ASBMB
JOURNAL OF LIPID RESEARCH

Genetic analysis of a polymorphism in the human apolipoprotein A-I gene promoter: effect on plasma HDL-cholesterol levels[1]

D. Edward Barre,* Rudy Guerra,† Richard Verstraete, Zifen Wang,* Scott M. Grundy,* and Jonathan C. Cohen[2,*,**]**

Center for Human Nutrition,* UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-9052; Department of Statistical Science,† Southern Methodist University, Dallas, TX 75275-0332; and Baylor/UT Southwestern Sports Science Research Center,** 411 N. Washington, Suite 3000, Dallas, TX 75246

Abstract Previous studies have indicated that a G to A substitution at position −76 in the gene encoding apolipoprotein A-I (apoA-I) confers increased plasma high density lipoprotein cholesterol (HDL-C). Increased HDL-C may be a direct consequence of the A allele, or may reflect the action of a locus in linkage disequilibrium with the A allele. To elucidate this question, we examined the effect of this polymorphism in a large sample (n = 409) of unrelated Caucasians and their nuclear families (n = 22). To eliminate the confounding effects of hypertriglyceridemia, individuals with triglyceride levels greater than 150 mg/dl were excluded from the study. ApoA-I genotype was determined by polymerase chain reaction (PCR) amplification of genomic DNA and restriction digestion with Msp I. Individuals were grouped by genotype (GG, GA or AA) and mean adjusted HDL levels of the three groups were compared by one-way analysis of variance. Our analysis indicates that HDL-C levels do not vary by genotype, and no gene dosage effect is apparent in men or in women. Analysis of 22 informative Caucasian nuclear families showed no significant difference between individuals with the A allele and their GG siblings. These data suggest that polymorphism at −76 in the apoA-I gene does not directly affect HDL levels. Therefore, the increased HDL-C levels reported in other populations must reflect linkage disequilibrium between the A allele and a putative HDL-raising allele. As we find no evidence for association between the A allele and high HDL levels, this putative allele must occur at a low frequency in the population sampled in this study.—**Barre, D. E., R. Guerra, R. Verstraete, Z. Wang, S. M. Grundy, and J. C. Cohen.** Genetic analysis of a polymorphism in the human apolipoprotein A-I gene promoter: effect on plasma HDL-cholesterol levels. *J. Lipid Res.* 1994. **35:** 1292–1296.

Supplementary key words high density lipoproteins • apolipoprotein A-I gene • DNA polymorphism • polymerase chain reaction

Familial aggregation of plasma high density lipoprotein cholesterol levels (HDL-C) has been reported in several studies (see ref. 1 for review). A component of the familial resemblance in HDL-C levels can be attributed to shared environment, but the major fraction appears to be genetically determined (2). Heritability estimates derived from twin and family data have indicated that genetic factors probably account for 40–60% of the inter-individual variation in plasma levels of HDL-C (3), but the specific genes involved have not been identified.

Candidate genes that potentially contribute to inter-individual variation in plasma HDL-C levels have been examined by comparing the HDL-C levels of unrelated individuals with different alleles of the gene under consideration (4). In most cases, alleles have been defined by restriction fragment length polymorphisms. Since most of these polymorphisms are in non-coding regions, an association between a candidate locus and plasma HDL-C levels is usually assumed to indicate linkage disequilibrium between the marker locus and another, unidentified, polymorphic sequence that actually confers phenotypic variation. Perhaps the most consistent association identified by these methods is that between apolipoprotein A-I (apoA-I) alleles bearing an adenine (A) at position −76, and high plasma levels of HDL-C. Five studies (5–9) have reported that individuals with the A allele, which occurs at a frequency of about .18 in Caucasian populations, have higher levels of HDL-C or apoA-I than do individuals homozygous for alleles bearing guanine (G) at −76, the common allele in these populations.

The magnitude and the gender distribution of the increase in plasma HDL-C levels associated with the A allele has differed among these studies. Jeenah et al. (5) reported that, in a sample of healthy English men, plasma apoA-I and HDL and HDL_2 cholesterol levels were

Abbreviations: HDL-C, high density lipoprotein cholesterol; PCR, polymerase chain reaction.

[1]Dr. John D. Brunzell served as Guest Editor for this paper.

