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Longitudinal analysis of haplotypes and polymorphisms of the APOA5 and APOC3 genes associated with variation in serum triglyceride levels: the Bogalusa Heart Study

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Abstract

Polymorphisms in the APOC3 and APOA5 genes, from the APOA1/APOC3/APOA4/APOA5 gene cluster on chromosome 11q23, have been associated with interindividual variation in plasma triglycerides. APOA5 polymorphisms implicated include 2 in the promoter region (−1131 T/C and −3 A/G) and 1 in exon 2 (+56 C/G). APOC3 polymorphisms implicated include 1 (*Sst*I) in the 3′ untranslated region and 1 (−2854 G/T) in the APOC3-APOA4 intergenic region. We analyzed the associations of haplotypes and multilocus genotypes of these polymorphisms on longitudinal serum triglyceride profiles in 360 African American and 823 white subjects from the Bogalusa Heart Study. Subjects were examined from 2 to 8 times (mean ± SD, 5.4 ± 1.3) between 1973 and 1996, at ages ranging from 4 to 38 years, with 1978 observations in African Americans and 4465 in whites. Serum triglycerides were significantly higher among whites across all ages. Allele frequencies differed significantly between African Americans and whites at all but the APOA5 +56 C/G locus. Linkage disequilibrium among the loci was higher in whites and haplotype diversity lower: 6 haplotypes had estimated frequencies of more than 1% in African Americans, 5 in whites. Individually, all polymorphisms except APOC3 −2854 G/T showed significant associations with triglyceride levels in the full sample. However, genotype models including all 5 loci showed significant triglyceride associations for only 3 (APOC3 *Sst*I, APOA5 −1131 T/C, and APOA5 +56 C/G); significant interactions among them indicated their effects were not independent. Neither APOC3 −2854 G/T nor APOA5 −3 A/G had significant effects when the other 3 loci were in the models. The EM algorithm was used to estimate haplotype frequencies and assign haplotype probabilities to individuals, which is conditional on their genotypes; individuals' haplotype probability vectors were then used as predictors in multilevel mixed models of longitudinal triglyceride profiles. Of haplotypes comprising, in order, APOC3 *Sst*I and −2854 G/T and APOA5 −1131 T/C, −3 A/G, and +56 C/G, 3 were significantly associated with higher triglycerides, even after adjusting for multiple tests: GGTAG ($P = .002$), GTTAG ($P < .0001$), and CGCGC ($P = .0002$). Each GGTAG haplotype carried would be expected to raise triglyceride levels (relative to those of GTTAC homozygotes) by ~19 mg/dL, each GTTAG haplotype by ~15 mg/dL, and each CGCGC haplotype by ~7 mg/dL. Haplotypes comprising the 3 loci implicated by genotype analyses (*Sst*I, −1131 T/C, and +56 C/G) were also tested: haplotypes C_C_C and G_T_G significantly raised triglycerides, even after adjustment for multiple comparisons ($P < .002$ for both), with each copy of C_C_C expected to raise triglycerides by ~7 mg/dL and each copy of G_T_G by ~15 mg/dL. Overall, our findings support those of others in associating specific polymorphisms and haplotypes in the APOA1/C3/A4/A5 gene cluster with higher serum triglyceride levels. However, the degree to which polymorphisms in the APOC3 and APOA5 genes may be independently associated with triglyceride levels remains to be determined.

1. Introduction

Interindividual variation in plasma levels of several lipids, including low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides, is associated with

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variation in risk of cardiovascular disease. In blood, circulating lipids are transported in lipoproteins composed of surface phospholipids, nonipolar lipids, such as cholesterol and triglycerides, and apolipoproteins, of which there are several types, including apolipoprotein (apo) A-I, A-II, A-III, A-IV, B, and E. In 2001, a fifth member of the apoA family, apoA-V, was discovered. In humans, the APOA5 gene is proximal to the APOA1/APOC3/APOA4 gene cluster on chromosome 11q23 [1].

