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Two Independent Apolipoprotein A5 Haplotypes Modulate Postprandial Lipoprotein Metabolism in a Healthy Caucasian Population

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Background: Apolipoprotein A5 (APOA5) plays an important role in plasma triacylglycerol (TG) homeostasis. Five polymorphisms (1131T>C, c.-3A>G, c.56C>G, IVS3+476G>A, and c.1259T>C) in the *APOA5* gene define three common haplotypes (*APOA5**1, *APOA5**2, and *APOA5**3) in Caucasian individuals. Our aim was to determine whether these haplotypes could modulate the postprandial response in young healthy males.

Design and Methods: Eighty-eight APO E3/3 volunteers [67 with (−1131T and 56C) *APOA5*1* haplotype, 12 with (−1131C and 56C) *APOA5*2* haplotype, and nine with (−1131T and 56G) *APOA5*3* haplotype] underwent a fat load test consisting of the consumption of 1 g of fat per kilogram body weight and 60,000 IU vitamin A. Blood samples were taken at time 0, at every hour until the sixth hour, and at every 2.5 h until the 11th hour. Total plasma cholesterol (C) and TG, and C, TG, apolipoprotein B-100, apolipoprotein B-48, and retinyl palmitate in lipoprotein fractions were determined.

Results: Subjects with the *APOA5*2* and *APOA5*3* haplotypes had a higher area under the curve of total plasma TG ($P = 0.03$), large TG-rich lipoprotein (TRL)-TG ($P = 0.02$), small TRL-TG ($P = 0.04$), small TRL-C ($P = 0.04$), large TRL-C ($P = 0.03$), and small apolipoprotein B100 ($P = 0.04$) than subjects with the APOA5*1 haplotype.

Conclusions: Our findings show that the presence of the *APOA5*2* and *APOA5*3* haplotypes in the *APOA5* gene is associated with a higher postprandial response that could be involved in the higher risk of coronary heart disease associated with the 56G and −1131C alleles. **(*J Clin Endocrinol Metab* 92: 2280–2285, 2007)**

THE POSTPRANDIAL STATE is the normal metabolic situation in human beings throughout the day in developed societies because the clearance of triacylglycerol (TG)-rich lipoprotein (TRL) particles of intestinal or hepatic origin continues for 6–8 h. Previous studies suggested that TRL plays an important role in the development of atherosclerosis because both coronary artery disease and myocardial infarcts have been associated with abnormal postprandial lipoprotein patterns (1). The basic mechanisms involved in postprandial lipemia are relatively well known, and the effects of different nutrients on the variability of the postprandial response are under active study. Less is known, however, about the dramatic interindividual variability observed during this period (2). In this respect, nutrigenomics is emerging as a multidisciplinary field that focuses on the interactions between nutritional and genetic factor and health outcomes (3). Several studies demonstrated that the presence of certain polymorphisms located in the A1-C3-A4 complex and other gene loci determine variation in the postprandial response (4–6).

Recently a gene that codes for apolipoprotein A5 (APOA5) has been identified in the proximity of cluster A1-C3-A4 on human chromosome 11 (7). Studies in knockout and transgenic mice revealed that its pattern of expression correlates negatively with plasma TG concentrations (8–10). These observations have also been verified in healthy subjects and patients with familial combined hyperlipidemia (11). Furthermore, the decrease in serum TG concentrations was associated with both diminished production of very low-density lipoprotein (VLDL) and a higher rate of VLDL catabolism (12).

Five polymorphisms (1131T>C, c.-3A>G, c.56C>G, IVS3 + 476G>A, c.1259T>C) were found to define three common haplotypes (denoted APOA5*1, APOA5*2, and APOA5*3) in Caucasian individuals (13). The presence of −1131C>T polymorphism, also known as SNP3, defines a haplotype called APOA5*2 (7, 14), whereas the 56C>G polymorphism, also known as S19W, defines another haplotype called APOA5*3 (7, 14) so that with these two variants we are able to obtain a total of three haplotypes, representing five polymorphic sites (14). These three haplotypes represented 82, 8, and 8% of the APOA5 chromosomes examined and thus comprise approximately 98% of APOA5 haplotypes in this population (Table 1) (13).

