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Population-based resequencing of *ANGPTL4* uncovers variations that reduce triglycerides and increase HDL

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Abstract

Resequencing genes provides the opportunity to assess the full spectrum of variants that influence complex traits. Here we report the first application of resequencing to a large population (n = 3,551) to examine the role of the adipokine ANGPTL4 in lipid metabolism. Nonsynonymous variants in *ANGPTL4* were more prevalent in individuals with triglyceride levels in the lowest quartile than in individuals with levels in the highest quartile (P = 0.016). One variant (E40K), present in ~3% of European Americans, was associated with significantly lower plasma levels of triglyceride and higher levels of high-density lipoprotein cholesterol in European Americans from the Atherosclerosis Risk in Communities Study and in Danes from the Copenhagen City Heart Study. The ratio of nonsynonymous to synonymous variants was higher in European Americans than in African Americans (4:1 versus 1.3:1), suggesting population-specific relaxation of purifying selection. Thus, resequencing of *ANGPTL4* in a multiethnic population allowed analysis of the phenotypic effects of both rare and common variants while taking advantage of genetic variation arising from ethnic differences in population history.

Adipocytes secrete a variety of proteins that regulate glucose and lipid metabolism[1]. The metabolic effects of these proteins have been largely deduced from studies in mice; less is

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

known about their importance in humans. As a first step toward elucidating the role of adipokines in lipid metabolism in humans, we examined the effects of sequence variation in *ANGPTL4*, a gene whose expression is induced in adipose tissue and liver during fasting[2]. Mice with a genetic deletion of *ANGPTL4* have lower plasma triglyceride levels[3], whereas hepatic overexpression of *ANGPTL4* causes hypertriglyceridemia[4], hepatic steatosis[5] and a reduction in fat mass[6]. Although the exact role of ANGPTL4 is not clear, it appears to inhibit lipoprotein lipase[4], an enzyme that hydrolyzes the triglycerides in circulating lipoproteins to release fatty acids for uptake by adjacent tissues[7]. Thus, ANGPTL4 may act in a paracrine manner to regulate the partitioning of fatty acids between sites of storage (adipose tissue) and sites of oxidation (heart, skeletal muscle and liver)[8].

To determine how sequence variations in *ANGPTL4* influence energy metabolism in humans, we sequenced the seven exons and the intron-exon boundaries of the gene (Supplementary Table 1 online) in 3,551 participants in the Dallas Heart Study, a population-based probability sample of Dallas County residents (1,830 African American, 601 Hispanic, 1,045 European American and 75 other ethnicities) whose lipid and glucose metabolism has been characterized in detail[9,10]. We identified a total of 93 sequence variations (65 in African Americans, 45 in European Americans and 31 in Hispanics), most of which were rare: more than half (n = 49) were found only in one subject, and 86% (n = 80) had a minor allele frequency below 3% (Supplementary Fig. 1 and Supplementary Table 2 online).

To examine the phenotypic effects of sequence variation in *ANGPTL4*, we stratified the population by race, sex and trait level and then compared the number of nonsynonymous variants in the top and bottom quartiles of the distribution. We excluded variants found in both quartiles. The first trait we examined was fasting levels of plasma triglyceride. Individuals with factors known to affect triglyceride levels (lipid-lowering drugs, diabetes mellitus and heavy alcohol use) were excluded from the analyses. After these exclusions, the number of individuals with nonsynonymous sequence variants in the bottom quartile (n = 13) was significantly greater than the number in the highest quartile (n = 2; P = 0.016) (Fig. 1); we found all sequence variations in separate individuals except R336C, which was present in three European Americans in the lowest quartile of triglycerides. Although European Americans comprised less than one-third of the sample (1,045 out of 3,551), 10 of the 13 individuals with a nonsynonymous sequence variant found in the low-triglyceride group were European Americans. Four of the sequence variations in the low-triglyceride group (IVS3+1 G > A, K217X, K245fs and S302fs) are predicted to truncate translation or interfere with splicing of the mRNA. In contrast to the nonsynonymous variants, the number of synonymous and noncoding variants in the upper and lower tails of the distribution was identical (n = 15).

