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ApoE Polymorphism Accounts for Only Part of the Genetic Variation in Quantitative ApoE Levels

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ApoE levels and chromosome 19 ApoE polymorphisms were measured in a sample of 156 Dutch families. Each pedigree consisted of parents aged 35–65 years and their twin offspring aged 14–21 years. A significant effect of the chromosome 19 apoE locus on quantitative plasma levels of apolipoprotein E was observed. The ApoE polymorphism explained 16% of the variance in ApoE levels. Tests of association of ApoE levels with the apoC1 locus, which is in complete linkage disequilibrium with the ApoE locus, also showed a significant effect, although the variance explained by ApoC1 was only 1%. Examination of the covariance between twins classified according to allele sharing indicates that the association is not due to population stratification, but to a genuine effect of the ApoE locus on levels. However, the ApoE locus accounts for only one-fourth of the genetic variation in ApoE levels. Genet. Epidemiol. 18:331–340, 2000. © 2000 Wiley-Liss, Inc.

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INTRODUCTION

Apolipoprotein (Apo) E plays a central role in the metabolism of cholesterol and triglycerides. It is one of the major protein constituents of chylomicrons, very low-density lipoprotein (VLDL), their remnant particles, and high-density lipoprotein (HDL). On these particles, ApoE serves as a ligand for the uptake by lipoprotein receptors [Havel et al., 1980; Sherril et al., 1980]. In humans, ApoE is a polymorphic protein with three major isoforms, ApoE2, ApoE3, and ApoE4 [Utermann et al., 1977]. In healthy individuals, between 5 and 15% of normal interindividual variation in plasma cholesterol levels can be attributed to this common ApoE polymorphism [Davignon et al., 1988]. In most human populations it is found that individuals with ApoE2 display high levels of ApoE and low levels of plasma cholesterol, low-density lipoprotein (LDL)-cholesterol and ApoB whereas those with ApoE4 show the opposite [Davignon et al., 1988; De Knijff et al., 1994b; Utermann, 1985]. Because of this influence of ApoE phenotypes on circulating levels of lipids, the ApoE gene is often seen as a major risk factor for atherosclerosis and coronary heart disease (CHD) [Luc et al., 1994; Tiret et al., 1994; Hixson, 1991], although no increased risk for CHD among ApoE4 carriers has been reported as well [Damaraju et al., 1995; De Knijff et al., 1992]. For the most part, these ApoE effects can be attributed to a more efficient catabolism of chylomicron remnants and intermediate density lipoprotein (IDL) particles in individuals with ApoE4 and a less efficient catabolism of these particles in individuals with ApoE2, due to a defect in binding of ApoE2 to hepatic lipoprotein receptors [Utermann, 1985, 1987; Weintraub et al., 1987]. In addition, Weisgraber [1990] has shown that there is a marked difference in the preference for the different lipoprotein particles between the common ApoE alleles, which could also explain part of differences in allelic influences of the common ApoE alleles. There is now evidence that in addition to differences in binding efficiencies between the ApoE forms, the number of ApoE molecules on a lipoprotein particle is also an important determinant of the clearance rate of these particles. The more ApoE is present on a lipoprotein particle, the faster it will be removed from the circulation [Dong et al., 1998]. It is, therefore, important to understand which factors (ApoE genotypes, background genes and environments) regulate the circulating levels of ApoE. Surprisingly few studies aimed at finding answers for this question have been published [Moll, 1993].

In an earlier report, heritabilities of ApoE levels were estimated from a sample of Dutch twins and their parents [Boomsma et al., 1996]. We found heritabilities of 87% (twins) and 31% (parents) for circulating levels of ApoE. Smit et al. [1988] demonstrated that in 35-year-old Dutch males about 18% of the variability of circulating ApoE levels can be explained by the ApoE polymorphism. Others have confirmed and refined these conclusions, indicating some interaction with age and sex [Zerba et al., 1996]. With these estimates, we are still far from any detailed insight. For example, it is still not known if the effect of the ApoE polymorphism on levels of ApoE is due to dominant or additive effects of the different alleles. Moreover, no studies to date show whether or not there are other

genes that also influence ApoE levels [Moll, 1993]. In order to answer some of these remaining questions, we report here the results of an analysis of the association between ApoE polymorphisms and ApoE levels in the Dutch twin family sample.

