APOE Modulates the Correlation Between Triglycerides, Cholesterol, and CHD Through Pleiotropy, and Gene-by-Gene Interactions

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ABSTRACT Relationship loci (rQTL) exist when the correlation between multiple traits varies by genotype. rQTL often occur due to gene-by-gene ($G \times G$) or gene-by-environmental interactions, making them a powerful tool for detecting $G \times G$. Here we present an empirical analysis of apolipoprotein E (*APOE*) with respect to lipid traits and incident CHD leading to the discovery of loci that interact with *APOE* to affect these traits. We found that the relationship between total cholesterol (TC) and triglycerides (ln TG) varies by *APOE* isoform genotype in African-American (AA) and European-American (EA) populations. The *e2* allele is associated with strong correlation between ln TG and TC while the *e4* allele leads to little or no correlation. This led to *a priori* hypotheses that APOE genotypes affect the relationship of TC and/or ln TG with incident CHD. We found that *APOE**TC was significant ($P = 0.016$) for AA but not EA while *APOE**ln TG was significant for EA ($P = 0.027$) but not AA. In both cases, *e2e2* and *e2e3* had strong relationships between TC and ln TG with CHD while *e2e4* and *e4e4* results in little or no relationship between TC and ln TG with CHD. Using ARIC GWAS data, scans for loci that significantly interact with *APOE* produced four loci for African Americans (one CHD, one TC, and two HDL). These interactions contribute to the rQTL pattern. rQTL are a powerful tool to identify loci that modify the relationship between risk factors and disease and substantially increase statistical power for detecting $G \times G$.

CORONARY heart disease [CHD (MIM 608901)] is challenging because it is a complex trait with a complicated genetic architecture. The MIM number is a reference in the Online Mendelian Inheritance in Man (OMIM) database. Recent genome-wide association studies (GWAS) have been successful in identifying regions of the genome with significant marginal effects. However, the combined effect of these loci explains only a small portion of the estimated total heritability. The traditional approach in association studies has been to test one phenotype at a time, even when multiple interrelated phenotypes are available for each individual. Because biological systems are organized in highly interactive pathways, changes at one level are likely to affect multiple traits throughout the system. Pleiotropy occurs when a single gene influences the variation of multiple phenotypes. Pleiotropic loci are common in complex biological systems (Stearns 2010) and tend to interact with other loci affecting traits within the same modular units (Wagner *et al.* 2007; Kenney-Hunt and Cheverud 2009). Pleiotropy is thought to play a primary role in the evolution of complex structures and systems (Wagner and Zhang 2011).

A conundrum in evolutionary biology is how a complex multitrait system with pleiotropy can evolve when a beneficial mutation for one trait may have detrimental consequences for another. Recent work (Pavlicev *et al.* 2011a; Pavlicev and Wagner 2012) has shown that relationship loci (rQTL) creates variation in pleiotropy that can be selected upon to further couple or uncouple trait variation and allow joint or separate evolution to occur in complex systems. rQTL occur

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when the correlation between multiple traits varies by genotype. They are the product of differential $G \times G$ or gene-by-environment interactions (see Pavlicev *et al.* 2011b, Figure 2). For example, differential gene interaction occurs when the pattern of interaction between two loci is different for multiple traits. This creates variation in the pleiotropic effects of a single locus. It also creates a pattern in which the correlation between two traits varies by genotype at the single locus level (rQTL). Empirical work has shown that most rQTL do not exhibit marginal effects., making them invisible to a typical association study. We can take advantage of this single locus pattern to identify rQTL and use them as *a priori* hypotheses to identify other loci that interact with them. This approach to detecting $G \times G$ greatly increases statistical power by reducing the multiple testing burden, and it connects multiple loci to each other and to multiple interrelated traits. A theoretical basis for and methodologies to detect relationship loci has been established in animal QTL work (Ehrich *et al.* 2003; Cheverud *et al.* 2004; Pavlicev *et al.* 2008, 2011b). An early example comes from a French population (Boerwinkle *et al.* 1987), where the correlation between total cholesterol and triglycerides varied among the most common genotypes of the three isoforms of apolipoprotein E (*APOE*) (MIM 107741). In another case (Sing *et al.* 1995), the relationship between the tertiles of cholesterol and coronary artery disease (CAD) changed with respect to *APOE* genotypes and the order of CAD risk among genotypes changed by cholesterol tertile.

A typical study would use genome-wide single locus tests to identify rQTL for a pair of traits. Each significant rQTL would then be used as a locus in a two-locus interaction model to identify other interacting loci for the two traits. In this article we work with a single rQTL by using the Atherosclerosis Risk in Communities Study (ARIC) to replicate in European-Americans the observation by Boerwinkle *et al.* (1987) that the correlation between total cholesterol (TC) and triglycerides (TG) varies by *APOE* genotype. This motivated three subsequent hypotheses. The first hypothesis is that *APOE* is also an rQTL for TC and TG in African Americans. The second is that *APOE* influences the relationship between TC and/or TG with incident CHD in both populations. Using genome-wide association data in ARIC, the last hypothesis is that other loci interact with *APOE* to effect these traits and produce the observed *APOE* rQTL patterns.

Materials and Methods

Study population

The ARIC study is a very well-phenotyped and ongoing prospective cohort study primarily focused on heart disease (ARIC Investigators 1989). Cohort members, totaling 15,792 persons aged 45–64 years at baseline (1987–89), were randomly chosen from four U.S. communities: Forsyth County, North Carolina; Jackson, Mississippi; suburban Minneapolis, Minnesota; and Washington County, Maryland. While the Jackson sample includes only African Americans, the other field centers samples are representative of the populations in these communities (*i.e.*, mostly non-Hispanic whites in Minneapolis and Washington County, and about 15% African American in Forsyth County). The ARIC study includes large numbers of African Americans ($N \sim 5000$) and non-Hispanic whites ($N \sim 11{,}000$).

During a baseline home interview, persons were invited to participate in the study, and information was collected on health status, selected risk factors, family medical history, employment and educational status, diet, and physical activity. Cohort members completed four clinic examinations, conducted 3 years apart, in 1987–89, 1990–92 (93% overall returned), 1993–95 (86% overall returned), and 1996–98 (80% overall returned; 90% of those who examined in 1993–95 returned). Surveillance of the ARIC cohort for morbidity and mortality has been carried out by annual phone interviews with subsequent abstraction of hospital records to validate cardiovascular events, and completeness of this annual follow-up has been high. For the last complete contact cycle available, 95% of still-living cohort members were contacted and completed a phone interview. The cardiovascular endpoints of interest to ARIC are CHD deaths, nonfatal myocardial infarction, coronary, revascularization, hospitalized congestive heart failure, and stroke.

We focus on incident CHD as an endpoint and measures of plasma levels of TC, TG, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). After excluding individuals on primary cholesterol medications, we used age, sex, body mass index (BMI), and medications with a secondary effect on cholesterol as covariates in all analyses. Most studies that use TG levels use a natural log transformation (ln TG) for analyses; however, the original analysis by Boerwinkle *et al.* (1987) did not. In the first model described below, we performed both for comparison while using only ln TG in the subsequent interaction analyses. Analyses with TG and ln TG were significant and comparable; in fact, ln TG had slightly smaller *P*-values. Table 1 and Table 2 give a summary of characteristics of individuals with each *APOE* genotype included in this study. The measure of incident CHD includes follow-up time and defined events as a definite or probable myocardial infarction (MI), fatal CHD, revascularization procedure, or electrocardiogram (ECG) evidence of MI. Follow-up time is from visit 1 until death, loss to follow-up, or censoring at 2007. Participants included in this study all gave written informed consent for study participation, including genetic research.

