# APOLIPOPROTEIN E POLYMORPHISM AND ATHEROSCLEROSIS: INSIGHT FROM A STUDY IN OCTOGENARIANS

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Apolipoprotein E is a polymorphic protein associated with plasma chylomicrons, very low (VLDL), intermediate (IDL) and high (HDL) density lipoproteins (1). It interacts with the surface "remnant receptor" (apo E receptor) and LDL-receptor (apo B/E receptor) of liver and peripheral cells, to modulate the catabolism of triglyceride-rich lipoprotein particles. Its three common isoforms, designated apo E4, E3 and E2, differ at two sites on the 299 amino-acid chain (112 and 158) (2). Apo E4 has arginine and apo E2 cysteine at both sites, while apo E3 has cysteine at 112 and arginine at 158. Apo E3 and E4 have 100 times more affinity for the apo B/E receptor in vitro than apo E2. In vivo, VLDL-bearing apo E2 are cleared from plasma more slowly (3) and VLDL-bearing E4 more rapidly (4) than apo E3 VLDL. Three alleles, designated $\epsilon 4$, $\epsilon 3$ and $\epsilon 2$, code at a single locus on chromosome 19 for the three separate isoforms and determine 6 apo E phenotypes (5, 6) which can be identified by isoelectric focusing of plasma VLDL on a polyacrylamide gel (7). Phenotypes E4/4, E3/3 and E2/2 are associated with a single circulating form of apo E and E4/3, E3/2 and E4/2 with two circulating forms. The most prevalent phenotype is E3/3 and the rarest is E2/2, the latter being found in nearly 90% of cases of familial hyperlipoproteinemia type III (8, 9).

Apo E polymorphism has a strong influence on the variation of plasma lipid levels (1, 7, 10) and can even modulate the response to a lipid-lowering drug (11). The presence of the $\epsilon 2$ allele appears to predispose to hypertriglyceridemia (1, 7, 12–15). A double dose of this allele provides the genetic background for the propensity to develop type III hyperlipoproteinemia (increased $\beta$-VLDL) in the presence, of an intervening factor (another hyperlipidemia gene, hypothyroidism, pregnancy, etc.) (1, 9). The absence of apo E (null allele) also results in hyperlipoproteinemia type III (12) and the frequency of the $\epsilon 2$ allele is increased in hypertri-

* Supported by grants from the Medical Research Council of Canada (MA-5427), the Ministry of Health and Welfare Canada (NHRDP- #6605-2087-52) and IRSST.

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glyceridemic individuals (13-15). On the other hand, the $\epsilon 2$ allele is associated with lower, and the $\epsilon 4$ allele with higher, plasma total cholesterol, LDL-cholesterol and apolipoprotein B concentrations than the $\epsilon 3$ allele (10). Since low density lipoproteins (LDL) are considered to be the most atherogenic of plasma lipoproteins, and because these two alleles have opposite impacts on LDL concentration, an atherogenic role for apo E4 and an antiatherogenic influence of apo E2 have been postulated. Studies attempting to relate differences in allele frequencies to coronary artery disease (CAD) so far are conflicting and inconclusive (16–21). The relative frequency of the $\epsilon 4$ allele in myocardial infarction was reported to be low in one study (16) and high in another (17). In a third report, in which both myocardial infarction and angiographic evidence of CAD were considered, the relative frequency of $\epsilon 4$ was not different from that of controls, but myocardial infarction occurred earlier in the presence of this allele (18). This observation prompted us to study apo E polymorphism in octogenarians (22).

We hypothesized that if the $\epsilon 4$ allele imparted an increased susceptibility to atherosclerosis, its frequency would be reduced among octogenarians who survived periods in life when CAD mortality is very high. Conversely, if the $\epsilon 2$ allele had a protective effect, it would be found with a higher frequency among octogenarians, provided another factor had not intervened during earlier life to induce an atherogenic form of hypertriglyceridemia. The study of 236 ambulatory autonomous octogenarians, reported here, demonstrates that the relative frequency of the $\epsilon 4$ allele is indeed reduced by about 50% in octogenarians. On the other hand, the expected increase in the $\epsilon 2$ allele frequency was observed only in men but did not reach statistical significance.

