Hum Genet (2004) 115: 331–345
DOI 10.1007/s00439-004-1165-z

ORIGINAL INVESTIGATION

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Gender- and age-specific contributions of additional DNA sequence variation in the 5′ regulatory region of the *APOE* gene to prediction of measures of lipid metabolism

Received: 30 April 2004 / Accepted: 9 June 2004 / Published online: 5 August 2004

Abstract In the present study of 9,000 individuals representative of the general population, we have considered whether the addition of common single nucleotide polymorphisms (SNPs) in the promoter region of *Apolipoprotein E* (*APOE*) improve the statistical explanation of variation in lipid traits and test the hypothesis that the estimated genotype effects are independent of factors indexed by gender and age. To address these questions, we have asked, for each gender and for each 20-year age strata (young: 20–39 years; middle-aged: 40–59 years; old: 60–79 years; very old: 80–100 years), how much trait variation is associated with the traditional ε2, ε3, and ε4 allelic variations defined by the g.2059T→C and g.2197C→T SNPs in the fourth exon of the *APOE* gene, and how much additional trait variation is associated with genotypes defined by combining the g.2059T→C and g.2197C→T SNPs with one, two, or three promoter SNPs. Our study demonstrates that the pleiotropic effects of genotype variation defined by the traditional ε2, ε3, and ε4 alleles on five plasma measures of lipid metabolism manifest differently in women and men and change significantly during the life cycle for high-density lipoprotein cholesterol in women. Multi-site genotypes defined by adding SNPs located in the 5′ promoter region to the traditional g.2059T→C and g.2197C→T SNPs doubled the estimate of genetic variance of high-density lipoprotein and apolipoprotein Al in middle-aged females.

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Introduction

The apolipoprotein E (APOE) protein plays a pivotal role in lipid metabolism and exerts its biological function as a surface component of triglyceride-rich lipoprotein particles. These include chylomicrons, chylomicron remnants, very-low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), and a subclass of high-density lipoprotein cholesterol (HDL-C) particles (Weisgraber 1994). The protein serves as a ligand for the low-density lipoprotein (LDL) receptor and the LDL receptor-related protein (LRP). It is responsible for a large fraction of the clearance of triglycerides (TG) and total cholesterol (TC) from the plasma compartment (Havel and Kane 2001). In humans, three major isoforms of APOE are present, APOE-2, APOE-3, and APOE-4 (Utermann et al. 1977). Differences are defined by cysteine–arginine interchanges at positions 112 and 158 in the 299-amino-acid APOE chain (Weisgraber 1994). Two variable sites, located in exon 4 of the *APOE* gene (positions g.2059T→C and g.2197C→T, Fig. 1, corresponding to Single nucleotide polymorphisms (SNPs) 3937 and 4075 in reference sequence AF261279) are responsible for these two amino acid changes. The SNPs at each site combine to determine three alleles (ε2, ε3, and ε4) and six common *APOE* genotypes (ε22, ε32, ε42, ε33, ε43, and ε44). In most populations, a stepwise increase in TC is associated with allelic changes from ε2 to ε3 to ε4 (Davignon et al. 1988; Mahley and Rall 2001). Carriers of the ε4 allele are at increased risk for cardiovascular disease (CVD) and Alzheimer's disease (Corder et al. 1993; Strittmatter et al.

1993; Tiret et al. 1994; Wilson et al. 1994; Stengård et al. 1995; Frikke-Schmidt et al. 2000b, 2001).

In the population at large, the six common *APOE* genotypes statistically explain 2%–5% and 11%–20% of the interindividual variation in TC and plasma APOE, respectively (Frikke-Schmidt et al. 2000a; Stengård et al. 2002). The fraction of interindividual phenotypic variability in TC and APOE statistically explained by the six common *APOE* genotypes is greater in females than in males (Frikke-Schmidt et al. 2000a; Stengård et al. 2002; Kaprio et al. 1991; Xhignesse et al. 1991; Kamboh et al. 1993; Heng et al. 1995; Zaman et al. 1997; Kamboh et al. 1999). The statistical correlation between measures of lipid metabolism among individuals with the same genotype differs between genders (Reilly et al. 1994). Interindividual trait variability associated with the common *APOE* genotypes and the correlations between traits within genotype classes also change as a function of age (Zerba and Sing 1993; Zerba et al. 1996, 2000). These findings suggest that statistical adjustment, for gender and age variation, of the quantitative traits that connect genome variation with variation in the risk of CVD (before making inferences about the impact of common genetic variations that influence measures of CVD risk) is not consistent with the biological reality of the natural history of CVD (Barrett-Connor et al. 1984; Hayward et al. 2000). Our study considers the impact of the genetic and environmental contexts indexed by gender and age on the contribution of the common "public" variations in the *APOE* gene to statistically explaining variation in measures of lipid metabolism.

Any strategy for evaluating the contribution of the *APOE* gene must consider the plethora of DNA sequence variation revealed by resequencing (Nickerson et al. 2000). Stengård et al. (2002) have compared the gender- and population-specific fraction of interindividual trait variability statistically explained by the common genotypes defined by the ε2, ε3, and ε4 alleles coded by the g.2059T→C and g.2197C→T SNPs with variability statistically explained by genotypes defined by considering one or more additional SNPs. Their study involves SNPs identified by resequencing 5.5 kb of the *APOE* gene

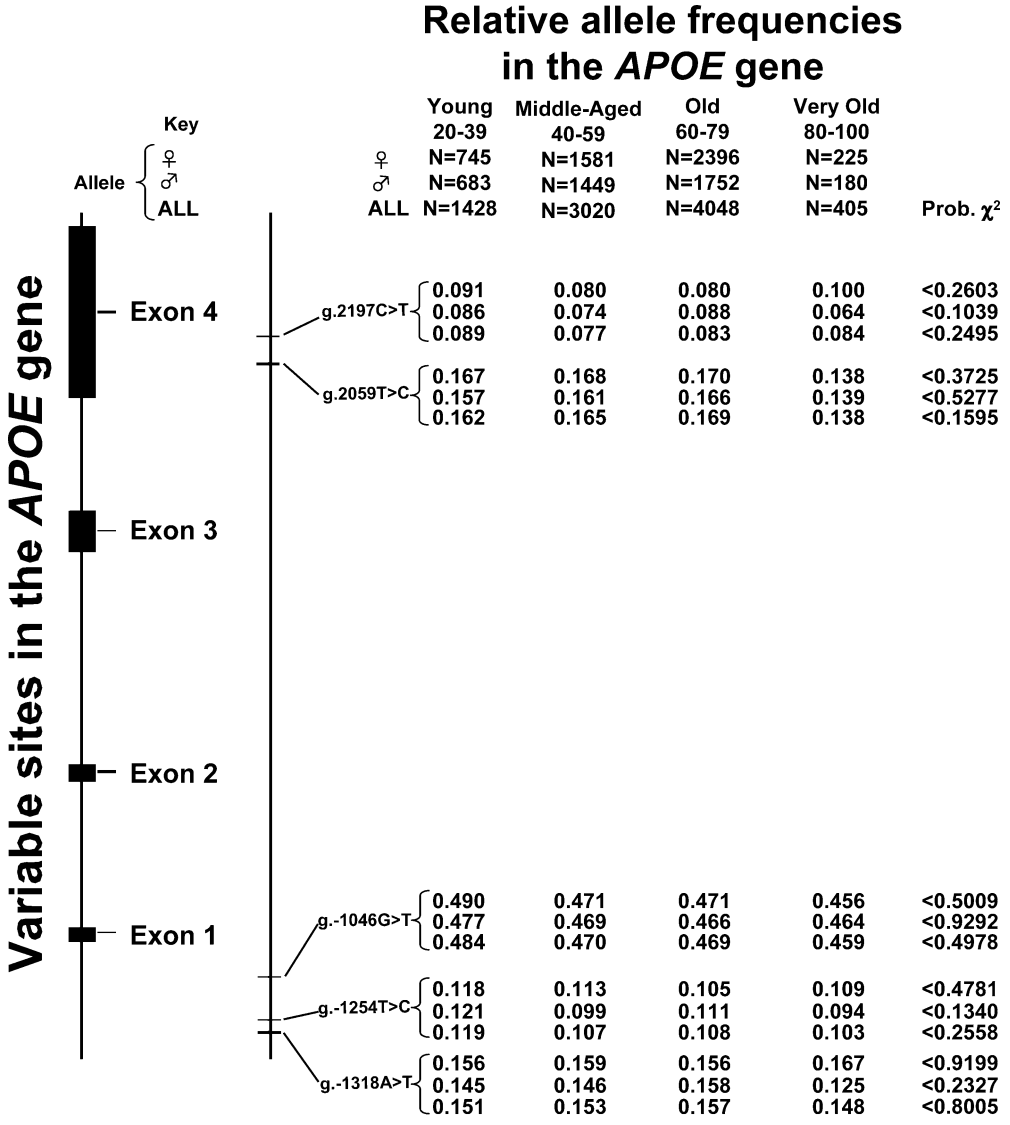


Fig. 1 Relative frequency of the least frequent allele at each of the five variable sites for each of four age categories separately for females and males

