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Apolipoprotein C3 Gene Variants in Nonalcoholic Fatty Liver Disease

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Abstract

BACKGROUND—Nonalcoholic fatty liver disease is associated with hepatic insulin resistance and type 2 diabetes mellitus. Whether this association has a genetic basis is unknown.

METHODS—In 95 healthy Asian Indian men, a group known to have a high prevalence of non-alcoholic fatty liver disease, we genotyped two single-nucleotide polymorphisms (SNPs) in the gene encoding apolipoprotein C3 (*APOC3*) that are known to be associated with hypertriglyceridemia (rs2854116 [T-455C] and rs2854117 [C-482T]). Plasma apolipoprotein C3 concentrations, insulin sensitivity, and hepatic triglyceride content were measured. We also measured plasma triglyceride concentrations and retinyl fatty acid ester absorption as well as plasma triglyceride clearance after oral and intravenous fat-tolerance tests. Liver triglyceride content and *APOC3* genotypes were also assessed in a group of 163 healthy non-Asian Indian men.

RESULTS—Carriers of the *APOC3* variant alleles (C-482T, T-455C, or both) had a 30% increase in the fasting plasma apolipoprotein C3 concentration, as compared with the wild-type homozygotes. They also had a 60% increase in the fasting plasma triglyceride concentration, an increase by a factor of approximately two in the plasma triglyceride and retinyl fatty acid ester concentrations after an oral fat-tolerance test, and a 46% reduction in plasma triglyceride clearance. The prevalence of nonalcoholic fatty liver disease was 38% among variant-allele carriers and 0% among wild-type homozygotes ($P < 0.001$). The subjects with nonalcoholic fatty liver disease had marked insulin resistance. A validation study involving non-Asian Indian men confirmed the association between *APOC3* variant alleles and nonalcoholic fatty liver disease.

CONCLUSIONS—The polymorphisms C-482T and T-455C in *APOC3* are associated with nonalcoholic fatty liver disease and insulin resistance.

Several studies have shown a strong relationship among nonalcoholic fatty liver disease, hepatic insulin resistance, and type 2 diabetes mellitus.^{1–3} Whether there is a genetic basis for this association remains unknown. Previous studies have suggested that two single-nucleotide polymorphisms (SNPs) in the gene encoding apolipoprotein C3 (*APOC3*) may be associated with hypertriglyceridemia.^{4–8} We therefore examined whether these SNPs might confer a predisposition to nonalcoholic fatty liver disease and insulin resistance.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We began by studying a cohort of healthy Asian Indian men, a group that has been shown to have a high prevalence of nonalcoholic fatty liver disease and insulin resistance.⁹ We screened this group for the two *APOC3* SNPs and measured their hepatic triglyceride content and whole-body insulin sensitivity. We then examined the effects of the *APOC3* SNPs on postprandial plasma triglyceride concentrations and retinyl fatty acid ester absorption after an oral fat-tolerance test and on plasma triglyceride clearance after an intravenous lipid infusion. In addition, we determined whether hepatic steatosis and whole-body insulin resistance in the risk-allele carriers could be reversed with weight loss. Finally, we performed a validation study in an independent group of healthy non-Asian Indian men.

METHODS

SUBJECTS

From 2004 through 2009, we prospectively recruited healthy, nonsmoking, sedentary Asian Indian men whose birth weights were known to be normal. These volunteers were drawn from the New Haven, Connecticut, community by means of local advertisement. Written informed consent was obtained from each subject, and the protocol was approved by the human subjects committee at Yale University. The participants answered a questionnaire about their usual daily intake of food and alcohol as well as any changes in body weight and eating habits over the previous 12 months. They were also asked to describe the food and snacks consumed on the day before the visit, and the composition of each participant's diet was assessed. Physical activity (during work, leisure time, and exercise) was measured by means of a pedometer and a questionnaire.¹⁰

MEASUREMENT OF PLASMA GLUCOSE, INSULIN, AND LIPIDS

Fasting plasma glucose concentrations were measured with the use of the YSI STAT 2700 Select Biochemistry Analyzer (YSI Life Sciences). Fasting plasma concentrations of insulin were measured with the use of a double-antibody radioimmunoassay kit (Linco). Fasting plasma triglyceride, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and apolipoprotein C3 concentrations were measured enzymatically (Roche Cobas Mira Plus, Roche Diagnostics).