## PATIENTS AND METHODS

Subjects for this study were ambulatory and autonomous, had completed their 80th year, were capable of answering a questionnaire and agreed to submit to a short physical examination and the drawing of blood. They were recruited from 6 homes for the aged and one Veterans Hospital. Individuals with chronic debilitating disease (cancer, Parkinson's disease, etc.), mental illness, alcoholism and untreated hypothyroidism were excluded, as well as subjects who had had major surgery, weight change ($\pm 10\%$) or a myocardial infarction within the previous 3 months. Of the 255 subjects evaluated, 236 unrelated octogenarians (118 men and 118 women) with a mean age of $84.7 \pm 4.2$ years, met the entry criteria and were included. The reference sample consisted of 102 normolipidemic (cholesterol <240 mg/dL, triglycerides <150 mg/dL) civil servants (55 men) participating in a health survey programme in the City of Ottawa with a mean age of $36.1 \pm 8.5$ years (10, 14). The apo E

phenotype and allele frequencies in this small reference group was virtually the same as that found in a larger (n = 400) randomly selected Caucasian population (17) and in other reference samples of Caucasians (1, 10, 23).

Blood samples were obtained after a 12 to 13-hour fast and plasma ($Na_2$-EDTA 1 mg/mL ± $NaN_3$ 0.02%) separated in the cold for automated enzymatic determination of cholesterol (24), triglycerides (25), glucose (26) and uric acid (27). Lipoprotein cholesterol was determined on fractions separated by combined ultracentrifugation and heparin-manganese precipitation (28). Apo B was measured in total plasma and in the 1.006 g/mL ultracentrifugal bottom fraction (LDL-B) and VLDL-B obtained by subtraction, using the method of Reardon, et al. (29) with the modification of Rosseneu, et al. (30). Apo E phenotyping was carried out on the washed VLDL fraction by unidimensional isoelectric focusing on polyacrylamide gel according to a technique developed in our laboratory (7).

In this preliminary report, the statistical significance of the difference in the mean level of lipids, lipoproteins and apoproteins between two groups was evaluated using Student's t test. The chi-square test was used to test the hypothesis that the relative frequencies of discrete outcomes are the same in the two groups being compared.

## RESULTS

In this sample, men (85.6 ± 4.8 y) were slightly older than women (83.8 ± 3.3, $p < 0.001$), had a lower systolic blood pressure (142 ± 20 vs 149 ± 24, $p < 0.02$), and smoked more (>10 cigarettes per day, 13.4% vs 4.2%, $p < 0.02$). They were comparable in terms of body mass index, diastolic blood pressure, plasma glucose and uric acid. Regarding current and previous illnesses, there were more women than men with treated hypothyroidism (14.4 vs 0.8%), hypertension (51.7 vs 27.1%), and coronary artery disease (angina pectoris and/or myocardial infarction) (40.6 vs 24.6%, $p < 0.01$). More men than women were physically active (walking, gymnastics and sports). Interestingly, there were significantly more single women (24.6 vs 13.6%) and more married men (25.4 vs 11%), but the widowed were equally distributed between both subsets. About 61% of men and 54% of women had one parent who had lived beyond the age of 80.

The lipid and lipoprotein profiles are given in Table 1 for octogenarian males, females and both combined. Among the octogenarians, 47 (19.9%) have hyperlipidemia including 15 (6.3%) with hypercholesterolemia alone (cholesterol >260 mg/dL), 19 (8.0%) with hypertriglyceridemia alone (triglycerides >200 mg/dL) and 12 (5.1%) with both. Lipid, lipoprotein and apolipoprotein variables are significantly higher in octogenarian

females than in octogenarian males except for triglycerides and VLDL-cholesterol. This reflects a higher prevalence of hyperlipidemia in women than in men (27.1 vs 12.7%, $p < 0.01$), especially hypercholesterolemia (19.5 vs 3.4%, $p < 0.001$) and hypercholesterolemia combined with hypertriglyceridemia (8.5 vs 1.7%, $p < 0.02$). As expected from its selection, young age and normolipidemia, most lipoprotein parameters are lower in the reference sample than in the octogenarians. Unexpectedly however, the mean HDL-cholesterol is significantly higher in the reference group, even though the proportion of males and females is similar (Table 1).