in samples of 24 individuals from each of three populations. Twenty-two variable sites have been detected (21 SNPs and one multi-allelic insertion/deletion polymorphism; Nickerson et al. 2000). Ten of the 21 SNPs vary in all three populations. This subset of "public" SNPs includes the g.2059T→C and g.2197C→T SNPs and three SNPs (g.−1318A→T, g.−1254T→C, and g.−1046G→T, Fig. 1, corresponding to SNPs 560, 624, and 832 in reference sequence AF261279) located in the 5′ promoter region of the *APOE* gene (Fullerton et al. 2000). Stengård et al. (2002) have demonstrated, in samples of individuals aged 45–64, that these three promoter SNPs improve the statistical explanation of variation in measures of lipid metabolism in a gender- and population-specific manner when included with the g.2059T→C and g.2197C→T SNPs in the definition of *APOE* genotypes. In the present study, we test the five common *APOE* SNPs in a very large data set, because validation in the population at large is of critical value in judging the relevance of genetic variation. We have asked, for each gender and for each 20-year age strata (young: 20–39 years; middle-aged: 40–59 years; old: 60–79 years; very old: 80–100 years) how much trait variation is associated with genotype variation defined by the g.2059T→C and g.2197C→T SNPs, and how much additional trait variation is associated with genotypes defined by combining the g.2059T→C and g.2197C→T SNPs with one, two, or three of the three promoter SNPs. Finally, we have asked whether there is statistically significant heterogeneity in the influence of *APOE* genotype effects among age strata for each lipid trait in each gender.

Materials and methods

Sample

We studied individuals who participated in the third examination of The Copenhagen City Heart Study from 1991 to 1994. The primary sample of 19,698 men and women was randomly drawn in January 1976 from the Copenhagen Population Register by using the special Central Personal Register Code, which includes date of birth and a registration number (Schnohr et al. 2001). The sample was drawn from a population of approximately 90,000 inhabitants 20 years and older living within ten wards surrounding the Rigshospitalet, The National University Hospital of Copenhagen. At the second examination (1981–1983), the population-based sample was supplemented with a new sample of 500 men and women of 20–24 years of age. At the third examination (1991–1994), the study sample was supplemented with 3,000 men and women aged 20–49, stratified into six 5-year age groups of 250 females and 250 males. Of the 16,563 individuals invited to the third examination, 10,135 participated, 9,259 gave blood, and 9,203 were genotyped for this study. Details on selection, invitation, and examination procedures are described in Schnohr et al. (2001). When individuals with missing trait values or trait values that exceeded five standard deviations from the overall gender sample means were excluded, 9,011 individuals (55% women) remained for the present study. For each gender, we subdivided this sample into four 20-year age strata (young: 20–39 years; middle-aged: 40–59 years; old: 60–79 years; very old: 80–100 years). All participants gave informed consent, and the study was approved by the Danish Ethics Committee for Copenhagen and Frederiksberg (no. 100.2039/91).

Laboratory analyses

TC, apolipoprotein B (APOB), HDL-C, apolipoprotein Al (APOAI), and TG (Boehringer Mannheim, Mannheim, Germany) were measured in non-fasting plasma. Typical day-to-day coefficients of variation were 3.0, 3.9, 2.5, 5.4 and 3.5% for TC (at the level of 3.9 mmol/l), APOB (at the level of 87 mg/dl), HDL-C (at the level of 1.0 mmol/l), APOAI (at the level of 155 mg/dl), and TG (at the level of 1.5 mmol/l), respectively. No seasonal variation in lipids, lipoproteins, and apolipoproteins was detectable. Genotypes of the common *APOE* polymorphism were determined by polymerase chain reaction (PCR) followed by digestion with *Hha*I as described (Hixson and Vernier 1990), with a slight modification (Frikke-Schmidt et al. 2000a). Genotypes of the three SNPs in the *APOE* promoter region (g.−1318A→T, g.−1254T→C, and g.−1046G→T, Fig. 1) were determined in a nested PCR design. First, a 952-bp fragment of the promoter was amplified (forward primer: 5′TGCTCAAGGTCACAACCAAA, reverse primer: 5′CAGGGTCCCAGCTCTTTCTA). The PCR product (5 μl) was diluted 1:20, and the rest was digested with *Alu*I, which recognized bases at the g.−1254T→C site (corresponding to site 427; Bullido et al. 1998), and produced fragments that clearly distinguished the CC, TC, and TT variants. Second, the diluted PCR product was used as the template for a second PCR amplifying two fragments spanning the g.−1318A→T and g.−1046G→T sites (corresponding to sites 491 and 219, respectively; Bullido et al. 1998). The following primers were used: site g.−1318A→T, forward primer: 5′TGTTGGCCAGGCTGGTCTCGA, reverse primer: 5′GCTGGGGGCGGTAGCTCAC; site g.−1046G→T, forward primer: 5′CCTACTTTCTTTCTGGGATCCAGG, reverse primer: 5′GAGGACACCTCGCCCAGTGAT. We designed two mismatches (underlined nucleotides) to create restriction sites for *Dpn*II at sites g.−1318A→T and g.−1046G→T. This digestion resulted in unique fragment lengths for each genotype at site g.−1318A→T (AA, AT, TT) and site g.−1046G→T (GG, GT, TT). To avoid preferential amplification of the largest fragment possible, between forward primer site g.−1318A→T and reverse primer site g.−1046G→T, we down-titrated the concentrations of these oligonucleotides to 1:30 and 1:20 of their corresponding primers. To eliminate a potential misclassification among AT and TT at site g.−1318A→T (most likely because of incomplete digestion), we performed a control assay for the subset of AT and TT

individuals. The diluted 952-bp PCR product was used as the template, and the following primers were designed: forward primer: 5′TGTTGGCCAGGCTGGTTTTAA, reverse primer: 5′CTCCTTTCCTGACCCTGTCC (underlined nucleotides indicate mismatches that create a restriction site for *Dra*I at site g.−1318A→T). This latter control assay resolved a minor misclassification between AT and TT individuals in the *Dpn*II assay and was modified according to a method previously described by Artiga et al. (1998).

Statistical analyses

Distributions of plasma TG were positively skewed in both genders. The application of the natural logarithm (ln) transformation decreased the skewness to less than 0.5 in each gender. To test whether variances of TC, APOB, HDL-C, APOAI, and ln TG differed between genders for each age strata we used the Fisher's *F*-ratio (Sokal and Rohlf 1995). To test whether trait means differed between genders for each age strata, Student's *t*-test was applied when the Fisher's *F*-ratio test did not show significant heterogeneity of the gender variances. When heterogeneity of variances was present, Student's *t*-test with the Satterthwaite's correction was used. Chi-square statistics were used to test homogeneity of relative allele and genotype frequencies across age strata in each gender.

The Boerwinkle and Sing (1986) biased corrected estimator of genetic variance (σ_G^2) in the population sample was used to estimate the interindividual phenotypic variability associated with genotype variation defined by different combinations of the five SNPs. This estimate summarizes deviations of the genotype means from the grand mean, weighted by their relative frequencies. This quantity is given by the following equation:

$$S_G^2 = \sum_{i=1}^{k} \frac{n_i(\bar{Y}_i - \bar{Y})^2}{n} - \frac{(k-1)}{n} \sum_{i=1}^{k} \sum_{j=1}^{n_i} \frac{(Y_{ij} - \bar{Y}_i)^2}{n-k} = \frac{SS_G}{n} - \frac{(k-1)}{n} MS_W$$

where n is the total sample size, k is the number of genotypes, $\bar{Y}$ is the sample grand mean, n_i is the number of individuals with the ith genotype, $\bar{Y}_i$ is the mean of the ith genotype, Y_{ij} is the phenotype of the jth individual in the ith genotype class and MS_W is the mean squared estimate of the phenotypic variability among individuals within genotype classes.