GENETIC SCREENING

Genomic DNA was extracted from whole blood, and participants were genotyped for the SNPs rs2854116 and rs2854117 in *APOC3* (for details, see the Supplementary Appendix, available with the full text of this article at NEJM.org). These SNPs have previously been shown to be associated with hypertriglyceridemia.^{4–8} Since earlier studies have shown that the SNP rs651821 in the gene encoding apolipoprotein A5 (*APOA5*) is also associated with hyperlipidemia, we excluded subjects who were identified as having the high-risk variant for this polymorphism and who were homozygous for the low-risk variants in *APOC3*. In addition, we genotyped all study participants for the SNP rs738409 in the gene encoding patatin-like phospholipase domain-containing 3 (*PNPLA3*), which has also been shown to be associated with increased hepatic triglyceride content.¹¹

GENETIC CLASSIFICATION

The two SNPs in *APOC3* (rs2854116 and rs2854117) are located in the promoter region (upstream of the transcription start site) and are in strong linkage disequilibrium. The variant alleles at rs2854116 (T-455C) and at rs2854117 (C-482T) are each associated with higher apolipoprotein C3 expression in vitro than are the respective wild-type alleles.¹² Because these associations are independent and not additive, we divided the study participants into two genetically defined groups for the purposes of analysis: those who were homozygous for the

low-expression (wild-type) allele at both SNPs and those who were carriers of one or more high-expression (variant) alleles at either SNP.

ASSESSMENT OF INSULIN SENSITIVITY

Whole-body insulin sensitivity was assessed by means of a 3-hour oral glucose-tolerance test. A 75-g dextrose drink (Glucola, Curtin Matheson Scientific) was administered, and blood samples were collected at 10, 20, 30, 60, 90, 120, 150, and 180 minutes to determine plasma glucose and insulin concentrations. The insulin-sensitivity index was calculated as previously described.¹³

MEASUREMENT OF HEPATIC TRIGLYCERIDE CONTENT

Hepatic triglyceride content was measured with proton magnetic resonance spectroscopy on a BioSpec 4T system (Bruker Daltonics). Localized spectra of the liver were obtained with the use of a coil assembly composed of twin elliptical proton radiofrequency coils (13 cm by 9 cm) arranged in quadrature. Triglyceride content was measured by proton respiration-gated, stimulated echo acquisition mode spectroscopy in a voxel (15 mm³ by 15 mm³ by 15 mm³) placed in three different locations within the liver to account for the heterogeneity of hepatic tissue.¹⁴

FAT-TOLERANCE TESTING

Plasma triglyceride concentrations and retinyl fatty acid ester absorption were measured in a sub-group of the Asian Indian subjects after they had consumed a high-fat liquid meal. In another subgroup of the Asian Indian subjects, plasma triglyceride clearance was assessed during an intravenous fat-tolerance test. (Details of these methods are described in the Supplementary Appendix.)

ASSESSMENT OF HEPATIC STEATOSIS WITH WEIGHT REDUCTION

A subgroup of subjects with hepatic steatosis (hepatic lipid content >5.5%)¹⁵ were studied after programmed caloric restriction (approximate intake, 1200 kcal per day).³ After an overnight fast, the subjects' body weight, plasma glucose and insulin concentrations, and liver triglyceride content were measured on a weekly basis. The weight-loss program continued until the hepatic triglyceride levels normalized. The hypocaloric period was followed by 4 weeks of weight stabilization on an isocaloric diet (35 kcal per kilogram of body weight; 60% carbohydrate, 20% protein, and 20% fat).

