

# Association analysis of 33 lipoprotein candidate genes in multi-generational families of African ancestry<sup>[S]</sup>

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**Abstract** African ancestry individuals have a more favorable lipoprotein profile than Caucasians, although the mechanisms for these differences remain unclear. We measured fasting serum lipoproteins and genotyped 768 tagging or potentially functional single nucleotide polymorphisms (SNPs) across 33 candidate gene regions in 401 Afro-Caribbeans older than 18 years belonging to 7 multi-generational pedigrees (mean family size 51, range 21–113, 3,426 relative pairs). All lipoproteins were significantly heritable ( $P < 0.05$ ). Gender-specific analysis showed that heritability for triglycerides was much higher ( $P < 0.01$ ) in women than in men (women,  $0.62 \pm 0.18$ ,  $P < 0.01$ ; men,  $0.13 \pm 0.17$ ,  $P > 0.10$ ), but the heritability for LDL cholesterol (LDL-C) was higher ( $P < 0.05$ ) in men than in women (men,  $0.79 \pm 0.21$ ,  $P < 0.01$ ; women,  $0.39 \pm 0.12$ ,  $P < 0.01$ ). The top 14 SNPs that passed the false discovery rate threshold in the families were then tested for replication in an independent population-based sample of 1,750 Afro-Caribbean men aged 40+ years. Our results revealed significant associations for three SNPs in two genes (rs5929 and rs6511720 in *LDLR* and rs7517090 in *PCSK9*) and LDL-C in both the family study and in the replication study. Our findings suggest that *LDLR* and *PCSK9* variants may contribute to a variation in LDL-C among African ancestry individuals. **■**

**Future sequencing and functional studies of these loci may advance our understanding of genetic factors contributing to LDL-C in African ancestry populations.**—Miljkovic, I., L. M. Yerges-Armstrong, L. H. Kuller, A. L. Kuipers, X. Wang, C. M. Kammerer, C. S. Nestlerode, C. H. Bunker, A. L. Patrick, V. W. Wheeler, R. W. Evans, and J. M. Zmuda. Association analysis of 33 lipoprotein candidate genes in multi-generational families of African ancestry. *J. Lipid Res.* 2010. 51: 1823–1831.

Lipoprotein abnormalities, characterized by elevated levels of LDL cholesterol (LDL-C) and triglycerides (TRIG) and low levels of HDL cholesterol (HDL-C), have a central role in the development of atherosclerotic coronary heart disease (CHD). A recent meta-analysis, including 3,000 individuals with CHD-related deaths, showed that HDL-C and LDL-C are independently associated with CHD risk (1). There is also considerable evidence that high levels of TRIG are an additional, independent risk factor for CHD (2, 3), although this is still controversial (4).

Individuals of African ancestry have a more favorable lipoprotein profile than Caucasians, characterized by lower levels of TRIG and higher levels of HDL-C (5–8). The mechanisms responsible for these ethnic differences remain to be defined. In particular, the differences in TRIG levels are independent of the greater degree of obesity among individuals of African ancestry and several other risk factors and appear to be consistent across African populations in different environments (9), indicating a possible role of genetic factors. Although genetic factors are important in determining lipoprotein levels, little data

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Abbreviations: BMI, body mass index; CHD, coronary heart disease; FDR, false discovery rate; HDL-C, HDL cholesterol; h2r, residual heritability; LDL-C, LDL cholesterol; MAF, minor-allele frequency; SNP, single nucleotide polymorphism; TRIG, triglyceride.

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exists regarding the importance of heredity and specific genetic factors in determining lipoprotein levels in populations of African ancestry, especially outside the US, and the findings from previous studies in African-Americans may not necessarily apply to other African ancestry populations. Recently, several genome-wide association studies identified a number of loci contributing to inter-individual variation in lipoprotein levels (10, 11). However, the majority of these studies were restricted to Caucasian populations. Given the ethnic differences in lifestyle and environmental factors, as well as in genetic background, it is important to examine genes related to lipoprotein metabolism in different ethnic groups. Therefore, we examined the heritability of fasting, serum levels of HDL-C, LDL-C, and TRIG and systematically screened for association with 33 positional and biological candidate genes in large, multigenerational families of African ancestry.

## METHODS

### The Tobago Family Health Study sample

The Tobago Family Health Study was designed to better understand the role of inheritance, lifestyle, and body weight and composition in the etiology of several common chronic diseases in a population of African ancestry. The population on the Caribbean island of Tobago was largely settled in the late 1700s during the transatlantic slave trade. The first official British records in 1770 enumerated 268 Whites and 3,110 African slaves on the island. There were 15,470 African slaves on the island in 1819 (12). The British occupied Tobago permanently in 1814 and slavery was officially abolished in 1833 (12). The most recent census indicates that there are approximately 54,000 inhabitants on the island. According to the 1990 census data based on self-report, the population of Tobago was 92% African descent, 4.5% mixed, 2% East Indian, 0.4% White, and 1% other (13). We confirmed with molecular markers that the Afro-Caribbean population of Tobago has a low level of non-African admixture (6%) (14) compared with the more genetically heterogeneous African-American population that has much higher degree of non-African admixture (17–23.9%) (15–17).

