

ORIGINAL PAPER

Concentrations of the atherogenic Lp(a) are elevated in FH

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Lipoprotein(a) (Lp(a)) is a complex in human plasma assembled from low-density lipoprotein (LDL) and apolipoprotein(a) (apo(a)). High plasma concentrations of Lp(a) are a risk factor for coronary heart disease (CHD) in particular in patients with concomitant elevation of LDL. We have analysed for elevated Lp(a) levels in patients with familial hypercholesterolaemia (FH), a condition caused by mutations in the LDL receptor (LDLR) gene and characterised by high LDL, xanthomatosis and premature CHD. To avoid possible confusion by the apo(a) gene which is the major quantitative trait locus controlling Lp(a) in the population at large, we used a sib pair approach based on genotype information for both the LDLR and the apo(a) gene. We analysed 367 family members of 30 South African and 30 French Canadian index patients with FH for LDLR mutations and for apo(a) genotype. Three lines of evidence showed a significant effect of FH on Lp(a) levels: (1) Lp(a) values were significantly higher in FH individuals compared to non-FH relatives ($p < 0.001$), although the distribution of apo(a) alleles was not different in the two groups; (2) comparison of Lp(a) concentrations in 28 sib pairs, identical by descent (i.b.d.) at the apo(a) locus but non-identical for LDLR status, extracted from this large sample demonstrated significantly elevated Lp(a) concentrations in sibs with FH ($p < 0.001$); (3) single i.b.d. apo(a) alleles were associated with significantly higher Lp(a) concentrations ($p < 0.0001$) in FH than non-FH family members. Variability in associated Lp(a) levels also depended on FH status and was highest when i.b.d. alleles were present in FH subjects and lowest when present in non-FH individuals. The study demonstrates that sib pair analysis makes it possible to detect the effect of a minor gene in the presence of the effect of a major gene. Given the interactive effect of elevated LDL and high Lp(a) on CHD risk our data suggest that elevated Lp(a) may add to the CHD risk in FH subjects.

Keywords: Lipoprotein(a); Familial Hypercholesterolaemia; sib pair analysis; Apo(a); genotype; LDL-receptor

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Introduction

Defects in the LDL receptor (LDLR) gene give rise to familial hypercholesterolaemia (FH), a condition characterised by high LDL cholesterol, xanthomatosis, and premature coronary heart disease (CHD).¹ High concentrations in plasma of Lp(a), a covalent complex of LDL and the plasminogen-related apolipoprotein(a), have also been considered a feature of FH and may confer additional risk of atherosclerosis on to FH subjects² but this has been a matter of continuous debate.³ Lp(a) is a quantitative genetic trait in human plasma showing extreme variation both within and between populations.⁴⁻⁶ In healthy Caucasians atherogenic Lp(a) plasma levels are almost exclusively controlled by the hypervariable *apo(a)* gene locus on chromosome 6q27,⁷⁻⁹ but several rare genetic conditions reportedly also affect Lp(a) levels (eg LPL deficiency,¹⁰ LCAT deficiency,¹¹ abetalipoproteinaemia).¹² Other variables like age, sex, body weight or diet have been excluded as significant determinants of Lp(a) concentrations.^{13,14}

Initial studies of unrelated FH subjects suggested that i) average Lp(a) levels are significantly elevated in FH¹⁵ and that ii) high Lp(a) confers additional atherosclerotic risk on to FH subjects.² Hyper Lp(a) was possibly a key feature of FH which adds to the risk of premature atherosclerosis in FH patients.² Subsequent family studies have challenged both suggestions and caused much confusion and debate.¹⁶⁻²¹ Some studies which compared Lp(a) in FH family members found no elevated Lp(a) in affected vs non affected family members,^{17,21,22} whereas others found an effect in some but not other families and suggested ethnic and/or mutation heterogeneity was the explanation.¹⁶ Two family studies also considered apo(a) protein phenotypes.^{16,22} The phenotyping methods used in the latter studies resulted in poor resolution of apo(a) isoforms which did not allow i.b.d. to be defined by this means in many cases.

It is, however, mandatory to stratify apo(a) allele effects in such studies because the apo(a) gene locus is the major quantitative trait locus (QTL) for Lp(a) plasma levels in the population at large.⁷⁻⁹ Random inheritance of apo(a) alleles may therefore severely distort the analysis. We have here used a variant of the sib pair approach. We first selected sib pairs i.b.d. at the apo(a) locus from a large family cohort of molecularly defined FH families, thus removing any potential effect of the apo(a) locus and also any phenocopies of FH from further analysis. We then compared Lp(a) levels in

FH-affected and non-affected sibs. We further determined Lp(a) concentrations associated with single apo(a) alleles in all apo(a) heterozygous family members. This allowed us to extend our analysis by comparing Lp(a) concentrations for i.b.d. alleles between FH and non-FH family members. Together these demonstrated a significant effect of LDLR mutation on Lp(a) levels.

