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# A New Locus for Autosomal Recessive Hypercholesterolemia Maps to Human Chromosome 15q25-q26

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## Summary

**High serum cholesterol is an established risk factor for cardiovascular disease and is the prime target for therapeutic intervention in large groups of patients. The development of modern treatments for this major risk factor was propelled by the early realization that forms of severe hypercholesterolemia could be caused by dominantly inherited defects in the LDL receptor or in the APOB gene. Further understanding of the mechanisms contributing to early atherosclerosis will allow for new targets for therapy. We therefore identified and investigated the genetics of families from Sardinia that have recessive inheritance of precocious hypercholesterolemia. We used five families in an analysis of linkage of the autosomal recessive hypercholesterolemia locus, termed "ARH1," to chromosome 15q25-q26. A genomewide search mapped the disease-causing gene with a LOD score of 3.3 and excluded major contributions to the phenotype of other genes. A candidate gene present in the mapped chromosome region—the ligand-activated liver-transcription-factor gene ARP1 (apolipoprotein regulatory-protein gene)—has been excluded after DNA sequencing. The close-bred nature of the Sardinian population offers unique opportunities for isolation of this hypercholesterolemia-causing gene.**

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## Introduction

An elevated plasma concentration of LDL-cholesterol (LDL-C) is one of the most important quantitative risk factors for coronary heart disease and is the target for therapeutic interventions in large groups of patients in urbanized societies (National Cholesterol Education Program 1994). Data from family and twin studies have shown the importance of genetic factors as regulators of cholesterol concentration (Austin et al. 1987; Bucher et al. 1988; Perusse et al. 1989; Rice et al. 1991), but only a few genes and their contributions to increased LDL-C levels have been characterized. Familial hypercholesterolemia (MIM 603813), an autosomal dominant disorder, is caused by a defective LDL-receptor gene located on chromosome 19 and is characterized by high levels of LDL-C, tendon xanthomas, and premature atherosclerosis with precocious cardiovascular disease (Goldstein and Brown 1973; Dammerman and Breslow 1995; Varret et al. 1998). A similar phenotype is observed in familial defective ApoB (FDB), as a result of a mutation in the APOB gene on chromosome 2 (Myant 1993). Recently, in families with dominantly inherited hypercholesterolemia, in which defects in the LDL receptor or the ApoB protein were excluded, a new hypercholesterolemia locus was mapped to chromosome 1 (Varret et al. 1999). These observations show the genetic heterogeneity of hypercholesterolemia and further emphasize the complex regulation of the cholesterol concentration.

In 1995, a consanguineous Sardinian family, characterized by members with severe hypercholesterolemia, was identified (Zuliani et al. 1995). The affected individuals showed clinical features that were indistinguishable from those present in patients with homozygous LDL-receptor defects. In contrast to heterozygous individuals with LDL-receptor defects, parents of the affected members of this family displayed levels of total cholesterol (total-C), triglycerides (TGs), LDL-C, and HDL-cholesterol (HDL-C) that were within the normal range for the Sardinian population. No defects were observed in binding of LDL isolated from patients or in

