The effect of apolipoprotein(a)-, apolipoprotein E-, and apolipoprotein A4- polymorphisms on quantitative lipoprotein(a) concentrations

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The effects of apolipoprotein (a), apolipoprotein-E, and apolipoprotein-A4 isoforms on quantitative lipoprotein(a) [Lp(a)] levels were assessed in a sample of 142 Dutch families consisting of two parents and their adolescent twin offspring. A total heritability of 95% was estimated for plasma Lp(a) concentrations. The largest part of this heritability was due to the apo(a) locus which explained 61% of the total variance in Lp(a) levels. The pattern of familial correlations for the residual part of the Lp(a) variance that could not be attributed to the apo(a) isoforms, suggested genetic influences on the residual variance. We addressed the question whether this residual genetic variance could be ascribed to the apoE or the apoA4 locus. A simultaneous analysis of all three loci showed that both the apoE and the apoA4 polymorphism did not contribute significantly to Lp(a) variation. Twin Research (2000) 3, 152–158.

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Introduction

Lipoprotein(a) [Lp(a)] is a low-density lipoprotein particle with an attached apolipoprotein(a) that was first described by Berg in 1963.[1] High plasma levels of Lp(a) are a major risk factor for premature atherosclerosis, cardiovascular heart disease and stroke;[2–7] but see also references[8–11]. Quantitative Lp(a) levels show large differences between individuals that are stable over time and are almost completely determined by genetic factors as shown by twin[12–15] and family studies.[16–20]

Independent studies have demonstrated that most of the genetic variability in Lp(a) levels can be explained by genetic heterogeneity of the apo(a) locus at chromosome 6.[21,22] Codominant alleles at this locus code for a large number of isoforms that differ in size. There is an inverse relationship between the size of these isoforms and quantitative Lp(a) concentrations. The basis for the size polymorphism is the number of kringle 4 encoding repeats in the apo(a) gene.[23] Utermann et al[24] originally identified 6 apo(a) isoforms and a null allele. Boerwinkle et

al[21] estimated that in Caucasians 42% of the variance in Lp(a) levels is accounted for by these different isoforms of apo(a). Gaubatz et al[25] found 11 different isoforms and Lackner et al[26] and Kamboh et al[27] reported methods that detect at least 20 allelic isoforms at the DNA level. Using these methods, Kraft et al[19] found that the KpnI polymorphism explained 46% of the variability in Lp(a) levels in Tyrolean subjects. Boerwinkle et al[28] estimated that the number of kringle 4 repeats in the apo(a) gene accounted for 69% of the Lp(a) variation in Caucasian American families.

The apo(a) size polymorphism is thus a major determinant of Lp(a) levels, but it is still unclear whether other genetic factors determine variance in Lp(a) concentrations that cannot be attributed to the apo(a) locus. Both the apoE and apoA4 polymorphisms have been reported to exhibit a low, but significant, influence on Lp(a) levels. De Knijff et al[29] hypothesised that involvement of the LDL receptor in Lp(a) catabolism would be supported by an effect of the apoE locus on Lp(a) levels and found that the apoE locus accounted for 4% of the phenotypic variance in Lp(a) levels. Eckardstein et al[30,31] unexpectedly observed an effect of the apoA4 locus on Lp(a) levels in healthy students and in male coronary heart patients. However, others,[4,19] have maintained that, given the Lp(a) linkage data, it would be

highly unlikely that other loci than the apo(a) locus influence plasma Lp(a) levels.

A simultaneous analysis of the influence of the genetic heterogeneity at the apo(a), apoE and apoA4 loci on the plasma levels of Lp(a) within the same population sample has not yet been attempted. Here we report the results of such a study. Quantitative plasma levels of Lp(a), and apo(a), apoE, and apoA4 size-polymorphisms were measured in 142 Dutch adolescent mono- and dizogytic twin pairs and both their parents, in order to determine whether, in addition to the apo(a) locus, Lp(a) levels are influenced by the apoE- and apoA4 locus.

