Role of Common Genetic Polymorphisms in the LDL Receptor Gene in Affecting Plasma Cholesterol Levels in the General Population

Young I. Ahn, M. Ilyas Kamboh, Christopher E. Aston, Robert E. Ferrell, Richard F. Hamman

Abstract
A large number of rare mutations in the low-density lipoprotein (LDL) receptor gene cause the autosomal dominant disorder familial hypercholesterolemia. In addition, a number of common DNA polymorphisms have been identified in the LDL receptor gene, but their significance in affecting plasma cholesterol levels in the general population has not been studied widely. We investigated the role of two common DNA polymorphisms, Ava II (exon 13) and Nco I (exon 18), at the LDL receptor locus in affecting plasma lipid profiles in normolipidemic Hispanics (n = 385) and non-Hispanic whites (NHWs; n = 543) from the San Luis Valley, Colorado. While the distribution of the Nco I polymorphism was comparable between Hispanics and NHWs, the allele frequencies at the Ava II restriction site differed significantly between the two ethnic groups (P < .001). The Ava II and Nco I polymorphisms were in linkage disequilibrium (P < .05) in both Hispanics and NHWs. Both polymorphisms revealed a gender-specific effect on total and LDL cholesterol (LDL-C) confined to women only in both ethnic groups. The Ava II polymorphism was associated significantly with total cholesterol and LDL-C in NHW women (P = .001 and P = .014) and in Hispanic women (P = .011 and P = .057). The effect of the Nco I polymorphism was significant on total cholesterol and LDL-C (P = .019 and P = .035) in Hispanic women only. Although a similar trend was observed in NHW women, the effect was not significant at the 5% level. There was a gene-dosage effect on total cholesterol and LDL-C levels among the Ava II and Nco I genotypes: these levels were low in the (-/-) genotype, intermediate in the (+/-) genotype, and high in the (+/+ ) genotype. The average effect of the Ava II (+) allele was to increase total cholesterol (LDL-C) by 6.52 mg/dL (4.95 mg/dL) in NHW women and 3.69 mg/dL (2.92 mg/dL) in Hispanic women; this polymorphism explained about 4% and 2% of the phenotypic variance in total cholesterol and LDL-C, respectively. The average effect of the Nco I (+) allele was to increase total cholesterol (LDL-C) by 3.66 mg/dL (3.07 mg/dL) in Hispanic women; this polymorphism explained 3.3% and 2.6% of the phenotypic variance in total cholesterol and LDL-C, respectively. When premenopausal and postmenopausal women were analyzed separately within each ethnic group, no evidence of physiological interaction was observed between the two LDL receptor polymorphisms and the menopausal status in affecting plasma lipid levels. After examination of an interaction effect between the LDL receptor/Ava II and apolipoprotein E polymorphisms, we found no evidence of interaction between these two genes in determining total cholesterol and LDL-C levels, indicating that the effects of these two genes on cholesterol levels are independent from each other. Thus, this study demonstrated a significant contribution of genetic variation at the LDL receptor locus in determining interindividual differences in plasma cholesterol levels in the general population.

Key Words: LDL receptor gene • restriction fragment length polymorphisms • plasma lipid levels

The low-density lipoprotein (LDL) receptor, a transmembrane cellular protein, plays a crucial role in a receptor-mediated pathway of cholesterol homeostasis.1 A relatively high plasma level of LDL cholesterol (LDL-C) has been recognized as a risk factor for coronary heart disease (CHD). The LDL receptor modulates plasma levels of LDL by regulating the uptake of LDL particles by the liver and delivers cholesterol to the adrenal glands and gonads for steroid hormone synthesis and to the liver for bile acid synthesis.1,2 Novel mutations in the LDL receptor gene that disturb the normal functions of the LDL receptor protein can cause familial hypercholesterolemia (FH), which is associated with elevated plasma LDL-C and premature CHD. FH, however, accounts for only about 5% of patients with CHD, and the contribution of genes to CHD in the remaining 95% of cases is still unknown. Common DNA polymorphisms in genes involved in lipid metabolism are potentially important genetic markers in affecting normal variation in the plasma lipid profile and thus determining susceptibility or resistance to CHD in the general population. So far, the major effect of a single gene in determining population variation of plasma cholesterol has been shown for the apolipoprotein (apo) E polymorphism.2 Since the LDL receptor gene plays an important role in cholesterol homeostasis, common genetic variations in this gene may also contribute to variation in plasma cholesterol levels in the general population.