The precise biologic function of apoA-V remains to be determined, but studies in transgenic mice expressing human APOA5 and in APOA5 knockout mice suggest that it may play a major role in determining plasma triglyceride levels, apart from any effects of apoC-III [1]. In humans, several APOA5 polymorphisms have been associated with altered plasma triglyceride levels. A T > C transition in the promoter region (−1131 T/C) has been associated with higher triglyceride levels in some populations [2-5], but not others [6]. In German patients with hyperlipidemia, the −1131 C allele was associated with higher triglyceride and lower high-density lipoprotein cholesterol levels, but only in subjects with a body mass index (BMI) greater than 25; the association with triglyceride levels was stronger in subjects who carried the ε4 allele of the common apoE polymorphism [7]. An A > G transition in the promoter region (−3 A/G), in linkage disequilibrium with the −1131 T/C polymorphism, has also been associated with variation in triglyceride levels [8]. A C > G transversion in exon 2 (+56 C/G) that changes a serine residue at codon 19 to tryptophan has also been associated with altered triglyceride levels, independent of triglyceride-associated APOC3 polymorphisms [2]. Two haplotypes carrying different alleles at the APOA5 −1131 T/C, −3 A/G, and +56 C/G loci (either C, G, and C, or T, A, and G, respectively) have been associated with higher triglycerides in whites [6], although only haplotypes with the former configuration were associated with higher triglycerides in several Asian populations [9].

ApoC-III, carried primarily on high-density lipoproteins, inhibits lipolysis of very low-density lipoproteins, both through inhibition of lipoprotein lipase and hepatic triglyceride lipase activity, and by interfering with lipoprotein binding to glycosaminoglycans on cell membranes [10]. Several polymorphisms in or near the APOC3 gene have been associated with hypertriglyceridemia. The most extensively studied of these, an *Sst*I restriction site polymorphism in the 3′ untranslated region, has been associated with hypertriglyceridemia in numerous studies and multiple populations [11,12]. A polymorphism in the APOC3-APOA4 intergenic region, −2854 G/T, was associated with variation in plasma triglyceride levels in 135 healthy adults participating in a study of postprandial lipid and glucose response; the T/T genotype was associated with lower fasting triglyceride levels in subjects younger than 55 years and in males [13]. In another study, the −2854 G allele was associated with increased postprandial triglyceride response [14]. However, a study of 2745 healthy men 50 to 61 years of age found no association between the −2854 G/T polymorphism and plasma triglycerides [15].

Although variation in both the APOA5 and APOC3 genes has been associated with effects on triglyceride levels, the proximity of the 2 genes to one another has made it difficult to determine whether the effects of variation in each are independent. We investigated this by analyzing the associations of both haplotypes and multilocus genotypes with serum triglyceride levels, using the 5 polymorphisms in the APOC3/APOA5 region discussed above. Because we also wished to investigate the possibility that changes in genotype effects may occur with age, we analyzed subjects from the Bogalusa Heart Study who had been measured multiple times at ages ranging from 4 to 38 years.

2. Subjects and methods

2.1. Subjects and examinations

Subjects were examined between 1973 and 1996, when schoolchildren in Bogalusa, LA, and older subjects previously examined as schoolchildren, were screened for cardiovascular disease risk factors approximately every 3 years. Participants in the present study had been examined at least 4 times between 1973 and 1996, and at least once between 1991 and 1996, when blood was collected for DNA. Participants gave informed consent at each examination; for those younger than 18 years, consent of a parent was also obtained. Study protocols were approved by institutional review boards at the institutions involved. Examinations were conducted by trained examiners following protocols published previously [16]. From 1973 to 1986, serum total cholesterol and triglycerides were measured chemically, following Lipid Research Clinics Program protocols [17]; after 1986, they were measured enzymatically [18,19]. Both chemical and enzymatic procedures gave comparable results and met standards of the Lipid Standardization Program of the Centers for Disease Control and Prevention, Atlanta, GA. Serum lipoprotein cholesterol fractions, including low-density lipoprotein cholesterol, were measured by heparin-Ca^{2+} precipitation followed by agar or agarose gel electrophoresis [20].

2.2. Genotyping

Genotyping of APOC3 *Sst*I (rs5128) used the TaqMan allele-discrimination assay (Applied Biosystems, Foster City, CA), using the ABI 7900HT and Sequence Detection System software (Applied Biosystems). Genotyping of APOC3 −2854 G/T (rs2542051) and APOA5 +56 G/T (rs3135506) used the TaqMan Assay-by-Design system (Applied Biosystems). Genotyping of APOA5 −1131 T/C (rs662799) and APOA5 −3 A/G (rs651821) used a multiplex reaction using the MassARRAY system (Sequenom, San Diego, CA), followed by a mini-sequencing reaction to extend the PCR product by a preset number of nucleotides