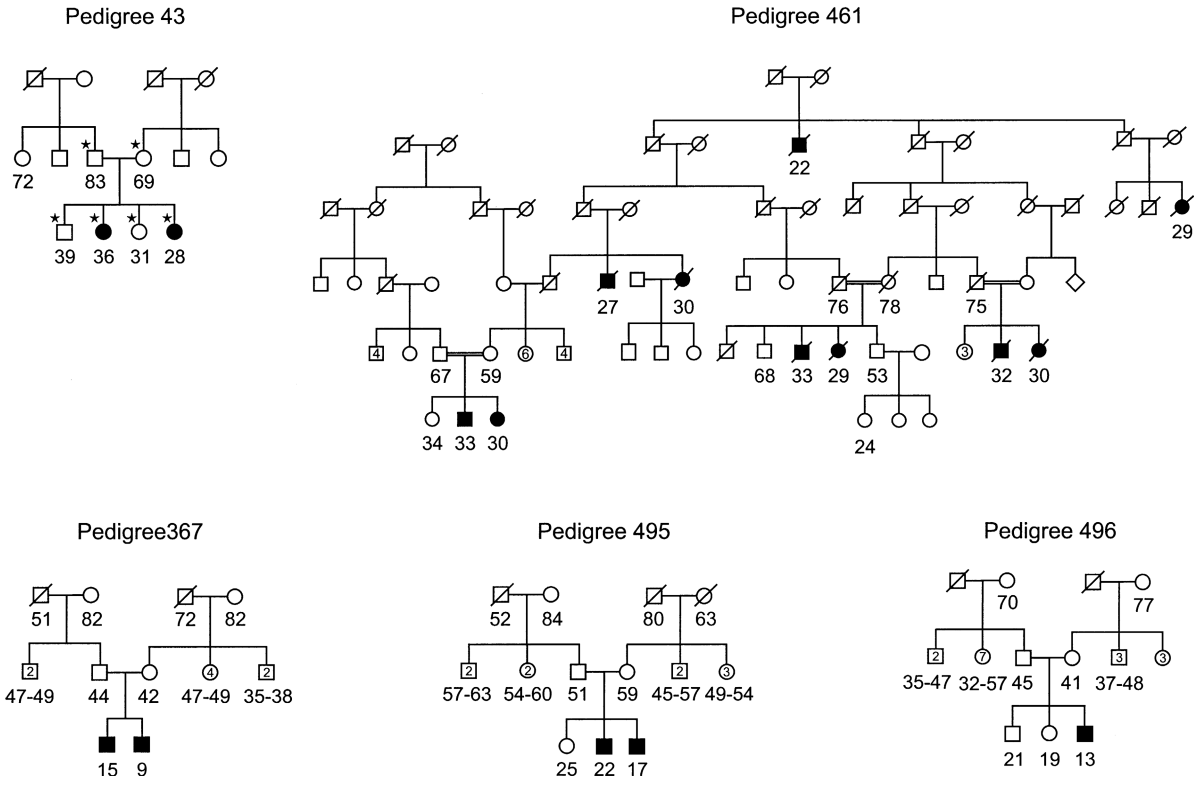


**Figure 1** Structure of the five Sardinian pedigrees with hypercholesterolemia. Circles denote females; squares, males; blackened symbols, affected individuals. Double lines indicate consanguineous matings. Numbers below each symbol indicate age at the time of this study or age at death; numbers inside symbols indicate the number of siblings with an identical phenotype. Stars above symbols denote individuals included in the linkage analysis.

LDL-receptor–mediated internalization and degradation of LDL, in fibroblasts from affected individuals. Other known causes of hypercholesterolemia, such as sitosterolemia and defects in ApoB and in ApoE, were excluded (Zuliani et al. 1995). In contrast, in vivo analysis of the LDL distribution in patients, compared with that in control individuals, indicated decreased LDL uptake in the liver, kidney, and spleen (Zuliani et al. 1999). Although affected family members display many clinical features in common with those of patients who are homozygous for LDL-receptor mutations, the clinical observations—in particular, the fact that obligate carriers display normal cholesterol concentrations—suggested that this family segregated a new genetic form of severe hypercholesterolemia that is distinct from hypercholesterolemia caused by LDL-receptor or ApoB defects. In the present study, we describe four additional Sardinian families with autosomal recessive hypercholesterolemia (ARH) that were used for the genetic mapping of the disease-causing gene, by means of genomewide linkage analysis.

