common *VLDLR* genetic variation (MAF > 10%) for further genotyping in the larger case-control CLEAR study cohort. A total of 20 tagSNPs were selected, and 17 of them were genotyped successfully in the CLEAR study cohort (**Table 2**).

Of the 17 SNPs genotyped, two tagSNPs, sites 1226 (rs1454626) and 12450 (rs1869592), were marginally significantly associated with CAAD case status (Table 2). The

TABLE 2. Associations between *VLDLR* tagSNPs and carotid artery disease among the CLEAR study participants

VLDLR SNP	rs Number	Case Status	*P*
		β	
1226	rs1454626	0.245	0.029
12450	rs1869592	0.282	0.045
1341	rs7043199	0.145	0.263
1678	rs7852409	0.038	0.753
2474	rs2219143	−0.076	0.483
3812	rs7032549	0.048	0.648
4383	rs7022122	−0.019	0.884
5369	rs10967213	0.026	0.815
6228	rs1545566	−0.148	0.319
6851	rs4741747	−0.009	0.931
7565	rs12551418	0.022	0.856
13748	rs10812379	0.002	0.989
17753	rs4740698	−0.077	0.478
17837	rs3516438	−0.151	0.219
18316	rs33967773	<−0.001	0.998
19026	rs7044155	0.057	0.593
28978	rs6148	−0.045	0.743

TagSNPs, single nucleotide polymorphisms; *VLDLR*, very low density lipoprotein receptor. Data shown are for case (n = 310) and control (n = 517) CLEAR study subjects.

first SNP (12450; rs1869592) is located in a nonconserved region of intron 1. The second SNP (1226; rs1454626) is located in the 5′ flanking region of *VLDLR* and is conserved with mouse. Both tagSNPs were the only SNPs in their tagSNP bin; therefore, neither was in strong linkage disequilibrium with other *VLDLR* SNPs. Both SNPs were significantly associated with case status at a false discovery rate of 40%; however, only SNP 1226 remained significant after adjustment for age, current smoking status, and the presence of diabetes. *VLDLR* 1226 was also significantly associated with BMI ($\beta = -0.046$, $P < 0.001$) and LDL B ($\beta = -0.045$, $P = 0.039$) among controls not on lipid-lowering medication (**Table 3**). These associations remained significant after adjusting for age, current smoking status, and the presence of diabetes (data not shown). SNP 12450 was not associated with BMI or any lipid trait and may represent a false-positive for case status (Table 3). Other SNPs were associated with lipid phenotypes LDL B (3812), HDL-cholesterol (7565), and apoA-I (6851 and 7565), but they were not associated with case status. No *VLDLR* SNP was marginally associated with LDL-Rf (data not shown).

VLDLR and APOE genetic interaction

Given that APOE is the VLDLR ligand, we tested for statistical interactions between the *VLDLR* 1226 SNP predicting CAAD after covariate adjustment and *APOE* genotype. A multiplicative interaction term for additive *VLDLR* and *APOE* effects yielded significant evidence for gene-gene interaction in the prediction of CAAD ($P = 0.0199$). When each *VLDLR* genotype and *APOE* genotype groups are modeled individually as 0, 1 variables to identify the pattern of interaction, only the *APOE* ε4-*VLDLR* 1226-CC interaction is significant ($\beta = -1.3747$, $P = 0.0331$) compared with the referent group *APOE* 3/3.

