Abstract—Heterozygous familial hypercholesterolemia (FH) is a highly atherogenic genetic disorder leading to premature coronary heart disease (CHD), usually before 60 years of age. We studied an extended multigenerational kindred with FH linked to chromosome 1p32 in which atherosclerotic complications were either delayed or prevented in individuals with elevated HDL cholesterol (HDL-C) levels or hyperalphalipoproteinemia (HA). Premature CHD was observed in FH individuals without HA. The study of this family established that the HA trait in the family also followed an autosomal dominant mode of inheritance with a pattern of segregation independent from FH. We identified a locus on chromosome 6 linked to elevated HDL-C levels (HA) in this family. Haplotype analysis refined the localization to a 7.32-cM interval (73 to 80 cM from pter) flanked by markers D6S1280 and D6S1275. Parametric 2-point and multipoint analyses yielded maximum LOD scores of 3.05 and 3.17, respectively. This finding was confirmed with a nonparametric multipoint score of 3.78 (P = 0.0009). We propose that this locus, linked to elevated HDL-C levels, confers protection against premature CHD within an FH context. (Circ Res. 2003;92:569-576.)

Key Words: LDL cholesterol ■ HDL cholesterol ■ genetics ■ familial hypercholesterolemia ■ hyperalphalipoproteinemia
FH is genetically heterogeneous with mutations in different genes affecting different families. The most common cause is mutations in the LDL receptor (LDLR) on chromosome 19 (19p13), with >800 mutations reported to date (LDLR Mutation Database; http://www.umd.necker.fr and http://www.umd.necker.fr are databases exclusive for LDLR mutations). A less common cause is mutations in the apoB gene on chromosome 2p23-p24 (FDB, MIM 144010), with only 3 reported mutations.7,8 Finally, genetic studies have identified in third locus on chromosome 1p34.1-32.9,10

As opposed to hypercholesterolemia, increased levels of HDL cholesterol (HDL-C) or hyperalphalipoproteinemia (HA) was admitted to the Instituto Nacional de Ciencias Médicas y Nutrición, where she was diagnosed with gastritis and arterial hypertension. The patient smoked from age 15 to 30 years. She also suffered transient ischemic episodes. A Doppler ultrasound of the carotid arteries showed multiple plaques on both sides, especially on the left carotid artery. The largest lesion caused a 50% obstruction of the vessel lumen. The symptoms disappeared after diprydamole treatment. An ECG showed a left bundle branch blockmate. Hypercholesterolemia was diagnosed (425 mg/dL), and the patient was referred to the Lipid Clinic. On physical examination, she had a body mass index of 24.6 and blood pressure of 140/80 mm Hg. Pulses at the lower limbs were normal. Symmetrical arcus corneae and bilateral xanthomata at the Achilles tendons were detected and confirmed by ultrasound. Blood chemistry and thyroid-stimulating hormone concentrations were normal. A complete lipid profile revealed the following: total cholesterol level 395 mg/dL, LDL-C 220 mg/dL, HDL-C 98 mg/dL, apoA-I 190 mg/dL, and triglycerides (TGs) 110 mg/dL. The LDL-C accumulation was mainly explained by increased concentrations of the smaller and denser LDL-C particles. The HDL-C elevation was mainly due to the HDL2 subclass (which represented 52% of the total). Increased lipoprotein(a) concentrations were also detected (136 mg/dL).

A low-fat low-cholesterol diet and lovastatin (20 to 40 mg/d) treatment were prescribed but were followed irregularly. A lipid profile during the follow-up was as follows: cholesterol 281±21 mg/dL, LDL-C 179±18 mg/dL, HDL-C 90±8 mg/dL, and TGs 131±29 mg/dL.

The family history is consistent with FH. Her brother (II3) had a similar lipid profile and tendinous xanthoma (see online Table, available at http://www.circresaha.org). The proband (II2) had 2 sons and 1 daughter. Her oldest son (III1) died at 52 years of age of a myocardial infarction, and his lipids and clinical profiles were consistent with FH. Her second son (III3) and her daughter (III4) are also affected, according to their lipid profiles and the presence of tendinous xanthomata.

Forty members of the family were studied (all available subjects gave informed consent). Of these, 12 were diagnosed as affected according to the criteria outlined by Kwiterovich:26 7 of these exhibited tendinous xanthoma. All affected individuals showed LDL-C levels above the 90th percentile according to age and sex in the Mexican population.27 As shown in Figure 1, the FH and HA phenotypes are consistent with an autosomal dominant pattern of transmission and independent segregation.

