Lipoprotein(a) in Homozygous Familial Hypercholesterolemia

H.G. Kraft, A. Lingenhel, F.J. Raal, M. Hohenegger, G. Utermann

Abstract—Lipoprotein(a) [Lp(a)] is a quantitative genetic trait that in the general population is largely controlled by 1 major locus—the locus for the apolipoprotein(a) [apo(a)] gene. Sibpair studies in families including familial defective apolipoprotein B or familial hypercholesterolemia (FH) heterozygotes have demonstrated that, in addition, mutations in apolipoprotein B and in the LDL receptor (LDL-R) gene may affect Lp(a) plasma concentrations, but this issue is controversial. Here, we have further investigated the influence of mutations in the LDL-R gene on Lp(a) levels by inclusion of FH homozygotes. Sixty-nine members of 22 families with FH were analyzed for mutations in the LDL-R as well as for apo(a) genotypes, apo(a) isoforms, and Lp(a) plasma levels. Twenty-six individuals were found to be homozygous for FH, and 43 were heterozygous for FH. As in our previous analysis, FH heterozygotes had significantly higher Lp(a) than did non-FH individuals from the same population. FH homozygotes with 2 nonfunctional LDL-R alleles had almost 2-fold higher Lp(a) levels than did FH heterozygotes. This increase was not explained by differences in apo(a) allele frequencies. Phenotyping of apo(a) and quantitative analysis of isoforms in family members allowed the assignment of Lp(a) levels to both isoforms in apo(a) heterozygous individuals. Thus, Lp(a) levels associated with apo(a) alleles that were identical by descent could be compared. In the resulting 40 allele pairs, significantly higher Lp(a) levels were detected in association with apo(a) alleles from individuals with 2 defective LDL-R alleles compared with those with only 1 defective allele. This difference of Lp(a) levels between allele pairs was present across the whole size range of apo(a) alleles. Hence, mutations in the LDL-R demonstrate a clear gene-dosage effect on Lp(a) plasma concentrations. (Arterioscler Thromb Vasc Biol. 2000;20:522-528.)

Key Words: lipoprotein(a) ■ apolipoprotein(a) ■ familial hypercholesterolemia ■ homozygous familial hypercholesterolemia ■ low density lipoprotein receptors

Whether mutations in the LDL receptor (LDL-R) gene affect lipoprotein(a) [Lp(a)] plasma levels has been addressed in several studies in the past.1–9 Because Lp(a) contains, in addition to apolipoprotein(a) [apo(a)], LDL (the principal ligand of the LDL-R), it was suggested that Lp(a) might be internalized and degraded via the LDL-R pathway. Consequently, it was postulated that Lp(a) concentrations should be increased in patients with familial hypercholesterolemia (FH), a condition caused by mutations in the LDL receptor (LDL-R), it was suggested that Lp(a) might be internalized and degraded via the LDL-R pathway. Consequently, it was postulated that Lp(a) concentrations should be increased in patients with familial hypercholesterolemia (FH), a condition caused by mutations in the LDL receptor (LDL-R). Several studies have focused on this theme, but conflicting results have been produced. One major reason for this dilemma might be the unique genetic control of Lp(a) levels.

Unlike all other lipoproteins, Lp(a) levels show an extreme interindividual variability and are largely controlled by variation in 1 major gene, which is the structural gene for apo(a).11,12 The apo(a) gene is highly polymorphic. One of the polymorphisms, the kringle IV (K-IV) polymorphism, which gives rise to the size polymorphism of the apo(a) protein, is responsible for a large part of the variation in Lp(a) plasma levels. In white populations, ~50% of the variation in the Lp(a) concentration is explained by the number of K-IV repeats in the apo(a) allele.11 The category of this effect is the same in every population studied to date, but the size of the effect differs between populations. The mechanism of this association was elucidated in in vitro cell studies that showed longer retention times for larger isoforms in the endoplasmic reticulum.13

However, isoforms with an identical number of K-IV repeats show a considerable divergence of Lp(a) levels, which is explained by sequence variation at or close to the apo(a) gene locus. Variation at the apo(a) gene locus explains from 70% to >90% of the variation in Lp(a) concentration.14 Hence, every study that intends to find additional factors/genes that affect Lp(a) levels has to consider and stratify for the strong impact of the apo(a) gene. One way to achieve this is by using sibpair analysis in which Lp(a) levels are compared in sibpairs who are identical by descent (IBD) for their apo(a) alleles. Recently, we have performed such an analysis for studying the influence of heterozygous FH on Lp(a) levels and found a significant influence of the disease state.5 In the present study, we expand this approach by analyzing families in which both parents are heterozygous for...
FH and which consequently also embody FH homozygous children. This scheme should allow us to determine whether there is a gene-dosage effect of the FH causing LDL-R mutations on the quantitative Lp(a) trait, thus providing further evidence for the effect of an LDL-R mutation on plasma levels of the atherogenic Lp(a).

