**Underexpression of the Apolipoprotein E4 Isoform in an Italian Population**

Richard W. James, Massimo Boemi, Roberto Giansanti, Paulo Fumelli, Daniel Pometta

Apolipoprotein (apo) E polymorphism was examined in a population of Italian blood donors. A significantly reduced frequency of the e4 allele was observed in comparison to a combined Caucasian population. Apo E polymorphism was also associated with significant differences in plasma lipid and lipoprotein levels. Notably, total and low-density lipoprotein cholesterol as well as triglycerides were increased, whereas high-density lipoprotein cholesterol was decreased in carriers of the E4 isoform. This is the first report of a significantly lower frequency of the apo E4 isoform in a European population. The reduced occurrence of an apo E isoform, which is associated with a more atherogenic lipid/lipoprotein profile, may be a contributory factor to the relatively lower incidence of cardiovascular disease in the Italian population. (Arterioscler Thromb. 1993;13:1456-1459.)

**Key Words** • apo E • lipoproteins • cholesterol • triglycerides • cardiovascular risk factors

Factors that influence plasma lipid levels have come under close scrutiny because of the role of the latter in the development of atherosclerotic disease. In this respect, apolipoprotein (apo) E, which is responsible for the elimination of potentially atherogenic remnant particles, is of particular interest. This apolipoprotein exists in three major isoforms: the ancestral and predominant E3 isoform and two mutant forms, E2 and E4. While apo E4 retains full and perhaps even increased receptor binding activity, the affinity of the E2 isoform for lipoprotein receptors is dramatically decreased. Polymorphism at the apo E gene locus has attracted a great deal of attention because of its consequences for plasma lipid levels. Thus, in comparison to homozygosity for E3, the E2 and E4 isoforms have, respectively, cholesterol-lowering and cholesterol-raising influences. This observation has led to suggestions that the E2 isoform may offer a measure of protection against cardiovascular disease. However, the cardioprotective effect can be corrupted by the presence of defects that result in increased production of triglyceride-rich lipoproteins and their consequent catabolic remnants, thus exposing the reduced receptor-binding capacity of apo E2. This diminished capacity is manifested in its extreme form as dysbetalipoproteinemia. The cholesterol-raising effect of E4, in part due to more efficient absorption of dietary cholesterol, would tend to designate E4 as an atherogenic isoform, and there exists evidence that it does indeed predispose to cardiovascular disease. Although further studies are necessary to consolidate this assertion, several groups have demonstrated a heterogeneity of apo E phenotype frequencies among populations, for example, a reduced frequency of the mutant isoforms in certain Asian populations compared with Caucasians. This raises a very interesting question, given the well-established differences in the incidence of cardiovascular disease between these populations. While other factors, notably dietary habits, are of prime consideration, apo E polymorphism may also contribute to interpopulation differences with respect to cardiovascular disease rates.

Within European populations, the Finns are distinguished by a significantly increased apo E4 allele frequency. They also have a relatively high incidence of cardiovascular disease that has been primarily attributed to their intake of saturated fats. Southern European countries enjoy a lower incidence of cardiovascular disease, and here again, dietary considerations are usually invoked. In the present study, we have examined apo E polymorphisms in an Italian population of blood donors. Our results demonstrate a reduced occurrence of the E4 isoform. As the latter is associated with a more atherogenic lipid profile, this observation raises the question of whether the relatively lower frequency of E4 is a contributory factor to the reduced incidence of cardiovascular disease in the Italian population.

**Methods**

**Subjects**

Subjects were recruited from those attending the blood donor center in Ancona, Italy, to act as a control population for ongoing studies of apo E polymorphisms in diabetic patients. A total of 365 subjects were enrolled, 296 men and 69 women, whose numbers are representative of the male-to-female ratio of patients attending the center. Blood was obtained from fasting subjects to allow liver and renal functions to be analyzed. Thus, subjects attended the center between 8 and 10 AM, which essentially accounts for the lower proportion of women (who are preoccupied at that time with domestic responsibilities). Light refreshment is offered, but there is no indication that this acts as an inducement
to donate blood. The average age (mean±SD) was 43.0±11.4 years (range, 20 to 64 years) for the whole population (males, 43.2±10.6 years; females, 42.0±14.3 years). Body mass index (BMI) was 25.2±3.2 (range, 18.7 to 39.6) for the whole population (males, 25.5±3.2; females, 23.8±2.8).