[2]To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 18, 2012

significantly higher in those individuals with the A allele than in those homozygous for the G allele. Pagani et al. (6) found that the A allele was over-represented in Italian women in the highest decile of HDL-C levels, but not in Italian men in the corresponding decile. These authors concluded that the A allele was associated with high HDL-C levels in women, but not in men. Paul-Hayase et al. (7) reported that plasma apoA-I levels were higher in Belgian men and boys (aged 7–23 years) with the A allele than in those homozygous for the G allele, although the magnitude of the difference reported by these authors (5 mg/dl) was much lower than that reported by Jeenah et al. (23 mg/dl). Siggurdsson et al. (8) reported that Icelandic male nonsmokers with the A allele had significantly higher HDL-C and apoA-I levels than did male nonsmokers homozygous for the G allele. In this study (8), the A allele had no effect on male smokers, or on women. Xu et al. (9) found no effect of the A allele on plasma HDL-C levels of Italian boys and girls aged between 8 and 11 years. ApoA-I levels were higher in boys, but not in girls with the A allele. The reasons for these discrepancies are not known.

It is also not clear whether the putative effect of the polymorphism on HDL levels is due to the G to A substitution per se, or to linkage disequilibrium between the A locus and a distinct as yet unidentified effector locus. In vitro analysis of the effects of this polymorphism on transcription has yielded conflicting results. Smith, Brinton, and Breslow (10) reported that substitution of an A for G at position −76 in the apoA-I promoter decreased in vitro transcription by about 30%. This finding was consistent with in vivo turnover studies that showed decreased apoA-I synthetic rates in individuals with the A allele, although plasma HDL levels did not differ between GG and GA individuals. Tuteja et al. (11) reported that substitution of A for G decreased transcription about 2-fold in a promoter construct spanning the region −330 to +1, but had no effect in a construct spanning the region −1469 to +397. Jeenah and Wells (12) reported that substitution of A for G increased transcription about 4-fold in a 350-bp fragment spanning the region from −256 to +100.

The present study was undertaken to elucidate the effects of the G to A substitution. We reasoned that if the A substitution at position −76 confers higher HDL-C levels, then a gene dosage effect should be evident. Homozygotes for the A allele should, on average, have higher HDL-C levels than GA heterozygotes, who should in turn have higher HDL-C levels than GG homozygotes. As the A allele is relatively infrequent, we studied a large sample of normolipidemic individuals in order to include a representative number of AA homozygotes. To reduce the possible confounding effects of genetic heterogeneity at other loci that affect HDL-C levels, we also compared the HDL-C levels of individuals bearing the A allele with those of their siblings who were homozygous for the G allele.

METHODS

Subjects

Blood samples were obtained from fasting, non-Hispanic whites living within 50 miles of the Dallas metropolitan area. Where possible, the nuclear families of these subjects were also ascertained. Blood lipid analysis was performed, and individuals with triglyceride levels greater than 150 mg/dl were excluded from the analysis.

Laboratory procedures

Plasma triglyceride, cholesterol, and HDL-C levels were determined enzymatically, as described previously (13). Genomic DNA was isolated using the procedure described by Lahieri and Nurnberger (14). The region of the apoA-I gene containing the G/A polymorphism was amplified by PCR. Each PCR reaction contained 15 pmol of each primer, 100 μM dNTP, 3.3 pmol 32P-dCTP (3000 mCi/mmol), 100 ng genomic DNA, and 0.25 U Taq polymerase in the buffer provided by the manufacturer. Amplifications were performed in 10 μl volumes. The PCR mixtures were overlaid with mineral oil and subjected to 30 cycles of denaturing at 94°C for 30 sec and annealing and extension at 68°C for 2 min in a thermocycler. The sample volume was then increased to 40 μl by addition of a restriction enzyme, Msp I, in the buffer recommended by the manufacturer, incubated twice for 2 h at 37°C, and subjected to electrophoresis on polyacrylamide gels.

Statistical methods

The observed HDL-C values were adjusted for the effects of age and sex using a procedure similar to that described by Sokal and Rohlf (15). The adjusted value for an observation y is $y_{adj} = \bar{y} + (y - \mu)$, where μ is the expected value of an individual of the observation's age and sex, and $\bar{y}$ is the sample average of the y values. The expected values were obtained from sex and age specific HDL-C tables reported by Heiss et al. (16). Within each sex-age specific class, the mean and standard deviation of our sample was similar to that of Heiss et al. (16).