## Families and Methods

### *Study Families and Diagnosis*

The criteria used to select families with ARH for linkage analysis were as follows: (i) at least one affected family member with precocious debut of the hypercholesterolemia phenotype (e.g., presence of high levels of LDL-C in untreated affected individuals who are following a Western-type diet [Thompson et al. 1989] and who have tuberous and/or tendon xanthomas or have precocious atherosclerosis), and (ii) obligate carriers (e.g., parents of affected individuals) who display lipid values within the normal range for the population. The influence on the cholesterol concentration of other diseases—for example, liver, kidney, or thyroid disease—has been considered. The study was approved by the ethics committee at the University of Sassari, and all subjects gave informed oral consent to participation in the study.

**Table 1**

**Clinical Characteristics of Family Members Affected with Early Hypercholesterolemia**

| | Clinical Characteristics of Affected Family Members | | | | | | |
|---|---|---|---|---|---|---|---|
| | In 1999 | | At Diagnosis of Early Hypercholesterolemia | | | | |
| Family Number and Patient Number (Sex) | Age (years) | BMI[a] ($kg/m^2$) | Age (years) | Total-C (mmol/liter) | LDL-C (mmol/liter) | HDL-C (mmol/liter) | TG (mmol/liter) |
| 43: | | | | | | | |
| 154 (F) | 36 | 26 | 23 | 16.2 | 14.8 | 1.24 | .55 |
| 157 (F) | 28 | 20 | 14 | 14.0 | 12.7 | 1.16 | .71 |
| 367: | | | | | | | |
| 1326 (M) | 9 | 16 | 4 | 12.3 | 11.2 | .90 | 1.06 |
| 1327 (M) | 15 | 20 | 9 | 11.0 | 10.0 | .80 | 1.06 |
| 461: | | | | | | | |
| 1776 (M) | 33 | 25 | 10 | 15.0 | 13.3 | 1.45 | .97 |
| 1777 (F) | 30 | 20 | 11 | 13.7 | 12.3 | 1.19 | 1.15 |
| 495: | | | | | | | |
| 2052 (M) | 22 | 23 | 11 | 16.6 | 15.7 | .69 | 1.26 |
| 2053 (M) | 17 | 25 | 8 | 13.8 | 12.9 | .72 | 1.00 |
| 496: | | | | | | | |
| 2059 (M) | 13 | 23 | 5 | 18.2 | 16.7 | 1.12 | 2.08 |

Note.—Lipid plasma concentrations (mean ± SD) in the general male population (age >20, $n = 4,552$) of Sardinia are as follows: total-C, 5.2 ± 1.0; HDL-C, 1.3 ± 0.3; LDL-C, 3.6 ± 0.9; and TG, 1.4 ± 0.9 (Maioli et al. 1989).

[a] BMI = body-mass index.

### *Plasma Lipid Analysis*

Venous-blood samples were drawn in EDTA-tubes, after a 12-h overnight fast, and were immediately centrifuged, at 3,000 *g,* for 15 min. Plasma samples were stored at −40°C, until lipid levels were determined (Maioli et al. 1989). Plasma LDL-C was calculated by use of the Friedewald formula, since plasma TGs were <4.5 mmol/liter (Friedewald et al. 1972).

### *Genotyping*

Genomic DNA was purified from peripheral lymphocytes, and 5 ng was amplified, by PCR, in 10 μl containing 0.15 μM each primer, 0.2 mM each dNTP, 1 × buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 150 mM KCl, and 0.1% Triton X-100), and 0.2 U DynaZyme. PCR cycles were as follows: 95°C for 5 min, then 35 cycles at 95°C for 30 s, and then annealing at primer-specific temperatures (53°C, 55°C, and 58°C) for 75 s and extension at 72°C for 15 s, followed by a final extension at 72°C for 10 min. We used 315 fluorescent microsatellite markers spanning all chromosomes (Murray et al. 1994). The PCR products were separated on 4% polyacrylamide gels, by use of an ABI377 Sequencer, and genotypes were identified by use of ABI GENESCAN/GENOTYPER software (PE Biosystems). All genotypes were checked for Mendelian inheritance, both by inspection and by use of a procedure in GENEHUNTER (Kruglyak et al. 1996). All inconsistent genotypes were resolved by repeating the PCR amplification and by typing the entire family in question for the relevant loci. The markers D15S652, D15S1004, D15S130, and D15S816 were typed twice in all subjects, for confirmation.