SUBJECTS AND MEASURES

Subjects

This study is part of a larger project in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents [Boomsma et al., 1993a,b, 1998; Kempen et al., 1991; de Knijff et al., 1994a]. Addresses of twins (between 14–21 years of age) living in Amsterdam and neighboring cities were obtained from City Council population registries. Twins living with both their biological parents were contacted by letter. A family was included in the study if the twins and both parents complied. In addition, a small number of families who heard of the study from other twins also volunteered to participate. Zygosity was determined by blood groups and DNA polymorphisms. Three series of triplets were included by discarding the data from the second born child. There were 35 monozygotic female (MZF), 35 monozygotic male (MZM), 30 dizygotic female (DZF), 31 dizygotic male (DZM) and 29 dizygotic opposite-sex pairs (DOS). Average age (with standard deviation in parentheses) of fathers and mothers was 48.1 (6.3) and 45.6 (5.9) years, respectively, and of the twins was: MZM, 16.57 (1.77); MZF, 16.04 (2.22); DZM, 17.16; (1.75); DZF 17.66 (2.06); DOS 16.39 (1.82).

Measures

EDTA blood was obtained between 8:30 and 10:30 AM by venipuncture after overnight fasting. Plasma was separated from cells after centrifugation for 10 minutes at 3,000 rpm. Part of the plasma was frozen at –20° ± 2°C for later use. ApoE levels were quantified from the frozen samples by enzyme-linked immunosorbent assays (ELISA) as described by Bury et al. [1986] . Blood samples were collected over a 2-year period with on average 10 families coming to the laboratory on the same occasion. ApoE levels were measured for all subjects simultaneously, with family members randomly distributed over measurement batches. In these same subjects, ApoE polymorphisms were assessed (ApoE isoforms E2, E3, and E4). ApoE phenotypes were determined by isoelectric focusing of delapidated plasma samples followed by immunoblotting as we have described previously [Havekes et al., 1987; De Knijff et al., 1988]. In four families no ApoE data were available.

In the Dutch population, the ApoE polymorphism is in complete linkage disequilibrium with a di-allelic Hpa1 polymorphism in the promotor region of the ApoC1 gene, 4.3 kb downstream of the ApoE gene [de Knijff, 1992; Smit et al., 1988]. The ApoE2 isoform is linked to the ApoC1 Hpa1-2 (c1-2) allele, ApoE3 is linked to c1-1, and ApoE4 is linked to c1-2. Hence, the six ApoE phenotypes can be replaced by three ApoC1 genotypes (e2/e2 is c1-2/c1-2; e2/e3 is c1-2/c1-1; e3/e3 is c1-1/c1-1; e2/e4 is c1-2/c1-2; e3/e4 is c1-1/c1-2 and e4/e4 is c1-2/c1-2). Assuming that this disequilibrium is preserved in the sample being analyzed, it permits a test of the effect of this di-allelic ApoC1 polymorphism on quantitative ApoE plasma levels, by recoding the ApoE genotypes as ApoC1 genotypes.

STATISTICAL METHODS

ApoE levels are known to vary with age and may vary with sex, so we developed a model for the influence of age, sex, and ApoE genotype on ApoE levels. Residual variation not explained by these factors was modeled by partitioning variation into additive genetic, shared environment, and specific environment components. Additive genetic factors give rise to a pattern of familial resemblance where MZ twins correlate twice as much DZ twins who correlate the same as parents and offspring, with spouses uncorrelated. Shared environmental factors, here modeled as a home environment, give rise to equal correlations for all relationships (spouses, parent-offspring, MZ and DZ twin). Specific environment factors, including error of measurement, make individuals different from their relatives. A path diagram of the model is shown in Figure 1. It is the job of model fitting to find the combination of these components that best matches the observed pattern of familial resemblance in the data.

Modeling the ApoE level for individuals requires the computation of a predicted level that may vary for each subject, according to their age, sex, and ApoE

