Influence of β^0-Thalassemia on the Phenotypic Expression of Heterozygous Familial Hypercholesterolemia

A Study of Patients With Familial Hypercholesterolemia From Sardinia

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Abstract—One of the genetic features of the Sardinian population is the high prevalence of hemoglobin disorders. It has been estimated that 13% to 33% of Sardinians carry a mutant allele of the α-globin gene (α-thalassemia trait) and that 6% to 17% are β-thalassemia carriers. In this population, a single mutation of β-globin gene (Q39X, β^0 39) accounts for >95% of β-thalassemia cases. Because previous studies have shown that Sardinian β-thalassemia carriers have lower total and low density lipoprotein (LDL) cholesterol than noncarriers, we wondered whether this LDL-lowering effect of the β-thalassemia trait was also present in subjects with familial hypercholesterolemia (FH). In a group of 63 Sardinian patients with the clinical diagnosis of FH, we identified 21 unrelated probands carrying 7 different mutations of the LDL receptor gene, 2 already known (313+1 g>a and C95R) and 5 not previously reported (D118N, C255W, A378T, T413R, and Fs572). The 313+1 g>a and Fs572 mutations were found in several families. In cluster Fs572, the plasma LDL cholesterol level was 5.76±1.08 mmol/L in subjects with β^0-thalassemia trait and 8.25±1.66 mmol/L in subjects without this trait ($P<0.001$). This LDL-lowering effect was confirmed in an FH heterozygote of the same cluster who had β^0-thalassemia major and whose LDL cholesterol level was below the 50th percentile of the distribution in the normal Sardinian population. The hypocholesterolemic effect of β^0-thalassemia trait emerged also when we pooled the data from all FH subjects with and without β^0-thalassemia trait, regardless of the type of mutation in the LDL receptor gene. The LDL-lowering effect of β^0-thalassemia may be related to (1) the mild erythroid hyperplasia, which would increase the LDL removal by the bone marrow, and (2) the chronic activation of the monocyte-macrophage system, causing an increased secretion of some cytokines (interleukin-1, interleukin-6, and tumor necrosis factor-α) known to affect the hepatic secretion and the receptor-mediated removal of apolipoprotein B–containing lipoproteins. The observation that our FH subjects with β^0-thalassemia trait (compared with noncarriers) have an increase of blood reticulocytes (40%) and plasma levels of interleukin-6 (+60%) supports these hypotheses. The lifelong LDL-lowering effect of β^0-thalassemia trait might slow the development and progression of coronary atherosclerosis in FH. **(*Arterioscler Thromb Vasc Biol*. 2000;20:236-243.)**

Key Words: familial hypercholesterolemia ■ low density lipoprotein receptor gene mutations ■ β-thalassemia ■ gene-gene interaction

Familial hypercholesterolemia (FH) is an autosomal codominant disorder that is due to defects of the low density lipoprotein receptor (LDL-R) that result in a defective removal of LDL from plasma. The accumulation of plasma LDL is thought to be the major cause of premature coronary artery disease (CAD) observed in FH.[1] The LDL-R defects are caused by a large number of mutations of the LDL-R gene.[2] In most white populations, the frequency of heterozygous FH is estimated to be 1:500 and that of homozygous FH is ≈1:1 000 000.[2] In most European countries, including continental Italy, FH is characterized by large allelic heterogeneity. However, in some ethnic groups living in Europe (eg, Finns) or of European descent (eg, French-Canadians and Afrikaners of South Africa), the frequency of heterozygous FH is higher, and the LDL-R defects are caused by a small number of mutations of the LDL-R gene (the founder effect).[2]

Sardinia is an island in the Mediterranean with an area of 24 000 km^2 and a population of ≈1 600 000 inhabitants. Sardinians belong to a genetically deviant population (like Basques, Icelanders, and Finns) among the other European populations.[3] They stem from various populations of the western and eastern basin of the Mediterranean that settled on the island 15 000 to 10 000 years BC.[4] Although over the centuries Sardinians had contacts with several foreign popu-

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lations (Phoenicians, Carthaginians, Romans, Vandals, Byzantines, the Genoese, the Spanish, and the Piedmontese), with the possible exception of the Romans, these foreign populations seem to have made little genetic contribution to the Sardinian genetic background.[3] Geographic isolation, inbreeding, genetic drift, and probably the selection induced by specific environmental factors (eg, the endemic malaria) have contributed to the genetic diversification of Sardinians from mainland Italians and other white populations.