Genetic data

APOE genotypes were obtained by using TaqMan assays (Applied Biosystems, Foster City, CA) to genotype the 112 (rs429358) and 158 (rs7412) amino acid variants from exon 4 of APOE (Morrison *et al.* 2002; Hsu *et al.* 2005). Genome-wide assocation data for the ARIC participants consist of

Table 1 Summary of characteristics of African-American individuals with *APOE* genotype data in the ARIC study after adjusting for age, sex, BMI, and secondary cholesterol medication

African American	All	*e2e2*	*e2e3*	*e2e4*	*e3e3*	*e3e4*	*e4e4*
Count	3149	37	427	160	1411	976	138
Genotype %	1	1.2	13.6	5.1	44.8	31.0	4.4
Sex (% male)	0.38	0.41	0.37	0.41	0.36	0.4	0.36
Age (mean ± SD)	53.35 ± 5.81	53.32 ± 6.12	53.26 ± 5.71	53.16 ± 5.64	53.4 ± 5.8	53.32 ± 5.91	53.64 ± 5.84
BMI	29.16 ± 5.81	29.01 ± 6.73	29.85 ± 6.3	29.38 ± 6.17	29.23 ± 5.89	28.8 ± 5.44	28.63 ± 5.12
Triglyceride (mg/dl)	1.2 ± 0.59	1.47 ± 0.75	1.19 ± 0.55	1.29 ± 0.61	1.18 ± 0.59	1.22 ± 0.58	1.25 ± 0.59
Total chol (mg/dl)	213.79 ± 43.69	193.30 ± 49.48	197.55 ± 39.05	210.70 ± 39.43	214.18 ± 42.53	219.59 ± 45.23	225.77 ± 45.62
LDL-C (mg/dl)	136.56 ± 42.15	104.99 ± 44.31	119.33 ± 36.75	132.4 ± 37.42	136.66 ± 41.11	144.15 ± 42.83	148.4 ± 43.43
HDL-C (mg/dl)	55.79 ± 16.69	62.35 ± 21.18	57.28 ± 16.81	55.71 ± 15.86	56.53 ± 17.13	53.9 ± 15.62	55.26 ± 16.36
inc CHD per 1000	135	108	126	151	133	142	138
ln(triglycerides)	53.35 ± 0.44	53.32 ± 0.48	53.26 ± 0.42	53.16 ± 0.44	53.4 ± 0.44	53.32 ± 0.43	53.64 ± 0.43

genotypes for nearly one million SNPs using the Affymetrix 6.0 platform (Ikram *et al.* 2009). An additional million SNPs were imputed in the Europoean-American sample using MaCH with HapMap as a reference panel (Dehghan *et al.* 2009). We used only observed genotypes in the African-American population. Quality control criteria for SNPs and individuals matched previous studies with these data (Dehghan *et al.* 2009; Ikram *et al.* 2009). All data use and analyses are approved by an institutional IRB HSC-SPH-11-0320. The GWAS data for the ARIC study are available in dbGAP.

Genetic analyses

All analyses were performed using the R statistical software environment (R Development Core Team 2012) with the addition of the Survival package (Therneau 2012). In addition, for the GWAS data, we used the Rserve (Urbanek 2012) package in R to link with the PLINK software package (Purcell *et al.* 2007; Purcell 2012). After excluding those on primary cholesterol medications, we fit the following linear model separately in both populations,

$$\begin{aligned}\mathrm{TC}_{ijk} = u + \mathrm{age} + \mathrm{sex} + \mathrm{bmi} + \mathrm{secondaryCholMed} \\ + APOE + \mathrm{lnTG} + APOE \times \mathrm{lnTG} + e_{ijk},\end{aligned} \quad (1)$$

where *APOE* is a variable with six levels (one for each genotype). The significance of the interaction term was assessed using full *vs.* reduced models. Significance of this test rejects the null hypothesis that the correlation between the two traits is equal across genotype classes and that the beta coefficients from each bivariate regression *within* genotypes do not differ. Because there are three alleles (*e2*, *e3*, *e4*), *APOE* isoform data are often collapsed to two alleles (*e4*, non-*e4*) to make it easier to paramaterize (*i.e.*, additive, dominance). We *did not* do this because there was no linear additive relationship among the genotypes in Boerwinkle *et al.* (1987). As a result, we used all five degrees of freedom available from the six possible genotypes. The ARIC study is large enough (see Table 1 and Table 2) to have many individuals for even the rarest genotypic classes (*i.e.*, *e2e2* and *e4e4*).

Because both TC and TG are positively correlated with CHD in the general population, we hypothesized that the *APOE* genotypes change the relationship of one or both of these lipids with CHD. A Cox proportional hazards model was used to test whether TC and/or ln TG interacts with *APOE* with respect to CHD separately in each population. A full *vs.* reduced-model likelihood-ratio test was used to test for significance of the *APOE**TC and *APOE**ln TG terms in each model. The Cox proportional hazards model is a semi-parametric survival method that uses a partial log-likelihood to estimate the effect of independent variables on the hazard function in relation to time to event data. It assumes a parametric form for the effect of the predictors on the hazard function; but unlike parametric models, it does not make any assumptions about the shape of the baseline hazard function (Agresti 2002). Below is the model including ln TG; the other model replaces ln TG with TC:

$$\begin{aligned}\mathrm{CHD}_{ijk} = \mathrm{age} + \mathrm{sex} + \mathrm{bmi} + \mathrm{secondaryCholMed} \\ + APOE + \mathrm{lnTG} + APOE \times \mathrm{lnTG} + e_{ijk}.\end{aligned} \quad (2)$$

The null hypothesis posits that TC or ln TG-related risk for incident CHD is equivalent across *APOE* genotypes.

For each phenotype (TC, ln TG, incident CHD), we performed a genome-wide scan for loci that interact with *APOE*. GWAS data were not available for many of the individuals with *APOE* genotypes (795 AA, 1376 EA), leaving 2354 AA and 7677 EA individuals for the *APOE**SNP interaction analyses. We used two different traditional genome-wide significance thresholds for the European-American ($P < 5 \times 10^{-8}$) and the African-American ($P < 4 \times 10^{-7}$) scans because of the large difference in the number of tests (~2.2 million for EA *vs.* ~800,000 for AA). Since there is an *a priori* hypothesis for each phenotype, we need correct for only genome-wide significance *within* each phenotype. Instead of creating two additive continuous variables (0, 1, 2) for the two SNPs that define the *APOE* isoforms, we treated it as a variable with six levels (one for each genotype) represented by five (0, 1) indicator variables for $n - 1$ genotypes and five degrees-of-freedom. The second locus paramaterized as a simple additive locus with one degree of

Table 2 Summary of characteristics of European-American individuals with *APOE* genotype data in the ARIC study after adjusting for age, sex, BMI, and secondary cholesterol medication

European American	All	*e2e2*	*e2e3*	*e2e4*	*e3e3*	*e3e4*	*e4e4*
Count	9053	66	1145	210	5399	2048	185
Genotype %	1	0.7	12.6	2.3	59.6	22.6	2.0
Sex (% male)	0.45	0.48	0.45	0.48	0.45	0.44	0.46
Age (mean ± SD)	54.15 ± 5.72	54.65 ± 6.26	54.06 ± 5.8	53.88 ± 5.63	54.11 ± 5.71	54.26 ± 5.72	54.67 ± 5.33
BMI	26.74 ± 4.72	26.8 ± 3.91	26.96 ± 4.83	26.22 ± 4.71	26.8 ± 4.73	26.54 ± 4.65	26.42 ± 4.66
Triglyceride (mg/dl)	1.43 ± 0.69	1.81 ± 0.9	1.51 ± 0.77	1.48 ± 0.71	1.4 ± 0.67	1.44 ± 0.66	1.64 ± 0.77
Total chol (mg/dl)	213.02 ± 39.05	194.46 ± 52.58	201.03 ± 38.66	203.74 ± 37.11	213.40 ± 38.27	219.59 ± 37.89	223.07 ± 35.57
LDL-C (mg/dl)	136.05 ± 36.82	113.23 ± 42.78	121.61 ± 35.78	124.48 ± 34.99	136.82 ± 36.24	143.19 ± 36.02	145.27 ± 34.81
HDL-C (mg/dl)	51.66 ± 14.21	49.24 ± 10.36	52.66 ± 13.99	53.1 ± 14.61	51.75 ± 14.15	51.03 ± 14.47	48.71 ± 13.08
Inc CHD per 1000	155	182	142	124	157	156	185
ln(Triglycerides)	54.15 ± 0.45	54.65 ± 0.49	54.06 ± 0.47	53.88 ± 0.45	54.11 ± 0.44	54.26 ± 0.43	54.67 ± 0.43

freedom limiting the interaction test to 5 degrees of freedom. Below is the general model where we are interested in the *APOE**SNPadd interaction term:

$$\mathrm{TC}_{ijk} = u + \mathrm{age} + \mathrm{sex} + \mathrm{bmi} + \mathrm{secondaryCholMed} + APOE + \mathrm{SNP}_{\mathrm{add}} + APOE \times \mathrm{SNP}_{\mathrm{add}} + e_{ijk}. \quad (3)$$

A standard linear model was used for TC and ln TG and an analogous Cox proportional hazards model for incident CHD. Secondarily, we performed analyses for LDL and HDL because they are components of TC and because *APOE* has known direct effects on both. The null hypothesis for the interaction term is that the relationship of the SNP with the trait is the same among *APOE* genotypes.