Materials and methods

Subjects

Plasma levels of Lp(a) were assessed in a sample of 160 Dutch mono- and dizygotic twin pairs and in both their parents.[13,32] Average age of the twins was 16.7 years (SD = 2.0); average age of their parents was 46.8 years (SD = 6.2). In these same subjects apo(a) polymorphisms were assessed (isoforms S1, S2, S3, S4, S5, B and F), as well as apolipoprotein E phenotypes (apoE isoforms E2, E3 and E4) and apolipoprotein A4 phenotypes (apoA4 isoforms A4-1, A4-2, A4-3 and A4-0). For five families no apoE data were available and for an additional eight families the apoA4 polymorphism was not measured. Five families were omitted from the analyses because one or both parents were non-Caucasian. This left 142 families with complete data. In these families there was an equal number of males and females. Zygosity of the twin offspring was determinated by blood group antigens and DNA fingerprinting. There were 58 monozygotic (MZ) and 84 dizygotic (DZ) twin pairs.

Methods

Fasting blood samples were taken between 8.30 and 10.30 am by venipuncture, using Becton-Dickinson Vacutainers containing sodium-EDTA (Becton Dickinson Vacutainer Systems Europe, Meylan, France). Plasma was immediately separated from the cells and stored in small portions at –20°C until further use. Apolipoprotein E and A4 phenotypes were determined by isoelectric focusing on delipidated plasma samples followed by immunoblotting.[33,34] Lp(a) levels were measured with a 'bi-site sandwich' ELISA.[13] Apo(a) isoforms were separated using the Phast System on a 4–15% gradient (PAGE) Phast Gel (Pharmacia, Uppsala, Sweden) under reducing conditions, for 75 min at 250 V at 15°C. Ten µl of the sample was mixed with 50 µl, 5% SDS (Biorad, Hercules, California, USA), 5 µl β mercapto-ethanol

(Biorad), 5 µl glycerol (80%) (Merck, Darmstadt, Germany) containing 0.01% bromophenolblue and 20 µl glycerol (80%). The sample was boiled for 3 min and loaded on the gel. After electrophoresis the gels were blotted by diffusion for 50 min at 70°C in a humid sealed petri dish on to nitrocellulose membranes (Hybond - C extra, Amersham-Pharmacia Biotech, Rainham, UK) and presoaked for 5 min in blotting buffer (192 mM glycine, 25 mM Tris HCl, 20% methanol at pH 7.4). Residual binding sites were blocked by incubation of the membrane in a buffer containing 3% BSA, 0.5 M NaCl, 200 mM Tris HCl, and 0.05% Tween pH 7.4. After washing in PBS buffer the membranes were incubated for 4 h at room temperature with the anti-Lp(a) antibody at 10 µg/ml in PBS buffer containing 1% BSA. Since the anti-Lp(a) antibodies were generated in rabbits by immunisation with Lp(a) the antisera were purified on an LDL-affinity column to eliminate cross-reacting anti-apo B antibodies. The membrane was washed in PBS buffer, containing 0.1% BSA and incubated for 2 h at 37°C with a peroxidase labelled swine anti-rabbit antibody (Dakopatts, Glostrup, Denmark) at a 1/300 dilution in PBS buffer + 0.1% BSA. The membrane was washed again and equilibrated for 10 mins in blotting buffer containing methanol. Apo(a) isoform bands were revealed by developing for 5–10 minutes with 10 mg 4-chloro–1 naphtol (Merck, Darmstadt, Germany), dissolved in 3.3 ml methanol, 10 µl H_2O_2 (Merck, Darmstadt, Germany) and brought to 17 ml with development buffer (50 mM Tris HCl, 200 mM NaCl pH 7.4). The reaction was stopped by rinsing with water. On each gel an 'in-house' serum mix containing the S1, S2, S4, B and F isoforms (according to the classification by Utermann et al[24]) was run. In some cases an extra band above the S4 was identified was as S5.

Statistical analysis

Fisher's exact test was used to determine if there were significant associations between phenotypes at the apo(a), apoE and apoA4 loci.[35]

To test the effects of the polymorphisms at the three measured loci on Lp(a) levels, a pedigree-based maximum likelihood method developed by Lange et al[36] was used. For a given pedigree of n individuals a vector of observations (x) was defined and a vector of expected values (E(x)), that depended on fixed variables such as age, sex or phenotype. The covariances between family members for the residual part of the observations, ie the part not accounted for by the fixed variables, depend on the relationships between the pedigree members and on the genetic model assumed for the observations. Throughout we modelled the variance not accounted for by the fixed variables as consisting of additive genetic and

random environmental variance. In an earlier analysis of plasma Lp(a) levels, we excluded the possibility that part of the familial variance may reflect environmental influences shared by family members.[13]

For a given E(x) and expected covariance matrix S, the log-likelihood of obtaining the observation vector x is:

$$L = -\tfrac{1}{2}\ln|S| - \tfrac{1}{2}(x - E(x))' S^{-1} (x - E(x)) + \text{constant}$$

where | | denotes the determinant of the matrix and ' denotes transpose.