We used permutation methods (Fischer 1935; Churchill and Doerge 1994; Good 2000) to test whether the six genotypes defined by the g.2059T→C and g.2197C→T SNPs contributed significantly to σ_G^2 (H_o: $\sigma_G^2=0$) in each gender-age group strata, to test whether the addition of one or more of the three promoter SNPs significantly increased σ_G^2 in each gender-age strata, and to test whether estimates of σ_G^2 differed significantly across age groups within each gender.

We constructed a test of the null hypothesis, $\sigma_G^2=0$, by randomly shuffling the phenotypes of each trait among the individuals 1,000 times and by calculating an estimate of σ_G^2 for each permuted sample. The 1,000 values of S_G^2 formed a null distribution of variance estimates. The null hypothesis was rejected if the original estimate of S_G^2 from the non-permuted sample exceeded the value of S_G^2 associated with the upper α-percentage of the null distribution of values obtained from the 1,000 permutations (Churchill and Doerge 1994).

To evaluate if the addition of one or more of the three promoter SNPs improved the prediction of variability in measures of lipid metabolism, we randomly shuffled the observed phenotypes among genotypic classes within each of the six genotypes classes defined by g.2059T→C and g.2197C→T 1,000 times and calculated the difference, V_d, between S_G^2 for genotypes defined by g.2059T→C and g.2197C→T and S_G^2 for genotypes defined by these two sites and one or more promoter SNPs. The null hypothesis of no improvement in prediction was rejected if the original value of V_d obtained from the non-permuted sample exceeded the value of V_d associated with the upper α-percentage of the distribution of values obtained from the 1,000 permutations (Stengård et al. 2002).

To test whether the genetic variability, explained by genotypes defined by a particular combination of SNPs, differed significantly as a function of age for each gender (H_o: σ_G^2 constant in all age strata), we carried out the following. First, we calculated a measure of the deviations of genetic variances estimated for the four age groups from the estimate of the genetic variance ignoring age grouping as follows:

$$\lambda_{observed} = \sum_{i=1}^{4} \left(S^2_{G_i} - S^2_{G_{pooled}} \right)^2$$

where $S^2_{G_i}$ is the estimate of genetic variance for the ith age category and $S^2_{G_{pooled}}$ is the genetic variance when all age groups are pooled. Second, we randomly shuffled the four age group identifiers among individuals and recalculated λ as follows:

$$\lambda_{permuted} = \sum_{i=1}^{4} \left(S^2_{G_{ipermuted}} - S^2_{G_{pooled}} \right)^2$$

The 1,000 values of λ permuted, obtained from the 1,000 permutations of the age group assignment, generated a null distribution. The null hypothesis was rejected if the $\lambda_{observed}$ (non-permuted data) exceeded the upper α-percentage of the values in the null distribution.

Pairwise linkage disequilibrium (LD) was measured with the LD parameter D, calculated as $D=P_{ij}-p_ip_j$, where P_{ij} is the frequency of the most common gametic type for a pair of sites, and p_i and p_j are the frequencies of the

nucleotides in that haplotype (Hartl and Clark 1997). The r^2 measure of LD was also considered (Pritchard and Przeqorsku 2001). Significance levels for pairwise linkage were evaluated by χ^2 statistics.

Unless otherwise denoted, we considered an α-level of 0.05 as the criterion for statistical significance.

Results

Age- and gender-specific distributions of the relative allele frequencies

The relative frequency of the least common allele at a variable site ranged from 0.06 to 0.49 (Fig. 1). Relative frequencies at four (SNPs at positions g.−1318A→T, g.−1254T→C, g.2059T→C, and g.2197C→T) of the five variable sites ranged from 0.06 to 0.17, whereas the minor allele was more common (0.46–0.49) in all age and gender strata at the fifth site (SNP at position g.−1046G→T). There was no statistically significant evidence of heterogeneity of relative allele frequencies across age strata in either gender for any of the five SNPs. The largest difference between age strata was observed for site g.2059T→C. The relative frequency of the minor allele ranges from 0.138 (very old) to 0.170 (old) in females and from 0.139 (very old) to 0.166 (old) in males. When very old individuals were compared with the other three pooled age strata, the very old individuals have a marginally significantly decreased relative frequency of the minor allele at site g.2059T→C in females (P=0.08), but not males (P=0.23). The relative allele frequencies of alleles at each of the five SNPs were not significantly different from those reported by Stengård et al. (2002) for the Rochester, Minn., USA population. Four of the five SNPs (g.2059T→C, g.−1046G→T, g.−1254T→C, g.−1318A→T) had relative allele frequencies that were significantly different from those estimated for the Jackson, Minn., USA population (Stengård et al. 2002) and three of the five (g.2197C→T, g.2059T→C, g.−1254T→C) had relative allele frequencies that were significantly different from those estimated for the population of North Karelia, Finland (Stengård et al. 2002).

The relative allele frequencies of the three 5′ SNPs were not different in demented participants vs. non-demented participants in a subsample of the cohort screened for dementia (participants above the age of 65). As expected, the ε4 allele was associated with dementia, as shown previously in the Copenhagen City Heart Study (Frikke-Schmidt et al. 2001).

Gender- and age-specific distributions of quantitative measures of lipid metabolism

Phenotypic characteristics are summarized in Table 1 for each of the eight gender-age group samples. Although only a difference of one year, the average age of females was significantly greater than males in the sample of old individuals. Except for TC in the middle-aged sample and lnTG in the very old sample, average levels of each of the five quantitative measures of lipid metabolism were significantly different between genders within each of the four age strata. On average, females had lower TC and APOB levels than males in the young group and higher levels than males in the old and very old groups. Females had higher average HDL-C and APOAI levels in all age groups and lower average lnTG in all but the very old age group. Interindividual variability of at least two of five lipid traits was significantly different between genders within each of the age strata.

Significant heterogeneity of the average level of each of the five measures of lipid metabolism across age strata was observed in males and for all but HDL-C in females. Significant heterogeneity of the phenotypic variance across age strata was observed for each of the five lipid traits in both genders, with the exception of TC in males. The average level and variance of each lipid trait was smallest in the young group and tended to larger values in the older age groups. Heterogeneity of the distribution of lipid traits among gender and age strata documented the importance of evaluating the age and gender influence on the relative role of genetic variation and justified the stratified analyses presented here.

Gender- and age-specific association of interindividual variation in measures of lipid metabolism with genotypes defined by the ε2, ε3, and ε4 alleles

The contribution of the six common genotypes defined by variable sites g.2059T→C and g.2197C→T to explaining interindividual variation in TC and APOB was statistically significant in both genders in each age group (Table 2). The percentage of phenotypic variation statistically explained was greater in females (TC ranged from 3.8% to 4.7%; APOB ranged from 7.4% to 10.6%) than males (TC ranged from 1.1% to 5.0%; APOB ranged from 4.5% to 6.4%) in each of the four age groups. Except for HDL-C in the old, the variation of HDL-C and APOAI associated with the six common genotypes was statistically significant in all age strata in females (HDL-C ranged from 0.1% in the old group to 6.5% in the very old; APOAI ranged from 0.1% in the old to 3.5% in the very old). In males, these six genotypes explained a small, but significant, amount of APOAI variation in the middle-aged (0.4%) and old (0.7%) groups, and HDL-C variation in the old age group (0.4%). The percentage of variance of lnTG statistically explained ranged from 0.2 to 2.5 in females and from 0.0 to 0.7 in males and was significant only in old and very old females, and in middle-aged and old males.

