Apolipoprotein E gene promoter −219G→T polymorphism increases LDL-cholesterol concentrations and susceptibility to oxidation in response to a diet rich in saturated fat\textsuperscript{1–3}

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ABSTRACT

Background: The apolipoprotein E (APOE) gene promoter polymorphism (−219G→T) has been associated with increased risk of myocardial infarction, premature coronary artery disease, and decreased plasma apolipoprotein E concentrations.

Objective: We aimed to determine in healthy subjects whether this polymorphism modifies the susceptibility of LDL to oxidation and the lipid response to the content and quality of dietary fat.

Design: Fifty-five healthy men with the APOE3/E3 genotype (7 GG, 38 GT, and 10 TT) completed 3 dietary periods, each lasting 4 wk. The first was a saturated fatty acid (SFA)-rich diet [38% fat—20% SFA and 12% monounsaturated fatty acid (MUFA)—and 47% carbohydrates (CHO)], which was followed by a CHO-rich diet (30% fat—<10% SFA and 12% MUFA—and 55% CHO) or a MUFA-rich diet (38% fat—<10% SFA and 22% MUFA—and 47% CHO) in a randomized crossover design. At the end of each dietary period, LDL oxidation susceptibility, lipids, and lipoproteins were measured.

Results: Compared with carriers of the G allele, TT subjects had a significantly (P < 0.05) shorter lag time after the SFA diet. The replacement of the SFA diet by the CHO or MUFA diet induced a greater increase (P < 0.05) in lag time in the TT subjects than in the GG or GT subjects. Carriers of the T allele had higher LDL-cholesterol (P < 0.05) and apolipoprotein B (P < 0.05) plasma concentrations after the SFA diet than did GG subjects. Compared with GG subjects, carriers of the T allele had a significantly (P < 0.05) greater decrease in LDL cholesterol and apolipoprotein B when they changed from the SFA to the CHO diet.

Conclusion: The −219G→T polymorphism may partially explain differences in individual responses to diet.

KEY WORDS Apolipoprotein E gene promoter polymorphism, −219G→T, APOE, dietary intervention, LDL oxidation, LDL cholesterol, cardiovascular disease risk

INTRODUCTION

Apolipoprotein E (apo E) is a structural component of several lipoproteins and serves as a ligand for the LDL receptor and the LDL-receptor-related protein (1, 2). Therefore, apo E plays an important role in lipid metabolism both by promoting efficient uptake of triacylglycerol-rich lipoproteins from the circulation and by taking part in cellular cholesterol efflux and reverse cholesterol transport (3). However, such functions are not uniformly effective because apo E is present in the population in 3 main isoforms (apo E2, apo E3, and apo E4) that determine apo E concentrations and differ in their affinity to bind to the specific receptors (4, 5). In addition to the effects on plasma lipids, apo E has been reported to influence LDL oxidation in vivo and in vitro. Apo E has antioxidant activity, and this activity differs in extent according to isoform and may be related to the ability of apo E to sequester copper in vitro (6). Other studies have shown that ApoE knockout mice are highly susceptible to developing atherosclerosis (7), at least partly because their LDL is more susceptible to oxidation (8) and also because they lose their normal resistance to cholesterol feeding. This effect is reversible by antioxidant supplementation of their diet (9).

In recent years, the interaction between lipoprotein responsiveness to dietary intervention and APOE genotypes has been analyzed; however, the results are controversial (10). Whereas some studies found a pronounced dietary responsiveness among APOE4 carriers, others reported no difference in response across APOE genotypes to changes in dietary fat or cholesterol content (11–14). This fact suggests that other genetic or environmental factors are likely to be responsible for the association of the APOE gene with individual response to diet.

In accordance with this hypothesis, polymorphisms in the proximal promoter region of the APOE gene were recently described at positions −491A→T, −427T→C, and −219G→T (15, 16). In particular, there is experimental evidence, both in vitro and in vivo, that the APOE gene promoter −219G→T polymorphism produces variations in the transcriptional activity

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of the gene. Specifically, the $-219G$ allele shows a higher transcriptional activity than does the $-219T$ allele (15). A study of a European population including control individuals and multi-infarct patients showed that the $-219G\rightarrow T$ polymorphism is also associated with differential plasma apo E concentrations (17), illustrating that this polymorphism influences APOE expression in vivo. Furthermore, the $-219T$ allele is associated with an increased risk of myocardial infarction (17) and with premature coronary artery disease (18).