Table 1
Frequencies (±SD) of the less common allele (based on frequencies in African Americans) for each APOC3 and APOA5 polymorphism, by group

Variant	Allele	African Americans	Whites
APOC3 *Sst*I	C	0.168 ± 0.016	0.095 ± 0.008*
APOC3 −2854	T	0.326 ± 0.020	0.634 ± 0.013*
APOA5 −1131	C	0.103 ± 0.013	0.073 ± 0.007*
APOA5 −3	G	0.119 ± 0.014	0.068 ± 0.007*
APOA5 +56	G	0.036 ± 0.008	0.052 ± 0.006

* $P < .05$, African Americans vs whites.

as determined by the Assay Design software (Sequenom). Mass spectrometry analysis of mini-sequencing reaction products was performed using the SpectroREADER (Bruker Biflex III) MALDI-TOF instrument, with the SpectroCALLER (Sequenom) being used to assign genotypes based on the mass spectra. Details on all reaction protocols and all primer and probe sequences used are available from the corresponding author by request.

2.3. Genetic analyses

Multilevel mixed models were used to analyze haplotype and genotype effects. Such models allowed the use of all observations by taking into account the correlations among multiple observations of the same individuals. We have described elsewhere our use of multilevel models to analyze genotype effects [21,22]. The vector of observed genotypes for a given individual was replaced by the vector of haplotype probabilities conditional on that individual's set of genotypes at the loci involved and the estimated haplotype frequencies in the population, to modify these models for haplotype analysis. Using haplotype probabilities as predictors avoids the problem of errors in assigning probable haplotypes to multiply heterozygous individuals and eliminates the need to discard subjects for whom the most probable haplotypes cannot be inferred with confidence. The EM algorithm, as implemented in the HelixTree program (Golden Helix, Bozeman, MT), was used to estimate haplotype frequencies and assign haplotype probabilities to individuals. Because genotype frequencies differed between African Americans and whites, all conditional haplotype probabilities were estimated for each group separately. Associations of haplotypes with longitudinal lipid profiles were tested using haplotype trend regression [23] in multilevel mixed models.

For each response variable, a full model was first fit that included all possible 2-way interactions involving race, sex, BMI, genotypes or haplotypes, and age terms through age cubed (age^3). Adding squared (age^2) and cubed (age^3) age terms to the model produced predicted profiles similar to those obtained from plotting observed values. Interactions were tested individually in a fixed order, using likelihood ratio tests; −2 times the difference in the model log likelihoods before and after removal of a term asymptotically follows a χ^2 distribution with 1 *df*. When all interactions of a given order involving an age term had been tested, the N nonsignificant terms were removed and a likelihood ratio test with N *df* conducted. For haplotypes, the null hypothesis was that including haplotype information in a model did not improve prediction of lipid profiles. Thus, haplotypes were tested as a group rather than individually; for example, in testing haplotype-by-BMI interactions, all haplotype-by-BMI terms were removed simultaneously to form the reduced model. Within models, the contributions of individual haplotypes to the overall effect were assessed using Wald tests. Apart from the estimation of haplotype probabilities as described above, all analyses were conducted using SAS (version 8, SAS Institute, Cary, NC). In all models and statistical tests involving triglycerides, triglyceride values were first transformed using natural logarithms.

3. Results

There were 360 African Americans and 823 whites with genotypes for at least 1 of the 5 APOC3 and APOA5 loci. Individuals were examined from 2 to 8 times each (mean ± SD, 5.4 ± 1.3); examination frequencies did not differ between African Americans and whites (data not shown). The total number of observations available was 6443, with 1978 in African Americans and 4465 in whites. Subjects were between 4 and 28 years of age when first examined, and between 14 and 38 years of age when last examined.

Allele frequencies differed significantly between African Americans and whites at all except the APOA5 +56 C/G locus, the least polymorphic locus in both groups (Table 1). Linkage disequilibrium existed among the 5 loci, but was more marked in whites (Table 2). In African Americans, the only statistically significant pairwise linkage disequilibrium existed between the 2 APOC3 loci, and between the −1131 and −3 loci of APOA5. However, $|D'|$ values between the APOA5 −1131 T/C and +56 C/G loci and between the APOA5 −3 A/G and +56 C/G loci in both African Americans and whites exceeded 0.9, close to the maximum of 1.0. That such high values were not statistically significant may be due largely to the low frequency of the