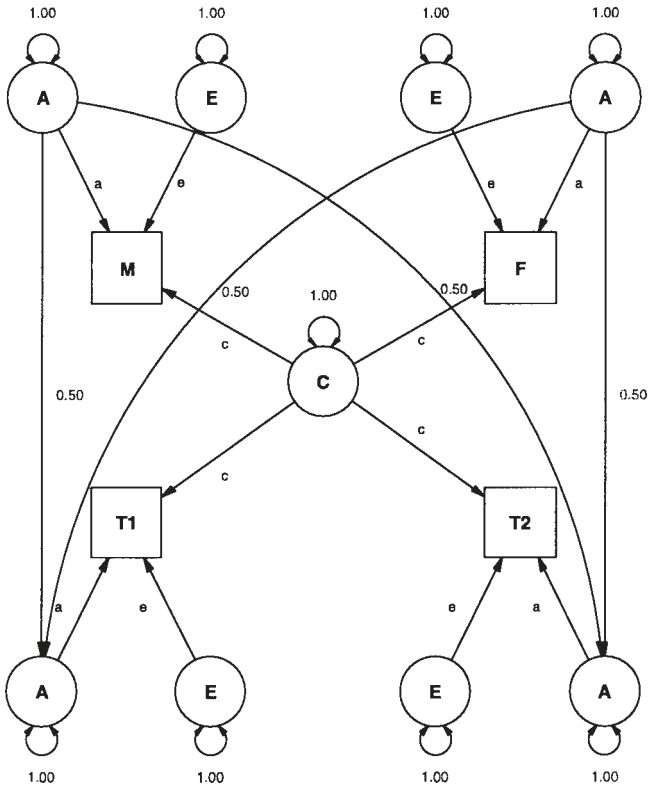

Fig. 1. Path diagram showing familial resemblance for ApoE levels as a function of additive genetic (a), shared (c), and non-shared environment (e) effects. In addition to these sources of variation, individual means are modeled as a function of ApoE genotype, age, and sex.

genotype. Using special features of the Mx [Neale, 1997], known as definition variables [Neale, 1998], we specified a model for the mean of subject *i* as

$$\mu_i = \mu + \mathrm{Age}_i\mu_a + \mathrm{Sex}_i\mu_s + \mu_{gi}$$

where for subject *i*: Age $_i$ is their age at observation; Sex $_i$ is their sex coded as 0 for females and 1 for males; a and s are the age and (male) sex deviations; and gi is the mean of genotype *i*. For the ApoE locus, six genotypes are recognized: e2/e2; e2/e3; e3/e3; e2/e4; e3/e4; and e4/e4. Comparison of parameter estimates and goodness of fit indices when age, sex, or genotypic effects are not included in the model allows estimation of the statistical significance of these effects via log-likelihood ratio tests. Mx scripts for these models and the raw data are available on the Mx website http://views.vcu.edu/mx. The method assumes multivariate normality of the data *conditional on the particular age, sex, and genotype of the individual*. That is, multivariate normality of the residuals is assumed, rather than of the raw distribution. Skewness and kurtosis of these residuals are small, and simulations have shown that the likelihood method is fairly robust to violations of this assumption [Allison et al., 1999].

It should be noted that mean differences in ApoE levels as a function of genotype could occur from either functional effects of the ApoE locus itself, or from a nearby locus in linkage disequilibrium (LD) with it, or artifactually through, e.g., population stratification [Fulker et al., 1998; Neale et al., 1999]. To examine whether the ApoC1 locus might be affecting levels, we constrained the means to be equal for ApoE phenotypes e2/e2, e2/e4, and e4/e4 (c1-2/c1-2), and for e2/e3 and e3/e4 (c1-1/c1-2), which, together with e3/e3 (c1-1/c1-1), results in only three ApoC1 genotypic means for additive and non-additive effects at this di-allelic locus. To test for possible stratification effects, we considered the sibling (DZ) intrapair similarity as a function of sharing alleles identical by state.

RESULTS

Table I gives an overview of the distribution of observed ApoE isoforms separately for parents (father and mother) and first- and second-born twins. There is a pronounced asymmetry in the genotypic frequencies: alleles 2 and 4 are relatively rare. This asymmetric distribution is in accordance with theory and observation of other polymorphic loci which typically display a Dirichlet-multinomial distribution of allele frequencies [Weir, 1996; Lange, 1995]. The net effect is that there is scant information on the means for genotypes 2=2; 4=4 and 4=2, while the other genotypes have quite large sample sizes.

TABLE I. Frequency Distribution of ApoE Isoforms

Isoforms	Father	Mother	Twin1	Twin2
2/2	2	1	0	1
3/2	21	26	32	29
3/3	86	94	81	85
4/3	42	25	36	38
4/4	3	2	3	3
4/2	2	8	4	0

Mean ApoE levels of the more frequent genotypes (Table II) follow the pattern of 3/2 > 3/3 > 4/3 consistently for fathers, mothers and both twins. The relatively small differences between fathers and mothers suggest that gender plays a minor role in ApoE levels, though the data should be corrected for age, which is properly accomplished with model-fitting. Results of model fitting are presented in Table III. The first, saturated model estimates the overall population mean at 6.5 with an increment of .03 per year of age.