The mechanisms by which the $-219T$ allele of the APOE gene enhances atherothrombosis remain to be elucidated. In a previous study, we observed that the presence of this polymorphism determines serum apo E concentrations and influences the metabolism of triacylglycerol-rich lipoproteins during the postprandial period (19). In other study, the $-219T$ allele was associated with insulin resistance (18). However, in the study by Lambert et al (17), the $-219G\rightarrow T$ polymorphism was not associated with plasma lipid or lipoprotein concentrations. Thus, the aim of the present study was to determine whether the APOE gene promoter $-219G\rightarrow T$ polymorphism modifies the susceptibility of LDL to oxidation and the lipid response to the quantity and quality of dietary fat in healthy men with the APOE3/E3 genotype.

SUBJECTS AND METHODS

Subjects

Because APOE genotypes have been implicated in a variable lipid response to dietary changes, we studied the effect of the $-219G\rightarrow T$ polymorphism in APOE3/E3 subjects, to the exclusion of other apo E isoforms. A group of 55 men (7 with the GG genotype, 38 with the GT genotype, and 10 with the TT genotype) were recruited from among students at the University of Cordoba. The subjects had a mean ($\pm$SD) age of $21 \pm 0.8$ y. All subjects underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrollment. The subjects showed no evidence of any chronic disease (hepatic, renal, thyroid, or cardiac dysfunction), obesity, or unusually high levels of physical activity (eg, sports training). None of the subjects had a family history of premature coronary artery disease or had taken medications or vitamin supplements in the 6 mo before the study. Physical activity and diet, including alcohol consumption, were recorded in a personal diary for 1 wk, and the data were used to calculate individual energy requirements. Mean body mass index (BMI; in kg/m$^2$) was $22.86 \pm 0.28$ at the onset of the study and remained constant throughout the experimental period. The subjects were encouraged to maintain their regular physical activity and lifestyle and were asked to record in a diary any event that could affect the outcome of the study, such as stress, change in smoking habits or alcohol consumption, and intake of foods not included in the experimental design. The study protocol was approved by the Human Investigation Review Committee at the Reina Sofia University Hospital, and informed consent was obtained from all participants.

Diets

The study design included an initial 28-d period during which all subjects consumed a saturated fatty acid (SFA)-rich diet containing 15% protein, 47% carbohydrates, and 38% fat ($<$10% SFA, 6% PUFA, and 22% MUFA) for 28 d. This diet was followed for 28 d by a carbohydrate (CHO)-rich diet containing 15% protein, 55% carbohydrates, and $<$30% fat ($<$10% SFA, 6% PUFA, and 12% MUFA). The other 27 subjects consumed the CHO diet before the MUFA diet. The cholesterol content remained constant ($<300$ mg/d) during the 3 periods. Eighty percent of the MUFA diet was provided by virgin olive oil, which was used for cooking, salad dressing, and as a spread. The carbohydrate intake of the CHO diet was based on the consumption of biscuits, jam, and bread. Butter and palm oil were used during the SFA dietary period.

The composition of the experimental diets was calculated by using the US Department of Agriculture (20) food tables and Spanish food-composition tables for local foodstuffs (21). All meals were prepared in the hospital kitchen and were supervised by a dietician. Lunch and dinner were consumed in the hospital dining room, whereas breakfast and an afternoon snack were eaten in the medical school cafeteria. Fourteen menus were prepared with regular solid foods and were rotated during the experimental period. Duplicate samples from each menu were collected, homogenized, and stored at $-70 \, ^\circ\text{C}$. The protein, fat, and carbohydrate contents of the diets were analyzed by standard methods (22). Dietary compliance was verified by analyzing the fatty acids in LDL cholesterol esters at the end of each dietary period (23). The study took place from January through March to minimize seasonal effects and academic stress.

Analysis of fatty acids in LDL cholesterol esters

Fatty acids were analyzed by gas-liquid chromatography. Lipids were transmethylated as previously described (23). The resulting fatty acid methyl esters were eluted with hexane and were analyzed by gas-liquid chromatography with a model 5890 series II gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector and an Omegawax 320 fused-silica capillary column (30 m x 0.32 mm internal diameter, 0.25-$\mu$m film) obtained from Supelco (Bellefonte, PA) with helium as the carrier gas.

Lipid analysis and biochemical determinations

Venous blood samples were collected at the beginning of the study and at the end of each dietary period into EDTA-containing (1 g/L) tubes from all subjects after they had fasted for 12 h overnight. Plasma was obtained by low-speed centrifugation (1500 $\times$ g) for $15 \, \text{min}$ at $4 \, \text{°C}$ within 1 h of venipuncture. To reduce interassay variation, plasma was stored at $-80 \, ^\circ\text{C}$ and was analyzed at the end of the study. Plasma cholesterol and triglyceride concentrations were measured by enzymatic techniques (24, 25). HDL cholesterol was measured after precipitation with phosphotungstic acid (26). Apo A-I and B were determined by immunonoturbidimetry (27). LDL-cholesterol concentrations were calculated by using the Friedewald formula (28).

