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Combined Association and Linkage Analysis Applied to the *APOE* Locus

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Combined association and linkage analysis is a powerful tool for pinpointing functional quantitative traits (QTLs) responsible for regions of significant linkage identified in genome-wide scans. We applied this technique to apoE plasma levels and the *APOEε2/ε3/ε4* polymorphism in two Dutch twin cohorts of different age ranges. Across chromosome 19, short tandem repeats and the *APOEε2/ε3/ε4* polymorphism were genotyped in adolescent (aged 13–22 years) and adult (aged 34–62 years) Dutch twins. In both samples, evidence for indicative linkage with plasma apoE levels was found (maximum LOD score (MLS)=0.8, MLS=2.5, respectively) at 19q13.32. These linkage regions included the *APOE* locus. As expected, the *APOEε2/ε3/ε4* polymorphism was strongly associated with apoE plasma levels in both samples. An extension of the between/within families association test developed by Fulker et al. ([1999] Am. J. Hum. Genet. 64:259–267) showed that these associations were not due to population stratification. The combined association and linkage analyses revealed that the association of the *APOEε2/ε3/ε4* polymorphism with apoE plasma levels completely explained the linkage in the adolescent twins and partly in the adult twins. © 2004 Wiley-Liss, Inc.

Key words: quantitative trait locus; fine-mapping; twin pairs; apoE level

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INTRODUCTION

Successful genome scans for quantitative traits in sibling pairs at best yield broad chromosomal regions, in which the loci influencing the quantitative traits (QTLs) need to be identified. These regions can hardly be narrowed by typing additional markers, since sib pairs regularly share large haplotypes of tens of centiMorgans [Atwood and Heard-Costa, 2003]. Testing polymorphisms for association with the quantitative trait is therefore essential for gene identification in positive linkage regions. Combined association and linkage analysis can give insights into which variant in a set of polymorphisms, located in a linkage region, explains the linkage result by association with the trait. By comparing allelic effects both within and between sibling pairs, this method also provides a direct test for the presence of population stratification [Fulker et al., 1999; Abecasis et al., 2000a].

We applied the combined association and linkage approach to apoE plasma levels and the *APOEε2/ε3/ε4* polymorphism in adolescent (aged 13–22 years) and adult (aged 34–62 years) Dutch twin pairs. Investigation of cohorts of different ages allows accounting for the finding that different genetic variations may influence lipid metabolism at different ages [Snieder et al., 1997]. The *APOEε2/ε3/ε4* polymorphism was previously found to explain between 9–20% of the total variance in apoE levels in the population at large

[Sing and Davignon, 1985; Boerwinkle and Utermann, 1988; Neale et al., 2000; Stengard et al., 2002]. However, more than 80% of the total variance in apoE levels is determined by genetic factors [Duggirala et al., 2000; Beekman et al., 2002], indicating that also other genetic variation must be involved. We investigated to what extent apoE plasma levels are influenced by the *APOEε2/ε3/ε4* polymorphism and whether other genetic variation influencing apoE levels is to be found at the *APOE* locus or elsewhere in the genome.

SUBJECTS AND METHODS

SUBJECTS

Subjects were part of an adolescent and adult Dutch twin cohort whose characteristics were described in detail previously [Beekman et al., 2002]. In this study, we used data from 90 dizygotic (DZ) and 70 monozygotic (MZ) adolescent Dutch twin pairs (aged 13–22 years), and 117 DZ and 96 MZ adult Dutch twin pairs (aged 34–62 years). The adolescent and adult twins were collected as separate samples; within the former twin sample, data were collected between 1988–1992, and within the latter twin sample between 1992–1996. Informed consent was obtained from all participants. Zygosity was confirmed with microsatellite data, using Graphical Representation of Relationship software [Abecasis et al., 2001]. Apolipoprotein E levels were assessed in plasma by enzyme-linked immunosorbent assays (ELISA) [Bury et al., 1986] in adolescent twins in 1994 and in adult twins in 1998. The Netherlands Heart Foundation and the National Institutes of Health approved this study.

GENOTYPING

In the DZ twins, 16 STRs with an average intermarker distance of 6.3 cM on chromosome 19 were genotyped (D19S247, D19S1034, D19S391, D19S865, D19S394, D19S714, D19S49, D19S433, D19S47, D19S420, D19S178, APOC2, D19S246, D19S180, D19S210, and D19S254). The average heterozygosity for these markers was estimated at 0.78. Information on the genetic map was obtained from the Marshfield linkage maps.

