Diverse effect of ethnicity on plasma lipoprotein[a] levels in heterozygote patients with familial hypercholesterolemia

Eran Leitersdorf,* Yechiel Friedlander,† Jean-Marie Bard,** Jean-Charles Fruchart,** Shlomo Eisenberg,* and Yechezkiel Stein*

Department of Medicine B,* Lipid Research Laboratory, Hadassah University Hospital, Jerusalem, Israel; Department of Social Medicine,† Faculty of Medicine, Hebrew University-Hadassah Medical School, Jerusalem, Israel; and Service de Recherches sur les Lipoprotéines et l'Artérosclérose et INSERM,** Institute Pasteur, 1 rue du Pr. Calmette, 59019, Lille Cédex, France

Abstract Plasma lipids, lipoproteins, and lipoprotein[a] (Lp[a]) levels were determined in 216 members of 14 families with familial hypercholesterolemia (FH). Ninety-nine subjects harbored a mutant low density lipoprotein (LDL) receptor allele as confirmed by molecular genetic analysis. Four different mutant alleles were identified, each in a defined genetic group, Druze, Christian-Arabs, and Ashkenazi and Sephardic Jews. The findings in FH subjects (cases) were compared with their nonaffected family members (controls). Plasma Lp[a] levels increased with age in the controls but not in cases and were different among the four genetic groups. Mean plasma Lp[a] levels were significantly higher in cases (33 mg/dl) than in controls (22 mg/dl). Plasma LDL cholesterol levels were raised in cases of the four genetic groups to a similar extent, in contrast to the mean plasma Lp[a] that varied. The Lp[a] level was higher by 30–33% in cases from the Druze, Christian-Arabs, and Jewish-Ashkenazi groups but by 110% in the Jewish-Sephardic group. Apo[a] isoform distribution was similar in cases and controls within each genetic group. Lp[a] levels were highest in subjects with Lp[a] isoform, in particular among the Jewish-Sephardic group. These data indicate that the higher Lp[a] levels in FH heterozygotes cannot be attributed solely to lack of functional LDL receptor molecules but possibly reflect multiple gene interactions. A clue to the understanding of the regulatory processes that determine plasma Lp[a] levels emerges from the observation that its levels are two- to threefold higher in heterozygote patients with FH as compared to controls (5-7). It has been suggested that the findings in FH reflect a multiplicative interaction of the apo[a] and the LDL receptor gene loci (5). In these studies, however, the entry criteria for FH were based on clinical grounds alone and both the cases and the control groups were genetically undefined.

Supplementary key words cholesterol metabolism • human genetics • polymerase chain reaction

Lipoprotein[a] (Lp[a]) has recently been recognized as an independent risk factor for ischemic heart disease (IHD). This association was shown in population groups of diverse origins and in individuals with familial hypercholesterolemia (FH) (1, 2). Lp[a] is a plasma lipoprotein particle that contains all the constituents of low density lipoprotein (LDL) and a specific glycoprotein molecule designated apo[a]. Apo[a] exhibits a high degree of homology with plasminogen and contains the protease domain, one kringle 5, and multiple repeats of kringle 4 domains (3). Lp[a] appears in plasma in several polymorphic forms that reflect the presence of genetically determined apo[a] isoforms (4). In spite of the growing interest in Lp[a], neither the site of synthesis and mode of secretion nor the pathways responsible for clearance of Lp[a] from the plasma compartment have been clarified.