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Abbreviations: Apo, Apolipoprotein; APOA5, apolipoprotein A5; AUC, area under the curve; ANCOVA, analysis of covariance; C, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; RP, retinyl palmitate; SNP, single nucleotide polymorphism; TG, triacylglycerol; TRL, TG-rich lipoprotein; VLDL, very low-density lipoprotein.

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TABLE 1. Haplotypes of the APOA5 chromosomes examined

Haplotypes	1131T>C (SNP3) rs662799	c.-3A>G (kozak) rs651821	c.56C>G (S19W) rs3135506	IVS3+476G>A (SNP2) rs2072560	c.1259T>C (SNP1) rs2266788
*APOA5*1*	T	A	C	G	T
*APOA5*2*	*C*	*G*	C	*A*	*C*
*APOA5*3*	T	A	*G*	G	T

The SNPs are depicted in the following order: −1131T>C, c.-3A>G, c.56C>G, IVS3+476G>A, and c.1259T>C. The minor alleles that define the haplotypes are highlighted in *italics* (13). *APOA5*1* haplotype: −1131T and 56C; *APOA5*2* haplotype: −1131C and 56C; *APOA5*3* haplotype**:** −1131T and 56G.

Previous studies suggest that two of these haplotypes, described by the rare alleles −1131T>C and 56C>G, are associated with higher TG levels than the most common haplotype *APOA5*1* (13, 15, 16). The association between the 56C>G polymorphism and plasma TG concentrations is independent of the −1131T>C polymorphism that has previously been shown to be associated with increased plasma TG levels (13). Thus, genotyping for 1131T>C or 56C>G essentially acts as tagging single-nucleotide polymorphisms (SNPs) (14, 17).

These data indicate that APOAV plays an important role in plasma TG homeostasis and that polymorphisms in the APOA5 gene are associated with variation in plasma TG levels in humans. Our aim now is to determine whether these haplotypes could modulate the postprandial response in healthy Caucasian males.

Subjects and Methods

Study volunteers

Eighty-eight healthy men were recruited from among 250 students at the University of Cordoba. They had a mean ± SD age of 23 ± 4.12 yr. Of these, 67 with (−1131T and 56C) *APOA5*1* haplotype, 12 with (−1131C and 56C) *APOA5*2* haplotype, and nine with (−1131T and 56G) *APOA5*3* haplotype. This distribution of genotypes was expected from the Hardy-Weinberg equilibrium. Informed consent was obtained from all participants, none of whom had diabetes or liver, renal, or thyroid disease. All were selected to have the APOE E3E3 genotype to avoid the potential confounding effect on postprandial lipemia that is associated with the presence of the other common *APOE* alleles (18). None of the participants was taking medication or vitamins known to affect plasma lipids. The fasting plasma lipids, lipoproteins, apolipoproteins (Apo), age, and body mass index are shown in Table 2. All studies were carried out in the Research Unit at the Reina Sofia University Hospital, and the experimental procedure was approved by the hospital's Human Investigation Review Committee.

Vitamin A fat-loading test

After a 12-h fast, subjects were given a fatty meal enriched with 60,000 U vitamin A per square meter of body surface area. The fatty meal consisted of two cups of whole milk, eggs, bread, bacon, cream, walnuts, and butter. The amount of fat given was 1 g of fat and 7 mg of cholesterol (C) per kilogram of body weight. The meal contained 60% of energy as fat, 15% protein, and 25% carbohydrates and was eaten in 20 min. After the meal, the subjects ate no energy for 11 h but were allowed to drink water. Blood samples were drawn before the meal, every hour until the sixth hour, and every 2.5 h until the 11th hour.