CONFIRMATION STUDY IN NON-ASIAN INDIAN MEN

To examine the relationship between the polymorphisms in *APOC3* and hepatic steatosis, we performed a confirmation study involving a cohort of healthy, sedentary, nonsmoking, multiethnic non-Asian Indian men of normal birth weight. The subjects were recruited from the New Haven community by local advertisement, with the use of the same inclusion and exclusion criteria that were applied to the Asian Indian subjects.

STATISTICAL ANALYSIS

Plasma and liver triglyceride concentrations were log-transformed to fit a normal distribution; normality was tested with the use of a Kolmogorov-Smirnov test. The base-10 logarithm of liver triglyceride, the natural logarithm of plasma triglyceride, and levels of plasma apolipoprotein C3 were then adjusted for age and body-mass index by means of linear regression. To test for association under an additive model, two methods were used: a linear-regression trend test for correlation with the genotypes at rs2854116 and rs2854117 (with genotypes at each SNP coded as 0, 1, or 2) and a one-way analysis of variance. To test for

association under a dominant model, we used a linear-regression trend test for correlation (with genotypes coded as 0 or 1) as well as an unequal-variance t-test. Nonparametric t-tests and Mann–Whitney U tests, as appropriate, were used to compare means between persons who were carrying one or more high-expression (variant) alleles for either or both of the *APOC3* SNPs and persons who were homozygous for the low-expression (wild-type) allele at both SNPs. Paired t-tests were performed to compare means before and after weight loss. All analyses were done on SPSS software, version 16.0, or StatView (SAS Institute), and all tests were two-tailed, with an alpha level of 0.05. The difference in the prevalence of nonalcoholic fatty liver disease between groups was determined by means of a two-sided Fisher's exact test, and linkage disequilibrium (*D'*) was calculated with the use of Haploview. All data are expressed as means \pm SD (except where otherwise noted).

RESULTS

SUBJECTS AND GENOTYPES

We performed initial clinical evaluations of 102 Asian Indian men. Seven had high-risk variants at rs651821 in *APOA5* and were therefore excluded. The clinical characteristics of the 95 subjects who met all the criteria for participation in the study are shown in Table 1. The mean age of the group was 32 ± 13 years, and the mean body-mass index (the weight in kilograms divided by the square of the height in meters) was 24.7 ± 3.6 .

Nineteen of the study subjects (20%) were homozygous for the common, low-expression (wild-type) alleles at both loci (CC at locus 482 and TT at locus 455); the other 76 carried at least one high-expression variant (C-482T, T-455C, or both). The number of subjects with each possible combination of haplotypes on the basis of these two loci are shown in Table 1 in the Supplementary Appendix. The 76 carriers of either high-expression variant allele were similar to the 19 wild-type homozygotes with respect to age, body weight, body-mass index, and physical activity level (see Table 2 in the Supplementary Appendix). The percentages of total daily caloric intake represented by fat, carbohydrate, and protein were also similar in the two groups.

PLASMA LIPIDS, HEPATIC LIPID CONTENT, AND INSULIN RESISTANCE

Plasma apolipoprotein C3 concentrations were approximately 30% higher in the variant-allele carriers than in the wild-type homozygotes (Table 2). In addition, fasting plasma triglyceride concentrations were approximately 60% higher in the carriers of variant alleles. There were no significant differences between the two groups in plasma concentrations of total cholesterol, HDL cholesterol, or LDL cholesterol.

The average hepatic triglyceride content was markedly higher in the *APOC3* variant-allele carriers than in the wild-type homozygotes (mean, $7.5\pm 10.3\%$ [median, 3.2%], vs. mean, $1.5\pm 1.3\%$ [median, 1.1%]; $P<0.001$) (Fig. 1A). These differences remained significant when the analysis was adjusted for age and body-mass index ($P = 0.005$ by Student's t-test; $P = 0.02$ by the Mann–Whitney test). The prevalence of nonalcoholic fatty liver disease (hepatic lipid content $>5.5\%$ ¹⁵) was 38% among the variant-allele carriers, whereas none of the wild-type homozygotes had nonalcoholic fatty liver disease ($P<0.001$) (Fig. 1A). Subjects with nonalcoholic fatty liver disease had marked insulin resistance as compared with those who did not have nonalcoholic fatty liver disease (insulin-sensitivity index, 2.0 ± 2.0 [lower-third percentile for insulin sensitivity⁹], vs. 3.5 ± 1.6 ; $P<0.001$).