Some types of $G \times G$ interactions can be classified as spreading or sign epistasis. Spreading epistasis occurs when the effect for locus 1 exists in one genotypic context but not another. Sign epistasis occurs when the allelic effects change direction across genetic backgrounds. Pavlicev *et al.* (2011b) found that rQTL have a higher proportion of sign epistatic interactions than non-rQTL. Sign epistasis sometimes involve compensatory mutations, which occur when the deleterious effect of a mutation at one locus is alleviated with a context dependency at another locus. In terms of pleiotropy, a mutation at a pleiotropic locus may have a beneficial impact on trait 1 while having a deleterious impact on trait 2. A mutation at another locus may create an interaction that alleviates the deleterious effects of the pleiotropci locus on trait 2 while preserving the beneficial impact on trait 1. Based on their pleiotropy and compensation model, Pavlicev and Wagner (2012) suggest that most adaptive signatures in genome scans could be the result of compensatory changes.

Results

Table 1 and Table 2 give counts and summary characteristics for the ARIC participants with *APOE* genotype data. The relationship between TC and TG significantly differed by *APOE* genotypes based on the model in Equation 1 (P = 10–7 EA, 10–5 AA) and the pattern was very similar in the two populations (see Figure 1). Boerwinkle *et al.* (1987) did not have enough individiuals with *e2e2* genotypes to estimate the correlation between TC and TG. Here we see that the correlation is very high (0.716 in both populations). The results using ln TG are virtually the same.

From the models based on Equation 2, *APOE**TC was significant for AA (P = 0.016) and not in EA (P = 0.57) and *APOE**TG was significant for EA (P = 0.027) but not AA (P = 0.34) (see Figure 1). All results were the same when using a natural log transformation of TG (P = 0.016 EA; P = 0.29 AA). The hazard ratio (exponential of the coefficient) from Cox regression within genotypes (including covariates) is shown in Figure 1. When the hazard ratio is >1 it denotes a positive correlation between CHD and TC or ln TG and values <1 suggest a negative correlation between CHD and TC or ln TG. The pattern of change in TC/ln TG correlation across APOE genotypes loosely resembles the hazard ratio changes in the CHD/ln TG and CHD/TC models with the primary differences related to where the heterozygotes lie with respect to the homozygotes.

In a post-hoc analysis with the components of TC, we found that LDL also has a strong relationship ($P = 9.6 \times 10^{-6}$ EA; $P = 1.4 \times 10^{-5}$ AA) with ln TG as observed before but the relationship of ln TG with HDL was weak or negligible (P = 0.013 EA; P = 0.264 AA). In African-Americans LDL had a stronger interaction (yet similar pattern) with APOE genotype to affect CHD than *APOE**TC (P = 0.0066 for *APOE**LDL *vs.* P = 0.016 for *APOE**TC) while there was no evidence for an *APOE**HDL interaction. There was no evidence for an *APOE**LDL or *APOE**HDL interaction in the European-American population.

The covariate adjusted correlation between TC and ln TG in the general population (0.24 for AA and 0.33 EA) is reflective of the *e3e3* genotype, which is by far the most common genotype. This is also true for hazard ratios for CHD/TC (in AA) and CHD/ln TG (in EA). The risk relationship seen in the general population is a weighted average with *e3e3* providing the largest influence while other less common genotypes pull in opposite directions (Figure 1). TC in AA and ln TG in AA have much a stronger positive

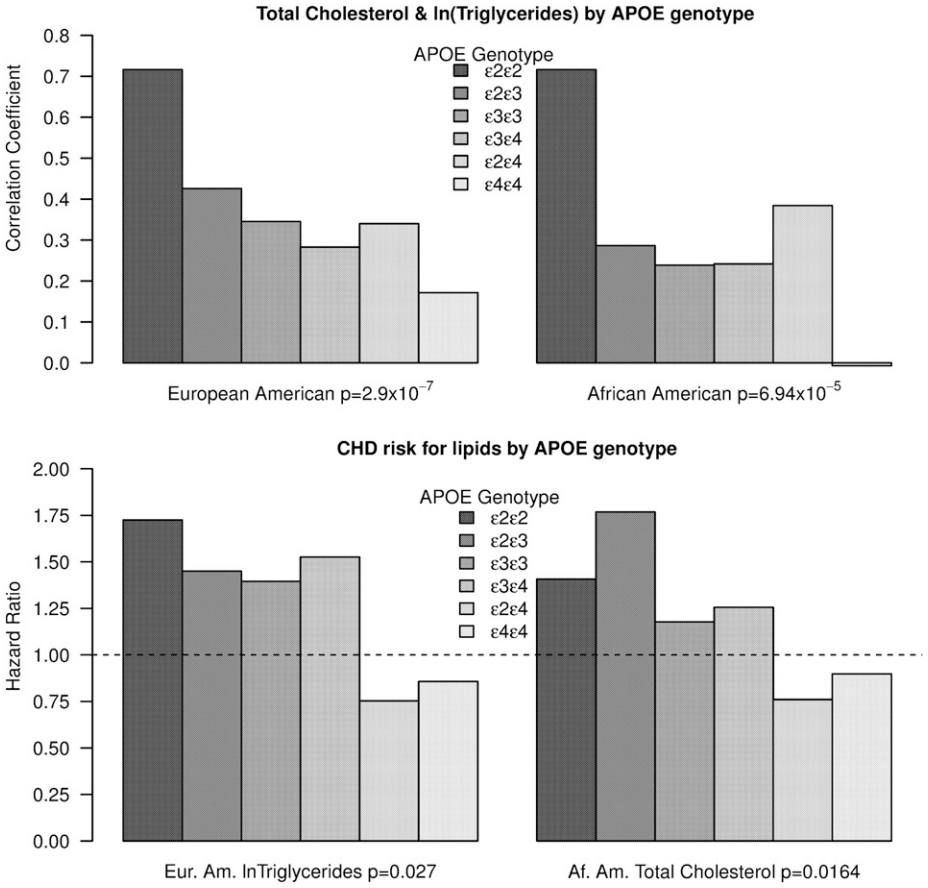

Figure 1 (Top) Within *APOE* genotype correlations between total cholesterol and triglycerides after correcting for age, sex, BMI, and secondary cholesterol medication for European-American (EA) and African-American (AA) populations in the ARIC study. The *P*-values are for the APOE*triglyceride interaction in the full model. (Bottom) Within each *APOE* genotype, a Cox proportional hazards model for incident CHD was performed with age, sex, BMI, and secondary cholesterol medication as covariates and total cholesterol for African Americans and triglycerides for European Americans. The hazard ratio is plotted where values >1 suggest risk and values <1 suggest a protective effect. The dotted line is at 1. The *P*-values are for the *APOE**triglyceride and *APOE**total cholesterol interaction in the full model. Here we report TG but results for ln TG are comparable.

CHD risk for individuals with the *e2e2* and *e2e3* genotypes while there is no risk (or negative) for those with *e2e3* or *e4e4*.

Initially, 10 loci that significantly interact with *APOE* in African Americans were found, one for incident CHD, six for TC, one for LDL, and two for HDL. Six loci were found for European Americans and they were all for TC. However, from QQ plots we found the scans for TC and LDL showed *P*-value inflation while CHD and HDL did not (see Supporting Information, Figure S3). The inflation does not appear to be due to stratificaiton for two reasons: because the use of principle compenents as covariates did not the affect the analyses (see below) and because traditional marginal single SNP tests with those traits did not show QQ plot inflation. $G \times G$ interaction tests sometimes exhibit type I error inflation due to underestimates of the covariance matrix (Voorman *et al.* 2011). Work done by Bůžková *et al.* (2011) and Voorman *et al.* (2011) showed that sandwich estimators and the parametric bootstrap can be used to obtain valid *P*-values, with the parametric bootstrap as the gold standard.

To obtain an empirical estimate of the *P*-values for the TC (six EA, six AA) and LDL (one AA) loci, for each locus we simulated 100 million parametric bootstraps to compare with the original statistic. Only one TC locus remained significant, leaving four significant loci that interact with *APOE* in African Americans (see Figure 2 and Table 3). Plots and descriptions of the other loci just under significance can be seen in Table S1, Figure S1, and Figure S2.

Stratification could be a source of confounding for analyses in the African-American population. Principal components were available for a subset of the individuals with *APOE* and GWAS data (1986 AA). Because of the loss of individuals (368), we decided to do the analyses with all of the available data (without the principal components) and for each genome-wide significant locus we used the smaller data set and the first two principal components as covariates to test for consistency. Using the smaller data set and including the principal components, each of the loci found in African Americans showed the same effects and remained highly significant. The original rQTL models with *APOE* in African Americans were also tested using the smaller data set and each retained significance.

There is no obvious functional information about the region around rs16828155 that connect to CHD risk but a study (Lunetta *et al.* 2007) using the Framingham Heart Study found a GWAS hit (rs1412337) 166 kb upstream for morbidity-free survival at age 65. rs16828155 has a strong additive relationship with CHD risk among African-American individuals that carry at least one APOE *e2* allele and none for those without an *e2* allele (see Figure 2).