The human LDL receptor gene has been cloned4 and mapped to chromosome 19p13.2.5 This gene is approximately 45 kb long and contains 18 exons separated by 17 introns.6 More than 12 restriction fragment length polymorphisms (RFLPs) within and near the LDL receptor gene are reported.7,8 A common Pvu II RFLP is associated with variation of cholesterol levels in several European populations from Norway,9 Germany,10 and the Czech Republic11 but not in a British population.12 Case-
control studies show that the frequency of the allele for the absence of the Nco I restriction site is significantly higher in FH patients than control subjects.12,13 Also, a high frequency for the absence of the Ava II restriction site is found in white Afrikaner FH patients.13 A linkage between the LDL receptor locus and an atherogenic lipoprotein profile suggests that a mutation in the LDL receptor gene might be responsible for this phenotype.14

To study the effect of common genetic variations in the LDL receptor gene on quantitative variation of plasma lipid levels in the general population, we examined the association of the Ava II and Nco I RFLPs in this gene with total, LDL, and high-density lipoprotein cholesterol (HDL-C) and triglyceride levels in a large sample of normolipidemic Hispanics and non-Hispanic whites (NHWs) from the San Luis Valley, Colorado. The Ava II polymorphic site is present in exon 13, which involves substitution of C for T in the third base of codon 632 (valine), which introduces a recognition site for the Ava II enzyme (GGTTC → GGTCC). However, since the mutation is synonymous it does not change the amino acid. The Nco I polymorphic site is in exon 18, which is transcribed but not translated and therefore does not involve any amino acid change.

Methods

Subjects

The study subjects consist of 385 Hispanics and 543 NHWs who are participants in the San Luis Valley Diabetes Study, which was designed to examine risk factors and prevalence of non-insulin-dependent diabetes mellitus by using a case-control approach in the biethnic population of the San Luis Valley in southern Colorado.15 Only normolipemic subjects between the ages of 20 and 74 years were screened for the LDL receptor Ava II and Nco I polymorphisms. Details of the clinical and biochemical features of the studied subjects are available.16

The NHW population immigrated to the San Luis Valley from several northern European countries in the mid 1800s. The Hispanic population has lived in this area since the time of the Spanish land grants. Today there is little immigration to the area, especially from Mexico, in contrast to conditions in Texas and southern California.15 During the 150-year settlement in this valley, there has been little direct Amerindian-Hispanic genetic admixture. Genetic data show that the proportion of Amerindian genes is about 3% in NHWs and 19% in Hispanics.17

DNA Typing

Fasting EDTA blood samples were collected by venipuncture, and the white blood cells were isolated, frozen at −70°C, and shipped to Pittsburgh, Pa. DNA was extracted by the procedure of Miller et al.18 DNA samples were subjected to amplification by polymerase chain reaction (PCR) in a Perkin-Elmer Cetus DNA thermal cycle. We used two sets of primers. One set was derived from the DNA sequences flanking an Ava II restriction site in exon 13 of the LDL receptor gene (the forward primer was 5'-GTATCCTTCTGTGCTGTGGTAG-3', and the reverse primer was 5'-GGTGTGCACAGGAGAGTTGCAAGT-3').19 The amplified fragment was 228 bp. The other set was from the DNA sequences flanking an Nco I restriction site in exon 18 (the forward primer was 5'-TCCGCTTTACCCATTGTGGGAGG-3', and the reverse primer was 5'-GTTGACACAGTGCACTGTGCAAGT-3').20 The amplified fragment in this case was 2490 bp.

The 50-μL reaction mixture contained 1× PCR buffer (10 mM Tris-hydroxymethylaminomethane, pH 8.3, 50 mM KCl, 1.5 mM MgCl2), dNTPs at 200 μM/L, 0.3 μM/L of each primer, 0.5 μg genomic DNA, and 1.25 units of Taq DNA polymerase. Amplification of the region flanking the Ava II site was performed for 28 cycles at 95°C for 1 minute, at 68°C for 2 minutes, and at 72°C for 1 minute. For amplification of the sequence around the Nco I site, the conditions were 95°C for 1 minute and 70°C for 3 minutes with 28 cycles. Amplified products were digested with Nco I or Ava II, and the resulting fragments were separated on 1% or 2% agarose gels.