The apo E phenotype distribution (%) and relative allele frequencies are given in Table 2 for the four groups reported in Table 1. Only one male subject had the E2/2 phenotype, he had a normal cholesterol of 205 mg/dL and moderate hypertriglyceridemia (350 mg/dL) with a lipopro-

TABLE 1
*Lipoprotein profile in octogenarians*

| mg/dl | Normolipidemics | Octogenarians | | |
|---|---|---|---|---|
| | n = 102 (55 M) | All n = 236 | Females n = 118 | Males n = 118 |
| Cholesterol | 174 ± 29 | 207 ± 44* | 224 ± 42** | 190 ± 38 |
| Triglycerides | 74 ± 30 | 134 ± 68* | 143 ± 71 | 126 ± 64 |
| VLDL-C | 24 ± 13 | 29 ± 16 | 31 ± 16 | 27 ± 15 |
| LDL-C | 103 ± 27 | 135 ± 37* | 148 ± 36** | 123 ± 33 |
| HDL-C | 47 ± 12 | 43 ± 12* | 46 ± 11** | 40 ± 12 |
| VLDL-B | 12 ± 10 | 20 ± 15* | 23 ± 14*** | 17 ± 16 |
| LDL-B | 83 ± 21 | 123 ± 34* | 135 ± 35** | 111 ± 29 |

* Differ from levels in normolipidemics ($p < 0.01$).
** Differ from levels in males ($p < 0.001$); *** ($p < 0.01$).

TABLE 2
*Apo E phenotype and allele frequencies in octogenarians*

| | Normolipidemics | Octogenarians | | |
|---|---|---|---|---|
| | n = 102 (55 M) | All n = 236 | Females n = 118 | Males n = 118 |
| Phenotypes (%) | | | | |
| E4/4 | 3.9 | 0.4 | — | 0.8 |
| E3/3 | 61.7 | 66.9 | 72.0 | 61.9 |
| E2/2 | 2.0 | 0.4 | — | 0.8 |
| E4/3 | 20.6 | 15.3 | 15.3 | 15.3 |
| E3/2 | 9.8 | 15.7 | 12.7 | 18.6 |
| E4/2 | 2.0 | 1.3 | — | 2.6 |
| Alleles | | | | |
| $\epsilon 4$ | 0.152 | 0.087* | 0.076* | 0.098 |
| $\epsilon 3$ | 0.770 | 0.824 | 0.860* | 0.788** |
| $\epsilon 2$ | 0.078 | 0.089 | 0.064 | 0.114 |

* Differ significantly from reference sample ($p < 0.025$).
** Differ significantly from female octogenarians ($p < 0.05$).

tein profile compatible with type III hyperlipoproteinemia (VLDL-C/TG ratio 0.277, LDL-C: 76, LDL-B: 59 and HDL: 32 mg/dL). Only one subject, an 85 year old normolipidemic man, had the E4/4 phenotype. The $\epsilon 4$ allele frequency was 43% lower in the octogenarian sample and 50% lower in the female subset than in the reference sample ($p < 0.025$). The $\epsilon 3$ allele frequency was significantly higher in female than in male octogenarians ($p < 0.05$). The trend for a higher $\epsilon 2$ allele frequency in males did not reach statistical significance whether it was contrasted with the reference sample or the female subset.

