Phenotypic Effects of Apolipoprotein Structural Variation on Lipid Profiles

III. Contribution of Apolipoprotein E Phenotype to Prediction of Total Cholesterol, Apolipoprotein B, and Low Density Lipoprotein Cholesterol in the Healthy Women Study

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The apolipoprotein (apo) E structural locus has been shown to influence concentrations of total cholesterol, apo B, and low density lipoprotein (LDL) cholesterol in population studies. Apo E has six phenotypes resulting from three common alleles at this locus. In the present study, we have typed 473 healthy white women for apo E. At baseline in 1984, all women were premenopausal. To date, 109 of these women have become postmenopausal and are not on hormone therapy. Statistical analyses were done on both pre- and postmenopausal groups to assess the influence of menopausal status in combination with the apo E locus on lipid profile. Nine lipoprotein lipids and apolipoproteins were categorized by three apo E phenotypes: apo E3-2, apo E3-3, and apo E4-3. These were compared in analysis of variance. At baseline, the apo E3-2 phenotype showed the lowest average concentrations of total cholesterol (170 mg/dl), apo B (80 mg/dl), and LDL cholesterol (91 mg/dl), while the apo E4-3 phenotype demonstrated the highest average concentrations of total cholesterol (192 mg/dl), apo B (104 mg/dl), and LDL cholesterol (116 mg/dl) (p<0.0004). Apo E3-3 homozygotes were intermediate on all three quantitative variables. The postmenopausal subset showed the same trends by phenotype, with overall increases in total cholesterol, apo B, LDL cholesterol, and triglycerides, regardless of phenotype. Women who remained premenopausal generally showed smaller increases in these same measures. Our results suggest that, on average, the lower lipoprotein values for the apo E3-2 phenotype are maintained through early menopause despite a worsening of lipid profiles for all women as they age. There did not appear to be a marked effect of apo E phenotype on change in lipoprotein lipids and apolipoproteins that might reflect an interaction between this locus and hormonal status. In addition, the effects of several environmental determinants of total cholesterol, apo B, and LDL cholesterol, along with apo E, were assessed by multivariate regression analysis. (Arteriosclerosis 10:379-385, May/June 1990)
density lipoprotein (VLDL) remnants, and a subgroup of high density lipoprotein (HDL) cholesterol, apo E functions as a mediator of endocytosis, and thus modulates the metabolism of atherogenic LDL cholesterol particles and may also function in reverse cholesterol transport.

Apo E exhibits structural heterogeneity due to single amino acid substitutions. Three alleles (apo E*2, apo E*3, apo E*4) determine six phenotypes. The E2 [arg158 -> cys] and E4 [cys121 -> arg] isoforms probably result from point mutations in the most common isoform, E3. The apo E structural locus is located on chromosome 19 in close proximity to the genes for apo C-I and apo C-II. The locus codes for a polypeptide of 299 amino acids with a calculated molecular weight of 34 145. The complete amino acid sequence of apo E has been published.

At the population level, the apo E protein polymorphism has been shown to have a consistent impact on total cholesterol, apo B, and LDL cholesterol concentrations. This influence exists in normolipemic persons as well as in patients with angiographic evidence of CAD. While evidence at both the population and molecular levels exists for the impact of this locus on cholesterol and apo B levels, angiographically documented studies in CAD patients have provided conflicting support for a causative role of apo E in myocardial infarction. Based on a study of patients undergoing coronary angiography versus controls, Menzel et al. proposed that, in the absence of hypercholesterolemia, the apo E*2 allele may provide protection against atherosclerosis.

In the present study, we confirm the effects of the apo E structural locus on lipids in a population of healthy premenopausal women, look at changes occurring with menopause, and assess the relative contributions of the apo E phenotype and several environmental predictors on total cholesterol, apo B, and LDL cholesterol.