Probands for the Tobago Family Health Study were identified from an ongoing population-based prostate cancer screening study (18). To be eligible, a proband had to be Afro-Caribbean, have had a spouse who was willing to participate in the study, and have at least six living offspring and/or siblings aged 18+ years who were residing in Tobago. Because we were interested in establishing a community-based sample of families, probands and their family members were recruited without regard to their health status. To date, 401 individuals aged 18–103 years (mean age 43 yrs) belonging to 7 multigenerational families (mean family size 51 individuals) of West African ancestry have been recruited. Among the families, we have the following relationships: 361 parent-offspring, 495 full siblings, 101 grandparent-grandchildren, 1,137 avuncular, 61 half-sibs, and 1,380 cousins (3,535 relative pairs). Written informed consent was obtained from every participant using forms and procedures approved by the Tobago Division of Health and Social Services and University of Pittsburgh Institutional Review Boards.

### Replication sample

Using an independent population-based cohort of 1,750 Afro-Caribbean men who live in the same geographic region as the family study, we attempted to replicate the associations with

single nucleotide polymorphisms (SNPs) that passed the false discovery rate (FDR) with  $\alpha = 0.2$ . The replication sample was a randomly selected subset of a larger study of body composition among 2,500 men aged 40 and older (mean age 59 years) on the island of Tobago (19), with the comprehensive data on anthropometric measures, demographic information, medical history, lifestyle information, and fasting lipoproteins. Men were recruited by word of mouth, via health care workers at the hospital, health centers, or private physicians as well as local advertising by poster, flyers, and public service announcements. Written informed consent was obtained using forms approved by the Institutional Review Boards of the University of Pittsburgh and the Tobago Ministry of Health.

### Data collection

Information on lifestyle habits [current smoking (yes/no), current alcohol intake (more than one drink per week, yes/no), walking (minutes per week), and TV viewing (hrs/week)], medical conditions, medication use, and reproductive characteristics in women (age at menarche, menopause, parity, oral contraceptive use) were assessed using standardized interviewer-administered questionnaires that were reviewed with participants in the study clinic. We recorded information on walking, because it is the predominant physical activity form on the island. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Weight was recorded to the nearest 0.1 kg without shoes on a balance-beam scale.

### Clinical and metabolic variables

All biochemical assays were performed in the Heinz Nutrition Laboratory at the University of Pittsburgh's Graduate School of Public Health, which has met the accuracy and precision standards of the Centers for Disease Control and Prevention and is CLIA accredited. Serum was prepared after morning, fasting phlebotomy and stored at  $-70^{\circ}\text{C}$  until assay. HDL-C was determined using the selective heparin/manganese chloride precipitation method, interassay CV 2.1% (20). LDL-C was calculated by means of the Friedewald equation. TRIG were determined enzymatically using the procedure of Bucolo and David, interassay CV 1.7% (21).

### Gene and SNP selection

We used a positional and biological candidate gene approach to prioritize genes. A positional candidate gene approach identified genes that lie under linkage peaks for lipid traits based on four studies that performed genome wide scans to identify loci that may affect lipoprotein metabolism in African ancestry populations: Hypertension Genetic Epidemiology Network (22), HERITAGE Family Study (23, 24), African-American Diabetes Mellitus Study (25), and Genetics of NIDDM Study (26). The strongest linkage signals reported were: 5q33 for LDL-C (25), 19p13 and 19q13 for TRIG (24, 26), 1q25 and 7p21 for HDL-C (25), and 21q22 for LDL-C, HDL-C, and TRIG (22). The genes included: 19q13.2 Cluster (*ApoE*, *ApoC1*, *ApoC2*, and *ApoC4*), *ABCG1* (ATP-binding Cassette, Subfamily G, Member 1), *GYS1* (glycogen synthase 1), *HSD11B1* [hydroxysteroid (11- $\beta$ ) dehydrogenase 1], *LDLR* (Low-density Lipoprotein Receptor), *LXR $\beta$*  (liver X receptor  $\beta$ ), *RETN* (resistin), *SOAT1* (sterol O-acyltransferase 1), and *USF2* (upstream stimulatory factor 2).

A biological candidate gene approach included known physiologically defined genes for lipid metabolism that were selected on the basis of published evidence. Many of these genes have not been previously studied in African ancestry populations. Some genes were identified from animal studies and have not been

investigated comprehensively in humans. The biological candidate genes included: *11q23.3* Cluster (*ApoA1*, *ApoA4*, *ApoA5*, and *ApoC3*), *ACDC* (Adiponectin), *AdipoR1* (Adiponectin Receptor 1), *AdipoR2* (Adiponectin Receptor 2), *ApoA2* (Apolipoprotein A2), *ApoB* (Apolipoprotein B), *LCAT* (Lecithin-cholesterol Acyltransferase), *DGAT2* (Acyl-CoA:diacylglycerol acyltransferase 2), *FABP4* (fatty acid binding protein 4), *FOXC2* (forkhead transcription factor C2), *GTF3A* (general transcription factor IIIa), *HNF4A* (Hepatic nuclear factor 4  $\alpha$ ), *LIPE* (hormone-sensitive lipase), *LPIN1* (lipin), *LIPG* (Endothelial Lipase), *LPL* (Lipoprotein Lipase), *MTP* (microsomal triglyceride transfer protein), *PBEF1* (Visfatin), *PCSK9* (Proprotein Convertase Subtilisin Kexin 9), *PLIN* (perilipin), *PPARA* (Peroxisome Proliferator-activated Receptor Alpha), *PPARG* (Peroxisome Proliferator-activated Receptor Gamma), *SCARB1* (Scavenger Receptor Class B Type I), and *SREBF2* (Sterol regulatory element binding transcription factor 2).