Evidence of a different genetic background for Sardinians and other European populations, including Italians, is given, for example, by the higher prevalence of some monogenic and polygenic diseases (eg, Wilson disease and type I diabetes) in Sardinia compared with continental Italy and other European countries.[5–7] One of the well-known genetic features of the Sardinian population is the high prevalence of some genetic disorders of erythrocytes, such as thalassemias, and deficiency of glucose-6-phosphate dehydrogenase.[8,9] It has been estimated that 13% to 33% of Sardinians carry 1 mutant allele of the α-globin genes, that 6% to 17% are β-thalassemia carriers,[8] and that 4% to 19% are carriers of glucose-6-phosphate dehydrogenase deficiency.[9] The high prevalence of these disorders appears to be the result of a selective advantage produced by malaria, which was endemic in Sardinia up to the late 1950s. It should be stressed that although in most Mediterranean populations β-thalassemia is caused by several mutations of the β-globin gene,[10] in the Sardinian population a single mutation (Q39X) accounts for $>$95% of the β-thalassemia chromosomes.[11] Finally, in Sardinia a vast proportion of the general population carries HLA haplotypes that are very rare elsewhere and that might have originated from a common ancestor (the founder effect).[12]

It is well established that β-thalassemia has a major impact on plasma lipids and lipoproteins. In severe β-thalassemia (thalassemia major and intermedia), hypocholesterolemia caused by a marked reduction of both LDL and HDL cholesterol has been consistently reported[13–15]; in β-thalassemia carriers (thalassemia minor), total and LDL cholesterol levels tend to be lower than those found in age- and sex-matched controls.[15,16] A wide study conducted in the Sardinian population not only showed that β-thalassemia carriers have a lower total and LDL cholesterol than do controls but also showed a small but significant reduction of apoB and apoA-I levels and borderline changes in lipid and protein composition in LDL and HDL.[16,17] It has been suggested that the mild hypocholesterolemia found in carriers of β-thalassemia might contribute to the protection of these individuals from the development of premature CAD.[18–20] Retrospective studies showed that the prevalence of thalassemia carriers among patients with myocardial infarction was much less than expected[18] and that in Italian males with β-thalassemia minor, myocardial infarction occurred $\approx$10 years later than in nonthalassemic subjects.[20]

The observed LDL-lowering effect of β-thalassemia trait raises the question as to whether this effect is maintained in FH heterozygotes. We decided to investigate this problem in Sardinian FH patients for the following reasons: (1) the presence in Sardinia of a single mutation of β-globin gene (Q39X), a null allele with a strong biological effect (β^0-thalassemia), which accounts for 95% of Sardinian β-thalassemia cases, and (2) the specific genetic background of Sardinians (as outlined above), which led us to expect to find a restricted number of mutations of the LDL-R gene in Sardinian FH patients, thus allowing us to compare the FH phenotype in a relatively large number of FH patients sharing the same mutation at the LDL-R locus and carrying or not carrying the β^0-thalassemia trait.

Methods

Our original series of Sardinian FH patients included 63 unrelated subjects (24 males and 39 females) with the clinical diagnosis of heterozygous FH. Most of them were from the northern part of Sardinia, and all were proven to stem from Sardinian ancestors living in the island for $\geq$1 century. The clinical diagnosis of heterozygous FH was based on the following criteria: (1) plasma LDL cholesterol level above the 95th percentile of the distribution in the Sardinian population, stratified for sex and age, (2) tendon xanthomatosis in the proband or in at least one first-degree relative or the presence of severe hypercholesterolemia in some children of the proband's family, and (3) identification of the LDL-R gene haplotype (at least 6 diallelic markers) cosegregating with hypercholesterolemia within the family. Informed consent was obtained from the patients or, in the case of children, from their parents. The study protocol was approved by the institutional human investigation committee of each participating institution.

Fasting plasma lipids were measured before any hypolipidemic drug treatment. Total cholesterol, triglyceride, and HDL cholesterol levels were measured enzymatically with commercial kits (Boehringer Mannheim GmbH) by use of an automated analyzer; LDL cholesterol was calculated by Friedewald's formula. Red blood cell parameters, including reticulocyte count, were determined with a Cell-Dyn 3500 system (Abbott); hemoglobin A_2 was quantified by the VARIANT Hemoglobin Testing system (Bio-Rad); and plasma interleukin (IL)-6 was measured by ELISA (Amersham Pharmacia Biotech).