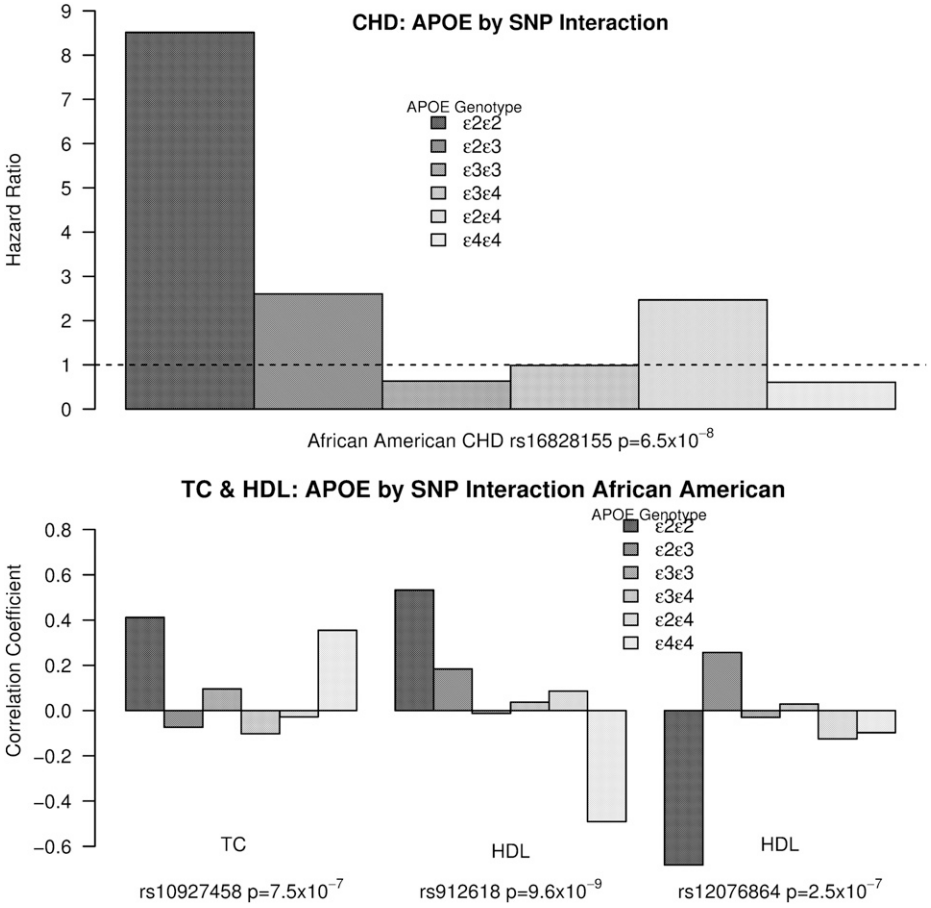

Figure 2 (Top) Interaction of rs16828155 with APOE genotypes in African Americans using a Cox proportional hazards model and incident CHD. The hazard ratio was plotted for the rs16828155 additive variable (0, 1, 2) from within the APOE genotype models. The dotted line is at 1. The *P*-value is for the *APOE**rs16828155 interaction term in the full model with the *APOE* genotypes as factors. (Bottom) Three SNPs referenced in the discussion with a genome-wide significant interaction in African Americans with *APOE* to affect total cholesterol (rs5758267) and HDL (rs912618 and rs12076864). Similar to the top, however, the traits are quantitative and we plot within genotype correlation coefficients after adjusting for the covariates. The *P*-values refer the to *APOE**SNP interaction term in the full model where the *APOE* genotypes are treated as factors and the SNP is treated as additive (0, 1, 2).

The HDL hit, rs912618, is found in protein kinase C, eta (PRKCH, 605437). A nonsynonymous variant in exon 9 (rs2230500) of PRKCH was shown to be associated with cerebral infarction in a Japanese case/control study, specifically lacunar infarction (Kubo *et al.* 2007). It is also associated with ischemic stroke (Li *et al.* 2012), LDL, and coronary heart disease (Zhu *et al.* 2012). According to the HapMap database, this SNP is of appreciable frequency only in Asian populations. Kubo *et al.* (2007) found that it was expressed in vascular endothelial cells and foamy macrophages in human atherosclerotic lesions and PKC-eta expression increased as the lesion type progressed. Using an analogous Cox proportional hazards model with incident CHD, the *APOE**rs912618 interaction term was not significant (P = 0.11); however, the direction of effects was as expected. In particular, individuals with *e4e4* genotypes had the strongest negative relationship (correlation = −0.49, $P = 2.47 \times 10^{-7}$; see Figure 2) between rs912618 and HDL and the expected positive association with CHD (hazard ratio = 2.19, P = 0.09) within the *e4e4* genotype. rs912618 was not associated with ischemic stroke directly or through interaction with *APOE* by use of a Cox proportional hazards model.

The TC hit, rs5758267, resides in an intron of L3MBTL2 (611865) and according to the ENCODE project using the RegulomeDB tool (Boyle *et al.* 2012), it has a score of 1f, which means it is an eQTL for the PHF5A and a transcription factor binding site/DNase peak. None of these are obvious clues to the biological nature of the interaction. However, as stated above rs5758267 also shows mild evidence for an interaction with *APOE* for TC in the European-American population. Unsurprisingly, it also shows strong evidence for an interaction with *APOE* for LDL ($P = 1 \times 10^{-5}$) in AA.

For comparison and possible replication of the interacting loci in African Americans, only rs5758267 had an appreciable minor allele frequency (MAF) in European Americans (AA = 0.27, EA = 0.28) and it also shows evidence for an interaction with *APOE* in EA (P = 0.035). For the other three, rs912618, the MAF in EA is 0.01 and the other two are <0.01, giving little or no power for replication. However, the remaining three interacting SNPs all had at least one nominally significant SNP interacting with *APOE* for their respective trait within a 40-kb region.

Differential epistasis can cause the pattern observed in an rQTL (Pavlicev *et al.* 2011b). The CHD and TC loci found to interact with *APOE* exhibit differential epistasis by interacting differently among the pairs of traits (TC/ln TG and CHD/TC), which in turn create different relationships between the traits across *APOE* genotypes (*i.e.*, the APOE rQTL pattern). Within *APOE* genotypes where the trait relationships are positively correlated, the interacting locus has the same direction of effects on the two traits, leading to

Table 3 The most significant SNP from each of the four genome-wide significant locations that interact with *APOE* in African Americans

Trait	CHD	TC	HDL	HDL
SNP	rs16828155	rs5758267	rs12076864	rs912618
Chromosome	1	22	1	14
Location (build 36.3)	167051924	39949296	110968915	61003344
Maj/Min (MAF)	T/C (0.475)	T/A (0.271)	C/T (0.216)	A/G (0.378)
P-value	6.51E-08	1.6–E–07[a]	2.54E–07	9.62E–09
Gene		*L3MBTL2* (intron)		*PRKCH* (intron)
Left gene	*DPT*	*C22:RP1-85F18.2*	*KCNA2*	*TMEM30B*
Right gene	*SUMO1P2*	*CHADL*	*KCNA3*	*LOC729637*

[a] *P*-value estimated with 100 million parametric bootstrap replicates and also. Significant in EA ($P = 0.03$).

a stronger correlation between the two traits *within* that genotype. Within APOE genotypes where the relationship between the traits is nonexistent (*i.e.*, *e2e4* and *e4e4*), the interacting loci have either opposing effects on the two traits or only an effect on one trait, which breaks up the correlation between the traits.

While the other 12 loci did not meet significance (Table S1, Figure S1, and Figure S2), they had patterns similar to that of the significant loci. Lumping all 16 loci together, for those loci with sufficient counts for *e2e4*, we found opposing effects for CHD and ln TG in EA (5 of 6) and CHD and TC in AA (4 of 7). All but one (12 of 13) found opposing direction of effects for TC and ln TG in *e4e4* while most showed opposing direction of effects for TC and CHD in EA (5 of 6) and ln TG and CHD in AA (5 of 7). It is not expected that all loci that interact with *APOE* will contribute to this specific rQTL, but these loci appear to contribute to the bivariate relationship differences among *APOE* genotypes (see Figure 1).

In addition to *APOE* itself, none of the other interacting loci have even a nominal association directly with the trait itself such that none of these loci would be found by a standard single-locus GWAS. This is consistent with other rQTL studies (Pavlicev *et al.* 2011b). *APOE* is the only locus to have a direct association with any of the traits (TC, LDL, ln TG, and HDL), which is already well established in the literature (Templeton *et al.* 2005). All of the observed interactions involve sign epistasis where the allelic effect changes directions across genetic backgrounds. (See Figure 2, Figure S1 and, Figure S2.)

