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Influence of apolipoprotein E genotype variation on the means, variances, and correlations of plasma lipids and apolipoproteins in children

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SUMMARY

The impact of the three most common apolipoprotein E (*APOE*) genotypes ($\epsilon 32$, $\epsilon 33$, and $\epsilon 43$) on means, variances, and correlations of nine plasma lipid and apolipoprotein traits (total cholesterol, lnTriglycerides, HDL cholesterol, and apolipoproteins AI, AII, B, CII, CIII, and lnE) was studied in 212 unrelated female and 219 unrelated male children aged 5–21.5 years from 278 pedigrees ascertained without regard to health status from Rochester, Minnesota. There was significant heterogeneity ($p \leqslant 0.05$) among genotypes for the mean plasma levels of lnApo E, Apo CII, Apo CIII, and lnTriglycerides (lnTrig) in females, and for the means of lnApo E, Apo B, and total cholesterol (Total-C) in males. Significant heterogeneity of intragenotypic variance was observed in males for Apo CII, lnTrig, and HDL-C; no significant heterogeneity was observed in females. Pairwise correlations between traits differed significantly among *APOE* genotypes in both females (6 of 36 pairs) and males (5 of 36 pairs). These results differ from those obtained from studies of the parental generation from the same sample of pedigrees. Our study further demonstrates that, with the exception of mean lnApo E levels, the univariate and bivariate distributions of traits that are measures of lipoprotein metabolism are influenced by variation in the *APOE* gene in a gender- and generation-dependent manner.

INTRODUCTION

Coronary heart disease (CHD, defined by a history of myocardial infarction and/or angina pectoris) affects 14 million individuals in the United States and was the primary cause of approximately half a million deaths in 1995 (AHA, 1998). CHD is the clinical manifestation of a lifelong disease process that begins in childhood, with the early stages characterized by lipid deposition and build-up in the walls of the coronary arteries at sites of intimal injury (PDAY, 1990; Ross, 1993; McGill *et al.* 1997). A number of studies of CHD risk factors in youth have been initiated to better understand the early natural history of the disease process (Lauer *et al.* 1975; Berenson *et al.* 1980; PDAY, 1990; Akerblom *et al.* 1991). The goals of such research are to identify risk factor patterns that characterize children at increased risk of manifesting CHD in adulthood and to develop disease intervention strategies for those children.

Variation in most CHD risk factors is influenced by genetic variation (Sing & Skolnick, 1979; Rao *et al.* 1984). Knowledge about the influence of genetic variation on variation in risk of CHD could provide more reliable methods for early detection of disease, provide insights into the etiology of CHD, and aid in the rational development of prevention and treatment

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strategies. However, identifying and evaluating the genetic variations that contribute to CHD risk in the population at large is complicated by the fact that the manifestations of disease aggregate in families, but do not segregate in accordance with Mendelian expectations. In other words, the statistical relationships between variations in any particular gene and the presence or absence of disease are not one-to-one (Badimon *et al.* 1993; Sing *et al.* 1996). Consequently, estimation of the statistical association of interindividual variation in established CHD risk factors with variation in candidate genes has become a widely used approach to estimating the relative contribution of measured genetic variations to variation in risk of CHD. This approach is used here to evaluate the contribution of the common genotypic variations in the gene (*APOE*) coding for the apolipoprotein E protein (Apo E) to predicting interindividual variation in quantitative levels of nine plasma lipid and apolipoprotein traits. The *APOE* gene has received considerable attention as a CHD susceptibility factor because of the involvement of its protein product in lipid metabolism and its association with CHD risk (see Davignon *et al.* 1988 and Mahley, 1988 for reviews). Many studies have established that interindividual variation in levels of various plasma lipid and apolipoprotein traits (Sing & Davignon, 1985; Davignon *et al.* 1988; Hallman *et al.* 1991; Kaprio *et al.* 1991; Xhignesse *et al.* 1991) as well as prevalence (reviewed in Davignon, 1993) and incidence (Stengård *et al.* 1995) of CHD are associated with variation in the *APOE* gene.

Nearly all studies that investigate the influence of genetic variability on interindividual variation in measures of cardiovascular health and disease focus on differences among genotype-specific trait means. Although, from a public health perspective, it is straightforward to assess genotypic information in terms of variation in average quantitative risk factor levels (Grundy *et al.* 1998), restricting attention to the genotypic means does not capture genotype specific influences on variation in risk factor levels among individuals within genotypic classes. Most studies of genotypic effects assume homogeneity of the intragenotypic variability and co-variability of traits among individuals across genotypic stratifications of the population. In this study we investigate the influences of the common *APOE* genotypes on intragenotypic variances and intragenotypic bivariate relationships of nine traits that are measures of lipoprotein metabolism, as well as their influence on the average level of each of these traits.

Lipid metabolism displays many properties characteristic of a complex system (Bar-Yam, 1997): (1) it consists of many agents that are organized hierarchically (e.g. genes that code for regulatory, structural, and enzymatic proteins influence protein products, plasma lipoproteins and lipids, and cellular and tissue physiology and morphology); (2) there is interaction between agents (e.g. formation of lipoprotein particles, lipolysis, and receptor-mediated endocytosis); (3) there is variability in the quality and quantity of the agents (e.g. variation in LDL subtype concentrations); (4) the system responds to variation in its environment (e.g. post-prandial lipemia); and (5) together the agents function in a non-linear fashion in the process of regulation, transport, and metabolism of lipids to produce the observed risk factor phenotype. Variability in CHD risk cannot be solely ascribed to variation in any particular agent. Furthermore, because the relationships among agents in a complex system like lipid metabolism can influence the risk of CHD (Ferrannini, 1991; Austin, 1992; Sing & Reilly, 1993; Reilly *et al.* 1994), understanding the influence of genetic variation on interindividual variation of a particular agent may not be as important as understanding its influence on interindividual variation in the relationships between the agents (Bailey, 1999).

We demonstrate in this paper that the influence of variation in the *APOE* gene on the means, variances, and correlations of plasma lipids and apolipoproteins is characterized by a combination of invariant and context (as defined by gender and generation) dependent genotype effects.

MATERIALS AND METHODS

Sample

As part of the first phase of the Rochester Family Heart Study (RFHS), 281 three generation pedigrees were ascertained without regard to health status through elementary school children in Rochester, MN. Details of the methods for ascertainment are described elsewhere (Moll *et al.* 1989; Turner *et al.* 1989). In these pedigrees, there were 415 females and 434 males ranging from 5 to 35 years of age in the child generation. To reduce the contribution of age to variation in lipid metabolism, we removed 89 individuals who were older than 21.5 years of age. Furthermore, children who did not possess one of the three most common *APOE* genotypes ($\epsilon 32$, $\epsilon 33$, and $\epsilon 43$), or had missing values for any of the traits considered in this study, were removed from the sample. We also removed from the study children with one or more measurements of plasma lipids and apolipoproteins greater than four standard deviations from their *APOE* genotype-specific means. This resulted in the removal of one $\epsilon 32$ female, five $\epsilon 33$ females and one $\epsilon 33$ male from further consideration. Finally, to remove the confounding effects of genetic relatedness and shared environment on the variation in the traits considered here, one child of each gender was randomly selected from each pedigree for inclusion in our study. The final sample consisted of 212 females (22 $\epsilon 32$, 136 $\epsilon 33$, and 54 $\epsilon 43$) and 219 males (33 $\epsilon 32$, 123 $\epsilon 33$, and 63 $\epsilon 43$) from 278 pedigrees.