SSCP Analysis of LDL-R Gene

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure.[21] Polymerase chain reaction (PCR) amplifications of the promoter region and exons 1 to 18 of the LDL-R gene were carried out by using the primers reported by Hobbs et al[2] in a total volume of 50 μL containing 100 ng of genomic DNA, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 100 μmol of each of the 4 nucleotides, 10 pmol of each primer, and 2 U of Taq DNA polymerase. Single-strand conformation polymorphism (SSCP) analysis was performed by using a vertical gel unit (Hoefer); 5 μL of PCR product was mixed with an equal amount of 95% formamide, 20 mmol/L EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol, denatured at 96°C for 5 minutes, snap-cooled in 4°C ice water, and then loaded onto an 8% polyacrylamide gel containing 5% glycerol. Electrophoresis was performed at room temperature in a standard Tris-borate-EDTA buffer, pH 8.0, at 150 V for 2 hours. After electrophoresis, gels were stained with silver stain.

Direct Sequencing of DNA

The samples showing an abnormal SSCP pattern were sequenced by using an automated fluorescent ABI Prism 310 Genetic Analyzer (Applied Biosystem Inc) according to the manufacturer's recommendations. Mutations identified by automated sequencer were confirmed by manual sequencing[22] with the use of appropriate primers.

Haplotype Analysis of LDL-R Locus

The intragenic haplotypes cosegregating with mutant alleles were constructed by use of 10 diallelic markers associated with the LDL-R gene. The genotyping was performed by using Southern blotting of genomic DNA (*Bst*EII, 5′*Apa*LI, *Pvu*II, and 3′*Apa*LI), by restriction enzyme digestion of amplified exons (*Stu*I, *Hinc*II, *Ava*II, and 5′ *Msp*I), or by SSCP analysis (*1413G/A* in exon 10, *2635G/A* in exon 18).[23–27]

TABLE 1. Mutations of LDL-R Gene Causing FH in Northern Sardinia

Exon/Intron	Nucleotide Position in cDNA	Codon/Intron Change	Mutation Designation	Newly or Previously Detected Mutation by Population and Reference No.
Intron 3	313+1 g>a	Aberrant splicing	FH Elverum	Norwegian,[29] Dutch,[34] English,[35] Danish,[36] Swedish,[37] and Korean[38]
Exon 4	346T>C	C95R	FH Alghero	Spanish[39]
Exon 4	415 G>A	D118N	FH Sassari-2	New
Exon 6	828C>G	C255W	FH Sassari-3	New
Exon 9	1195G>A	A378T	FH Nuoro	New
Exon 9	1301C>G	T413R	FH Sassari-4	New
Exon 12	1778delG	Fs572>Term643	FH Sassari-1	New

Rapid Screening Methods for Detection of Some Mutations

The rapid screening method for the D118N mutation, which eliminates a *Dde*I site in exon 4, was based on the amplification of the 5′ half of exon 4,[2] followed by the digestion with *Dde*I. For the screening of C255W and T413R mutations, we amplified exon 6 and exon 9 by using 2 sense-mismatched primers: 5′-CTCTGGCTCTCACAGTGACACTCCG-3′ and 5′-CTGAGG-AACGTGGTCGCTCTGGCC-3′, respectively, and SP65 and SP71[28] as antisense primers, respectively. In the presence of the mutation, these mismatched primers introduce an *Msp*I and an *Eco*RV cutting site in exon 6 and exon 9, respectively. The mutation Fs572, caused by a G deletion in exon 12, was easily detectable by heteroduplex analysis of the amplified exon 12 on 10% polyacrylamide gel. In brief, 1 μL of 0.25 mol/L EDTA was added to the amplification product, which was submitted to the thermal treatment mentioned below to obtain completion of heteroduplex formation that was begun during PCR cycles. The incubation conditions were 95°C for 3 minutes, followed by a slow cooling to room temperature over a 40-minute period. Next, 8 μL of the product was mixed with 2 μL of gel loading buffer and electrophoresed on 10% polyacrylamide gel, which was made from a stock solution containing acrylamide and *N*,*N*′-methylene-bis-acrylamide (49:1) in 1× Tris-borate-EDTA buffer. After the run (40 minutes at 180 V), the gel was stained with ethidium bromide and UV-visualized. The C95R and A378T mutations were screened by SSCP analysis and confirmed by direct sequencing. The 313+1 g>a mutation was screened with the use of a mismatched primer, as suggested by Leren et al.[29]