We performed similar analyses for body mass index (BMI), high-density lipoprotein cholesterol (HDL-C) levels and fasting plasma insulin levels. We did not find any significant differences in the number of nonsynonymous sequence variants in *ANGPTL4* in the lowest and highest quartiles of any of these traits (Supplementary Table 3 online).

Next, we determined if any of the sequence variants in *ANGPTL4* with a minor allele frequency (MAF) > 1% were associated with trait levels in the three ethnic groups. Only one variant, E40K, which had a MAF of 1.3% in European Americans, was significantly associated with plasma triglyceride levels (Supplementary Table 4 online). The median plasma triglyceride level was 29 mg/dl (27%) lower in carriers than in noncarriers (P = 0.004) in the sample (Table 1). Plasma triglyceride levels cluster with other metabolic risk factors for cardiovascular disease, including adiposity, blood pressure, lipoprotein size distribution, HDL-C levels and insulin sensitivity[11]. We did not find any significant differences between the carriers and noncarriers in the Dallas Heart Study in mean BMI; blood pressure; fasting levels of glucose, insulin or cholesterol; or hepatic triglyceride content (Table 1). Plasma levels of low-density

lipoprotein cholesterol (LDL-C) were significantly higher in carriers than the noncarriers (P = 0.002). The frequencies of the E40K allele in African Americans and Hispanics in the DHS were too low to allow meaningful statistical analysis in these populations (Supplementary Table 5 online).

To confirm these findings, we tested for association between *ANGPTL4*[E40K] and metabolic phenotypes in two larger population-based studies: the Atherosclerosis Risk in Communities (ARIC) study[12] (n = 15,792) and the Copenhagen City Heart Study (CCHS) (n = 10,135)[13]. In European Americans in the ARIC population, the median plasma triglyceride level was reduced by 16 mg/dl (15%) in the 343 heterozygous carriers ($P = 2.2 \times 10^{-10}$, Table 1). Carriers had significantly lower levels of fasting insulin and LDL-C and significantly higher levels of HDL-C (56 ± 16 versus 51 ± 17 mg/dl, $P = 4.0 \times 10^{-7}$) (Table 1). In the CCHS population, median plasma levels of triglyceride were also significantly lower (141 mg/dl versus 158 mg/dl; $P = 1.0 \times 10^{-5}$) and HDL-C levels significantly higher (63 ± 18 mg/dl versus 60 ± 19 mg/dl, P = 0.0005) in carriers than in non-carriers (Table 1). The E40K heterozygotes also had significantly lower plasma levels of LDL-C. Plasma insulin levels were not available in the CCHS population. As in the Dallas Heart Study, *ANGPTL4*[E40K] was not associated with BMI, blood pressure or plasma levels of glucose in either ARIC or CCHS.

Thus, the E40K variation was systematically associated with lower plasma triglyceride concentrations in three independent populations. The effects of the variant on other metabolic phenotypes were apparent in the two larger study populations, ARIC and CCHS. Taken together, our data indicate that sequence variation in *ANGPTL4* primarily affects plasma levels of triglycerides but also affects other related metabolic parameters, including HDL-C, LDL-C and possibly fasting insulin levels. Furthermore, it is possible that the promoter region and other segments of *ANGPTL4* that were not resequenced in this study may contain functionally significant sequence variants.

A notable finding in our population-based study was that loss-of-function alleles in *ANGPTL4* were much more common in European Americans than in African Americans. The ratio of nonsynonymous to synonymous variants was substantially greater in European Americans (4:1) than in African Americans (1.3:1) (Fig. 2). To determine if these population differences were a result of natural selection, we analyzed the frequency distribution of the *ANGPTL4* sequence variations identified in the Dallas Heart Study using three test statistics (Tajima's D, Fu and Li's D and Fu and Li's Ds)[14,15]. All three tests showed a significant excess of rare (new) alleles in African Americans, European Americans and Hispanics, consistent with purifying selection acting at the *ANGPTL4* locus (Supplementary Table 6 online). Tests for selection have the potential to be confounded by demographic factors such as population expansion, which can also give rise to an excess of rare variants[15]. However, demographic factors would be expected to affect functional and neutral variants similarly. The disproportionate accumulation of nonsynonymous variants in European Americans is unlikely to result from population expansion and is most consistent with relaxation of purifying selection in this population. We speculate that loss-of-function variants in *ANGPTL4* may have been less deleterious to reproductive fitness in Europeans than in Africans. As ANGPTL4 has a role in triglyceride transport and fatty acid metabolism[2], changes in selective pressure on *ANGPTL4* may reflect shifts in energy use associated with acclimation to colder climates or changes in diet.