Methods

Blood and plasma samples were drawn from 69 individuals belonging to 22 families who had at least 1 family member with homozygous FH by the Department of Medicine, University of Witwatersrand, Johannesburg, South Africa. The diagnosis of homozygous FH was based on the presence of (1) serum LDL cholesterol consistently >12 mmol/L, (2) the appearance of xanthomas in the first decade of life, (3) documentation of hypercholesterolemia or clinical features of the heterozygous state in both parents, and (4) confirmation of LDL-R gene mutations by DNA analysis. For some types of analysis, the sample was expanded by including data from South African families described previously.8

Screening for Mutations in the LDL-R Gene

Genomic DNA was extracted from whole blood collected in EDTA-containing tubes according to standard techniques.15,16 DNA screening for 3 founder-related Afrikaner mutations, D206E (Afrik), V408 (Afrik), and D154N (Afrik), was performed in a single reaction by a multiplex amplification refractory mutation system—polymerase chain reaction, as previously described.17 The DNA samples were also screened for mutation P664L (FH-Gujerat) previously identified in South African Indians.18 After screening for familial defective apoB, subjects negative for these mutations underwent a more extensive search by heteroduplex and/or single-strand conformational polymorphism analysis.17

Lp(a)/Apo(a) Analysis

Samples were shipped on dry ice to Innsbruck for Lp(a) and apo(a) analysis. Lp(a) levels were determined by ELISA,19 and the assignment of the plasma level to the 2 alleles of 1 individual was performed after SDS–agarose gel electrophoresis and immunoblotting as described.8

The number of K-IV repeats in the apo(a) alleles was determined by pulsed-field gel electrophoresis and Southern blotting.20 For the latter, genomic DNA was prepared as DNA-containing agarose plugs, which were subjected to a digestion with KpnI. The fragmented DNA was size-separated in a pulsed-field gel electrophoresis CHEF-mapper (Bio-Rad) and then transferred to a nylon membrane by alkaline blotting. The visualization of K-IV–containing fragments was performed with a DIG-labeled (Boehringer-Mannheim) cDNA probe11 followed by chemiluminescence detection. The size determination of the individual alleles was accomplished by comparison with secondary standards.

Statistical Analysis

Only nonparametric statistics were used because of the positively skewed distribution of Lp(a) levels. To compare median Lp(a) levels between groups, the Wilcoxon Mann-Whitney test was used, and pairwise comparisons were performed with the Wilcoxon matched-pairs signed rank test. A 2-tailed significance of P<0.05 was considered to be statistically significant: for multiple comparisons, the Bonferroni correction was applied. Apo(a) K-IV allele frequencies were compared by \( \chi^2 \) analysis after binning the alleles in the following way: for group 1, the number of K-IV repeats was 15 to 20; for group 2, 21 to 25; for group 3, 26 to 30; for group 4, 31 to 35; and for group 5, >35. By this procedure an underrepresentation of individual cells was avoided. Other forms of binning have also been performed (small versus large apo(a) alleles and 5 groups containing equal numbers of apo(a) alleles), which did not change the outcome.

Univariate ANOVA calculations were performed to search for factors affecting Lp(a) levels. All calculations were performed by using the SPSS (version 8.0 for Windows) program.

Results

Sixteen nuclear families, 4 families containing 3 generations, and 2 single homozygous FH individuals (Figure 1) were recruited for the present study. On average, the families had 1.6 children. In all nuclear families, the parents were heterozygous for FH. In addition, in the 3-generation families, 3 parents were FH–non-FH, and 1 parent pair was homozygous FH–non-FH. The sample included 26 homozygous individuals and 43 heterozygous relatives (parents, sibs, and children). Eighteen of the homozygous FH patients were homozygous or compound heterozygous for FH Afrikaner mutations 1, 2, or 3. These 3 founder mutations together accounted for >80% of FH in Afrikaners and have been confirmed to cause defective LDL-R function at the cellular level.17 Two subjects were homozygous for the FH-Gujerat mutation.18 LDL-R mutations in the remaining 6 subjects are yet to be fully characterized, but these 6 patients fulfilled all the clinical criteria for homozygous FH. Most of the family members who did not have heterozygous or homozygous FH were children. Therefore, non-FH family members were not included in the study for ethical reasons.