Methods

Samples
Serum samples, which had been frozen at -20°C for approximately 1 year, were used for apo E genotyping. Lipid, lipoprotein, and apolipoprotein determinations were done on blood drawn at a baseline clinic visit occurring between November, 1983 and May, 1985. All samples come from the Healthy Women Study, a prospective study of cardiovascular risk factors in 541 premenopausal women (ages 42 to 50) residing in Pittsburgh, Pennsylvania. A total of 4153 women randomly sampled from women with drivers’ licenses in selected zip codes of Allegheny County, Pennsylvania, were recruited by mail. Of these, 2405 completed a telephone recruitment survey. Approximately 58% of this latter group were excluded from the study because of hysterectomy, amenorrhea, hypertension, or other problems with eligibility. Of women contacted and meeting criteria for inclusion, 63.7% (541) agreed to participate in the study, which involved initial home and clinic visits and follow-up over a 7-year period. Initially, each woman had 60 ml of blood drawn for determination of blood biochemistry profiles. A total of 473 Caucasian subjects were typed for apo E. Women not typed were either of other racial origins or did not have serum available. Blood was also drawn at follow-up visits.

Quantitative Lipid and Apolipoprotein Measures
Participants fasted for at least 12 hours before blood drawing for determination of lipoprotein lipids, apolipoproteins, and triglycerides. Blood was centrifuged, and serum was separated. HDL cholesterol (total HDL cholesterol) was measured by a heparin-manganese precipitation method, and the HDL cholesterol subfractions, HDL2 and HDL3, were separated by using dextran sulfate. HDL2 was computed as the difference between total HDL cholesterol and HDL3. Triglycerides were measured enzymatically. LDL cholesterol was calculated by using the Friedewald formula. Electrophoresis was used for determination of apo A-I and apo B concentrations. The intraassay coefficient of variation for apo A-I was 6.4% and for apo B, 10.6%. Apo A-II was measured by an enzyme-linked immunoabsorbant assay. The intraassay coefficient of variation for apo A-II was 10.5%.

Apolipoprotein E Typing
Isoelectric focusing (IEF) followed by immunoblotting was used to detect apo E phenotypes. Serum samples were delipidated by using the guanidine-HCl method proposed by Havekes et al. IEF was performed on 5% (wt/vol) flat bed polyacrylamide gels containing 3 M urea. The gels were prepared as follows: 5.4 g of urea was dissolved in 5 ml of monomer solution (29.1% wt/vol), 5 ml of bisacrylamide solution (0.9% wt/vol), 0.45 ml of Pharmalyte ampholines (pH 4.5 to 5.4), and 0.90 ml of Pharmalyte ampholines (pH 5 to 8), and this was adjusted to a total volume of 30 ml with deionized water. An aliquot of 30 ml of a 0.1% riboflavin solution was added directly to this solution to initiate the polymerization reaction. Each gel was photopolymerized under fluorescent light overnight.

Electrode strips (6 mm x 25 cm) were saturated with 1 M of NaOH (cathode) and 1 M of H3PO4 (anode) and were placed on opposite sides of the gel. Wicks (5 mm x 3 mm made of 3 MM filter paper) were saturated with delipidated serum, were blotted on filter paper, and were loaded approximately 0.5 cm from the cathode. The gel was run for 3 hours on an LKB 217 Ultraphor electrophoresis unit at 10 W, 2000 V, and 250 mA at 10°C. After 1 hour, the wicks were removed, and the electrofocusing continued. At the conclusion of electrofocusing, the proteins were blotted onto a nitrocellulose filter of 0.2 µm pore size by simple diffusion. This transfer was allowed to proceed for 1 hour. The filter was then removed from the gel, washed in Tris-buffered saline (TBS) solution (0.25 M NaCl, 0.03 M Tris-HCl pH 8.0), and was incubated overnight with 5% (wt/vol) powdered skim milk to block the remaining protein-binding sites.

A double antibody technique was used to visualize the apo E phenotypes. The filter was rinsed in TBS briefly and then exposed to goat-antihuman apo E polyclonal antibody (Daichi Chemical, Tokyo, Japan) diluted 1:500 in TBS buffer for 3 hours. The filter was washed for 45 minutes with three changes of TBS buffer. The second
incubation was with rabbit-antigoat IgG conjugated with the enzyme alkaline phosphatase and lasted 1 hour (1:5000 dilution in TBS). Again, the filter was washed for 45 minutes with three changes of TBS buffer.