PLASMA TRIGLYCERIDE AND RETINYL FATTY ACID ESTER CONCENTRATIONS

A total of 28 variant-allele carriers and 10 wild-type homozygotes underwent oral fat-tolerance testing. The average area under the curve (AUC) for the plasma triglyceride concentration was

approximately doubled in the variant-allele carrier group as compared with the wild-type homozygote group (746±102 vs. 383±41 mg per deciliter per hour, $P = 0.002$) (Fig. 2A). Similarly, among the variant-allele carriers, the changes in the AUC for plasma concentrations of retinyl fatty acid ester (reflecting chylomicron remnants) was approximately twice those among the wild-type homozygotes (60±36 vs. 36±19 μmol per liter per hour, $P = 0.01$) (Fig. 2B).

PLASMA TRIGLYCERIDE CLEARANCE

A total of 15 variant-allele carriers and 4 wild-type homozygotes underwent intravenous fat-tolerance testing. The variant-allele carriers had a 46% increase in the AUC for the relative plasma triglyceride concentration, reflecting decreased plasma triglyceride clearance, as compared with the wild-type homozygotes (171±21 vs. 249±71% per hour, $P = 0.046$) (Fig. 3).

EFFECT OF WEIGHT LOSS ON HEPATIC STEATOSIS AND INSULIN RESISTANCE

Seven of the subjects with hepatic steatosis and insulin resistance underwent a hypocaloric dietary intervention.³ Over a period of 3 to 6 months, their average weight decreased from 80.4±10.7 kg to 74.9±10.2 kg ($P = 0.003$). This weight loss was accompanied by a significant reduction in the liver triglyceride content (from 14.0 to 3.8%, $P = 0.05$). After weight loss, there was a marked improvement in insulin sensitivity on oral glucose-tolerance testing, with reductions in plasma glucose and plasma insulin concentrations (Fig. 1 in the Supplementary Appendix) and an increase in the insulin-sensitivity index (from 1.8±0.8 to 3.7±1.4, $P < 0.01$).

EFFECT OF *PNPLA3* GENOTYPE ON HEPATIC STEATOSIS

The liver triglyceride content was also increased in subjects of the CG and GG genotypes at rs738409 in *PNPLA3*, as compared with subjects of the CC genotype, with the use of both a dominant model ($P = 0.002$ by Student's *t*-test; $P = 0.003$ by linear regression) and an additive model ($P = 0.008$ by analysis of variance; $P = 0.04$ by linear regression).¹¹ Variants in *APOC3* alone, *PNPLA3* alone, and the two types combined accounted, respectively, for 11.0, 6.5, and 13.1% of the variance in the risk of nonalcoholic fatty liver disease.

CONFIRMATION STUDY IN NON-ASIAN INDIAN MEN

A confirmation study was performed in 163 healthy non-Asian Indian men (108 of whom were white, 26 Asian, 15 Hispanic, and 14 black) (Table 1, and Table 2 in the Supplementary Appendix). As was the case with the Asian Indian men, the prevalence of nonalcoholic fatty liver disease was higher among the *APOC3* variant-allele carriers than among the wild-type homozygotes in this group (9% vs. 0%, $P = 0.02$) (Fig. 1B). Similarly, the non-Asian Indian men with nonalcoholic fatty liver disease had marked insulin resistance, as reflected by their lower insulin-sensitivity index as compared with that in the non-Asian men without nonalcoholic fatty liver disease (2.92±1.13 vs. 4.93±2.20, $P < 0.001$).