To further explore these effects, we contrasted unadjusted and covariate-adjusted odds ratios for CAAD case status for *VLDLR* 1226 risk genotypes CC and AC versus AA in the entire cohort as well as stratified by *APOE* genotypes (**Figs. 1 and 2**). The unadjusted odds ratios for the CC and AC genotypes compared with the referent *VLDLR* 1226 AA genotype were 1.62 [95% confidence interval (CI) = 0.96–2.72] and 1.28 (95% CI = 0.96–1.73), respectively. Stratification of *VLDLR* 1226 genotypes by *APOE* genotypes generally strengthened the *VLDLR* 1226 effect in the *APOE* 3/3 and ε2 allele carriers; however, no *VLDLR* effect was observed in the *APOE* ε4 allele carriers. The odds ratio for *VLDLR* CC within *APOE* 3/3 was 2.68 (95% CI = 1.28–5.74), and that for *VLDLR* AC within *APOE* ε2 carriers was 2.84 (95% CI = 1.09–7.88; Fig. 1 and 2). The odds ratio for *VLDLR* CC versus AA within *APOE* ε2 allele carriers also increased to 4.13; however, the CI in this small group includes 1 (0.70–22.50). After adjustment for age, current smoking status, and the presence of diabetes, the *VLDLR* CC *APOE* 3/3 association with CAAD remained statistically significant (95% CI = 1.06–5.25; $P = 0.04$), whereas the *VLDLR* AC *APOE* ε2 association with CAAD became marginally significant (95% CI = 0.94–37.35; $P = 0.05$).

The adjusted change in lipid traits and BMI per *VLDLR* 1226 risk allele stratified by *APOE* genotypes is shown in **Fig. 3**. After adjustment for age, current smoking status, and diabetes, both BMI ($\beta = 0.956$, $P = 0.019$) and triglycerides ($\beta = 0.850$, $P = 0.005$) were significantly associated with *VLDLR* 1226 within the *APOE* 3/3 genotype group (Fig. 3). Within the *APOE* ε2 allele carriers, LDL-cholesterol ($\beta = 0.874$, $P = 0.049$) and LDL B ($\beta = 0.854$, $P = 0.012$) were statistically significant (Fig. 3). LDL-Rf was significantly associated with *VLDLR* 1226 within *APOE* 3/3 and *APOE* ε4 carriers in both adjusted and unadjusted tests of association (Kruskal-Wallis; $P < 0.05$). Formal 1 degree of freedom tests of interaction between *VLDLR* 1226 and *APOE* on the quantitative traits were not signifi-

TABLE 3. Associations between *VLDLR* tagSNPs and BMI and lipid traits among the CLEAR study participants

Trait	1226	1226	12450	12450
	e^{β} (β)	*P* (*SEM*)	e^{β} (β)	*P* (*SEM*)
ln(BMI)	0.955 (−0.046)	<0.001 (0.013)	0.992 (−0.008)	0.607 (0.016)
ln(LDL-cholesterol)	0.9627 (−0.038)	0.078 (0.022)	0.981 (−0.019)	0.448 (0.025)
ln(LDL B)	0.956 (−0.045)	0.039 (0.022)	0.999 (<−0.001)	0.973 (0.025)
ln(HDL)	1.025 (0.025)	0.266 (0.022)	0.970 (−0.031)	0.237 (0.027)
ln(triglycerides)	0.943 (−0.059)	0.157 (0.042)	1.106 (0.016)	0.751 (0.049)
ln(APOAI)	1.015 (0.015)	0.278 (0.013)	0.977 (−0.023)	0.145 (0.016)

Data shown are for controls from the CLEAR study not on lipid-lowering medication (n = 412).