Lipoprotein separation

Blood was collected in tubes containing EDTA to give a final concentration of 0.1% EDTA. Plasma was separated from red cells by centrifugation at 1500 × *g* for 15 min at 4 C. The chylomicron fraction of TRL (large TRL) was isolated from 4 ml of plasma overlayered with 0.15 mol/liter NaCl, 1 mmol/liter EDTA (pH 7.4, density < 1.006 kg/liter) by a single ultracentrifugal spin (20,000 rpm, 30 min, 4 C) in a 50-type rotor (Beckman Instruments, Fullerton, CA). Large TRLs, contained in the top layer, were removed by aspiration after cutting the tubes and the infranatant was centrifuged at a density of 1.019 kg/liter for 24 h at 45,000 rpm in the same rotor. The nonchylomicron fraction of TRL (also referred as small TRL) was removed from the top of the tube. All operations were done in subdued light. Large and small TRL fractions were stored at −70 C until assayed for retinyl palmitate (RP).

Lipid analysis

C and TG in plasma and lipoprotein fractions were assayed by enzymatic procedures (19, 20). Apo A-I and Apo B were determined by turbidimetry (21). High-density lipoprotein (HDL)-C was measured by analyzing the supernatant obtained after precipitation of a plasma aliquot with dextran sulfate-Mg^{2+}, as described by Warnick *et al.* (22). Low-density lipoprotein (LDL)-C was obtained as the difference between the C from the bottom part of the tube after ultracentrifugation at 1.019 kg/liter (23).

RP assay

The RP content of large and small TRL fractions was assayed using a method previously described (24). The RP concentration in each sample was expressed as the ratio of the area under the RP peak to the area under the retinyl acetate peak (25).

Determination of ApoB-48 and ApoB-100

ApoB-48 and ApoB-100 were determined by SDS-PAGE as described by Karpe and Hamsten (26). Gels were scanned with a video densitom-

TABLE 2. Baseline characteristics of plasma lipids and Apo according to the APOA5 haplotypes

	*APOA5*1* (n = 67)	*APOA5*2* (n = 12)	*APOA5*3* (n = 9)	*P*[a]
Age (yr)	22.77 ± 6.13	22.36 ± 2.29	23.62 ± 4.28	0.87
Body mass index (kg/m^2)	24.75 ± 3.59	27.04 ± 3.88	25.04 ± 2.69	0.18
Total C (mmol/liter)	3.84 ± 0.53	4.19 ± 0.42	4.11 ± 1.02	0.11
Total TGs (mmol/liter)	0.84 ± 0.40	0.96 ± 0.30	1.01 ± 0.29	0.44
LDL-C (mmol/liter)	2.25 ± 0.47	2.55 ± 0.21	2.54 ± 0.91	0.15
HDL-C (mmol/liter)	1.21 ± 0.26	1.17 ± 0.30	1.16 ± 0.21	0.82
Apo B (g/liter)	0.65 ± 0.14	0.70 ± 0.19	0.66 ± 0.16	0.52
Apo A-I (g/liter)	1.08 ± 0.19	0.99 ± 0.17	0.96 ± 0.17	0.13

Values are given as mean ± SD. *APOA5*1* haplotype: −1131T (rs 662799) and 56C (rs3135506); *APOA5*2* haplotype: −1131C and 56C; *APOA5*3* haplotype**:** −1131T and 56G.

[a] One-way ANOVA or the Kruskal-Wallis test.

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eter scanner (TDI, Madrid, Spain) connected to a personal computer for integration of the signals. Background intensity was calculated after scanning an empty lane. The coefficient of variation for the SDS-PAGE was 7.3% for apoB-48 and 5.1% for apoB-100.