The joint log-likelihood of obtaining all 142 pedigrees is the sum of the log-likelihood of the separate pedigrees. Estimation involves selection of parameter values under a specific model which maximizes the joint likelihood of all pedigrees. The FISHER package[36] was used for genetic modelling. The effects of the apo(a), the apoE and the apoA4 locus on logarithmically transformed Lp(a) levels were considered simultaneously by estimating 107 parameters for all possible combinations of the phenotypes at the three loci. Next, a mean effect for each phenotype at each of the three loci (25, 6 and 5 different means for apo(a), apoE and apoA4, respectively) was estimated and the effect of each locus summed across loci. The difference in likelihood between these two models provides a test of interaction (epistasis) between the three loci. The significant contribution of each locus to variation in Lp(a) levels was evaluated separately by testing submodels that specified no effects of the apo(a), the apoE or the apoA4 locus, respectively. The likelihoods obtained for different models were compared with chi-squared difference tests where $\chi^2 = 2(L_1 - L_0)$. L_1 and L_0 denote the log-likelihoods of the general (H_1) and a constrained (H_0) hypothesis. The degrees of freedom (df) for this test are equal to the number of constrained parameters between H_1 and H_0.[37]

Results

In the total sample of parents and children we observed 25 different apo(a) phenotypes, with the S4 phenotype being the most frequent (20.6%). The relative frequencies of the apo(a) phenotypes and the corresponding Lp(a) levels for each apo(a) phenotype are given in Table 1. Table 1 also presents the relative frequencies for the apoE and the apoA4 loci, as well as the observed average Lp(a) concentrations for each of these phenotypes. There were no significant associations between apo(a), apoE and apoA4 phenotypes (Fisher's exact test, P = 0.49 for apo(a)-apoA4, P = 0.13 for apo(a)-apoE, and P = 0.68 for apoA4-apoE association).

Log-likelihoods for several models testing the effects of sex and age on Lp(a) levels and for models

Table 1   Average Lp(a) concentrations in mg/dl for apo(a), apoE and apoA4 phenotypes in 568 subjects from 142 families

	By apo(a) Phenotype				By apoE Phenotype				By apoA4 Phenotype		
	Mean	Cases			Mean	Cases			Mean	Cases	
		N	%			N	%			N	%
null	1.03	70	12.3	2/2	5.03	4	0.7	1/1	14.30	486	85.6
S1	24.27	12	2.1	3/2	10.38	97	17.1	1/2	12.17	75	13.2
S2	28.17	58	10.2	3/3	14.53	324	57.0	2/2	1.53	3	0.5
S3	10.67	67	11.8	4/3	16.41	124	22.0	3/1	33.80	3	0.5
S4	8.80	117	20.6	4/4	22.46	7	1.2	1/0	0.70	1	0.2
S5	4.43	64	11.3	4/2	2.67	11	1.9				
B	18.85	2	0.4								
F	50.30	3	0.5								
S1/S2	28.55	2	0.4								
S1/S3	39.60	2	0.4								
S1/S4	31.31	9	1.6								
S1/S5	31.71	8	1.4								
S2/S3	24.16	16	2.8								
S2/S4	25.10	26	4.6								
S2/S5	16.95	19	3.3								
S3/S4	11.32	27	4.8								
S3/S5	9.65	24	4.2								
S4/S5	7.61	24	4.2								
B/S2	104.80	2	0.4								
B/S3	14.90	1	0.2								
B/S4	41.44	5	0.9								
B/S5	28.00	4	0.7								
B/F	46.00	2	0.4								
F/S4	40.25	2	0.4								
F/S5	141.00	2	0.4								

testing different hypotheses about familial resemblance are given in Table 2. There were no sex or generation differences in Lp(a) levels, and no associations with age. The spouse correlation between Lp(a) levels of fathers and mothers was not different from zero. The MZ correlation (r = 0.95) was twice the DZ correlation (r = 0.47), and the parent–offspring correlation could be set equal to the DZ correlation, indicating a heritability of 95% for quantitiatve Lp(a) levels, that was the same in both generations.