Screening for Familial Defective ApoB-100

Familial defective apoB-100, resulting from R3500Q substitution in apoB-100, was ruled out in all probands by using the method of Motti et al.[30]

Screening for Mutation Q39X in β-Globin Gene

The presence of the Q39X mutation in the β-globin gene was investigated in all FH families with the identified LDL-R gene mutation by using the allele-specific amplification method.[31]

Screening for Mutations in α-Globin Gene

The presence of the most common mutations of the α-globin gene present in the Mediterranean area were investigated in 1 homozygous FH patient and his daughter by using a PCR-based method reported by Foglietta et al.[32]

ApoE Genotyping

The apoE genotype was determined by PCR amplification of genomic DNA according to the procedure of Hixon and Vernier.[33]

Statistical Evaluation

Lipid values were adjusted for mean age, sex, mean body mass index (BMI), and apoE genotype by multiple linear regression analysis. Lipid values were also adjusted for the β^0-thalassemia carrier status. We arbitrarily scored apoE genotypes as follows: ϵ2/3 was scored 2, ϵ3/3 was 3, and ϵ2/4+ϵ3/4 was 4 (ϵ2/2 and ϵ4/4 were not found in our series); the β^0-thalassemia carrier status was scored 1; and the noncarrier status was scored 0. The comparison between FH subjects with and without the Q39X mutation of the β-globin gene was performed after adjustment for mean age, sex, mean BMI, and apoE genotype and for the effect of each LDL-R gene mutation on lipid values (ie, the lipid values for each mutation were adjusted to the grand mean of the whole sample). BMI values were adjusted for sex and age; IL-6 levels were adjusted for age.

The statistical significance of the differences between the means of adjusted values was assessed by Student *t* test for unpaired data. A value of $P<0.05$ was considered significant.

Results

The systematic analysis of the LDL-R gene, which was completed in 21 unrelated probands (20 heterozygotes and 1 homozygote), led to the identification of 7 point mutations of this gene, 5 of which have not been reported previously (Table 1).[29,34–39] By using rapid screening methods, we have been able to collect several families carrying 2 specific mutations, namely, 313+1 g>a and Fs572. These 2 clusters have been located in the eastern (313+1 g>a) and the western (Fs572) districts of northern Sardinia, respectively.

Unadjusted plasma total and LDL cholesterol levels found in our FH patients (Table 2) were within the range previously reported in molecularly defined FH heterozygotes, with the possible exception of a single subject carrying the A378T mutation, whose plasma total cholesterol and LDL cholesterol levels were 5.43 and 3.54 mmol/L, respectively. This subject (a 14-year-old girl) was the daughter of a patient with severe hypercholesterolemia (LDL cholesterol 10.11 mmol/L), consistent with the diagnosis of heterozygous FH, who had developed CAD at the age of 42 and underwent coronary bypass surgery for single-vessel disease. DNA analysis revealed that this subject was homozygous for the A378T mutation, whereas the LDL-R activity in his skin fibroblasts was 55% of the value found in control cell lines. One factor that might play a role in reducing the clinical expression of FH in the A378T proband and his daughter is the presence of a 3.7-kb deletion of one copy of the α_2-globin gene ($--/-\alpha^{3.7}$, α_2-thalassemia carrier). It is likely that α_2-thalassemia trait reduces plasma LDL cholesterol as does β^0-thalassemia trait (see below).

Effect of β^0-Thalassemia Trait on FH Phenotype

The systematic screening of the Q39X mutation of the β-globin gene in all probands and their FH relatives with identified mutations of the LDL-R gene revealed the presence of 19 carriers of the β-globin gene mutation of 73 FH subjects investigated. The distribution of the β-thalassemia carriers

TABLE 2. Clinical Features of FH Subjects With Identified Mutations of LDL-R Gene