### *Linkage Analysis*

To localize the disease locus, we genotyped all five families, with the use of 310 markers that were evenly spaced across the genome and that were selected from the Cooperative Human Linkage Center (CHLC) database. Five additional markers (cen-D15S963, D15S1004, D15S130, D15S207, and D15S157-tel) were identified from the Généthon map. The chromosomal order of non-CHLC markers was taken from Généthon. The order of all markers on chromosome 15 was checked by use of the program CRIMAP, version 2.4; our results did not differ from the Généthon data. The average spacing of microsatellite markers was 12 cM (range 0.2–37 cM). On chromosome 15, the average distance between the 16 markers was 6.8 cM (range 0.2–15 cM), and the markers showed an average heterozygosity of .7 (range .48–.82). In particular, D15S1004 had a heterozygosity of .66 in a Sardinian population sample ($n = 60$). After analysis of all available data (e.g., affected individuals have parents with cholesterol levels within the normal range, both sexes are affected, consanguineous matings occur, and parents originate from the same village or its vicinity), we selected to calculate linkage, under

**Table 2**

**Phenotypes of Parents (Heterozygous Carriers) of Patients with Hypercholesterolemia**

| Family Number and Patient Number (Sex) | Phenotypes, in 1999, of Parents of Affected Individuals | | | | | |
|---|---|---|---|---|---|---|
| | Age (years) | BMI ($kg/m^2$) | Total-C (mmol/liter) | LDL-C (mmol/liter) | HDL-C (mmol/liter) | TG (mmol/liter) |
| 43: | | | | | | |
| 152 (M) | 83 | 24 | 5.4 | 3.6 | 1.6 | .8 |
| 153 (F) | 69 | 39 | 5.8 | 3.9 | 1.6 | 1.1 |
| 367: | | | | | | |
| 1324 (M) | 44 | 26 | 6.2 | 4.9 | .8 | 2.3 |
| 1325 (F) | 42 | 20 | 3.8 | 2.6 | 1.1 | .7 |
| 461: | | | | | | |
| 1774 (M) | 67 | 27 | 5.9 | 4.0 | 1.7 | .9 |
| 1775 (F) | 59 | 31 | 4.4 | 2.7 | 1.5 | .7 |
| 495: | | | | | | |
| 2054 (M) | 51 | 29 | 5.1 | 3.8 | 1.1 | 1.0 |
| 2055 (F) | 59 | 24 | 4.6 | 3.3 | 1.1 | .9 |
| 496: | | | | | | |
| 2060 (M) | 45 | 24 | 5.1 | 3.7 | 1.2 | 1.1 |
| 2057 (F) | 41 | 23 | 5.1 | 3.6 | 1.3 | 1.0 |

Note.—Data are as in table 1.

a recessive model with full penetrance, no phenocopies, and the assumption of a gene frequency of .001, by use of GENEHUNTER software (Kruglyak et al. 1996). Two-point linkage analysis was performed by use of both the MLINK program of the LINKAGE package and FASTLINK, version 2.2 (Ott 1991; Cottingham et al. 1993).