Materials and Methods

Subjects

The Department of Human Genetics, University of Stellenbosch, South Africa recruited for this study 30 Afrikaner FH families, which carry one of the three common LDLR mutations (D206E, V408M, D154N).²³ EDTA-blood was taken from 203 family members (103 FH patients, 57 relatives and 43 spouses) for analysis of their FH status. To determine Lp(a) concentrations and apo(a) genotype and phenotype samples were shipped on dry ice by air to Innsbruck. They were kept frozen at -20°C until they were used for the laboratory procedures. The 30 French Canadian FH families have been described previously.²⁰

Determination of LDLR Mutations

Identification of the Afrikaner mutations in the LDLR gene was performed in all 203 Afrikaner family members by a nonradioactive multiplex PCR screening method as published elsewhere.²⁴ The French Canadian 10 kb deletion was determined as described.²⁰

Plasma concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured by standard techniques as previously described.²⁵ Low-density lipoprotein cholesterol (LDL-C) values were calculated according to the Friedewald formula.²⁶

Plasma Lp(a) Immunoassay and Determination of apo(a) Isoforms by Horizontal SDS Agarose Gel Electrophoresis (AGE)

Plasma Lp(a) quantification was performed with a double antibody ELISA^{12,27} using an affinity-purified polyclonal apo(a) antibody for coating and the horseradish peroxidase-conjugated monoclonal antibody 1A2 for detection.

Apo(a) phenotyping by high resolution SDS-AGE was performed using a modification²⁸ of the method of Kamboh *et al*.²⁹

Apo(a) immunoblots were developed using the apo(a) MAB 1A^{2,30}. Extreme care was taken to avoid incomplete transfer from the gels to the immunoblots and also to avoid blotting of apo(a) isoforms through the membranes (which may both result in a change in the relative amounts of apo(a) determined in heterozygotes by the subsequent densitometric evaluation especially if the two isoforms in a subject differ significantly in size).

Optimal conditions for complete transfer were established in pilot experiments. We further controlled for 'blotting through the membrane' by using a second membrane in each experiment. Densitometric scanning of apo(a) blots was

performed using a video system and the program E.A.S.Y. (Herolab, Wiesloch, Germany).

DNA Isolation, Plug Preparation and PFGE

Genomic DNA was prepared as agarose plugs from frozen EDTA-blood samples (5 ml) exactly as described.⁶ For separation of the apo(a) alleles by pulsed field gel electrophoresis (PFGE), a plug containing about 3 µg genomic DNA was washed twice in TE buffer for 30 min and was digested with 2×40 U KpnI restriction enzyme (Boehringer Mannheim, Mannheim, Germany) for 2 h at 37°C. The restriction fragments were separated in a 1% LE agarose gel with 0.5% TAE buffer (40 mM TRIS-acetate, 1 mM EDTA) at 14°C in the Chef mapper system (Biorad, Hercules, CA, USA). The fragment size limits were 28–200 kb. After electrophoresis the gel was subjected to Southern blotting analysis using a chemiluminescence detection system with a DIG-labelled (Boehringer Mannheim, Mannheim, Germany) apo(a) specific probe as described.⁸

Statistical Methods

We used the analysis of variance for comparison of the total cholesterol (TC), HDL-cholesterol (HDL-C) and triglyceride (TG) values between patients and the controls. The non-parametric Mann-Whitney U test was applied to compare Lp(a) concentrations between the two groups because of the highly skewed distribution of Lp(a) levels. No adjustments of Lp(a) levels for age, sex, body mass or other variables were performed before any of the analyses because Lp(a) was not affected by these variables.

The sib pair analysis was performed in three ways

- (1) The Lp(a) concentrations of all sib pairs i.b.d. for both apo(a) alleles but with different FH status were compared by the nonparametric Wilcoxon signed rank test for pair differences.
- (2) Average Lp(a) concentrations associated with individual apo(a) alleles i.b.d. were compared between FH and non-FH groups by the Wilcoxon matched pairs signed rank test.
- (3) Lp(a) concentration differences (Delta) were calculated separately for FH-discordant and FH-concordant sib pairs, which were all i.b.d. for their apo(a) alleles. The Delta values were then compared between the FH discordant and concordant groups using the Mann-Whitney U test. In addition we determined the influ-

ence of both apo(a) alleles (difference of the sum of the number of K-IV repeats) of a sib pair and of its FH status on the Delta Lp(a) values by univariate linear regression analysis. Regression analysis was performed using the SAS system; all other calculations were performed using the SPSS program.