A reference panel of SNPs that spanned 5 kb upstream of the transcriptional start site and 5 kb downstream of the 3' region of each gene or gene cluster was identified from the International HapMap database ([www.hapmap.org](http://www.hapmap.org)) (Phase II, Yoruba population of Ibadan, Nigeria). We used the program HClust to select tag SNPs with a minor-allele frequency (MAF)  $\geq 5\%$  that predicted the remaining SNPs with an  $r^2 \geq 0.8$  (27). In addition, potentially functional SNPs that were either nonsynonymous coding variants, predicted to alter a putative transcription factor binding site in the promoter region, or a putative exon splice enhancer with MAF  $\geq 5\%$  in an African reference population were selected for genotyping using the dbSNP database (Build 125) and the PupaSNP (28).

### Genotyping in the Tobago Family Health Study

Genotyping of 768 SNPs was performed on genomic DNA using the Illumina GoldenGate Custom assay system. Eight blind duplicates were run and a 100% reproducibility rate was observed. Of the 768 SNPs attempted, 91 SNPs were dropped before statistical analysis, because genotypes were not able to be determined ( $N = 39$ ), they were monomorphic or had a MAF  $< 1\%$  ( $N = 48$ ), or they did not conform to the expectations of Hardy-Weinberg equilibrium in unrelated individuals ( $N = 4$ ,  $P < 0.005$ ). The 677 remaining SNPs were used in statistical analysis.

### Genotyping of SNPs for replication in the Tobago population-based study of men

Fourteen SNPs that passed the FDR (rs5929, rs7517090, rs8030806, rs389261, rs6511720, rs3746575, rs11608456, rs11890442, rs2569540, rs11574739, rs1028583, rs3212198, rs5927, and rs6073435) were genotyped using genomic DNA with the fluorogenic 5'-nuclease TaqMan allelic discrimination assay system (Applied Biosystems, Foster City, CA). The assays were performed under standard conditions on a 7900HT real-time PCR instrument with probes and reagents purchased from Applied Biosystems. The best proxy SNP (rs6031593) was used in place of rs3746575 in the population study ( $D' = 0.92$ ,  $r^2 = 0.72$  HapMap Database, phase II release 24) due to rs3746575 assay failure. rs389261 (in the *19q13.2* Cluster) could not be genotyped by TaqMan, and the TaqMan Genotyping Assay for rs11608456 (in the *AdipoR2*) was not able to be manufactured (no proxies were identified for this SNP; the highest  $r^2$  was 0.61, HapMap Database, phase II release 24). All successfully genotyped SNPs conformed to the expectations of Hardy-Weinberg equilibrium ( $\chi^2$  test of allele frequencies:  $P > 0.01$ ). The average genotyping completeness rate was 95.8% and the average genotyping consensus rate among the  $> 5\%$  blind replicate samples was 99.2%.

### Statistical analyses

First, the distributions of all the traits were assessed for non-normality, and data were transformed before statistical analysis to reduce nonnormality. Subsequently, all outliers ( $\pm 3.5$  SD) were removed for each trait, and no more than four values were removed for a single variable. We calculated site-specific allele frequencies by gene counting and tested for departures from Hardy-Weinberg equilibrium using a goodness-of-fit statistic. Pairwise estimates of linkage disequilibrium were measured as  $D'$  and  $r^2$  from the diploid data (29). Quantitative genetic methods were used to model the total variation in all phenotypic parameters as a function of the mean trait value [additive genetic effects, heritability residual ( $h^2r$ )], effects attributed by the measured covariates, and the uncertain variation due to residual genetic and unmeasured environmental impact plus random errors. We also tested whether the heritabilities in phenotypes differed between men and women (i.e.,  $h^2r$  men =  $h^2r$  women) by comparing the difference between the heritability estimates in the two sexes with the estimated variance of the difference. Briefly, we expanded the basic variance component model to allow the genetic variances in male and female to differ (30). Then we use the likelihood ratio test to compare the basic versus the expanded model. All analyses were performed using the SOLAR software package (Solar, version 2.1.4; Southwest Foundation for Biomedical Research, San Antonio, TX) (31) under the variance components analytical framework, which accounts for the nonindependence among family members. Covariates of probable importance for lipoprotein metabolism, including age, gender, body mass index (BMI), current smoking, current alcohol intake, minutes walking per week, postmenopausal status, parity, age at menarche, and oral contraceptive use, were included in all models. Based on participants' self-report, none of the participants reported using lipid-lowering medication. After assessment of covariates, association analyses were performed to compare mean trait levels by genotype assuming an additive model.