The Cy5-labeled PCR products were electrophoretically separated on an automated-fluorescence DNA sequencer, ALF*express* (Amersham Pharmacia Biotech). Analysis and assignment of marker alleles were performed with Fragment Analyser 1.02 (Amersham Pharmacia Biotech). For monitoring possible genotyping errors, one known genotype was present on each gel, 5% of the genotypings were repeated, and two independent observers performed the allele calling. SIBMED [Douglas et al., 2000] was used to check for unlikely double recombinants indicative of genotyping errors. After running SIBMED and checking possible genotyping errors in the raw data, approximately 0.2% of the total genotypings appeared to be erroneous. These genotypes were corrected or set to missing values.

The *APOEε2/ε3/ε4* genotypes were determined on genomic DNA in both MZ and DZ twins and in parents of adolescent twins, as described previously [O'Dell et al., 1995]. Digestion products were separated on 5% agarose gels. For monitoring possible genotyping errors, two observers independently assessed the *APOEε2/ε3/ε4* genotypes. In addition, a randomly chosen 10% of the samples was reamplified and genotyped. In all cases, the previous genotype was confirmed. Previously, in the adolescent twin sample including their parents, *APOEε2/ε3/ε4* isoforms (i.e., *APOE* phenotypes) were determined by the use of iso-electric focusing instead of DNA analysis [Neale et al., 2000]. In 4.7% of the cases, the original *APOE* phenotypes did not correspond to the genotype, which is in agreement with previous reports [Kim et al., 1997; Kardaun et al., 2000].

STATISTICAL ANALYSIS

As the plasma levels of apoE had a skewed distribution, all values were transformed by natural logarithm prior to analysis. Since the data on these twin samples are separately collected during two different time spans and since in adolescence and adulthood lipid and apolipoprotein levels may be influenced by different genes [Snieder et al., 1997], the two cohorts were analyzed separately. Allele frequencies of STRs and the *APOEε2/ε3/ε4* polymorphism were estimated per twin sample using marker data for DZ twins, ignoring their relationships [Broman, 2001]. The full distribution of multipoint identity-by-descent (IBD) sharing probabilities was estimated in DZ twins every centiMorgan, using Genehunter 2.1 [Kruglyak et al., 1996]. All analyses were performed with the use of a variance components approach implemented in the software package Mx 1.50d [Neale et al., 1999].

Linkage analysis. The linkage model for the observed apoE levels is represented as:

$$y_{ij}=\mu + (\beta_1 \times age_{ij}) + (\beta_2 \times sex_{ij}) + e_{ij}$$

where y_{ij} is the observed apoE level for sib *j* in the *i*-th family, μ denotes the grand mean, β_1 denotes the regression coefficient for age, β_2 denotes the deviation of females, age_{ij} and sex_{ij} denote the age and sex (male=0; female=1), respectively, of sib *j* from the *i*-th family, and e_{ij} denotes a residual term that is not explained by the age and sex effects. The variance of e_{ij} is decomposed into additive genetic variance (A), nonshared environmental variance (E), and additive genetic variance due to a QTL in the vicinity of the marker (Q). No variation due to shared environmental influences was included, as we previously determined the absence of this influence on variation in apoE level [Beekman et al., 2002]. A weighted likelihood approach, which uses the full distribution of IBD probabilities [Neale et al., 2000], was employed to estimate variation due to the QTL.

Association analysis. The association model for observed apoE levels as a function of the genotyped *APOEε2/ε3/ε4* polymorphism is represented as:

$$y_{ij}=\mu+(\beta_1\times age_{ij})+(\beta_2\times sex_{ij})+(a_b\times A_{bi})+(d_b\times D_{bi})+(a_w\times A_{wij})+(d_w\times D_{wij})+e_{ij}$$

where y_{ij} is the observed score for sib *j* in the *i*-th family, μ denotes the grand mean, β_1 denotes the regression coefficient for age, β_2 denotes the deviation of females, age_{ij} and sex_{ij} denote the observed age and sex, respectively, of sib *j* in the *i*-th family. A_{bi} is the derived coefficient for the additive genetic effect of the *APOEε2/ε3/ε4* polymorphism between families for the *i*-th family, A_{wij} denotes the coefficient as derived for the additive genetic effects of the APOEε2/ε3/ε4 polymorphism within families for sib *j* from the *i*-th family, D_{bi} is the coefficient for the dominant genetic effect of the *APOEε2/ε3/ε4* polymorphism between families for the *i*-th family, and D_{wij} denotes the coefficient for the dominant genetic effects of the *APOEε2/ε3/ε4* polymorphism within families for sib *j* from the *i*-th family. a_b and a_w are the estimated additive effects between and within families, d_b and d_w are the estimated dominance effects between and within families, and e_{ij} denotes a residual that is not explained by the age, sex, and allelic effects of the *APOEε2/ε3/ε4* polymorphism. The variance of e_{ij} is decomposed into A and E components.