The estimate of μ_s is –.67 suggesting a lower mean of males. The estimated mean parameters for 3/2, 3/3, and 4/3 agree with the observed pattern in the data shown in Table I, and indicate approximately 2.4 difference between those with 3/2 and those with 4/3 genotypes. Residual variation appears largely (71%) genetic with no effect of shared family environment. In Model II, the sex difference is eliminated, but doing so causes a significant loss of fit, as seen in the $\Delta\chi^2$. Model III tests the age increment, which is also found to be highly significant. Model IV eliminates the effects of the ApoE locus, which again produces a highly significant loss of fit, indicating that the ApoE locus accounts for a significant proportion of variation in ApoE levels.

Model V, which attempts to explain variation in ApoE levels by equating the parameters according to the most likely ApoC1 genotypes based on LD, does not produce a significantly better fit than Model IV ($\Delta\chi^2_2$ = 0:88). This result supports the hypothesis that ApoE levels are controlled by the ApoE locus rather than the Hpa1 site in the promotor region of than the ApoC1 locus. The effects of age, sex, and genotype are therefore all highly significant and are required for a parsimonious explanation of the data.

The maximum likelihood estimate of the ApoE level variance, δ^2 varies according to which model is fitted. It is lowest for Model I because the model for the means takes into account the effects of age, sex, and genotype, which in turn leaves less variance to explain. From these estimates it is possible to assess the proportion of variance accounted for by the difference sources. ApoE genotype accounts for (6.68–5.62)/6.68=15.9% of the variance. In contrast, ApoC1 accounts for (6.68–6.62)/6.68=0.8% of the variance, and yields a substantially poorer fit to the data.

Estimates of the proportion of variance accounted for by residual additive genetic factors, α^2, are approximately .70 for models in which the effect of the ApoE locus are included, and are .81 when it is omitted. These results suggest that there is substantial genetic variation not accounted for by the ApoE polymorphisms nor by

TABLE II. Mean ApoE Levels for the apoE Isoforms

	Fathers		Mothers		Twin1		Twin2	
Group	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2/2	11.50	5.36	17.75	—	—	—	7.20	—
3/2	9.43	1.62	10.04	2.84	9.67	2.47	9.17	2.84
3/3	7.57	2.23	7.51	2.39	6.50	1.70	6.46	1.71
4/3	6.95	2.19	6.58	2.10	5.46	1.71	5.45	1.67
4/4	6.14	2.78	4.07	0.68	4.20	1.20	5.66	3.80
4/2	9.47	.43	9.98	2.54	9.38	1.89	—	—
Total	7.71	2.34	7.93	2.81	6.94	2.43	6.71	2.34

TABLE III. Parameters Estimates and Goodness of Fit Statistics for a Variety of Models and Submodels Applied to the Twin-Family ApoE Genotype and Phenotype Data*

	Model							
Parameter	I		II	III	IV	V	VI	VII
μ	6.50	(6.01; 6.99)	6.26	7.74	6.64	6.55	6.46	9.45
μ_a	0.03	(0.02; 0.04)	0.03	[0.00]	0.03	0.03	0.03	0.03
μ_s	–0.67	(–1.05; –0.30)	[0.00]	–0.55	–0.82	–0.84	–0.60	–0.69
$\mu_{2/2}$	0.86	(–1.60; 3.33)	0.71	1.42	[0.00]	0.08†	2.84	0.00
$\mu_{3/2}$	1.49	(0.93; 2.04)	1.45	1.36	[0.00]	0.21‡	1.84	–1.47
$\mu_{3/3}$	0.00	(0.00; 0.00)	[0.00]	[0.00]	[0.00]	0.00	0.00	–2.94
$\mu_{4/3}$	–0.87	(–1.39; –0.35)	–1.06	–1.21	[0.00]	0.21‡	–0.94	–3.74
$\mu_{4/4}$	–1.64	(–3.17; –0.11)	–1.85	–2.02	[0.00]	0.08†	–1.81	–4.54
$\mu_{4/2}$	2.02	(0.24; 3.80)	1.96	1.66	[0.00]	0.08†	2.07	–2.27
a^2	0.71	(0.52; 0.84)	0.70	0.59	0.81	0.81	[0.00]	0.68
c^2	0.00	(0.00; 0.08)	0.00	0.00	0.00	0.00	0.20	0.00
e^2	0.29	(0.16; 0.46)	0.30	0.41	0.19	0.19	0.80	0.32
$\hat{\sigma}^2$	5.62	(4.87; 6.58)	5.70	5.68	6.68	6.62	5.13	5.56
$\Delta\chi^2$			12.39	48.65	53.90	53.02	34.87	5.93
df			1	1	5	3	1	3
p			0.00	0.00	0.0	0.00	0.00	.12

*$\Delta\chi^2$ denotes the difference in fit against the first model. The parameters are: μ, the global mean; μ_a, the age deviation; μ_s, the sex deviation; μ_i/j, the deviation specific for individuals with genotype i/j; a^2, c^2 and e^2 are the proportions of vartiance associated with additive genetic, common environment and specific environment sources, respectively; and $\hat{\sigma}$ is the total background variance from these sources. Parameters fixed ex hypothesi are denoted by brackets.

loci in linkage disequilibrium with the ApoE locus. This impression is confirmed by Model VI, which fixes the residual genetic variance at zero; doing so results in a substantial loss of fit ($\Delta\chi^2 = 34.87$).