Mutation	No. of Families	No. of Subjects	Mean±SD, mmol/L				No. of Families With Clinical Feature	
			TC	LDL-C	HDL-C	TG	Tx	pCAD
313+1 g>a	7	20 Het (7 M, 13 F)	8.37±1.71	6.51±1.69	1.21±0.24	1.44±0.86	0	1
C95R	1	6 Het (3 M, 3 F)	8.28±1.26	6.72±1.36	1.03±0.15	1.17±0.50	0	0
D118N	2	4 Het (2 M, 2 F)	8.64±1.06	6.82±1.24	0.89±0.38	2.15±0.97	1	2
C255W	1	1 Het (1 F)	10.57	9.07	1.06	1.21	1	0
A378T	1	1 Hom (1 M)	11.74	10.11	0.98	1.43	1	1
		1 Het (1 F)	5.43	3.54	1.24	1.80		
T413R	1	1 Het (1 F)	12.93	11.53	0.88	1.13	1	1
Fs572	8	39 Het (12 M, 27 F)	9.19±2.31	7.56±2.19	1.15±0.23	1.10±0.62	7	6

TC indicates total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglyceride; Tx, tendon xanthomatosis; pCAD, premature CAD (before 55 y in males or 65 y in females); Het, heterozygote; Hom, homozygote; M, male; and F, female. One patient heterozygous for Fs572 and homozygous for Q39X mutation of the β-globin gene is not included in the table.

was as follows: 3 cases in 2 families with the 313+1 g>a mutation, 4 cases in a single family with the C95R mutation, and 12 cases in 2 families with the Fs572 mutation. In addition, in 1 family with Fs572, 1 FH subject was homozygous for the Q39X mutation of the β-globin gene (β-thalassemia major). The hematologic parameters of these FH patients with and without β^0-thalassemia trait are shown in Table 3.

The FH heterozygote with β-thalassemia major was a 27-year-old female in a good clinical state who had been receiving red blood cell transfusions since infancy (≈36 transfusions per year). Her hematologic parameters are shown in Table 3. All routine laboratory tests (blood glucose, prothrombin time, blood urea nitrogen, serum creatinine, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, alkaline phosphatase, albumin, and fibrinogen) were within the normal range. Serum bilirubin was 1.4 mg/dL; serum iron, 195 μg/dL; and ferritin, 1435 mg/dL. At the age of 10 years, she had had hepatitis B, from which she fully recovered. Liver ultrasound examination did not reveal abnormalities in organ size and structure.

To ascertain the effect of β^0-thalassemia trait on FH phenotype, we compared plasma lipids between β^0-thalassemia carriers and noncarriers in the Fs572 cluster. After adjustment for age, sex, BMI, and apoE genotype ($\epsilon3\epsilon3$, n=37; $\epsilon3\epsilon4$, n=3), plasma total and LDL cholesterol levels in β^0-thalassemia carriers were 27% and 30% lower than those in noncarriers (Table 4). β^0-Thalassemia trait had no effect on HDL cholesterol and triglyceride levels (Table 4). In the FH patient affected by thalassemia major, plasma total and LDL cholesterol levels were below the 50th percentile of the distribution in subjects of the same sex and age from the general population.

In view of the LDL-lowering effect associated with the β^0-thalassemia trait observed in the Fs572 cluster, we decided to adjust the lipid values found in all FH patients not only for age, sex, BMI, and apoE genotype but also for the presence of β^0-thalassemia. This adjustment was adopted to eliminate the interference of these parameters with the effect of LDL-R mutation per se on the plasma lipid profile. The adjusted lipid values are shown in Table 5. The analysis of these data allowed us a more accurate comparison of the phenotypic expression of FH in the 2 clusters identified in our series. Carriers of the Fs572 mutation were found to have higher total and LDL cholesterol levels than carriers of the 313+1 g>a mutation. These differences are not so striking in the unadjusted lipid data shown in Table 2 because of the uneven number of β^0-thalassemia carriers in the 2 clusters.

To define the effect of the β^0-thalassemia trait on plasma lipids in all FH subjects, regardless of the type of LDL-R

TABLE 3. Hematological Parameters of FH Patients With and Without β^0-Thalassemia