### *Sequence of the Apolipoprotein Regulatory-Protein Gene (ARP1)*

Genomic DNA (100 ng) was PCR amplified in 50 $\mu$l containing 0.36 $\mu$M each primer, 0.2 mM each dNTP, 0.15 $\mu$M 7-deaza-dGTP, 1 × DZB (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 150 mM KCl, and 0.1% Triton X-100), and 1.4 U of DynaZyme. The PCR conditions were as follows: consisting of 40 cycles for 60 s at 96°C, followed by annealing for 30 s at 52°C (for exon 1), at 60°C (for exon 2), and at 56°C (for exon 3) and then by extension at 72°C for 120 s. The DNA sequence was determined from 40 $\mu$l of PCR product, by use of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems), and was separated on an ABI377 DNA Sequencer. The DNA sequence of the ARP1 gene was determined for all nine affected individuals, for one unaffected family member, and for one unaffected, unrelated individual from Sardinia. The two unaffected individuals (age 33 years and age 34 years) had normal lipid values (in particular, LDL-C was <2.6 mmol/liter for both individuals), and they had no clinical features of hypercholesterolemia.

## Results

### *Phenotypes*

Applying the criteria as described above, we identified four new Sardinian families with ARH. A total of 29 individuals from five families—four families with two affected offspring each and one family with one affected offspring—were included in the linkage analysis (fig. 1).

In all families selected, the disease phenotype was inherited in a recessive mode; cholesterol levels displayed bimodal distribution within each family, and affected individuals had total-C levels ⩾11 mmol/liter, in combination with normal TG levels (table 1). Moreover, parents of affected individuals displayed concentrations of TGs, total-C, LDL-C, and HDL-C that were within the normal distribution (table 2).

### *Linkage Analysis*

We applied genomewide linkage analysis, using 310 markers that were evenly spaced throughout the genome. At the q arm of chromosome 15, a suggestive LOD score for linkage (LOD score 2.2) was observed, with the marker D15S816. To extract more data from the available pedigrees, we elected to type the families by use of five additional informative markers located in this region of chromosome 15. Because of the structure of the pedigrees, the haplotypes cannot be unequivocally determined in all instances; the haplotypes resolved by use of GENEHUNTER software are presented in figure 2. When additional markers were added to the linkage analysis, we were able to map the hypercholesterolemia