This association model for the observed apoE levels includes effects of *APOEε2/ε3/ε4* genotype on the observations. Allelic effects were calculated both between and within sib pairs, using the genotypic mean of a sib pair and the deviation of a sib from the genotypic mean of a sib pair, respectively. The derivation of genotypic means of sib pairs and the differences of sibs from the genotypic means, for diallelic loci, can be found in Fulker et al. [1999]. The extensions to dominance effects, multiallele loci, and the use of parental genotypes if available, were described by Posthuma et al. [2004]. For the adolescent twins, parental *APOEε2/ε3/ε4* genotypes were available and were used to derive between- and within-families coefficients A_{bi}, A_{wij}, D_{bi}, and D_{wij}. For the adult twins, parental *APOEε2/ε3/ε4* genotypes were not available, and the sibling genotypes were used to derive between- and within-families coefficients. Although MZ pairs are uninformative for linkage and for genotypic effects on observed scores within sib pairs, the inclusion of MZ twins allows proper estimation of the additive genetic variance component and provides information for the genotypic effects on the observed scores between sib pairs.

Statistically testing the equivalence of the between- and within-families effects ($a_b=a_w$ and $d_b=d_w$) provides a test of the presence of population stratification, since in the absence of population stratification, genotypic effects operating within families are equal to the genotypic effects between families. The former represent the true genetic effects, whereas the latter contain both the true and the spurious genetic effects [Fulker et al., 1999].

The presence of nonadditive allelic effects of *APOEε2/ε3/ε4* genotype on apoE levels was evaluated by constraining the dominance coefficients to equal zero ($d_b=d_w=0$). The evidence for an effect of the *APOEε2/ε3/ε4* polymorphism on apoE level is examined by constraining all association parameters to equal zero ($a_b=a_w=d_b=d_w=0$).

Combined association and linkage analysis. The association model that provided the most parsimonious fit to the data, as determined by likelihood ratio test, was taken as a starting point for the combined association and linkage analyses. In the combined model, the variance in apoE levels that was not accounted for by age, sex, and allelic effects of the *APOEε2/ε3/ε4* polymorphism (e_{ij}) was decomposed into A, E, and Q components. It was thus tested for each position across chromosome 19 whether linkage with apoE levels was still present when modelled simultaneously with association of the *APOEε2/ε3/ε4* polymorphism.

RESULTS

Table I gives the descriptive statistics of MZ and DZ twins, and their phenotypic resemblance

TABLE I. Descriptive statistics of the adolescent and adult Dutch monozygotic and dizygotic twin-pair samples of which both members provide data on apoE level and *APOE* genotype

	Adolescent twins		Adult twins	
	MZ	DZ	MZ	DZ
Number of pairs	65	83	88	114
Percentage males	46.2	49.4	47.7	48.2
Age, years—mean (range)	16 (13–22)	17 (13–22)	44 (34–62)	44 (34–59)
ApoE, mg/dL—mean (SD)	7.12 (2.42)	6.59 (2.39)	2.53 (1.03)	2.54 (1.02)
Ln(apoE)—mean (SD)	1.91 (0.34)	1.82 (0.36)	0.86 (0.39)	0.86 (0.37)
Correlation	0.88	0.37	0.87	0.41