Besides the proband, 3 other individuals (III3, aged 84 years; III4, aged 57 years; and III18, aged 49 years) exhibited elevated total cholesterol and LDL-C levels but no evidence of premature CHD. Individual II5, (proband’s brother) died of natural causes at 75 years of age. Of his offspring, 1 of his daughters (II16) had the HA phenotype, another (III20) had the HF phenotype, and his son had both phenotypes, which would support the assumption that this individual also carried both traits. A closer examination of the lipid profiles of the entire family led us to the observation that several members of the family had high levels of serum HDL-C. These data are available in the online data supplement, which can be found at http://www.circresaha.org. The criteria for HA was considered to be HDL-C >60 mg/dL because that level has been established to confer protection against CHD.28–30 This value corresponds to the 90th percentile (according to age and sex) in the Mexican population.27

Biochemical Determinations

Plasma lipids and blood chemistry were performed in 33 available individuals through fully automated tests with commercially available reagents. Serum concentrations of total cholesterol and TGs were determined by enzymatic methods (Boehringer-Mannheim). The HDL-C and LDL-C particle distributions were characterized through density gradient ultracentrifugation as described.31 The double precipitation method was used to measure the HDL-C subclasses as described.32 ApoA-I was measured by immunophelomtrix.33 Sitosterol levels were determined by capillary-gas-liquid chromatography.34

The diagnosis or status assignment for all family members was carefully established. Members with a single measurement of lipid
levels or borderline lipid values were considered as “unknown” for linkage analysis.

Analysis of Candidate Genes
DNA was extracted from whole blood using a phenol-free extraction protocol adapted from Buffone and Darlington. Analysis of microsatellite markers was performed by polymerase chain reaction amplification using end-labeled (\[\gamma\]32P]dCTP) primers. The role of different candidate genes in the expression of FH and HA was evaluated using genetic markers closely linked to these genes selected from various databases (Center for Medical Genetics, available at http://research.marshfieldclinic.org/genetics, and Genome Database, available at http://www.gdb.org). Candidate genes analyzed were as follows: LDLR and apoB for HF and apoA-I, LDLR and apoB for HA, apoA-II/apoC-III/apoA-IV cluster, apoC-II, apoE, ATP-binding cassette A type 1, peroxisome proliferator-activated receptor-\gamma, scavenger receptor class B type I, hepatocyte nuclear factor-4 \alpha, cholesterol ester transfer protein, lipoprotein lipase, and paraoxonase 1 for HA. In addition, apoE haplotyping was performed as described.

Genomewide Scan
Thirty-four family members were genotyped with a set of 238 autosomal markers (average spacing of 15 to 20 cm). Most markers used were trinucleotide or tetranucleotide repeats. Polymerase chain reaction products were analyzed on an ABI 377 sequencer through linkage analysis.

Linkage Analysis
A model assuming an autosomal dominant pattern of inheritance was used with a gene frequency of 0.001 and 90% penetrance. The maximum expected LOD score for this family was estimated through SIMULATE (LINKAGE software) using 1000 replicas. Two-point LOD scores were calculated with the use of FASTLINK. Allele frequencies for all markers were calculated from unrelated individuals from the Mexican population. Parametric multipoint analysis was performed with the use of GENEHUNTER-PLUS. To avoid any potential uncertainty in the exact mode of inheritance of HA, the data were analyzed with the model-free multipoint allele-sharing method implemented in this same program. Haplotypes were obtained with this same program and adjusted manually.

Results
Mapping of the FH Locus
We identified a kindred (CGZ) displaying all clinical characteristic of FH, including high total cholesterol and LDL-C, tendon xanthomata, arcus corneae, and premature CHD. We estimated the power of the family for linkage analysis through simulation. With the use of 1000 replicates, a maximum expected Z max of 5.9 was obtained (the average expected Z max was 2.1, with 57% >3.00).

Through linkage analysis, both LDLR and the apoB genes were ruled out as the underlying cause of this condition. For the apoB gene, screening for the 2 most frequent mutations (R3500Q and R3531C) was also performed for the proband and an affected brother. The possibility of sitosterolemia was excluded by direct sitosterol measurement in the proband.