Table 2
Pairwise D' values for APOC3 and APOA5 polymorphisms in African Americans (above diagonal) and whites (below diagonal)

	APOC3 *Sst*I	APOC3 −2854 G/T	APOA5 −1131 T/C	APOA5 −3 A/G	APOA5 +56 C/G
APOA5 +56 C/G	0.112	−0.204	−0.947	−0.904	1.000
APOA5 −3 A/G	0.204	−0.324	**0.980**	1.000	−0.954
APOA5 −1131 T/C	0.096	−0.056	1.000	**1.000**	−0.959
APOC3 −2854 G/T	**−0.972**	1.000	**−0.859**	**−0.830**	**0.780**
APOC3 *Sst*I	1.000	**−0.945**	**0.759**	**0.742**	−0.775

Values in bold type were significant at $\alpha = .05$.

Table 3
Estimated haplotype frequencies (±SE), by race

5-Locus haplotype	Estimated frequency	Corresponding 3-locus haplotype	Estimated frequency
African Americans			
GTTAC	0.267 ± 0.019	G_T_C	0.721 ± 0.018
GGTAC	0.422 ± 0.021		
GGTGC	0.037 ± 0.008		
GTTGC	0.002 ± 0.003		
GGTAG	0.016 ± 0.007	G_T_G	0.024 ± 0.007
GTTAG	0.008 ± 0.005		
GGCAC	0.002 ± 0.007	G_C_C	0.089 ± 0.012
GGCGC	0.046 ± 0.012		
GTCGC	0.031 ± 0.009		
CTTAC	0.003 ± 0.004	C_T_C	0.141 ± 0.015
CGTAC	0.139 ± 0.015		
CGCGC	0.018 ± 0.007	C_C_C	0.016 ± 0.007
CGTAG	0.009 ± 0.005	C_T_G	0.009 ± 0.005
Whites			
GTTAC	0.579 ± 0.013	G_T_C	0.834 ± 0.010
GGTAC	0.259 ± 0.012		
GGTGC	–	–	
GTTGC	–	–	
GGTAG	0.003 ± 0.003	G_T_G	0.053 ± 0.006
GTTAG	0.050 ± 0.006		
GGCAC	0.001 ± 0.003	G_C_C	0.016 ± 0.003
GGCGC	0.009 ± 0.003		
GTCGC	0.006 ± 0.003		
CTTAC	0.002 ± 0.001	C_T_C	0.038 ± 0.005
CGTAC	0.036 ± 0.005		
CGCGC	0.054 ± 0.006	C_C_C	0.057 ± 0.006
CGTAG	0.002 ± 0.002	C_T_G	0.002 ± 0.002

Haplotype locus order: (1) APOC3 *Sst*I G/C; (2) APOC3 −2854 G/T; (3) APOA5 −1131 T/C; (4) APOA5 −3 A/G; (5) APOA5 +56 C/G.

+56 G allele. Eight haplotypes had estimated frequencies of more than 1% in African Americans, compared with 5 in whites (Table 3).

Triglyceride levels differed significantly between African Americans and whites within 5-year age groups, with levels tending to be higher in whites (Table 4). Of the individual loci, only the APOC3 −2854 G/T polymorphism (data not shown) showed no evidence of significant effects on triglyceride levels in the full sample. Of the remaining 4 loci, all but the APOA5 +56 C/G polymorphism showed significant race-by-genotype interactions affecting overall triglyceride levels, whereas the APOA5 +56 C/G polymorphism alone showed a significant (P = .02) BMI-by-genotype interaction (Table 5); the similar interaction coefficients for the first 3 polymorphisms may largely reflect the high linkage disequilibrium among them in whites. The lack of a significant race-by-APOA5 +56 C/G interaction may be due in part to the very small number of African Americans (n = 22) who carried the G allele; for no other polymorphism was the rare allele found in fewer than 70 individuals in either group (Table 2). None of the polymorphisms showed significant interactions with age, age^2, or age^3 terms.

Multilevel analyses showed significant effects of 5-locus haplotypes on triglyceride levels. The best-fitting model included main effects for race, sex, BMI, age, age^2, age^3, and haplotypes, and interactions of race with sex, race with BMI, sex with BMI, sex with age, BMI with age, sex with age^2, and BMI with age^2. Removing all haplotype effects from the model significantly reduced its fit (χ^2_{11} = 62.9, P < .00001), but there were no significant interactions involving haplotypes. This may be due in part to the fact that tests of haplotype interactions involved multiple degrees of freedom—up to 11—for 5-locus haplotypes.