When plasma concentrations are expressed as a function of apo E phenotypes, the stepwise decrease in total cholesterol or LDL-cholesterol concentrations (Figure 1) from phenotypes E4/3 to E3/3 to E3/2 observed in the reference population (10) is not seen in octogenarians. The concentrations remain the same for E4/3 and E3/3. This similarity between the E4/3 and E3/3 is observed for all parameters in the 236 subjects including total cholesterol (214 vs 211), triglycerides (136 vs 135), HDL cholesterol (43 vs 43) and LDL-apo B (130 vs 128). One might speculate that E4/3 subjects with high LDL-cholesterol may have failed to reach the ninth decade on the basis of our findings and of the observation that myocardial infarction tends to occur earlier in individuals with the $\epsilon 4$ allele (18).

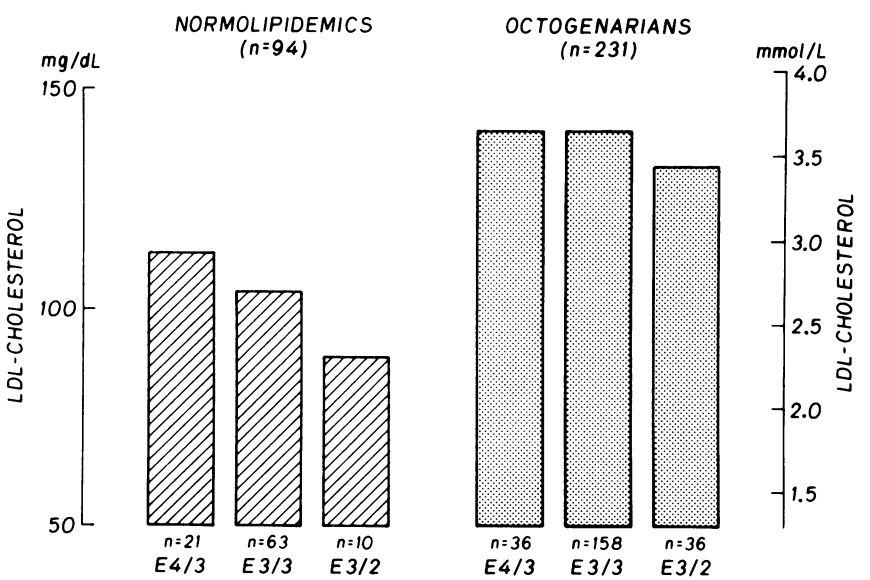


FIG. 1. Mean LDL-Cholesterol as a function of apo E phenotypes in young normolipidemic volunteers (10) and in octogenarians: the gradient between E4/3 and E3/3 is not observed in the older age group. The means are within the normal range in both groups and in all phenotypes.

The prevalence of hyperlipidemia is given in Table 3 for each of the three common apo E phenotypes. The high prevalence of hyperlipidemia across all three phenotypes in females and its very low prevalence in males reemphasize the findings reported in Table 1 that women have higher plasma lipid and lipoprotein levels than men in this age group. More specifically, it shows that 1) there were more women than men with hypercholesterolemia and hypertriglyceridemia across all three phenotypes, 2) in women there was a greater proportion of hypercholesterolemic subjects with the E4/3 phenotype and of hypertriglyceridemic subjects with the E3/2 phenotype, as expected, but the numbers are too small for this trend to be significant, 3) in men, hypercholesterolemia was rare and the frequency of hypertriglyceridemia was less than 5% among bearers of the $\epsilon 2$ allele; this indicated that hypercholesterolemic men in general and hypertriglyceridemic men bearing the $\epsilon 2$ allele had not survived, tending to confirm the working hypothesis.

## DISCUSSION AND CONCLUSIONS

One of the most striking features of this group of octogenarians is the marked contrast between men and women. There are many more women than men with coronary artery disease and with cardiovascular risk factors especially hypercholesterolemia, hypertriglyceridemia, hypertension, and physical inactivity. These women seem to have a level of risk factors and disease that the men may have had years earlier, perhaps around the age of 60. At the age of 80 the major risk factors may have