The filter was stained histochemically by using 25 mg of β-naphthyl phosphate, 25 mg of Fast Blue BB salt, and 60 mg of magnesium sulfate in 50 ml of stock buffer (1.8 g NaOH, 3.7 g boric acid/l).

Collection of Other Variables

Other variables [i.e., age, race, body mass index (BMI), cigarette smoking, leisure time energy expenditure, and alcohol consumption] used in the analyses were collected at the baseline examination. BMI, an index of obesity, was calculated using weight in kilograms divided by height in meters squared (kg/m²). Cigarette smoking was measured as cigarettes per day. The Paffenbarger questionnaire was used to estimate energy expenditure in leisure time physical activity and was measured as kilocalories per week. Alcohol intake, in grams per day, was based on self-reported frequency, amount, and type of alcoholic beverages normally consumed.

Statistics

Statistical analyses were done with the computer package, SPSSx. Analysis of variance (ANOVA) with nine quantitative lipid measures used individually (total cholesterol, total HDL cholesterol, HDL2, HDL3, triglycerides, apo A-I, apo A-II, apo B, and LDL cholesterol) as dependent variables and three apo E phenotypes (apo E 3-2, apo E 3-3, and apo E 4-3) as categorical variables was performed on residuals. Residuals were saved after use of stepwise regression analysis to adjust quantitative measures for age and BMI, since both of these attributes vary concomitantly with lipid levels. Not every quantitative lipid measure needed to be adjusted for both age and BMI; thus, adjustments were only done if age and/or BMI influenced the outcome variable. One quantitative residual variable, triglycerides, was transformed using natural logarithms, because this improved the normality of the distribution. ANOVA was then done on the transformed triglyceride residuals. The average allelic effects for apo E were calculated with the formula of Boerwinkle et al.

A multivariate linear model was used to determine the contribution of the apo E locus along with other determinants of lipid levels in premenopausal women. These latter variables included cigarette smoking, BMI, age, alcohol consumption, and leisure time energy expenditure. With the exception of apo E and smoking, all variables were continuous. Three phenotypes of apo E (apo E 3-2, apo E 3-3, and apo E 4-3) and cigarettes/day (1 to 20 and 21 or more) were entered as dummy variables. The remaining three phenotypes had too few individuals for modeling. Multiple regression equations were generated for three dependent variables: total cholesterol, apo B, and LDL cholesterol.

ANOVA was also done on a group of postmenopausal women not using hormones and on change in quantitative measures based on the difference between pre- and postmenopausal status. Postmenopausal is defined as at least 12 months ammenorrheic. Change variables for each subgroup were computed over a 3- to 3.5-year period.

Results

A total of 473 white women were typed for apo E, and their phenotypes were used in the statistical analyses. Allele frequencies in this sample were: apo E*2=0.059, apo E*3=0.819, and apo E*4=0.122 (Table 1). Alleles at the apo E locus are in Hardy-Weinberg equilibrium. The distribution of phenotypes is presented in Table 1. These frequencies are comparable to those obtained on a white diabetic population from the same geographical area (Kamboh, unpublished data).

Because lipid and apolipoprotein levels are influenced by age and BMI, these latter two variables were controlled for before the analysis of variance was performed. Adjustments in the quantitative variables in premenopausal women were made as follows: age and BMI accounted for 3% of the variance in total cholesterol, 28% of the variance in triglycerides, and 12% of the variance in apo B. BMI accounted for 15% of the variance in HDL cholesterol, 16% of the variance in HDL2, 5% of the variance in HDL3, 2% of the variance in apo A-I, and 3% of the variance in LDL cholesterol. Age explained 2% of the variance in apo A-II. ANOVA was done on residual values after adjustments. Because of small cell sizes for three phenotypes (apo E 2-2=2; apo E 4-2=5, and apo E 4-4=6), these were excluded from the analyses. The mean adjusted values were obtained by adding the overall mean to the phenotype residual means, thus restoring recognizable values. Table 2 presents phenotypic means and standard errors for the major three apo E phenotypes. The mean values of relevant quantitative measures for the remaining three phenotypes are discussed in the text.