Most of the haplotype effects were attributable to 3 haplotypes associated with higher triglyceride levels even after a Bonferroni adjustment for the number of haplotypes tested (Fig. 1). Haplotypes GGTAG (P = .002) and GTTAG (P < .0001) had relatively similar effects and were associated with higher triglyceride levels than CGCGC (P = .0002). Based on the model coefficients, each GGTAG haplotype carried would be expected to raise triglyceride levels above those in GTTAC homozygotes by approximately 19 mg/dL, each GTTAG haplotype by approximately 15 mg/dL, and each CGCGC haplotype by approximately 7 mg/dL.

To determine whether a subset of the 5 loci could account for the observed haplotype associations, we analyzed both multilocus genotypes and haplotypes, using different combinations of the 5 loci. Genotype models derived either from all 5 loci or all possible 4-locus combinations strongly implicated only 3 loci (APOC3 *Sst*I, APOA5 −1131 T/C, and APOA5 +56 C/G) as contributing to variation in serum triglyceride levels. After all nonsignificant terms were removed, all 4- and 5-locus combinations that included these 3 loci converged on the same model, which included a significant interaction between BMI and APOA5 +56 C/G, as well as significant interlocus interactions between APOC3 *Sst*I and APOA5 +56 C/G and between APOA5 −1131 T/C and APOA5 +56 G/C (Table 6).

Neither the APOC3 −2854 G/T nor the APOA5 −3 A/G loci were significant when the remaining 3 loci were included

Table 4
Serum triglycerides (in mg/dL) by race, within 5-year age groups

Age (y)	Race	Sex (M/F)	Mean	SD
4-8	African American	87/156	58.2	23.5***
	White	179/323	65.7	28.9
9-13	African American	195/322	61.9	27.4***
	White	402/632	72.6	34.2
14-18	African American	196/301	65.7	29.6***
	White	445/607	77.1	34.3
19-23	African American	102/175	77.3	44.1***
	White	250/430	95.7	51.8
24-28	African American	81/147	86.3	64.7***
	White	250/379	107.5	67.0
29-33	African American	57/81	108.2	93.1*
	White	156/183	120.8	104.0
34-38	African American	34/44	103.7	73.4**
	White	99/130	135.9	115.3

* P < .05.
** P < .01.
*** P < .001.

Table 5
Model coefficients (±SE) for polymorphisms significantly associated with serum $\log_e$ triglyceride levels, taken from the best-fitting model for each polymorphism

Locus	Genotypes	Genotype effect	Genotype interactions with	
			Race	BMI
APOC3 *Sst*I	C/C + C/G	0.0767 ± 0.0258	−0.1095 ± 0.0422	NS
APOA5 −1131	C/C + C/T	0.0889 ± 0.0295	−0.1305 ± 0.0477	NS
APOA5 −3	G/G + A/G	0.0901 ± 0.0304	−0.1160 ± 0.0464	NS
APOA5 +56	G/G + C/G	0.1370 ± 0.0300	NS	0.0082 ± 0.0036

NS indicates not significant.

in a model. We therefore examined 3-locus haplotypes comprising the APOC3 *Sst*I, APOA5 −1131 T/C, and APOA5 +56 C/G loci. The correspondence between specific 3- and 5-locus haplotypes is shown in Table 4. Five 3-locus haplotypes (C_C_C, C_T_C, G_C_C, G_T_G, and G_T_C, with blanks indicating the omitted APOC3 −2854 G/T and the APOA5 −3 A/G loci, respectively) had frequencies high enough for analysis. The best-fitting model for 3-locus haplotypes included the same interactions found in the 5-locus haplotype model. Relative to haplotype G_T_C, haplotypes C_C_C and G_T_G had significant triglyceride-raising effects, even after adjustment for multiple comparisons ($P < .002$ for both), with the model coefficients showing a stronger effect for G_T_G (0.428 ± 0.061) than C_C_C (0.217 ± 0.060). The correspondence of the predicted results for the 3 individually significant 5-locus haplotypes (GTTAG, GGTAG, CGCGC) and the 2 individually significant 3-locus haplotypes (G_T_G and C_C_C) is shown in Fig. 1 for the largest group of subjects, white females.