TABLE 3

*Excess cholesterol and triglycerides as a function of the three most common apo E phenotypes*

| | | Total | Females | | | Males | | |
|---|---|---|---|---|---|---|---|---|
| | | | E4/3 | E3/3 | E3/2 | E4/3 | E3/3 | E3/2 |
| Number | | 236 | 18 | 85 | 15 | 18 | 73 | 22 |
| Chol > 260* | n | 27 | 4 | 17 | 2 | 0 | 4 | 0 |
| | % | 11.4 | 22.2 | 20.0 | 13.3 | 0 | 5.5 | 0 |
| TG > 200* | n | 32** | 2 | 14 | 3 | 2 | 9 | 1 |
| | % | 13.5 | 11.1 | 18.8 | 20.0 | 11.1 | 12.3 | 4.5 |
| Chol↑ + TG↑ | n | 12 | 1 | 7 | 2 | 0 | 2 | 0 |
| | % | 5.1 | 5.6 | 8.2 | 13.3 | 0 | 2.7 | 0 |
| Total HLP | n | 47** | 5 | 24 | 3 | 2 | 11 | 1 |
| | % | 19.9 | 27.8 | 28.2 | 20.0 | 11.1 | 8.5 | 4.5 |

* The rounded upper normal limit selected is near the 90th percentile for both men and women (Lipid Research Clinics Prevalence study) (29).

** These figures include a hypertriglyceridemic E2/2 male with type III hyperlipoproteinemia. Subjects with both hypercholesterolemia and hypertriglyceridemia appear in the 3 subsets (high chol, high TG and both).

taken their toll and are no longer observed in men. The male survivors have relatively fewer major CAD risk factors, without any relative increase in protective factors. Indeed, there is no increase in HDL-cholesterol in octogenarians of either sex and the concentrations are significantly lower than in the sample of younger, normolipidemic, healthy volunteers. The HDL-cholesterol frequency distribution is very similar in the reference and in the octogenarian samples and does not seem to contribute much to longevity in this setting. This is in contrast with the report by Glueck et al. (32) of a higher frequency of elevated HDL levels in octogenarians as assessed by kindred studies. Their observation was not confirmed by Alvarez et al. (33), and by Heckers et al. (34) who attributed Glueck's findings to a selection bias. Our findings concur with this interpretation.

The reduced frequency of the $\epsilon 4$ allele in octogenarians is in agreement with our working hypothesis. It supports the notion that the presence of the $\epsilon 4$ allele, because of its association with higher LDL-cholesterol concentrations, may confer an increased susceptibility to atherosclerosis. In this respect it is in agreement with the findings of Cumming and Robertson (17) of an increased frequency of the $\epsilon 4$ allele in myocardial infarction, and the observation that bearers of this allele tend to have their myocardial infarction earlier in life (18). In the study of Cumming and Robertson (17), the increased relative frequency of the $\epsilon 4$ allele in patients with myocardial infarction was significant only in women; in octogenarians the lowered frequency of this allele was significant only in women. This might indicate that the effect of the $\epsilon 4$ allele is stronger in women than in men. The lower frequency of the $\epsilon 4$ allele in octogenarians is also in agreement with the higher frequency of the $\epsilon 4$ allele reported in Finns (35), who have the highest CAD mortality rates and the highest cholesterol levels in the world, and the low frequency of this allele in Asian populations where CAD mortality rates are the lowest (1, 36). It remains at variance with the findings of Utermann et al. (16). Failure to find an association between apo E polymorphism and the occurrence of coronary atherosclerosis may depend on population selection or experimental design. Age appears to have a major influence on the impact of apo E alleles on CAD (18). Another source of error may be uneven distribution of risk factors. The relative proportion of hypercholesterolemics (more of whom have the $\epsilon 4$ allele) and hypertriglyceridemics (more of whom have the $\epsilon 2$ allele) included in the sample could influence the outcome. A bias could also be introduced by the selective influence of major risk factors (hypertension, diabetes, cigarette smoking, etc.) which are stronger than, and act independently of the impact of apo E alleles. The definition of endpoints could also contribute to the confusion when relating apo E phenotypes and coronary lesions; several studies have equated coronary "atherosclerosis" with the presence of 50% stenosis or

more, considering arteries "normal" when less than 50% narrowing of the arterial lumen was present.