We used the FDR with  $\alpha = 0.2$  to adjust for multiple hypothesis testing. The FDR method controls the expected proportion of false-positives among all positive results over multiple studies (14). The FDR can be regarded as a post hoc maximizing procedure and is more powerful than multiple-comparison procedures based on the family-wise error rate. SNPs with  $P$ -values more significant than the expected FDR distribution with  $\alpha = 0.2$  were considered significant.

Using an independent population-based sample of African ancestry men from the same geographic region, we attempted to replicate associations of LDL-C and TRIG with 14 SNPs that passed the FDR in the family study. The single SNPs were tested for their association with the lipoproteins assuming an additive model. Linear regression was used to test for association between the number of rare alleles and the levels of lipoproteins. All models were adjusted for age, BMI, minutes walking per week, current smoking, and current alcohol intake. Analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

## RESULTS

### Subject characteristics

Mean age of the family study participants was approximately 43 years and ranged from 18 to 103 years (Table 1). Participants were predominantly women (60.3%). The prevalence of obesity was particularly high among women ( $\sim 43\%$ ). More men than women smoked (11% vs. 1%) and drank alcohol (29% vs. 9%)



TABLE 1. General characteristics of the Afro-Caribbean participants in the Tobago Family Health Study

	Total (N = 471)	Men (N = 187)	Women (N = 284)
Age (years)	42.7 ± 16.8	42.8 ± 16.9	42.6 ± 16.8
Anthropometric			
BMI (kg/m <sup>2</sup> )	28.3 ± 6.4	26.7 ± 4.9	29.4 ± 7.0*
Waist circumference (cm)	89.9 ± 15.4	90.1 ± 12.4	89.7 ± 17.2
Lifestyle			
Alcohol use (>1 drink/week) (%)	13.2	28.9	2.8*
Current smoking (%)	4.9	11.4	0.7*
TV viewing (h/week)	15.9 ± 8.0	16.6 ± 8.5	15.5 ± 7.7
Time spent walking per week (min/week)	47.9 ± 96.7	52.6 ± 72.7	44.8 ± 109.8
Reproductive			
Use of oral contraceptives (%)	N/A	N/A	33.1
Ever pregnant (%)	N/A	N/A	76.7
Number of pregnancies	N/A	N/A	3.5 ± 2.6
Postmenopausal status (%)	N/A	N/A	31.6
Lipoprotein levels			
TRIG (mg/dl)	89 ± 46	92 ± 50	86.2 ± 43.6
HDL-C (mg/dl)	40 ± 13	41 ± 12	39.6 ± 13.2
LDL-C (mg/dl)	133 ± 42	130 ± 40	134 ± 43
Medical conditions			
Obesity <sup>a</sup> (%)	33.5	19.8	42.6*
Diabetes <sup>b</sup> (%)	15.4	12.4	17.2
Hypertension <sup>c</sup> (%)	28.6	30.4	27.4

Values are unadjusted means (SEM). \* *P*-value for gender difference <0.001.

<sup>a</sup> BMI ≥ 30 kg/m<sup>2</sup>.

<sup>b</sup> Fasting glucose of ≥126 mg/dl and/or use of insulin/hypoglycemic medications.

<sup>c</sup> Systolic blood pressure of ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg or currently taking antihypertensive medications.

on a regular basis. Approximately one-third of women were postmenopausal and one-third used oral contraceptives. BMI was significantly greater in women, but waist circumference was similar in men and women. The mean levels of TRIG, HDL-C, and LDL-C were 88.6 ± 46.4 mg/dl, 40.3 ± 12.8 mg/dl and 132.4 ± 42.4 mg/dl, respectively, and all lipoprotein levels were similar in men and women (Table 1).