Oxidation of LDL

LDL was isolated from fresh plasma samples by sequential ultracentrifugation with a Beckman model LE-70 ultracentrifuge with a type NYT65 rotor (Beckman, Palo Alto, CA) for 2 h at 405 000 $\times$ g and $4 \, ^\circ\text{C}$. The formation of conjugated dienes was measured by incubating 100 $\mu$g LDL protein with 5 $\mu$mol...
increase in oleic acid in the cholesterol esters was also seen after LDL cholesterol esters than were observed after the CHO and significantly greater (P < 0.001) after the SFA diet: apo A-I, and apo B after the 3 diets are shown in Table 3. Changes in diet were associated with significantly (P < 0.05) longer lag times and significantly lower concentrations of total (P < 0.001), LDL (P < 0.001), and HDL (P < 0.05) cholesterol; apo A-I (P < 0.05); and apo B (P < 0.001) after the CHO and MUFA diets. However, in comparison with the MUFA diet, the CHO diet was associated with significantly shorter lag times (P < 0.01) and significantly lower plasma concentrations of HDL cholesterol (P < 0.05) and apo A-I (P < 0.01). No significant differences in triacylglycerol concentrations were observed after the different diets (P = 0.695).

A significant diet-by-genotype interaction effect was observed on apo B (P = 0.038), HDL-cholesterol (P = 0.005), and LDL-cholesterol (P = 0.048) concentrations (Table 3). Carriers of the T allele had significantly (P < 0.05) higher concentrations of apo B and LDL cholesterol after the SFA diet than did subjects homozygous for the G allele. Thus, in TT and GT subjects, the decrease in apo B plasma concentrations was significantly (P = 0.049) greater than that in the GG subjects when the subjects switched from the SFA diet to the CHO one (TT: −14%; GT: −16%; GG: −5%). In carriers of the T allele, the decrease in LDL-cholesterol concentrations was significantly (P = 0.048) greater than that in GG subjects when the subjects switched from the SFA diet to the CHO diet (TT: −21%; GT: −17%; GG: −5%). However, when the SFA diet was compared with the MUFA diet, no significant differences in apo B or LDL-cholesterol concentrations were observed between genotypes.

CuSO₄/L in 1.0 mL phosphate-buffered saline medium. Absorbance at 234 nm was measured continuously every 3 min for 4 h at 37 °C in a spectrophotometer as previously described (29). Results are expressed as the duration of the lag time before propagation of the LDL oxidation reaction, which was determined by the absolute increase in absorbance above the initial value.

DNA amplification and genotyping
Genomic DNA extraction and APOE E2, E3, E4 (30) and −219G→T APOE (12–14) genotypes were determined as previously described. Digested DNA was separated by electrophoresis on an 8% nondenaturing polyacrylamide gel at 150 V for 2 h. Bands were visualized by silver staining. Samples containing the T allele of the −219G→T polymorphism were amplified a second time to verify the genotype.

Statistical analysis
We used analysis of variance for repeated measures to test for effects of the APOE −219G→T polymorphism on plasma total cholesterol, LDL-cholesterol, HDL-cholesterol, triacylglycerol, apo A-I, and apo B concentrations in each dietary stage. When statistical significance was found, Tukey’s post hoc comparison test was used to identify between-group differences. Statistical analyses were carried out by using SPSS statistical software, version 8.0 (SPSS Inc, Chicago).

RESULTS
We observed no significant differences in any of the variables studied when we compared the baseline characteristics of the subjects who were homozygous for the G allele (GG; n = 7) with the subjects heterozygous for the T allele (GT; n = 38) and the subjects homozygous for the T allele (TT; n = 10) (Table 1). The composition of the participants’ mean daily intake is shown in Table 2. Analysis of LDL cholesterol esters obtained after each dietary period showed good adherence during the different intervention stages. After the SFA diet period, we observed a significantly greater (P < 0.005) increase in palmitic acid in the LDL cholesterol esters than were observed after the CHO and MUFA diets: 27.3 ± 1.4% compared with 19.8 ± 3.9% and 15.2 ± 0.4%, respectively. A significantly greater (P < 0.05) increase in oleic acid in the cholesterol esters was also seen after