Discussion

We were able to replicate the work of Boerwinkle *et al.* (1987) and establish that *APOE* acts as an rQTL between TC and ln TG in both EA and AA populations. This led to significant *a priori* tests establishing that APOE modulates the relationship between CHD and ln TG in European Americans and CHD and TC in African Americans. The *a priori* tests allow for multiple testing to be done only *within* each genome scan analogous to work on controlling false positives for epistatic QTL done by Wei *et al.* (2010).

The rQTL approach, as demonstrated here, is a powerful way to identify loci that effect relationships between important biological risk factors and the relationship between these factors and disease. In our case, the rQTL (*APOE*) was already known; however, the same model used to validate *APOE* as an rQTL can be used in a genome-wide scan to identify other previously unknown rQTL for a given pair of traits. These loci would typically be undetected in a normal GWAS analysis and even if "seen," their role in pleiotropic variation and gene-by-gene interactions would not be evident. It is also a unique, efficient, and powerful approach to identifying gene-by-gene interactions. It enhances statistical power by defining *a priori* loci (rQTL) that are likely to be involved in an interaction and reducing the number of tests to the order of a GWAS instead of all pairwise tests.

Biologically, it provides a framework to link multiple traits together and with the rQTL and other interacting loci. In the context of human medicine, these loci can lead to further insights about conditions where the magnitude of risk for a known risk factor changes. In the case of triglycerides in European Americans and total cholesterol (and LDL) in African Americans, their importance to CHD risk depends on what *APOE* genotype an individual has. This raises issues of importance for treatments targeting risk factor levels. If treatments for TC and ln TG are based on their association with CHD, then lipid-lowering drugs may be necessary or useful only for individuals with *APOE* genotypes where the CHD risk is strong for TC or ln TG. In particular, the level of triglycerides matters, in terms of CHD risk, for an EA carrying the *e2e2* genotype while it does not for those carrying an *e2e4* or *e4e4* genotype. The level of cholesterol matters for an AA carrying an *e2e3* or *e2e2* genotype while it does not for those carrying an *e2e4* or *e4e4* genotype.

It is well established that both TC and ln TG have positive CHD risk in the general population. The three *APOE* alleles (*e2*, *e3*, *e4*) are associated with low, medium, and high TC and ln TG levels. These same alleles are associated with low, medium, and high CHD risk. It is tempting to think that the relationship between *APOE* and CHD is strictly through its linear influence on lipid levels. Our results suggest that this explanation is too simple and masks important relationships between *APOE* and CHD independent of or at least not

linearly related to lipid levels. Here we suggest that *APOE* genotypes influence the relationship between lipid levels and CHD, not just the actual lipid level itself.

Various studies have shown that *APOE* alleles respond differently to different types of LDL-lowering treatments. Response (reduction in LDL) to exercise is greater for those with the *e3* allele than the *e4* allele. Statins produce a similar pattern while Probucol, which has a different target and mechanism for lowering LDL than statins, has the opposite effect with a greater response for those carrying the e4 allele (Hagberg *et al.* 2000). Gustavsson *et al.* (2012) found that *APOE* genotypes interact with both smoking and physical inactivity with respect to CHD. They determined that these interactions were independent of LDL levels and concluded that something other than a direct effect on lipid levels is responsible for this relationship with CHD.

While *APOE* has a similar effect on the correlation between TC and ln TG in both populations, it is surprising to see that *APOE* affects the relationship between only TC and CHD in African Americans and only ln TG and CHD in European Americans. This difference between African Americans and European Americans may be another example of observed yet not understood differences in the behavior of lipids and CHD between the two populations (Haffner *et al.* 1999). In another study (T. J. Maxwell and C. M. Ballantyne, unpublished results) the authors have observed that *CETP* promoter variants associated with *CETP* protein concentration in European-Americans are strongly associated with HDL levels, yet those same variants in African-Americans are associated with only *CETP* protein concentration and not HDL.

Relationship loci present a powerful approach to uncovering the complex genetic architecture of common diseases. They establish a foothold into the world of pleiotropy and interactions, which are the basis for modularity and the inherent organization of biological systems in pathways of interacting factors. Because rQTL create variation in pleiotropy, selection can act upon them to couple and uncouple traits enabling evolution to change multiple traits jointly or separately (Pavlicev *et al.* 2011a).

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APOE Modulates the Correlation Between Triglycerides, Cholesterol, and CHD Through Pleiotropy, and Gene-by-Gene Interactions

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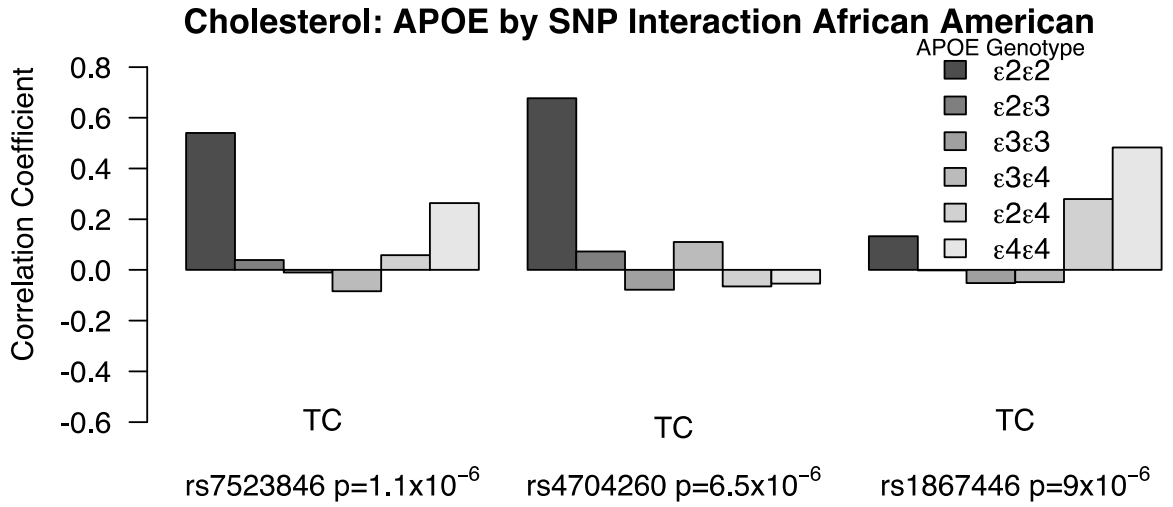


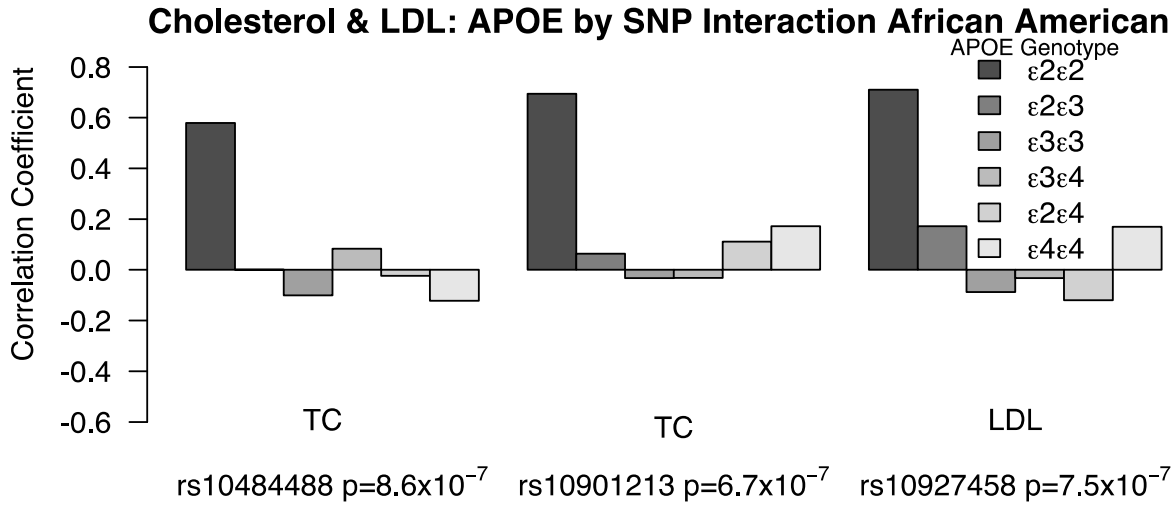
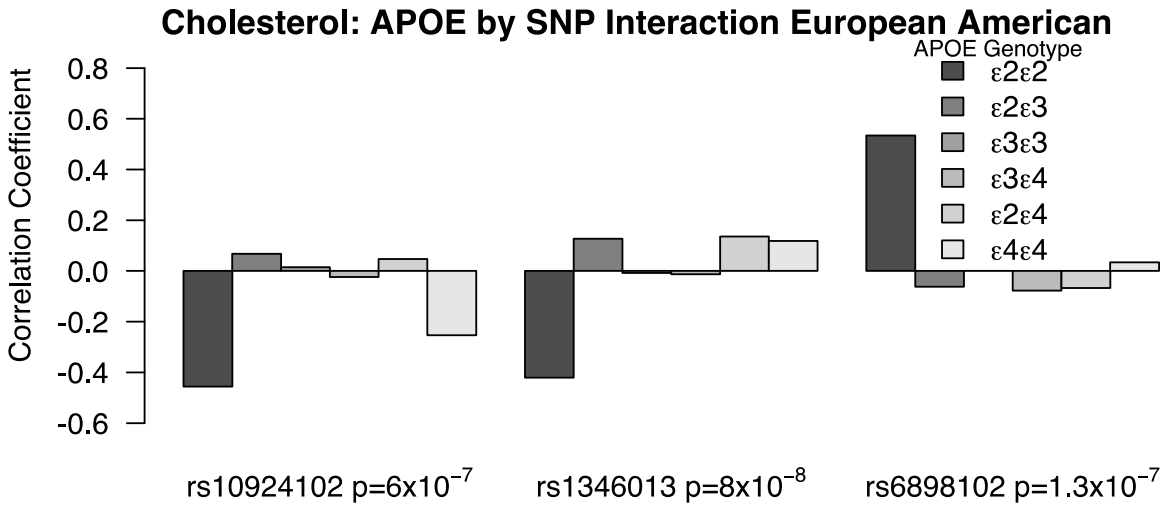