TABLE II. Fit statistics of nested models in adolescent and adult Dutch twins

IIA Model	df	−2ll	vs.	Δdf	χ^2	*P*
Adolescent twins						
No linkage, no association						
1: AE+μ+β_1+β_2	**289**	**78.14**				
2: AE+μ+β_1	290	103.28	2 vs. 1	1	25.14	0.00
3: AE+μ+β_2	290	84.84	3 vs. 1	1	6.71	0.01
4: AE+μ	291	109.21	4 vs. 2	1	24.37	0.00
Linkage at maximum χ^2 difference						
5: AEQ+μ+β_1+β_2	**288**	**74.65**	**1 vs. 5**	**1**	**3.49**	**0.06**
Association						
6: AE+μ+β_1+β_2+a_b+a_w+d_b+d_w	279	−44.58	1 vs. 6	10	122.72	0.00
7: AE+μ+β_1+β_{2+}(a_b=a_w)+ (d_b=d_w)	**284**	**−35.46**	**7 vs. 6**	**5**	**9.12**	**0.10**
			1 vs. 7	**5**	**113.60**	**0.00**
8: AE+μ+β_1+β_{2+}(a_b =a_w)+ (d_b=d_w =0)	287	−21.42	8 vs. 7	3	14.05	0.00
Linkage at maximum χ^2+Association						
9: AEQ+μ+β_1+β_2+(a_b=a_w)+ (d_b=d_w)	**283**	**−36.75**	**7 vs. 9**	**1**	**1.29**	**0.26**
IIB						
Adult twins						
No linkage, no association						
1: AE+μ+β_1+β_2	399	191.76				
2: AE+μ+β_1	**400**	**191.97**	**2 vs. 1**	**1**	**0.21**	**0.65**
3: AE+μ+β_2	400	203.31	3 vs. 1	1	11.55	0.00
4: AE+μ	401	203.39	4 vs. 2	1	11.42	0.00
Linkage at maximum χ^2 difference						
5: AEQ+μ+β_1	399	180.26	2 vs. 5	1	11.71	0.00
Association						
6: AE+μ+β_1+a_b+a_w+d_b+d_w	390	71.13	2 vs. 6	10	120.84	0.00
7: AE+μ+β_1+(a_b=a_w)+(d_b=d_w)	395	73.76	7 vs. 6	5	2.63	0.76
8: AE+μ+β_1+(a_b=a_w)+(d_b=d_w=0)	**398**	**78.46**	**8 vs. 7**	**3**	**4.70**	**0.20**
			2 vs. 8	2	113.51	0.00
Linkage at maximum χ^2+association						
9: AEQ+μ+β_1+(a_b=a_w)+(d_b=d_w=0)	**397**	**70.70**	**8 vs. 9**	**1**	**7.76**	**0.01**

The most parsimonious models are shown in bold. In models, A represents additive genetic variance; E, non shared environmental variance; Q, additive genetic variance due to a QTL in vicinity of a marker; μ grand mean; β_1, regression weight of age; β_2, female deviation; df, degrees of freedom; −2ll, −2 times log likelihood fit statistic; vs., versus, representing model to which comparison is made; Δdf, difference in degrees of freedom between two tested models; χ^2, difference in −2ll between two tested models, which follows a χ^2 distribution; *P*, *P*-value.

(correlation). The high MZ correlations and the large differences between MZ and DZ correlations indicate substantial heritability for apoE levels. To find the most parsimonious model to describe the data on apoE plasma levels in adolescent and adult twins (Table I), a model is fitted to the data, in which the grand mean with regression deviations for age and sex is included, and in which the residual variance is decomposed into additive genetic variance (A) and unique environmental variance (E). The results of the model-fitting procedure are shown in Table II. Per analysis, the most extended model was simplified by leaving one factor from the model; when the simplification of the model has a significant effect ($P<0.05$), the factor has a significant effect on apoE levels and should not be excluded from the model. The models of the linkage and association analyses were compared to the model of the "no linkage, no association" analysis to test whether the QTL or the association had significant effect on the observed apoE levels. In the combined association and linkage analysis, we tested whether the QTL had a significant effect on apoE levels in the presence of association.

In adolescent twins, sex (Table IIA, model 2) and age (Table IIA, model 3) both have a significant effect on apoE levels. ApoE levels significantly decrease with older age ($\beta_1=-0.03$) and are significantly higher in women ($\beta_2=0.22$). Model 1 thus describes the apoE levels observed in adolescent twins most parsimoniously, and shows that 87% of the total variance in apoE levels is determined by additive genetic factors (Table IIIA, model 1). In adult twins, sex has no significant effect on apoE levels (Table IIB, model 2), and age has a significant effect on apoE levels (Table IIB, model 3). Adult apoE levels increase significantly with older age ($\beta_1=0.01$). Model 2 describes the apoE levels observed in adults most parsimoniously, and shows that 85% of the total variance in adult apoE levels is determined by additive genetic factors (Table IIIB, model 2). These best-fitting models (i.e., model 1 for adolescents and model 2 for adults) were extended for linkage, association, and combined association and linkage analyses.

For linkage analysis, the variance of the residuals is decomposed into A and E, and additionally, in additive genetic variance due to a QTL (Q). The adolescent twins provide an indication for linkage on chromosome 19q13.3, 70 cM from pter, with a maximum LOD score (MLS) of 0.8 (χ^2 of 3.49; $P=0.06$; Table IIA, model 5; Fig. 1, "Linkage

TABLE III. Variance components estimates in most parsimonious models for situations of no linkage and no association, linkage at 70 cM from pter, association with APOEε2/ε3/ε4 polymorphism, and simultaneous linkage at 70 cM from pter and association in adolescent and adult Dutch twins