In Table 3 likelihood-ratio tests for the effects of the apo(a), apoE and apoA4 polymorphism on Lp(a) concentrations are summarised. Table 3 first gives the log-likelihood for a general model that estimated 107 separate means for all possible combinations of the phenotypes at the apo(a), apoE and apoA4 loci. The next model specified the effects of the three loci to act additively. The relatively large decrease in likelihood for this model is accompanied by a large number of degrees of freedom and is not significant. This test indicates that there is no interaction between the three loci with respect to Lp(a) levels. That is, the effect of the apo(a) locus on Lp(a) concentrations is not dependent on a subject's apoE or apoA4 phenotype. The apo(a) isoforms had a highly significant effect on Lp(a) concentrations, as indicated by the significant decrease in likelihood, for the model in which all apo(a) phenotypes were constrained to have the same average Lp(a) concen-

tration. The apo(a) locus explained 61% of the variance in Lp(a) levels. The MZ, DZ and parent–offspring correlations for the residual part of the variance were estimated as 0.87, 0.32, and 0.33, respectively. The next two entries in Table 3 show that both the apoE and the apoA4 locus did not contribute significantly to Lp(a) variation, as there was a non-significant decrease in likelihood when these effects were omitted from the model.

Discussion

We found an additive genetic heritability of 95% for quantitative Lp(a) plasma levels in unselected Dutch families consisting of parents and their twin offspring. This heritability estimate is very similar to the estimates we obtained previously in this[13] and other Dutch samples[15] using a structural modelling approach to analyse the data and also closely resembles estimates from other family studies. Size heterogeneity of the Lp(a) polypeptide chain was associated with quantitative Lp(a) plasma levels. A major part (61%) of the variance in Lp(a) levels in this sample could be attributed to the difference apo(a) isoforms. The detection of apo(a) isoforms at the protein level as used by us, is not as sensitive as the pulsed-field electrophoretic method developed by Lackner et al[26] that can detect 20 or more alleles at the DNA level and represents a size category

Table 2 Log-likelihoods for different models testing the significance of sex and age on log-transformed Lp(a) levels. Tests on the covariance structure between family members include a test of assortative mating (correlation between spouses), of additive genetic influences ($r_{MZ} = 2r_{DZ}$) and of equal heritability in parental and offspring generations ($r_{DZ} = r_{parent-offspring}$)

| | | Difference test | |
	Log-likelihood	df	χ^2
General model	198.178	–	–
Same means for both generations and both sexes	196.292	3	3.772
No association with age	196.159	1	0.266
No correlation between spouses	195.951	1	0.416
MZ correlation equals 2 * DZ correlation	195.915	1	0.072
DZ correlation equals parent-offspring correlation	194.707	1	2.416

All models were tested against the preceding model. All decreases in likelihood were non-significant (critical value for χ^2 with 3 df is 7.18 and for 1 df 3.84). The general model estimated separate means for fathers, mothers, sons and daughters, a linear regression of Lp(a) levels on age and separate correlations between spouses, parents and offspring, MZ and DZ twins.

Table 3 Simultaneous analyses of the effects of the apo(a), apoE and apoA4 loci on log-transformed Lp(a) levels. Separate MZ, DZ and parent–offspring correlations and no correlation between spouses were estimated for the residual variances

| | | Difference test | |
	Log-likelihood	df	χ^2
1 General model	452.689	–	–
2 Additive effects of phenotypes at 3 loci	412.112	73	81.154
3 No effect of apo(a) locus	213.962	24	396.30[a]
4 No effect of apoA4 locus	408.780	4	6.664
5 No effect of apoE locus	408.388	5	7.448

[a]significant decrease in likelihood (critical value for χ^2 with 73 df is 93.94, for 4 df 9.48, and for 5 df 11.07). Model 2 is tested against model 1; models 3, 4 and 5 are tested against model 2. The general model estimated separate means for all possible combinations of the phenotypes at the apo(a), apoE and apoA4 loci.

rather than one distinct species. However, the percentages of variance explained by the apo(a) size polymorphism, as measured by the different apo(a) isoforms in our study and by the 16 different apo(a) alleles in the study of Boerwinkle et al[28] are remarkably similar. Boerwinkle et al reported that 69% of the variation in Lp(a) levels was explained by the number of kringle 4 repeats in the apo(a) gene, whereas we found 61%. This similarity in results agrees with the perfect correlation reported by Kraft et al[19] between apo(a) protein isoforms and apo(a) allele size.