Results

FH Families

Thirty Afrikaner FH families were identified by probands carrying one of the 3 Afrikaner founder mutations in the LDLR gene (*D206E*, *V408M* and *D154N*) which have all been demonstrated by cosegregation and functional studies to cause FH.²³ A total of 203 family members (including 43 spouses) of these probands were analysed for the respective LDLR mutations. This resulted in the identification of 103 carriers of LDL receptor mutations, 57 non-affected blood relatives and 43 non-affected spouses. Average lipid and lipoprotein concentrations for FH-affected and non-affected relatives and spouses are given in Table 1; the distribution of Lp(a) concentration in the three groups is shown in Figure 1. As predicted from the LDLR mutation TC and LDL-C were significantly higher in the FH group ($p < 0.001$). Lp(a) concentrations were also significantly higher in the FH group as compared to both non-FH relatives ($p = 0.0047$) and spouses ($p = 0.0387$). However the latter, though in agreement with some previous studies,^{15,16,19} might represent a chance finding caused by random inheritance of apo(a) alleles associated with high Lp(a) by FH affected subjects.

Therefore we considered it not justified to conclude from such data alone that Lp(a) is elevated in FH and have as a first step stratified for the effect of the apo(a) gene locus.

Table 1 Lipid and lipoprotein data of Afrikaner FH family members

Variables mean (S.D.)	FH (1)	non-FH relatives (2)	spouses (3)	p-value ^c (1-2)	(1-3)	(2-3)
n (male:female)	103 (45:58)	57 (26:31)	43 (20:23)			
TC (mM/l)	8.66 (1.89)	5.27 (1.3)	5.47 (1.11)	<0.001	<0.001	n.s.
LDL-C (mM/l)	6.87 (1.83)	3.26 (1.2)	3.51 (1.13)	<0.001	<0.001	n.s.
HDL-C (mM/l)	1.21 (0.3)	1.37 (0.33)	1.28 (0.3)	n.s.	n.s.	n.s.
TG (mM/l)	1.28 (0.85)	1.40 (0.93)	1.47 (0.7)	n.s.	n.s.	n.s.
Lp(a) (mg/l)	35.4 (31.0)	20.7 (18.1)	26.4 (29.7)	0.0014 ^a	0.0052 ^a	n.s.
Median Lp(a) (mg/dl)	27.7	16.3	15.5	0.0047 ^b	0.0387 ^b	n.s.
25th; 75th percentile	(10.5; 52.6)	(7.3; 30.6)	(6.8; 41.7)			

^aANOVA; ^bMann-Whitney U test; ^cp-value calculated for the combinations of the three groups (FH, non-FH relatives, spouses).

Sib Pair Analysis

Afrikaner FH Families Apo(a) genotypes as defined by the number of kringle IV repeats on each allele were determined for all 203 family members by PFGE/genomic blotting. A total of 26 apo(a) size alleles was represented in the sample with kringle IV repeat numbers ranging from 11 to 45; 96% of individuals were heterozygotes carrying two different sized apo(a) alleles. The rest were homozygous for a distinct apo(a) allele. The distribution of apo(a) allele frequencies were virtually identical between FH and non-FH family members considering either the overall distribution (t-test; $p = 0.997$) or the ratio of small (K-IV 11–22) vs large (K-IV > 22) apo(a) alleles among the groups ($\chi^2 = 0.15$, $p > 0.5$). This makes it unlikely that the observed differences in Lp(a) concentrations are caused by differences in apo(a) allele frequencies between the groups. However, this possibility cannot be ruled out completely since apo(a) alleles

of identical size may be associated and segregate with dramatically different Lp(a) concentrations.³¹

Apo(a) DNA typing allowed us to identify all sib pairs which were i.b.d. at the apo(a) locus. Figure 2 shows a pedigree of FH family No. 67 and the corresponding apo(a) PFGE/Southern blot. The mother (I/1) is heterozygote for the Afrikaner-1 (D206E) LDLR mutation and three of her four children have inherited this mutation. The children present three of the four possible apo(a) allele combinations. Two of the sibs (II:1; II:2) are i.b.d. for apo(a) alleles but non-identical for LDLR status. Together our sample which consisted of 118 sib pairs included 16 such informative sib pairs which were i.b.d. for apo(a) but non-identical for LDL receptor status (Table 2). Median Lp(a) concentrations were higher in the FH-affected (16.1 mg/dl) than the non-affected sibs (14.6 mg/dl). This difference was of borderline significance only ($p = 0.077$). In 12 of the pairs the FH-affected sib had the higher Lp(a) concentration.

French Canadian FH Families Sib pairs i.b.d. at the apo(a) locus and non-identical for FH status were further recruited from 30 French Canadian families