In females, but not males, there was statistically significant evidence of heterogeneity across age strata for the estimates of the genetic variance of HDL-C associated with variation in the six common genotypes (from 0.6 in old to 22.3 in very old, P=0.015). There was no statistically significant evidence of heterogeneity of the

Table 1 Phenotypic characteristics of females and males from the population at large in age strata of 20 years

Variable	Females (n=745)		Males (n=683)		P-values	
	Mean	Variance	Mean	Variance	t-test Mean	F-test Variance
Young, age 20–39						
Age (years)	32	24	32	24	0.69	0.93
Plasma measure						
TC (mg/dl)	194	1,370	203	1,754	<0.0001	0.001
APOB (mg/dl)	65	311	74	371	<0.0001	0.02
HDL-C (mg/dl)	66	236	51	169	<0.0001	<0.0001
APOAl (mg/dl)	143	599	123	451	<0.0001	<0.0001
lnTG (mg/dl)	4.55	0.22	4.87	0.30	<0.0001	<0.0001
Variable	Females (n=1,581)		Males (n=1,449)		P-values	
	Mean	Variance	Mean	Variance	t-test Mean	F-test Variance
Middle-aged, age 40–59						
Age (years)	51	33	51	31	0.95	0.13
Plasma measure						
TC (mg/dl)	235	2,130	235	1,932	0.84	0.06
APOB (mg/dl)	83	502	89	466	<0.0001	0.15
HDL-C (mg/dl)	66	343	53	261	<0.0001	<0.0001
APOAl (mg/dl)	149	758	131	610	<0.0001	<0.0001
lnTG (mg/dl)	4.81	0.25	5.09	0.33	<0.0001	<0.0001
Variable	Females (n=2,396)		Males (n=1,752)		P-values	
	Mean	Variance	Mean	Variance	t-test Mean	F-test Variance
Old, age 60–79						
Age (years)	69	27	68	29	0.008	0.07
Plasma measure						
TC (mg/dl)	261	2,133	236	1,845	<0.0001	0.001
APOB (mg/dl)	94	481	90	452	<0.0001	0.16
HDL-C (mg/dl)	67	381	54	279	<0.0001	<0.0001
APOAl (mg/dl)	154	811	132	616	<0.0001	<0.0001
lnTG (mg/dl)	4.99	0.21	5.08	0.27	<0.0001	<0.0001
Variable	Females (n=225)		Males (n=180)		P-values	
	Mean	Variance	Mean	Variance	t-test Mean	F-test Variance
Very old, age 80–100						
Age (years)	83	6	83	6	0.59	0.54
Plasma measure						
TC (mg/dl)	263	2,538	229	1,801	<0.0001	0.02
APOB (mg/dl)	95	477	87	432	0.0004	0.49
HDL-C (mg/dl)	66	345	55	264	<0.0001	0.06
APOAl (mg/dl)	151	716	130	616	<0.0001	0.29
lnTG (mg/dl)	5.02	0.19	4.97	0.23	0.30	0.16

Variable	P-values Mean	*F-test* Variance	P-values Mean	*F-test* Variance
TC (mg/dl)	<0.0001	<0.0001	<0.0001	0.5051
APOB (mg/dl)	<0.0001	<0.0001	<0.0001	0.006
HDL-C (mg/dl)	0.2518	<0.0001	0.0006	<0.0001
APOAl (mg/dl)	<0.0001	<0.0001	<0.0001	<0.0001
lnTG (mg/dl)	<0.0001	0.0002	<0.0001	<0.0001

Table 2 Estimates of genetic variance (σ_G^2) and percentage phenotypic variance associated with genotypes defined by the ε2, ε3, and ε4 *APOE* alleles coded by the g.2059T→C and g.2197C→T SNPs for each gender and age strata of 20 years. Six genotypes were observed in all gender and age strata samples except for the very old males. In the very old male age-group, only five genotypes were present; the rare ε22 was absent

	Females g.2059T→C/ g.2197C→T SNPs		Males g.2059T→C/ g.2197C→T SNPs	
	σ_G^2	(%)	σ_G^2	(%)
Young, age 20–39				
TC (mg/dl)	55.6***	4.1	62.4***	3.6
APOB (mg/dl)	25.1***	8.1	22.0***	5.9
HDL-C (mg/dl)[a]	6.3***	2.7	0.0	0.0
APOAl (mg/dl)	7.7*	1.3	0.0	0.0
lnTG (mg/dl)	0.001	0.6	0.0	0.1
Middle-aged, age 40–59				
TC (mg/dl)	87.1***	4.1	22.2***	1.1
APOB (mg/dl)	37.1***	7.4	20.8***	4.5
HDL-C (mg/dl)	2.9**	0.8	0.0	0.0
APOAl (mg/dl)	7.1***	0.9	2.5*	0.4
lnTG (mg/dl)	0.001	0.3	0.002*	0.6
Old, age 60–79				
TC (mg/dl)	101.1***	4.7	56.3***	3.1
APOB (mg/dl)	44.2***	9.2	28.9***	6.4
HDL-C (mg/dl)	0.6	0.1	1.1*	0.4
APOAl (mg/dl)	3.8***	0.5	4.2***	0.7
lnTG (mg/dl)	0.001*	0.2	0.002**	0.7
Very old, age 80–100				
TC (mg/dl)	96.0*	3.8	89.3*	5.0
APOB (mg/dl)	50.4***	10.6	20.2*	4.7
HDL-C (mg/dl)	22.3**	6.5	0.0	0.0
APOAl (mg/dl)	24.8*	3.5	1.8	0.3
lnTG (mg/dl)	0.005*	2.5	0.0	0.0

Significant at *$\alpha \leq 0.05$, **$\alpha \leq 0.01$, ***$\alpha \leq 0.001$
[a]Significant heterogeneity across age strata in females (P=0.015)

estimate of genetic variance among age strata for the other four traits in either gender.

Gender- and age-specific contributions of one additional *APOE* promoter SNP to the statistical explanation of variation in measures of lipid metabolism

The gender- and age-specific estimates of the genetic variance statistically explained by the six genotypes defined by the g.2059T→C and g.2197C→T SNPs and the three SNP genotypes defined by including one additional promoter SNP are presented for each trait in the left panel of Fig. 2. In young, old, and very old females, none of the combinations of three SNP genotypes statistically explained significantly more genetic variance in any of the five lipid traits than did the six common genotypes defined by the g.2059T→C and g.2197C→T SNPs. In middle-aged females (40–59 years), all three combinations of three SNP genotypes significantly improved the prediction of genetic variance in HDL-C and APOAI. The largest increase in the genetic variance of HDL-C was associated with the genotypes defined by combining the SNP at position g.−1046G→T with the g.2059T→C and g.2197C→T SNPs, from 2.9 to 6.2 ($P \leq 0.01$). When combined with g.2059T→C and g.2197C→T to define three SNP genotypes, SNP g.−1254T→C gave the largest increase in the genetic variance of APOAI, from 7.1 to 12.7 ($P \leq 0.05$).

In young males, two different combinations of three SNP genotypes significantly increased the explanation of trait variation. Adding the SNP at g.−1046G→T significantly increased the genetic variance of TC and APOB (from 62.4 to 100.5 and from 22.0 to 25.9, $P \leq 0.01$, $P \leq 0.05$, respectively). The SNP at position g.−1254T→C significantly increased the estimate of genetic variance of APOAI from 0.0 to 5.2 ($P \leq 0.05$). In old males, the SNP at position g.−1318A→T significantly increased the estimate of the genetic variance of APOAI from 4.2 to 9.3 ($P \leq 0.01$).

Gender- and age-specific contributions of the four and five SNP combinations to the statistical explanation of variation in measures of lipid metabolism

The gender- and age-specific estimates of amount of variation statistically explained by genotypes defined by combining the g.2059T→C and g.2197C→T SNPs with two or three additional promoter SNPs are presented for each trait in the right panel of Fig. 2. In females, none of the four or five SNP combinations predicted significant additional variability in any of the lipid traits beyond that predicted by the best three-site combination. In young, middle-aged, and very old males, none of the four or five SNP combinations defined genotypes that predicted significant additional genetic variance in any lipid trait beyond that predicted by the best three-site. In old males, one four-site combination (SNPs g.−1254T→C, g.−1046G→T, g.2059T→C, and g.2197C→T) defined genotypes that significantly explained more genetic variability in lnTG when compared with the genotypes defined by the best three-site combination (SNPs g.−1254T→C, g.2059T→C, and g.2197C→T; 0.005 vs. 0.003, $P \leq 0.038$).

Of the 48 ε22 individuals, only two developed overt type III hyperlipoproteinemia with increased levels of both TG (6.27 and 5.44 mmol/l) and TC (11.8 and 11.2 mmol/l). Neither SNPs g.−1318A→T, g.−1254T→C, or g.−1046G→T, nor any combination thereof was selectively clustered in the two (ε22) type III hyperlipoproteinemic individuals.