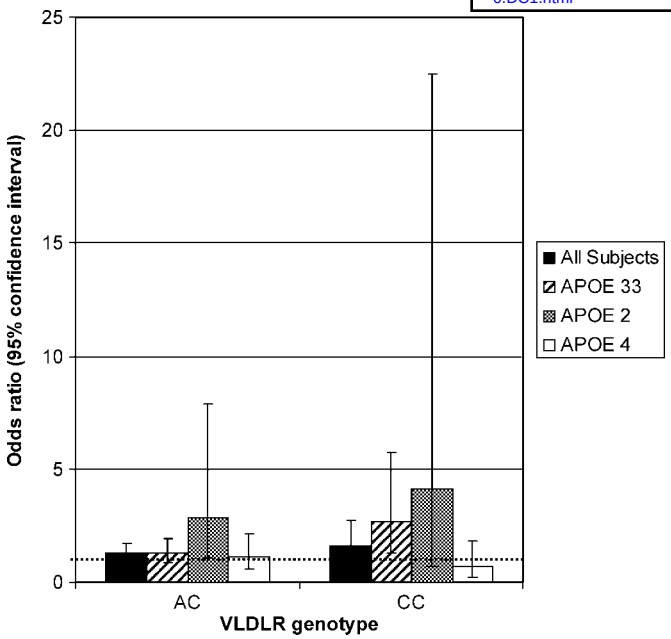

Fig. 1. Carotid artery disease (CAAD) risk by very low density lipoprotein receptor (*VLDLR*) 1226 stratified by apolipoprotein E (*APOE*) genotype. Male cases (n = 310) and controls (n = 517) of European descent are included in this analysis. The *y* axis represents the odds ratio. The *x* axis represents the *VLDLR* 1226 genotypes not stratified (all subjects) or stratified by *APOE* genotypes. The *APOE* genotype groups include 3/3 (n = 170 cases and 277 controls), ε2 (2/3 and 2/2; n = 26 cases and 77 controls), and ε4 (3/4 and 4/4; n = 71 cases and 91 controls). *VLDLR* 1226 AA is the reference genotype. The dashed line represents an odds ratio of 1.0. Error bars are 95% confidence intervals.

cant at the 0.05 level. We found no evidence of a genetic interaction between *VLDLR* 12450 and *APOE* genotypes for case status, BMI, or any of the lipid traits in either the unadjusted or the adjusted models (data not shown).

DISCUSSION

We identified a statistical interaction between *VLDLR* 5′ flanking region SNP 1226 and *APOE* genotypes for CAAD case status prediction. *VLDLR* 1226 was not in strong linkage disequilibrium with other *VLDLR* SNPs in the sequence discovery set; thus, it is a strong candidate for being the functional SNP underlying the associations. Also, *VLDLR* 1226 was not in strong linkage disequilibrium with *VLDLR* SNPs recently associated with age-related macular degeneration (35). Given that VLDLR expression is high in heart, muscle, and adipose, but not in liver, and the detection of a *VLDLR* genotype effect on BMI, the effects found may be related to expression differences in these tissues. The *VLDLR* 1226 risk allele interacted with *APOE* genotype and did not predict CAAD risk only in *APOE* ε4 allele carriers.

Over all *APOE* genotypes and within the *APOE* ε3ε3 group, of which ~50% of the population is constituted (36), the *VLDLR* 1226-C risk allele was associated with reduced BMI and triglycerides and a trend toward a favorable lipid profile. It is likely that the effects of this allele on lipids, which are not observed in the ε2 carriers that also have increased *VLDLR* 1226-C-related CAAD risk, are not determinative of CAAD risk. Rather, based on evidence that increased VLDLR expression is correlated with foam cell formation (37–39), one could hypothesize that the endothelial effects are more important in disease risk. Indeed, this may be a polymorphism that predicts disease risk in persons with favorable lipid profiles.

This is the first report of evidence for a genetic interaction between *VLDLR* and *APOE* on disease prediction in mice or humans. The APOE isoform has been shown to affect VLDLR processing, with ordered effects in which E2 is greatest, then E3, and E4 least (40). The ε4 allele product has reduced conformational stability and increased domain interaction (41) and consists of arginines at positions 112 and 158 of the amino acid sequence versus ε2 (cytosines at these positions) and ε3 (cytosine and arginine at positions 112 and 158, respectively). Previous studies have suggested that individuals with the *APOE* 2/3 genotype have a lower risk for CAAD and individuals with the *APOE* 3/4 genotype have a higher risk for CAAD compared with individuals with the *APOE* 3/3 genotype (42–65), and our results are consistent with these marginal effects. Yet, risk differences between the *APOE* ε4 allele carriers and the *APOE* 3/3 genotype subjects decrease with each *VLDLR* 1226 risk allele.