For the sample of unrelated individuals, a one-way analysis of variance (17) was performed on the deviations $y - \mu$ to test the null hypothesis that phenotypic variation is unaffected by the marker genotype polymorphism. Separate analyses for men and women were conducted. For the sample of nuclear families, a generalization of a comparison based on paired data was performed. Comparison of phenotypes within families accounts for genetic correlations and thereby removes biases that might otherwise be incurred. The adjusted HDL-C levels were ranked within families and the ranking scores were analyzed

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org by guest, on June 18, 2012

across families. Due to the paucity of A homozygote individuals, the AA individuals were combined with the AG individuals to form one class, A+ genotypes. We thus compared HDL-C levels from AA and AG genotypes within families. Let $S_{i1} < \ldots < S_{in_i}$ denote the n_i A ranks from the ith family, and $W_A^{(i)} = S_{i1} + \ldots + S_{in_i}$ the Wilcoxon rank-sum statistic computed for the ith family. For unequal family sizes the test statistic $W_A = \Sigma\{W_A^{(i)}/(N_i + 1)\}$, where N_i is the number of siblings in the ith family, is appropriate for testing the significance of the effects of the marker polymorphism on adjusted HDL-C levels. This test, including its exact null distribution, is discussed by Lehmann (18). Two analyses were performed, one comparing all sibs within a family, and another comparing same sex sibs within families. The statistical computer package StatXact (19) was used to determine exact *P*-values.

RESULTS

HDL-C levels from 205 women (aged between 14 and 86 years, mean 45 years), and 204 men (aged between 16 and 84 years, mean 45 years) were included in this analysis. Mean values of adjusted HDL-C levels for each genotype in the sample of unrelated individuals are given in **Table 1.** We found no evidence to suggest that the mean HDL-C levels of the genotype groups were significantly different in women (P = 0.84) or in men (P = 0.77).

Furthermore, there was no evidence for a gene dosage effect in either sex; mean HDL-C levels for AA homozygotes were not higher than those of GG homozygotes in men or in women (Table 1). **Figure 1** shows the HDL-C distributions associated with the three genotypes in women; it is clear that the between-genotype variation is negligible compared to the within-genotype variation. A corresponding plot for the men, **Fig. 2,** leads to the same conclusion. To evaluate whether our findings were biased by the procedure used to adjust HDL-C levels, we performed identical analyses on unadjusted HDL-C data, and on HDL-C data adjusted by regression. No effect of apolipoprotein A-I genotype was evident is either of these analyses.

Of the 80 nuclear families examined, 22 included sibships with both GG and GA genotypes. Four of these sibships included three children, eight sibships included four children, seven sibships included five children, and three

TABLE 1. Mean values of adjusted HDL levels among marker genotypes

Sex		GG	GA	AA	P^a
		Genotype			
Females[b]	n	131	64	10	
	$\bar{Y}_{adj}$	57.4	58.9	57.5	
	$\overline{Y - \mu}^c$	-0.9	0.6	-0.7	0.84
	SD(Y − μ)	15.48	16.99	17.69	
Males[b]	n	130	66	8	
	$\bar{Y}_{adj}$	45.8	46.8	43.8	
	$\overline{Y - \mu}^c$	-0.2	0.7	-2.3	0.77
	SD(Y − μ)	12.35	14.02	7.03	

[a] *P*-value associated with analysis of variance F-ratio.
[b] From sample of unrelated individuals.
[c] Y − μ denotes observed HDL value minus expected value conditional on age and sex.

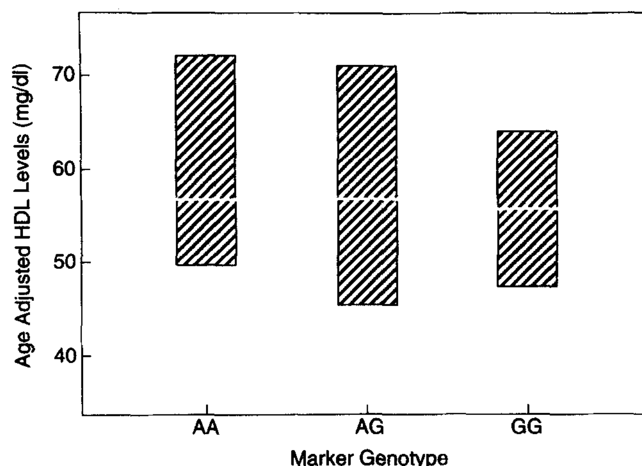

Fig. 1. Distributions of HDL-C levels for the marker genotypes AA (n = 10), AG (n = 64), and GG (n = 131) in a sample of 205 unrelated Caucasian women. Median values are indicated by the white bars within the boxes; the upper and lower quartiles are indicated by the top and bottom of the boxes, respectively.