Pairwise LD in the *APOE* gene

The LD structure for the five common variable sites in *APOE* is given in Table 3 for females pooled and males pooled. In general, the age-stratified pairwise LDs

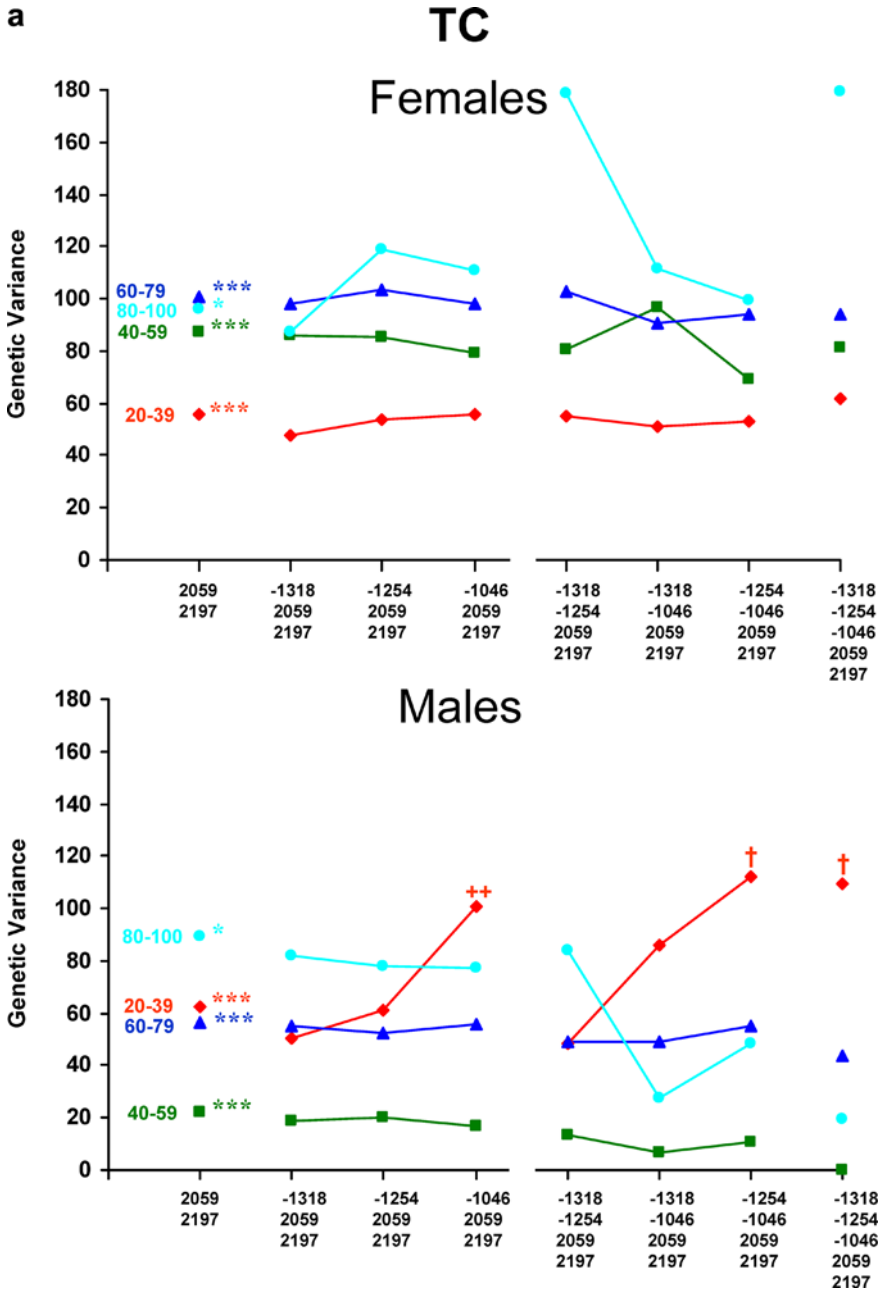
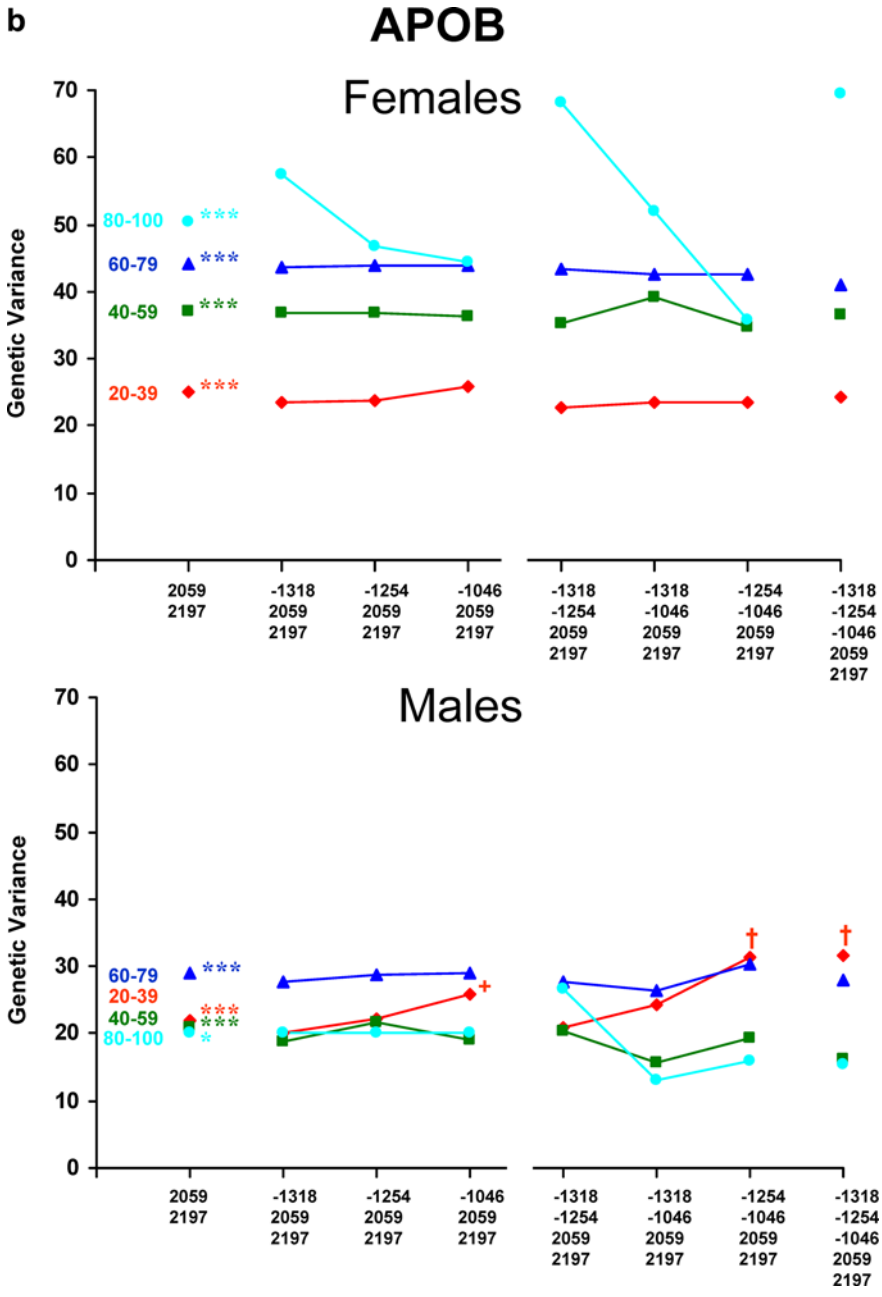


Fig. 2a–e The gender- and age-specific estimates of the genetic variance for the genotypes defined by the g.2059T→C and g.2197C→T SNPs and for each possible combination of one or more additional promoter SNPs are presented (*−1318* g.−1318A→T, *−1254* g.−1254T→C, *−1046* g.−1046G→T, *2059* g.2059T→C, *2197* g.2197C→T). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ for the genotypes defined by the g.2059T→C and g.2197C→T SNPs. +$P \leq 0.05$, ++$P \leq 0.01$ for the genotypes defined by combinations of three sites that improved the prediction in trait variability beyond that explained by the genotypes defined by the g.2059T→C and g.2197C→T SNPs. † $P \leq 0.05$, †† $P \leq 0.01$ for the four and five site combinations that improved the explanation of trait variability beyond that explained by genotypes defined by g.2059T→C and g.2197C→T SNPs. ‡ $P \leq 0.05$ for the four and five site combinations that improved the explanation of trait variability beyond that explained by genotypes defined by the best three site combination