The pattern of familial correlations for the residual part of the Lp(a) variance that could not be attributed to the apo(a) isoforms suggested genetic influences on the residual variance, as the MZ correlation (0.87) was larger than the DZ and parent–offspring correlations (0.32 and 0.33). We addressed the question whether the residual genetic variance that is not accounted for by the apo(a) size polymorphism is caused by genetic heterogeneity at other loci. Two other loci have been suggested, the apoE locus at chromosome 19 and the apoA4 locus at chromosome 11. In an earlier analysis of the Lp(a) levels in the parents from our twin families, we found that a small part (4%) of the Lp(a) variance was due to the apoE locus.[29] Similar effects have been reported by others. In the EARS study, Tiret et al[38] found a significant lowering effect on the apoE2 allele on Lp(a) concentrations in almost 1900 young adults from 11 European countries. Lindahl et al[39] used a crude form of correcting Lp(a) levels for apo(a) phenotype, using part of a sample of 149 patients with familial hypercholesterolaemia. No effect of apoE phenotype was seen on residual Lp(a) levels, but the number of patients with residual Lp(a) levels below the median was larger in the E2 + group and lower in the E4 + group. In contrast, in the Framingham offspring study apoE phenotype was not associated with plasma Lp(a) concentrations.[40] Two other studies also did not observe this association. In patients with myocardial infarction[41] and in patients with familial hypercholesterolaemia[42] no apoE effect on Lp(a) levels was detected.

Eckardstein et al[30,31] obtained evidence that the apoA4 locus influences Lp(a) concentrations. The effect was shown in two independent samples, consisting of healthy males and females and of male coronary heart patients. ApoA4 explained around 5% of the Lp(a) variance in patients. However, a negative result was found in the EARS study. No association between apoA4 phenotype and Lp(a) levels was seen.[43]

The data on the influences of the genetic heterogeneity at the apoE and the apoA4 locus on plasma Lp(a) levels thus are inconsistent. We found in our

data that the apoE locus explained 6% of the apoA4 locus 3% of the variation in Lp(a) concentrations where we analysed the effects of apoE and apoA4 independent from those of the apo(a) locus. However, when the effect of the apo(a) locus was included in the model (after obtaining evidence that the effects of the apo(a), apoE and apoA4 loci did not interact) the effects of apoE and apoA4 phenotype on Lp(a) levels were not significant. This suggests that the earlier positive findings, obtained in data that were not adjusted for differences in apo(a) phenotypes between subjects, may have been artifacts.

If genetic factors account for more than 90% of the variance in Lp(a) levels and the apo(a) size polymorphism explains 61% of the total Lp(a) variation, then a substantial part of the genetic variance must be caused by genetic factors other than the size polymorphism itself. Evidence from the studies by Kraft et al[19] and Boerwinkle et al[28] suggests that genetic factors linked to the apo(a) gene determine Lp(a) variation. Both these studies observed that the correlation for Lp(a) levels among siblings who shared both apo(a) alleles identical by descent, was as high as Lp(a) heritability, whereas correlations among siblings sharing only one or no alleles identical by descent were significantly lower. These observations and the results from our own study suggest that the remainder of the Lp(a) variance is not accounted for by the apoE or apoA4 locus, but may be caused by differences in the whole process of Lp(a) production, eg transcription of the apo(a) gene, including trans-acting factors and cis-acting promoter sequences at the apo(a) locus, stability of the apo(a) mRNA, apo(a) protein translation, intracellular processing, transport and secretion of the protein, and assembly into a Lp(a) particle. This view is supported by the observation that the substantial variation in Lp(a) levels among individuals with the same apo(a) phenotype is caused primarily by differences in Lp(a) production rate.[44]

Acknowledgements

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