A genomewide scan (chromosomes 1 to 22) was performed with 238 markers. Positive pairwise LOD scores were obtained for adjacent markers on chromosome 1. The maximum pairwise LOD score obtained was 2.94 at \(\theta=0.05\) for marker D1S197. Further analysis with a higher density of markers in this region showed positive LOD scores at \(\theta=0.0\) for 2 additional markers closely linked to D1S197 (D1S386 and D1S1661) (Table 1). We also analyzed the data set under an “affecteds-only” model (Table 1). Multipoint analysis gave a maximum LOD score of 3.29 between markers D1S2134 and D1S200 (Figure 2). Haplotype reconstruction showed that all 12 affected individuals for FH share a common haplotype encompassing markers D1S197 through D1S417. The centromeric boundary of this interval was defined by a recombination event between markers D1S417 and D1S200 in individual II 3, and the telomeric boundary was defined by a recombination event between markers D1S197 and D1S2134 in individual II 3 (Figure 3). This interval corresponds to a 6.75-cM region located 75.6 to 82.4 cM from pter. Five asymptomatic individuals and 2 with unknown affection status also share this same haplotype, implying incomplete penetrance.

Table 1. Pairwise LOD Scores for Chromosome 1p32 Markers and HF

<table>
<thead>
<tr>
<th>Locus</th>
<th>Distance*</th>
<th>(\theta=0.00)</th>
<th>(Z_{\text{max}})</th>
<th>(\theta_{\text{max}})</th>
<th>(\text{Affecteds Only})</th>
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<tr>
<td>D1S3721</td>
<td>72.59</td>
<td>-5.27</td>
<td>0.00</td>
<td>0.49</td>
<td>-1.56</td>
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<tr>
<td>D1S2134</td>
<td>75.66</td>
<td>-1.21</td>
<td>1.00</td>
<td>0.18</td>
<td>0.25</td>
</tr>
<tr>
<td>D1S197</td>
<td>76.27</td>
<td>-1.59</td>
<td>2.94</td>
<td>0.05</td>
<td>1.68</td>
</tr>
<tr>
<td>D1S386</td>
<td>77.18</td>
<td>0.80</td>
<td>1.18</td>
<td>0.10</td>
<td>2.41</td>
</tr>
<tr>
<td>D1S1661</td>
<td>76.25</td>
<td>1.82</td>
<td>1.82</td>
<td>0.00</td>
<td>1.60</td>
</tr>
<tr>
<td>D1S417</td>
<td>79.80</td>
<td>-1.41</td>
<td>0.14</td>
<td>0.30</td>
<td>1.90</td>
</tr>
<tr>
<td>D1S200</td>
<td>82.41</td>
<td>-2.99</td>
<td>0.60</td>
<td>0.22</td>
<td>-0.23</td>
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<td>D1S2867</td>
<td>85.68</td>
<td>-7.91</td>
<td>0.12</td>
<td>0.30</td>
<td>-1.00</td>
</tr>
</tbody>
</table>

*Distance in Kosambi centimorgans (cM) from pter.
Mapping of the HA Locus

On further clinical evaluation of the proband and other family members, we identified 10 individuals with elevated levels of HDL-C. Examination of the extended family led to the observation that HA segregated as an independent trait with an autosomal dominant pattern of inheritance and independent of FH (Figure 1). Thus, we tested the hypothesis that a locus associated with elevated HDL-C levels is present in this family.

Thirteen candidate genes were examined through linkage analysis to evaluate their possible contribution to the HA phenotype, including genes for several apolipoproteins, cholesterol ester transfer protein, the scavenger receptor class B type I, and transcription factors, such as hepatocyte nuclear factor-4α and peroxisome proliferator-activated receptor-γ (see Materials and Methods). No evidence of linkage was found to any of the analyzed genes.

Genome-wide multipoint analysis showed a single statistically significant locus, with a maximum LOD score of 3.17 and a maximum nonparametric (NPL) score of 3.78 ($P=0.0009$) between markers D6S1280 and D6S1275. The maximum 2-point LOD score and NPL values in this region were 3.05 and 3.08 ($P=0.004$), respectively, for the marker D6S1662 (Figure 4 and Table 2).