DISCUSSION

We studied the relationship between genetic variants in *APOC3* and the risk of nonalcoholic fatty liver disease and insulin resistance. The *APOC3* variants we studied (C-482T and T-455C) were associated with approximately a 30% increase in the fasting plasma apolipoprotein C3 concentration, approximately a 60% increase in the fasting plasma triglyceride concentration, and an increase by a factor of approximately two in postprandial plasma triglyceride and retinyl fatty acid ester concentrations after an oral fat-tolerance test. Thirty-eight percent of the Asian Indian men with *APOC3* variants had nonalcoholic fatty liver disease and marked insulin

resistance, whereas no *APOC3* wild-type homozygotes had nonalcoholic fatty liver disease. This association was confirmed in an independent group of non-Asian Indian men.

We also measured plasma triglyceride concentrations after an intravenous lipid infusion and found a 46% reduction in plasma triglyceride clearance in carriers of *APOC3* variant alleles as compared with wild-type homozygotes. These findings support the hypothesis that the *APOC3* variants we studied promote both fasting and postprandial hypertriglyceridemia by reducing triglyceride clearance. These data are consistent with previous studies showing that transgenic mice with overexpression of human apolipoprotein C3 had hypertriglyceridemia due to decreased lipoprotein lipase activity,¹⁶ as well as a recent study by Pollin et al. showing that a different mutation in the apolipoprotein C3 gene resulted in lower plasma triglyceride levels and had cardioprotective effects in Amish subjects.¹⁷

Taken together, these results suggest that the *APOC3* variants C-482T and T-455C lead to increased plasma concentrations of apolipoprotein C3, which in turn inhibit lipoprotein lipase and triglyceride clearance, thus conferring a predisposition to both fasting and postprandial hypertriglyceridemia due to an increase in chylomicron-remnant particles. Increased concentrations of circulating chylomicron-remnant particles are then preferentially taken up by the liver by means of a receptor-mediated process,^{18–22} resulting in non-alcoholic fatty liver disease and hepatic insulin resistance.

There is now strong evidence that nonalcoholic fatty liver disease has an important role in causing hepatic insulin resistance.^{1–3,23–27} Recent studies have shown that increased hepatocellular concentrations of diacylglycerol in nonalcoholic fatty liver disease lead to activation of the protein kinase C epsilon isoform, resulting in reduced insulin signaling and hepatic insulin resistance.^{23–27} Furthermore, the reversal of nonalcoholic fatty liver disease by modest weight reduction corrects hepatic insulin resistance and fasting hyperglycemia in patients with type 2 diabetes mellitus.³ Consistent with these studies was our finding that modest weight reduction (approximately 6 kg) in Asian Indian men eliminated hepatic steatosis and markedly improved whole-body insulin sensitivity, as reflected by marked reductions in their plasma glucose and insulin concentrations and a doubling of their insulin-sensitivity index.

We found that the prevalence of variant *APOC3* alleles was similar among Asian Indian men and non-Asian Indian men, suggesting that other factors are responsible for the increased prevalence of nonalcoholic fatty liver disease among the Asian Indian men.⁹ In this regard, we also genotyped the Asian Indian men for the SNP rs738409 in *PNPLA3*, which has recently been shown to be associated with nonalcoholic fatty liver disease by means of an unknown mechanism.¹¹ We found that the CG and GG genotypes of this SNP were also associated with increased liver triglyceride content, as compared with the CC genotype in this population.

We also examined the fraction of variance in the risk of nonalcoholic fatty liver disease attributable to *APOC3*, *PNPLA3*, or both and found that they accounted for 11.0, 6.5, and 13.1%, respectively, of the variance, suggesting that these gene variants have an interactive effect. However, in contrast to the *APOC3* variants described here, the *PNPLA3* variants have not been found to be associated with insulin resistance.¹¹

In summary, we found that genetic variants in *APOC3* were associated with nonalcoholic fatty liver disease and insulin resistance in a cohort of Asian Indian men. We confirmed this observation in a second cohort of non-Asian Indian men.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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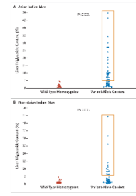


Figure 1. Scattergrams of Liver Triglyceride Content in Asian Indian and Non-Asian Indian Men Carriers of *APOC3* variant alleles (C-482T, T-455C, or both) were compared with *APOC3* wild-type homozygotes (C-482 and T-455) in terms of liver triglyceride content. Results are shown for two groups of Asian Indian men (Panel A) and for two groups of non-Asian Indian men (Panel B). The boxes delineate subjects who had nonalcoholic fatty liver disease (hepatic lipid content >5.5%), all of whom were carriers of the variant allele.

