ApoCIII Gene Variants Modulate Postprandial Response to Both Glucose and Fat Tolerance Tests

Dawn M. Waterworth, PhD; Josep Ribalta, PhD; Viviane Nicaud, MA; Jean Dallongeville, PhD; Steve E. Humphries, PhD; Philippa Talmud, PhD; on behalf of the EARS Group

Background—We investigated the relationship between variation in the apolipoprotein (apo) AI-CIII-AIV gene cluster and response to an oral glucose test (OGTT) and oral fat load test (OFTT) in the EARSII group of young, healthy male offspring whose fathers had had a myocardial infarction before the age of 55 years (cases, n=407) compared with age-matched controls (n=415). The apoCIII variations examined were C3238G (SsrI) in the 3′-UTR, C1100T in exon 3, C-482T in the insulin response element (IRE), and T-2854G in the apoCIII-AIV intergenic region.

Methods and Results—The postprandial response was regulated by variation at the T-2854G and C3238G sites. After the OFTT, carriers of the rare alleles had delayed clearance of triglyceride (Tg) levels; G-2854 carriers showed the largest response (AUC, 24% greater, P<0.002; peak, 19% greater, P<0.005), and G3238 carriers showed a smaller response (AUC, 13% greater, P<0.05; peak, 13% greater, P=0.03). However, after adjustment for fasting level of Tg, only the effect with the T-2854G remained significant. Variation at the C-482T (IRE) determined response to the OGTT, with carriers of the rare T-482 having significantly elevated glucose (28.7% AUC, P=0.013) and insulin (20.5% AUC, P<0.01) concentrations.

Conclusions—These data suggest that specific genetic variants at the apoCIII gene locus differentially affect postprandial response and suggest a novel mechanism for the effects of variation at this locus on risk for atherosclerosis. (Circulation. 1999;99:1872-1877.)

Key Words: genes ■ apolipoproteins ■ diet

Plasma apolipoprotein CIII (apoCIII) is a major component of triglyceride (Tg)-rich lipoproteins (chylomicrons and VLDL) and a minor component of HDL. The mature 79-amino-acid apoCIII protein is synthesized predominantly in the liver but also to a lesser extent in the intestine. In vitro studies have indicated that apoCIII is a noncompetitive inhibitor of lipoprotein lipase (LPL), thereby suggesting an important role in the catabolism of Tg-rich lipoproteins. Postprandial studies have found that delayed clearance of Tg-rich lipoproteins (TRLs) are associated with myocardial ischemia, carotid artery atherosclerosis, and atherosclerosis risk.

The majority of evidence supporting the role of apoCIII in the clearance of TRL has come from transgenic mouse studies. Disruption of the apoCIII gene in mice results in protection from postprandial hypertriglyceridemia, and correspondingly, the overexpression of human apoCIII results in hypertriglyceridemia with a positive linear relation between apoCIII levels and Tg concentration. This elevated Tg in human apoCIII transgenic mice is due to an increased number of VLDL particles in the circulation, which contain more Tg and apoCIII and less apoE, thus diminishing apoE-mediated lipoprotein uptake. ApoCIII overexpression also reduces VLDL glycosaminoglycan binding, decreasing lipolysis at the cell surface.

It is therefore during the postprandial period that apoCIII exerts its maximal effects, when it is a potent modulator of Tg metabolism. Thus, a postprandial study design would be the most appropriate for investigating the functional impact of variation in the apoCIII gene. Many studies have examined the relationship between the SsrI site in the 3′-untranslated region (UTR) and hypertriglyceridemia; however, none of these have incorporated postprandial tests (reviewed in References 9 and 10).