A statistically significant effect of the apo E phenotype on total cholesterol, apo B, LDL cholesterol, and apo A-II was observed. Apo E 3-2 heterozygotes had lower average total cholesterol, apo B, and LDL cholesterol than the apo E 3-3 homozygotes. Apo E 4-3 heterozygotes had higher average total cholesterol, apo B, and LDL cholesterol than the apo E 3-3 homozygotes. Although there were only five apo E 4-2 heterozygotes at baseline, their
average total cholesterol (160.3±10.7 mg/dl) and LDL cholesterol (86.8±7.4 mg/dl) were the lowest of all phenotype classes. The concentration of apo B (95.7±15.6 mg/dl), on the other hand, was close to the overall average. The apo E 4-4 homozygotes, while only six in number, had the highest average cholesterol (202.9±12.6 mg/dl) and LDL cholesterol (124.7±9.1 mg/dl) levels among all phenotype classes. This was not true for apo B concentration (90.6±13.1 mg/dl). There were two individuals with the apo E 2-2 phenotype with an average total cholesterol of 165.1±2.1 mg/dl, an average apo B of 147.3±1.0 mg/dl, and an average LDL of 90.9±3.6 mg/dl.

In this population, the average effect of the apo E*2 allele was to lower total cholesterol by 16 mg/dl, apo B by 11 mg/dl, and LDL cholesterol by 18 mg/dl. The average effect of the apo E*4 allele was to raise cholesterol by 6 mg/dl, apo B by 8 mg/dl, and LDL cholesterol by 6 mg/dl. At baseline in premenopausal women, after adjusting for age and BMI, the apo E structural locus accounted for 3% of the variation in total cholesterol, 8% of the variation in apo B, and 5% of the variation in LDL cholesterol. The apo E phenotype also appeared to influence concentration of apo A-II, with the apo E 4-3 heterozygotes showing a higher average concentration than the apo E 3-3 homozygotes and 3-2 heterozygotes (Table 2). However, this was no longer true in the postmenopausal subset.

Multivariate analyses demonstrate apo E to be an important predictor of total cholesterol, apo B, and LDL cholesterol levels in premenopausal women given additional information about risk factors for CAD. Determinants of total cholesterol, apo B, and LDL cholesterol are presented in Table 3 with their beta coefficients and standard errors. Each determinant is statistically significant at p≤0.05. Beta coefficients give an idea of the impact of each independent variable on the dependent variable. Cumulative adjusted r² values give the proportion of variance accounted for by the combined variables (i.e., apo E3-2, apo E4-3, BMI, cigarettes/day, age, and alcohol intake). For total cholesterol, r²=8%; for apo B, r²=22%; and for LDL cholesterol, r²=14%.

Because the Healthy Women Study is a longitudinal study, repeat measures of lipids were done approximately 3.5 years after entry and as women became postmenopausal. Of this group, 109 postmenopausal women who were not on hormone replacement therapy showed the same effects of apo E phenotype on total cholesterol, apo B, and LDL cholesterol (Table 4),
Effects of Apo E Phenotype

Table 4. Phenotype Specific Means and Hypothesis Testing Results in White Postmenopausal Women Not Taking Hormones