### Heritability analyses

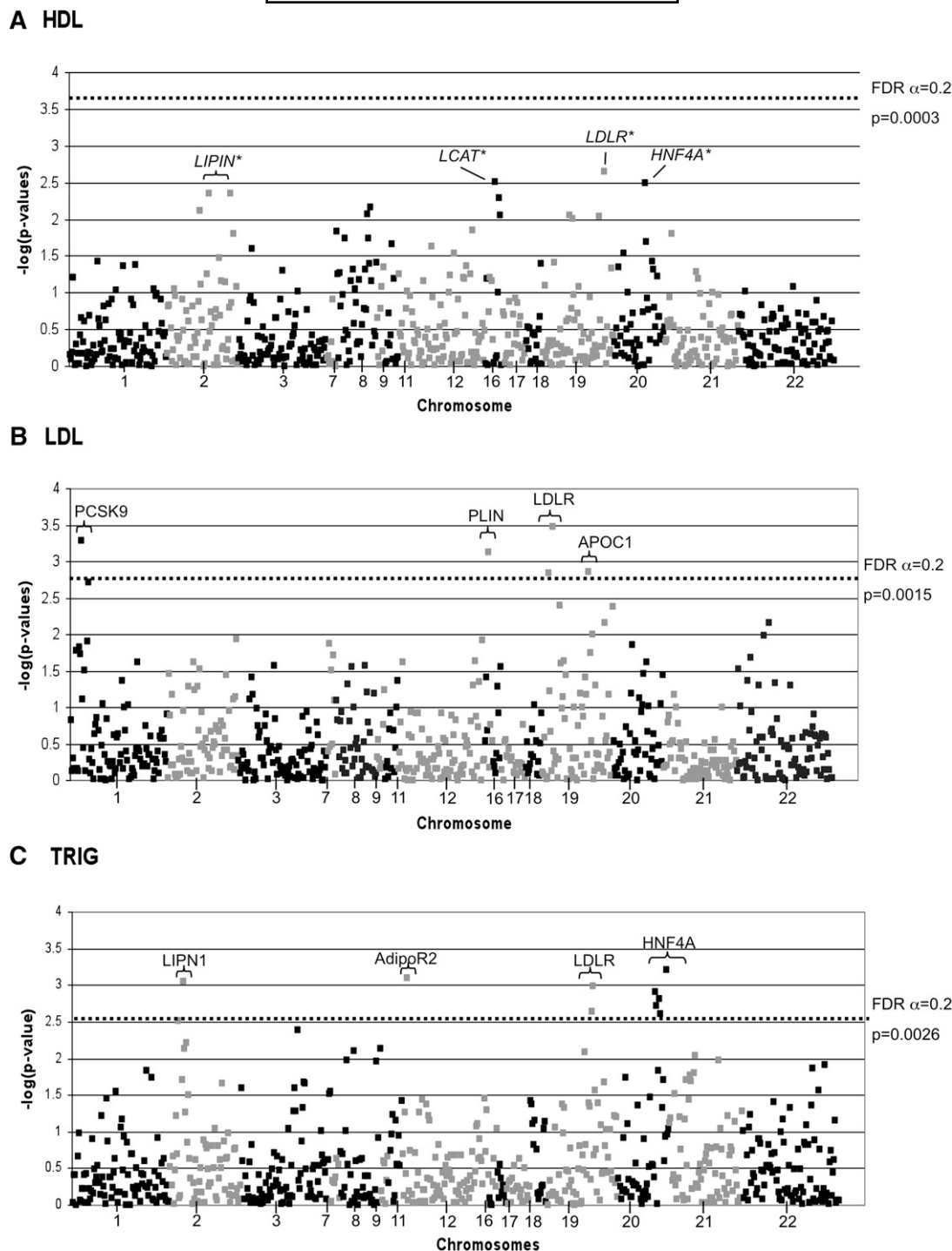
h<sup>2</sup>r, the proportion of variance due to additive genetic effects, was estimated after removing the variation attributable to significant covariates. All lipid and lipoproteins were significantly heritable (h<sup>2</sup>r ± SE; TRIG, 0.28 ± 0.11; HDL-C, 0.48 ± 0.11; and LDL-C, 0.44 ± 0.11; *P* < 0.05 for all). Significant covariates accounted for 24% of the total phenotypic variation for TRIG (age, gender, BMI, menopause, parity, and current oral contraceptive medication intake were significant covariates), 6% of the total phenotypic variation for HDL-C (BMI and current alcohol intake were significant covariates), and 18% of the total phenotypic variation for LDL-C (age, BMI, and menopause were significant covariates).

Gender-stratified analysis showed that the residual heritability for TRIG was much higher (*P* < 0.01) in women than in men (women, 0.62 ± 0.18, *P* < 0.01; men, 0.13 ± 0.17, *P* > 0.10). In contrast, the residual heritability for LDL-C was higher (*P* < 0.05) in men than in women (men, 0.79 ± 0.21, *P* < 0.01; women, 0.39 ± 0.12, *P* < 0.01). Heritability of HDL-C tended to be higher in men but the difference was not significantly different (*P* = 0.20) (men, 0.51 ± 0.18, *P* < 0.01; women, 0.33 ± 0.14, *P* < 0.01).

### Family-based association analyses

No SNPs passed the FDR cutoff of 0.2 for associations with HDL-C in the families (Fig. 1A). However, SNPs in the *LDLR*, *LCAT*, *HNF4A*, and *LIPN1* showed the most suggestive nominal associations with HDL-C (supplementary Table I and Fig. 1A). Using an FDR cutoff of 0.20, five SNPs in four genes (*LDLR*, *PCSK9*, *PLIN*, and *APOC1*) passed the *P*-value threshold (*P* = 0.0015) for associations with LDL-C levels, and nine SNPs in four genes (*HNF4A*, *ADIPOR2*, *LIPN1*, and *LDLR*) passed the *P*-value threshold (*P* = 0.0026) for associations with TRIG levels in the families, after adjustment for potential covariates (Supplementary Tables II and III; Fig. 1B, C).