Figure S1 A plot similar to **Figure 2** in the main text for each of the SNPs in **Table S1** that interact with APOE to affect TC and LDL in African Americans. These tests were significant with the parametric test yet dropped below significance after calculating empirical p-values with 100 million parametric bootstrap replicate for each SNP. Plots are based on *within* APOE genotype models after adjusting for covariates while the p-values are based on the APOE*SNP interaction in the full model where the APOE genotypes are treated as factors and the SNP is treated as a (0,1,2) additive variable. The beta coefficients from the linear model provide the same story but the correlation coefficient is easier to visualize.



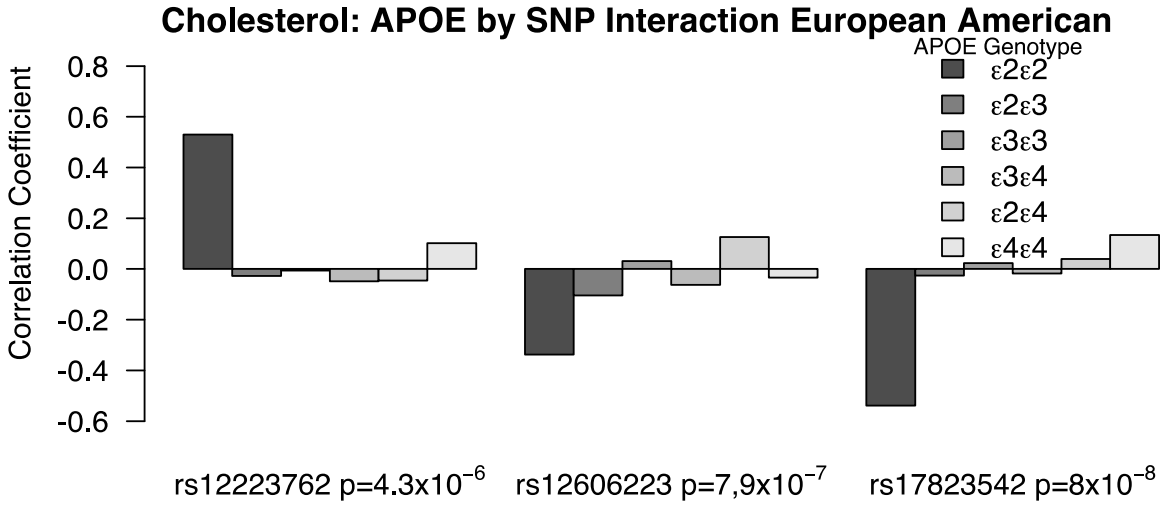
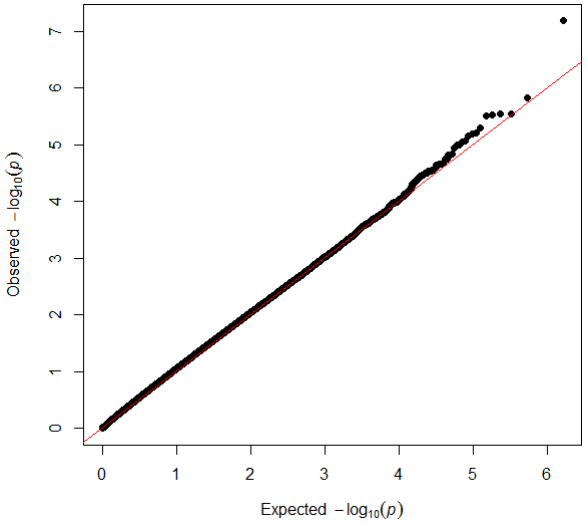
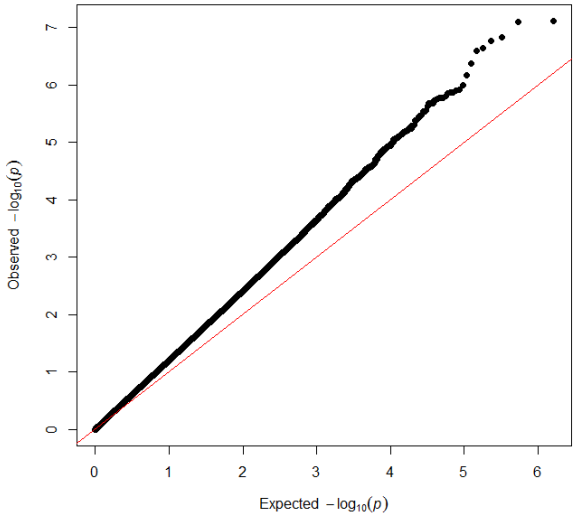


Figure S2 A plot similar to **Figure 2** in the main text for each of the SNPs in **Table S1** that interact with APOE to affect TC in European Americans. These tests were significant with the parametric test yet dropped below significance after calculating empirical p-values with 100 million parametric bootstrap replicate for each SNP. Plots are based on *within* APOE genotype models after adjusting for covariates while the p-values are based on the APOE*SNP interaction in the full model where the APOE genotypes are treated as factors and the SNP is treated as a (0,1,2) additive variable. The beta coefficients from the linear model provide the same story but the correlation coefficient is easier to visualize.