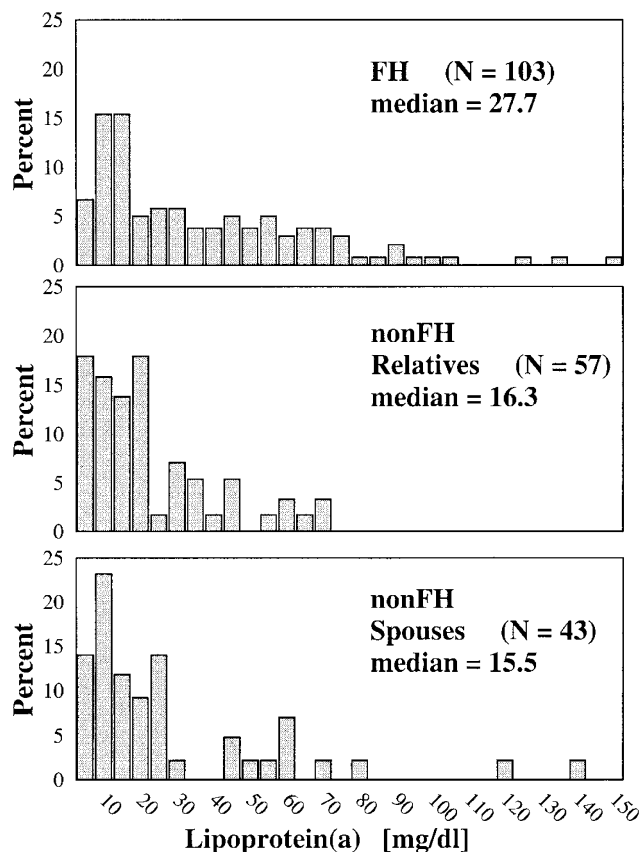


Figure 1 Distribution of Lp(a) concentration (mg/dl) in South African FH subjects, non-FH relatives, and spouses of FH patients

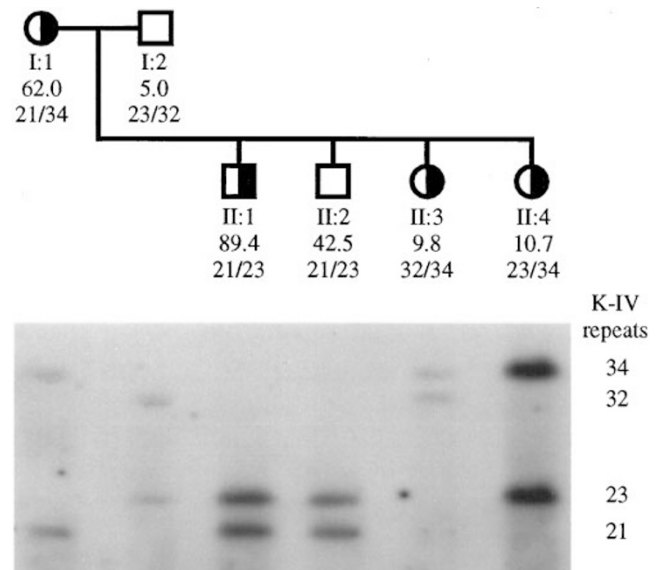


Figure 2 Pedigree of an Afrikaner family with the D206E mutation in the LDL receptor. Under the pedigree symbols the ID number, the Lp(a) concentration, and the apo(a) genotype (number of K-IV repeats in the apo(a) alleles) are given. Below the pedigree the corresponding chemoillumination image of the PFGE/Southern blot is shown. The number of K-IV repeats present in the respective apo(a) alleles are indicated at the right. Individuals II:1 and II:2 have both inherited apo(a) alleles 21 and 23 and are thus i.b.d. for apo(a) but discordant for FH

with the 10 kb LDLR deletion;³² 12 sib pairs fulfilled these criteria. Only apo(a) protein isoforms (see below) were available from these families. Median Lp(a) concentrations were significantly higher (46.0 mg/dl) in FH than in non-FH (10.0 mg/dl) sibs ($p = 0.0076$). For all except one sib pair Lp(a) levels were higher in the FH-affected sibs.

Combined FH Families For the combined Afrikaner and French Canadian sib pairs ($n = 28$), median Lp(a) concentrations were 22.8 mg/dl in FH-affected sibs which is significantly higher than the 13.7 mg/dl in non-affected sibs ($p = 0.0013$). The mean difference of Lp(a) levels between FH and non-FH sibs was 11.5 mg/dl. Subjects with a LDLR mutation had on average 85% higher Lp(a) levels than non-affected sibs. This difference was highly significant ($p < 0.01$).

Comparison of Apo(a) Allele Associated Lp(a) Concentrations in South African FH and non-FH Family Members

The number of sib pairs in the above analysis was limited despite the large amount of family material analysed. To increase the number of informative pairs we performed a second type of analysis and determined the Lp(a) concentrations for the single apo(a) allele in each family member. The Lp(a) concentration in plasma is the sum of the concentrations determined independently by each apo(a) allele. Therefore, apo(a) allele associated Lp(a) concentrations can be determined after separation of apo(a) isoforms, eg by electrophoresis, immunoblotting followed by densitometric scanning. Apo(a) phenotypes were determined by high resolution SDS-AGE/immunoblotting in all family members and the relative intensities of apo(a)

Table 2 Lp(a) concentrations in FH discordant sib pairs with identical apo(a) alleles