The associations identified here are somewhat consistent with previous observations in mice and humans. We observed an association with *VLDLR* 1226 and BMI, and lower BMI was observed in both VLDLR-deficient mice (7) and humans (11). However, we did not identify an association with *VLDLR* 1226 and increased triglycerides, whereas increased triglycerides were reported in VLDLR-deficient mice compared with their normal littermates (8). This difference is not unexpected given the fact that *VLDLR* 1226 does not abolish VLDLR expression, which would mimic VLDLR deficiency. Also, other investigators have reported that a high-fat, high-calorie diet and LDLR deficiency are necessary to observe a lipid phenotype in VLDLR-deficient mice (9), emphasizing the complex relationship between genetic background and the environment in the expression of the phenotype.

One limitation of our design is that the lipid effects can only be reliably tested in subjects who are not on lipid-lowering medications. It would be of interest to know the genotype effects in an unselected, untreated sample rather than in subjects known to not have CAAD. A second limitation of this study is that it is limited to men of European descent. Other populations must be studied to determine whether the associations and genetic interaction identified here would be found. Demonstration of *VLDLR* 1226 expression effects in relevant tissue such as adipocytes or endothelial cells would also be supportive. We were unable to demonstrate reliable expression in monocytes, despite reports of macrophage VLDLR expression correlating with atherosclerosis in mice (66) (data not shown).

Another limitation of our study is that the results must be replicated in other independent cohorts. Replication is

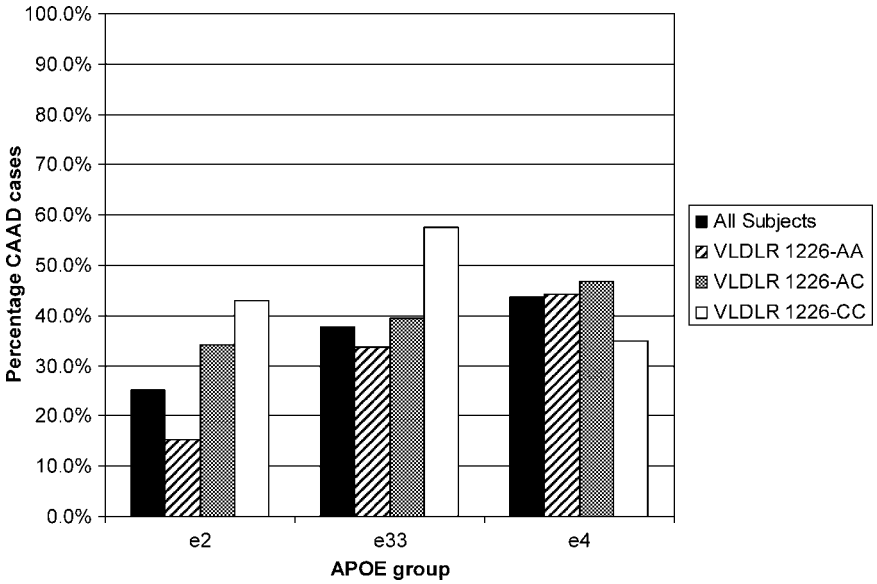

Fig. 2. Proportion of CAAD cases per *APOE* group stratified by *VLDLR* 1226 genotype. Male cases (n = 310) of European descent are included in this analysis. The *y* axis represents the percentage of CAAD cases per *APOE* group stratified by *VLDLR* 1226 genotype. The *x* axis represents *APOE* genotype groups ε2 (2/2 and 2/3), ε3 (3/3), and ε4 (3/4 and 4/4).