Metabolic Determinations

Lipid levels were determined in the laboratories of the Clinical Research Center, University Hospital, Denver, Co, from blood samples collected after an overnight (>8 hours) fast. Triglycerides were determined by enzymatic assay21 and total cholesterol by the esterase-oxidase method.22 HDL-C levels were measured by enzymatic assay after dextran sulfate and magnesium precipitation.23 LDL-C was calculated by using the Friedewald equation.24 Individuals with triglyceride levels ≥400 mg/dL were excluded from the analysis.

Statistical Analysis

Allele frequencies for each polymorphic site were estimated by gene counting. Heterozygosity25 and polymorphism information content (PIC)26 were computed to estimate the informativeness of each polymorphism. Goodness of fit to Hardy-Weinberg proportions was tested by the log-likelihood ratio (LLR).27 The degree of nonrandom association between the two polymorphic sites at the LDL receptor locus was determined by calculating the delta (pairwise) value28 using maximum-likelihood estimates of the haplotype frequencies. Haplotype frequencies were also computed by direct counting in individuals heterozygous at a single site.

To evaluate the effect of each polymorphism on the variation of quantitative variables of lipid, ANCOVA was performed. The sampling distributions of all the variables were tested for normality. HDL-C and triglycerides were logarithmically transformed to induce approximate normality. The determination of the effects of covariates was done by stepwise regression. Age, smoking, and body mass index (BMI) were considered as covariates. BMI was computed as weight in kilograms divided by height in meters squared.29 Men and women were analyzed separately within each ethnic group. Women were further categorized into premenopausal and postmenopausal, and the data were reanalyzed. Statistical analyses were done using the statistical software package SAS. The average effects of LDL receptor alleles for each polymorphism were estimated according to the method of Templeton.30

Results

Allele Frequencies

The digestion of the PCR-amplified product with the restriction enzyme Ava II revealed two fragments of 141 and 87 bp, indicating the presence of the restriction site (+ allele). Similarly, the digestion with the restriction enzyme Nco I yielded two fragments of 1570 and 920 bp when the Nco I restriction site was present (+ allele) (data not shown). Table 1 presents allele frequencies for each polymorphic site along with estimated heterozygosity and PIC for the normolipidemic Hispanics and NHWs. For the Nco I restriction site, comparable allele frequencies were observed across all ethnic and sex categories. On the other hand, for the Ava II restriction site, there was a significant difference in allele frequencies (0.56 and 0.43 for the [+ ] allele, P<.001, Hispanics and NHWs, respectively). However, the heterozygosity and the PIC values were similar at both polymorphic sites in both ethnic groups. The Nco I genotypes were in Hardy-Weinberg equilibrium in both ethnic groups. The Ava II polymorphism, however, was in equilibrium in
Ava II Polymorphism and Lipid Profile

To exclude the possibility of misclassification in Hispanics, all gels were reread blindly by two persons without any change. When men and women were analyzed separately the sample was in Hardy-Weinberg equilibrium in both genders. Therefore we concluded that the marginal departure from Hardy-Weinberg equilibrium in the combined sample may be due to chance. Alternatively, a large proportion of Amerindian admixture in Hispanics may have caused this departure from Hardy-Weinberg equilibrium. However, since men and women were analyzed separately for the genetic effects of Ava II and Nco I polymorphisms on quantitative lipid traits in both ethnic groups, this phenomenon did not bear significantly on the interpretation of results.

Linkage Disequilibrium

Table 2 presents the maximum-likelihood estimates of the haplotype frequencies and the delta values reflecting the extent and significance of nonrandom associations between the Ava II and Nco I restriction sites. Also shown are the haplotype frequencies estimated by direct count in heterozygous individuals at one polymorphic site. The two estimates of the haplotype frequencies are not significantly different. The delta values indicate that there is a statistically significant association between the Ava II and Nco I polymorphisms (P<.05) in both ethnic groups.

