

# Defects in the low density lipoprotein receptor gene affect lipoprotein (a) levels: Multiplicative interaction of two gene loci associated with premature atherosclerosis

(familial hypercholesterolemia)

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**ABSTRACT** The lipoprotein (a) [Lp(a)] contains two non-identical protein species, apolipoprotein (apo) B-100 and a specific high molecular weight glycoprotein, apo(a). Lp(a) represents a continuous quantitative genetic trait, the genetics of which are only poorly understood. Genetic variation at the apo(a) locus affects plasma Lp(a) levels and explains at least 40% of the variability of this trait. Lp(a) levels were found to be elevated 3-fold in the plasma from patients with the heterozygous form of familial hypercholesterolemia who have one mutant low density lipoprotein receptor gene. This elevation was not due to a higher frequency of those apo(a) types that are associated with high Lp(a) levels in familial hypercholesterolemia patients. Rather Lp(a) levels were elevated for each of the apo(a) phenotypes examined. The effects of the apo(a) and low density lipoprotein receptor genes on Lp(a) levels are not additive but multiplicative. This is a situation not commonly considered in quantitative human genetics. We conclude that Lp(a) levels in plasma may be determined by variation at more than one gene locus.

Recent advances in molecular biology have facilitated our understanding of the role of particular genes in the etiology of several human diseases. Variation in these genes may produce monogenic disorders considered to represent the extremes of the variation of a particular phenotype (e.g., phenylketonuria). On the other hand detailed knowledge about the genes involved in the expression of quantitative traits, such as blood pressure or plasma cholesterol levels, is minuscule by comparison. Some of these traits are risk factors for the common chronic diseases (e.g., atherosclerosis).

The role of elevated plasma cholesterol levels in the etiology of coronary atherosclerosis has been impressively demonstrated by studies of the monogenic disorder familial hypercholesterolemia (FH; ref. 1). Mutations in the low density lipoprotein (LDL) receptor gene that cause FH, though not uncommon, explain only a small fraction of the genetic variability in plasma cholesterol levels and likewise only a fraction of the mortality from coronary heart disease in the general population. Polymorphic gene loci affecting plasma cholesterol levels and explaining a substantial fraction of the variability of this trait are being identified and characterized. The best known example is the polymorphism of apolipoprotein (apo) E (2-5) but other polymorphic genes affecting plasma cholesterol levels have also been suggested (6). One of these is the lipoprotein (a) [Lp(a)] gene locus (7). Lp(a) was detected as an antigenic property of LDL by Berg (8) and believed to be transmitted as an autosomal dominant trait. A series of subsequent studies have demonstrated that

Lp(a) levels in plasma represent a quantitative rather than a qualitative trait. Numerous studies have demonstrated an association of elevated Lp(a) levels with premature atherosclerosis (9). The distribution of plasma levels of Lp(a) in Caucasian subjects is skewed toward higher levels with most values in the lower concentration range (10, 11). Statistical genetic studies have advanced several hypotheses to explain the inter-individual variability and intra-individual constancy of Lp(a) levels, most of which assume one major autosomal gene contributing to plasma Lp(a) levels (12-14).

Lp(a) is assembled from a lipoprotein closely resembling or identical with LDL (containing apoB-100) and from the high molecular weight Lp(a) glycoprotein or apo(a) (15, 16). Apo(a) structure is composed of three elements all of which have a high degree of sequence homology with plasminogen [i.e., the protease domain, one Kringle 5 domain, and multiple repeats of Kringle 4 domains (17, 18)]. The Lp(a) glycoprotein exhibits a genetically determined size polymorphism controlled by at least seven alleles (19, 20) at a locus closely linked to the plasminogen gene on chromosome 6 (21-23). The apo(a) locus has been identified (19, 24) as a major gene affecting Lp(a) plasma levels. However, at present only as much as 40% of the variability in Lp(a) levels is explained by the measured variation at the apo(a) gene locus (7). Hence other genes and/or environmental factors may affect plasma Lp(a) levels. We show here that defects in the LDL receptor gene resulting in FH have highly significant effects on Lp(a) levels. We, therefore, propose the Lp(a) system as a model that allows one to study the interaction of different genes in producing a quantitative phenotype associated with premature coronary heart disease.