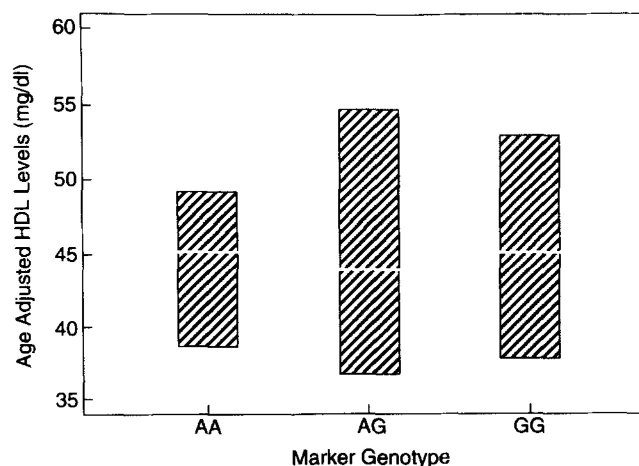

Fig. 2. Distributions of HDL-C levels for the marker genotypes AA (n = 8), AG (n = 66), and GG (n = 130) in a sample of 204 unrelated Caucasian males. Median values are indicated by the white bars within the boxes; the upper and lower quartiles are indicated by the top and bottom of the boxes, respectively.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

sibships included six children. The sample of informative nuclear families included few A homozygotes, therefore we combined the AA and AG individuals into one class, A+. The within-family comparisons rank G homozygotes with respect to the A+ genotypes. Comparing all sibs within families resulted in a P-value of 0.19 and thus we failed to reject the null hypothesis assuming equality of A+ and GG phenotypic means ($\alpha = 0.05$). The investigation comparing same sex sibs reduced the number of informative families to 12 and 10 for the female and male analyses, respectively. The sibships that did qualify for the analysis typically were of size 3. In neither case, male or female, did we reject ($\alpha = 0.05$) the null hypothesis: male P-value = 0.39, female P-value = 0.15.

DISCUSSION

In this study we have examined the influence of a G to A substitution in the 5' flanking region of the apoA-I gene on plasma levels of HDL-C. Previous studies (5-9) have reported that the A allele is associated with higher HDL levels than the G allele, but the magnitude and gender specificity of the effects have not been consistent. Our study design differed significantly from that used by previous investigators in that we exploited the effects of gene dosage and segregation.

First, to maximize the possibility of detecting variation in HDL levels that may be associated with this polymorphism, we compared the HDL-C levels of individuals homozygous for the A allele with those of individuals homozygous for the G allele. Our analysis indicated that HDL-C levels did not vary by genotype, and no gene dosage effect was apparent in men or in women.

Second, to evaluate the possibility that the slight difference in HDL-C levels between GA heterozygotes and GG homozygotes reflects a systematic effect of the A allele, and that this effect is obscured by random variation in HDL-C levels due to genetic polymorphism at other loci, we compared the HDL-C levels of GA heterozygotes with those of their siblings who were homozygous for the G allele. On average, siblings share 50% of their genetic material identical by descent, therefore this comparison should significantly reduce variation due to polymorphism at other genetic loci. Analysis of 22 informative sibships revealed no effect of the A allele on HDL-C levels, a result consistent with our finding in unrelated individuals. These findings indicate that the G to A substitution at −76 in the apoA-I gene does not, per se, confer higher HDL-C levels. Accordingly, the association between the A allele and increased HDL levels observed in other populations (5-9) must reflect linkage disequilibrium between the A allele and a locus that raises HDL-C levels. Since we find no evidence for association between the A allele and high HDL-C levels, this putative HDL-raising locus must occur at a low frequency in the population sampled in this study.

We thank Kathy Schutt, Kevin Robinzine, and Anika Hochstein for excellent technical assistance, and Dr. Jim Stray-Gundersen for his support of this work. This work was supported by NIH grants HL-29252 and MO1-RR-00633, the Southwestern Medical Foundation, The Moss Heart Foundation of Dallas TX, and an unrestricted grant from Bristol-Myers-Squibb.

Manuscript received 13 December 1993 and in revised form 11 February 1994.