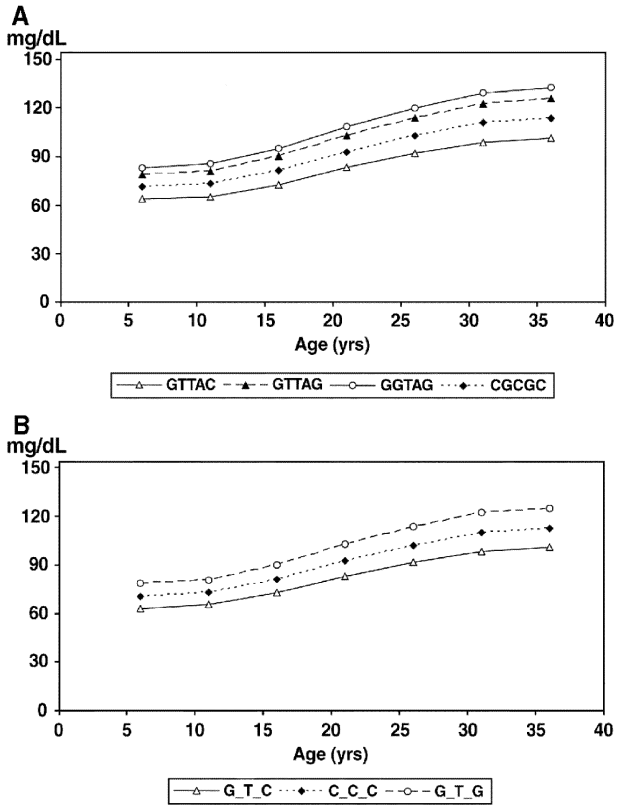

Fig. 1. Predicted triglyceride profiles by APOC3/APOA5 haplotype. Results for white females are shown. Profiles for the reference haplotypes (GTTAC or G_T_C) are for homozygous individuals; other profiles are for individuals carrying one copy of the indicated haplotype and one of the reference haplotype. A, Five-locus haplotypes comprising the APOC3 *Sst*I, APOC3 −2854 G/T, APOA5 −1131 T/C, APOA5 −3 A/G, and APOA5 +56 C/G polymorphisms. B, Three-locus haplotypes comprising the APOC3 *Sst*I, APOA5 −1131 T/C, and APOA5 +56 C/G polymorphisms. Only profiles for the reference haplotype and those significantly different from it are shown.

Although 3 of the loci showed significant race-by-genotype interactions when analyzed individually, we found no significant race-by-haplotype interactions. However, because statistical power to detect haplotype interactions is likely to be low, we also tested separate haplotype models for African Americans and whites. In African Americans, only 3-locus APOC3 *Sst*I/APOA5 −1131/APOA5 +56 haplotypes showed significant effects ($P = .01$), due almost entirely to one haplotype, G_T_G. In whites, both the 5-locus haplotypes, GTTAG and CGCGC, and the corresponding 3-locus haplotypes, G_T_G and C_C_C, contributed to significant haplotype associations overall

Table 6
Coefficients and SEs from the best model for $\log_e$ triglycerides

Variable	Genotype group	Model coefficient	SE
Intercept		4.1496	0.01557***
Race		−0.1360	0.02047***
Sex		−0.07275	0.02034***
BMI		0.03665	0.00328***
Age		−0.00951	0.00333**
Age^2		0.00204	0.00033***
Age^3		−0.00006	0.00001***
Race × BMI		−0.01479	0.00219***
Sex × BMI		0.01141	0.00312***
BMI × age		−0.00130	0.00042**
BMI × age^2		0.00006	0.00002***
Sex × age		−0.00298	0.00401
Sex × age^2		0.00034	0.00014*
APOC3 *Sst*I	C/C + C/G	0.04069	0.02329
APOA5 −1131	C/C + T/C	0.02195	0.02580
APOA5 C56G	G/G + G/C	0.1654	0.03358***
BMI × C56G		0.00905	0.00371*
*Sst*I × C56G		−0.2186	0.07731**
−1131 × C56G		0.3576	0.1181**

Starting model included all 5 APOC3 and APOA5 polymorphisms.
* $P < .05$.
** $P < .005$.
*** $P < .0005$.

($P < .0001$ for either 5- or 3-locus haplotypes). Haplotype GGTAG was significantly associated with triglycerides only when both African Americans and whites were included in the model.