Table 3 Pairwise LDs. $D=P_{ij}-p_ip_j$; r^2 is the correlation coefficient for alleles at site 1 and site 2. The χ^2 statistic was used to test significance of r^2

Site 1/site 2	Females			Males		
	D	r^2	*P*-values	*D*	r^2	*P*-values
g.−1318A→T/g.−1254T→C	−0.013	0.014	<0.0001	−0.013	0.014	<0.0001
g.−1318A→T/g.−1046G→T	0.005	0.001	0.050	0.005	0.001	0.074
g.−1318A→T/g.2059T→C	−0.025	0.034	<0.0001	−0.024	0.033	<0.0001
g.−1318A→T/g.2197C→T	0.015	0.023	<0.0001	0.015	0.023	<0.0001
g.−1254T→C/g.−1046G→T	−0.008	0.002	<0.0001	−0.009	0.004	<0.0001
g.−1254T→C/g.2059T→C	−0.004	0.001	0.022	−0.001	0.000	0.592
g.−1254T→C/g.2197C→T	0.024	0.077	<0.0001	0.021	0.062	<0.0001
g.−1046G→T/g.2059T→C	0.045	0.058	<0.0001	0.038	0.044	<0.0001
g.−1046G→T/g.2197C→T	0.039	0.079	<0.0001	0.037	0.073	<0.0001
g.2059T→C/g.2197C→T	−0.014	0.018	<0.0001	−0.013	0.017	<0.0001

Fig. 2a–e (continued)

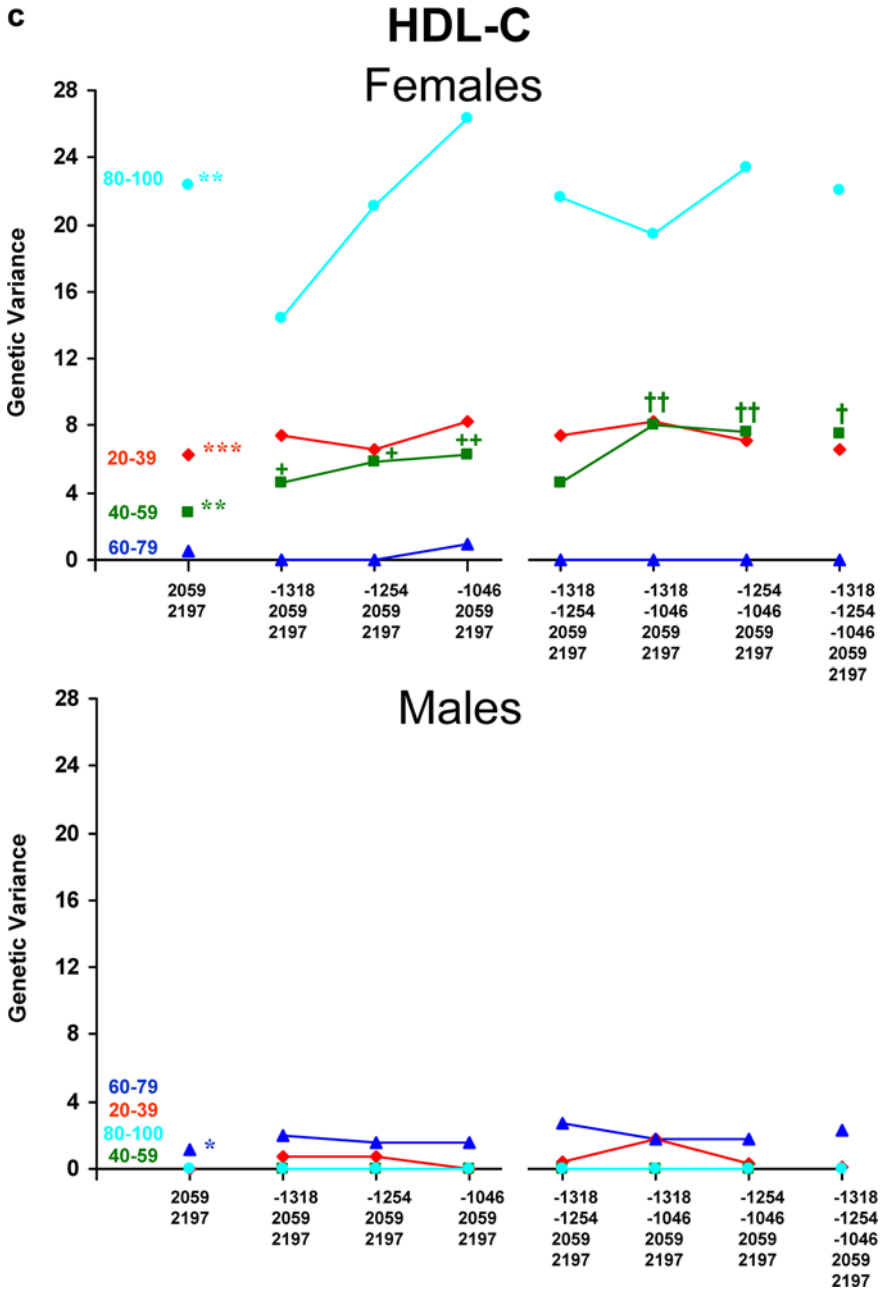


resembled the pooled estimates for both females and males, with the exception of the very old male group. All LDs involving the g.2197C→T SNP, which defines the ε2 allele, were markedly different in this group, most likely because of the absence of the rare ε22 genotype among these very old males. The three pairs with strongest correlation in both females and males were g.−1254T→C vs. g.2197C→T, g.−1046G→T vs. g.2059T→C, and g.−1046G→T vs. g.2197C→T (r^2 0.044–0.079, P<0.0001 for all comparisons).

Discussion

We observed pleiotropic effects of the traditional ε2, ε3 and ε4 *APOE* polymorphism, defined by the g.2059T→C and g.2197C→T SNPs, on five plasma measures of lipid metabolism. The explained variance was greatest for TC and APOB and smallest for HDL, APOAI, and TG in all age-gender strata. The traditional *APOE* genotypes explained more phenotypic variance in females than in males in all lipid traits, except TG. Only the genetic variance of HDL-C explained by the six traditional *APOE* genotypes differed significantly across age groups, and this was observed in females only. In no case did we observe an improvement in prediction associated with adding a 5′ SNP that was consistent across age or gender

Fig. 2a–e (continued)