Recently, population-based samples have been proposed as an alternative to case-control cohorts for association and resequencing studies[16,17]. The present study, the first large-scale sequencing survey of unselected individuals in which the resulting genetic data have been coupled to phenotype information, illustrates some of the strengths and limitations of this approach. A major advantage of unselected, well-characterized samples is that sequence

variants identified can be tested against multiple phenotypes. The detailed phenotype database available on the Dallas Heart Study participants allowed us to test the spectrum of metabolic consequences associated with genetic variation in *ANGPTL4*. The use of a multiethnic population for resequencing capitalizes on genetic variation that arises from ethnic differences in population history. Previously, in the Dallas Heart Study we found highly informative sequence variants in *PCSK9* and *NPC1L1* that were largely confined to African Americans[18–20]. In contrast, the *ANGPTL4* variants identified in this study were more informative in European Americans. Another advantage is that both rare and common sequence variants can be evaluated using multiple approaches.

The increased flexibility afforded by an unselected sample is achieved at the expense of efficiency, as much of the information and statistical power is provided by individuals in the tails of the distribution. Of 23 European Americans heterozygous for *ANGPTL4*[E40K] in the Dallas Heart Study, 11 had plasma triglyceride levels in the bottom quartile of the distribution, whereas only one was in the top quartile ($P = 0.008$, Fig. 3). In the ARIC study, 52 of the carriers (49 heterozygotes and 3 homozygotes) had plasma triglyceride levels below the 5th percentile of the population distribution, whereas only six were above the 95th percentile ($P = 1.1 \times 10^{-9}$, Fig. 2). Thus, comparison of the extremes of the population distribution constitutes a powerful and efficient analytical strategy to capture the effects of both common and rare sequence variants on complex traits.

METHODS

Study populations

The coding regions of the *ANGPTL4* gene were sequenced in those participants of the Dallas Heart Study (DHS) ($n = 3,551$) from whom fasting venous blood samples were obtained (Supplementary Note online). The DHS is a population-based probability sample of Dallas County (52% African American, self-identified as ‘black’; 29% European American, self-identified as ‘white’; 17% Hispanic and 2% other ethnicities) in which ethnicity was self assigned according to US census categories[9]. The study was approved by the institutional review board of University of Texas Southwestern Medical Center, and all subjects provided written informed consent before participation.

The genetic associations observed in the DHS were validated in the ARIC and CCHS studies (Supplementary Note). The ARIC study is a prospective study of atherosclerosis initiated in 1987 (ref. [12]) in four communities in the USA (Jackson, Mississippi; Minneapolis, Minnesota; Forsyth County, North Carolina and Washington County, Maryland). A randomly selected cohort of approximately 4,000 persons, ages 45–64 years, was selected from each community[12]. The protocol for the study was approved by the institutional review boards of all centers, and all participants provided written informed consent that included consent for genetic studies. The Copenhagen City Heart Study is a prospective study of ischemic heart disease initiated in 1976. At the third examination (1991–1994), 10,135 individuals participated, and 9,255 gave blood for DNA analysis[13]. A total of 9,247 individuals were genotyped in the present study. The study was approved by local ethical committees, and all subjects provided written informed consent.

DNA sequencing

The exons and flanking introns of *ANGPTL4* were sequenced in both directions in 3,551 participants in the Dallas Heart Study as described[21]. The oligonucleotide primers used for sequencing are shown in Supplementary Table 1. All sequence variants identified were verified by manual inspection of the chromatograms, and missense changes were confirmed by an independent resequencing reaction.

Genotyping assay

Fluorogenic 5′-nucleotidase assays for the *ANGPTL4* alleles encoding E40K or the wild-type protein were developed with the use of the TaqMan assay system (Applied Biosystems). The assays were performed on a 7900HT Fast Real-Time PCR instrument with probes and reagents purchased from Applied Biosystems.