Blood samples were centrifuged to obtain plasma, which was rapidly frozen and stored at −70°C. The frozen samples were transferred on dry ice to Geneva every 1 to 2 months for apo E phenotyping and measurement of blood lipid and apolipoprotein levels. In preliminary studies, we established that storage of plasma samples under such conditions (up to at least 6 months) did not affect subsequent phenotyping of apo E.

**Apo E Phenotyping**

Phenotyping of apo E was achieved by using a modified version of the procedure described by Menzel et al. Delipidated whole plasma was isoelectrofocused, and apo E isoforms were revealed by immunoblotting with a high-affinity, anti-apo E monoclonal antibody produced in our laboratory.

**Lipid and Apolipoprotein Assays**

Plasma cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol (after precipitation of lower-density lipoproteins with phosphotungstate) were assayed enzymatically, and apop A-I and B were measured by electroimmunoassay as described previously. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula for samples with triglyceride values <400 mg/dL.

**Statistical Analyses**

Allele frequencies were estimated by the gene-counting method. Statistical analyses of frequency distributions were performed with the χ² goodness-of-fit test. Analysis of variance (ANOVA) was employed to compare lipid and apolipoprotein levels according to apo E type. Analyses of triglyceride measurements were performed after logarithmic transformation of the values.

**Results**

The observed phenotype (in Hardy-Weinberg equilibrium; χ²=6.75, df=5; P=.24) and allele frequencies of the Italian population are summarized in Table 1. When compared with those of an average Caucasian population (Table 1), significant differences were observed both for phenotypes (χ²=23.35, df=5, P<.001) and alleles (χ²=18.37, df=2, P<.001), due to a reduced occurrence of the E4 isofrom.

A second series of analyses examined the influence of apo E polymorphism on various plasma and apolipoprotein parameters after grouping the subjects as E2 carriers (genotypes e2/2 plus e2/3), E3 homozygotes (e3/3), and E4 carriers (e4/3 plus e4/4).

Preliminary analyses established that between the subgroups, there were no significant differences in the distribution of the three factors known to influence blood lipid levels, namely gender (χ²=0.745, P=.86), age (five groups: 20 to 29, 30 to 39, 40 to 49, 50 to 59, and >60 years; χ²=8.57, P=.38), and BMI (categories: ≤24.9, ≥25 but ≤29.9, and ≥30; χ²=7.74, P=.1). Apo E polymorphism had a highly significant (P<.0001) influence on total and LDL cholesterol as well as on apo B, with concentrations rising from e2 to e3 to e4 carriers.

**Table 1. Phenotype and Allele Frequencies of Italian and Caucasian Populations**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Italian n</th>
<th>Italian Percent</th>
<th>Caucasian n</th>
<th>Caucasian Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/2</td>
<td>3</td>
<td>0.8</td>
<td>41</td>
<td>0.7</td>
</tr>
<tr>
<td>E3/2</td>
<td>43</td>
<td>11.8</td>
<td>720</td>
<td>12.4</td>
</tr>
<tr>
<td>E3/3</td>
<td>257</td>
<td>70.4</td>
<td>3425</td>
<td>59.0</td>
</tr>
<tr>
<td>E4/3</td>
<td>51</td>
<td>14.0</td>
<td>1341</td>
<td>23.1</td>
</tr>
<tr>
<td>E4/4</td>
<td>7</td>
<td>1.9</td>
<td>133</td>
<td>2.3</td>
</tr>
<tr>
<td>E4/2</td>
<td>4</td>
<td>1.1</td>
<td>145</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The allele frequency of the Caucasian population is an average determined by Davignon et al (Table 1 of Reference 8). Phenotype frequencies for the Caucasian population were calculated from the allele frequency based on Hardy-Weinberg equilibrium. Italian, N=365; Caucasian, N=5805.

(Tables and Figure). From these data, the average effect of the e allele variation on plasma cholesterol levels was determined. Whereas E3 had little overall influence on cholesterol levels (−0.24 mg/dL), E2 lowered levels by 17.0 mg/dL and E4 raised cholesterol by 16.8 mg/dL.