4. Discussion

Using analytical methods that allowed us to use data from 6443 examinations of 1183 subjects who participated in the Bogalusa Heart Study multiple times over a 23-year period, we found both haplotypes and individual genotypes involving 5 loci in the APOC3/APOA5 gene cluster to be associated with interindividual variation in serum triglyceride levels. Our haplotype analyses may be the first to combine longitudinal models, which account for correlations among serial measurements, with the use of conditional haplotype probabilities of individuals as predictors, which avoids the loss of data and potential errors inherent in methods that assign probable haplotypes to individuals. We were thus able to use all the available data to enhance the power of our study. That our results tend to confirm some of those reported for APOA5 haplotypes in cross-sectional studies supports the validity of our approach, although we found no evidence in our longitudinal analyses that any of the associations change with age.

Because the APOA1/C3/A4/A5 cluster occupies a restricted chromosomal region and involves a number of genes with related functions, phenotypic effects that appear to be due to mutations in one member of the cluster might actually be due to allelic associations with functional variants in another member. In particular, it has been difficult to determine whether polymorphic variants in the APOC3 and APOA5 genes have independent effects on triglycerides, although evidence for this has been reported [2]. In our analyses, however, the effects of the APOC3 and APOA5 variants on serum triglycerides were not independent: the 5-locus haplotypes significantly associated with higher triglycerides included variants in both genes. Attempts to separate the effects of loci in the 2 genes by analyzing genotypes, rather than haplotypes, implicated only 3 of the 5 loci (APOC3 *Sst*I, APOA5 −1131 T/C, and APOA5 +56 C/G), but these included loci in both genes, with significant interactions among them. Haplotype analyses using various subsets of the 5 loci also implicated the same 3 loci as the genotype analyses.

All 3 of the 5-locus haplotypes significantly associated with higher serum triglyceride levels in our analyses shared APOA5 alleles with haplotypes previously associated with higher triglycerides [6,9]. Haplotypes GG**TAG** and GT**TAG** shared 3 alleles (T, A, and G at the APOA5 −1131, −3, and +56 sites, respectively, denoted in boldface type) with the APOA5*3 haplotype of Pennacchio et al [6], whereas haplotype CG**CGC** shared alleles at these same sites with the APOA5*2 haplotype. In our analyses, however, the −3 A/G polymorphism was superfluous when the −1131 T/C and +56 C/G polymorphisms were measured: when both −1131 T/C and −3 A/G genotypes were included in models for genotype effects, only the former was significant. The −1131 T/C and −3 A/G polymorphisms were in virtually complete linkage disequilibrium in our sample, with D' values of 1.00 for whites and 0.98 for African Americans. It has been suggested that the −3 A/G polymorphism, which occurs in the Kozak sequence preceding the initiation codon, is more likely than the −1131 T/C polymorphism to be functional [4], but our results provide no evidence for this.

To further complicate attempts to separate APOC3 and APOA5 effects, the haplotypes corresponding to APOA5*2 and APOA5*3 were associated with specific APOC3 *Sst*I alleles. Almost all haplotypes having the APOA5*3 motif also carried the *Sst*I G allele and were associated with higher triglycerides. Of the haplotypes that carried the APOA5*2 motif, the only one significantly associated with higher triglycerides, haplotype CGCGC, carried the *Sst*I C allele. Strong associations of haplotype APOA5*2 with the *Sst*I C allele, and of APOA5*3 with the *Sst*I G allele, have been reported by others. Because both APOA5*2 and APOA5*3 were associated with higher triglycerides, but different *Sst*I alleles, it was suggested that the haplotype effects were independent of the *Sst*I locus [24]. However, the overall evidence from our study and those of others seems insufficient to establish whether haplotypes APOA5*2 and APOA5*3 would have the same effects if coupled with different APOC3 alleles. In our sample, the only haplotype with both the APOA*3 motif and the *Sst*I C allele, CGTAG, was too rare in both African Americans and whites for tests of phenotypic associations. In whites, 2 haplotypes combining the APOA5*2 motif with the *Sst*I G allele, GGCGC and GTCGC, occurred at such low frequencies (0.009 and 0.006, respectively) that no phenotypic associations with either were detectable. Although their frequencies were much higher in African Americans (0.046 and 0.031, respectively), neither those two nor haplotype CGCGC (with the *Sst*I C allele on an APOA5*2 background) were significantly associated with higher triglycerides in this group. Even if APOC3 variants can modulate the effects of the APOA5*2 and APOA5*3 haplotypes, it seems doubtful that the *Sst*I locus itself would be responsible, as it occurs in the 3′ -untranslated region [12]. Recent in vitro studies have suggested that the apparent effects of the APOA5*3 haplotype may be due at least in part to linkage disequilibrium with functional APOC3 single nucleotide polymorphisms [25], even if the *Sst*I locus is unlikely to be involved.