Sib pair (mutation type) ^a	Apo(a) genotype (K-IV repeats)	Lp(a) mg/dl FH	Lp(a) mg/dl non-FH	(mg/dl)
1 (3)	20/25	69.7	66.7	3.0
2 (3)	20/34	53.4	44.4	9.0
3 (1)	20/37	69.4	64.8	4.6
4 (1)	20/39	68.8	56.4	12.4
5 (1)	21/23	89.4	42.5	46.9
6 (2)	23/28	7.0	6.5	1.5
7 (1)	24/29	12.7	16.1	-3.4
8 (3)	24/33	11.4	9.6	1.8
9 (1)	27/37	0.7	0.3	0.4
10 (1)	27/37	(0.7)	0.3	0.4
11 (3)	28/29	28.7	12.3	16.4
12 (1)	28/32	4.5	7.9	-3.4
13 (1)	29/37	16.8	14.6	2.2
14 (1)	29/37	(16.8)	9.9	6.9
15 (1)	30/30	15.4	17.7	-2.3
16 (1)	32/34	13.4	14.6	-1.2
(1-3)	median	16.1 (10.3; 68.95) ^b	14.6 (8.3; 43.9) ^b	mean 5.9
17 (4)	19/0	104.1	72.1	32.0
18 (4)	19/34	11.0	7.5	3.5
19 (4)	20/33	99.5	66.2	33.3
20 (4)	20/34	12.2	7.1	5.1
21 (4)	21/23	68.6	60.0	8.6
22 (4)	21/23	69.0	(60.0)	9.0
23 (4)	23/27	5.2	3.7	1.5
24 (4)	23/34	30.0	10.0	20.0
25 (4)	23/34	62.0	(10.0)	52.0
26 (4)	23/34	79.0	(10.0)	69.0
27 (4)	25/28	4.6	1.8	2.8
28 (4)	28/28	5.2	13.7	-8.5
(4)	median	46.0 (6.7; 76.5) ^b	10.0 (5.4; 63.1) ^b	mean 19.0
Total median		22.8	13.7 ^c	mean 11.5

Bold letters designate the higher Lp(a) level in each sib pair. Values in brackets were not included for the estimation of the median because of redundant usage for constructing a sib pair. ^aLDL-R mutations: 1 = *D206E*, 2 = *V408M*, 3 = *D154N*, 4 = 10 kb deletion; ^bValues in brackets give the 25th and 75th percentile; ^c $p = 0.0013$ by Wilcoxon matched pairs signed rank test; '0' shows the presence of a 'null' allele that could not be detected by immunoblotting.

isoforms in heterozygotes were measured by scanning densitometry of the immunoblots.

Together with the knowledge of total Lp(a) concentration and the K-IV repeat number from DNA typing this allowed an Lp(a) concentration to be assigned to each single apo(a) allele; 68% of family members showed two different apo(a) isoforms each with a distinct Lp(a) concentration. In the plasma of the other 32% we identified only one isoform. According to the DNA genotyping 4% were true homozygotes for K-IV repeat number. In these it was not possible to assign an Lp(a) concentration to each allele; 28% expressed only one of their two alleles and the total Lp(a) was assigned to this allele. The frequency of non-expressed alleles was not different between FH-affected and non-affected blood relatives (27.5% vs 28.1%). The data allowed us to compare the average Lp(a) concentrations for a distinct apo(a) allele (again defined as i.b.d.) between FH and non-FH individuals.

The strategy is illustrated in Figure 3. In that family there is no sib pair with two identical apo(a) alleles. Apo(a) allele #20 is, however, present in FH-affected (I:1, II:2) and non-affected (II:3) family members and apo(a) allele #23 is also present in FH-affected (II:2) and non-affected (I:2) subjects. Therefore three allele pairs could be deduced from this family which had been uninformative on the basis of sib pair analysis.

Using this approach we obtained a total of 202 allele pairs i.b.d. where one of the apo(a) alleles was present in an FH-heterozygote (FH+) and the other in a non-affected family member (FH-). Average Lp(a) concentrations were significantly higher for apo(a) alleles that were present in an FH environment (Wilcoxon test for pair differences, $p = 0.0001$). This difference was present over the whole range of apo(a) size alleles. When Lp(a) concentration was plotted against the number of K-IV repeats in an allele for FH and non-FH family members separately, we observed the well-known inverse correlation in both groups. The regression line was, however, shifted towards higher Lp(a) levels in the FH group (Figure 4).

Variability of Lp(a) for Identical Alleles Depends on LDLR Status

Whilst analysing our material we noted that apo(a) allele associated Lp(a) levels vary considerably in members from the same family with identical LDLR status, even for apo(a) alleles i.b.d. A similar observation has been reported by Perombelon *et al.*¹⁸ To analyse whether this variation is affected by LDL receptor status, we calculated the mean difference (Δ)

between Lp(a) concentrations for apo(a) alleles i.b.d. in the three different combinations of pairs FH+/FH+, FH+/FH- and FH-/FH- (ie the mean difference between pairs of alleles i.b.d. for apo(a) which were both in FH-heterozygote and in non-FH members or one in an FH and the other in a non-FH subject).