SNP genotyping

DNA was extracted from 10 ml of EDTA-containing blood using DNA blood midi kits (QIAGEN, Hilden, Germany) according to the vendor's recommended protocol. Two APOA5 SNPs, − 1131T>C (rs662799) (7) and 56C>G (rs3135506) (27), were used to define the three APOA5 haplotypes studied. Our nomenclature is in agreement with that suggested by the Human Genome Variation Society. The methods, primer, and probes used for genotyping were described previously (7, 27). Standard laboratory practices were used to ensure the accuracy of genotype data. Internal controls and repetitive experiments were used.

Statistical analysis

Several variables were calculated to characterize the postprandial responses of plasma TG, large TRLs, and small TRLs to the test meal. The area under the curve (AUC) was defined as the area between the plasma concentration *vs.* time curve and a line drawn parallel to the horizontal axis. These areas were calculated by a computer program using the trapezoidal rule. Data were tested for statistical significance between genotypes by ANOVA (one-way ANOVA) or the Kruskal-Wallis test and between genotypes and time by general linear model for repeated measures [analysis of covariance (ANCOVA)], adjusted for the following covariates: CT and TG plasma. In this analysis, we studied the effect of the interaction of both factors (genotype and time), which is indicative of the magnitude of the postprandial response in each group of subjects. The contrast statistic used when the sphericity assumption was not satisfied was Greenhouse-Geisser.

We also carried out a multivariate ANOVA (multiple analysis of covariance) to analyze the AUC by means of a general linear model, using cholesterol and basal triglycerides as covariates.

$P < 0.05$ was considered significant. All data presented in the text and tables are expressed as means ± SD. SPSS 11.0 (SPSS Inc., Chicago, IL) was used for the statistical comparisons.

Results

The baseline characteristics of the subjects are shown in Table 2. Significant differences were not observed in any of the variables analyzed at baseline between subjects who were carriers of the common alleles −1131T and 56C (*APOA5*1* haplotype, n = 67), carriers for the −1131C and 56C rare allele (*APOA5*2* haplotype, n = 12), and subjects with the −1131T and 56G rare allele (*APOA5*3* haplotype, n = 9), located in the *APOA5* gene.

Analysis of the interaction between genotype and time showed that carriers of the *APOA5*1* haplotype have a lower postprandial response in total plasma TG ($P = 0.024$), large TRL-C ($P = 0.028$), and large TRL-TG ($P = 0.015$) than subjects who are carriers of the *APOA5*2* and *-3* haplotypes (Fig. 1). In addition, carriers of *APOA5*1* have a lower postprandial response in small TRL-C ($P = 0.031$) and small TRL-TG ($P = 0.038$) than carriers for the *APOA5*2* and *-3* haplotypes (Fig. 2). Furthermore, the AUC TG, TG, and C in both large and small TRLs were all greater in carriers of the *APOA5*2* and *-3* haplotypes than carriers of the *APOA5*1* haplotype ($P < 0.05$) (Table 3).

ApoB-100 and ApoB-48 in large and small TRLs were also analyzed. Significant differences were not found in the ApoB-48 in large or small TRLs or for the ApoB-100 in large TRLs among the three haplotypes (Table 3). However, a significant effect of the interaction between genotype and time was observed for the ApoB-100 levels in small TRLs ($P < 0.05$) (Fig. 2C). Carriers of the *APOA5*2* haplotype showed a higher postprandial response in ApoB-100 levels in the small TRL than carriers of the common allele (*APOA5*1*). Moreover, the AUC of ApoB-100 in small TRLs ($P < 0.05$) was greater in carriers of the *APOA5*2* haplotype than carriers of the *APOA5*3* haplotype (Table 3).