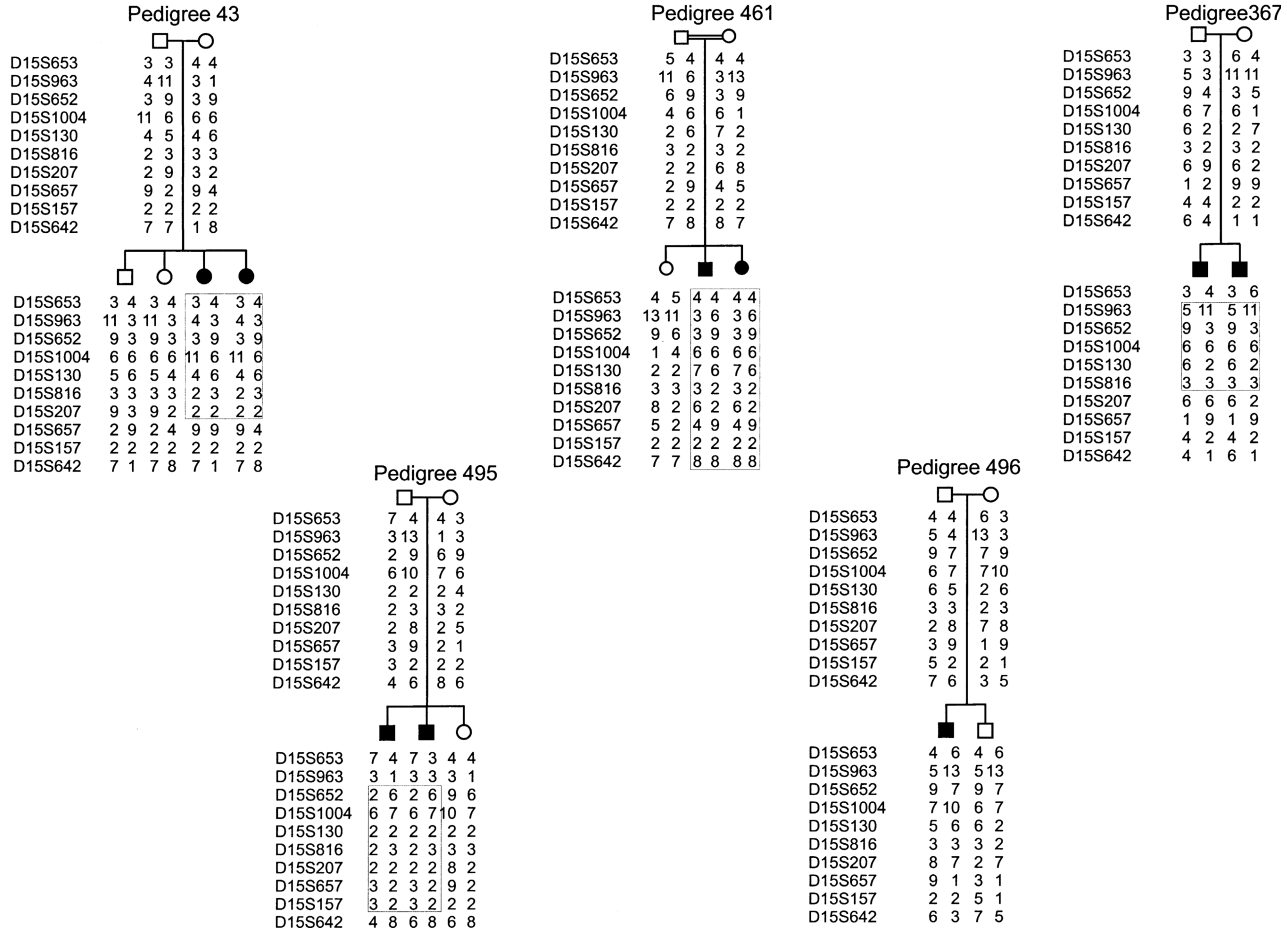


**Figure 2** Suggested haplotypes of chromosome 15q24-15qter in families with ARH. The common region shared in affected individuals within each family is boxed. Displayed haplotypes were calculated by use of the GENEHUNTER program.

locus to 15q25-q26 (figs. 2 and 3). The marker D15S1004 was the most informative marker in the region, and it generated a LOD score of 3.3, by use of two-point linkage analysis. The LOD score remained >3, even if the penetrance was changed to .9 and/or the disease-gene frequency was changed to .01. In the region of 15q25-q26, we identified, from public databases, a candidate gene—the transcription-factor gene ARP1. The LOD scores for all chromosomes in the multipoint analysis are shown in figure 4.

### *Sequence of ARP1*

Comparison of the genomic DNA sequence of ARP1 with published human sequences (Ladias and Karathanasis 1991) revealed homozygous differences in intron 1 that were present in the two unaffected individuals as well. No DNA sequence variants were identified in either the three exons, the conserved splicing sequences, the promoter region up to −221, or the 3′ UTR of the gene.

## Discussion

In the present report, we have shown that ARH is linked to a locus, termed "ARH1," located in the chromosome region 15q25-q26. The initial set of genetic markers indicated suggestive linkage. A denser set of markers, including nine markers selected from this region of the q arm of chromosome 15, showed genomewide significance for linkage (LOD score 3.3), by means of two-point linkage analysis with marker D15S1004. When multipoint linkage was calculated, the right part of the largest pedigree (family 461) was removed, as a result of software limitations. The maximum multipoint LOD score was therefore 3.05 (fig. 3). At the telomeric side, a crossover in family 367, between the markers D15S816 and D15S207, delimited the linked 16-cM region, and, at the centromeric side, a crossover between markers D15S963 and D15S652 delimited the region in family 495. With this set of markers, common haplotypes were observed within each family but not between