Ava II Polymorphism and Lipid Profile

The relatively high PIC values of the two polymorphisms enabled us to examine the impact of the LDL receptor genetic variation on the quantitative variation of lipid traits. We looked for associations between lipid phenotypes and LDL receptor genotypes using ANCOVA. Since not all the subjects in this study could be unambiguously haplotyped (Table 2), the effects of the genotypes on quantitative traits were examined for each restriction site separately. The analyses were performed for each ethnic and gender category separately because the lipid levels under study vary by ethnic group and gender.

Table 3 shows adjusted mean lipid levels among the three Ava II genotypes (−/−, +/+, and +/−) in NHW and Hispanic men and women. The gender-specific effect was significant for HDL-C (P<.001) and triglycerides (P=.001). Ethnicity affected only the levels of triglycerides (P=.02). Significant variability among Ava II genotypes was observed for total cholesterol and LDL-C in NHW women (P=.0015 and P=.014) and total cholesterol in Hispanic women (P=.011) and was highly suggestive for LDL-C in Hispanic women (P=.057). For both NHW and Hispanic women the trends seen in the cholesterol levels were similar: low in the (−/−) genotype, intermediate in the (+/−) genotype, and high in the (+/+) genotype. Neither significant nor directional results were seen in men in either ethnic group. In NHW women the estimated average effect of the Ava II (+) allele was to raise total cholesterol (LDL-C) by 6.52 mg/dL (4.95 mg/dL), and the average effect of the (−) allele was to lower total cholesterol (LDL-C) by 4.84 mg/dL (3.68 mg/dL); this variation explained 3.9% of the phenotypic variance in total cholesterol and 2.3% in LDL-C (Table 4). In Hispanic women the estimated average effect of the Ava II (+) allele was to increase total cholesterol (LDL-C) by 3.69 mg/dL (2.92 mg/dL), and the Ava II (−) allele lowered levels by 4.81 mg/dL (3.81 mg/dL); this variation explained 3.7% and 2% of the phenotypic variance in total cholesterol and LDL-C, respectively. However, the observation that the (−/−) and (+/+) levels were nearly identical (Table 3) suggests that the estimated average effect for the (+) allele may not be reliable for Hispanic women. The Ava II polymorphism showed gender- and ethnic-specific effects on LDL-C in NHW men (P=.017) and Hispanic women (P=.028) but in opposite directions (Tables 3 and 4). Triglyceride levels also varied significantly among Ava II genotypes in NHW women (P=.035).

Among women, two-way ANOVA revealed that in addition to the effect of the Ava II polymorphism on total cholesterol and LDL-C, the effect of menopausal status was also significant on these traits in both NHW (P=.037 and P=.040, respectively) and Hispanic (P=.021 and P=.071) women (Table 5). When we further categorized women into premenopausal and postmenopausal groups (Table 6), the effect of the Ava II polymorphism was still significant on total cholesterol and LDL-C in both groups of NHW women. Probably because of the small sample sizes, the effect was no longer statistically significant in Hispanic women. These
TABLE 3. Age-, Smoking-, and BMI-Adjusted Values of Quantitative Variables Among LDL Receptor/Ava II Genotypes by Ethnicity and Gender

<table>
<thead>
<tr>
<th>Trait</th>
<th>NHW</th>
<th>Hispanic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
</tr>
<tr>
<td>Trait</td>
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<td>+/+  144</td>
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<td>Chol, mg/dL</td>
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<td>HDL-C, mg/dL</td>
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<td>LDL-C, mg/dL</td>
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<tr>
<td>Tg, mg/dL</td>
<td>134.6</td>
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</table>

BMI indicates body mass index; LDL, low-density lipoprotein; NHW, non-Hispanic white; Chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, LDL cholesterol; and Tg, triglycerides. Values are mean (SEM).

data showed no physiological interaction between the Ava II polymorphism and menopausal status in affecting total cholesterol and LDL-C in Hispanics and NHWs (Table 5).