Laboratory methods

Blood samples were collected by venipuncture in EDTA after the subjects had fasted overnight. Plasma apolipoprotein (Apo) AI, AII, CII, CIII, and E levels were measured in multiple replicates by radioimmunoassay (Kottke *et al.* 1991). Apo B was measured by enzyme-linked immunosorbent assay (Kottke *et al.* 1991). Plasma total cholesterol (Total-C) and triglycerides (Trig) levels were measured by standard enzymatic methods (Barham & Trinder, 1972; Barr *et al.* 1981). High density lipoprotein cholesterol (HDL-C) was measured after precipitation of Apo B containing lipoproteins with polyethelene glycol (Izzo *et al.* 1981). The *APOE* genotypes were inferred from isozyme phenotypes (Weisgraber *et al.* 1981; Mailly *et al.* 1992) determined by isoelectric focusing plasma samples on polyacrylamide gels, followed by immunoblotting with human Apo E antiserum, as described by Kamboh *et al.* (1988).

Statistical methods

Because the natural history of CHD is gender dependent (Barrett-Conner, 1997), all analyses were carried out separately in females and males. For the test of homogeneity of the lipid and apolipoprotein means, variances, or correlations among the most common *APOE* genotypes we considered two null hypotheses, $\epsilon 32 = \epsilon 33$ and $\epsilon 43 = \epsilon 33$, which focus our attention on the differences among individuals with the less common heterozygous genotypes ($\epsilon 32$ and $\epsilon 43$) and individuals with the most common homozygous $\epsilon 33$ genotype. Throughout this report, we use a p-value of less than 0.05 as our criterion for statistical significance; however, we also report the probability corresponding to each test statistic to enable the readers to apply their own criterion of statistical significance.

To test the equality of genotype-specific means, we used Student's t-test unless there was evidence of significant heterogeneity of variance, which was assessed using the F-ratio test (Neter *et al.* 1990). When the null hypothesis of homoscedasticity was rejected, Satterthwaite's approximate t-test – which is robust to heterogeneity of interindividual variances – was used in place of Student's t-test (Satterthwaite, 1946). Intragenotypic pairwise correlations between all pairs of plasma lipid and apolipoprotein traits were estimated by Pearson's product moment correlation coefficient. The test of homogeneity of a particular pairwise correlation between two genotype classes was carried out using a chi-squared test on transformed correlation coefficients as described by Sokal & Rohlf (1981).

The total correlation, computed as the sum of the absolute values of pairwise correlations, was

used to summarize the complex patterns of correlation observed for each *APOE* genotype class and to provide a measure of cohesiveness among the nine interrelated and interacting traits of lipid metabolism (Reilly *et al.* 1994). Because the distribution of the total correlation statistic is unknown, we tested the null hypothesis of homogeneity of total correlations between *APOE* genotypes using the bootstrap method (Efron & Tibshirani, 1993). To carry out these tests, 1000 bootstrap samples were created by randomly sampling with replacement from the original sample. The *APOE* genotype frequencies in each bootstrap sample were constrained to be the same as in the original sample (see sample description above). For each bootstrap sample the genotype-specific total correlations were estimated and the differences in the total correlation between the $\epsilon 33$ and the $\epsilon 32$ or the $\epsilon 43$ were computed. Quantiles from the empirical distribution of the 1000 bootstrap estimates of the difference in total correlation produced approximate 95% and 99% confidence intervals about the original sample estimate. If the resulting confidence intervals did not include zero the null hypothesis of homogeneity of total correlations was rejected with $p \leqslant 0.05$ and $p \leqslant 0.01$, respectively.

The validity of the parametric tests of homogeneity of means, variances, and pairwise correlations among genotypes used here relies on the assumption that the genotype-specific distribution of each trait is approximately normal. After adjusting all plasma lipid and apolipoprotein values for variation due to date of assay, we assessed the resulting genotype-specific distributions for normality with the Shapiro–Wilk test statistic (Shapiro & Wilk, 1965). Most of the traits, except Apo E and Trig, did not deviate significantly from normality for at least five of the six gender-genotype strata. We chose not to transform these deviant gender-genotype distributions for reasons of comparability between genotypes. Plasma Apo E and Trig showed the greatest deviations from normality. In five of the six genotype-gender strata the null hypothesis of normality was rejected for both traits. The distributions of plasma Apo E and Trig were generally skewed ($0.5 < g_1 < 4.0$) and leptokurtic ($0.3 < g_2 < 23.9$). A natural logarithm transformation applied to Apo E and Trig reduced both the skew and the kurtosis ($-1.5 < g_1 < 0.8$ and $-0.3 < g_2 < 6.0$). All analyses were performed on the natural logarithm transformed values of Apo E and Trig, which we denote lnApo E and lnTrig.

RESULTS

The descriptive statistics for the concomitants age, height, weight, body mass index (BMI), and waist to hip ratio (WHR), and plasma levels of lnApo E, Apo CII, Apo CIII, lnTrig, Apo AI, Apo AII, HDL-C, Apo B, and Total-C are given in Table 1 by gender. Females were not significantly different from males for either average age or variability in age. However, on average, males were significantly taller, heavier, and had a greater WHR than females. Of the plasma lipid and apolipoprotein means, only Apo CII differed significantly between genders, with females having lower average plasma levels than males. Males showed significantly greater interindividual variation in height, Apo CII, and Total-C, and significantly less variation in WHR. The average age, height, weight, BMI, and WHR did not differ significantly among *APOE* genotypes in females or in males (analyses not shown).

Influence of APOE genotype variation in children

The plasma lipid and apolipoprotein means and variances, stratified by gender and *APOE* genotype, are given in Tables 2 and 3 for females and males, respectively. In females, mean levels of lnApo E, Apo CII, Apo CIII, and lnTrig were significantly greater in $\epsilon 32$ children than in those with the $\epsilon 33$ genotype. The mean level of lnApo E in $\epsilon 43$ females was significantly lower and the mean level of lnTrig was significantly higher than for $\epsilon 33$ females. In males, the mean level of lnApo E in $\epsilon 32$ children was significantly greater and the mean level of Total-C was significantly lower than for those with the $\epsilon 33$ genotype. The $\epsilon 43$ males had a significantly lower mean lnApo

Table 1. *Summary statistics for concomitants, plasma lipids, and apolipoproteins by gender*

	Females $n = 212$		Males $n = 219$		p-Values t-test†	F-test
Trait	Means	Variances	Means	Variances	Means	Variances
Concomitants						
Age (yr)	13.9	15.9	13.8	13.7	0.729	0.286
Height (cm)	154.9	256.1	159.8	340.1	0.004	0.039
Weight (kg)	49.4	257.2	52.8	319.4	0.038	0.114
BMI (kg/cm$^2 \times 1000$)	2.0	0.14	2.0	0.12	0.972	0.159
WHR	0.80	0.004	0.83	0.002	< 0.001	< 0.001
Plasma Traits (mg/dl)						
lnApo E	1.4	0.16	1.4	0.17	0.214	0.610
Apo CII	1.8	0.28	2.0	0.37	< 0.001	0.041
Apo CIII	12.2	9.9	12.5	9.45	0.482	0.711
lnTrig	4.4	0.13	4.4	0.14	0.320	0.293
Apo AI	129.0	296.1	127.6	256.9	0.380	0.299
Apo AII	32.9	18.5	32.5	23.5	0.464	0.081
HDL-C	47.8	104.8	46.3	113.8	0.131	0.546
Apo B	66.9	108.4	65.9	89.5	0.334	0.161
Total-C	149.5	576.3	149.7	754.5	0.929	0.049

† If the variances were found to be significantly different ($p \leqslant 0.05$) by the F-test, then Satterthwaite's approximate t-test was used.