REFERENCES

1. Breslow, J. L. 1989. Familial disorders of high density lipoproteins. *In* The Metabolic Basis of Inherited Disease. 6th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Book Co., New York. 1251-1264.
2. Namboodiri, K. K., P. P. Green, E. B. Kaplan, H. A. Tyroler, J. A. Morrison, G. A. Chase, R. C. Elston, B. M. Rifkind, and C. J. Glueck. 1983. Family aggregation of high density lipoprotein cholesterol. *Arteriosclerosis.* **3:** 616-626.
3. Heller, P. A., U. De Faire, N. Pederson, G. Dahlen, and G. E. McClearn. 1993. Genetic and environmental influences on serum lipid levels in twins. *N. Engl. J. Med.* **328:** 1150-1156.
4. Humphries, S. E. 1988. DNA polymorphusms of the apolipoproteins — their use in the investigation of the genetic component of hyperlipidemia and atherosclerosis. *Atherosclerosis.* **72:** 89-108.
5. Jeenah, M., A. Kessling, N. Miller, and S. Humphries. 1990. G to A substitution in the promoter region of the apolipoprotein A-I gene is associated with elevated serum apolipoprotein A-I and high density lipoprotein cholesterol concentrations. *Mol. Biol. Med.* **7:** 233-241.
6. Pagani, F., A. Sidoli, G. A. Giudici, L. Barenghi, C. Vergani, and F. E. Baralle. 1990. Human apolipoprotein A-I gene promoter polymorphism: association with hyperalphalipoproteinemia. *J. Lipid Res.* **31:** 1371-1377.
7. Paul-Hayase, F., M. Rossenneu, D. Robinson, J. Van Bervliet, J. P. Deslypere, and S. E. Humphries. 1992. Polymorphisms in the apolipoprotein (apo) A-I-C-III-A-IV gene cluster: detection of genetic variation determining plasma apoA-I, apoC-III and apoA-IV concentrations. *Hum. Genet.* **88:** 439-446.
8. Sigurdsson, G., V. Gudnason, G. Sigurdsson, and S. E. Humphries. 1992. Interaction between a polymorphism of the apoA-I promoter region and smoking determines plasma levels of HDL and apoA-I. *Arterioscler. Thromb.* **12:** 1017-1022.
9. Xu, C-F., F. Angelico, M. Del Ben, and S. E. Humphries. 1993. Role of genetic variation at the apoA-I-C-III-A-IV gene cluster in determining plasma apoA-I levels in boys and girls. *Genet. Epidermiol.* **10:** 113-122.
10. Smith, J. D., E. A. Brinton, and J. L. Breslow. 1992. Polymorphism in the human apolipoprotein A-I gene promoter region. Association of the minor allele with decreased production rate in vivo and promoter activity in vitro. *J. Clin. Invest.* **89:** 1796-1800.
11. Tuteja, R., N. Tuteja, C. Melo, G. Casari, and F. Baralle.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

1992. Transcription efficiency of human apolipoprotein A-I promoter varies with naturally occurring A to G transition. *FEBS Lett.* **304:** 98–101.
12. Jeenah, M. S., and C. Wells. 1992. Functional significance of a point mutation in the apoA-I promoter. *Circulation.* **(Suppl)86:** 1-279 (abs).
13. Cohen, J. C., and S. M. Grundy. 1992. Normal post-prandial lipemia in men with low plasma HDL concentrations. *Arterioscler. Thromb.* **12:** 972–975.
14. Lahiri, D. K., and J. I. Nurnberger. 1991. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res.* **19:** 5444.
15. Sokal, R. R., and F. J. Rohlf. 1969. Biometry. W. H. Freeman and Company, San Francisco.
16. Heiss, G., N. J. Johnson, S. Reiland, C. E. Davis, and H. A. Tyroler. 1980. The epidemiology of plasma high density lipoprotein cholesterol levels. The Lipid Research Clinics Program Prevalence Study. Summary. *Circulation.* **Suppl. 4:** 116–136.
17. Scheffe, H. 1959. The Analysis of Variance. John Wiley & Sons, Inc., New York.
18. Lehmann, E. L. 1975. Nonparametrics: Statistical Methods Based on Ranks. Holden-Day, San Francisco.
19. StatXact. 1989. A Statistical Package for Exact Nonparametric Inference. Cytel Software Corporation, Cambridge, MA.

Downloaded from www.jlr.org by guest, on June 18, 2012