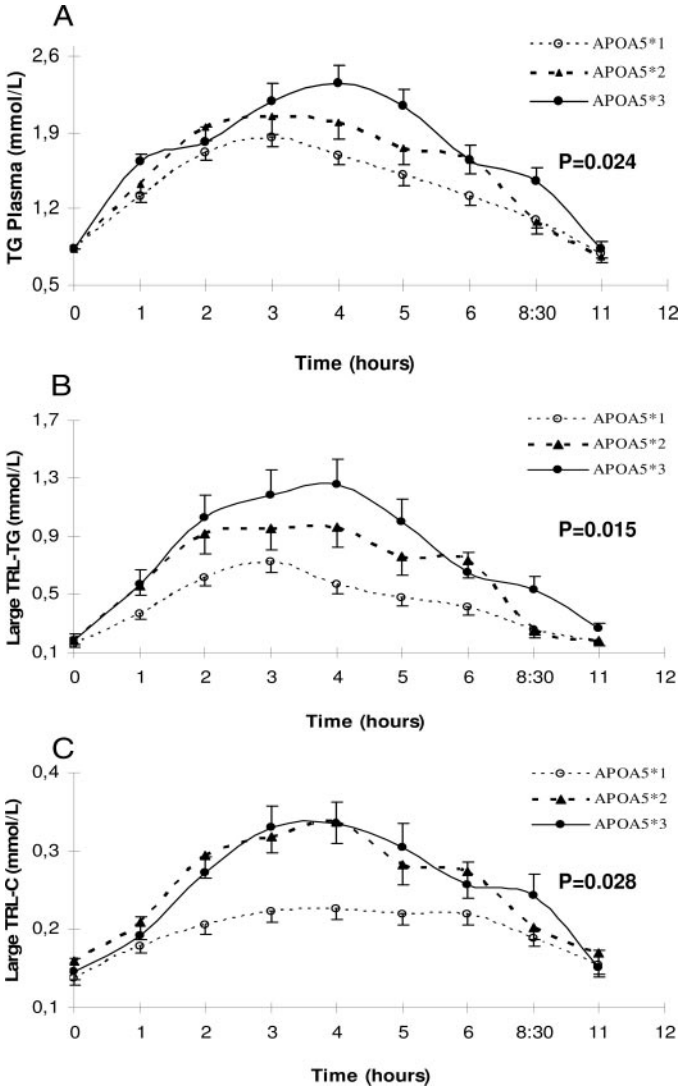

FIG. 1. Line plots of postprandial TG plasma (A), large TRL-TG (B), and large TRL-C (C) response in APOA5*3 subjects (n = 9, *solid line*, *black circles*), APOA5*2 subjects (n = 12, *dashed line*, *black triangles*), and APOA5*1 subjects (n = 67, *dotted line*, *white circles*). *P*, Genotype by time interaction; general linear model for repeated measures (ANCOVA); mean ± SEM.

Discussion

Our results show that, after a fatty meal, subjects carrying the *APOA5*2* and *APOA5*3* haplotypes present a higher postprandial response in plasma TG, large and small TRL-TG, and Apo B-100 in small TRLs than subjects with the *APOA5*1* haplotype.

Several studies demonstrated that the presence of polymorphisms in the A1-C3-A4 cluster and other gene loci de-