<table>
<thead>
<tr>
<th>Variable</th>
<th>Apo E 3-2 (n=12)</th>
<th>Apo E 3-3 (n=79)</th>
<th>Apo E 4-3 (n=18)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol*</td>
<td>189.4 (10.9)</td>
<td>206.3 (3.6)</td>
<td>213.3 (10.2)</td>
<td>1.78</td>
<td>0.1734</td>
</tr>
<tr>
<td>Total HDL cholesterol†</td>
<td>56.2 (2.8)</td>
<td>57.7 (1.6)</td>
<td>56.3 (3.3)</td>
<td>0.12</td>
<td>0.8880</td>
</tr>
<tr>
<td>HDL₃*⁺⁺⁺⁺⁺†</td>
<td>17.5 (2.7)</td>
<td>18.3 (1.1)</td>
<td>15.4 (2.1)</td>
<td>0.62</td>
<td>0.5380</td>
</tr>
<tr>
<td>HDL₄*⁺⁺⁺⁺⁺†</td>
<td>39.4 (1.7)</td>
<td>39.3 (0.9)</td>
<td>41.0 (2.2)</td>
<td>0.32</td>
<td>0.7255</td>
</tr>
<tr>
<td>Triglycerides⁺⁺⁺⁺⁺†</td>
<td>100.5 (13.2)</td>
<td>105.7 (7.5)</td>
<td>119.6 (15.5)</td>
<td>0.35</td>
<td>0.7047</td>
</tr>
<tr>
<td>LDL cholesterol⁺⁺⁺⁺⁺†</td>
<td>113.3 (10.1)</td>
<td>127.3 (3.3)</td>
<td>132.6 (9.3)</td>
<td>1.37</td>
<td>0.2579</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>151.8 (5.0)</td>
<td>144.3 (2.5)</td>
<td>148.5 (5.7)</td>
<td>0.77</td>
<td>0.4669</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>49.1 (3.9)</td>
<td>50.8 (1.1)</td>
<td>53.3 (2.8)</td>
<td>0.58</td>
<td>0.5592</td>
</tr>
<tr>
<td>Apo B⁺⁺⁺⁺⁺†</td>
<td>95.5 (7.9)</td>
<td>108.4 (2.7)</td>
<td>113.3 (7.4)</td>
<td>1.81</td>
<td>0.1683</td>
</tr>
</tbody>
</table>

The values for the phenotypes are given in mg/dl and are the means and standard errors. Quantitative variables were adjusted for age* and body mass index.† Indicates a log₁₀ transformation of residuals for calculation of F statistic.

HDL=high density lipoprotein, LDL=low density lipoprotein, Apo=apolipoprotein.

Table 5. Mean Change in Quantitative Measure by Apolipoprotein E Phenotype in White Women Who Experienced Natural Menopause

<table>
<thead>
<tr>
<th>Variable</th>
<th>Apo E 3-2 (n=12)</th>
<th>Apo E 3-3 (n=78)</th>
<th>Apo E 4-3 (n=18)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>14.2 (4.6)</td>
<td>15.7 (2.6)</td>
<td>15.7 (7.1)</td>
<td>0.02</td>
<td>0.9778</td>
</tr>
<tr>
<td>Total HDL cholesterol</td>
<td>-6.0 (2.8)</td>
<td>-0.7 (0.9)</td>
<td>-3.2 (2.7)</td>
<td>2.17</td>
<td>0.1194</td>
</tr>
<tr>
<td>HDL₃*⁺⁺⁺⁺⁺†</td>
<td>-6.0 (2.0)</td>
<td>-2.3 (0.8)</td>
<td>-7.2 (2.0)</td>
<td>3.86</td>
<td>0.0242</td>
</tr>
<tr>
<td>HDL₄*⁺⁺⁺⁺⁺†</td>
<td>0.0 (1.9)</td>
<td>1.7 (1.0)</td>
<td>4.0 (2.0)</td>
<td>0.94</td>
<td>0.3940</td>
</tr>
<tr>
<td>Triglycerides⁺⁺⁺⁺⁺†</td>
<td>15.8 (13.1)</td>
<td>13.3 (5.3)</td>
<td>7.7 (11.5)</td>
<td>0.14</td>
<td>0.8738</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>17.0 (4.6)</td>
<td>13.7 (2.3)</td>
<td>17.4 (6.0)</td>
<td>0.31</td>
<td>0.7366</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>6.0 (4.7)</td>
<td>1.1 (2.7)</td>
<td>-1.1 (7.0)</td>
<td>0.31</td>
<td>0.7343</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>-1.3 (3.8)</td>
<td>-1.5 (1.2)</td>
<td>-1.8 (2.3)</td>
<td>0.01</td>
<td>0.9898</td>
</tr>
<tr>
<td>Apo B⁺⁺⁺⁺⁺†</td>
<td>15.9 (6.6)</td>
<td>10.4 (2.8)</td>
<td>-0.7 (3.9)</td>
<td>2.13</td>
<td>0.1244</td>
</tr>
</tbody>
</table>

Values for the phenotypes are given as mg/dl and are means and standard errors. HDL=high density lipoprotein, LDL=low density lipoprotein, Apo=apolipoprotein.