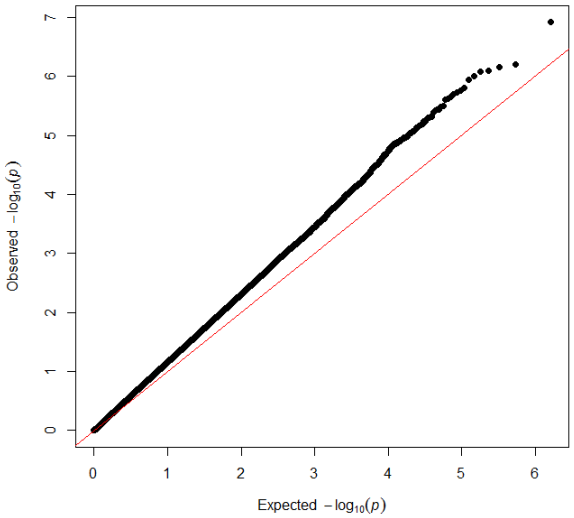
APOExSNP CHD AA



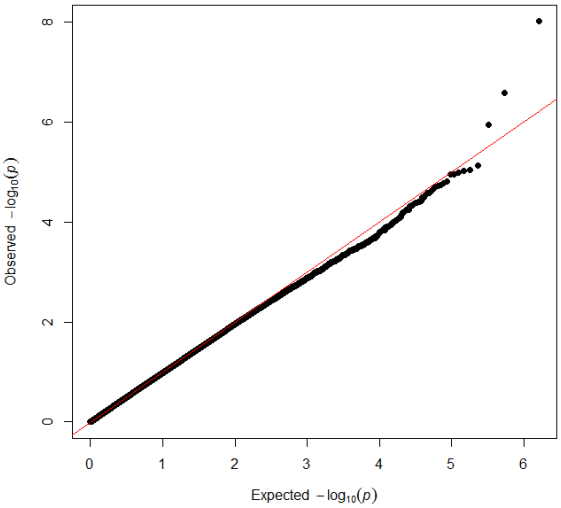
APOExSNP TC AA



APOExSNP LDL AA



APOExSNP HDL AA



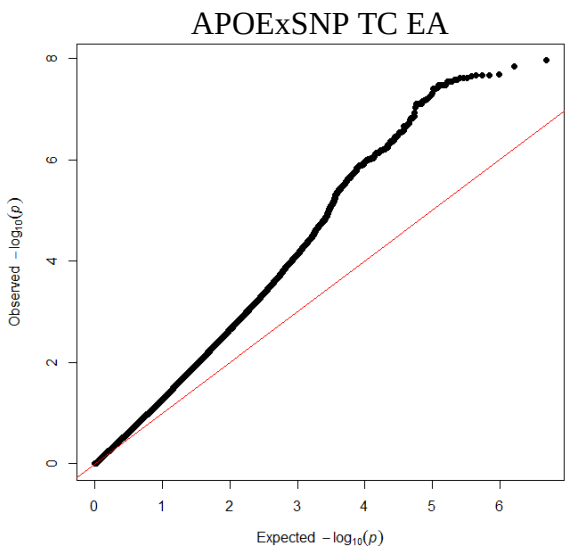
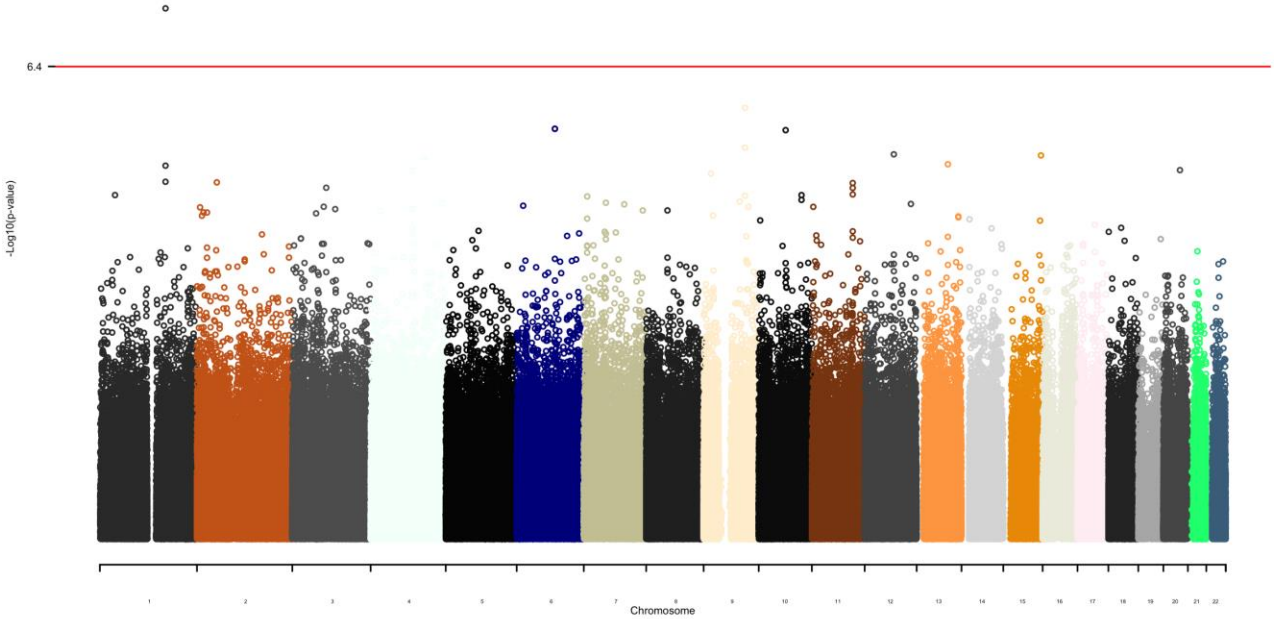
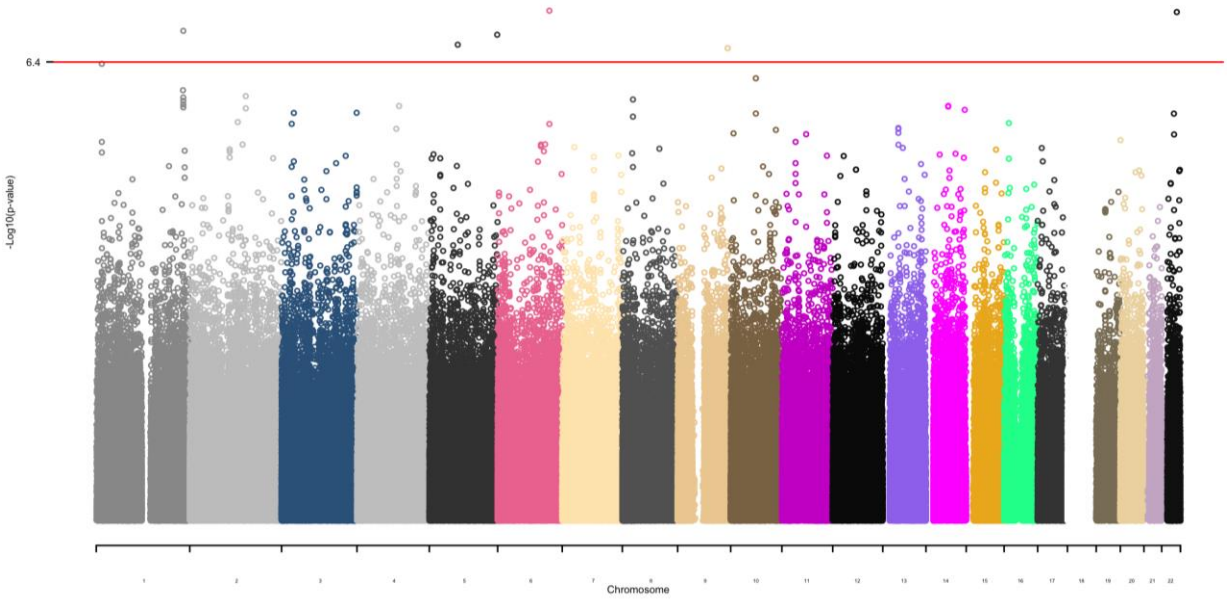


Figure S3 QQ Plots for each of the APOExSNP interaction scans from the parametric tests.