Statistical analysis

The prevalence of nonsynonymous variants in the upper and lower quartiles of the Dallas Heart Study was compared using Fisher's exact test. Individuals who had diabetes, used lipid-lowering drugs or consumed more than 30 g alcohol per day for a man or 20 g per day for a woman were excluded from the analyses. Risk factor levels between carriers of each *ANGPTL4* variant and noncarriers were compared by analysis of variance (ANOVA). For comparison of plasma lipid levels, we included age, sex and BMI as covariates in the model. Plasma levels of triglyceride and insulin were log transformed before analysis. Selective neutrality for the sequence variants identified was tested by Tajima's method[14] and Fu and Li's methods[15].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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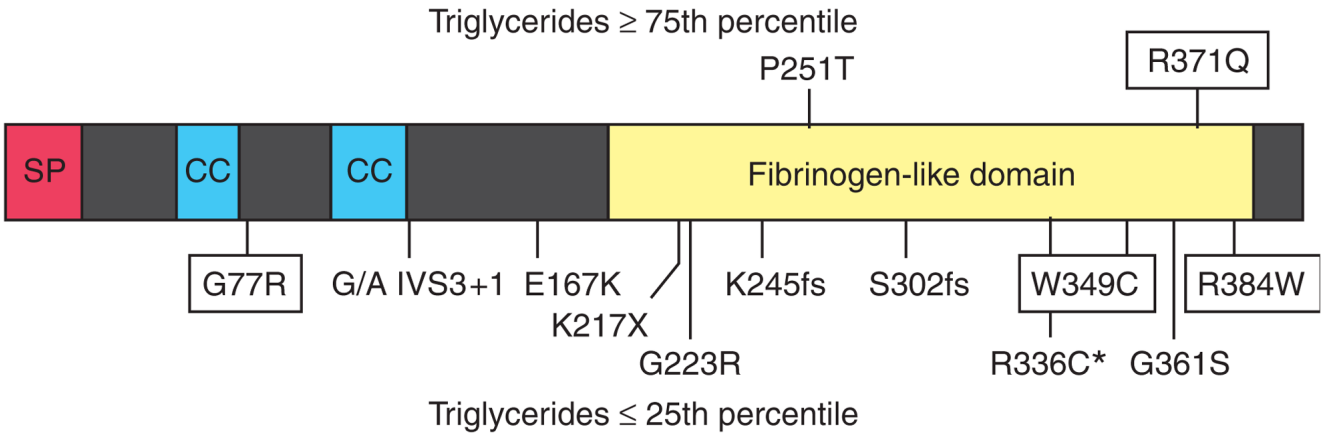

Figure 1.

Schematic of the *ANGPTL4* gene with location of nonsynonymous sequence variations identified in the upper and lower quartiles of Dallas Heart Study. The 406-residue protein comprises a signal sequence (SP), a coiled-coil domain (CC) and a fibrinogen-like domain. Sequence variants found among individuals in the low-triglyceride group (plasma triglyceride levels less than or equal to the 25th percentile) but not in individuals in the high-triglyceride group (plasma triglyceride levels greater than or equal to the 75th percentile) are shown below the protein. Sequence variants found in the high-triglyceride group but not in the low-triglyceride group are shown above the protein. Sequence variants found in African Americans are indicated by boxes. All variants were found in separate individuals in each group, except for R336C, which was found in three individuals in the low group (asterisk indicates $n = 3$). Thus, the number of individuals with nonsynonymous sequence variants in the bottom quartile ($n = 13$) was significantly greater than the number in the highest quartile ($n = 2$; $P = 0.016$). fs, frameshift.