Table 2. *Means and variances of plasma apolipoproteins and lipids by APOE genotype in females*

				p-Values†	
Trait	$\epsilon 32$ $n = 22$	$\epsilon 33$ $n = 136$	$\epsilon 43$ $n = 54$	$\epsilon 32 = \epsilon 33$	$\epsilon 33 = \epsilon 43$
Means (mg/dl)					
lnApo E	1.7	1.5	1.2	0.009	< 0.001
Apo CII	2.2	1.8	1.8	0.002	0.758
Apo CIII	14.4	11.9	12.2	0.001	0.633
lnTrig	4.6	4.4	4.5	0.002	0.046
Apo AI	130.0	128.8	129.2	0.743	0.892
Apo AII	33.2	32.5	33.6	0.497	0.135
HDL-C	49.3	48.2	46.2	0.647	0.232
Apo B	66.6	66.3	68.2	0.913	0.260
Total-C	145.4	148.9	152.7	0.436	0.333
Variances					
lnApo E	0.2	0.1	0.1	0.123	0.121
Apo CII	0.2	0.3	0.3	0.949	0.257
Apo CIII	9.7	9.5	9.3	0.894	0.940
lnTrig	0.1	0.1	0.1	0.746	0.741
Apo AI	167.9	284.6	186.6	0.164	0.164
Apo AII	17.6	18.1	19.8	0.999	0.662
HDL-C	124.5	109.3	85.6	0.632	0.312
Apo B	118.6	116.2	86.0	0.887	0.213
Total-C	327.8	611.7	587.7	0.098	0.888

† If the variances were found to be significantly different ($p \leqslant 0.05$) by the F-test, then Satterthwaite's approximate t-test was used.

E and significantly higher mean Apo B levels than $\epsilon 33$ males.

In contrast to the results of comparisons of plasma lipid and apolipoprotein means between genotypes, significant heterogeneity of the intra-genotypic interindividual variances among genotypes was observed only in males for three traits. Interindividual variability in lnTrig and Apo CII was significantly greater among $\epsilon 32$ males than among males with the $\epsilon 33$ genotype. The interindividual variance of HDL-C was significantly smaller among $\epsilon 43$ males than among males with the $\epsilon 33$ genotype.

Estimates of the 36 pairwise correlations among the nine lipid and apolipoprotein traits for each *APOE* genotype were computed and the tests of significance were carried out. Figures 1A and 2A offer a graphical representation of those pairwise correlations that have an absolute value, $|r|$, that exceeds 0.3 in females and males, respectively. We chose to display only those correlations with values greater than 0.3 (i.e. $r^2 > 0.09$, where r^2 can be loosely interpreted as the amount of variation in one trait that is explained by a second trait) to focus attention on those pairwise associations of greater biological rel-

Table 3. *Means and variances of plasma apolipoproteins and lipids by APOE genotype in males*

Trait	ϵ32 $n = 33$	ϵ33 $n = 123$	ϵ43 $n = 63$	p-Values† ϵ32 = ϵ33	ϵ33 = ϵ43
Means (mg/dl)					
lnApo E	1.6	1.4	1.1	0.007	< 0.001
Apo CII	2.1	2.0	2.1	0.495	0.448
Apo CIII	12.5	12.3	12.7	0.753	0.453
lnTrig	4.5	4.4	4.5	0.184	0.330
Apo AI	123.6	128.2	128.4	0.153	0.944
Apo AII	31.4	32.4	33.5	0.280	0.148
HDL-C	45.1	47.0	45.6	0.318	0.394
Apo B	63.5	65.4	68.3	0.316	0.043
Total-C	136.9	150.3	155.3	0.012	0.242
Variances					
lnApo E	0.1	0.2	0.1	0.712	0.902
Apo CII	0.7	0.3	0.3	0.008	0.366
Apo CIII	9.7	10.3	7.8	0.877	0.217
lnTrig	0.2	0.1	0.2	0.017	0.332
Apo AI	239.7	278.1	222.2	0.642	0.328
Apo AII	24.3	22.1	25.0	0.690	0.549
HDL-C	79.9	141.4	78.6	0.063	0.011
Apo B	86.1	82.9	97.8	0.847	0.436
Total-C	554.4	770.7	730.9	0.282	0.830

† If the variances were found to be significantly different ($p \leqslant 0.05$) by the F-test, then Satterthwaite's approximate t-test was used.

evance (statistically significant correlations as small as $r = 0.18$, i.e. $r^2 = 0.03$, were observed). The magnitude of each correlation coefficient is represented by the thickness of the line connecting two traits. The sign of the correlation is represented by the line type; solid lines indicate positive correlations and broken lines indicate negative correlations. The results of the tests of the equality of the Pearson product-moment correlation coefficients between the ϵ33 and the ϵ32 or the ϵ43 genotypes are presented in Figures 1B and 2B. These figures display all statistically significant differences between genotype-specific correlation coefficients (ϵ33–ϵ32 and ϵ33–ϵ43) for females and males, respectively.

The patterns of pairwise correlation (Figures 1A and 2A) suggests that some relationships between traits are common to most gender-genotype strata and some are specific to a single stratum. In females (Figure 1A) the strongest relationships conserved across genotypes ($|r| \geqslant 0.45$) were between Apo CII : Apo CIII and between Apo B : Total-C. Examination of the difference correlation plots in Figure 1B indicates that the correlations between Apo AI : HDL-C and between lnApo E : Apo B were significantly greater in ϵ33 than in ϵ32 females. The pairwise correlations between lnApo E : Apo AI, Apo CII : Apo B, Apo AI : Apo B, and Apo AII : Apo B were significantly greater in ϵ43 than in ϵ33 females.

Figure 2A shows the genotype-specific patterns of correlation in males. There were multiple correlations with $|r| \geqslant 0.45$ within all three *APOE* genotypes: Apo CII : Apo CIII, Apo AI : HDL-C, Apo AI : Apo AII, and Apo B : Total-C. In contrast to females, more significant pairwise heterogeneity was observed between ϵ32 and ϵ33 males. The difference correlation plots shown in Figure 2B indicate that ϵ33 males had significantly greater correlations between Apo AI : HDL-C and between Apo AI : Total-C and significantly lower correlations between lnApo E : Total-C and between Apo CII : Apo CIII than ϵ32 males. Also, the correlation between Apo B : lnTrig was significantly greater in ϵ43 than ϵ33 males. We note that the correlation between Apo AI : HDL-C was significantly greater in ϵ33 than ϵ32 in both females and males.

To aid in summarizing the complex patterns of correlation observed within each *APOE* genotype and to provide a summary measure of the cohesiveness among these nine interrelated and interacting components of the lipid metabolic pathway we employed the total correlation (TC) statistic suggested by Reilly *et al.* (1994). In females, the total correlations for the ϵ32, ϵ33, and ϵ43 genotypes were 10.83, 10.10, and 14.16, respectively. The total correlation for the ϵ43 genotype was significantly greater than the reference ϵ33 genotype ($p \leqslant 0.05$). In males, the total correlations for the ϵ32, ϵ33, and ϵ43 genotypes were 11.42, 11.33, and 12.26, respectively. The differences between these estimates of total correlation were not statistically significant ($p > 0.05$).