The apoCIII gene is flanked by the genes for apoAI and apoAIV in a 15-kb cluster on chromosome 11q23. The apoCIII gene polymorphisms investigated are shown in Figure 1. In addition to the C3238G (SsrI) site in the 3′-UTR, we examined the C1100T in exon 3, the C-482T in the promoter insulin-response element (IRE), and the T-2854G in the apoCIII-AIV intergenic region. The G3238 allele (SsrI) has been found to be associated with raised apoCIII and Tg levels. The T-482 variant promoter is particularly interesting because it has been found to be constitutively active at
all concentrations of insulin compared with the wild-type promoter, which is transcriptionally downregulated by insulin.14 The simultaneous examination of 4 different polymorphisms in the same study, which are distributed across the apoCIII gene region, will allow the determination of the relative importance of each variant in determining postprandial Tg levels and response to an oral glucose load.

Methods

Study Population

The European Atherosclerosis Research Study (EARS) II was undertaken to compare postprandial responses (oral glucose tolerance test [OGTT] and oral fat tolerance test [OFTT]) of male case subjects (n=407) between 18 and 28 years old whose father had a proven myocardial infarction before the age of 55 years and male control subjects (n=415) who were age-matched and were recruited irrespective of family history. The subjects were recruited from 14 European university student populations from 11 European countries, which were divided into 4 regions: Baltic (Estonia and Finland); United Kingdom; Middle Europe (Belgium, Denmark, Germany, and Switzerland); and South Europe (Greece, Italy, Portugal, and Spain). The study design has been described previously.15

Postprandial Tests

A standard 75-g oral glucose load was administered after a 12-hour overnight fast. Venous blood samples were drawn at 0, 30, 60, 90, and 120 minutes for determination of insulin and glucose concentrations. One week later, the participants had an OFTT. The standard fat meal consisted of 1493 kcal of energy: 21.6 g dairy milk protein, 56.2 g carbohydrates, and 65.5 g dairy milk fat (of which 41.6 g was fat). The meal was administered after a 12-hour overnight fast. Blood samples were withdrawn at 0, 2, 3, 4, and 6 hours for determination of Tg concentrations. Subjects were instructed to keep physical activity to a minimum and to refrain from smoking during the 6 hours of the test.

Laboratory Measurements

Laboratory measurements were performed centrally. Plasma Tg was measured in Glasgow (UK) according to the Lipid Research Clinics Manual of Laboratory Operations and standardized to criteria of the Centers for Disease Control and Prevention. Glucose was measured in Glasgow by the hexokinase/glucose 6-phosphate dehydrogenase end-point method. Insulin was measured with an in-house radioimmunoassay in Glasgow.

Polymorphism Detection

SsrI (C3238G) and BstNI (C-492T) are naturally occurring restriction enzyme sites. Restriction enzyme sites were “forced” for the C1100T and the T-2854G by incorporating single-base changes into one of the pair of primers used in the PCR reaction. Primer sequences, PCR conditions, and restriction enzyme digestions were as follows (oligonucleotides were synthesized by Gibco BRL). For SsrI: forward, 5'-CATGGTTGCTTACAGGAGGT-3'; reverse, 5'-TGACCTTCGCCAACAAAGCTTG-3'. The product was carried out in 30 µL containing 200 ng genomic DNA, 100 ng of each primer, 0.2 mmol/L dNTPs (Amersham Pharmacia Biotech), 1.75 mmol/L MgCl2, PCR buffer (Gibco BRL), 1.5 µL 1% W1 (Gibco BRL), and 0.25 µL Taq polymerase (Gibco BRL). After an initial denaturation of 5 minutes, denaturing was at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes for 35 cycles. Ten microliters of the PCR product was digested with 2 U of SsrI (New England Biolabs, NEB) at 37°C overnight. For C1100T: forward, 5'-AGAGGCCGTCACCCACCCCAACCTAGCC-3'; reverse, 5'-GGCTTTGGTGCCGCTGCTCAGG-3'. PCR conditions were as described above, except that MgCl2 was changed to 3.25 mmol/L, 1% W1 was omitted, and an annealing temperature of 68°C and extension time of 1 minute were used. Samples were digested with 2 U of BstEI (NEB) for 4 hours at 60°C. For C-492T: forward, 5'-GTTGCTGCTTGGAGGGCGGTGAGCTCACG-3'; reverse, 5'-CCCTCCAACGACCCCAAAGGGCCAGACACG-3'. PCR conditions were as described for C1100T except for the use of NH4 polymerase buffer (Bioline). A 2-step PCR was used with a combined annealing and extension step of 72°C for 1 minute. The PCR product was digested with 2 U of BstNI (NEB) for 4 hours at 60°C. For T-2854G: forward, 5'-CACCAGAGGTGCTTCTCTGCTAGTCG-3'; reverse, 5'-GGTCTAGTCCAGAGGTGCTAGAAGGAGAG-3'. The PCR was as described for the IRE apart from cycling conditions, which were 58°C annealing for 1 minute and 30 seconds of extension at 72°C for 1 minute. PCR products were digested with 2 U of Alw26I (MBI Fermentas) at 37°C overnight. In all cases, the rare allele was detected by the presence of a cutting site. The fragments were separated by use of 5% to 10% polyacrylamide microtiter array diagonal gel electrophoresis (MADGE).16