	Males			Females			*β^0-Thalassemia Homozygote
	Noncarriers (n=16)	Carriers (n=8)		Noncarriers (n=36)	Carriers (n=11)	*P*	(n=1)
RBC, $\times10^6/\mu$L	5.07±0.37	6.30±0.45	<0.001	4.86±0.32	5.82±0.16	<0.001	4.46
Hb, g/dL	14.4±0.6	13.5±1.0	<0.001	13.9±0.9	11.8±0.7	<0.001	13.20
Ht, %	42.3±2.0	41.8±3.5	<0.001	40.9±2.9	36.3±1.6	<0.001	37.60
Reticulocytes, %	1.44±0.13	2.01±0.10	<0.001	1.43±0.18	1.94±0.12	<0.001	0.27
MCV, fL	83.9±4.6	66.1±3.4	0.001	84.6±5.4	62.3±2.5	<0.001	84.40
MCH, pg	28.5±2.0	21.4±1.4	<0.001	28.7±1.9	20.2±0.9	<0.001	29.70
MCHC, g/dL	34.0±1.2	32.4±0.7	<0.05	33.9±0.9	32.5±1.2	0.001	35.20
HbA_2, %	2.45±0.57	5.07±1.15	<0.001	2.67±0.29	5.67±0.49	<0.001	2.90

RBC indicates red blood cell; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular Hb; and MCHC, mean corpuscular Hb concentration.

*RBC transfusions every 10–12 d.

TABLE 4. Plasma Lipid Values in Heterozygous FH Subjects With Fs572 Mutation of LDL-R Gene With and Without Q39X Mutation in β-Globin Gene

	Noncarriers (n=27)	Q39X Heterozygotes (n=12)	*P*	Q39X Homozygote (n=1)
BMI, kg/m^2	22.7±2.5	22.3±2.3	NS	18.7
TC, mmol/L	9.96±1.71	7.25±0.99	<0.001	3.67
LDL-C, mmol/L	8.25±1.66	5.76±1.08	<0.001	2.61
HDL-C, mmol/L	1.19±0.21	1.10±0.23	NS	0.67
TG, mmol/L	1.20±0.51	0.86±0.61	NS	0.87

BMI values (mean±SD) are adjusted for sex and age; lipid values (mean±SD) are adjusted for age, sex, BMI, and apoE genotype. NS indicates not significant.

mutation, we arbitrarily adjusted plasma lipids for age, sex, BMI, and apoE genotype (ε2/3, n=5; ε2/4, n=2; ε3/3, n=56; and ε3/4, n=8) as well as for the effect of the LDL-R gene mutations (see Methods for details). This analysis showed that plasma total and LDL cholesterol levels in FH patients with β^0-thalassemia trait were 23% and 27% lower than those observed in FH patients without β^0-thalassemia. In absolute terms, this corresponds to a reduction of ≈2.0 mmol/L of total and LDL cholesterol (Table 6). In addition, we looked at the effect of β^0-thalassemia in all unrelated FH heterozygotes of our series (62 subjects), with and without identified mutations of the LDL-R gene. In carriers of β^0-thalassemia, plasma total and LDL cholesterol levels were 74% and 68%, respectively, of the values observed in noncarriers (Table 7).

Clinical Features of FH

We tried to quantify the prevalence of tendon xanthomatosis and premature CAD in the families belonging to the 2 clusters identified in the present study. Families were considered positive for tendon xanthomatosis and premature CAD if one or both these clinical features were present in at least 1 family member (Table 2). For this comparison, we considered only FH subjects >40 years of age (because tendon xanthomatosis and premature CAD usually occur after that age) who were not carriers of the β^0-thalassemia trait. Cluster Fs572 included 15 FH subjects (mean age 56.8 years) belonging to 8 families (1.87 subjects per family). Nine (60%) of these subjects had tendon xanthomatosis, and 7 (47%) had premature CAD. Cluster 313+1 g>a included 12 FH subjects (mean age 59.3 years) belonging to 7 families (1.71 subjects per family). None of them had tendon xanthomatosis, and only 1 (8%) had premature CAD. These findings, in addition to the higher LDL cholesterol level found in Fs572 carriers (Table 5), strongly suggest that the latter mutation is more severe than the 313+1 g>a mutation.

Haplotypes Cosegregating With 313+1 g>a and Fs572 Mutations

Intragenic haplotype analysis showed that the 313+1 g>a mutation cosegregated with haplotype A in 4 families and with haplotype B in 3 families. Haplotype A was as follows: *Stu*I (+), *G/A nt 1413* (G), *Hin*cII (−), *Ava*II (+), *Msp*I 5′ (+), *Apa*LI 5′ (+), *Pvu*II (−), *Nco*I (+), and *Apa*LI 3′ (+). Haplotype B was as follows: *Stu*I (+), *G/A nt 1413* (A), *Hin*cII (+), *Ava*II (−), *Msp*I 5′ (−), *Apa*LI 5′ (−), *Pvu*II (+), *Nco*I (+), and *Apa*LI 3′ (+).