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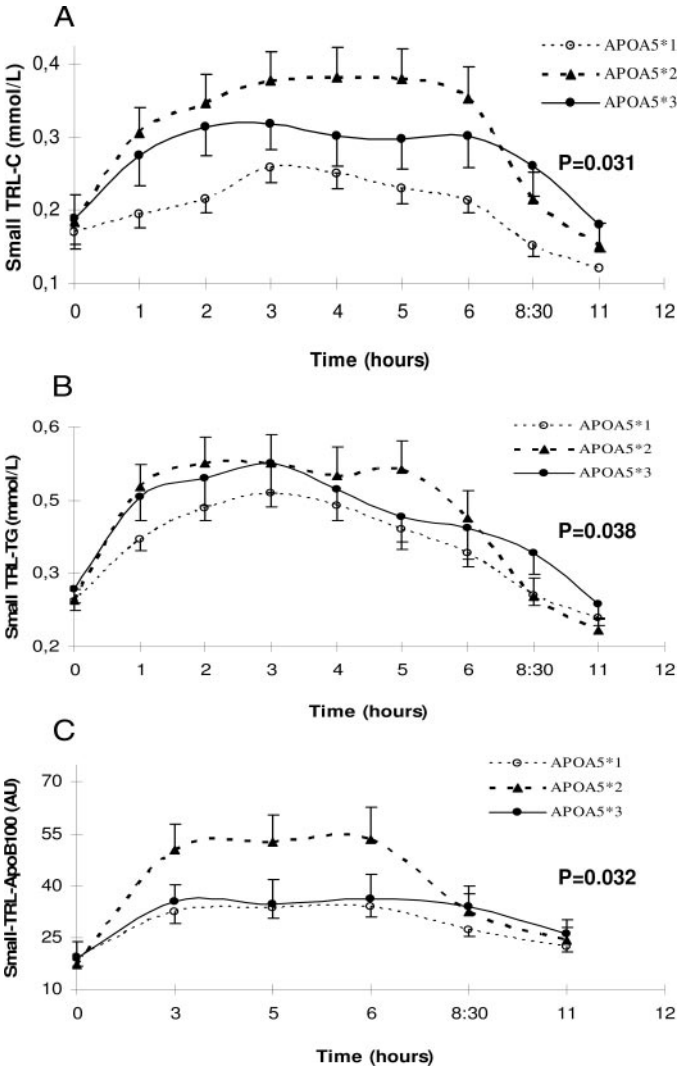

FIG. 2. Line plots of postprandial small TRL-C (A), small TRL-TG (B), and small TRL-apoB-100 (C) response in APOA5*3 subjects (n = 9, *solid line*, *black circles*), APOA5*2 subjects (n = 12, *dashed line*, *black triangles*), and APOA5*1 subjects (n = 67, *dotted line*, *white circles*). *P*, Genotype by time interaction; general linear model for repeated measures (ANCOVA); mean ± SEM.

termines the variability of the postprandial lipoprotein response (5, 28). Recently a gene coding for APOA5 was identified in this cluster, and this is emerging as a main candidate gene for modulating TG metabolism in humans (7). Two polymorphisms, − 1131T>C and 56C>G, have been extensively studied and are independently associated with higher TG levels (13, 15, 16). Previous studies have shown that plasma TG concentrations were 69% higher in CC subjects than TT subjects with the −1131T>C polymorphism (29, 30) and 20–30% higher in CG than CC subjects with the 56C>G polymorphism (13, 14). We demonstrated that carriers of the −1131C allele (−1131T>C) displayed a higher postprandial response in plasma TG concentrations (31). However, association studies using haplotypes should increase our ability to detect true associations and interactions. To the best of our knowledge, this study is the first to determine whether different haplotypes of APOA5 gene modulate the postprandial response. Thus, our data show markedly higher postprandial responses in both large and small TRLs in subjects with the *APOA5*2* and *APOA5*3* haplotypes, which may explain the higher risk of coronary heart disease associated with the 56G and −1131C alleles (14, 32).

In our study, both the *APOA5*2* and *APOA5*3* haplotypes were significantly associated with increases in TRLs and had roughly similar frequencies (13 and 10%). However, these haplotypes exhibit different associations with the postprandial response of ApoB-100 at TRL concentrations, whereas the *APOA5*3* haplotype has no effect on ApoB-100 in small TRL levels. *APOA5*2* displayed a higher postprandial increase in small TRL ApoB-100 than the *APOA5*1* haplotype, which is indicative of an increase in the number of small TRL particles. These results thus suggest that each of these haplotypes may be associated with different mechanisms that enhance the postprandial response and the risk of atherogenesis.