Abbreviations: LDL-C, low density lipoprotein-cholesterol; cLDL-C, corrected LDL-C; FH, familial hypercholesterolemia; IHD, ischemic heart disease; PCR, polymerase chain reaction; Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; HDL, high density lipoprotein; VLDL, very low density lipoprotein; TG, triglyceride.
MATERIALS AND METHODS

Materials

Restriction endonucleases, DNA polymerase I (Klenow fragment), and other enzymes were obtained from New England Biolabs (Beverly, MA), Boehringer Mannheim (Mannheim, Germany), and Pharmacia (Piscataway, NJ). Thermus aquaticus DNA polymerase I was obtained from Perkin-Elmer Cetus Co. (Norwalk, CT). $[^{32}P]dCTP$ (3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Biotrans nylon membranes were purchased from ICN Biochemical (Irvine, CA). Oligonucleotides were synthesized by Biotechnology Inc. (Rehovot, Israel). DNA was amplified using the Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). Plasma lipids were determined on Vitatron model FPS-A batch-analyzer (Vital Scientific, The Netherlands).

Recruitment of patients and families

The following inclusion criteria were used for patient and family recruitment. Index cases were patients with hypercholesterolemia (plasma LDL-cholesterol level above the 95th percentile compared with the age- and sex-specific LDL-C distribution) (8) with at least one first degree relative with ischemic heart disease (IHD), tendon xanthomas and/or corneal arcus. All available family members of these probands were recruited. FH, defined as an LDL receptor mutation or LDL receptor haplotype associated with hypercholesterolemia, was present in 14 families included in the present analysis.

Biochemical determinations

Plasma total triglyceride, cholesterol, and HDL-cholesterol levels were determined using commercially available diagnostic kits (Boehringer Mannheim, Germany). Plasma LDL-cholesterol levels were calculated according to the Friedewald-Levy formula (9). Plasma Lp[a] concentrations were determined on frozen (-70°C) samples using a double antibody technique. A selective bi-site ELISA assay was used (10). LDL-C levels were corrected for the relative contribution of the cholesterol content of the Lp[a] particle. The following formula was used: corrected LDL-C (cLDL-C) = LDL-C - 0.31 x Lp[a] mass; assuming an average molecular mass of apo[a] of 500 kDa. Apo[a] isoforms were determined on the same plasma samples using 6.6% SDS-polyacrylamide gel electrophoresis under reducing conditions as described by Utermann et al. (4). Apo[a] bands were visualized by immunoblotting with a mixture of monoclonal antibodies to apo[a] (K07, K09). The apparent mass of the isoforms ranged from 400 to 700 kDa. Six major apo[a] isoforms could be detected and were designated Lp$^{S_1}$, Lp$^{S_2}$, Lp$^{S_3}$, Lp$^{S_4}$, Lp$^{B}$, and Lp$^{F}$. Subjects without a detectable apo[a] band were designated Lp$^{0}$. Family data were used to confirm the autosomal co-dominant segregation of the apo[a] isoforms.

Determination of LDL-receptor RFLPs

Genomic DNA was extracted from blood leukocytes (11) and diluted to a final concentration of 0.1 mg/ml. Ten RFLP sites were determined (12). Eight sites (BsmI, Spel, ApaLI-5', PvuII, NcoI, PsI, ApaLI-3') were analyzed using the Southern blotting technique (13) and the other two (StuI and AvaII) were determined using the polymerase chain reaction (PCR) (14) and restriction analysis.

Determination of haplotypes from multilocus RFLP data

Genotypes at 10 RFLP sites were determined for individual members of the families whose LDL receptor mutations were yet unknown. Haplotypes for each individual were constructed by analysis of the joint segregation of the RFLPs in all members of each pedigree. Construction of the haplotypes was based on the assumption that there had been no recombination within the LDL receptor locus in these families.

Direct detection of the “Lebanese” allele

The “Lebanese” mutation is a single nucleotide substitution that produces a premature termination codon and a truncated LDL receptor (15). We used PCR amplification and restriction analysis for direct detection of this mutation. Oligonucleotides LM-I(upstream) (5'–GAGTGAACTGGTGTGACCACGAGGAGCCAC-3') and LM-II(downstream) (5'–CTGTGAGGCAGCTCTTACATGTCGCTG-3') complementary to exon 14 sequences that flank the mutation were used. The amplified DNA was subjected to restriction analysis using Hinfl enzyme (New England Biolabs) and electrophoresis on a 6% polyacrylamide gel. Results were read directly from gels following ethidium staining.