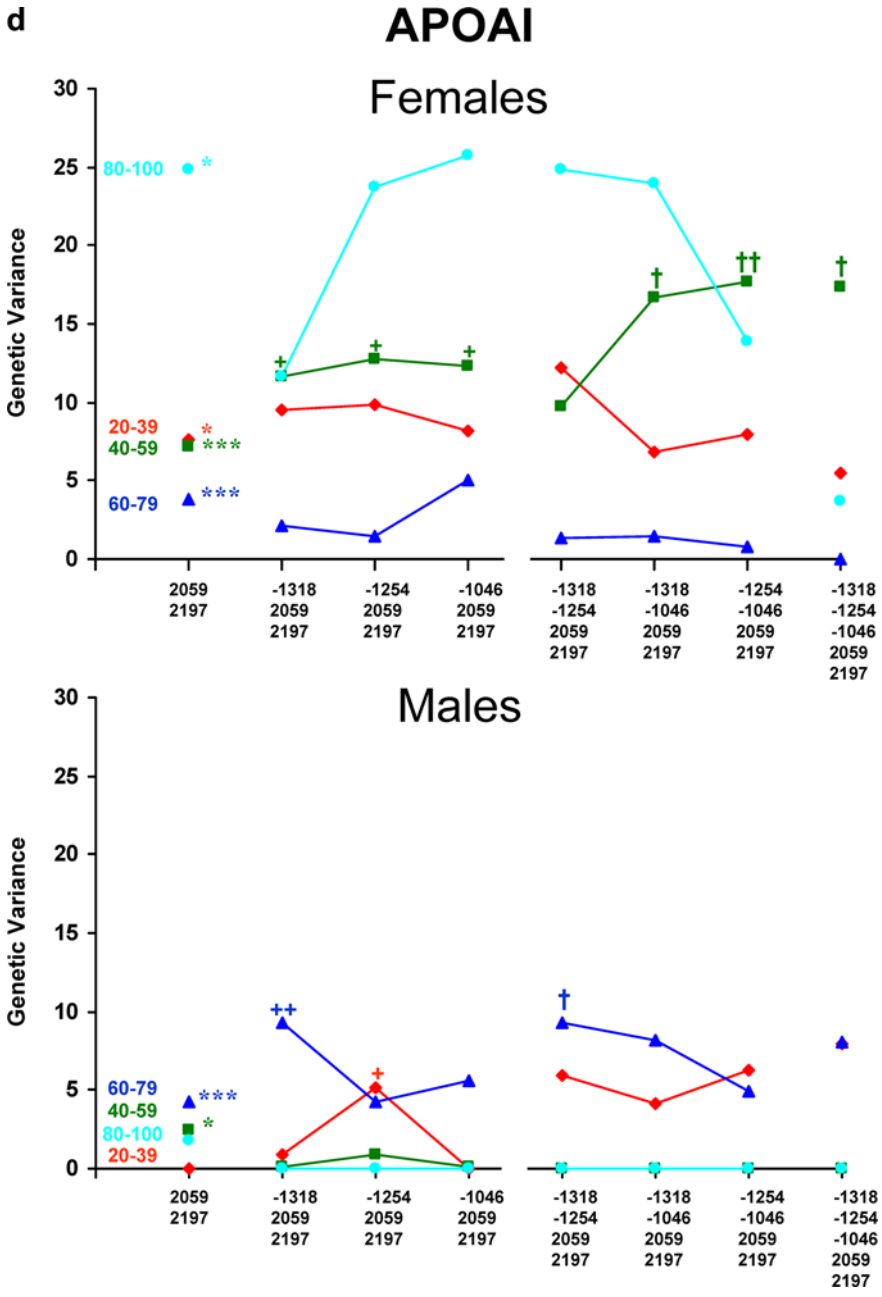
strata. Multi-site genotypes defined by adding SNPs located in the 5′ promoter region to the traditional g.2059T→C and g.2197C→T SNPs doubled the estimate of genetic variance of HDL and APOAI in middle-aged females. Adding 5′ SNPs increased the genetic variance of TC, APOB, and APOAI in young and old males.

Pleiotropic, age-, and gender-dependent effects of genotypes defined by the ε2, ε3, and ε4 alleles on variation in measures of lipid metabolism

Over the last two decades, numerous studies have documented pleiotropic effects of the *APOE* gene on interindividual variation in TC, APOB, APOE, and TG (Hallman et al. 1991; Kaprio et al. 1991; Reilly et al. 1991; Sing and Davignon 1985; Xhignesse et al. 1991). Our population-based study is the largest to date to document these pleiotropic effects. In particular, we have confirmed a significant influence of the genotypes defined by the ε2, ε3, and ε4 alleles on additional lipid traits, such as HDL-C and APOAI (Frikke-Schmidt et al. 2000a).

Age and gender index a broad spectrum of physiological and environmental conditions that the individual experiences through life (Barrett-Connor et al. 1984; Hayward et al. 2000; Zerba and Sing 1993), examplified by the present heterogeneity with phenotypic variance change across age-strata in all lipid traits in both genders, except for TC in men. The present age-specific prediction of HDL-C by the g.2059T→C and g.2197C→T SNPs in

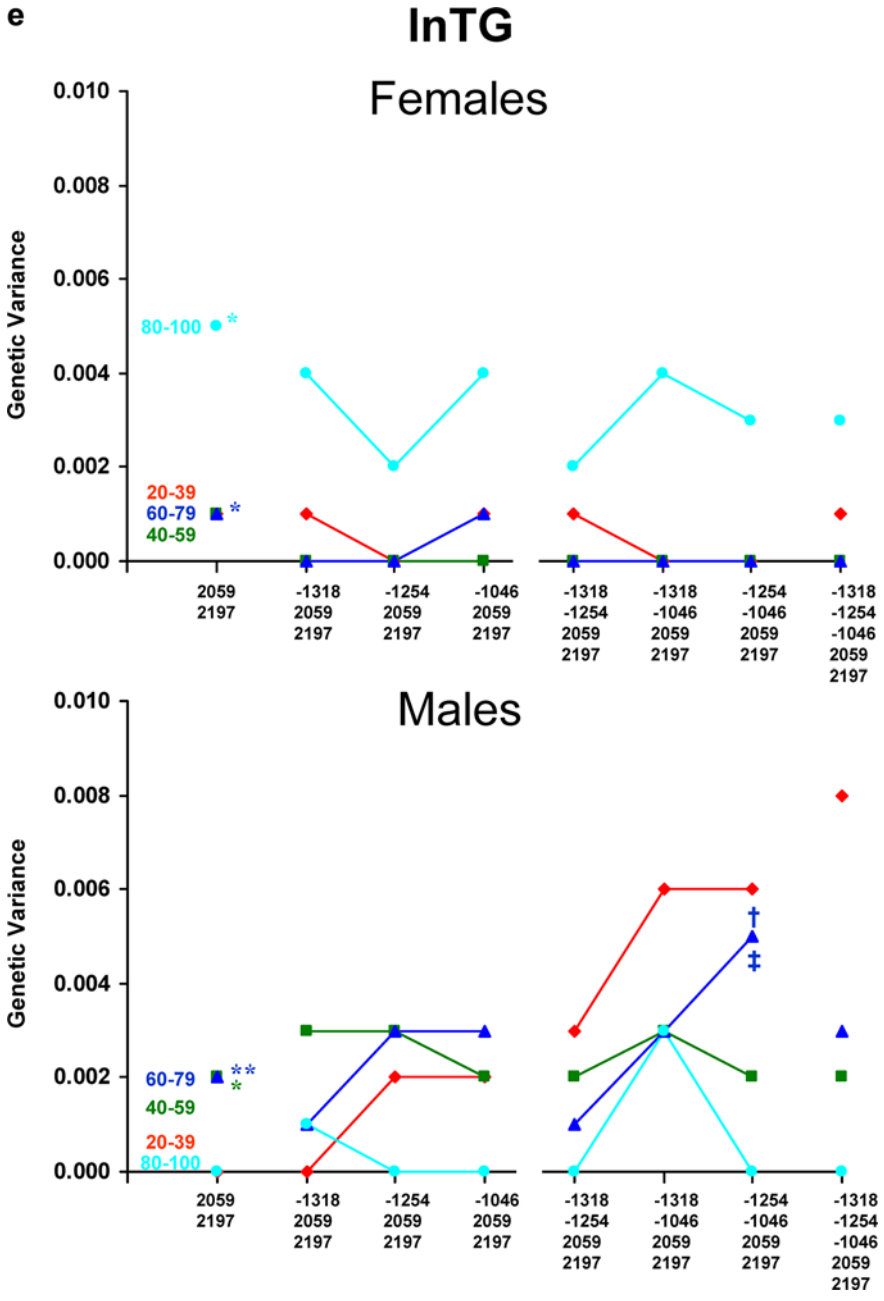
Fig. 2a–e (continued)



females is supported by previous work studying *APOE* genotype-phenotype relationships as a function of age (Zerba et al. 1996, 2000). In this case, both the genetic variance and the phenotypic variance for HDL-C change over age-strata; however, the relative change in genetic variance across age strata (3.5-fold) is several fold larger than the relative change in phenotypic variance (1.6-fold). Differences in exposure to environmental factors between genders (Schnohr et al. 2001) probably contributes to observed gender differences in genotype effects on means and variances or to observed gender differences of genotype-specific correlations between lipids and anthropometric factors (Frikke-Schmidt et al. 2000a; Reilly et al. 1994; Lussier-Cacan et al. 2002).