This analysis is shown in the boxplot in Figure 5. Notably, variation (expressed as delta) was largest if both apo(a) alleles were present in FH-heterozygotes, intermediate if one was present in a FH-heterozygote and the other not, and smallest if both alleles of the pair

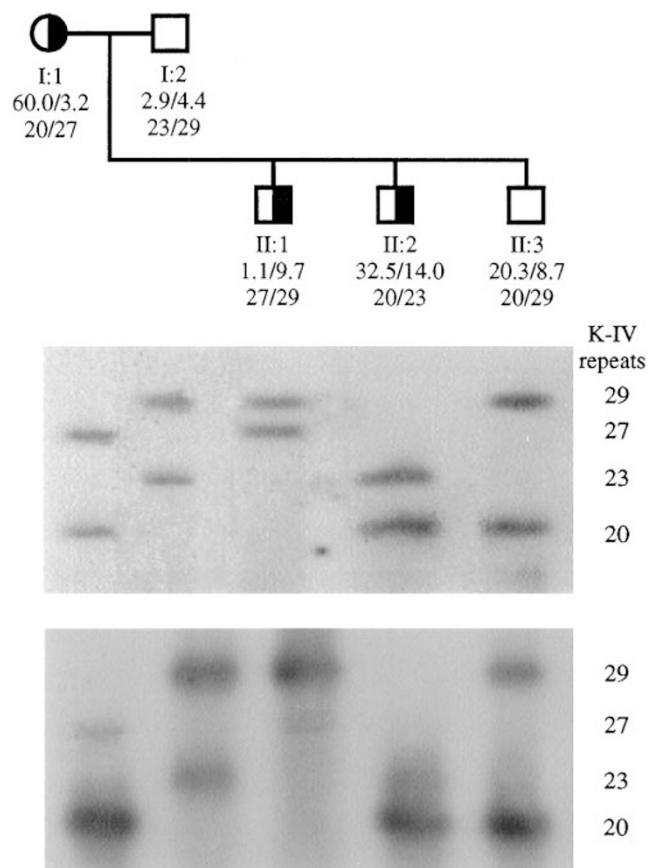
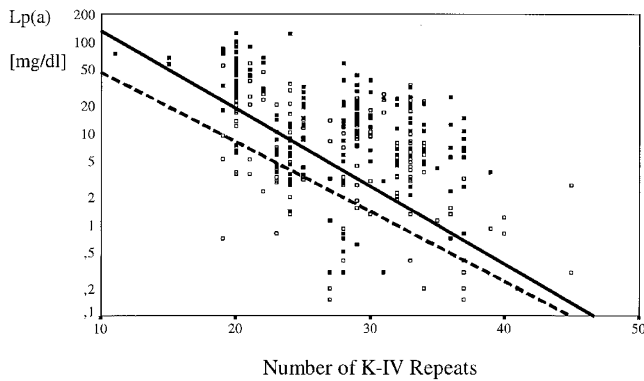


Figure 3 Pedigree of an Afrikaner family with the D154N mutation in the LDLR. Within the pedigree the ID numbers, the apo(a) allele associated Lp(a) concentrations, and the apo(a) genotypes are shown. The middle panel represents the chemoillumination of the corresponding PFGE/Southern blot and the lower panel shows the immunoblot. An apo(a) allele with 20 K-IV repeats is present in the mother (I:1) and two of her sons (II:2 and II:3). One of these three individuals (II:3) does not have FH, the other two are heterozygous for the D154N mutation. Another apo(a) allele containing 23 K-IV repeats is also present in a non-FH (I:2) and in an FH (II:2) individual. Thus three informative pairs of alleles can be obtained from this family. There is only low expression of alleles 23 and 27 resulting in weak bands on the immunoblots

were in FH non-affected subjects. This correlation was significant ($p = 0.033$) and it suggests that the variation in Lp(a) concentration is larger in FH than in non-FH subjects and that this larger variation is caused by the LDLR status rather than by the apo(a) locus or other factors.



Spearman's rank correlation coefficients: FH (■) $R = -0.462$

non-FH (□) $R = -0.429$

Figure 4 Histogram demonstrating the inverse correlation of log Lp(a) concentration with K-IV repeat number in FH (filled squares) and non-FH (open squares) family members. Lp(a) concentrations are higher for each repeat length in FH than non-FH subjects as indicated by the regression lines. Each symbol represents the Lp(a) concentration associated with a single allele (--- regression line for FH, ---- regression line for non-FH)