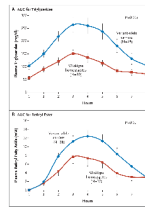


Figure 2. Plasma Triglyceride and Retinyl Fatty Acid Ester Concentrations after an Oral Fat-Tolerance Test in Asian Indian Men

Panel A shows the area under the curve (AUC) for plasma triglyceride concentrations after an oral fat-tolerance test in 28 carriers of *APOC3* variant alleles (C-482T, T-455C, or both) as compared with 10 *APOC3* wild-type homozygotes. Panel B shows the AUC for plasma retinyl ester concentrations in the two groups. Data are means \pm SE.

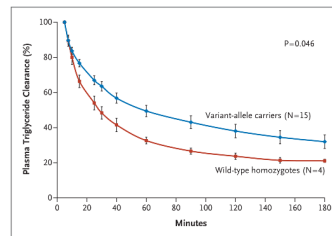


Figure 3. Relative Plasma Triglyceride Concentrations after an Intravenous Fat-Tolerance Test in Asian Indian Men

The relative plasma triglyceride concentrations are shown for 15 carriers of *APOC3* variant alleles and 4 wild-type homozygotes. (Relative refers to the fact that starting plasma triglyceride levels were normalized to 100% for each subject.) The area under the curve was significantly higher for the variant-allele carriers ($P = 0.046$). Data are means \pm SE.

Table 1

Clinical Characteristics of the Study Participants.*

Characteristic	Asian Indian Cohort (N = 95)	Non-Asian Indian Cohort (N = 163)
Age (yr)	32±13	28±13
Height (m)	1.73±0.09	1.77±0.07
Weight (kg)	74±11	75±10
Body-mass index [†]	24.7±3.6	24.1±2.9
Fat intake (%) [‡]	27±12	31±9
Carbohydrate intake (%) [‡]	57±13	49±11
Protein intake (%) [‡]	15±6	18±5
Alcohol intake (g/day)	2±5	5±8
Pedometer activity (km/day) [§]	5.38±3.06	6.42±2.95
Exercise index	2.5±1.0	2.5±0.9
Fasting glucose (mg/dl)	100±14	95±8
Fasting insulin (μU/ml)	13±1	9±5
Fasting triglycerides (mg/dl)	109±78	82±50

* Plus-minus values are means ±SD.

[†] The body-mass index is the weight in kilograms divided by the square of the height in meters.

[‡] Values given are a percentage of total daily caloric intake.

[§] To convert kilometers to miles, multiply by 0.62.

Table 2

Fasting Plasma Concentrations of Apolipoprotein C3, Triglycerides, and Cholesterol in *APOC3* Wild-Type Homozygotes and Carriers of Variant Alleles.*

Variable	Wild-Type Homozygotes (C-482 and T-455) (N = 19)	Variant-Allele Carriers (C-482T, T-455C, or both) (N = 75) [†]	P Value [‡]	
			Log-Transformed	Mann-Whitney Test
Apolipoprotein C3 (mg/dl)	8.9±2.6	11.4±3.5	0.003	0.03
Triglycerides (mg/dl)	74±31	118±87	0.02	0.053
Cholesterol (mg/dl)				
Total	146±31	160±35	0.28	0.35
HDL	49±9	47±17	0.64	0.17
LDL	83±26	90±26	0.68	0.99

* To convert the values for triglycerides to millimoles per liter, multiply by 0.01129. To convert the values for cholesterol to millimoles per liter, multiply by 0.02586. HDL denotes high-density lipoprotein cholesterol, and LDL low-density lipoprotein cholesterol.

[†] Data were not available for one carrier.

[‡] P values were adjusted for age and body-mass index.