The expected increase in the $\epsilon 2$ allele frequency was not observed in this study but a trend, almost reaching statistical significance, was found surprisingly only in men and, as expected, in men free of hypertriglyceridemia. This is in agreement with the concept of a protective role for the $\epsilon 2$ allele put forth several years ago by Utermann et al. (38), but the sex differential is intriguing and warrants further study. Because of their active ovarian function, women are perhaps more susceptible to induction of atherogenic triglyceride-rich lipoprotein particles by estrogens in the presence of the $\epsilon 2$ allele, becoming more likely to suffer a heart attack in later years before reaching the ninth decade. The fact that the only E2/2 found in 236 octogenarians sampled had hyperlipoproteinemia type III is compatible with the view that, with aging, E2/2 subjects are at risk for CAD and perhaps likely to develop hyperlipidemia with time. In the study of Utermann et al. (16) of over 500 myocardial infarction survivors, all 5 subjects with an E2/2 phenotypes had type III hyperlipoproteinemia in contrast to none of the 21 individuals with this phenotype in the control group.

In octogenarians, the influence of the apo E alleles on plasma lipids was not very striking, although in women the E4/3 phenotype was associated with a higher frequency of hypercholesterolemia and the E3/2 phenotype with a higher frequency of hypertriglyceridemia. There were very few hyperlipidemic men. Furthermore, in the whole sample the E4/3 phenotype was not associated with higher concentrations of LDL-cholesterol (Figure 1) as observed in normolipidemics (10), randomly selected control populations (1, 17) or CAD patients (18). We speculate that an earlier demise of the bearer of the $\epsilon 4$ allele with high LDL-cholesterol is responsible for this but other explanations exist, such as a greater resistance to CAD of the E3/3 individuals.

In conclusion, our finding of a decreased frequency of the $\epsilon 4$ allele in octogenarians is consistent with the hypothesis that the presence of the $\epsilon 4$ allele predisposes to atherosclerosis and constitutes a risk factor for CAD. The burden of proof now lies on carefully controlled studies of CAD patients where care is taken to exclude the "background noise" imparted by the major classical risk factors, and on prospective epidemiologic studies. The putative protective role of the $\epsilon 2$ allele, in the absence of factors promoting hypertriglyceridemia, is strengthened by our findings but might apply only to males. There is no evidence in this study that high HDL cholesterol accounts for longevity. The persistence of hyperlipidemia and other major risk factors in octogenarian females probably reflects the 15 to 20-year reprieve in CAD risk afforded by active ovarian function until the menopause. Their absence in octogen-

arian men conversely reflects their increased susceptibility in earlier years.

## ACKNOWLEDGEMENTS

The authors wish to express their most sincere thanks to Mrs. Suzanne Quidoz, RN and Angèle Richard, RN for obtaining the blood samples, to Ann Chamberland, Louis-Jacques Fortin, Lucie Boulet and Michel Tremblay for their technical assistance.

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## DISCUSSION

**James** (Galveston): Dr. Davignon, could you tell us how many of the octogenarians were married to each other. Were most of them single examples of widows or widowers, or were they married pairs?

**Davignon:** No. None of these octogenarians were related, either as spouses or in the same family. We took good care of excluding those. As relates to widowers, there were equal numbers of widowers among the males and widows among the females.

**McIntosh** (Lakeland): Do you have any information about the replacement of estrogen therapy in your females? Did this have any effect, or were any of these 80 year olds on estrogen?

**Davignon:** No, they were not. Most of the women were not on estrogen but I think estrogens are probably important in accounting for the sex differential regarding the frequency of clinically apparent CAD. What happens is that before the menopause the presence of estrogen will favor an increase in triglycerides, and probably change the proportion of cholesterol esters. After the menopause the female is probably more exposed to cholesterol ester, triglyceride, and VLDL; then atherosclerosis will slowly develop whose consequences will be revealed twenty years later.