Minor alleles of two SNPs (rs5929 and rs6511720) in *LDLR* were associated with lower LDL-C in the families (Table 2). The minor allele of *PCSK9* rs7517090 was also associated with lower LDL-C (Table 2). On the other hand, minor alleles of *APOC1* rs389261 and *PLIN* rs8030806 were associated with greater LDL-C (Table 2). Three additional SNPs in both *LDLR* and 19q cluster and seven additional SNPs in *PCSK9* showed nominal associations with LDL-C (*P* < 0.05), but no additional SNPs in *PLIN* showed nominal associations with LDL-C (supplementary Table II). Each of the five SNPs accounted for 1.5–5.2% of the total phenotypic variability in LDL-C (Table 2). The minor allele frequencies for all significant SNPs in the *LDLR*, *PCSK9*, *APOC1*, and *PLIN* were comparable to those reported in African ancestry individuals from the Yoruba population of Ibadan, Nigeria (www.hapmap.org). Interestingly, the *LDLR* rs6511720 has shown the strongest association with HDL-C (nominal *P*-value = 0.0022), but no other SNPs in the *LDLR* gene were associated with HDL-C.



**Fig. 1.** Association results are presented for HDL (A), LDL (B), and TRIG (C). The  $-\log$  of the  $P$ -value is presented on the  $y$  axis and SNPs are presented across the  $x$  axis in order of chromosome position. The dashed lines in B and C represent the  $P$ -value for significant SNPs and gene symbols are presented for those SNPs. No SNPs in HDL were associated beyond the FDR threshold, and the dashed line in A represents the level of significance, which would have been needed. Top HDL findings are indicated in A.

A significant association with TRIG was observed for the five SNPs in *HNF4A*, one SNP in *AdipoR2* and *LPIN1*, and two SNPs in *LDLR* in the families. Nine additional SNPs in *HNF4A*, two additional SNPs in *AdipoR2*, five additional SNPs in *LPIN1*, and six additional SNPs in *LDLR* were nominally significant ( $P < 0.05$ ; supplementary Table III). The minor allele frequencies (MAF)

for all SNPs were comparable to those reported in African ancestry subjects from the Yoruba population of Ibadan, Nigeria ([www.hapmap.org](http://www.hapmap.org)). Each of these 14 SNPs accounted for 3.5–6.7% of the total phenotypic variability in TRIG levels. The major alleles of all significant *HNF4A* SNPs were associated with lower TRIG levels.

TABLE 2. Adjusted mean ( $\pm$  SE) LDL-C levels for SNPs that showed significant associations with LDL-C after adjusting for multiple comparisons

Gene Name	SNP rs no.	Chromosome	Position (bp)*	Study	Adjusted Means $\pm$ SE			Variation in LDL-C Explained by SNP	Additive <i>P</i> value
					1/1	1/2	2/2		
LDLR	rs5929	19	11087800	Family	134 $\pm$ 2.3	124 $\pm$ 4.3	99 $\pm$ 14.7	4.6%	<b>0.00033</b>
				Replication	134 $\pm$ 1.1	127 $\pm$ 2.2	128 $\pm$ 7.6	0.5%	<b>0.004</b>
LDLR	rs6511720	19	11063306	Family	136 $\pm$ 2.2	117 $\pm$ 4.3	93 $\pm$ 13.3	4.8%	<b>0.0014</b>
				Replication	135 $\pm$ 1.1	127 $\pm$ 2.0	124 $\pm$ 6.6	0.77%	<b>0.0002</b>
PCSK9	rs7517090	1	55287544	Family	138 $\pm$ 2.4	120 $\pm$ 3.4	98 $\pm$ 10.6	5.2%	<b>0.0005</b>
				Replication	134 $\pm$ 1.2	132 $\pm$ 1.7	121 $\pm$ 5.0	0.9%	<b>0.05</b>
PLIN	rs8030806	15	88005145	Family	128 $\pm$ 2.4	137 $\pm$ 3.8	168 $\pm$ 12.8	1.5%	<b>0.0007</b>
				Replication	133 $\pm$ 1.1	133 $\pm$ 2.0	118 $\pm$ 7.5	1.1%	0.44
19q13.2	rs389261	19	50112183	Family	124 $\pm$ 2.8	137 $\pm$ 3.1	140 $\pm$ 6.7	3.2%	<b>0.0013</b>
				Replication	ND	ND	ND	ND	ND

1, major allele; 2, minor allele. Bold indicates significant *P*value. ND, Not determined due to assay failure.

\* Chromosomal position was based on the National Center for Biotechnology Information dbSNP, build 36.2.

### Population-based replication analyses

Twelve out of 14 SNPs were successfully genotyped in an independent population-based sample of 1,750 African ancestry men, participants of the large body composition study among 2,500 men from the same geographic region as the family study. Our replication results revealed significant associations for three successfully genotyped SNPs (in *LDLR* and *PCSK9*), which were associated with LDL-C level in families, but not for rs8030806 in *PLIN* (Table 2). However, no association with TRIG levels was replicated for eight successfully genotyped significant SNPs (Table 3).