The e4 allele also distinguished itself from the other two alleles by the significantly lower HDL cholesterol and significantly higher triglyceride levels (Table 2). Elimination of subjects with severe hypertriglyceridemia (triglycerides >400 mg/dL; see the footnote to Table 2) did not modify this conclusion: ANOVA again demonstrated a highly significant difference between mean triglyceride values (E2, 114.7±55.7 mg/dL; E3, 113.5±58.8 mg/dL; and E4, 140.2±65.2 mg/dL; P=.006).

**Discussion**

The present results are the first indication of an underrepresentation of the cholesterol-raising apo e4 allele in a Caucasian cohort. These findings underline the heterogeneity of apo E allele frequencies within the European community and provide a stark contrast to certain northern European populations (Finland and, to a lesser extent, Iceland), where overexpression of apo E4 is evident. It is in this context that the observations are of particular interest if one considers that Finnish and Italian populations represent the extremes of cardiovascular disease rates on a European scale.

The contribution of apo E polymorphisms to the incidence of cardiovascular disease across populations remains an open question, in part because of a paucity of relevant studies. Presently available data are suggestive of a beneficial influence of the e2 allele on cardiovascular disease, while the e4 allele appears to predispose to cardiovascular disease (see References 8 and 14 for a discussion). As regards the e4 allele, this proposal is based on its underexpression in octogenarians, its overexpression in patients with documented cardiovas-
TABLE 2. Plasma Lipid, Lipoprotein, and Apolipoprotein Levels as a Function of Apolipoprotein E Alleles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total</th>
<th>e2</th>
<th>e3</th>
<th>e4</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n, M/F</td>
<td>361 (293/68)</td>
<td>46 (37/9)</td>
<td>257 (211/46)</td>
<td>58 (45/13)</td>
<td>. . .</td>
</tr>
<tr>
<td>Age, y</td>
<td>43.0±11.4</td>
<td>43.2±12.8</td>
<td>42.6±11.2</td>
<td>44.0±11.5</td>
<td>.70</td>
</tr>
<tr>
<td>BMI, weight/height</td>
<td>25.2±3.2</td>
<td>26.0±3.6</td>
<td>25.2±3.2</td>
<td>24.7±2.7</td>
<td>.10</td>
</tr>
<tr>
<td>Cholesterol*</td>
<td>204.2±40.2</td>
<td>186.5±39.8</td>
<td>203.2±38.7</td>
<td>221.5±41.0</td>
<td>.0001</td>
</tr>
<tr>
<td>Triglycerides†</td>
<td>123.3±79.7</td>
<td>115.8±55.7</td>
<td>119.5±82.2</td>
<td>146.7±82.9</td>
<td>.003</td>
</tr>
<tr>
<td>HDL cholesterol*</td>
<td>40.5±9.4</td>
<td>41.5±10.4</td>
<td>41.0±9.2</td>
<td>37.9±9.3</td>
<td>.024</td>
</tr>
<tr>
<td>LDL cholesterol*†</td>
<td>138.4±36.1</td>
<td>120.5±35.7</td>
<td>137.8±34.0</td>
<td>155.0±38.3</td>
<td>.0001</td>
</tr>
<tr>
<td>Apo A-I*</td>
<td>1.19±0.17</td>
<td>1.20±0.15</td>
<td>1.20±0.17</td>
<td>1.17±0.17</td>
<td>.33</td>
</tr>
<tr>
<td>Apo B*</td>
<td>0.75±0.20</td>
<td>0.67±0.20</td>
<td>0.75±0.20</td>
<td>0.83±0.23</td>
<td>.0001</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apolipoprotein. e2 includes E2/2 plus E3/2; e3 is E3/3; e4 is E4/3 plus 4/4. Subjects with the E4/2 phenotype (n=4) were not considered.

*Values are mean±SD and are in milligrams per deciliter for lipids and grams per liter for apolipoproteins. An analysis of variance was employed to examine differences in mean values between E allele carriers.

†Triglyceride values were subjected to logarithmic transformation before analysis.