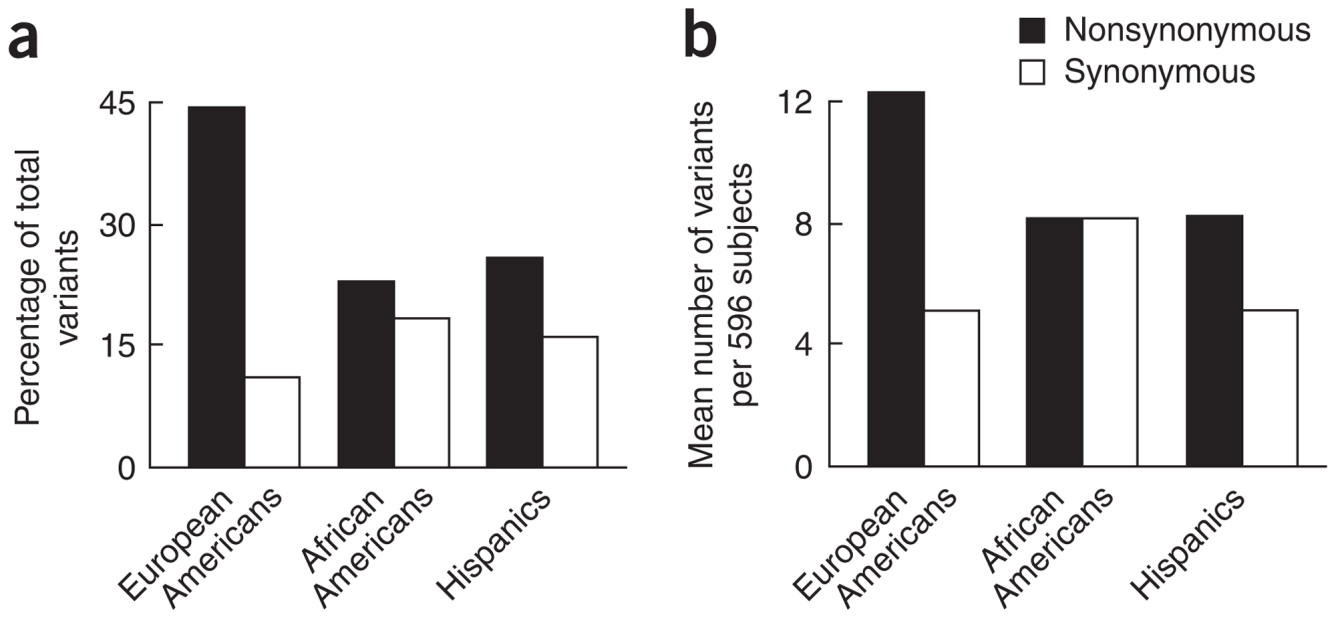

Figure 2.

Proportion of nonsynonymous and synonymous sequence variants in European Americans, African Americans and Hispanics in the Dallas Heart Study. (**a**) Number of nonsynonymous and synonymous variants expressed as a percentage of the total number of variants identified in the three ethnic groups in the DHS. (**b**) Number of nonsynonymous and synonymous variants identified per 596 individuals in each population. To adjust for the different numbers of individuals in each group, five subsamples of 596 individuals (corresponding to the total number of Hispanics from whom sequence data were obtained) were drawn at random from the 1,045 European Americans and from the 1,830 African Americans in the sample. The number of nonsynonymous and synonymous variants was determined in each subsample, and the average number across the five subsamples was calculated. This random selection process was repeated once, with essentially identical results.

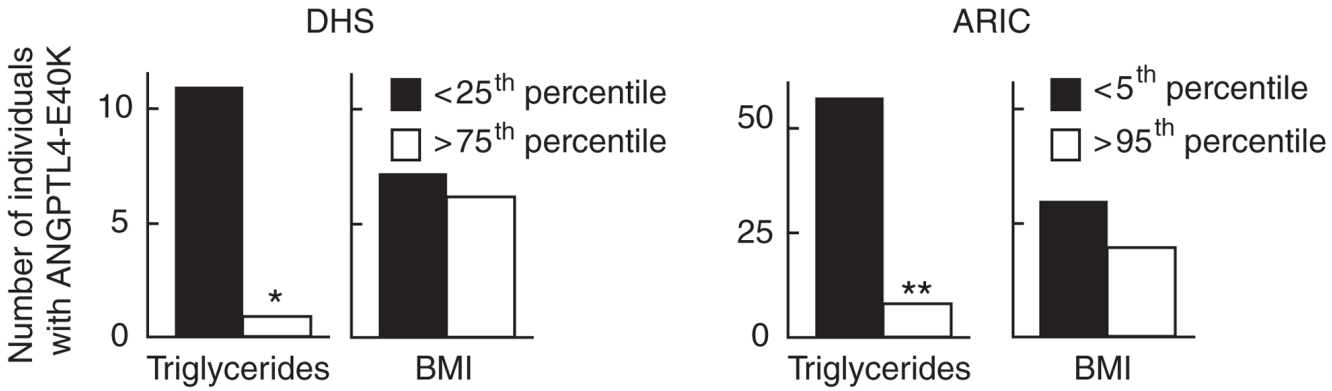

Figure 3.