Nco I Polymorphism and Lipid Profile

Table 7 presents the plasma lipid variation among the three Nco I genotypes (−/−, +/−, and +/+ ) in NHW and Hispanic men and women. Similar to the Ava II polymorphism, the Nco I polymorphism also showed a gene-dosage effect on total cholesterol and LDL-C in women from both ethnic groups, but now the effect was significant in Hispanic women only (P = .019 for total cholesterol and P = .035 for LDL-C). In Hispanic women the Nco I (+−) allele was associated with 3.66 mg/dL (3.07 mg/dL) higher total cholesterol (LDL-C), and the Nco I (−−) allele was associated with 8.99 mg/dL (7.55 mg/dL) lower total cholesterol (LDL-C); this variation explained 3.3% and 2.6% of the phenotypic variance in total cholesterol and LDL-C, respectively (Table 8). The HDL-C level showed significant variability among the Nco I genotypes in NHW women (P = .019): the (−−) genotype was associated with lower levels of HDL-C than the other two genotypes. Although a similar trend was seen in men from both ethnic groups, the difference was not statistically significant. The average allelic effect associated with the (−) allele of the Nco I polymorphism was −2.21 mg/dL in NHW and Hispanic men and women; this variation explained 2.4% of the phenotypic variance in HDL-C (Table 8). Similar to the Ava II polymorphism, no evidence of interaction between the Nco I polymorphism and menopausal status was observed on any lipid trait among NHW and Hispanic women (data not shown).

LDL Receptor Gene–ApoE Gene Interaction

The apoE polymorphism has a significant effect on cholesterol levels in both Hispanics and NHWs.31 We
examined the possibility of joint effects (a genotype-genotype interaction) of the two unlinked loci, apoE and LDL receptor, in determining total cholesterol levels. The adjusted means for total cholesterol levels separated by gender and ethnicity are shown for the joint LDL receptor/Ava II and three common apoE genotypes (3-2, 3-3, and 4-3) in Fig 1 and for the joint LDL receptor/Nco I and apoE genotypes in Fig 2. Separate two-way ANOVA using apoE and LDL receptor for each gender and ethnicity showed no evidence of interaction with either Ava II or Nco I polymorphisms.

Individuals with the Ava II (−/−) and apoE 3-2 genotypes usually have the lowest mean levels, while those with the Ava II (+/+ ) and apoE 4-3 genotypes have the highest mean levels of total cholesterol. A similar pattern was also observed between the LDL receptor/Nco I and apoE genotypes, but this trend was more apparent in women from both ethnic groups than in men (Fig 1). The interaction studies for LDL-C also yielded results similar to those for total cholesterol (data not shown). These results indicate that the effects of the LDL receptor polymorphisms on total cholesterol and LDL-C are independent of the well-defined effect of the apoE polymorphism on these traits.

Discussion

We determined the distribution of two DNA polymorphisms in the LDL receptor gene and evaluated their significance in affecting plasma cholesterol concentrations in the general population of Hispanics and NHWs residing in the San Luis Valley of Colorado. The distribution pattern of the Nco I polymorphism was comparable between the two ethnic groups but varied significantly for the Ava II polymorphism. Hispanics showed a high frequency of the Ava II (+) allele compared with NHWs, and this difference may reflect a substantial amount of Amerindian admixture in the Hispanic population. Our data provide strong evidence that common genetic variation at the LDL receptor contributes to determining cholesterol levels in normolipidemic individuals. The Ava II polymorphism was significantly associated with both total cholesterol and LDL-C differences, but this effect was gender specific and confined to women in both ethnic groups. Women with the (+) allele at the Ava II site tended to have high levels of total cholesterol and LDL-C compared with the individuals with the (−) allele. There was a clear gene-dosage effect in the NHW women, the heterozygotes having cholesterol levels intermediate between the two types of homozygous genotypes. The genetic variation at the LDL receptor/Ava II polymorphism explains about 4% of the variance in total cholesterol and about 2% of the variance in LDL-C in women from both ethnic groups. The Nco I polymorphism also showed gender-specific and gene-dosage effects on total cholesterol and LDL-C in women, but these differences were statistically significant only in Hispanic women. In Hispanic women the Nco I polymorphism explains 3.3% of the phenotypic variance in total cholesterol and 2.6% of the variance in LDL-C. The ethnic- and gender-specific effects of the Nco I polymorphism on cholesterol levels probably reflect its nonrandom association with the Ava II polymorphism in both Hispanics and NHWs as noted in this study and by Leitersdorf et al.