a necessary step in progressing from the initial discovery to establishing a robust association worthy of further characterization. Both candidate gene studies (67–69) and genome-wide association studies (70–74) are plagued with reports of nonreplication, and a recent meta-analysis of genome-wide association studies suggests that independent replications reported in the same body of work as the initial discovery do not guarantee a robust association (71). Thus, although a second cohort presented here would support our initial findings, additional cohorts would still

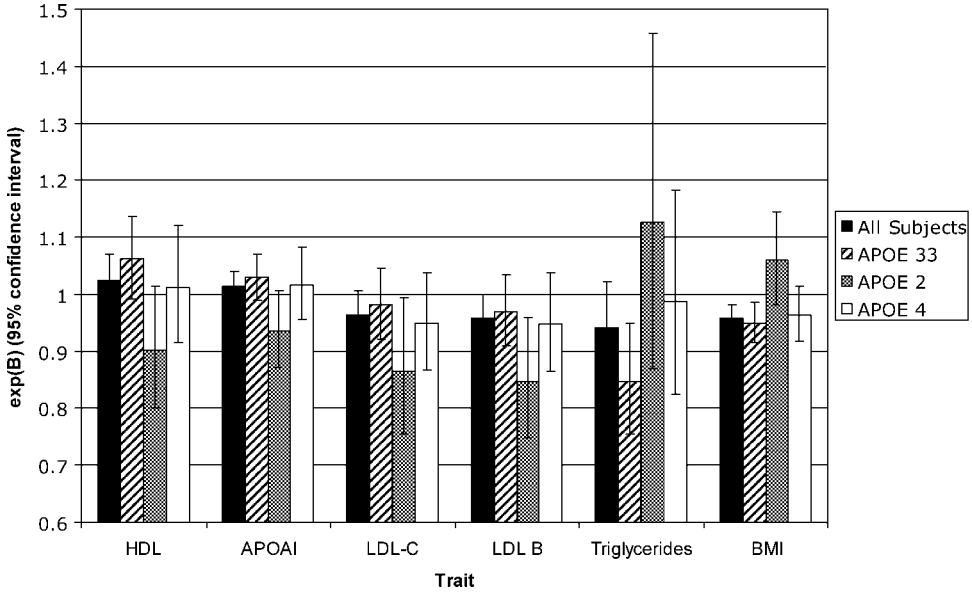

Fig. 3. Change in body mass index (BMI) and lipid traits per *VLDLR* 1226 risk allele stratified by *APOE* genotype. The *x* axis represents BMI or the lipid traits measured in the male controls of European descent not on lipid-lowering medication from the Carotid Lesion Epidemiology and Risk study (n = 412). The *y* axis represents the exponential change in BMI or the lipid trait per *VLDLR* 1226 risk allele. The *APOE* genotype groups include 3/3 (n = 218), ε2 (2/3 and 2/2; n = 69), and ε4 (3/4 and 4/4; n = 67). LDL B, apolipoprotein B associated with LDL-cholesterol; LDL-C, LDL-cholesterol. Error bars are 95% confidence intervals.

be needed to establish the *VLDLR* 1226 association with CAAD, BMI, and lipid traits as well as the more complex interaction with *APOE*.

Study strengths include the relatively large sample size and homogeneity of the study population. The additional presence of the lipid effects and the *APOE* interaction are all consistent with a true effect of *VLDLR* 1226 on CAAD status. Because *VLDLR* was resequenced in both a subset of the study population and a reference sample of similar race/ethnicity, we were able to characterize linkage disequilibrium across the gene to choose SNPs that would adequately represent all common genetic variation across the gene. Knowledge of linkage disequilibrium, along with the position of the *VLDLR* SNP and conservation with mouse, was essential in the interpretation of the observed associations and suggest the 5′ flanking *VLDLR* 1226 SNP as functional. This combination of strengths allowed us to identify *VLDLR* as a modifier of risk for CAAD, with its risk dependant on the genotype of its ligand *APOE*. These findings warrant further study to establish and better define the role of *VLDLR* genotypes in vascular disease.

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