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline anthropometric characteristics and plasma lipid and apolipoprotein (apo) concentrations according to −219G→T APOE promoter polymorphism^</th>
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<tbody>
<tr>
<td></td>
<td>GG (n = 7)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>20.6 ± 1.6</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 2.4</td>
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<tr>
<td>Cholesterol (mmol/L)</td>
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<tr>
<td>Total</td>
<td>4.1 ± 0.5</td>
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<tr>
<td>LDL</td>
<td>2.4 ± 0.3</td>
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<tr>
<td>HDL</td>
<td>1.2 ± 0.2</td>
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<tr>
<td>Apo A-I  (g/L)</td>
<td>1.2 ± 0.2</td>
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<tr>
<td>Apo B    (g/L)</td>
<td>0.5 ± 0.1</td>
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<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.0 ± 0.4</td>
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^ All values are x ± SD. There were no significant differences between genotype groups by ANOVA.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Daily intake during each experimental diet period^</th>
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<tr>
<td></td>
<td>SFA diet</td>
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<tr>
<td>Protein (% of energy)</td>
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</tr>
<tr>
<td>Calculated</td>
<td>15</td>
</tr>
<tr>
<td>Analyzed</td>
<td>18.0</td>
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<tr>
<td>Fat (% of energy)</td>
<td></td>
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<tr>
<td>Saturated</td>
<td>20</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>22.2</td>
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<tr>
<td>Carbohydrates (% of energy)</td>
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<tr>
<td>Calculated</td>
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<tr>
<td>Analyzed</td>
<td>44.2</td>
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<tr>
<td>Fiber (g/d)</td>
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<td>Calculated</td>
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<tr>
<td>Analyzed</td>
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</tr>
<tr>
<td>Energy (MJ)</td>
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<tr>
<td>Calculated</td>
<td>10.2</td>
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</tbody>
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^SFA diet, saturated fatty acid–rich diet; CHO diet, low-fat, high-carbohydrate diet; MUFA diet, monounsaturated fatty acid–rich diet.

The −219G→T polymorphism had a significant effect on the lag time before propagation of the LDL oxidation reaction (P = 0.025). Compared with carriers of the G allele, TT subjects had significantly (P < 0.05) shorter lag times after the SFA diet. In addition, there was a significant diet-by-genotype interaction effect on lag times (P = 0.032). Thus, the replacement of the SFA diet by the CHO diet induced a greater increase (P = 0.047) in the lag time in TT subjects (14.2 min, or 72%) than in carriers of the G allele (10.3 min, or 34%). In the same way, a significantly (P < 0.001) greater increase in lag time (29.25 min, or 154%) was observed in TT subjects than in carriers of the G allele (21.3 min, or 129%). The SFA diet was compared with the MUFA diet. In addition, we observed a significant correlation (r = −0.27, P = 0.005) between the increase in LDL cholesterol and the decrease in lag time.

**DISCUSSION**

Our results show that the presence of the T allele in the APOE −219G→T polymorphism increases the susceptibility of plasma LDL to oxidative modifications and enhances the response of apo B and LDL cholesterol to the presence of SFA in the diet of healthy men. Previous studies suggested that differences in individual response to diet exist. Therefore, the influence of the genetic loci of the principal apolipoproteins, such as the A1-CIII-AIV complex and the APOE gene, has been studied. The allelic variations of these genes influence the degree of response by both HDL and LDL cholesterol (31, 32). Thus, the hyperresponse of LDL-cholesterol concentrations associated with the E4 allele occurs only when the fat content of the diet is varied (33). However, the inconsistencies observed in several studies (10–14) suggest that other genetic or environmental factors may interact with the APOE gene in determining the individual response to diet. Thus, in our study, we observed that carriers of the T allele showed a higher response of LDL cholesterol and apo B when consuming an SFA-rich diet. Furthermore, in carriers of the T allele, the decrease in LDL cholesterol and apo B was significantly (P < 0.05) higher when they changed from the SFA to the CHO diet.