The Fs572 mutation cosegregated with a single haplotype in all families. This haplotype was as follows: *Stu*I (+), *G/A nt 1413* (G), *Hin*cII (−), *Bst*EII (−), *Ava*II (+), *Msp*I 5′ (+), *Apa*LI 5′ (+), *Pvu*II (−), *G/A nt 2635* (A), and *Apa*LI 3′ (+).

Discussion

In a group of 63 FH probands of Sardinian ancestry, we identified 21 unrelated FH patients carrying 7 mutations of the LDL-R gene (Table 1). Against our predictions, these findings suggest that in Sardinians, as in other populations, FH is caused by many mutations of the LDL-R gene. In the present study, we have been able to investigate the clinical phenotype of 71 molecularly defined FH heterozygotes (52 of whom belonged to 2 major clusters of mutations: 313+1 g>a and Fs572) and to demonstrate the LDL-lowering effect of β^0-thalassemia. The analysis of the 2 clusters deserves some consideration.

TABLE 5. Clinical Features and Lipid Parameters of Heterozygous FH Subjects With Identified Mutations of LDL-R Gene

Mutation	No. of Families	No. of Heterozygous Subjects	Mean±SD, mmol/L TC	LDL-C	HDL-C	TG
313+1 g>a	7	20	8.48±1.50*	6.63±1.44*	1.22±0.19	1.45±0.61
C95R	1	6	9.87±0.64	8.28±0.59	1.07±0.12	1.18±0.41
D118N	2	4	8.86±0.76	7.04±1.03	0.94±0.33	2.01±0.53
C255W	1	1	10.20	8.70	1.00	1.26
T413R	1	1	12.29	11.02	0.86	0.90
Fs572	8	39	10.03±1.57	8.36±1.55	1.16±0.23	1.18±0.55

Lipid values are adjusted for age, sex, BMI, apoE genotype, and β^0-thalassemia carrier status.
*P<0.01 vs Fs572.

TABLE 6. Plasma Lipids in Heterozygous FH Subjects With and Without Q39X Mutation of β-Globin Gene

	Noncarriers (n=52)	Heterozygous Carriers (n=19)	P
BMI, kg/m^2	22.8±3.0	22.9±3.8	NS
TC, mmol/L	9.48±1.73	7.27±1.08	<0.001
LDL-C, mmol/L	7.78±1.53	5.64±1.16	<0.001
HDL-C, mmol/L	1.15±0.19	1.12±0.25	NS
TG, mmol/L	1.30±0.52	1.19±0.59	NS

BMI values (mean±SD) are adjusted for age and sex; lipid values (mean±SD) are adjusted for age, sex, BMI, and apoE genotype and for effect of LDL-R gene mutations. The patient heterozygous for A378T mutation and carrier of α-thalassemia trait and the patient heterozygous for Fs572 mutation and affected by β^0-thalassemia major were not included.

The 313+1 g>a in intron 3, which is associated with a receptor-defective phenotype,[35] was first reported in FH patients from Norway (FH Elverum),[29] and it was subsequently observed in other populations.[34–38] One of the interesting clinical features of the Sardinian patients with the 313+1 g>a mutation is the relatively mild elevation of LDL cholesterol (6.51±1.69 mmol/L) compared with the corresponding value found in Norwegian patients (7.96±1.65 mmol/L).[40] This difference, which is not explained by the presence of β^0-thalassemia carriers among the Sardinian patients (only 3 of 20 of these patients were β^0-thalassemia carriers), suggests that other genetic or environmental factors have a strong influence on the phenotypic expression of this mutation.

The Fs572 mutation in exon 12 (FH Sassari-1) is a novel mutation consisting of a single nucleotide deletion, which leads to the formation of a truncated protein of 642 amino acids (receptor-negative phenotype). Compared with the patients with the 313+1 g>a mutation, Fs572 subjects have higher total and LDL cholesterol levels and a higher prevalence of tendon xanthomatosis and premature CAD (Tables 2 and 5).