	Model	Variance (corrected for age and/or sex)	% of variance due to association	% of variance due to A	% of variance due to E	% of variance due to Q
IIIA						
Adolescent twins						
No linkage, no association	1	0.1106		87	13	
Linkage at maximum LOD score	5	0.1106		51	13	36
Association	7	0.1106	39	49	13	
Association+linkage at peak linkage only (70 cM from pter)	9	0.1106	39	49	13	0
Association+linkage at maximum LOD score (43 cM from pter)	9	0.1106	4	64	13	19
IIIB						
Adult twins						
No linkage, no association	2	0.1319		85	15	
Linkage at maximum LOD score	5	0.1319		0	15	85
Association	8	0.1319	30	56	15	
Association+linkage at peak linkage only (63 cM from pter)	9	0.1319	32	0	15	53
Association+linkage at maximum LOD score (60 cM from pter)	9	0.1319	30	0	15	55

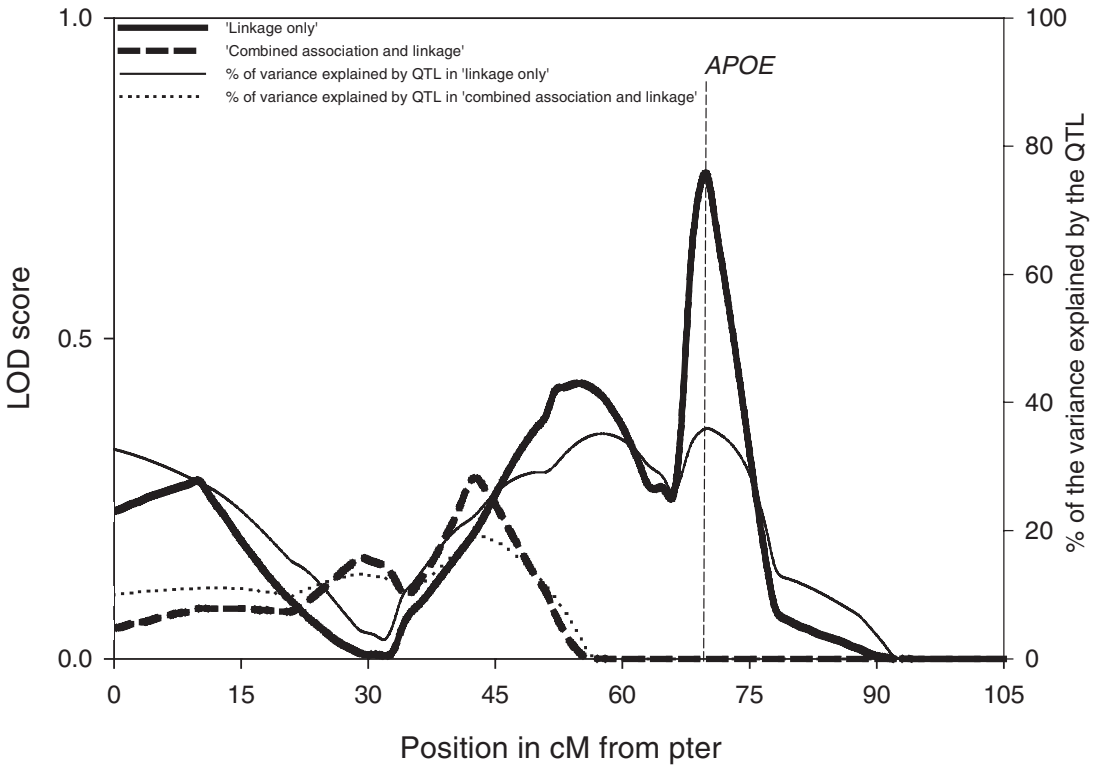

Fig. 1. "Linkage only" analysis (solid lines) and "Combined association and linkage" analysis (dashed lines) of chromosome 19 with apoE levels in adolescent Dutch twins. Thick lines correspond with left axis, indicating LOD score. Thin lines correspond with right axis, indicating percentage of variance (corrected for age and sex) accounted for by QTL.

only''). This putative QTL is estimated to explain 36% of the variance in adolescent apoE levels (Table IIIA, model 5). The adult twins provide evidence in favor of linkage at 63 cM from pter, with an MLS of 2.5 (χ^2 of 11.71; $P < 0.000$; Table IIB, model 5; Fig. 2, ''Linkage only''). Both linkage peaks in adolescent and adult twins largely overlap the location of the *APOE* gene (19q13.32). This putative apoE QTL is estimated to explain 85% of the total variance in adult apoE levels (Table IIIB, model 5).