Data Analysis

The database is held on an IBM RISC System/6000 in Paris, and the analyses were performed with SAS statistical software (SAS Institute Inc.). Hardy-Weinberg equilibrium was tested by a χ2 test with 2 degrees of freedom separately in cases and controls from the 4 European regions described above. Allele frequencies were deduced from the genotype frequencies, and the differences between regions and case/control status were tested by a χ2 test. Pairwise linkage disequilibrium coefficients between polymorphisms were estimated by log-linear analysis (A), and their extents were expressed as the ratio of the unstandardized coefficients to their maximal value [(+DB')['16,17,18]

Lipid levels according to genotype were studied by ANOVA (SAS-PROC GLM) adjusted for age, recruitment center, and case/control status. The homogeneity of the results was systematically tested in cases and controls by entering the corresponding interaction term.

The postprandial response was compared across genotypes by ANOVA (SAS-PROC GLM) (1) for each time point; (2) for the area under the curve (AUC) above the fasting concentration, calculated by the trapezoidal rule; (3) for the peak, calculated as the highest value minus the fasting value; and (4) time at peak at which the highest value was observed (difference across genotypes tested by the Kruskal-Wallis rank-sum test). For distributions positively skewed, a power transformation (log or square-root) was applied for tests, but untransformed values are given in the tables and graphs. Statistical significance was reached when P<0.05. Total numbers were for Tg at OFTT, n=770; for glucose at OGTT, n=787; and for insulin at OGTT, n=752.

Results

Allele Frequencies

The approximate positions in the AI-CIII-AIV gene cluster of the 4 polymorphisms studied are shown in Figure 1. In this
sample, there were no significant case/control differences in any baseline biochemical measures (see below) or allele. The cases and controls were therefore combined for all analyses presented. Allele frequencies for the 4 polymorphisms in subjects from the 4 regions of Europe studied are shown in Table 1 and were similar for cases and controls. There were some regional differences in allele frequencies in that the T-482 (IRE) allele and the G3238 allele were more frequent in the Baltic and South Europe regions than in the UK or Middle Europe region (P < 0.002 and P < 0.011, respectively).

Linkage Disequilibrium Across the ApoCIII Region

Pairwise linkage disequilibrium coefficients (Δ) of the apo-CIII polymorphisms are shown in Figure 1. The linkage disequilibrium is high across the whole gene region except for the C1100T site, which shows low values between both the C-482T (Δ=0.21, P<0.001) and T-2854G sites (Δ=0.10, P=NS).

Clinical and Biochemical Characteristics

Clinical and biochemical characteristics of the subjects divided by case/control status are shown in Table 2. There were no differences between cases and controls for either age, body mass index, or any of the fasting biochemical variables.

Oral Fat Tolerance Test

Postprandial changes in Tg concentration after an OFTT, according to the apoCIII polymorphisms, are shown in Table 3. There were no differences between cases and controls for either age, body mass index, or any of the fasting biochemical variables.