Direct detection of the “Lithuanian” allele

The “Lithuanian” mutation is a 3-bp deletion in exon 4 of the LDL receptor gene (16) previously designated FH Piscataway (17). We used PCR amplification and heteroduplex analysis for direct detection of this LDL receptor mutation in FH heterozygotes. Oligonucleotides D1(upstream) (5'–CCCCAGCTTGGGGCGCGACACG-3') and D2(downstream) (5'–CGCCCCATACCCAAGTCGGGAG-3') were used for amplification of the 3' end of exon 4 of the LDL receptor gene. Following amplification, the DNA was size fractionated on a 6% polyacrylamide gel and subjected to ethidium staining.
Statistical analysis

We first investigated the effect of age and gender on plasma LDL-C and Lp[a] concentrations. Next, in order to compare these levels between individuals, subjects were stratified into sex and age groups, 20 years each. Stratum specific mean ($\bar{x}$) and standard deviations (SD) were computed. For each study participant adjusted values were computed as follows:

$$x' = \left( \frac{x - \bar{x}}{SD} \right) \ast SD' + \bar{x'}$$

where: $X'$ is the adjusted value; $X$ is the unadjusted trait value; $\bar{x}$ and $SD'$ are the means and the standard deviations for the "standard" group, males aged 20–39. In addition we have used two other methods for sex and age adjustment: 1) instead of using the means and the standard deviations we applied the same formula to the medians and the inter-quartile ranges; and 2) a regression analysis with LDL-C and Lp[a] as dependent variables and sex, age (introduced as a continuous variable), age*sex, and sex*age as independent variables. Since all three methods showed similar results in subsequent analyses, we decided to present those obtained by the mean variance method. The null hypothesis of equality of sex- and age-adjusted mean Lp[a] levels in cases and controls was tested by using the non-parametric Mann-Whitney test (18). The equality of Lp[a] levels among the different types of mutations in the LDL receptor was tested by using the Kruskal-Wallis test (18). Since plasma Lp[a] levels were significantly skewed, the Box and Cox power transformation (19) was applied to produce approximately normal distribution of the data prior to the parametric multivariate analysis. Analysis of variance and multiple regression were applied to estimate the independent contribution of an LDL receptor mutation, the different types of mutations, and the different apo[a] isoforms to the variability of Lp[a] levels. The homogeneity of the effects of different types of LDL receptor mutations on plasma Lp[a] levels was tested through these models by introducing interaction terms between the LDL receptor mutation and the type of the mutation. Specifically, the following independent variables were included in the regression model: 1) a dummy variable indicating the presence of a mutation in the LDL receptor; 2) three dummy variables defined for the different types of LDL receptor mutations keeping those with the Druze mutation as a reference group; 3) five dummy variables defined for characterization of the isoform groups keeping the null isoform as a reference group; and 4) interaction terms between the different types of mutations and the different isoforms. The likelihood of this saturated model was compared to a reduced model that included only the main effects as predictors of the adjusted and transformed plasma Lp[a] levels. Since the values of the members within a family are not independent of each other, significance tests will have to be interpreted as estimates of strength of association between the variables.

RESULTS

Haplotype analysis at the LDL receptor gene locus was performed on four Jewish-Sephardic and two Druze families with familial hypercholesterolemia. Co-segregation analysis revealed that LDL receptor haplotype number 1 (BsmI+, Sphi+, Stul+, AvaII+, Psl-, ApaLI-5'+, PvuII-, Ncol+, PstI+, and ApaLI-3' +) and number 16 (BsmI-, Sphi+, Stul-, AvaII-, Psl-, ApaLI-5', PvuII+, Ncol+, PstI-, and ApaLI-3' +) co-segregated with hypercholesterolemia in the Druze and the Jewish-Sephardic FH families, respectively. The presence of the "Lebanese" mutation and of the "Lithuanian" mutation was confirmed by PCR in individuals from five Arab and three Jewish-Ashkenazi families, respectively.