Furthermore, we find that estimates of phenotypic variance explained by the g.2059T→C and g.2197C→T SNPs defining the six traditional *APOE* genotypes are approximately twice as high in females than in males in most age strata. We and others have repeatedly observed gender-specific patterns of influence of variation in genes that influence measures of lipid metabolism (Frikke-Schmidt et al. 2000a, 2000b; Stengård et al. 2002; Reilly et al. 1991, 1992, 1994; Agerholm-Larsen et al. 2000; Wittrup et al. 2000). The selected age strata correspond well with menopausal status in women, a major determinator of plasma lipid levels (Hayward et al. 2000; Betteridge and Morrell 1998). Several promoters in lipid genes contain hormone responsive elements (Zannis et al. 2001), and effects of variations in these genes are thus

Fig. 2a–e (continued)



likely to be modulated by hormonal status, indexed by gender status, pre- or post-menopausal status, or exogenous hormone treatment status. The gender-specific effects of the *APOE* gene observed in our study may be attributable to differences in environmental contexts, as suggested by the abolishment of the traditional *APOE* genotype effects on HDL-C levels by hormonal replacement treatment in postmenopausal women (Frikke-Schmidt et al. 2000b). *APOE* genotype-specific correlations between lipids and body mass index are also highly dependent on gender, alcohol intake, and smoking (Reilly et al. 1994; Lussier-Cacan et al. 2002). These specific findings substantiate that gender- and age-specific estimates of gene effects are pivotal for understanding and predicting the contribution of genetic variation to inter-individual phenotypic variation in measures of lipid metabolism.

Population- and gender-specific effects of additional *APOE* promoter SNPs on variation in measures of lipid metabolism

Our study supports previously reported population-dependent effects of the *APOE* promoter SNPs. Recently, Stengård et al. (2002) have presented evidence of context dependency of variations in *APOE* promoter SNPs. The effects are expressed in particular populations and genders; this is most pronounced in Finnish (North Karelia sample) and African–Americans (Jackson sample) and less

pronounced in Caucasian–Americans (Rochester sample; Stengård et al. 2002). The promoter variants are located in regulatory regions and are thus prone to modulation by differences in environmental contexts, which are likely to differ between populations. Unsurprisingly, we have consequently found different amounts of additional trait variation predicted by the three *APOE* promoter SNPs in the Danish population, a population with different environmental contexts than the Finnish and African–American samples. When 1,581 females and 1,449 males aged 40–59 years from the present study are compared with a population with similar relative allele frequencies and age (456 females and 398 males from the Rochester sample; Stengård et al. 2002), the estimates of phenotypic variance for TC, HDL-C, and lnTG associated with the promoter SNPs and with the traditional *APOE* genotypes are similar and small in magnitude. The lack of additional phenotypic variance predicted by the three promoter SNPs throughout all age and gender strata in the present study is probably not attributable to a lack of statistical power, because six out of eight age-gender strata include more than 700 individuals.

An important context difference between the present study and many other *APOE* studies is that lipid values in The Copenhagen City Heart Study are non-fasting. However, these lipid values are not postprandial but are measured at different time points after the last meal, thus giving a more natural picture of the normal metabolic cycle. The non-fasting state does not seem to be an important enhancer or inhibitor of associations of plasma lipid levels with the *APOE* g.2059T→C and g.2197C→T SNPs, because associations are of similar magnitude in both the fasting (Stengård et al. 2002; Rochester study) and non-fasting state (present study).

Pairwise LDs

The three pairwise comparisons with highest correlations (g.−1254T→C/g.2197C→T, g.−1046G→T/g.2059T→C, g.−1046G→T/g.2197C→T) in the present study have also been reported in other populations (Fullerton et al. 2000). Although most of the 10 pairwise comparisons in each gender are highly significant, the magnitude of the LD is low. Fullerton et al. (2000) have reported that the high degree of homoplasy and the large number of low frequency variants explain the low level of LD observed among site pairs at the *APOE* locus. Consequently, most marker SNPs in the *APOE* gene are probably not useful for identifying a particular functional variant within the gene. It is unlikely that the observed weak LD between SNP pairs affects the estimates of genetic variance presented here.

Boerwinkle and Sing bias-corrected estimate of genetic variance

We used the Boerwinkle and Sing bias-corrected estimate of genetic variance (Boerwinkle and Sing 1986). This bias correction gives a per observation variance estimate by adjusting the genotypic sum of squares, from the standard one-way analysis of variance, by a quantity that increases with the number of genotypes, providing that the estimate of the mean square within genotypes (MS_W) does not decrease as additional genotypes are considered. If this bias correction term is ignored, as is the case when the often-reported naive standard r^2 estimate of genotypic variance is employed, the genetic variance estimates will be overestimated (Boerwinkle and Sing 1986). In some situations, the Boerwinkle and Sing bias-corrected estimator is, however, also biased (Wijsman and Nur 2001). This bias is small in magnitude in the present study because of the large sample size and low trait heritability. In a few cases, we have observed a small decrease in the estimate of genetic variance when additional sites were added. In most of these cases, this occurred when genotypes with large interindividual variances were added, thus increasing MS_W and resulting in a decreased estimate of the genetic variance.

Estimation of haplotype effects versus genotype effects

Recent emphasis has been placed on haplotype–phenotype relations in studies examining multiple SNPs (Klerkx et al. 2003). However, these haplotype–phenotype relations are estimated with great error. Indeed, it may be impossible to estimate the effects of a haplotype when there are many variable sites, because all genotypes that could have the haplotype do not exist in the sample, or because the homozygous haplotype is so rare that a reliable estimate of the average effect is impossible to obtain. To overcome the problem with rare haplotypes and the huge variation of their estimated effects, a pooling approach is often used (Klerkx et al. 2003). The decision regarding which alleles to pool is arbitrary, and although the number of haplotypes one has to deal with decreases, this only confounds a particular haplotype effect with the effects of the haplotypes with which it is pooled.

In the present study, we wanted to evaluate whether additional sites improved the prediction of trait variability, and not which haplotype carries the functional combination of the DNA variations measured. One can only capture the linear additive effects with studies of alleles; the dominance and the epistatic effects that influence the phenotype reside in the genotypes. Those who seek to identify haplotype effects are most interested in defining and understanding allele effects; this can be most important in plant and animal breeding where gametic effects are manipulated from generation to generation. In medicine, however, the primary goal is to identify the subset of SNPs that defines the set of genotypes that

predict variation in a particular phenotypic outcome or discriminate between those who will remain healthy or those who have or will develop disease.

Conclusion

The pleotropic effects of the *APOE* gene on measures of lipid metabolism and in several pathways in the central nervous system (Mahley et al. 1995; Dietschy and Turley 2001) emphasize the role of *APOE* as an important susceptibility gene for risk assessment in common diseases having a complex multifactorial etiology. Both invariant and context-dependent inferences concerning the impact of variation in the *APOE* gene have emerged from over 20 years of study. Our study of five common polymorphisms in the *APOE* gene in a large European cohort, representing the population at large, documents that the pleiotropic effects of variation at the traditional g.2059T→C and g.2197C→T sites in the fourth exon of the *APOE* gene on measures of lipid metabolism manifest differently in women and men throughout life. Multi-site genotypes defined by adding SNPs located in the 5′ promoter region to the traditional g.2059T→C and g.2197C→T SNPs double the estimate of genetic variance of HDL and APOAI in middle-aged females. The practical implications of these 5′ SNPs for the clinician need, however, to be established. The association between an endpoint phenotype and a predictor should be much stronger than in the present findings before it is worthwhile taking it into consideration in risk assessment.

One reservation that should be noted is that our findings may not apply to other population groups in which other SNPs ("non-public") may have significant effects. At present, the widely accepted research strategy of most candidate gene studies involves only those polymorphisms that segregate in all populations or environmental contexts and assumes that such "public" polymorphisms are relevant for the prediction of phenotypic variability in any population or context (Reich et al. 2001). Heterogeneity of relative genotype frequencies among populations and context dependency of genotype effects should not be ignored, because "non-public" SNPs may have a large impact in specific populations and contexts. Otherwise, the quantitative estimates of phenotypic variability attributable to a candidate gene are not representative of any population, gender, age, or environmental context.

Acknowledgements We wish to thank Mette Refstrup and Hanne Damm for expert technical assistance and Kenneth G. Weiss for his continual attention to the details of the data management and statistical analyses. The technical help of Lynn Illeck in preparing the manuscript is deeply appreciated. This work was supported by the Danish Heart Foundation (grant 97-2-5-69-22531), Danish Research Council (grant 9701975), and in part by National Institute of Health Grant Genomic Approaches to Common Chronic Disease (grant GM-065509) and Genetic Epidemiology of Coronary Artery Disease (grant HL-039107).

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