For association analyses, models 1 and 2 for the adolescent and adult twins, respectively, were extended with additive and nonadditive allelic effects of the *APOEε2/ε3/ε4* polymorphism between and within families. These extensions to model 1 in the adolescent twins resulted in a significantly better fit to the observed apoE levels (Table IIA, model 6). To test whether this association was confounded by population stratification, the allelic effects of the *APOEε2/ε3/ε4* polymorphism between families were equated to the effects within families. This simplified association model describes the observed apoE levels in adolescents not significantly worse than model 6 (Table IIA, model 7), indicating that the association of the *APOEε2/ε3/ε4* polymorphism with adolescent apoE levels is not confounded by population stratification. Furthermore, dominant effects of the *APOEε2/ε3/ε4* polymorphism on adolescent apoE levels could not be equated to zero (Table IIA, model 8), indicating that dominance plays a role in adolescent apoE levels. Carriers of the *ε3/ε4* and *ε4/ε4* genotype had lower levels of apoE compared to the common *ε3/ε3* genotype (Table IVa). Carriers of the *ε2* allele have higher levels as compared to the common *ε3/ε3* genotype, but the effect size of the *APOEε2* allele appears to depend on the accompanying allele. When an *ε2* allele accompanies the *ε2* allele, apoE levels are intermediate high as compared with an accompanying *ε3* or *ε4* allele. In adolescent twins, the *APOEε2/ε3/ε4* polymorphism is strongly associated with apoE levels (Table IIA, model 1 vs. model 7), which explains 39% of the total apoE variance (Table IIIA).

The extensions to model 2 in adult twins also resulted in a significantly better fit to the observed apoE data (Table IIB, model 6). This association of the *APOEε2/ε3/ε4* polymorphism with apoE levels in adults is not confounded with population

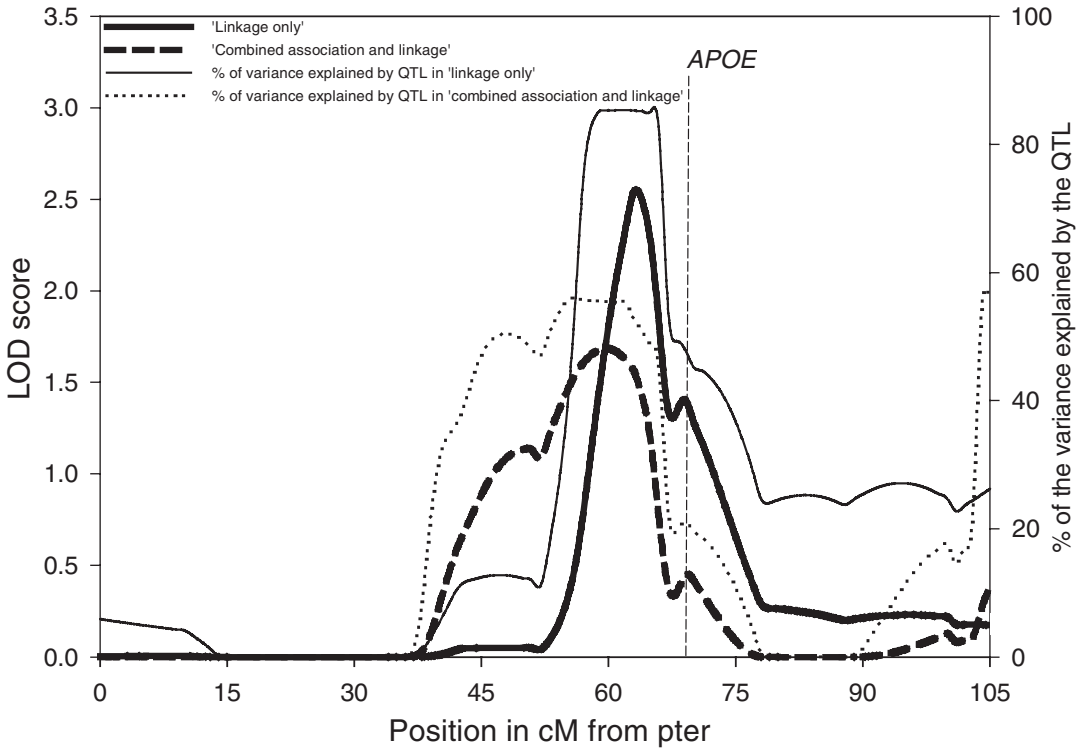

Fig. 2. "Linkage only" analysis (solid lines) and "Combined association and linkage" analysis (dashed lines) of chromosome 19 with apoE levels in adult Dutch twins. Thick lines correspond with left axis, indicating LOD score. Thin lines correspond with right axis, indicating percentage of variance (corrected for age) accounted for by QTL.