The mean age of the cases (family members with FH) and controls (family members without FH) was almost identical, 30.0 (range 1–86) and 30.4 (range 1–83) years, respectively (Table 1). In the cases, 49 were males and 50 were females; in the controls, there were 54 males and 63 females. Sex- and age-adjusted mean plasma lipid and lipoprotein levels are shown in Table 1. Total plasma and LDL-C levels are shown in Table 1. Total plasma and LDL-C levels were significantly higher in the cases. After correction for the relative contribution of Lp[a]-cholesterol, LDL-C was more than twofold higher in the cases as compared to controls, 298.8 and 136.3 mg/dl, respectively ($P < 0.001$). Plasma TG levels were higher in the cases while HDL-C levels were significantly lower. Mean plasma Lp[a] level was 50% higher in cases as compared to controls, 33.0 and 22 mg/dl respectively ($P < 0.001$). In both cases and controls mean plasma Lp[a] concentrations did not differ between males and females, 31.4 and 35.5 in cases ($P = 0.20$) and 23.9 and 21.2 in controls ($P = 0.26$), respectively.

<table>
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<tr>
<th>TABLE 1. Subject characteristics</th>
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<tr>
<td>Controls n = 116</td>
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<tr>
<td>Age (yr)</td>
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<tr>
<td>TC (mg/dl)</td>
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<tr>
<td>LDL-C (mg/dl)$^a$</td>
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<td>cLDL-C (mg/dl)$^a$</td>
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<td>HDL-C (mg/dl)</td>
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Data are means ± SD; TC: Plasma total cholesterol; TG, Plasma triglycerides; ns, not significant.

$^a$Calculated as described (9).

$^b$Lp[a] corrected for the contribution of Lp[a] cholesterol according to the formula described in Materials and Methods.
Corrected LDL-C levels increased significantly with age both in cases and in controls (Fig. 1A). It is interesting to note that in a few subjects with genetically defined FH the cLDL-C was close to the normal range while a few controls showed elevated cLDL-C levels. In such subjects phenotypic expression could result in misclassification. As expected, Lp[a] levels both in cases and controls showed marked variation from very low to extremely high levels (Fig. 1B). In the controls a moderate age-related increase of plasma Lp[a] was found \( (r = 0.35; P < 0.001) \). In contrast to this finding, Lp[a] levels did not change with age in the FH patients \( (r = 0.07; \text{ns}) \).

Corrected LDL-C and plasma Lp[a] levels in cases and controls in the four genetic groups are shown in Fig. 2. Corrected LDL-C levels were significantly elevated \( (P \leq 0.001) \) in cases as compared to controls, approximately to the same extent in all four groups (Fig. 2A). Sex- and age-adjusted plasma Lp[a] levels for the total group were significantly different between cases and controls \( (P = 0.001) \) as well as among the four origin groups \( (P = 0.007) \). Similar significant origin differences were found for the cases \( (P = 0.03) \) and the controls \( (P = 0.008) \). Due to the asymmetric distribution of Lp[a] levels in our sample, the analysis that utilized parametric statistical tests was carried out after an appropriate transformation of the data. In the total sample, mean sex- and age-adjusted plasma Lp[a] levels were significantly different \( (P = 0.02) \) across the four origin groups.

When the control groups were analyzed separately, mean Lp[a] levels were significantly different among the origin groups \( (P = 0.032) \) being lowest in the Druze and highest in the Ashkenazi (Fig. 2B). In cases, Lp[a] levels also differed across the groups being, however, highest in the Sephardics \( (P = 0.08) \). In a multivariate analysis the effects of genetic background (origin groups) and of the presence of a mutation in the LDL receptor gene maintained their significance levels \( (P_{\text{origin}} = 0.003, P_{\text{mutation}} = \ldots) \).