Prevalence of the *ANGPTL4*[E40K] allele among individuals with low and high plasma triglyceride levels in the Dallas Heart Study and ARIC study. European American participants in the DHS were stratified by plasma triglyceride level (which was significantly associated with the E40K variant in all three populations) and by BMI (which was not associated with the variant in any of the three populations). The number of *ANGPTL4*[E40K] carriers in the upper and lower quartiles for each trait was determined. A corresponding analysis was performed in the ARIC study using the upper and lower 5% of the population distribution for the two traits. $*P = 0.008$; $**P = 1.1 \times 10^{-9}$.

Table 1

Clinical and laboratory characteristics of European Americans and Danes

	European Americans, Dallas Heart Study (*n* = 838)			European Americans, ARIC (*n* = 8,726)				Danes, Copenhagen City Heart Study (*n* = 8,750)			
	EE	**EK**	***P* value**	**EE**	**EK**	**KK**	***P* value**	**EE**	**EK**	**KK**	***P* value**
n	815	23		8,376	343	7		8,239	505	6	–
Number of males/ number of females	352/423	12/11	0.68	3,785/4,591	144/199	2/5	0.34	3,584/4,655	247/258	1/5	–
Age (years)	45 ± 10	47 ± 9	0.21	54 ± 6	54 ± 6	57 ± 7	0.63	58 ± 15	60 ± 15	47 ± 17	0.004
BMI (kg/m^2)	28.7 ± 6	27.0 ± 7	0.20	26.6 ± 5	26.3 ± 5	27.9 ± 4	0.29	25.5 ± 4.2	25.4 ± 4.4	23.6 ± 1.3	0.50
SBP (mm/Hg)	123 ± 14	127 ± 20	0.17	117 ± 17	116 ± 17	124 ± 22	0.39	138 ± 22	139 ± 23	124 ± 17	0.12
DBP (mm/Hg)	77 ± 9	78 ± 10	0.67	71 ± 10	71 ± 10	74 ± 15	0.50	84 ± 12	84 ± 12	71 ± 12	0.03
Glucose (mg/dl)	91 ± 10	93 ± 11	0.14	99 ± 9	98 ± 9	101 ± 10	0.89	100 ± 18	100 ± 18	92 ± 25	0.56
Insulin (mIU/l)	9.7 (10.2)	7.8 (12.6)	0.87	8.3 (6.2)	7.2 (7.2)	8.3 (16)	0.003	–	–	–	–
Cholesterol (mg/dl)	184 ± 38	198 ± 52	0.16	213 ± 40	208 ± 35	231 ± 17	0.073	237 ± 50	235 ± 46	203 ± 48	0.16
Triglyceride (mg/dl)	106 (91)	77 (46)	0.004	110 (76)	94 (64)	75 (105)	2.2×10^{-10}	158 (97)	141 (80)	101 (20)	1.0×10^{-5}
LDL-C (mg/dl)	109 ± 34	130 ± 49	0.002	136 ± 37	131 ± 34	145 ± 22	0.026	161 ± 47	157 ± 43	132 ± 37	0.04
HDL-C (mg/dl)	49 ± 15	51 ± 13	0.74	51 ± 16	56 ± 16	59 ± 19	4.0×10^{-7}	60 ± 18	63 ± 19	62 ± 19	0.0005
HTGC (%)	5 ± 5	5 ± 5	0.56	–	–	–	–	–	–	–	–

Values are mean ± s.d., except for insulin and triglyceride, which are medians (with interquartile ranges in parentheses). *P* values were calculated using ANOVA. Variables with highly skewed distributions (plasma insulin and triglycerides) were log transformed before analysis, and individuals with diabetes or those who used lipid-lowering drugs were excluded from all analyses. For analysis of triglyceride and HDL-C levels, men consuming more than 30 g alcohol per day and women consuming more than 20 g alcohol per day were also excluded. Additional analyses were performed with the alcohol users included, and with age, sex, and BMI in the model, with essentially identical results. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HTGC, hepatic triglyceride content. EE, homozygotes for the wild-type allele; EK, heterozygotes for *ANGPTL4*[E40K]; KK, homozygotes for *ANGPTL4*[E40K].

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