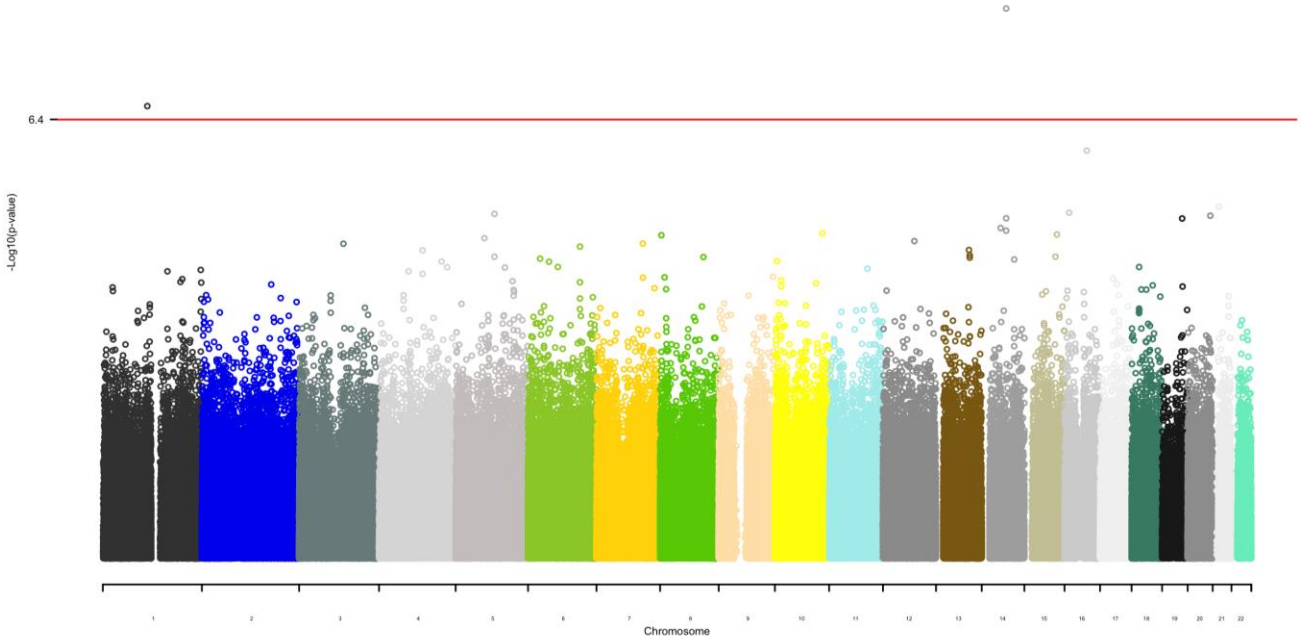
AA APOExSNP Interactions for CHD



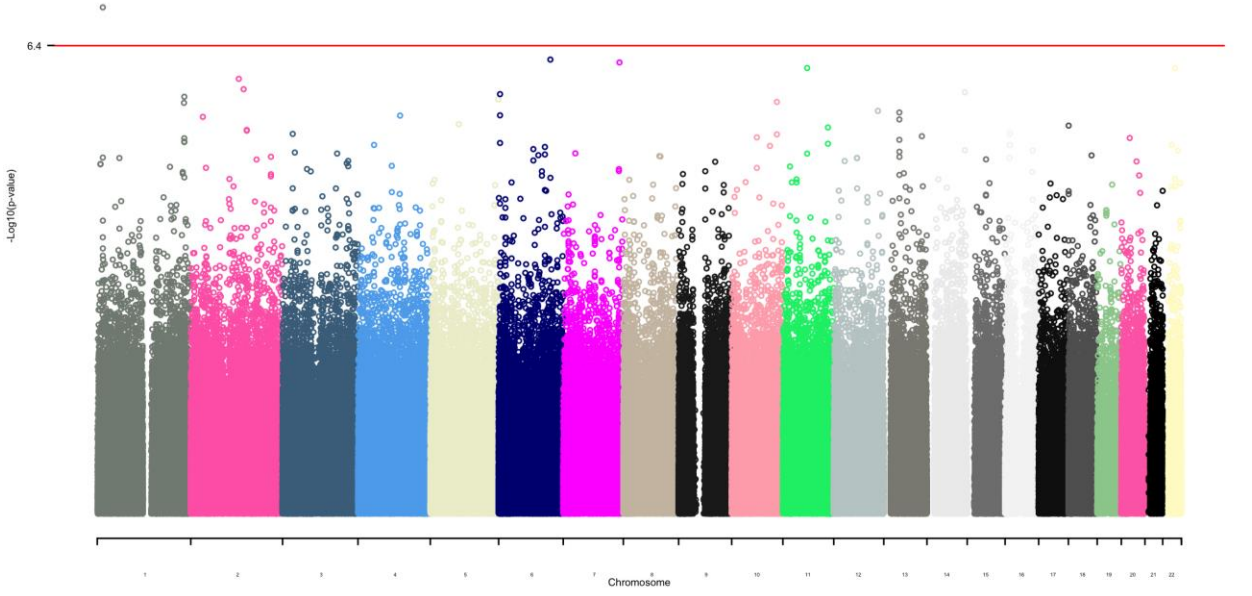
AA APOExSNP Interactions for Total Cholesterol



AA APOExSNP Interactions for HDL



AA APOExSNP Interactions for LDL



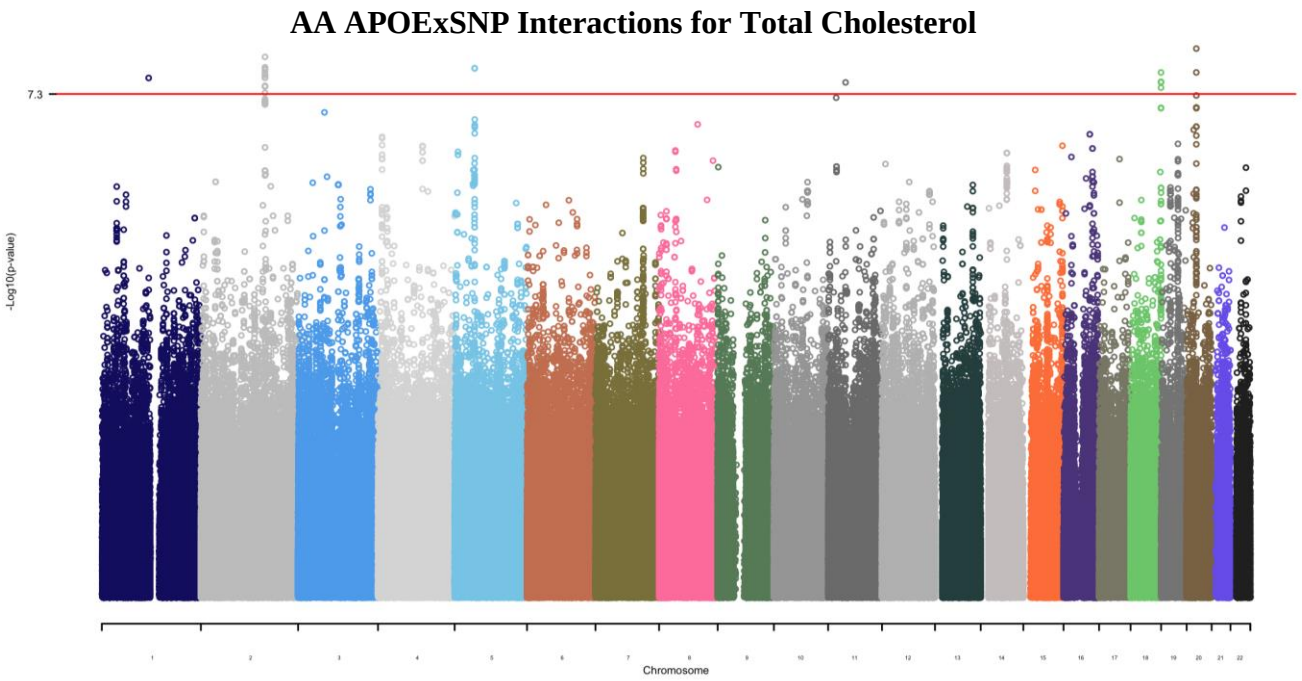


Figure S4 Manhattan plots for each of the APOExSNP scans. These plots are for the original parametric tests. Note that the SNP density in the European American population is about three times that of the African American population. This would lead to greater clustering around highly significant SNPs.

Table S1 SNPs from the TC and LDL scans initially significant with the parametric test yet dropped below significance after doing 100 million parametric bootstrap replicates.

Population	AA	AA	AA	AA
Trait	TC	TC	TC	TC
SNP	rs7523846	rs4704260	rs1867446	rs10484488
Chromosome	1	5	5	6
Location (build 36.3)	230075483	75223049	179439720	136713787
Maj/Min (MAF)	G/A (0.098)	A/T (0.035)	T/C (0.032)	T/G (0.104)
Parametric p-val	1.47E-07	2.29E-07	1.66E-07	7.69E-08
Par Bootstrap p-val	1.05E-06	6.49E-06	8.99E-06	8.60E-07
Gene	*DISC1* (intron)			*MAP7* (intron)
Left Gene	*DISC2*	*LOC391798*	*RNF130*	*BCLAF1*
Right Gene	*SIPA1L2*	*LOC100132039*	*LOC646058*	*LOC100128745*
Population	AA	AA	EA	EA
Trait	TC	LDL	TC	TC
SNP	rs10901213	rs10927458	rs10924102	rs1346013
Chromosome	9	1	1	2
Location (build 36.3)	132237274	14731263	116832505	160021586
Maj/Min (MAF)	A/C (0.122)	C/A (0.185)	C/T (0.302)	G/A (0.486)
Parametric p-val	2.56E-07	7.50E-07	2.93E-08	1.45E-08
Par Bootstrap p-val	6.70E-07[a]	1.20E-07	6.00E-07	8.00E-08
Gene	*HMCN2* (intron)			*BAZ2B* (intron)
Left Gene	*LOC392395*	*PRDM2*	*LOC148766*	*LOC100127929*
Right Gene	*ASS1*	*RP1-21O18.1*	*CD58*	*LOC728059*
Population	EA	EA	EA	EA
Trait	TC	TC	TC	TC
SNP	rs6898102	rs12223762	rs12606223	rs17823542
Chromosome	5	11	18	20
Location (build 36.3)	49910087	43546197	75914383	24071100
Maj/Min (MAF)	G/A (0.487)	T/A (0.040)	T/G (0.289)	G/C (0.486)
Parametric p-val	2.12E-08	3.40E-08	2.44E-08	1.10E-08
Par Bootstrap p-val	1.30E-07	4.27E-06	7.90E-07	8.00E-08
Gene				
Left Gene	*EMB*	*LOC120449*	*C18orf22*	*POM121L3*
Right Gene	*PARP8*	*LOC100131381*	*ADNP2*	*LOC100128232*

[a]nominally significant in EA (p=0.008)