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**Fig. 1.** Panel A: Sex-adjusted, age-related plasma cLDL-C levels (mg/dl) in 99 individuals with molecularly defined LDL receptor mutant alleles (solid circles, \( y = 1.74x + 223.7, r = 0.50, P < 0.001 \)) and 116 normal family members (open circles, \( y = 1.00x + 90.6, r = 0.56, P < 0.001 \)). Panel B: Sex-adjusted, age-related plasma Lp[a] levels in the same individuals (cases, \( y = 0.11x + 30.1, r = 0.07, \text{ns} \); controls, \( y = 0.31x + 12.0, r = 0.35, P < 0.001 \)).

**Fig. 2.** Sex- and age-adjusted plasma cLDL-C and Lp[a] levels (mg/dl) in FH individuals and normal controls from four different genetic groups. The number of FH individuals (solid bars) and controls (open bars) in each group, respectively, were as follows: Druze (20, 18), Lebanese (45, 54), Ashkenazi (10, 15), and Sephardic (24, 32). The group-specific means (+SE) for cLDL-C are shown in panel A and for Lp[a] in panel B. The median Lp[a] levels are indicated with an asterisk.
In addition, the interaction term between mutation in the LDL receptor gene and the type of mutation in the four genetic groups was borderline significant ($P = 0.054$). Plasma Lp[a] levels within each of the genetic groups were higher in cases than in their respective controls, approximately by 33% in cases with the Druze, Lebanese, and Lithuanian alleles and 110% higher in the Sephardic group (Fig. 2B).

Apo[a] isoforms were determined in all subjects. In the total group the distribution of apo[a] isoforms was similar in cases and controls (chi-square = 17.8, df = 11; $P = 0.09$), except for a low proportion of subjects with Lp0 and a higher proportion of subjects with LpSI in the cases (Table 2). This difference could be due to relatively low sensitivity of the assay used for detection of apo[a] isoforms.


data is means ± SD of the number of subjects in each category.

### DISCUSSION

Two recently published reports demonstrated two- to threefold higher plasma Lp[a] levels in patients with FH.
as compared to healthy individuals (5, 7). In the first study, FH patients in the city of London were compared with a random sample of blood donors from the province of Tyrol, Austria. In the second study the FH patients recruited in Göteborg and Stockholm were compared to a normocholesterolemic control sample from Göteborg. Neither the patients nor their controls were genetically characterized. The present investigation demonstrates similar, albeit less pronounced, elevation of plasma Lp[a] levels in FH individuals characterized at the molecular level. The controls were their family members shown to be free of the respective LDL receptor mutation. Moreover, with the approach used in the present investigation, it was possible to compare subjects with defined LDL receptor mutant alleles. The four alleles characterized represent four discernable populations in Israel: Druze, Christian-Arabs of Lebanese origin, and Ashkenazi and Sephardic Jews. Higher Lp[a] levels were found in cases of all four genetic groups although to a different extent.

The design of the present investigation allowed the analysis of plasma lipids and Lp[a] levels in subjects over a wide range of age. Variation of Lp[a] plasma levels with age has not been reported so far. In the control group we found an age-related increase of plasma Lp[a] level that was similar to that of plasma LDL. This was true for all control subjects when analyzed together as well as for each of the genetic groups. This finding indicates that at least in this respect the behavior of Lp[a] is similar to that of the apoB-100-containing lipoproteins, VLDL and LDL (8). A different relationship was observed in cases where Lp[a] levels did not increase with age. A possible explanation for this phenomenon in the FH group is a nearly complete absence of cases above the age of 54 years with plasma Lp[a] levels above 30 mg/dl. Noteworthy, many FH individuals older than 54 years were identified whose plasma Lp[a] levels were below 30 mg/dl. Assuming that the main cause of death in FH is IHD, this observation supports the suggestion that the association of high plasma LDL-C with high plasma Lp[a] is particularly atherogenic (6).