### DISCUSSION

The current study examined the heritability of lipoprotein phenotypes in a well-characterized collection of extended multi-generational families of West African ancestry on the Caribbean island of Tobago. This relatively isolated and homogeneous population has a low level of non-African ancestry as determined by population ancestry informative molecular markers (32). We found that all lipoprotein traits were significantly heritable, with heritability estimates ranging from 28% to 48%. Significant demographic, lifestyle, reproductive, and medical factors accounted for only 6–24% of the total phenotypic variation in lipoprotein traits. Heritability estimates obtained in our study are in the range of those previously reported in African-Americans [TRIG: 0.14–0.41 (22, 26, 33); LDL-C: 0.39–0.55 (22, 26); HDL-C: 0.38–0.65 (26, 33)]. Our results also suggest that among women, genes may have a much stronger influence on TRIG levels but less strong influence on LDL-C levels than in men. This difference could be related to gender differences in the ability to metabolize fat, interactions with genes on the sex chromosomes, effects related to female reproduction or differences arising from gender-specific hormonal factors, or other environmental exposures, such as diet.

We observed very low levels of TRIG in this Afro-Caribbean population. This finding is consistent with the results from other studies in African ancestry populations (6). On the other hand, we observed lower HDL-C levels than those reported in other studies of African ancestry

populations (6). Lower HDL-C observed in our sample might be due to nutritional factors. Also, obesity is strongly associated with low HDL-C (34). The prevalence of overweight and obesity were high in our families, especially among women, which could be an additional explanation for the observed low HDL-C levels. Excessive adiposity and accumulation of body fat is strongly associated with a low concentration, adverse distribution pattern, and abnormal metabolism of HDL particles (35). However, TRIG levels were not influenced by the high prevalence of obesity as TRIG levels were low even among women.

A second objective of our analysis was to determine the association of lipoprotein variation with tagging SNPs spanning 33 candidate gene regions. We used a pathway-driven approach to select positional and biological candidate genes, the majority of which have not yet been investigated comprehensively in an African ancestry population. We successfully replicated an association between LDL-C and two SNPs in the *LDLR* gene (rs5929 and rs6511720). rs6511720 has also been associated with LDL-C in a large genome-wide association study of lipoproteins (11). Notably, using an independent sample, we replicated the *LDLR* SNP, rs5929, which showed the strongest association with LDL-C levels in the Tobago Family Study. Although this SNP was not previously reported to be associated with LDL-C, it has a very low frequency in populations of European ancestry (MAF = 0.8%), which could be a potential explanation why it has not been identified in previous population studies of European ancestry individuals. In silico analysis using FASTSNP (36) (<http://fastsnp.ibms.sinica.edu.tw/>) indicated a possible impact of rs5929, located within a coding region, on splicing regulation. The *LDLR* SNPs, rs2304182 and rs2738464, which were nominally associated with LDL-C in our study, were previously associated with LDL-C in the Multi-Ethnic Study of Atherosclerosis (37) and the Framingham Offspring Study (38).

A SNP in *PCSK9*, rs7517090, was associated with LDL-C in the families and was replicated in the population-based sample. Minor alleles of an intronic SNP (rs7517090) in *PCSK9* were associated with decreased LDL-C levels in both the family- and population-based Afro-Caribbean samples. This SNP has not been previously associated with

TABLE 3. Adjusted mean ( $\pm$  SE) TRIG levels for SNPs that showed significant associations with TRIG after adjusting for multiple comparisons

Gene Name	SNP rs no.	Chromosome	Position (bp)*	Study Population	Adjusted Means $\pm$ SE			Variation in TRIG Explained by SNP	Additive P-value
					1/1	1/2	2/2		
HNF4A	rs3746575 <sup>a</sup>	20	42491510	Family	82 $\pm$ 4.3	93 $\pm$ 3.3	114 $\pm$ 6.6	6.7%	<b>0.0006</b>
HNF4A	rs11574739	20	42476364	Population-based Family	111 $\pm$ 2.6	112 $\pm$ 2.0	109 $\pm$ 2.9	0.21	0.57
HNF4A	rs1028583	20	42484175	Population-based Family	88 $\pm$ 3.1	94 $\pm$ 4.3	146 $\pm$ 11.7	5.7%	<b>0.0012</b>
HNF4A	rs3212198	20	4247776	Population-based Family	112 $\pm$ 1.7	110 $\pm$ 2.5	110 $\pm$ 6.9	0.16	0.47
HNF4A	rs6073435	20	42487002	Population-based Family	85 $\pm$ 4.3	91 $\pm$ 3.4	112 $\pm$ 6.4	4.5%	<b>0.0015</b>
LDLR	rs2569540	19	11099239	Population-based Family	111 $\pm$ 2.6	111 $\pm$ 1.9	110 $\pm$ 2.9	0.011	0.96
LDLR	rs5927	19	11094941	Population-based Family	84 $\pm$ 3.9	93 $\pm$ 3.6	112 $\pm$ 6.5	5.1%	<b>0.0019</b>
AdipoR2	rs11608456	12	1701455	Population-based Family	112 $\pm$ 2.3	110 $\pm$ 2.0	112 $\pm$ 3.2	0.03%	0.78
LPIN1	rs11890442	2	11828454	Population-based Family	87 $\pm$ 3.7	92 $\pm$ 3.6	119 $\pm$ 7.6	4.3%	<b>0.0025</b>
					113 $\pm$ 2.2	109 $\pm$ 2.0	114 $\pm$ 3.4	0.11%	0.87
					106 $\pm$ 4.7	90 $\pm$ 3.6	83 $\pm$ 5.1	3.6%	<b>0.001</b>
					114 $\pm$ 2.5	114 $\pm$ 20.0	111 $\pm$ 3.19	0.38	0.20
					86 $\pm$ 3.7	95 $\pm$ 3.9	108 $\pm$ 7.2	3.5%	<b>0.0023</b>
					111 $\pm$ 2.0	112 $\pm$ 2.1	108 $\pm$ 4.3	0.28%	0.76
					97 $\pm$ 2.9	79 $\pm$ 5.3	70 $\pm$ 18.3	3.8%	<b>0.0007</b>
					ND	ND	ND	ND	ND
					85 $\pm$ 3.2	98 $\pm$ 4.3	119 $\pm$ 7.6	6.1%	<b>0.0009</b>
					111 $\pm$ 2.0	110 $\pm$ 2.1	118 $\pm$ 4.1	0.61	0.31