All 10 individuals with HA shared a common haplotype encompassing markers D6S2410 and D6S1053 (Figure 5). The centromeric boundary of this interval is defined by a recombination event between markers D6S1053 and D6S1275 in individual II2, and the telomeric boundary is defined by a recombination event between markers D6S1280 and D6S2410 in individual II3, defining a 7.32-cM interval on chromosome 6p located 73.1 to 80.4 cM from pter. There are no obvious candidate genes that directly regulate HDL metabolism in this region.
However, there are two proinflammatory cytokine genes in this interval: interleukin-17 (IL-17) and interleukin-17F (IL-17F). 40,41 Several proinflammatory cytokines have been implicated in the overall regulation of plasma HDL-C concentrations.42–44 Sequence analysis identified two polymorphisms in the promoter region of the IL-17 gene and one polymorphism (E126G) in exon 3 of IL-17F. None of these nucleotide changes cosegregates with elevated HDL levels in this family.

Discussion
Atherosclerosis is a multifactorial entity in which genetics and environmental factors play a role in the pathophysiology of the disease.45 Mutations in genes associated with FH, as well as mutations in genes that lower HDL-C levels, such as ABC1, are known to cause accelerated atherogenesis.2,8,46,47 On the other hand, factors such as cigarette smoking, arterial hypertension and dietary cholesterol consumption are associated with an increased risk of CHD.3,48,49 Several epidemiological and genetic studies confirm the association between elevated HDL-C levels and protection against atherogenesis.15,50,51 In the Framingham Heart Study, individuals with HDL-C concentrations of 1.5 mmol/L (60 mg/dL) or higher are protected against the development of CHD even in the presence of elevated LDL-C serum levels.28–30 In a different study, an association was found between an increase of HDL-C >45% and a decreased frequency of atherosclerosis.52

In addition, animal models have demonstrated the role of HDL-C as cardioprotective particles. For instance, hypercholesterolemic Watanabe heritable hyperlipidemic rabbits overexpressing the apoA-I protein showed a reduction in the formation of atherosclerotic plaques.53 Furthermore, overexpression of human apoA-I in transgenic mice led to an increase in HDL-C levels >150 mg/dL and a 95% reduction of the atherosclerotic plaques in C57BL/6 mice on a high-fat diet.54

We identified an FH kindred in which elevated HDL-C levels (HA) are displayed as an independent trait within the family. Finding concurrent expression of FH and HA within a family is highly unusual because elevated LDL-C levels are generally inversely correlated with HDL-C levels.55,56 Therefore, this kindred provides a unique opportunity to dissect the genetic component underlying the HA trait in a background of atherogenicity.

A striking feature found in this family is that 2 affected individuals aged >80 years (proband II2, aged 94 years, and her brother II3, aged 84 years) do not display the expected

<table>
<thead>
<tr>
<th>Locus</th>
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<th>Distance in Kosambi centimorgans (cM) from pter.</th>
</tr>
</thead>
<tbody>
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<td>D6S2410</td>
<td>73.13</td>
<td>1.53 1.52 1.45 1.32 0.97 0.54 0.14 1.53 0.00</td>
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<td>75.45</td>
<td>1.58 1.54 1.40 1.19 0.86 0.49 0.17 1.58 0.00</td>
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<tr>
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<td>76.62</td>
<td>1.50 1.78 2.15 2.21 1.92 1.34 0.60 2.21 0.10</td>
</tr>
<tr>
<td>D6S1662</td>
<td>77.78</td>
<td>3.05 3.03 2.89 2.67 2.09 1.39 0.60 3.05 0.00</td>
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<td>D6S1276</td>
<td>78.85</td>
<td>2.20 2.21 2.16 2.02 1.60 1.05 0.43 2.21 0.01</td>
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<td>D6S1952</td>
<td>79.92</td>
<td>0.39 0.38 0.34 0.30 0.22 0.14 0.07 0.39 0.00</td>
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<tr>
<td>D6S1053</td>
<td>80.45</td>
<td>1.92 1.88 1.71 1.50 1.06 0.61 0.21 1.92 0.00</td>
</tr>
<tr>
<td>D6S1275</td>
<td>80.45</td>
<td>1.86 0.74 0.12 0.47 0.60 0.45 0.17 0.61 0.18</td>
</tr>
<tr>
<td>D6S1031</td>
<td>88.63</td>
<td>7.35 3.11 1.25 0.43 0.14 0.23 0.12 0.23 0.30</td>
</tr>
</tbody>
</table>

*Distance in Kosambi centimorgans (cM) from pter.
atherosclerotic manifestations associated with this condition in spite of their elevated total cholesterol and LDL-C levels and additional risk factors [hypertension, elevated Lp(a), or hypertriglyceridemia]. The absence of symptomatic CHD in these individuals is in stark contrast to the observed incidence of CHD in FH heterozygotes aged >60 years.1