The novel observation made in this study is that the magnitude of the elevation of Lp[a] associated with FH differs among the four genetic groups, all with defined LDL receptor mutations. For example, when cases with the Lebanese mutation and cases with the Sephardic allele are compared to their respective controls, Lp[a] levels are higher by only 33% in the former group but by 110% in the latter. Yet in the controls of these two groups, the plasma Lp[a] level is similar or even slightly lower in the Sephardic group. Also, in both groups apo[a] isoform distribution was similar when comparing the cases to controls, except for Lp54 which is more prevalent in cases of both groups. Therefore, the marked difference in plasma Lp[a] levels found in the cases of these two groups is most probably not due to different regulation at the apo[a] gene locus. Another possible explanation of the high Lp[a] levels in cases as compared to controls is the expression of only one functional LDL receptor allele in heterozygote FH subjects that differs between genetic groups. In cases from these two groups, however, cLDL-C was elevated to a similar extent, 210% and 219% as compared to their respective normal controls. This indicated that the expression of the one functional LDL receptor allele in the heterozygous FH is similar in cases with the Lebanese mutation and with the Sephardic allele. We would have expected similarly higher plasma Lp[a] levels in the two groups if the elevated Lp[a] levels resulted solely from lack of functional LDL receptor in FH heterozygotes. This was not observed. Could different mutations in the LDL receptor account for the difference in the Lp[a] levels between the two groups? The Lebanese mutation results in the synthesis of a truncated LDL receptor and hence in its complete absence from the cell surface (15). The nature of the mutation in subjects with the Sephardic allele is not yet known but human skin fibroblasts from homozygous patients do not bind, internalize, or degrade LDL (Leitersdorf, E., et al., unpublished observation). One could speculate that if, in the subjects with the Sephardic allele, there are defective LDL receptors that can bind but not internalize Lp[a], the normal catabolism of Lp[a] could be impeded. However, it seems unlikely that it would have caused such a marked elevation of Lp[a] levels in comparison to patients that completely lack the mutant receptors on the cell surface. In addition, it appears that cases with Lp54 and the Sephardic allele exhibit higher plasma Lp[a] levels as compared to those with the Lebanese allele or to cases with the same Sephardic allele but with other apo[a] isoforms. If this observation is confirmed it would further indicate that Lp[a] elevations in FH may reflect processes that are independent, at least in part, of the presence of the LDL receptor mutation. However, the possibility that the Lp54 phenotype includes several distinct apo[a] alleles that respond differently to specific LDL receptor mutations cannot be ruled out.

The considerations discussed above lead us to conclude that the higher Lp[a] levels in FH heterozygotes are not simply due to retarded clearance of Lp[a] from the plasma through the LDL receptor pathway. This view is supported by observations that in human cells the affinity of Lp[a] towards the LDL receptor is lower than that of LDL (20–22), although studies in transgenic mice indicated that the receptor may play a significant role in the clearance of Lp[a] from the plasma (23). In humans, turnover studies suggested that plasma Lp[a] levels are primarily regulated by the rate of synthesis of the apo[a] protein (24). Hence, a possible mechanism that may cause high plasma Lp[a] levels in patients with FH is accelerated synthesis of apo[a] in the liver. If that is the case, interactions between the products of the apo[a] gene and of other genes occur specifically in FH. Such genes should be sensitive to the “FH state” and to the activity of LDL recep-
tors but apparently do not include the LDL receptor gene. They could be regulated differently in FH patients of diverse genetic groups. In accordance with their respective mode of inheritance, they may even demonstrate differential expression in individuals from the same genetic background. In our sample, the wide distribution of plasma Lp[a] levels in cases supports this notion. Genes regulated by sterols in the cholesterol biosynthetic pathway and genes that control the intracellular packaging, transport, and secretion of Lp[a] are possible candidates. Clearly, additional studies involving molecular genetic analysis of the apo[a] and other genes are needed for further elucidation of processes that regulate plasma Lp[a] levels.

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