The mean and median Lp(a) levels in the homozygous versus the heterozygous family members were 49.9 and 36.6 mg/dL versus 29.9 and 14.4 mg/dL, respectively (Table 1). This difference was significant (P=0.004, Wilcoxon Mann-Whitney test). Because this difference could have been caused by a difference in the apo(a) allele frequencies between the 2 groups, we compared the K-IV allele frequencies between the FH homozygous and FH heterozygous individuals. A \( \chi^2 \) analysis of binned apo(a) alleles by size (Table 2) showed that the distribution of apo(a) K-IV alleles was indistinguishable between the 2 groups (\( \chi^2 \) 0.64, df 4, \( P>0.95 \)). Also, the frequency of “null” alleles was not different between FH homozygotes (13.5%) and FH heterozygotes (11.5%).

Because most of the families had only 1 child, it was not possible to compare Lp(a) levels in sibpairs IBD for apo(a) alleles but nonidentical regarding their FH state. Instead, we compared Lp(a) levels associated with alleles IBD, which were present either in an FH homozygote or FH heterozygote. This was accomplished by scanning of the apo(a) immunoblot from the apo(a) heterozygotes, which allowed assignment of the respective fraction of the total plasma Lp(a) concentration to each allele [allelic Lp(a) levels]. Forty such IBD allele pairs could be drawn from the sample. Again, higher average Lp(a) levels were found in association with IBD alleles if these were present in FH homozygous individuals (mean 22.6 mg/dL, median 12.55 mg/dL) as when present in FH heterozygous individuals (mean 12.7 mg/dL, median 5.8 mg/dL). Also, this difference was statistically significant (P=0.005, Wilcoxon matched-pairs signed rank test). On average, allelic Lp(a) levels were higher by 9.91 mg/dL in FH homozygotes than in FH heterozygotes. This difference between FH homozygotes and heterozygotes was present over the whole range of K-IV alleles and hence was not affected by the number of K-IV repeats (P=0.613 for the Spearman correlation coefficient between K-IV repeat number and \( \Delta \)Lp(a), Figure 2).

Because of the structures of the families, which were ascertained through FH homozygous children and in which
both parents were FH heterozygotes, we were unable to recruit a sufficient number of adult nonaffected blood relatives. To allow a comparison with FH unaffected individuals from the same South African population, we combined the data from the present study with those from our previous study in families with heterozygous FH. The nonaffected individuals from this previous family study consisted of nonaffected relatives (n = 57) and spouses (n = 43). From these 100 independent individuals, we were able to determine 190 allelic Lp(a) values. The box plot in Figure 4 shows allelic Lp(a) levels for unaffected individuals (n = 190), heterozygous FH individuals (n = 280), and homozygous FH individuals (n = 52). Median allelic Lp(a) levels rise from 4.95 mg/dL in unaffected individuals to 8.0 mg/dL in heterozygous FH individuals to 13.2 mg/dL in homozygous FH individuals. Lp(a) levels associated with individual apo(a) alleles are thus almost twice as high in homozygous FH compared with heterozygous FH and 3 times as high when homozygous FH individuals are compared with unaffected individuals. This difference is highly significant (P < 0.001). In addition to the average Lp(a) levels, the variance also increased with the number of mutated LDL-R alleles in a subject (Figure 4). The influence of the K-IV size polymorphism on allelic Lp(a) levels in the 3 genotypic groups is illustrated in Figure 3, and the values are given in Table 3. Apo(a) alleles were again binned into 5 groups for this presentation. In every size group, there is a gradual increase in average allelic Lp(a) levels from non-FH, to heterozygous FH, and further to homozygous FH. The intragroup difference was significant for 3 groups (15 to 20, 31 to 35, and >35 mg/dL).

### Table 1. Basic Description of FH Groups

<table>
<thead>
<tr>
<th></th>
<th>Present Study</th>
<th>Combined Data (From Present and Previous Study)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous FH</td>
<td>Heterozygous FH</td>
</tr>
<tr>
<td>n</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>Age mean, y</td>
<td>21.9</td>
<td>43.1</td>
</tr>
<tr>
<td>Sex ratio, male:female</td>
<td>1:1</td>
<td>1:1.7</td>
</tr>
<tr>
<td>Lp(a) mean, mg/dL</td>
<td>49.9</td>
<td>29.9</td>
</tr>
<tr>
<td>Lp(a) median, mg/dL</td>
<td>36.6</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Figure 1. Pedigrees of the 20 families, together with age (years), plasma Lp(a) level [mg/dL], apo(a) genotype (expressed as number of K-IV repeats), and LDL-R genotype of all 69 individuals. Homozygous and heterozygous individuals are illustrated as filled or half-filled symbols, respectively. In 4 FH heterozygous individuals, no plasma was available for Lp(a) determination. An asterisk indicates the homozygous presence of an LDL-R mutation in an individual. FH Afrikaner mutations are expressed as 1, 2, or 3, ex16 and ex9 indicate that the mutation is present in exon 16 and 9, respectively; prom indicates the presence of the mutation in the promoter region of the LDL-R gene; and 664 is the abbreviation for the Gujarati mutation.16
TABLE 2. Distribution of Apo(a) K-IV Alleles