Although the results did not reach statistical significance, it is possible that hormonal status may temporarily obscure the more consistent differences seen by apo E phenotype in premenopausal women. As the postmenopausal group increases and more data are available, these estimates will become more stable.

The change variable, which is based on the difference between pre- and post-lipoprotein lipid and apolipoprotein levels in this small group of women who became postmenopausal demonstrates, in general, an increase in total cholesterol, LDL cholesterol, apo B, and triglycerides (Table 5). There is, however, no clear evidence that apo E phenotype is related to the degree of change over time. Change in HDL₃ in postmenopausal women, which had a p value of 0.0242 in ANOVA, did not show this difference in women who remained premenopausal (Tables 5 and 6). More data are needed to determine if this is a true association related to menopause and not due to small cell sizes.

Table 6 presents change variables in 250 premenopausal women typed for the most common apo E phenotypes. These women who remained premenopausal after approximately 3.5 years of follow-up also demonstrated changes in lipoprotein lipids and apolipoproteins but not as dramatically as those experiencing a natural menopause. Apo E phenotype did not show a statistically significant effect on change in women who remained premenopausal.

Discussion

Many variables were collected on each subject in the Healthy Women Study to help characterize the complex nature of CAD risk. Because of this, it was possible to assess the impact of the apo E locus, given other determinants of lipid and apolipoprotein levels. The multiple linear regression analyses demonstrate the contribution of both genetic and environmental factors in predicting quantitative lipid and apolipoprotein levels. It is interesting to note that the greatest amount of variance accounted for, 22% for apo B, required four environmental variables, BMI, age, cigarettes/day, and alcohol/day, and one genetic variable, apo E phenotype. Each of the attributes made a small independent contribution to the...
concentration of apo B. Still, 78% of the variation was not associated with these known risk factors. The apo E locus provided only modest additional information in determining cholesterol, apo B, and LDL cholesterol concentrations.

Estrogen, which offers protection from CAD risk, does not mask the allelic effects of the apo E locus. At baseline, all women were premenopausal. Subsequent analyses in a group of postmenopausal women not on estrogen replacement therapy demonstrated similar, albeit not statistically significant, phenotypic effects on total cholesterol, apo B, and LDL cholesterol (Table 4). Lack of statistical significance is most likely due to the smaller sample size, but might also be due to the decline in estrogen output. In this group of women who became postmenopausal, the average age at baseline was 47.9 years and the average BMI, 25.3. For their postmenopausal visit, the mean age was 51.1 with a mean BMI of 26.3.

Results from the Healthy Women Study, as well as from other longitudinal studies, have shown a greater increase in total cholesterol over time among women who became postmenopausal than among those who remained premenopausal. In the Healthy Women Study, along with increases in age and BMI, there were concomitant increases in total cholesterol, triglycerides, apo B, and LDL cholesterol. Total HDL cholesterol was reduced, largely due to HDL₂. In between 3 to 4 years, there has been an overall worsening of the lipid profiles of the study subjects. This was not entirely due to cessation of menses since a control group of women, who remained premenopausal at follow-up, showed similar trends (Table 6). A smaller group of women who began hormone replacement therapy also demonstrated increases in total cholesterol and apo B; however, LDL cholesterol was not as dramatically increased. Contrary to the pre- and postmenopausal groups, women on hormone therapy showed increases in HDL cholesterol and apo A-I. Triglycerides rose in all groups, especially in those using hormones.

Apo E phenotype clearly influenced total cholesterol, apo B, and LDL cholesterol in cross-sectional analyses. However, in preliminary analyses, phenotype did not markedly affect changes in lipid parameters either in women who remained premenopausal or in those who experienced a natural menopause. Knowledge of the combined effects of hormones on lipids and genes that function in regulatory roles may aid in understanding the complex influences on lipid profiles in women as they age.

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References


Index Terms: apolipoprotein E • lipoproteins • Healthy Women Study • apolipoprotein polymorphisms
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