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Oral Fat Tolerance Test

Postprandial changes in Tg concentration after an OFTT, according to the apoCIII polymorphisms, are shown in Table 3.
3. Carriers of the G3238 (SstI) allele exhibited significantly elevated Tg levels (AUC, 13% greater, \( P = 0.05 \); peak, 13% greater, \( P = 0.03 \)), but the largest effect was found in carriers homozygous for the G-2854 allele (AUC, 24% greater, \( P = 0.002 \); peak, 19% greater, \( P = 0.005 \)). The plot of the OFTT divided by T-2854G genotype is shown in Figure 2.

After adjustment for fasting Tg levels, only the association with the T-2854G site remained statistically significant. Table 4 shows the effect of the T-2854G polymorphism on postprandial AUC Tg divided into cases and controls. The effects were similar between the 2 groups (test for heterogeneity, \( P = 0.91 \)); however, Tg levels were slightly higher in cases than in controls.

### Oral Glucose Tolerance Test

Postprandial changes in plasma insulin and glucose concentrations according to the C-482T (IRE) variant after an OGTT are shown in Table 5. None of the other 3 apoCIII polymorphisms showed any significant association with insulin or glucose levels. The C-482T polymorphism showed a significant association with insulin (\( P = 0.005 \)), which remained statistically significant after adjustment for fasting insulin level (\( P < 0.01 \)). This effect appeared to be codominant, with T-482 heterozygotes having levels 7% higher and homozygotes 34% higher than C-482 homozygotes. As shown in Figure 3, the difference among the genotypes was greatest at the last time point (120 minutes, \( P < 0.0001 \)). A similar association was observed with the C-482T polymorphism and AUC for glucose (\( P = 0.013 \)), which remained significant even after adjustment for fasting level (\( P = 0.013 \)). C-482 heterozygous individuals had glucose levels 25% higher and C-482 homozygotes had levels 32% higher than T-482 homozygotes. The plot of glucose levels after the OGTT is shown in Figure 4. There was no evidence for heterogeneity (\( P > 0.5 \)) between cases and controls for either AUC for insulin or AUC for glucose.

### Discussion

The most novel aspect of the EARSII study is the large numbers of subjects who had undergone both an OGTT and a OFTT and thus were ideal for investigating the effect of apoCIII gene variants on postprandial and glucose clearance. The combining of the case and control groups was justified by the absence of statistical differences between the groups in allele frequencies, baseline characteristics, and biochemical measures. Although the investigation of genotype/phenotype effects could have been conducted only in the controls, the associations observed were similar in cases and controls, and it was statistically justifiable to combine the groups to gain power. The offspring study design considerably dilutes the genetic differences between cases and controls but has the advantage of investigating healthy subjects and therefore the examination of predisease phenotypes. However, the limitation of this approach is that it renders the interpretation

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### Table 4. Postprandial AUC Tg by T-2854G Genotype and Case/Control Status

<table>
<thead>
<tr>
<th>Status</th>
<th>T-2854G Genotype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T/T</td>
<td>T/G</td>
<td>G/G</td>
</tr>
<tr>
<td>Cases</td>
<td>3.91 (0.19)</td>
<td>3.91 (0.18)</td>
<td>4.81 (0.33)</td>
</tr>
<tr>
<td></td>
<td>( n = 142 )</td>
<td>( n = 159 )</td>
<td>( n = 46 )</td>
</tr>
<tr>
<td>Controls</td>
<td>3.61 (0.19)</td>
<td>3.73 (0.17)</td>
<td>4.59 (0.36)</td>
</tr>
<tr>
<td></td>
<td>( n = 142 )</td>
<td>( n = 189 )</td>
<td>( n = 39 )</td>
</tr>
</tbody>
</table>

Values are mean (SEM).

Test of heterogeneity between cases and controls: \( P = 0.91 \).