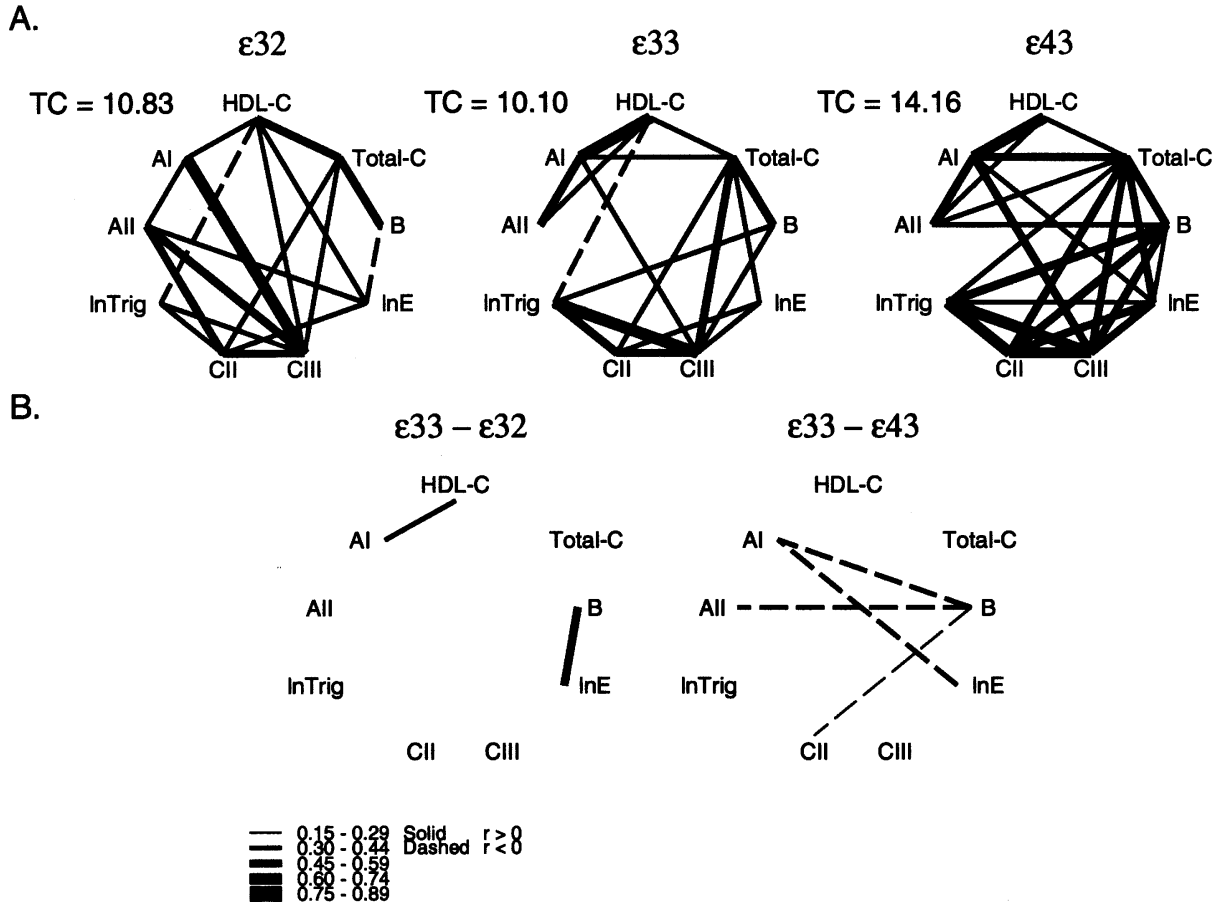

Fig. 1. (A) Correlation diagrams showing the bivariate correlations between traits where $|r| > 0.3$ for each *APOE* genotype in females. TC is the sum of the 36 pairwise correlations. (B) Difference correlation diagrams showing the statistically significant differences between genotype-specific bivariate correlations.

Comparison of the influence of APOE genotype in children and parents

Analyses of the influence of *APOE* genotype on means, variances, and correlations similar to those reported in this paper were conducted previously on a sample from the parental generation (ages 26–64) from the same sample of pedigrees from Rochester, MN (Reilly *et al.* 1991; 1994). Rather than carry out a statistical comparison of the genotype-specific plasma lipid and apolipoproteins in children and parents, we offer a qualitative comparison of the generation-specific phenotypic response to variation in *APOE* genotype. The results of the tests of homogeneity of means, variances, and correlations in the children and parents are summarized in Figure 3.

First, we compared the influence of variation in the *APOE* gene on the trait means in each generation. As shown in Figure 3 (first column), of the nine lipid and apolipoprotein traits studied in parents and children, only the mean transformed levels of the protein product of the *APOE* gene itself, lnApo E, differed significantly among *APOE* genotypes in both genders and generations. In all four gender- and generation-specific strata, significantly higher levels of lnApo E were associated with the $\epsilon 32$ genotype and significantly lower levels were associated with the $\epsilon 43$ genotype. For females only, the Apo CIII means were significantly higher in individuals with the $\epsilon 32$ genotype than the $\epsilon 33$ genotype in both parents and children. Surprisingly, we found the greatest similarity of genotype-specific effects on trait means in female children and male parents.

Second, we compared the influence of variation

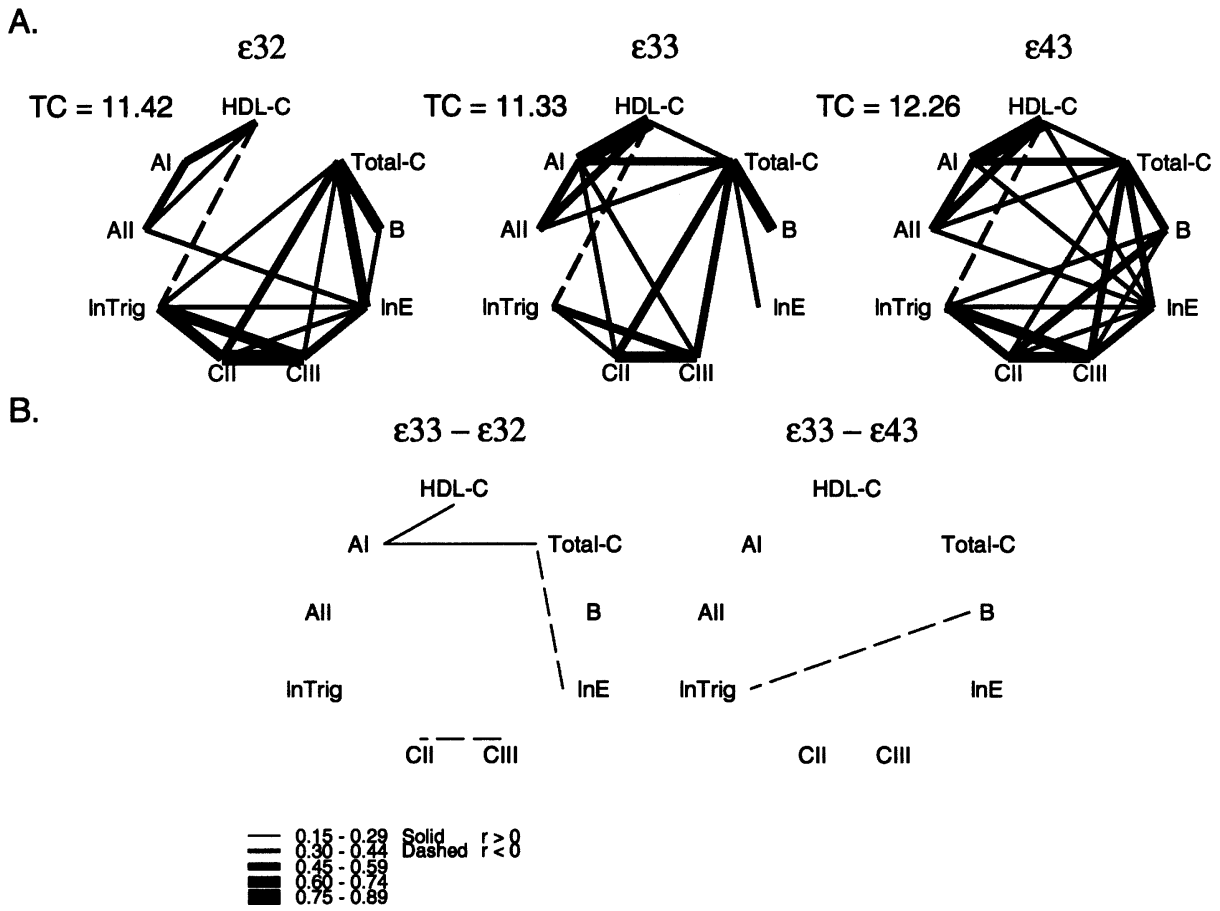

Fig. 2. (A) Correlation diagrams showing the bivariate correlations between traits where $|r| > 0.3$ for each *APOE* genotype in males. TC is the sum of the 36 pairwise correlations. (B) Difference correlation diagrams showing the statistically significant differences between genotype-specific bivariate correlations.

in *APOE* genotype on the trait variances in each generation. Children differed more from parents with respect to inferences about the homogeneity of genotype-specific interindividual variances (Fig. 3, second column) than homogeneity of trait means. In parents, 11 of 36 tests of homogeneity of variance were rejected with $p \leqslant 0.05$, with eight of those 11 significant results observed in females. In children, only three of 36 tests were significant, all observed in males. The most striking comparison of the influence of *APOE* on the variances in children with adults was with respect to Total-C. In both female and male children, the variance of Total-C among individuals with the $\epsilon 32$ or the $\epsilon 43$ genotypes was smaller than for those with the $\epsilon 33$ genotype, though the differences were not statistically significant. However, in parents the *APOE* genotype had a very strong influence on the variance of Total-C with the variance among those with the $\epsilon 32$ or the $\epsilon 43$ genotype being significantly greater than $\epsilon 33$ and more than twice the magnitude observed in children.