TABLE IVA. Observed and expected means of apoE levels according to *APOEε2/ε3/ε4* genotype in adolescent Dutch twins

APOEε2/ε3/ε4 genotype	Observed mean (mg/dl)	Expected mean (mg/dl)
ε2ε2 (n=1)	7.20	7.17
ε2ε3 (n=58)	9.16	9.87
ε2ε4 (n=4)	9.24	10.59
ε3ε3 (n=152)	6.27	6.62
ε3ε4 (n=75)	5.15	5.47
ε4ε4 (n=6)	4.50	4.90

TABLE IVB. Observed and expected mean of apoE levels according to *APOEε2/ε3/ε4* genotype in adult Dutch twins

APOEε2/ε3/ε4 genotype	Observed mean (mg/dL)	Expected mean (mg/dL)
ε2ε2 (n=3)	5.40	5.37
ε2ε3 (n=55)	3.48	3.49
ε2ε4 (n=12)	3.43	3.06
ε3ε3 (n=218)	2.30	2.27
ε3ε4 (n=109)	1.99	1.99
ε4ε4 (n=7)	1.51	1.75

stratification (Table IIA, model 7). Furthermore, there is no evidence that dominant effects of the *APOEϵ2/ϵ3/ϵ4* polymorphism play a significant role in adult apoE levels (Table IIB, model 8). The alleles thus showed an additive effect on apoE levels, with a decreasing pattern from *ϵ2* to *ϵ3* to *ϵ4*. Carriers of the *ϵ2* allele had higher apoE plasma levels, and carriers of the *ϵ3/ϵ4* and *ϵ4/ϵ4* genotype had lower levels of apoE compared to the common *ϵ3/ϵ3* genotype (Table IVb). The *APOEϵ2/ϵ3/ϵ4* polymorphism is also strongly associated with apoE levels in adult twins (Table IIB, model 2 vs. 8), which explains 30% of the total apoE variance (Table IIIB).

When the allelic association effects are simultaneously modelled with linkage, models 7 for adolescents and 8 for adults are extended with the decomposition of the residual variance into A, E, and additionally Q. In this combined association and linkage analysis, all linkage at the peak location of the "linkage only" analysis in the adolescent twins (70 cM from pter) is explained by the association of the *APOEϵ2/ϵ3/ϵ4* polymorphism with apoE levels, since evidence for linkage completely disappears (Table IIa, model 9; Fig. 1, "Combined association and linkage"). Table IIIA also shows that at 70 cM from pter, no variance is left due to a QTL and that 39% of the apoE

variance can be explained by its association with the *APOEε2/ε3/ε4* polymorphism in adolescent twins.

In adult twins, this combined association and linkage analysis shows that part of the apoE variance linked to 19q13.3 can be explained by its association with the *APOEε2/ε3/ε4* polymorphism (Table IIB, model 9; Fig. 2, ''Combined association and linkage''). Table IIIB shows that at 63 cM from pter, 53% of the variance is still linked to 19q13.3, and that 32% of the apoE variance can be explained by its association with the *APOEε2/ε3/ε4* polymorphism in adult twins. All residual genetic variance in adult apoE levels is still linked to chromosome 19q13.3 (Table III), indicating that this chromosomal region harbors additional genetic variation influencing apoE plasma levels in adults.

DISCUSSION

A crucial step, following a positive linkage result from a genome scan for quantitative traits, is to identify which gene variants in the chromosomal region explain the linkage. Fulker et al. [1999] developed a method for simultaneous modelling of association and linkage for quantitative traits, using sib pair data that controlled for population stratification. Until now, most studies performed linkage analyses to find QTLs, but some studies also carried out combined association and linkage analyses [Klos et al., 2001; Soria et al., 2002]. However, these studies did not control for population stratification. Recently, the combined association and linkage analysis that includes a test for population stratification [Fulker et al., 1999] was extended to incorporate effects of multiallelic loci, parental genotypes, and genetic dominance by Posthuma et al. [2004; see also Abecasis et al., 2000b]. We applied the extended approach for quantitative traits in sib pairs to the *APOE* locus and its effect on apoE plasma levels.

We found evidence for linkage of chromosome 19q13.3 with apoE levels in both adolescent and adult Dutch twins, although it did not reach genome-wide significance according to the criteria of Lander and Kruglyak [1995]. The peak LOD score in adolescents was exactly on the location where the *APOE* gene is mapped. The peak LOD score in adults is located 7 cM pter from the *APOE* gene, but the linkage region included the *APOE* gene.