## MATERIALS AND METHODS

**Subjects.** Plasma samples were collected at the Hammersmith Hospital, London, from 102 Caucasian patients with FH, 55 men and 47 women, ages 18-71 years and a mean age of 39.6 years. The diagnosis of FH was based on hypercholesterolemia (serum cholesterol, >7.5 mmol/liter) together with the presence of tendon xanthomata in subjects or a first-degree relative. Plasma was collected from 279 consecutive blood donors from the province Tyrol (117 men and 162 women from 18 to 64 years old with a mean age of 36.8 years), representing a healthy Caucasian random population sample. All subjects were unrelated. The serum samples from London were shipped by air on dry ice to Innsbruck.

**Laboratory Procedures.** Lp(a) levels were determined in both groups by electroimmunodiffusion using polyclonal anti-Lp(a) antibodies as described (19). Apo(a) phenotyping

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Abbreviations: apo (prefix), apolipoprotein; FH, familial hypercholesterolemia; LDL, low density lipoprotein; Lp(a), lipoprotein (a).

was performed by SDS/polyacrylamide gel electrophoresis followed by immunoblotting according to Utermann *et al.* (19) with modifications. Plasma (10  $\mu$ l) was delipidated by extraction with 2.5 ml of ethanol/ether, 4:1 (vol/vol) for 16 hr at  $-20^{\circ}\text{C}$ . Proteins were collected by centrifugation in an Eppendorf centrifuge at  $10,000 \times g$  for 3 min and were washed once with ether. The protein pellet was dissolved by addition of 200  $\mu$ l of 5% (wt/vol) SDS/0.02 M ethylmorpholine, pH 8.6, 5  $\mu$ l of 2-mercaptoethanol, plus 10  $\mu$ l of 1.5% (wt/vol) bromophenol blue in glycerol and boiled for 5 min at  $100^{\circ}\text{C}$ . The mixture (50  $\mu$ l) was applied to 8% polyacrylamide gels. Immunoblotting was performed as described (19) using the monoclonal anti-apo(a) antibodies 1A<sup>2</sup> or 5' that are both specific for apo(a) and do not cross react with plasminogen or other plasma proteins (ref. 25 and H.D. and G.U., unpublished data). As the second antibody we used a goat anti-mouse peroxidase conjugate (Dako) at a 1:500 dilution.

The frequencies of apo(a) types reported here (Table 1) differ from published values (19, 24, 25). The frequencies of S4, S3, and double-band types were increased and the frequency of the null type was decreased. This is due to a higher sensitivity of the immunoblotting method with the monoclonal anti-apo(a) antibodies and the peroxidase reaction. Because small differences in the assay conditions may result in considerable differences in apo(a) type frequencies, the random population and patient samples were typed in parallel under identical conditions.

**Statistical Methods.** Pearson's  $\chi^2$  statistic was used to test the independence of the apo(a) type frequencies between the sample of FH patients and healthy subjects. The equality of Lp(a) levels among strata was tested using the analysis of variance (26) and the Mann-Whitney rank sum or Kruskal-Wallis nonparametric tests (27). A two-way analysis of variance and nonsequential hypothesis testing methods (28) were used to test hypotheses about the interaction of variation in the LDL receptor and apo(a) loci as they affect Lp(a) levels. These methods yield a valid test of interaction in the presence of an unbalanced design as we have here. Fisher's  $z$ -transformation was used to test hypotheses about the estimated correlation coefficients (29).

## RESULTS

Lp(a) levels were determined in 102 Caucasian patients with FH and in 279 healthy subjects, representing a random

Table 1. Lp(a) phenotype frequencies in healthy Tyroleans and in patients with FH

Phenotype	Random population		FH patient(s)	
	<i>n</i>	Frequency, %	<i>n</i>	Frequency, %
B	3	1.08	1	0.98
S1	8	2.87	4	3.92
S2	50	17.92	24	23.53
S3	58	20.79	17	16.67
S4	89	31.90	28	27.45
0	16	5.73	5	4.90
B/S1	—	0	—	0
B/S2	—	0	—	0
B/S3	—	0	—	0
B/S4	1	0.36	2	1.96
S1/S2	1	0.36	1	0.98
S1/S3	—	0	1	0.98
S1/S4	—	0	1	0.98
S2/S3	18	6.45	5	4.90
S2/S4	8	2.87	8	7.84
S3/S4	27	9.68	5	4.90
Total	279	100	102	100

Pearson's  $\chi^2$  test,  $P = 0.12$  (not significant).