The observed protection against premature CHD in these 2 individuals is correlated with a concurrent elevation of their HDL-C levels. Conversely, there are 2 documented affected FH individuals with premature CHD: (1) individual III3, who does not have the HA trait or the haplotype associated with it and has presented with several ischemic episodes since 52 years of age, and (2) individual III1, who died at 52 years of age as a result of myocardial infarction. Even though HDL-C levels were not available for the latter individual, he did not have the haplotype linked to HA (his haplotype was unequivocally inferred). Also, none of his 3 daughters has either elevated HDL-C levels or the chromosome 6 haplotype, which would support the assumption that his HDL-C levels were below what is regarded as protective. In light of these observations, we propose that in this family, the HDL-C elevation explains the antiatherogenic effect seen in those FH individuals also carrying the HA trait.

Through a genome-wide scan, we mapped the FH trait to a region on chromosome 1p32, a region previously linked to FH in 2 separate studies.9,10,57 Based on recombination events in 2 affected individuals, our results localize the responsible gene to an interval of 6.75 cM flanked by markers D1S2134 and D1S200. The integration of the mapping data from all 3 studies yields a common interval of 0.61 cM flanked by markers D1S2134 to D1S197. Some likely candidate genes in the vicinity of this region are EPS15 (an epidermal growth factor receptor pathway substrate),58 APOER2 (an apoE receptor),59 and SCP2 (a sterol carrier protein).60

In the family we studied, this locus displays incomplete penetrance, because in addition to all 12 affected individuals sharing a common 4 marker haplotype, several other members share the same region but appear to be asymptomatic. Of these, 5 are individuals with total cholesterol and LDL-C levels within the normal range, and 2 are individuals who showed either borderline lipid values according to age and sex or are individuals with unknown status (see Materials and Methods). The affecteds-only analysis showed 5 consecutive markers (D1S2134 through D1S417) with positive LOD scores (all at \(\theta=0\)). A comparison of the results obtained under the 2 models used (whole family versus affecteds only) is consistent with the observed incomplete penetrance in this kindred. Although incomplete penetrance for this FH locus has been reported for both French and Spanish families,9 the penetrance found in our kindred may be lower. This suggests the possible involvement of additional loci influencing the phenotype.

For the HA trait, a genome-wide scan identified a locus on chromosome 6p. Haplotype analysis defined a region spanning 7.32 cM between markers D6S1280 and D6S1275. This haplotype is shared by all individuals with HA and 1 individual with levels within the normal range, implying a penetrance of 90% in this family. Our region on chromosome 6p (73.13 to 80.45 cM from pter) overlaps the region reported by Knoblauch et al61 influencing LDL levels in an FH Arab family and an independent sample of healthy white monozygotic and dizygotic twins from Germany. This same region has also been linked with TG/HDL levels in a study in a population from Minnesota,62 and it is close to a peak for TG levels (LOD score 1.24 at 71.3 cM) reported in African American families from the HyperGEN Study.63 Therefore, it is possible that the locus mapped in chromosome 6p12.3-q13 in our family for high HDL levels may also influence lipid concentrations in other populations. The critical interval defined in our family has \(\sim 30\) known genes, with interleukin-17 and interleukin-17F as the only candidate genes showing a possible biologically related function. Sequence analysis of these genes showed no evidence that they were responsible for the HA phenotype. We have not ana-
lyzed other positional candidate genes within this chromosomal region.

Although the 6p12.3-q13 locus has already been associated with the modulation of cholesterol, TG, and TG/HDL levels, it has not been previously linked to high HDL levels or to an antiatherogenic effect in humans. As to the extent of the observed protective effect conferred by this locus in the family we studied, it is noteworthy that there are independent cardiovascular risk factors besides FH in affected individuals with no manifestations of CHD. This suggests that the antiatherogenic effect conferred by this locus may also provide protection against atherogenesis-promoting conditions other than FH. In this regard, it would be of interest to determine the possible influence of this locus on lipid concentrations and its associated cardiovascular risk in Mexican population.

The identification of the responsible gene within the 6p12.3-q13 locus will provide new information regarding the role of the HDL-C particles as antiatherogenic agents and may result in the identification of new therapeutic targets for the prevention and treatment of atherosclerotic disease.

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Locus on Chromosome 6p Linked to Elevated HDL Cholesterol Serum Levels and to Protection Against Premature Atherosclerosis in a Kindred With Familial Hypercholesterolemia

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