Third, we present in Figure 3 the influence of variation in the *APOE* gene for each gender-generation stratum on correlations. Overall, the lipid and apolipoprotein traits were more highly correlated in parents (TC = 13.402, 12.055, and 13.042 for $\epsilon 32$, $\epsilon 33$, and $\epsilon 43$ females, respectively and TC = 13.567, 12.753, and 13.894 for $\epsilon 32$, $\epsilon 33$, and $\epsilon 43$ males, respectively) than they were in the children (TC = 10.832, 10.095, and 14.156 for $\epsilon 32$, $\epsilon 33$, and $\epsilon 43$ females, respectively and TC = 11.421, 11.326, and 12.260 for $\epsilon 32$, $\epsilon 33$, and $\epsilon 43$ males, respectively). In parents there was a very strong negative correlation between lnTrig and HDL-C and very strong positive correlations between lnTrig and Total-C and Apo B. These

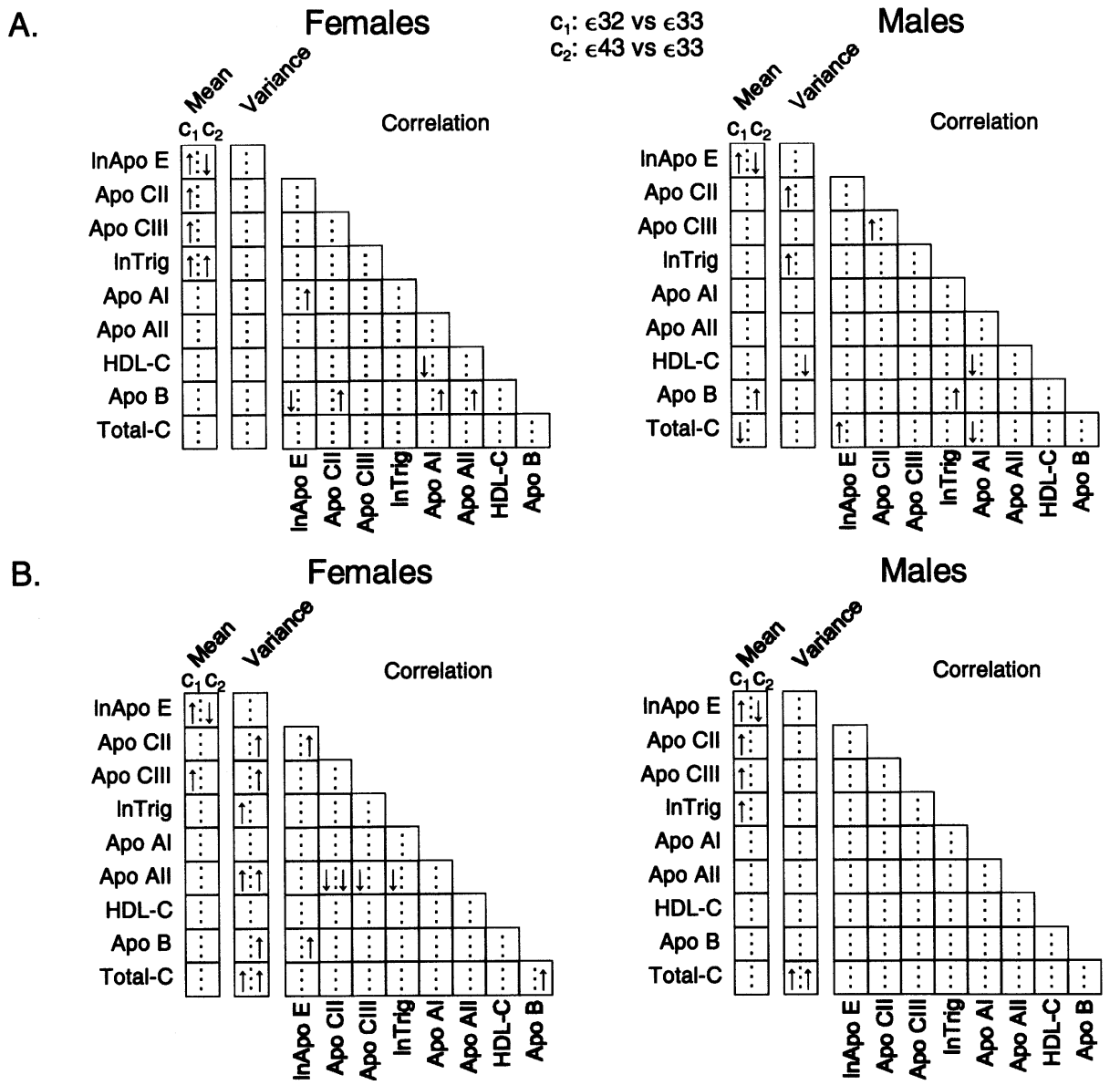

Fig. 3. Summary of significant results of the tests of homogeneity of means, variances, and correlations in children (A) and parents (B). The statistics for the ϵ32 and ϵ43 genotypes are indicated by ↑ if they are significantly higher and ↓ if they are significantly lower than the reference ϵ33 genotype.

three correlations were consistent across *APOE* genotypes but they were much weaker in female and male children than in the parents. The significant differences in pairwise correlations among genotypes were also very different in parents and children. There was more genotype-specific heterogeneity of correlation in male children than in male parents. None of the gender- and genotype-specific significant differences in correlation were observed in both generations.

Influence of APOE genotype on multivariate measures in children and parents

A visualization of the total impact of variation in *APOE* genotype on the means, variances, and correlations in children and parents is provided through multivariate graphs in Figures 4 and 5 for females and males, respectively. These multivariate graphs combine information from each of the nine generation-, gender-, and genotype-specific means and variances, as well as on the