There are several mechanisms potentially capable of explaining why these polymorphisms may be responsible for our findings. The APOA5 −1131 T>C polymorphism (*APOA5*2*) was found to be associated with significant differences in APOAV plasma concentration, and the presence of the C allele has been linked to lower plasma APOAV concentrations (33). On the other hand, he *APOA5*3*, which is defined by the minor allele of the 56C, results in a nonsynonymous change at position 19 of APOAV from serine to tryptophan, which may possibly change the structure and function of APOAV (13). The 56C>G allele thus causes a change in the secondary structure of APOAV, with a concomitant change in its tertiary structure, as it lengthens the initial α-helix segment by increasing the tendency of residues 14–16 to adopt an α-helix conformation. The structure alteration may therefore lead to the malfunction of APOAV in lipid metabolism, probably via an alteration in the efficiency either of the insertion of the nascent polypeptide chain into the endoplasmic reticulum lumen or cleavage of the signal peptide or the altered lipid affinity (34).

Another possibility is related to TRL catabolism. In this sense TG concentrations increased dramatically with the suppression of APOA5 expression, whereas overexpression of this apoprotein had the opposite effect (7). Furthermore, VLDLs that lack APOAV do not bind as well to the LDL receptor as normal or APOAV-enriched VLDL, and this may explain the lower rate of removal of small TRLs observed in mice lacking APOA5 (35). Higher levels of plasma APOAV may additionally be related to enhanced lipoprotein lipase (LPL) activity, increasing the conversion of TRLs and stimulating remnant formation and hepatic clearance (12). This phenomenon may be capable of explaining the higher postprandial response observed in carriers of the *APOA5*2* and *APOA5*3* haplotypes. Thus, APOAV induced both an increase in lipolysis, through the increase in the LPL activity, and more active removal of VLDL particles (12). Our results also show that the effect of APOAV on LPL decreases the hydrolysis of TG in large TRLs without a change the number of these particles, and this fact may be due to the lack of significant differences in RP or apoB-48 in TRL because ApoB is a marker of particle number. Although these results are preliminary, the fact that our study was carried out on

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TABLE 3. Area under the postprandial curve according to the APOA5 haplotypes gene

	*APOA5*1*	*APOA5*2*	*APOA5*3*	*P*
Large TRL-RP (ng·sec/ml)	1293 ± 670	1301 ± 909	1321 ± 960	0.69
Small TRL-RP (ng·sec/ml)	690 ± 426	688 ± 556	685 ± 510	0.94
Plasma TG (mmol·sec/liter)	0.22 ± 0.02	0.28 ± 0.05	0.32 ± 0.07	**0.03**
Large TRL-TG (mmol·sec/liter)	0.08 ± 0.03	0.15 ± 0.07	0.18 ± 0.08	**0.02**
Small TRL-TG (mmol·sec/liter)	0.10 ± 0.04	0.15 ± 0.07	0.14 ± 0.06	**0.04**
Large TRL-C (mmol·sec/liter)	0.02 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	**0.03**
Small TRL-C (mmol·sec/liter)	0.04 ± 0.03	0.07 ± 0.02	0.06 ± 0.04	**0.04**
LDL-C (mmol·sec/liter)	0.43 ± 0.07	0.48 ± 0.08	0.47 ± 0.10	0.38
HDL-C (mmol·sec/liter)	0.22 ± 0.04	0.21 ± 0.05	0.21 ± 0.05	0.48
Large TRL apo B-100 (AU)	9.88 ± 10.04	8.21 ± 13.98	8.71 ± 12.91	0.32
Small TRL apo B-100 (AU)	182 ± 65	628 ± 256	219 ± 138	**0.04**
Large TRL apo B-48 (AU)	5.13 ± 3.99	3.28 ± 2.85	6.71 ± 5.33	0.64
Small TRL apo B-48 (AU)	5.11 ± 5.01	3.25 ± 4.03	5.64 ± 7.07	0.32

General linear model for multivariate (multiple analysis of covariance). *Bold* font indicates statistically significant differences ($P < 0.05$). Values are given as (mean ± SD) × 10^{-5}, except for ApoB-48 and ApoB-100 (mean ± SD) × 10^{-2} in both large and small TRLs. *5*1* haplotype: −1131T (rs 662799) and 56C (rs3135506); *APOA5*2* haplotype: −1131C and 56C; *APOA5*3* haplotype: −1131T and 56G. AU, Arbitrary units.

healthy individuals in a controlled intervention trial could be the key to a better understanding of APOA5 activity and its role in lipoprotein metabolism.