To test for the effects of non-additivity in the genotypic effects, the model was parameterized in terms of two allele deviations μ_3 and μ_4 for the e3 and e4 alleles, respectively, while the deviation μ_2 for the e2 allele was fixed at zero. Model VII shows the results of fitting this model; estimates of the allele means were $\mu_3 = 1.47$ and $\mu_4 = 2.27$. The loss of fit of this model compared to Model I is not statistically significant, which suggests that genetic dominance at the ApoE locus is either small or non-existent.

To investigate possible stratification, we grouped the DZ twins in the sample according to the number of alleles that they shared identical by state (IBS) at the ApoE locus. There were 127 pairs sharing two alleles IBS, 33 pairs sharing one allele, and no pairs sharing zero alleles IBS, a pattern that is not unexpected given the unequal allele frequencies at this locus. The correlation for ApoE level between siblings sharing two alleles IBS is .809 ($P < .0001$) and between siblings sharing 1 allele IBS it is -0.011 ($P = .95$). The difference between these correlations is highly significant ($\chi^2 = 29.7$, df = 1, $P < .0001$). These results support the hypothesis of an allele effect at the ApoE locus, rather than population stratification. Pure stratification would act like a shared environment and generate approximately equal correlations for the two groups.

CONCLUSION

The aims of this article were to increase understanding about the relationship between polymorphisms at the ApoE locus and ApoE levels in the blood. These aims were achieved by developing a statistical model for the effects of age, sex, and ApoE genotype on circulating levels of ApoE. Several variants of the model were fitted to data collected from Dutch twins and their parents.

One clear result is that there is significant familial resemblance that is not accounted for by variation at the ApoE locus. This residual resemblance appears to be genetic rather than environmental in origin, and may be responsible for more than 50% of variance in ApoE levels (non-ApoE genotype variance is 84% and this variation has 70% heritability; .7*.84 = .59). These results would appear to justify a genome scan for other loci that may determine ApoE levels. It is possible that some of the residual genetic variation is behaviorally mediated via pathways such as dietary preference. However, complex behavioral phenotypes may be less likely to be influenced by loci of large effect.

A large and robust effect of the ApoE locus on ApoE levels was found. The 4/4 genotype has the lowest and the 4/2 genotype has highest sample mean. The confidence intervals on the genotypic means vary quite markedly because the sample size differs substantially across genotypes. There is no evidence for genetical dominance of the alleles at the ApoE locus; genetic variation at the ApoE locus appears additive. This may suggest that ApoE levels have not been under selective pressure in the evolutionary history of mankind [Fisher, 1929].

We explored the extent to which variation in ApoE levels might be accounted for by the ApoC1 locus, which is in strong linkage disequilibrium with ApoE in the Dutch population. Variation in ApoE levels does not appear to be associated with the diallelic ApoC1 genotype. These results suggest that, despite the fact that this diallelic polymorphism appears to be in complete LD with, and only 4.3 kb downstream from, a major gene (ApoE), it would not have been detected in an association analysis using ApoC1 genotypes. This finding is consonant with recent reports of difficulties using single-nucleotide polymorphisms (SNPs) to detect disease-related genes and quantitative trait loci for LDL [Nickerson et al., 1998; Pennisi, 1998]. In part this may be due to the ApoC locus being less informative. It also remains possible that the complete LD observed in other Dutch samples is not preserved here; only direct assessment of the ApoC locus in this sample could resolve this question. Nonetheless, caution would seem to be required when trying to exclude regions based on genome-wide association studies, as nearby loci in substantial LD can have quite large effects.

The association with the ApoE locus does not appear to be an effect of population stratification. DZ twin pairs were classified as sharing either one or two alleles identical by state at the ApoE locus; no pairs shared zero alleles. Pairs sharing two alleles IBS showed remarkable similarity in ApoE level, correlating .81, whereas those sharing one allele were essentially uncorrelated. A more sophisticated partitioning of variance into within and between family components of the association, such as that proposed by Fulker et al. [1998] and Neale et al. [1999] might be used to explore this finding further.

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