The −219T allele is associated with an increased risk of myocardial infarction, premature coronary artery disease, and decreased plasma apo E concentrations (17, 18). These facts suggest that the basal ability of cells to synthesize and secrete apo E may be of particular relevance in atherothrombosis. Studies have shown that the genetic expression of APOE modifies lipoprotein metabolism in animals. In transgenic mice, an increase in APOE expression produces an increase of up to 300% in the uptake of chylomicron remnants, VLDL, and LDL (34). In addition, the uptake of apo B–containing lipoproteins was increased by the LDL receptor, probably through an interaction with the LDL-receptor-related protein. On the contrary, the total suppression of APOE expression results in a massive accumulation of cholesterol-rich VLDL-like remnants and also LDL-like particles. The higher plasma LDL concentrations and the prolonged circulation of these atherogenic lipoproteins in the plasma of ApoE knockout mice (E0) cause an oxidative stress that is associated with the increased susceptibility of the lipoprotein to oxidation, especially when the animals are stressed by an atherogenic diet. This phenomenon could explain our results, wherein TT subjects, with lower apo E plasma concentrations (17, 19) and higher plasma LDL concentrations, had a higher susceptibility of plasma LDL to oxidative modifications when consuming an SFA-rich diet. In accordance with this hypothesis, we observed a significant correlation between increases in LDL cholesterol and decreases in lag time. In addition, apo E can protect against atherosclerosis because it acts as an antioxidant (35). Thus, studies in E0 mice have reported an increase in LDL oxidation (8, 36). Moreover, E0 mice that were fed an antioxidant-rich diet showed
greater resistance to in vitro oxidation, and the extent of atherosclerosis was significantly lower than that in untreated mice. E0 mice fed high-fat, low-cholesterol diets enriched with olive oil showed a reduction in atherosclerotic lesions and a decrease in hepatic lipid peroxidation (37).

We recently observed that the presence of the $-219G \rightarrow T$ polymorphism influences the metabolism of triacylglycerol-rich lipoproteins during the postprandial phase, thus prolonging postprandial lipemia in subjects with the TT genotype (19). Postprandial lipemia affects several steps in lipoprotein metabolism that may mediate effects on LDL susceptibility to oxidation in addition to those related to variations in plasma triacylglycerol-rich lipoproteins. Thus, the increased plasma triacylglycerol-rich lipoprotein concentration during the postprandial phase causes cholesterol exchange between triacylglycerol-rich lipoproteins and LDL and HDL mediated by cholesteryl ester transfer protein. In the postprandial state, it is postulated that the extent of exchange may be determined by particle residence time in the circulation (38). This implies an enhanced exchange in subjects with prolonged postprandial lipemia, as in TT subjects. The resultant triacylglycerol-enriched LDL and HDL particles are subject to lipolysis by hepatic lipase, thus forming small, dense particles that are more susceptible to lipid peroxidation and therefore more atherogenic (39). These facts may partially explain the mechanisms by which the $-219T$ allele of the APOE gene enhances the susceptibility of LDL to oxidation.

LDL susceptibility to oxidation is determined by antioxidant content, fatty acid composition, and the size of the particle. As shown previously, we observed that a shift from an SFA-rich diet or a diet rich in carbohydrates to a MUFA-rich diet increases the resistance of LDL particles to oxidation (40). MUFA-rich diets contain variable amounts of antioxidant micronutrients and phytochemicals, which may beneficially increase LDL oxidation resistance beyond that due to fatty acid composition (41). The mechanism of the increased oxidative stress of LDL when subjects consume an SFA diet, given that these fatty acids are not prone to oxidation, probably involves other factors, such as the LDL concentration and the particle residence time in the circulation. Thus, the SFA diet was associated with significantly higher plasma LDL-cholesterol concentrations than was either hypolipidemic diets. Furthermore, we observed a correlation between increases in LDL cholesterol and decreases in lag time.

Our data seem to indicate that the $-219G \rightarrow T$ polymorphism influences lipoprotein concentrations and LDL susceptibility to oxidation according to the presence of SFAs in the diet of healthy, young, normolipemic men. We included only healthy, young, normolipemic men to avoid the effect of other factors (age, sex, BMI, etc) on lipid response to the content and quality of dietary fat. Studies conducted with conditions representing impaired metabolism, such as in dyslipoproteinemic subjects, will generally be more successful in finding differential effects across APOE genotypes, and such studies may be helpful in clarifying APOE-nutrition relations. In contrast, the results of the present study suggest the use of genotyping of the APOE $-219G \rightarrow T$ polymorphism in the design of more precise dietary counseling and intervention and more efficacious primary and secondary coronary artery disease prevention.

In conclusion, healthy normolipidemic men who were carriers of the T allele were more susceptible to the presence of SFA in the diet because of a greater increase in apo B, LDL-cholesterol concentrations, and oxidative modifications in LDL. The allelic variability in the APOE gene promoter polymorphism may partially explain the differences in individual response to diet.

JAM was responsible for data collection, data analysis, and writing of the manuscript. CM and PG contributed to the collection and analysis of data. PP-M provided statistical advice and contributed to the writing of the manuscript. CB and RM contributed to the collection of data and the writing of the manuscript. FF was responsible for the design of the study and analysis of the data. FP-J and JL-M were responsible for the conception and design of the study, analysis of data, and writing of the manuscript. None of the authors had any conflicts of interest.

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INTERACTION OF DIET AND APOE POLYMORPHISM