‡To estimate LDL cholesterol levels by the Friedewald equation, subjects with triglycerides >400 mg/dL were excluded. Here, n=45 for e2, n=233 for e3, and n=56 for e4.

cullar disease, and a tendency for subjects with the e4 allele to experience myocardial infarction at a younger age. This study confirms the cholesterol-raising effect of E4, analyzed as a function of either allele or phenotype (Figure). The cholesterol-raising effect of E4 was of a similar magnitude to the cholesterol-lowering influence of E2. This is somewhat in contrast to the overall results of other studies in which the impact of E2 appeared to be double that of E4; however, in certain populations (Hungarian and Tyrolean) their impacts were, as in this study, quite similar. Thus, with respect to cholesterol, the results reported herein correspond to those obtained in numerous other studies (reviewed in Reference 8). However, two features of the present study are of particular clinical relevance.

The first concerns the significant differences in triglyceride levels associated with the different apo E alleles. This applies notably to the e4 allele, whose carriers have plasma lipid levels that are significantly higher than those of e2 carriers and e3/3 homozygotes. Previous studies have proved inconclusive with regard to this question, although a more recent meta-analysis of pooled data arrived at the conclusion that E4 has a propensity to raise plasma triglyceride levels. Ghiselli et al had earlier concluded that apo E4 was associated with severe hypertriglyceridemia, a proposal supported by subsequent studies but with some dissenting opinion. The significant differences that we observed with respect to triglycerides were still apparent when subjects with triglyceride levels >400 mg/dL were discounted, suggesting that the triglyceride-raising effect of E4 is a general phenomenon in this population.

The second feature of the present study is the difference in HDL cholesterol levels between apo E allele carriers. Again this applies particularly to e4 allele carriers, whose plasma HDL cholesterol levels were significantly lower. As with triglyceride levels, previous studies had not been able to show a consistent impact of apo E polymorphism on HDL cholesterol until subjected to meta-analysis. This recent examination of pooled data has led to the proposal that the E4/3 phenotype has a deleterious influence on HDL cholesterol levels. Whether the effect is subordinate to the raised triglyceride levels remains to be established: there is a well-known inverse relation between plasma
concentrations of triglycerides and HDL cholesterol. It should be noted that no significant differences in apo A-I concentrations were observed. Divergences between plasma levels of HDL cholesterol and apo A-I have been previously reported.

Why the Italian population studied herein should manifest such clear-cut influences of the apo E4 allele on triglyceride and HDL cholesterol levels is not presently evident. One possibility is that the more healthful, Mediterranean-type diet may limit or remove confounding dietary factors that could mask the influence of the e4 allele on these lipids.

Thus, in this particular Italian population, the apo E4 isofrom is associated with a more atherogenic lipid profile when compared with apo E3/3 homozygotes or carriers of the e2 allele. Higher levels of total and LDL cholesterol are evident and represent a rising concentration gradient from the E2 to the E3 to the E4 isoforms. Raised triglyceride and concurrently decreased HDL cholesterol concentrations are also manifested by e4 carriers. Moreover, they appear to be a particular feature of the apo E4 isoform, as similar concentrations of these lipids were present in E2 carriers and apo E3/3 homozygotes. Finally, there was a highly significant difference in the total cholesterol to HDL cholesterol ratio when examined as a function of e alleles (e2, 4.8±1.6; e3, 5.1±1.4; and e4, 6.1±1.7: ANOVA: F=13.91, P=.0001). There is now substantial evidence that the influence of apo E alleles on cholesterol levels is fairly constant across and within populations. Thus, the potential importance of apo E polymorphism in cardiovascular risk is related to differences in the relative frequencies of apo E alleles. Our observation that the e4 allele is associated with a more atherogenic lipid profile supports the contention that it predisposes to cardiovascular disease. Furthermore, its underrepresentation in a cohort with a relatively low incidence of cardiovascular disease is consistent with the proposal that apo E polymorphism may contribute to population differences in disease rates. The latter can only be substantiated and its extent determined by further studies. The European community, with its divergent dietary habits, cardiovascular disease rates, and perhaps apo E allele frequencies, may provide the background for such studies.

Acknowledgments

The study was supported by a grant from the Fonds National Suisse de la Recherche Scientifique (No. 32-30782.91). The authors are grateful for the skilful technical assistance of Marie-Claude Bruhlhart, Freda Ruinard, and Brigitte Wojtek.

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doi: 10.1161/01.ATV.13.10.1456
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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