1, Major allele; 2, minor allele.

<sup>a</sup> In the population-based Afro-Caribbean replication study rs6031593 was genotyped as a proxy SNP for rs3746575 genotyped in the family study. Bold indicates significant P-value. ND, Not determined due to assay failure.

\* Chromosomal position was based on the National Center for Biotechnology Information dbSNP, build 36.2.

Supplemental Material can be found at:  
<http://www.jlr.org/content/suppl/2010/03/22/jlr.M003897.DC1.html>



LDL-C, but it is absent in populations of European and Asian ancestries (MAF = 0%), whereas in the current sample and in the Yoruba population from HapMap, the MAF is 13.9–19.9%. PCSK9 has recently emerged as a potential target for reducing LDL-C levels, as genetic variation in this gene strongly contributes to variation in LDL-C, particularly among African-Americans (39). PCSK9 post-transcriptionally downregulates the LDLR in the liver and thereby controls the level of LDL-C (40). For example, two rare nonsense mutations in *PCSK9* with a combined frequency of 2% in African ancestry individuals were associated with a 28% reduction in mean LDL-C and an 88% reduction in CHD risk (41). Although several variants of *PCSK9* have been identified so far, their effect on PCSK9 activity has not been determined.

We report for the first time a potential association of multiple common variants in a transcription factor gene, hepatic nuclear factor 4 $\alpha$  (*HNF4A*), with decreased TRIG levels in African ancestry population. However, we were unable to replicate these findings in an independent sample of men. Taking into consideration that we observed significant gender differences in heritability estimates for TRIGs and in obesity levels, future studies should test for similar *HNF4A* associations in an independent sample of African ancestry women and those with a wider range in body weight. Nonetheless, similar to our findings, another family study showed that rs3212198 was associated with TRIG in families of European and Mexican ancestry (42).

Our study has several potential limitations. We only genotyped tagging SNPs with a MAF  $\geq$  0.05 from the Yoruban population in HapMap. It is plausible that SNPs with a minor allele frequency of 1–5%, rare variants with MAF < 1%, such as those in *PCSK9* from previous studies in African-Americans, which we did not genotype, and structural variants also influence lipoproteins in African ancestry populations. Additionally, because we only studied individuals of Afro-Caribbean ancestry, our findings may not be generalizable to populations of other ethnicities. Although none of our participants reported using lipid-lowering medications, some under-reporting of lipid-lowering medication may have occurred. Furthermore, the small number of pedigrees in our analysis may have influenced our heritability estimates and association results. However, previous studies (43, 44) have shown that extended multi-generational pedigrees, such as those in the present study, may provide more precise heritability estimates and may be more powerful than nuclear pedigrees or sib-pairs in detecting and locating disease loci and with fewer false positives. Therefore, our multigenerational families, which contained over 3,000 relative pairs, should have been sufficient to provide a robust estimate of heritability. Finally, the African ancestry population of Tobago appears to have a low level of non-African admixture (6%), but we did not genotype individual ancestry-informative markers in the family or population samples. However, the MAF of all significant SNPs were similar to those reported in the Yoruba population. Our study also has notable strengths, including its inclusion of very large, multi-generational pedigrees of African ancestry from a

relatively isolated and homogeneous population with a low level of non-African ancestry. We also assessed a broad array of potential covariates for lipid and lipoprotein levels. Finally, to directly replicate the most promising SNP associations, we genotyped an independent population-based sample derived from the same geographic region and with the same genetic background as our families, which increases the validity and generalizability of our findings.

In conclusion, this study suggests that genetic factors are a significant source of inter-individual differences in fasting lipid and lipoprotein levels among Afro-Caribbeans and that genes may have much stronger influence on the distribution of TRIGs in women than in men of African ancestry. We have confirmed that *LDLR* and *PCSK9* are strong candidate genes for LDL-C in African ancestry individuals. Our study also suggests a potentially novel association between common *HNF4A* variants and TRIG levels in Afro-Caribbean families. Future sequencing and functional studies of these loci may advance our understanding of the genetic factors contributing to the low LDL-C and TRIG phenotypes in African ancestry populations.

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