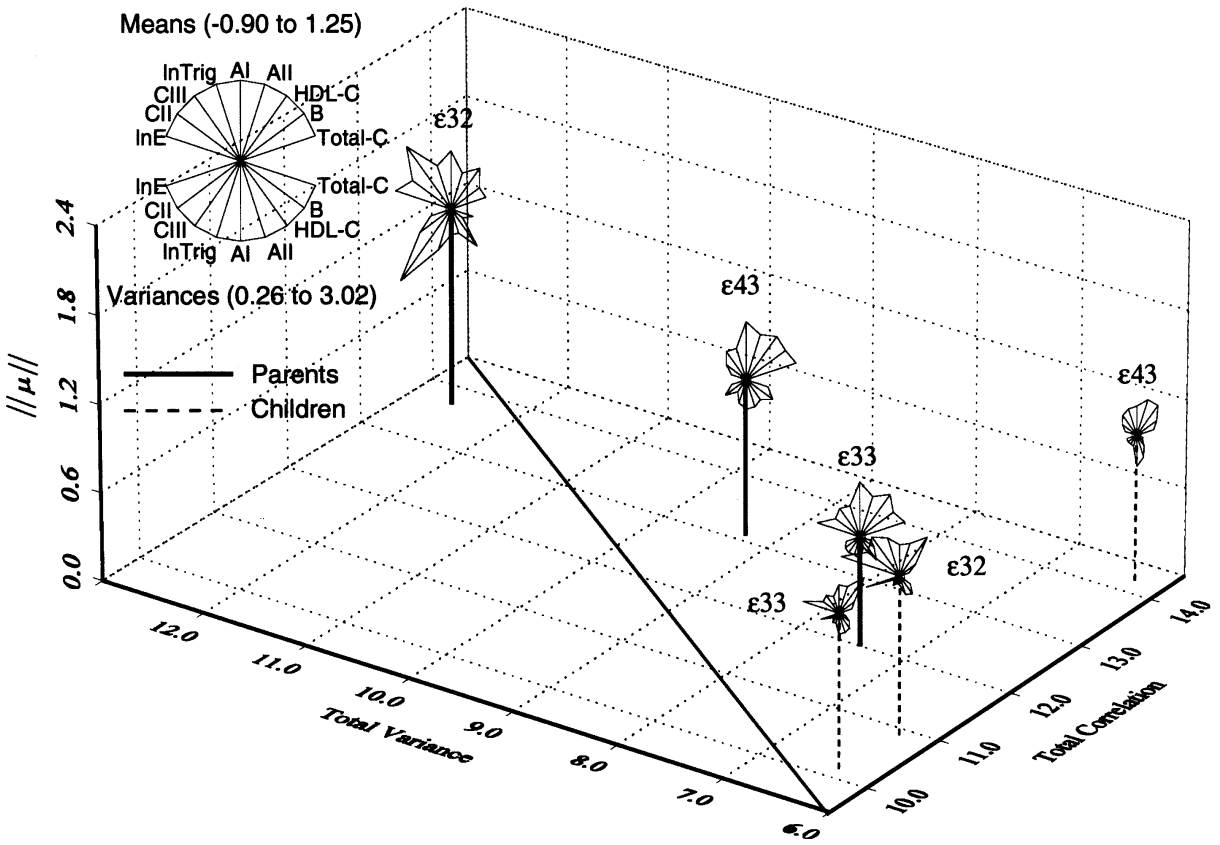

Fig. 4. Multivariate profile of female children and parents. See text for description.

combined contribution of the traits to multivariate measures of the mean and variance vectors and the correlation matrix. To remove scale differences between traits, the pooled data from all genotype-gender-generation strata was adjusted to a standard normal scale (mean = 0.0 and S.D. = 1.0). This re-scaling also permits direct comparisons of the deviations of each of the 12 generation-, gender-, and genotype-strata from the reference established by the pooled data. The six multivariate stars (parents and children for each of the three genotypes) in Figures 4 and 5 depict the mean (upper half) and variance (lower half) values for each re-scaled trait, which are given in Tables A 1 and A 2 in the Appendix. The length of each ray is proportional to the deviation of each generation-, gender-, and genotype-specific mean and variance from the minimum scaled mean and variance for all traits. The orientation of each ray indicates the corresponding trait, as shown in the upper left-hand corner of each Figure. Stars corresponding to parents are indicated by a solid drop line and those for children by a dashed drop line.

The location of the centre of each star in the three dimensional graph shows the Euclidean distance of the means from the origin, total variance, and total correlation for each genotype in females and males. The Euclidean distance is the straight line distance between two points in space. It is used here to measure the distance of the point defined by the vector of trait means from the origin in nine-dimensional space. It is computed by taking the square root of the sum of the nine squared re-scaled means (details can be found in Reilly *et al.* 1991). The total variance is computed by summing the variances of the re-scaled data for all traits across the nine traits. The method for calculating the total correlation is presented above in the *Statistical methods* section. Estimates of the total correlation are not changed by the re-scaling.

In females (Fig. 4), the Euclidean distances of

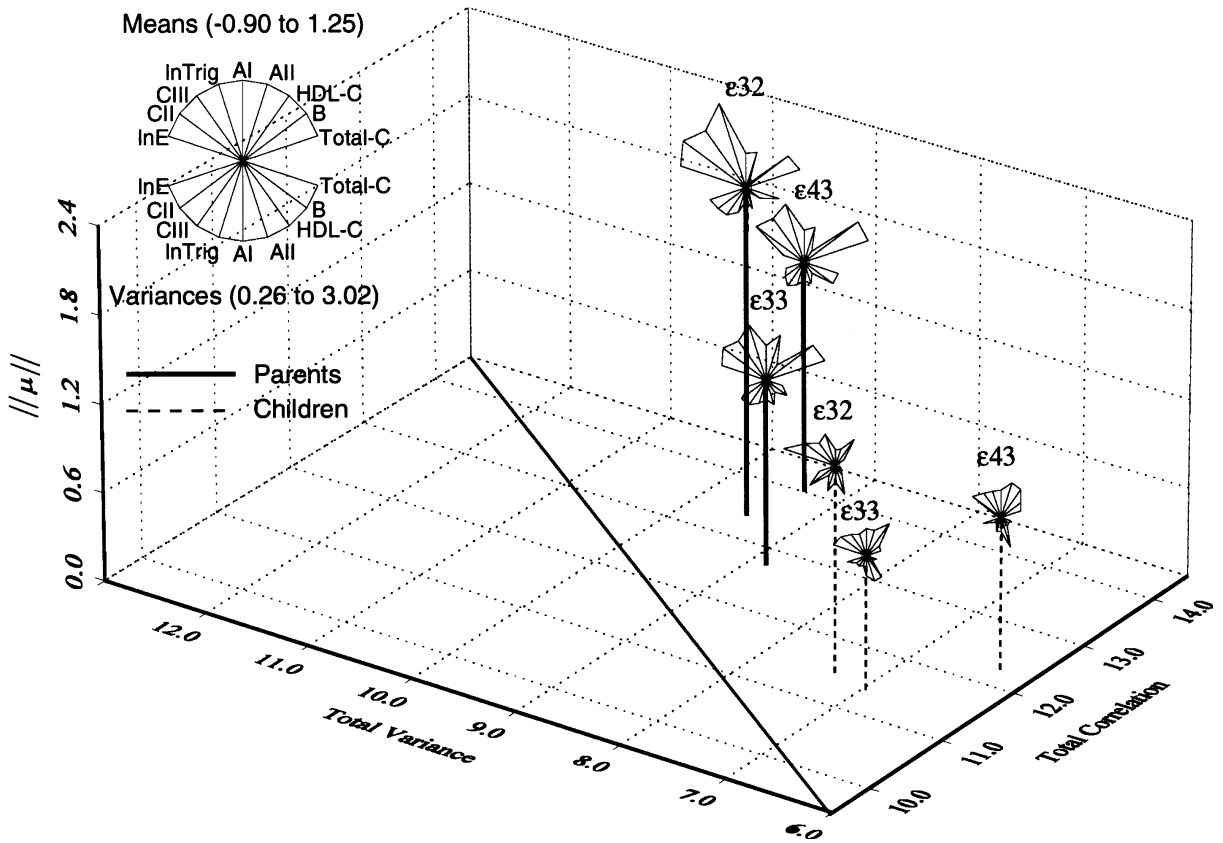

Fig. 5. Multivariate profile of male children and parents. See text for description.

the means in the children were approximately equal, while in parents it was smallest in $\epsilon 33$ females, and largest in $\epsilon 32$ females. The Euclidean distances of the means in children were intermediate between the $\epsilon 33$ and $\epsilon 32$ Euclidean distances of the means in parents. Similar patterns were observed within generations with respect to the total variance. However, female parents had a greater total variance than female children for each genotype. The shapes of the stars differed both in size and shape among *APOE* genotypes and generations. The total correlations in children and parents were discussed in the preceding sections.

In males (Fig. 5), the generation-specific Euclidean distances were smallest in the $\epsilon 33$ strata and largest in the $\epsilon 32$ strata, and the genotype-specific distances were smaller in children than parents. As in females, the total variances were uniformly greater in parents than children. The $\epsilon 43$ male children had the smallest total variance and the $\epsilon 32$ male children had the largest total variance, and the $\epsilon 33$ and $\epsilon 43$ male parents had similar total variances but substantially smaller than the total variance of the $\epsilon 32$ male parents.