### Table 5. Changes in Plasma Insulin and Glucose Concentrations After OGTT According to C-482T (IRE) Polymorphism

<table>
<thead>
<tr>
<th>C-482T (IRE) Genotype</th>
<th>P, Significance Test Adjusted for Fasting Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>C/C ( n = 365 )</td>
</tr>
<tr>
<td>Insulin</td>
<td>56.82 (1.88)</td>
</tr>
<tr>
<td>AUC</td>
<td>55.44 (1.90)</td>
</tr>
<tr>
<td>Peak</td>
<td>0.74 (0.02)</td>
</tr>
<tr>
<td>Time at peak, min</td>
<td>0.57 (0.01)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.64 (0.10)</td>
</tr>
<tr>
<td>AUC</td>
<td>2.61 (0.03)</td>
</tr>
<tr>
<td>Peak</td>
<td>0.57 (0.01)</td>
</tr>
</tbody>
</table>

Peak indicates peak height minus fasting level. Time at peak was tested by the Wilcoxon rank-sum test. Values are means adjusted for age, center, and case/control status.

*Test performed on square-root values.

†Test performed on log-transformed values.
of these results, in terms of risk of myocardial infarction, more inconclusive.

The greatest effect on postprandial Tg was in subjects homozygous for the G-2854 allele in the apoCIII-AIV intergenic region, who exhibited a significantly delayed clearance compared with subjects with a T-2854 allele. A significant effect of lower magnitude was also observed with the C3238G site (SstI), although significance was lost after adjustment for fasting Tg level. It is likely that this effect of the C3238G variant on postprandial Tg levels is a result of allelic association with the T-2854G variant. It is possible that the effect observed with the T-2854G site may be due to linkage disequilibrium with another functional polymorphism, perhaps further upstream in the apoCIII-AIV region. However, the T-2854G site lies within a recently identified apoCIII and apoAIV enhancer element, in close proximity to a HNF-4 binding site,19 supporting the view that the site itself may be functionally important.

The most surprising result of the study was the observation of an association with the C-482T (IRE) site and both insulin and glucose levels after an OGTT. Although the IRE is involved in the expression of apoCIII (and possibly the expression of apoAI and apoAIV), the molecular mechanism responsible for these effects on insulin and glucose is unclear. The following hypothesis could explain the observed effects of the C-482T site on both glucose and insulin. In the postabsorptive state, the majority of lipolysis takes place in adipose tissue by hormone-sensitive lipase, which converts Tg stores to nonesterified fatty acid (NEFA) for use as fuel during fasting and refeeding.22 How apoCIII may affect these functions is unclear; however, apoCIII overexpression in apoE-knockout mice produced a marked decrease in VLDL glycosaminoglycan binding.8 Thus, even though the catalytic activity of LPL may be partly inhibited by inappropriate apoCIII expression, the elevated levels of circulating lipoprotein caused by decreased uptake could result in a higher absolute circulating NEFA concentration. Consequently, compared with those subjects with the wild-type promoter, subjects with the T-482 variant would have more circulating NEFA, which will compete with glucose for use as fuel in the muscles. Not only will less glucose be utilized by the muscles, but the increased NEFA availability will stimulate gluconeogenesis.26 If the glucose levels remain high, there will be a compensatory increase in insulin secretion.

Thus, different apoCIII variants modulate Tg metabolism according to the nutritional state and the composition of the challenge meal. Given that these variants are often coinherited, those subjects with the combination of rare variants will have a particularly unfavorable lipoprotein/lipid profile. The application of postprandial tests in this study has allowed us, at least in part, to dissect the complex interactions between the apoCIII variants and lipid metabolism, interactions that are both substrate-specific and temporally different. The data suggest a novel mechanism whereby apoCIII genetic variants predispose to increased atherosclerotic risk, namely, that there may also be an increased risk of impaired glucose tolerance and non–insulin-dependent diabetes mellitus. Proof of these proposed mechanisms will require further in vitro and clinical studies.

Appendix

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Acknowledgments

Drs Waterworth, Talmud, and Humphries are funded by the British Heart Foundation (RG/95007, PG/95190, and PG/96184). The authors thank J.C. Vallvé for technical assistance.

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Circulation. 1999;99:1872-1877
doi: 10.1161/01.CIR.99.14.1872

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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