In conclusion, our data suggest that haplotypes *2* and *3* in the APOA5 gene may influence TRL metabolism, inducing a higher postprandial lipemia response than in subjects with haplotype *1*. Studies of the association between these haplotypes and coronary heart disease may provide insights into the role of hypertriglyceridemia in atherogenesis.

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Erratum

In the article "Effect of Monitoring Bone Turnover Markers on Persistence with Risedronate Treatment of Postmenopausal Osteoporosis" by Pierre D. Delmas, Bernard Vrijens, Richard Eastell, Christian Roux, Huibert A. P. Pols, Johann D. Ringe, Andreas Grauer, David Cahall, Nelson B. Watts, on behalf of the Improving Measurements of Persistence on Actonel Treatment (IMPACT) Investigators (*The Journal of Clinical Endocrinology & Metabolism* **92:**1296-1304), there was an error in the tabular portion of Fig. 5. The arrows next to 'BTM' (a downward arrow in the third row and an upward arrow in the fifth row) were omitted. The corrected figure and legend are printed *below*. *The printer regrets the error*.

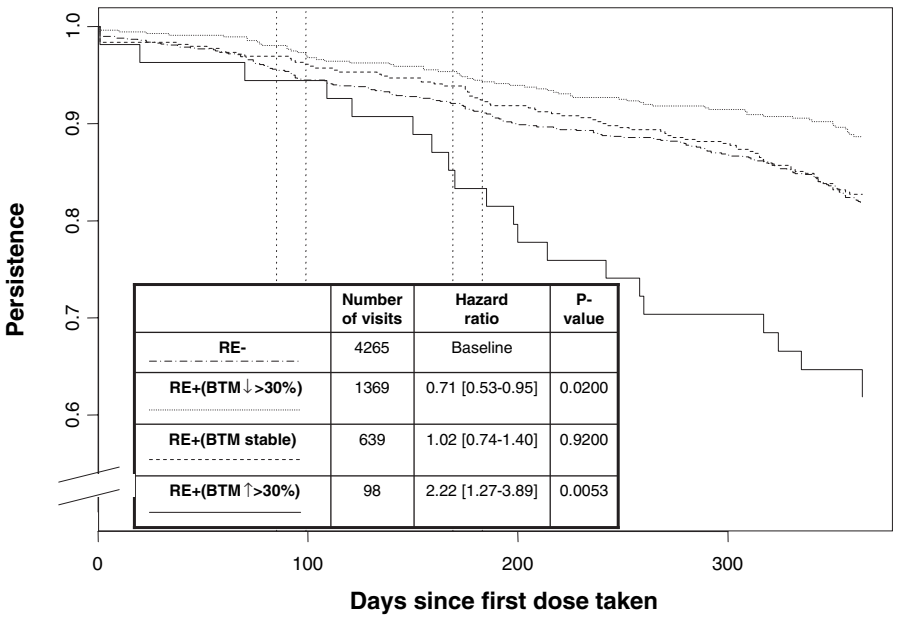

FIG. 5. Kaplan-Meier survival curves to show effect of feedback (uNTX) based on persistence (n = 2302). Because the reinforcement message delivered at wk 13 and 25 could be different for the same patient, graphical representation of the model was assessed by classifying the patients in RE+ into three message categories: good, more than 30% decrease from baseline in uNTX at wk 10 and 22; stable, at least one stable uNTX response at wk 10 or 22 and no increase in uNTX more than 30%; and poor, at least one uNTX increase more than 30% at wk 10 or 22. The numbers of visits corresponding to good uNTX response (1369 visits) or stable uNTX response (639 visits) were higher than those corresponding to a poor uNTX response (98 visits).

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