It has been reported that APOA5 and APOC3 haplotype blocks are separated by a region of increased recombination, such that no haplotype blocks exist that include both genes [24]. In our sample, linkage disequilibrium between APOC3 and APOA5 polymorphisms was much lower in African Americans than in whites: the highest $|D'|$ value between any pair of APOC3 and APOA5 loci in African Americans was 0.324, whereas the lowest of such value in whites was 0.742. Our results in African Americans are

consistent with the absence of haplotype blocks encompassing both genes, although it seems possible that such blocks could exist in whites. However, significant pairwise linkage disequilibrium between distant loci in the APOA1/C3/A4/A5 cluster (eg, between variants in APOA1 and APOA5) has been reported even where there was evidence of increased recombination between APOA5 and the other genes in the cluster [26].

Our analyses indicated that just 3 of the 5 loci measured were sufficient to account for the associations of specific haplotypes and multilocus genotypes with higher serum triglycerides. In the present case, both haplotype and genotype analyses converged on the same set of polymorphisms, although there can sometimes be instances in which analyzing haplotypes reveals associations that analyzing genotypes would miss [27]. By using each individual's full haplotype probability vector in the multilevel models, we avoided potential errors in assigning haplotypes to individuals [23,28], allowing us to make full use of the available information. However, as the number of loci involved increases, the number of degrees of freedom needed for global tests of haplotype effects may reduce statistical power, possibly obscuring the effects of specific haplotypes. Further work is needed to establish the conditions in which haplotype analysis will be most useful.

Neither our haplotype nor genotype analyses indicated that the genetic associations changed as subjects aged. This is consistent with most of the limited evidence from other studies of APOC3 or APOA5 in different age groups. In Japanese schoolchildren 9 to 13 years of age, the −1131 C allele was associated with higher triglycerides [29]. This is consistent with findings in adults in a number of populations [1-5,7-9,30], suggesting that the effects of this variant are similar in children and adults. In a study of Hispanic and non-Hispanic white nuclear families, a significant association between triglyceride levels and the APOC3 −455 T/C polymorphism was found only in adults, although in analyses combining generations, there were no significant interactions between parent/child status and genotype [31]. A study of APOA1/C3/A4 polymorphisms in Brazilian children 5 to 15 years of age found no variants associated with triglyceride levels, but the polymorphisms differed from those in the present study [32].

Few studies have examined the relationship between APOA5 variants and indices of body size or obesity, such as BMI. Homozygosity for the rare allele of a polymorphism in the region between the APOA4 and APOA5 genes (−12238 T/C) has been associated with higher waist-hip ratio in European men [4]. In a Han Chinese sample, homozygosity for the −1131 C allele was associated with higher BMI [30]. However, until more is learned about the biologic function of apoA-V, it will be difficult to determine the nature of the relationship between APOA5 variation and body fat composition. The interaction we observed between the +56 C/G polymorphism and BMI would result in a greater triglyceride-raising effect of the G allele at higher BMI levels. It has been suggested that APOA5 reduces plasma triglycerides by targeting VLDL particles to proteoglycan-bound LPL for lipolysis [33], although plasma levels of apoA-V are so low that few VLDL particles could contain it [34]. Several studies have suggested that apoA-V may interact with LPL to affect lipolysis [35,36]. It seems possible that excess adipose tissue could change the balance between APOA5 levels and adipocyte-bound LPL, reducing lipolysis and raising plasma triglycerides. That APOA5 levels may be affected by adipose tissue amount is suggested by studies showing that APOA5 is regulated by the peroxisome proliferator-activated receptor α [37,38], which may play a role in the control of body weight [39].

Overall, our findings on the relationship between variation in the APOA1/C3/A4/A5 gene cluster and serum triglyceride levels support those reported by others and provide more direct evidence than previously available that the association between variants in this gene cluster and triglycerides do not change with age. However, the degree to which phenotypic associations involving polymorphisms in the APOC3 and APOA5 genes can be considered independent remains to be determined.

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