Table 2. Lp(a) concentration in the Tyrolean population and in patients with FH

Phenotype	Random population		FH patient(s)	
	<i>n</i>	Lp(a), mg/dl	<i>n</i>	Lp(a), mg/dl
B	3	61.7 $\pm$ 33.8	1	79.0
S1	8	34.4 $\pm$ 20.7	4	58.7 $\pm$ 32.0
S2	50	24.5 $\pm$ 24.2	24	62.8 $\pm$ 34.8
S3	58	10.2 $\pm$ 9.7	17	35.3 $\pm$ 31.6
S4	89	5.7 $\pm$ 7.6	28	23.4 $\pm$ 14.0
0	16	0.4 $\pm$ 1.3	5	2.0 $\pm$ 4.5
B/S1	—	—	—	—
B/S2	—	—	—	—
B/S3	—	—	—	—
B/S4	1	41.0	2	71.0 $\pm$ 8.5
S1/S2	1	84.0	1	36.0
S1/S3	—	—	1	79.0
S1/S4	—	—	1	52.0
S2/S3	18	27.9 $\pm$ 27.0	5	45.4 $\pm$ 11.9
S2/S4	8	34.1 $\pm$ 30.7	8	61.4 $\pm$ 36.9
S3/S4	27	8.8 $\pm$ 8.6	5	40.2 $\pm$ 24.1
Total	279	14.1 $\pm$ 19.3	102	41.3 $\pm$ 32.5

Lp(a) values are mean  $\pm$  SD, when indicated.

Caucasian population. Lp(a) levels were significantly different between the two groups. The mean Lp(a) concentration in the patients was 41.3 mg/dl (Table 2) compared to 14.1 mg/dl in the healthy subjects ( $P < 0.001$ ). The major gene affecting quantitative Lp(a) levels is the polymorphic apo(a) structural gene locus (7, 19, 20, 23, 24) that determines a genetic size polymorphism of apo(a) (Fig. 1). Differences in Lp(a) levels, therefore, might be due to apo(a) allele frequency differences between the FH and the healthy subjects, either randomly or due to association. We, therefore, have determined apo(a) phenotypes in the FH patients and compared them to those in the random sample (Table 1). Apo(a) phenotype frequencies were not significantly different between the two groups ( $P = 0.12$ ; not significant). In particular none of those types associated with high Lp(a) levels in the populations was significantly overrepresented in the patients. Any small differences between the frequencies shown in Table 1 are not significant and could not explain the large differences in Lp(a) levels.

The most revealing comparison was that of Lp(a) levels between patients and the random population for each phenotype separately (Table 2). This comparison clearly showed

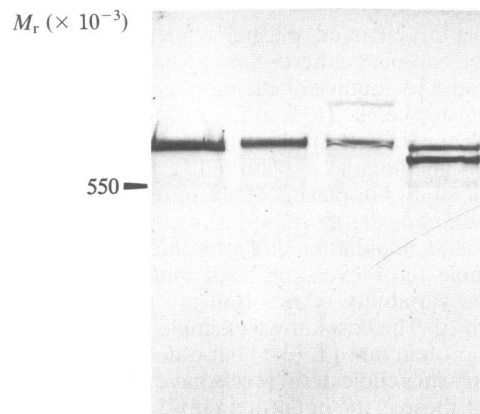


FIG. 1. Principal apo(a) phenotypes. Delipidated plasma samples were subjected to SDS/polyacrylamide gel electrophoresis. Apo(a) bands were visualized by immunoblotting using monoclonal anti-apo(a) antibody 1A<sup>2</sup>. Single-band types, representing homozygotes or heterozygotes with a null allele [lanes 1 and 2 (phenotype S2)], double-band types, representing heterozygotes [lanes 3 (phenotype S2/S4) and 4 (phenotype S1/S2)], are shown.