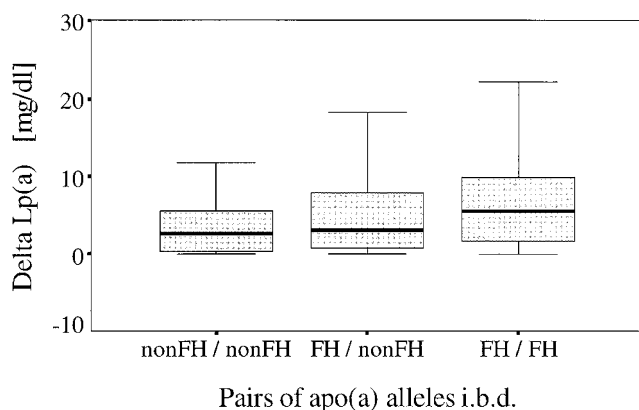


Figure 5 Boxplot of the mean difference (Delta) in Lp(a) concentration for paired apo(a) alleles. The Lp(a) concentration associated with single i.b.d. apo(a) alleles was compared between pairs with normal LDL receptor activity (non-FH/non-FH), pairs nonidentical for LDLR status (FH/non-FH), and pairs where both were heterozygous for FH (FH/FH)

Regression Analysis

Multiple linear regression analysis was carried out using the difference of Lp(a) levels between all possible sib pairs ($n = 110$) as depending variable. The difference in the number of K-IV repeats between the sib pairs as well as the FH status of the sib pairs (FH/FH, FH/non-FH, non-FH/non-FH) were taken as independent variables. This demonstrated a significant effect of the apo(a) K-IV VNTR ($p = 0.0001$) and of the FH status ($p = 0.0150$) on Lp(a) level differences between sib pairs ($R^2 = 0.233$, $p = 0.0001$).

Relation of the LDL Receptor Mutation Type with Lp(a) Concentration

Three different types of LDL receptor mutations were present in the 30 FH Afrikaner families. Two of them are in the ligand binding domain (*D206E*, *D154N*), the third (*V408M*) is in the EGF homology region. To determine if the type of LDLR mutation has an influence on Lp(a) levels, we compared the mean concentrations for the three Afrikaner mutation types (*D206E* = 38.1 ± 36.9 mg/dl; *V408M* = 33.1 ± 21.3 mg/dl; *D154N* = 33.5 ± 28.2 mg/dl). The distribution of the apo(a) K-IV alleles was similar among the three groups. Only in the group with the *D206E* mutation was a slightly larger number of small apo(a) alleles (K IV < 23) found, consistent with the slightly increased mean Lp(a) level in this subgroup. The median Lp(a) concentration was not significantly different (Mann-Whitney U test) between the groups. Because of the different genetic background no comparison was performed with the >10 kb deletion mutation in the French Canadians.

Discussion

A number of studies have analysed Lp(a) in FH patients but results have not been consistent. None of these studies has considered apo(a) genotypes. In view of the enormous variation in apo(a) concentration in the population, which is almost entirely explained by the apo(a) locus and is present even in subjects with apo(a) isoforms identical by size, it is essential to check for any potential effect of apo(a) locus variation in the analysis.

We have rigorously checked for apo(a) gene effects using

- (1) comparison of Lp(a) levels in FH and non-FH relatives with matching distribution of apo(a) alleles in the two groups;

- (2) a sib pair approach including exclusively sibs with apo(a) alleles i.b.d. and
- (3) an extension of this approach comparing Lp(a) concentrations for apo(a) alleles i.b.d. between family members who differed by LDLR status.

All three types of analysis demonstrated that average Lp(a) concentrations are elevated in FH heterozygotes. On average, Lp(a) was 85% higher in sibs with FH compared with sibs without FH which were i.b.d. for both apo(a) alleles. This is less than the two or three fold rise observed in our initial study of non-related FH and non-FH groups, but is nevertheless highly significant.

Our conclusions are supported by similar findings in families with familial defective apolipoprotein B (FDB), a form of familial hypercholesterolaemia which is caused by a mutation in the apolipoprotein B rather than in the LDLR. Sib pair analysis consistently revealed higher Lp(a) in FDB-affected than non-affected siblings.³³

Our results are at variance with a previous family study¹⁸ which determined Lp(a) concentrations for individual apo(a) isoforms and thus used a strategy similar to part of this work. There are, however, distinct differences between our analysis and the work of Perombelon¹⁸ which may explain the different results. Those authors used

- (1) only protein isoform information,
- (2) a technique with less power for resolution of isoforms,
- (3) analysed a smaller sample and, most importantly
- (4) replaced 'allele pairs with large differences in Lp(a)' with identical alleles from their analysis.

The latter may be of particular relevance in view of our demonstration that the LDLR status affects variability and not only levels of Lp(a). Furthermore, many of the allele pairs in the work of Perombelon *et al* were not unequivocally i.b.d. but rather i.b.s. (identical by state).