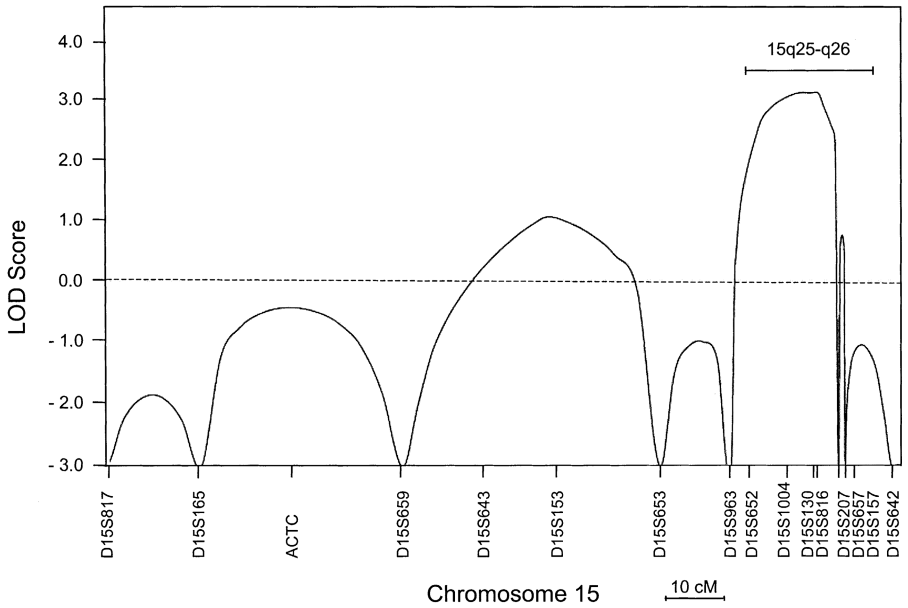


**Figure 3** Multipoint linkage analysis of ARH, displaying chromosome 15 results. Markers are indicated below the *X*-axis. The ARP1 gene is located at 15q25-15q26.

families (fig. 2). This could be the result of several factors, either alone or in different combinations: (i) the five families come from different geographic regions of Sardinia that, given the close breeding often observed in Sardinian villages, have most likely been genetically separated for a considerable fraction of the time since the colonization of the Sardinian island; (ii) the disease-carrying haplotypes originated outside Sardinia but are detected in the Sardinian population, as a result of the higher degree of close breeding/inbreeding in combination with the recessive nature of the disease; and/or (iii) the available published markers in the linked region do not permit a map that is dense enough to detect a common haplotype in Sardinians from different villages, even though there might be a common origin of the hypercholesterolemia mutation(s).

With the exception of chromosome 15, no significant or suggestive indications of linkage to the ARH phenotype were revealed by linkage analysis (fig. 4). Particular emphasis has been placed on genotyping of chromosome regions that contain candidate genes for hypercholesterolemia, such as LDLR (19p13.2-p13.1), APOB, A-I/C-III/A-IV (11q23), HMGCR (5q13.3-q14), APOE (19q13.2), and LRP (12q13.1-q13.3). None of the markers flanking these candidate genes showed any evidence for linkage to the disease, confirming—also from the genetic point of view—that this disease is distinct from other known or suspected causes of hypercholesterolemia. Moreover, our data show that ARH in these five families is not linked to the recently described familial-hypercholesterolemia locus on chromosome 1, confirming, for this locus, the negative linkage obtained for two other Sardinian families with ARH (Varret et al. 1999).