that Lp(a) levels are elevated in the patients when compared in each apo(a) phenotype. As in other samples studied (19, 24, 25), average Lp(a) levels are significantly different also among apo(a) types in the Tyrolean sample studied here. Lp(a) levels are also significantly different among apo(a) types in the group of FH patients ( $P < 0.001$ , Table 2). It is noteworthy that the rank order of apo(a) phenotypes according to average Lp(a) levels was the same in patients as in the population sample (Table 2). Hence, apo(a) gene effects on plasma Lp(a) levels are also demonstrable in the FH patients. However, considering each of the common apo(a) phenotypes, average Lp(a) levels were significantly higher in the FH patients than in the random sample. Considering only the common phenotypes S2, S3, S4, S2/S3, and S2/S4, the effects of the apo(a) polymorphism are indeed significantly different between the patients and the normal subjects. Using a two-way analysis of variance with an interaction term, there is a significant interaction between apo(a) variation and LDL receptor gene variation as they affect Lp(a) levels ( $P < 0.05$ ). The effects of the apo(a) types on Lp(a) levels are not the same in the sample of healthy subjects and the sample of FH patients. These gene loci do not combine in an additive fashion. Upon examination of Table 2, it appears that the variation at the apo(a) and LDL receptor genes affects Lp(a) levels in a multiplicative way. Mean Lp(a) levels were found to be elevated by  $\approx 3$ -fold in the patients. The multiplicative mode of interaction may be demonstrated by simply transforming the Lp(a) levels of the FH sample for each of the common phenotypes by dividing them by 2.99 to give the patient's sample the same grand mean as the population sample. There is then no significant difference between Lp(a) levels in the transformed FH sample and the controls and no significant interaction between the FH and apo(a) genes affecting plasma Lp(a) levels (Table 3). These data suggest that the LDL receptor defect increases Lp(a) levels 2.99 times regardless of the apo(a) type.

## DISCUSSION

The major findings of this study are that patients with FH have significantly higher Lp(a) levels than controls and that variations at the LDL receptor locus and the apo(a) locus act multiplicatively to affect plasma Lp(a) levels. The 3-fold higher Lp(a) plasma levels cannot be explained by a difference in apo(a) phenotype frequencies between the patients and random subjects. We have estimated the expected Lp(a) levels in the FH patients from the determined apo(a) type frequencies in the FH patients (Table 1) and from the phenotype-specific means in Lp(a) levels in the population (Table 2). This value was close to that in the controls and different from the observed value (data not shown). More-

Table 3. Mean Lp(a) levels in the random population and transformed mean Lp(a) levels in FH patients for the common apo(a) phenotypes

Phenotype	Lp(a), mg/dl	
	Random population	FH patients
S2	24.5	20.7
S3	10.2	11.8
S4	5.7	7.8
0	0.4	0.7
S2/S3	27.9	15.2
S2/S4	34.1	20.5
S3/S4	8.8	13.4

Lp(a) levels in FH patients were divided by 2.99 to give the random population and the patients the same grand mean.  $P = 0.17$  (not significant).

over, the gene loci for apo(a) and the LDL receptor are not linked, the locus for apo(a) being on chromosome 6 (21–23) and that for the LDL receptor on chromosome 19 (30), excluding the possibility that the observed effects are due to nonrandom allelic association between the LDL receptor and the apo(a) genes. Hence, the only reasonable conclusion from our data is that variation at the LDL receptor gene locus affects Lp(a) levels in plasma. The most striking argument for this is that Lp(a) levels were elevated for each of the apo(a) types (Table 2). To exclude the possibility that some of the differences in Lp(a) levels between the population and FH heterozygotes may relate to ethnic differences, we determined apo(a) types and levels also in a group of healthy Londoners ( $n = 61$ ). Lp(a) levels in the total group ( $16.7 \pm 17$  mg/dl) and in phenotype-matched subjects from London were not significantly different from those in Tyrol (data not shown).

Clearly the effect of the LDL receptor and the apo(a) genes on Lp(a) levels is not simply additive. In an additive model one would expect the same constant increase in average Lp(a) levels for each apo(a) phenotype. Rather, the findings in the FH patients are best explained by assuming a multiplicative interaction of the apo(a) and LDL receptor gene loci. This is a situation not commonly considered in quantitative genetics.