Both adolescent and adult apoE levels were strongly associated with the *APOEε2/ε3/ε4* polymorphism, and these associations were not confounded by population stratification. Using the combined association and linkage approach, we showed that linkage of 19q13.32 with apoE plasma levels is completely explained by the *APOEε2/ε3/ε4* polymorphism in adolescents, but only partly in adults. This finding at a young age indicates that the *APOEε2/ε3/ε4* polymorphism is the only relevant genetic variant in *APOE*, not taking into account variants in complete LD. Later in life, however, other genetic variations may become relevant, such as other polymorphisms in the *APOE* gene, which were shown to influence the variance in apoE levels in addition to the *APOEε2/ε3/ε4* polymorphism in adults [Stengard et al., 2002]. Of special interest are *APOE* promotor polymorphisms, −491A/T and −219G/T, that may influence transcription of the *APOE* gene [Artiga et al., 1998]. Previously, the −219G/T polymorphism was associated with apoE plasma levels [Lambert et al., 2000; Stengard et al., 2002]. This SNP is in partial LD with the *APOEε2/ε3/ε4* polymorphism [Fullerton et al.; 2000; Heijmans et al., 2002], and thus may have contributed to the association we observed.

In adolescent twins, not all variance linked to chromosome 19 can be explained by the *APOEε2/ε3/ε4* polymorphism, since at 43 cM from pter, a QTL explaining 19% of the total variance in apoE levels remains, although this is not a significant QTL. Possibly, on chromosome 19p13, additional genetic variants influence adolescent apoE levels, but for a complete explanation of the genetic variance in adolescent apoE levels, additional genetic variation should be found on other chromosomes. Since in adult twins, part of the variance linked to chromosome 19 can be explained by the *APOEε2/ε3/ε4* polymorphism and the remaining putative apoE QTL at 60 cM from pter explains 55% of the total variance, no residual heritability remains to be explained outside chromosome 19. The genetic architecture of adolescent apoE levels thus seems to differ from that of adult levels. This is consistent with previous studies, describing that lipid and apolipoprotein levels may be influenced by different genes in adolescence and adulthood [Snieder et al., 1997], and that a quantitative trait locus on chromosome 19 seems to play only a role in adult and not in adolescent LDL cholesterol levels [Beekman et al., 2003]. Such differential influence of genes at different ages might also be the case for apoE levels.

We found that age has a decreasing effect on the plasma levels of apoE in adolescent twins, while it has an increasing effect in our adult twin cohort. This differential effect of age may reflect the different interactions of genes influencing apoE levels with the environment at different ages (gene-environment interaction), as assumed by Zerba et al. [1996, 2000]. This finding may also refer to the influence of different genes, including *APOE*, at different ages on apoE levels and their interactions (*APOE* gene-gene interaction), reinforcing our observation that genes have differential influence on measures of lipid metabolism at different ages.

Much is known about the association of the *APOEϵ2/ϵ3/ϵ4* polymorphism in adult apoE levels. Adolescent apoE levels, however, are rarely investigated. Neale et al. [2000] also studied this adolescent Dutch twin cohort, and found that the *APOEϵ2/ϵ3/ϵ4* polymorphism explained 16% of the total variance in apoE levels, without evidence for nonadditive allelic effects. In their analyses, Neale et al. [2000] used *APOEϵ2/ϵ3/ϵ4* phenotypes, which led to a genotype discrepancy, which may have influenced the proportion of variance due to the *APOEϵ2/ϵ3/ϵ4* polymorphism. Furthermore, Neale et al. [2000] included parental apoE levels and equated the association effects in the parental and offspring generation, which diluted the nonadditive allelic effect in the adolescent twins to nonsignificance. The absence of such effects in the parental generation converges with our results from the adult Dutch twin sample.

To our knowledge, this is the first time that a nonadditive allelic effect of the *APOEϵ2/ϵ3/ϵ4* polymorphism on apoE levels was found in adolescents. Additionally, the finding that the *APOEϵ2/ϵ3/ϵ4* polymorphism is the only variant in the *APOE* gene influencing apoE levels in adolescents (not taking into account variants in complete LD) is different from the findings in adults. This differential architecture across cohorts, and the differential effects of age across cohorts, reveal the dynamic nature of genetic and environmental effects.

We showed that using a combined association and linkage analysis, linkage results can be explained by genetic variation in positional candidate genes. It is possible to find out whether the variant tested is the only genetic variant influencing the quantitative trait, or if additional variants have to be found for a complete explanation of the linkage result.

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ELECTRONIC DATABASE INFORMATION

The Marshfield linkage map is available at http://research.marshfieldclinic.org/genetics/.

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