There are several general features of these graphs that summarize the impact of generation, gender, and genotype on scale, variability and co-variability. First, while the females tended to cluster more by the Euclidean distance of the means and vary more by their total variances and total correlations, males tended to cluster more by their total variances and total correlations and vary more by their Euclidean distances. Second, we call attention to the observation that the shapes of the portion of the stars summarizing means tended to resemble the portion summarizing the variances, reflecting the positively correlated nature of the means and variances. Third, we found an unexpected positive correlation between the total variance and the total correlation, illustrated by the location of 11 of the 12 stars above the diagonal line.

Since the correlation is computed by dividing the covariance by the product of the standard deviation for each trait, if the covariance remains constant the correlation will be reduced as the variance for each trait increases. The observed positive correlation must result as a consequence of the covariances between traits generally increasing more rapidly than their variances.

discussion

Because the natural history of CHD begins relatively early in life (Hixson, 1991; McGill *et al.* 1997), much attention has been devoted to the study of CHD risk factors in children. In addition to traditional CHD risk factors, a better understanding of the role that common genetic variation plays in the early pathogenesis can offer insights into the biology of the natural history of disease, may suggest improved treatment strategies, and promises more accurate identification of subgroups within the population at large that are at increased risk for developing heart disease. In this paper, we have evaluated the influence of variation in one gene, *APOE*, on the univariate distributions of nine participants in plasma lipoprotein metabolism and the bivariate correlations between them in children, and compared these results to similar studies conducted previously in parents from the same sample of pedigrees. We found that the influence of each of the three common *APOE* genotypes on the trait means and the intra-genotypic variances and correlations was gender and generation specific. We discuss below four implications of these findings for our understanding of the etiology of interindividual variation in quantitative risk factor variation, and for the utilization of genetic information to predict interindividual variation in risk of CHD.

First, the pleiotropic effects of each *APOE* genotype on the multivariate vector of nine trait means are dependent on context indexed by gender and age. Our study of children further documents that the pleiotropic influences of genotypic variation on interindividual variation in the levels of quantitative traits that are plasma measures of lipid metabolism are dependent on context defined by gender. Combined with the results of our previous studies of adults from the same sample of pedigrees (Reilly *et al.* 1991) it is clear that the influence of genetic variation on levels of plasma measures of the lipid metabolism should be studied separately in females and males. The heterogeneity of inferences about *APOE* genotype means between children and adults in females and males establishes that age is also an index of variation in agents that alter genotype effects. Variability in the contribution of the nine traits to the height of the centroid that is portrayed by the variability in the shape of the upper halves of the multivariate stars in Figures 4 and 5 represents variation in the pleiotropic effects of each genotype on the nine traits among the twelve generation-, gender-, and genotype-strata. The only exception to the general observation of context dependency of genotype effects is the consistent statistically significant differences among genotypes for the average level of lnApo E in both genders in children and parents. These findings further validate the concern by Sing *et al.* (1996) that genotype-phenotype models for quantitative traits must accommodate a mix of invariant genotypic effects and genotypic effects that are dependent on interindividual variation in the contemporary ecological context, the history of exposures to environmental agents and the epigenetic phenomena that operate throughout the life cycle. Evidence that effects of the *APOE* genotypes have invariant average effects only on the level of the primary gene product that is physiologically closer to the *APOE* gene, and hence less susceptible to the influences of exposure to external environmental variations, lends biological credence to the need for more realistic statistical models of genotype-phenotype relationships for quantitative CHD risk factors than have been traditionally employed in genetic epidemiological studies.

One other study has investigated the influence of *APOE* on plasma lipid and apolipoprotein means in female and male children separately. In

a study of 1544 Finnish children, ages 3 to 18, Porkka *et al*. (1994) found significant variation in Total-C among four *APOE* genotypes (ϵ32, ϵ33, ϵ43, and ϵ44) in both females and males. We found statistically significant differences among *APOE* genotypes in males only and, although not statistically significant, the ranks of the genotype means were the same in females as in males. They also observed the same rankings among genotypes of plasma lnTrig in females as reported here, though the differences were not statistically significant. Given the differences between these two studies in the characteristics of the populations considered, the sampling design, and the statistical methods that were used, the gender-specific influence of variation in the *APOE* genotype on trait means in children was remarkably consistent.

The primary influence of the *APOE* protein product on lipid metabolism involves intestinal cholesterol absorption, hepatic clearance of triglyceride rich chylomicron and VLDL particles, and metabolism of LDL particles. Adults carrying the ϵ2 allele tend to absorb cholesterol from the intestine and clear chylomicron and VLDL particles more slowly than ϵ33 individuals and catabolize LDL particles more quickly (Mahley, 1988). Adults carrying the ϵ4 allele tend to have higher rates of cholesterol absorption and chylomicron and VLDL clearance and lower rates of LDL catabolism than ϵ33 individuals (see Davignon *et al*. 1988; Davignon, 1993). In our study of children, a statistically significant influence of variation in the *APOE* gene on chylomicron and VLDL related trait means was only observed in females, and the influence on LDL related trait means was only observed in males. Female children with the ϵ32 genotype had significantly higher chylomicron and VLDL related trait means. Paradoxically, female children with the ϵ43 genotype also had significantly increased levels of lnTrig. How these fundamental physiological processes are influenced by agents that are indexed by gender and age is the major hurdle in establishing biologically meaningful interpretations of the statistical evidence for context dependent genotype effects. In this regard, efforts to develop an ability to identify and measure the interacting agents and the epigenetic processes in population based study designs, as easily and accurately as it is possible to measure genotypes, are long overdue.

The existence of context dependent genotype effects on mean levels brings into question the routine statistical adjustment of quantitative risk factors for gender and age variation before estimating genotype effects. Inferences from genotype means about trait levels estimated from adjusted data may not be appropriate for any age or either gender. Furthermore, when there are context dependent genotype effects, the evaluation of replication of the estimates of genotype effects across studies to establish invariant biological truths, or the estimation of genotypic effects from a meta-analysis of pooled data, can lead to inappropriate inferences about the utility of genetic information for most individuals, families, age groups, or populations.

Second, the intragenotypic variance of each trait and correlation between traits is dependent on context defined by genotype, gender, and age. Most studies of the pleiotropic effects of genotypic variation assume homogeneity of intragenotypic variability of each trait and covariability between the members of the set of traits of interest and hence ignore the possibility of estimating, or testing hypotheses about, the influence of genetic variation on phenotypic plasticity (Bradshaw, 1965; Schlichting & Pigliucci, 1998). Our gender and trait specific findings in children support the generality of the argument that the gender dependent influences of genetic variation on the characteristics of a complex system, such as lipid metabolism, is not solely limited to average levels of the component traits. The general observation that there is greater heterogeneity of the intragenotypic trait variance among *APOE* genotypes in parents than children, and that trait variances and correlations are greater in parents than in children for both genders, clearly documents a genotype, gender, and generation influence on intragenotypic phenotypic plasticity. These results argue that *APOE* genotypes vary in their

biological influence on the range of phenotypic expression of each component trait and the coherence, or biological connectedness, between traits. The means, variances, and correlations that are characteristic of each genotype are measures of the 'norm of reaction' or 'adaptive response' to variation in unmeasured genetic and environmental agents.

Heterogeneity in the adaptive response may be a consequence of genotype by environment interaction, different interacting agents, or alternative forms of the same interacting agents being non-randomly distributed among the different gender and generation strata. Studies that consider interaction effects of the *APOE* genotypes with other genes and the effects of exposures to environmental agents are required. Relatively few studies have addressed the role of gene-gene interactions on measures of lipid metabolism. However, numerous studies have documented interactions in adults between *APOE* genotype effects and effects of various environmental agents, including smoking (Kaprio *et al*. 1989), diet (Mänttäri *et al*. 1991; Cobb *et al*. 1992), exercise (Taimela *et al*. 1996), measures of body size (Reilly *et al*. 1992), drug treatment (Nestruck *et al*. 1987; Carmena *et al*. 1993), and age (Jarvik *et al*. 1997). The observed heterogeneity of variance for seven of nine traits and larger correlations in parents is consistent with an increasing role of environmental agents with age. However, it is unlikely that the same variations in any one factor or the same factors are involved in interactions with *APOE* in all gender and age strata.