In the region 15q25-q26, there is one candidate gene, identified in public databases, that can be connected to lipid metabolism. The liver transcription factor Arp-1 is involved in the regulation of APOB and other lipoprotein genes (Qiu et al. 1995). Arp-1 was shown to regulate APOB transcription, acting either alone or with other transcription factors. Moreover, that Arp-1 is one of several regulators of transcription in hepatic cells suggests that the effect on APOB transcription could depend on their relative intracellular levels and/or affinities (Malik and Karathanasis 1995). In conclusion, both the documented activity of Arp-1 as a repressor or an activator of hepatic apolipoprotein-gene transcription and the observation that this form of hypercholesterolemia is expressed mainly as an LDL-catabolism defect in hepatocytes (Zuliani et al. 1999) have led us to investigate the structure of the gene in some detail. The analysis of the ARP1 sequence in affected members did not reveal any DNA-sequence variation in either the three exons or their conserved splicing sequences, in the promoter region, or in the 3′ UTR of the gene. These results make it unlikely that ARP1 is involved in the hypercholesterolemia phenotype.

Other reported cases of ARH not linked to LDLR or

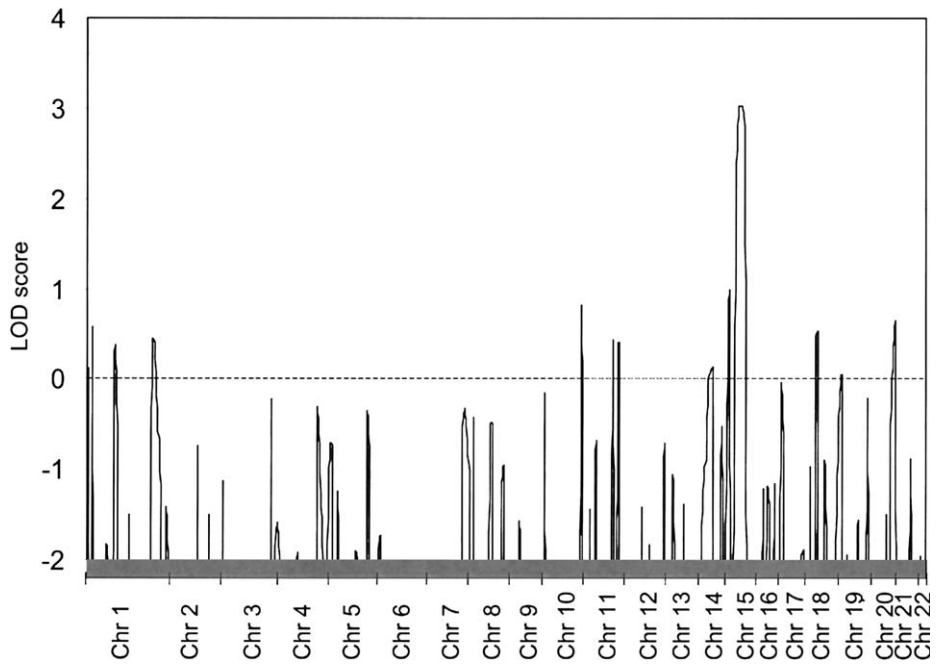


**Figure 4** Multipoint-linkage-analysis data for all chromosomes except the sex chromosomes. LOD scores >−2 are hidden and indicate that the chromosome region is excluded for linkage. Chromosome X was excluded by single-point analysis, with use of LINKAGE software, version 5.1.

APOB suggest the existence of as-yet-unknown genes for hypercholesterolemia (Sirtori et al. 1991; Harada-Shiba et al. 1992; Schmidt et al. 1998). In these families, the hypercholesterolemia phenotype—expressed as elevated LDL-C, xanthomas, or coronary heart disease—was not reported in parents.

The data presented in this report support the notion that this form of ARH is the result of an as-yet-unknown gene, at chromosome 15q25-q26, that has a major effect on cholesterol metabolism and atherosclerosis etiology. In addition, our results provide genetic evidence in support of the notion that ARH1 is a distinct form of monogenic hypercholesterolemia.

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## Electronic-Database Information

The accession number and URLs for data in this article are as follows:

Cooperative Human Linkage Center, http://www.chlc.org/ (for 310 markers used for genotyping)
Généthon, http://www.genethon.fr/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for familial hypercholesterolemia [MIM 603813])

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