It should also be noted in this context that no study, whether comparing unrelated FH subjects with controls or FH-affected or non-affected family members, has

ever observed lower Lp(a) concentrations in the FH group (which is to be expected if deviations were random). In all studies, including all reported family studies, Lp(a) concentrations were higher in FH-affected subjects though the difference was not statistically significant in most cases. We believe that in these studies the power to detect the effect of the LDLR status on Lp(a) was limited by sample size in conjunction with the high variability in Lp(a) concentration and by the lack of rigorous checking for the effects of the apo(a) locus. The most robust analysis performed in our work is the sib pair approach based on information for both LDLR and apo(a) genetic status. This analysis which was based on a total of 28 sib pairs clearly demonstrated a significant effect of FH status on Lp(a) concentration. The higher Lp(a) in FH-affected sibs can be explained neither by differences in age nor sex distribution between the pairs. The average age difference between FH and non-FH sibs was 0.56 years and the male:female ratios were 1:1.6 and 1:1.2 respectively. Furthermore, it is well established that age and sex have no effect on Lp(a) in adults, with the possible exception of the postmenopausal state in women which has been reported to be associated with elevated Lp(a) in some but not all studies.^{34,35} Only two postmenopausal women were present within the sib pairs and both belonged to the non-FH group. Thus if there had been any effect it would have been to reduce rather than increase the difference in Lp(a) levels between FH and non-FH sibs.

A further explanation for the smaller than expected difference was found by chance in the sampling of informative sib pairs in the Afrikaner population. The median Lp(a) level in Afrikaner FH individuals within the sib pairs (Table 2) was much lower compared with the total group (16.1 mg/dl and 27.7 mg/dl respectively). The predominance of individuals with low Lp(a) may explain the marginal significance of the difference in Lp(a) concentrations between FH and non-FH sibs in this population.

The opposite was true of the French Canadian sample. Here the selection for informative sib pairs yielded an over-representation of FH sibs with high Lp(a) levels, which resulted in a highly significant difference in Lp(a) levels in sib pairs who are i.b.d. for apo(a) but discordant for LDLR mutation (Table 2). The pooling of the two data sets reconciled these two divergent biases and the analysis by pairs had a significant effect of FH on Lp(a) levels.

Results of studies concerning Lp(a) in FH have frequently been used to ascertain whether or not the LDLR clears Lp(a) *in vivo*.^{16,18,19} We do not conclude from our study that Lp(a) is cleared – at least in part – by the LDLR pathway, although this is one possible explanation of the data. Several other interpretations, however, are more likely. The role of LDLR in Lp(a) catabolism has been a subject of continuous debate. *In vitro* binding studies have generated conflicting results. One elegant study in transgenic mice for the human LDL receptor³⁶ has suggested that the LDLR does contribute to LDL catabolism *in vivo*. In contrast, *in vivo* turnover studies of Lp(a) have found identical decay curves in FH subjects and healthy controls suggesting that the Lp(a) receptor is not involved in Lp(a) removal.³⁷ Indirect evidence also supports this conclusion. Modulation of LDLR activity *in vivo* by HMG-CoA reductase inhibitors seems to have no effect on Lp(a) levels.^{38,39}

Our finding that Lp(a) is elevated in FH caused by LDLR gene defects seems to conflict with the *in vivo* turnover studies and the analysis of HMG-CoA reductase inhibitor effects. One explanation might be that the effect of the LDLR mutations on Lp(a) is indirect rather than direct and is on synthesis rather than on catabolism. Such a mechanism is supported by kinetic studies of Lp(a) in FH patients after LDL apheresis.⁴⁰ Our observation of a larger variation of Lp(a) levels in FH may also suggest such a scenario. Such an indirect effect might well be modulated by interacting environmental factors, thus explaining the larger variation in FH subjects. A larger variation and fluctuation of Lp(a) concentrations in FH heterozygotes compared to controls has not yet been reported but may have contributed to the conflicting reports in the literature.

Another unexplained finding may also have contributed to the lack of significant differences in Lp(a) levels between FH-affected and non-affected subjects in family studies. This is the higher than expected Lp(a) concentration in non-affected family members. In both the Afrikaner and French Canadian FH families studied here, Lp(a) levels were significantly higher in the non-affected family members compared with a reference population (²⁰ and A Lingenhel and G Utermann, 1996, unpublished data). The reason for this is presently unclear. It is particularly intriguing that Lp(a) is high in spouses (Table 1 and ²⁰). This was seen in both the Afrikaner and French Canadian families. Excessive Lp(a) (>100 mg/dl) is extremely rare in all Caucasian populations studied by us (<1%) but was present in

two out of 43 Afrikaner and in one out of 23 French Canadian spouses (Figure 1). If for any reason there is preferential mating of FH individuals with 'high Lp(a)' subjects this would also explain the higher Lp(a) in FH families. This would offer a further intriguing explanation for the high Lp(a) in FH.

The conflicting results in the literature regarding the influence of LDLR mutations on Lp(a) levels presumably arise from the difficulty in detecting a minor gene effect in the presence of a major gene effect. The apo(a) locus which determines >90% of the variation of Lp(a) levels in Caucasians⁷⁻⁹ is an outstanding example of a major gene effect on a quantitative trait. The search for additional effectors must address this perplexing factor. The type of sib pair analysis performed here provides a solution to that problem.

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