Also, a history of interactions with environmental factors may influence contemporary variability and covariability of traits. The children studied have been exposed to a much more limited number of environmental agents over a shorter period of time than their parents, who are on average 29 years older, providing fewer opportunities for interactions to have occurred. Alternatively, if variation in a particular environmental agent is generation-specific, such as particular dietary components, quality of medical care, or type of environmental pollutants, there may be genotype-specific influences that remain unique to each generation. We might expect the genotype-specific differences in variances and correlations observed in what is currently the child generation to begin to resemble the parent generation as they age if the responsible environmental exposures are common to the aging process, such as sexual maturation, child bearing, and changes in physical activity.

Another etiological explanation for the heterogeneity of intra-genotypic variances and correlations among generation, gender, and genotype strata is possible. There may be combinations of genotypes in the genes that interact with *APOE* that occur in the parents that were not reproduced in the children, particularly if the samples being studied represent a recently admixed population.

Third, the context dependent penetrance function for each *APOE* genotype foretells complex biological relationships between genomic variation and variation in measures of lipid metabolism. The complexity of biological relationships between genotype and phenotype have been recognized for nearly 100 years (Garrod, 1908; Fisher, 1918; Wright, 1923). Most of the molecular and statistical studies in biomedicine over the past four decades have either not asked about the nature of these relationships, or have made oversimplified assumptions about them that severely limit biologically meaningful inferences. It has been established from statistical studies of the sort reported here that the relationships between genome variations and phenotypic variations are not one-to-one. The digital genotype information at the genome level influences both the quality and quantity of the agents at the intermediate biochemical level which interact with the internal physiological, and external environmental, agents to produce emergent qualitative properties that are probability functions of the genetic, biochemical, and environmental information in the hierarchy. It is imperative to conclude that neither genes nor environments are the causes of phenotypic variation (Lewontin, 1992).

Realistic models for the statistical relationships between genome and phenome that take these complexities into account have yet to be devised. Such models will include a mix of invariant phenotypic properties that do not depend on context (e.g. correlation between Apo B and Total-C), context dependent phenotypic effects that depend on genotype (e.g. higher lnTrig in the ϵ43) and genotype effects that depend on context (e.g. higher correlation between Apo AI and HDL-C in the ϵ33 than ϵ32 in children but not parents). Such a model must also take into account the increasing importance of context on the influence of phenotypic variation as the biological distance between the genome and each trait in the hierarchical organization of a complex system like lipid metabolism increases.

Fourth, the implications of genotype, gender, and age dependency of the distribution of quantitative risk factors are central to the utilization of genomic information for understanding the biology of health, selecting therapies for disease, and predicting those subgroups at risk. There is a tendency among geneticists and non-geneticists alike to make very general statements about the influence of particular genetic variants based on few studies with little regard to the contexts associated with the referenced studies. This leads to the mistaken understanding that a genotype has an intrinsic invariant biological effect. The work reported here and elsewhere suggests that for complex traits, such as quantitative CHD risk factors, the effect of a genotype is dependent on the context in which the genotype is embedded. Only after genotypic influences are studied over a range of contexts (the norm of reaction for each genotype) can we develop conceptual models to describe the biology of health. These models can subsequently be applied to predict those population subgroups that are at increased risk of disease, as well as aid in the selection of more efficacious disease treatment strategies.

In summary, our research demonstrates that variation in the *APOE* gene influences the distributions of each of nine measures of plasma lipoprotein metabolism, as well as the bivariate relationships between them. Moreover, our study establishes that the influence of variation in the *APOE* gene is characterized by a combination of a few invariant effects that are consistent regardless of gender and generation context (variation in mean levels of lnApo E), and many context dependent properties, wherein the influence of variation in the *APOE* gene *must* be considered separately for each gender and generation context. Our work to estimate the norm of reaction associated with each of the *APOE* genotypes suggests the complexity that we can expect in the relationships between genome variations and variations in measures of health in the population at large.

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For Appendix tables A 1 and A 2 see overleaf.

appendix

Table A 1. *Means and variances in female children and parents after adjustment of data to a N(0,1) scale*

	Children			Parents		
Adjusted values	$\epsilon 32$	$\epsilon 33$	$\epsilon 43$	$\epsilon 32$	$\epsilon 33$	$\epsilon 43$
Means						
lnApo E	0.63	0.04	−0.60	0.55	0.21	−0.52
Apo CII	0.08	−0.42	−0.46	0.28	−0.15	−0.29
Apo CIII	0.27	−0.37	−0.30	0.98	−0.05	−0.06
lnTrig	0.07	−0.47	−0.23	0.21	−0.10	−0.10
Apo AI	−0.14	−0.21	−0.19	0.52	0.44	0.51
Apo AII	−0.07	−0.22	0.01	0.02	0.13	0.25
HDL-C	0.27	0.17	0.00	0.33	0.38	0.40
Apo B	−0.41	−0.43	−0.29	−0.02	0.13	0.31
Total-C	−0.66	−0.57	−0.46	0.02	0.27	0.41
Euclidean distance	1.09	1.08	1.02	1.33	0.72	1.05
Variances						
lnApo E	1.38	0.87	0.60	0.95	0.72	0.75
Apo CII	0.45	0.48	0.61	1.50	0.84	1.10
Apo CIII	0.62	0.61	0.59	3.02	0.87	0.73
lnTrig	0.50	0.58	0.62	1.60	0.84	1.03
Apo AI	0.54	0.91	1.24	1.07	0.98	1.19
Apo AII	0.87	0.89	0.98	1.05	0.60	1.16
HDL-C	0.95	0.84	0.65	1.69	1.15	1.04
Apo B	0.68	0.67	0.49	0.72	0.81	1.31
Total-C	0.26	0.49	0.47	1.02	0.63	1.00
Total variance	6.26	6.32	6.25	12.62	7.44	9.29

Table A 2. *Means and variances in male children and parents after adjustment of data to a N(0,1) scale*

	Children			Parents		
Adjusted values	$\epsilon 32$	$\epsilon 33$	$\epsilon 43$	$\epsilon 32$	$\epsilon 33$	$\epsilon 43$
Means						
lnApo E	0.46	−0.04	−0.79	0.97	0.85	0.87
Apo CII	0.02	−0.12	−0.03	1.22	0.92	1.05
Apo CIII	−0.21	−0.26	−0.17	1.74	0.93	0.85
lnTrig	−0.09	−0.35	−0.23	1.27	0.92	1.02
Apo AI	−0.50	−0.24	−0.23	1.03	0.99	1.09
Apo AII	−0.48	−0.25	−0.01	1.03	0.78	1.08
HDL-C	−0.10	0.06	−0.05	1.30	1.07	1.02
Apo B	−0.65	−0.51	−0.28	0.85	0.81	1.14
Total-C	−0.90	−0.53	−0.39	1.01	0.80	1.00
Euclidean distance	1.41	0.93	1.00	2.23	1.25	1.58
Variances						
lnApo E	0.80	0.90	0.87	1.47	0.85	0.87
Apo CII	1.28	0.64	0.52	1.37	1.25	1.01
Apo CIII	0.62	0.66	0.50	1.31	1.21	1.17
lnTrig	1.08	0.58	0.72	1.07	1.02	1.00
Apo AI	0.77	0.89	0.71	0.67	0.87	0.84
Apo AII	1.20	1.09	1.24	0.95	1.06	0.97
HDL-C	0.61	1.08	0.60	0.42	0.72	0.69
Apo B	0.50	0.48	0.56	0.82	1.08	1.40
Total-C	0.44	0.61	0.58	1.38	0.80	1.41
Total variance	7.29	6.93	6.30	9.46	8.85	9.35