The effect of the LDL receptor defect on Lp(a) levels is not unreasonable because Lp(a) contains apoB-100, one of the two ligands for the LDL receptor. Hence defects in the LDL receptor gene might result in a delayed catabolism of Lp(a) in the same manner as it does for LDL (1). The  $\approx 3$ -fold increase in Lp(a) levels is close to the 2.5-fold increase typically observed in LDL caused by the LDL receptor defect (1). In the FH patients studied, LDL-cholesterol level (8.14 mmol/liter) was 2.2-fold elevated over healthy controls from London (3.7 mmol/liter). The marked effects of the heterozygous LDL receptor defect on Lp(a) levels imply that more moderate changes in LDL receptor activity might affect Lp(a) levels. Hence the contribution of genetic variation at the LDL receptor gene to variation in lipid metabolism in the population at large might be more important than is obvious from the relatively rare frequency of FH heterozygotes. Though such a mechanism seems plausible there is unfortunately a controversy on whether or not the Lp(a) lipoprotein binds to the LDL receptor. Results from *in vitro* binding studies are conflicting (31–33). Drugs that lower LDL levels by increasing the number of LDL receptors on the liver cell surface [e.g., bile acid sequestrants (34) and inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase] reportedly do not affect Lp(a) levels in plasma (35). These findings challenge the hypothesis that variation in the number of active LDL receptors directly affects Lp(a) levels in plasma. We, therefore, cannot exclude the possibility that there is increased synthesis of Lp(a) in FH in the same manner as it has been postulated for LDL (36). Studies of the turnover of Lp(a) of known apo(a) phenotype in healthy subjects and FH patients will be necessary to finally clarify the *in vivo* significance of the LDL receptor for Lp(a) removal.

The higher Lp(a) levels in the patients might be a direct consequence of their elevated LDL levels. This may be expected if plasma Lp(a) particles were derived from existing LDL or LDL-precursor particles. There was, however, no significant correlation between Lp(a) levels and plasma levels of total cholesterol ( $-0.130$ ; not significant) or LDL-cholesterol ( $-0.126$ ; not significant) in the total group of FH patients. This strongly argues against the concept that the Lp(a) levels in FH patients are simply a function of their elevated LDL levels. Our results do, however, imply that there exists a metabolic relationship between LDL and Lp(a).

Lp(a) is believed to be highly atherogenic but the mechanisms by which it exerts its deleterious effects are presently

unclear. The functions of apo(a) and Lp(a) are also unclear. The high homology of apo(a) with plasminogen has led to the speculation that Lp(a) is a link between the lipoprotein and clotting systems with effects on atherogenesis and thrombosis (37).

The atherogenic effect of elevated LDL levels is undisputed, whereas Armstrong *et al.* (38) have provided evidence that Lp(a) is highly atherogenic only in the presence of concomitantly elevated LDL levels, suggesting that LDL and Lp(a) act synergistically in producing atherosclerosis. This implies that FH patients of certain apo(a) types and with elevated Lp(a) levels would be especially prone to premature atherosclerosis. Indeed, we have observed a nonrandom association of coronary heart disease morbidity with apo(a) types and Lp(a) levels in the group of FH patients studied here (M.S., G.T., and G.U., unpublished data).

Apart from the possible practical implications of our finding for risk assessment in FH patients, we believe that the Lp(a) trait also represents an excellent model system to dissect genetic and environmental factors that govern a quantitative phenotype. Phenotypes like high blood pressure, blood glucose levels, or cholesterol levels are believed to be controlled by many genes interacting with environmental factors. Only a few genes have been identified that affect any of these traits. Nothing is known of how many genes control such phenotypes. So far cholesterol levels in plasma are one of the best examples for a continuous, disease-associated quantitative phenotype where variation at single gene loci significantly affects the variance of levels within populations. Among these are the genes for apoE and apo(a) (3–5, 7). Apo(a) levels in plasma by themselves represent a quantitative trait that may be easier to study than cholesterol levels. A goal of these studies is to relate single genes to lipoprotein and cholesterol levels and finally to the common chronic diseases themselves by understanding their interactions. The present study provides insights into the multifactorial cause of a quantitative phenotype associated with premature atherosclerosis.

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