<table>
<thead>
<tr>
<th>K-IV Repeats</th>
<th>Heterozygous FH</th>
<th>Homozygous FH</th>
</tr>
</thead>
<tbody>
<tr>
<td>15–20</td>
<td>9.3</td>
<td>11.5</td>
</tr>
<tr>
<td>21–25</td>
<td>19.8</td>
<td>21.2</td>
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<tr>
<td>26–30</td>
<td>23.3</td>
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<tr>
<td>31–35</td>
<td>38.4</td>
<td>36.5</td>
</tr>
<tr>
<td>36–45</td>
<td>9.3</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Analysis yielded χ² 0.64 (P>0.95).

K-IV repeats). In the 2 intermediate size groups (21 to 25 and 26 to 30 K-IV repeats), the significance level was not reached. The largest relative increase in average allelic Lp(a) levels (298%) was seen in the largest apo(a) size group (>35 K-IV repeats), and the largest absolute increase (34.5 mg/dL) was in the smallest group (15 to 20 K-IV repeats).

An additional method, univariate ANOVA, was applied to determine the factors that contribute to Lp(a) levels. This showed that Lp(a) levels in the sample of 20 families were explained significantly by 2 factors: the number of K-IV repeats in the smaller apo(a) allele (explaining 46% of the variation) and the number of mutated LDL-R alleles in a given individual (explaining 9.7% of the variation). Variables that were tested but gave no significant contribution included age, sex, K-IV repeat number of the larger apo(a) allele, and additional polymorphisms in the apo(a) gene (pentanucleotide repeat polymorphism, Met/Thr, and +93 C/T polymorphism).

Discussion

FH is a frequently occurring semidominant disease with a prevalence of 1:500 in the white population. Hence, for this condition, homozygotes who show very severe symptoms of the disease (very early onset of coronary artery disease with early myocardial infarction and giant xanthomas) can also be observed. They are estimated to have a frequency of 1:1 000 000. The disease is caused by a plethora of mutations in the LDL-R gene (>300 mutations are listed in the Human Gene Mutation Database: http://www.uwcm.ac.uk/search/mg). In some populations (eg, French Canadians, Afrikaners in South Africa, and Lebanese), FH frequencies are much higher, and a few specific mutations in the LDL-R are frequent because of founder effects. We took advantage of the high frequency of FH in Afrikaners to study the effect of FH homozygosity on Lp(a) levels and to compare FH homozygotes and heterozygotes.

Most previous studies have analyzed the situation in FH heterozygotes. Case-control studies that compared Lp(a) levels in heterozygotes and controls uniformly found higher Lp(a) in FH, but these findings changed when FH families were studied. Results from some studies were in line with those from the case-control studies, but others were not. Furthermore, some authors have concluded that LDL-R mutations that result in heterozygous FH do not affect Lp(a) levels. However, none of these studies controlled rigorously for the effect of the apo(a) gene locus by apo(a) genotyping. We have recently performed a large study of South African and French-Canadian families with heterozygous FH. Using apo(a) typing and a sibpair approach, we observed a significant effect of the FH status on Lp(a) levels, which were significantly higher in the apo(a) identical FH heterozygous siblings.

In the present study, we have extended our investigation to FH homozygotes. This not only confirmed our conclusions from the analysis of FH heterozygotes but also demonstrated a significant gene-dosage effect. Not only were Lp(a) concentrations higher in the combined FH homozygotes from the 20 families analyzed in the present study than in almost any other sample of the white population [only patients with nephrotic syndrome have been reported to have higher Lp(a)] but average Lp(a) concentrations were also significantly higher in the FH homozygotes (mean 49.9 mg/dL) than in the FH heterozygotes (mean 29.9 mg/dL) from the same families.