The significant association of HDL-C variation with the Ava II and Nco I polymorphisms was not consistent

### Table 5. P Values From Two-Way ANOVA (LDL Receptor/Ava II x Menopause Status)

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<th>Hispanic</th>
<th>LDLR</th>
<th>Meno</th>
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<td>.265</td>
<td>.086</td>
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<td>LDL-C</td>
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<td>.048</td>
<td>.858</td>
<td>.071</td>
<td>.834</td>
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<tr>
<td>Tg</td>
<td>.042</td>
<td>.861</td>
<td>.418</td>
<td>.725</td>
<td>.892</td>
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<td>.725</td>
<td>.892</td>
</tr>
</tbody>
</table>

- LDL indicates low-density lipoprotein; NHW, non-Hispanic white; LDLR, LDL receptor; Meno, menopausal status; Chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, LDL cholesterol; and Tg, triglycerides.

### Table 6. Adjusted Total and LDL Cholesterol by LDL Receptor/Ava II Genotype in Premenopausal and Postmenopausal Women

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<td>(9.5)</td>
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<td>59</td>
<td>42</td>
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<td>(6.3)</td>
<td>(4.3)</td>
<td>(5.9)</td>
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<td>26</td>
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<td>26</td>
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<td>Chol</td>
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<td>200.9</td>
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</tr>
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<td>(7.8)</td>
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<td>.276</td>
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TABLE 7. Age-, Smoking-, and BMI-Adjusted Values of Quantitative Variables Among LDL Receptor/Nco I Genotypes by Ethnicity and Gender

| Trait        | NHW | Hispanic |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |     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interactions, through the influence of environmental factors. The apoE genetic variation strongly affects total cholesterol and LDL-C levels in Hispanics and NHWs. In contrast to Pedersen and Berg, who note a significant interaction between the Pvu II polymorphism at the LDL receptor locus and the apoE polymorphism in affecting variation in total cholesterol and LDL-C levels, we did not observe a significant interaction between these two genes in determining plasma cholesterol levels in this sample. Taking advantage of the large sample size in this study, we identified a subset of individuals who were homozygous for the common apoE 3-3 genotype and reexamined the effect of the LDL receptor polymorphisms against a common apoE background. We found a significant effect of LDL receptor polymorphisms in the apoE 3-3 subgroup (Figs 1 and 2). Similar results were obtained when the effect of the LDL receptor polymorphisms was analyzed among the apoE 3-2 and 4-3 genotypes separately (Figs 1 and 2). Thus, our study clearly shows that the effects of the apoE and LDL receptor/Ava II polymorphisms on total cholesterol and LDL-C are independent of each other; together the two polymorphisms explain about 6% and 14% of the phenotypic variance of total cholesterol.
cholesterol levels in NHW men and women, respectively, and about 3% and 9% in Hispanic men and women, respectively. Similar trends were observed for apoE and LDL receptor/Nco I genotypes, although the effects were significant only in women, in whom the two polymorphisms explained about 9% of the phenotypic variance in total cholesterol. The different effect of the allelic variation between men and women seen in this study has been well documented for the apoE polymorphism (for review, see Reference 31). Since the risk of cardiovascular disease is different among men and women, the difference in gender allele effect on quantitative traits is expected. These differences may have a significant effect on cardiovascular disease risk prediction between men and women.

In conclusion, this study demonstrated that genetic variation at the LDL receptor locus contributes significantly to the determination of plasma cholesterol levels in the general population.

Acknowledgments
Support for this study was provided in part by National Institutes of Health (NIH) grant HL-44672 from the National Heart, Lung, and Blood Institute, and by the National Dairy Board and administered in cooperation with the National Dairy Council. Core funding for the San Luis Valley Diabetes Study was from NIH grant DK-30747 and Clinical Research Center core grant CRC-RR00005. We thank Lori Kelly for her excellent technical assistance and Kimberley Smithwick for clerical assistance.

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Role of common genetic polymorphisms in the LDL receptor gene in affecting plasma cholesterol levels in the general population.
Y I Ahn, M I Kamboh, C E Aston, R E Ferrell and R F Hamman

doi: 10.1161/01.ATV.14.5.663
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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