It is noteworthy that average Lp(a) levels in South African FH heterozygotes from our previous study (mean 35.4 mg/dL) were not different (P>0.05) from what we found in the present study in an independent sample from the same population (Afrikaners from South Africa). This is important because it allowed us to use Lp(a) levels in the FH heterozygotes as the common standard against which Lp(a) levels in FH homozygotes and in non-FH blood relatives could be compared (in our previous study, there were no FH homozygotes included, whereas in the present study, there were no nonaffected family members included, with both situations being due to the ascertainment and resultant structure of the analyzed families). When the data from our previous and the present study were combined, we obtained a very large data set, which showed a stepwise and significant increase from unaffected to heterozygous and homozygous family members (Table 1, Figures 3 and 4).

As previously mentioned, because of the small family size, it was not possible in the present study to compare sibpairs IBD for apo(a) alleles. Instead, we compared Lp(a) levels associated with alleles IBD between groups, a strategy we also had used in our earlier study.

There are some previous reports on Lp(a) levels in FH homozygotes, but the numbers of patients were low, no stratification for apo(a) gene effects was performed, and no
comparison with FH heterozygotes or nonaffected family members was made. Guo et al. analyzed Lp(a) and apo(a) phenotypes in 8 homozygous FH subjects and compared them with 40 normolipidemic subjects. Their homozygous FH individuals had exactly the same elevated mean Lp(a) level (50 mg/dL) as in the present study. They did not detect an association of the Lp(a) level with the size of the apo(a) isoforms, which is not surprising in view of the very low resolution of their phenotyping method (only 3 different isoforms were detected in the 8 homozygous FH subjects). If only intermediate-sized apo(a) alleles (21 to 35 K-IV repeats) had been detected in the present study, we also would have seen no influence of apo(a) allele size on Lp(a) levels (Figure 3), but in the apo(a) heterozygous FH individuals, Guo et al. also found a higher Lp(a) level associated with the smaller isoform.

Because of the peculiar genetic regulation and large interindividual variation of Lp(a) levels, studies intended to detect differences in Lp(a) concentrations between groups must obey certain rules. Because Lp(a) levels are determined to a very large degree by the apo(a) gene, the gold standard is to compare Lp(a) levels between individuals with both apo(a) alleles IBD. Because this was not possible within this group, we have used the second best approach: comparing allele-associated Lp(a) levels of IBD apo(a) alleles present in either homozygous or heterozygous FH individuals (Figure 1). In addition, it is necessary to investigate sufficiently high numbers of individuals to reduce the influence of fluctuations created by chance. The significant gene dosage effect would have been missed if, by chance, only individuals with very low differences (<5 mg/dL) who represented 47.5% of all pairs had been analyzed.

Rader et al. performed a turnover study that used radioactively labeled Lp(a) and LDL in 5 homozygous FH individuals, 4 heterozygous relatives, and 8 normolipidemic controls. They followed the disappearance of radioactivity from the plasma and calculated the fractional catabolic rate (FCR). They concluded that the catabolism of Lp(a) was not significantly different in the homozygous FH individuals compared with the heterozygous parents and controls. This is certainly true for the homozygous FH patient 2 shown in Figure 2 of their study, but reanalysis of the kinetic parameters in Table 1 of Rader et al. gave a different result. The FCR of Lp(a) was significantly reduced in homozygous and heterozygous FH individuals when compared with normal controls (P = 0.020). The effect of mutations in the LDL-R gene on FCR of Lp(a) was not as pronounced as it was on the FCR of LDL, but it was present! This new finding is also more compatible with a further finding of that study. Rader et al. showed that a fraction of the Lp(a) appears in the LDL density range and that this newly Lp(a)-derived LDL, also designated as Lp(a), has the same reduced FCR in FH individuals as does LDL. If this conversion of Lp(a) to LDL is a significant part of Lp(a) catabolism and is not due to the labeling treatment, as suggested earlier by Knight et al., then one should also expect a reduced FCR of Lp(a) in FH subjects.

Together, our data clearly demonstrate that average Lp(a) concentrations in FH homozygotes are in a range above the 90th percentile of Lp(a) in healthy white populations and twice as high as in FH heterozygotes. This leaves little doubt that LDL-R mutations that result in FH with elevated LDL also result in hyperlipoprotein(a).
The challenging question now is what is the mechanism underlying this effect may be. In vivo the LDL-R seems to play a minor role in the uptake of Lp(a), although this is still under debate. Possibly, the elevated levels of Lp(a) in FH are not a consequence of the LDL-R defect but result from poorly understood metabolic changes in FH. These might include the metabolism of fatty acids, which is known to affect lipoprotein synthesis or the so-called direct synthesis of LDL. Potentially, elucidation of this mechanism will shed light not only on the regulation of Lp(a) metabolic pathways but also on secondary metabolic disturbances in FH.

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