In the present study, we have had the chance to ascertain whether the mild LDL-lowering effect produced by the β^0-thalassemia trait in the general population of Sardinia[16] was still effective in heterozygous FH. Because different LDL-R mutations are associated with different phenotypic expression of FH (producing a mild, moderate, or severe phenotype),[41] at first we looked at the patients carrying the same LDL-R mutation (ie, Fs572, FH Sassari-1), which is expected to result in a receptor-negative phenotype. We then determined that the phenotypic expression of FH Sassari-1 is strongly affected by the presence of a specific mutation of the β-globin gene (Q39X), known to cause β^0-thalassemia in the homozygous state (β^0 39). The mean LDL cholesterol level in FH patients who are β^0-thalassemia carriers was 30% lower than that observed in the patients without the β^0-thalassemia trait (Table 4). Further evidence of this LDL-lowering effect is given by the observation of an FH patient heterozygous for FH Sassari-1 who was affected by β^0-thalassemia major. Plasma LDL cholesterol of this patient, who had no clinical or laboratory signs of liver disease (a condition that might have reduced the hepatic production of apoB-containing lipoproteins), was much lower than that observed in the FH patients of the same cluster and was below the mean LDL cholesterol level found in the Sardinian population. A similar LDL-lowering effect induced by β^0-thalassemia trait was observed when all FH patients with identified mutations of the LDL-R gene were considered together (Table 6) and when we analyzed the entire group of 62 unrelated FH subjects, irrespective of whether the molecular defect of LDL-R gene had been identified (Table 7).

TABLE 7. Plasma Lipids in Unrelated Heterozygous FH With and Without Q39X Mutation of β-Globin Gene

	Noncarriers (n=47; 18 M, 29 F)	Heterozygous Carriers (n=15; 5 M, 10 F)	P
TC, mmol/L	9.93±1.46	7.30±0.80	<0.001
LDL-C, mmol/L	8.06±1.51	5.44±0.88	<0.001
HDL-C, mmol/L	1.21±0.28	1.31±0.26	NS
TG, mmol/L	1.47±0.49	1.27±0.47	NS
IL-6, pg/mL	2.06±0.42	3.30±0.46	<0.001

Lipid values (mean±SD) are adjusted for age, sex, and BMI; Il-6 values (mean±SD) are adjusted for age. The patient homozygous for A378T mutation (Table 2) was not included.

Two major mechanisms might account for the LDL-lowering effect of β^0-thalassemia in FH. First, the mild anemia, frequently observed in β^0-thalassemia carriers[42] and documented in Table 3, is expected to induce the secretion of erythropoietin, which stimulates the differentiation of the erythroid progenitor cells in the bone marrow and promotes their proliferation, leading to a mild erythroid hyperplasia.[42] Because cell proliferation is associated with an increased requirement for cholesterol, as documented in several cell systems in vitro,[43–49] one way to meet these requirements is to increase the expression of the LDL-R (ie, the number of the LDL-Rs on the cell surface).[43–49] In this context, it is reasonable to assume that in heterozygous FH with β^0-thalassemia trait, the proliferation of erythroid progenitor cells might be associated with an overexpression of the normal LDL-R allele. The combined effect of erythroid hyperplasia and the increased number of wild-type LDL-R per cell might increase the receptor-mediated removal of plasma LDL in the bone marrow, thus reducing the expected elevation of plasma LDL caused by the presence of a mutant allele of the LDL-R gene. A similar overexpression of the LDL-R has been postulated to cause hypocholesterolemia in patients with some forms of leukemia or myeloproliferative disorders.[50–53]

The second mechanism may be related to the activation of the monocyte/macrophage system in various districts of the body. Erythrokinetic measurements indicate that in β^0-thalassemia carriers, red blood cell survival is slightly shortened, presumably because abnormal red blood cells (anisopoikilocytosis) are removed more rapidly from the circulation by the macrophages in spleen, bone marrow, and liver.[42] This chronic mild activation of the macrophage system might be associated with an increased release of some cytokines, generating a situation similar to that found in a mild chronic inflammation. It is well established that chronic inflammation causes hypocholesterolemia through a reduction of LDL and, to some extent, HDL.[54] There is evidence that some cytokines like, IL-1, IL-6, and tumor necrosis factor-α, may contribute

to these lipoprotein changes.[54] In HepG2 cells, the effect of these cytokines is 2-fold: they reduce the secretion of apoB-containing lipoproteins and increase the expression of the LDL-R.[55–58] The observation that in our probands the plasma levels of IL-6 was higher in β^0-thalassemia carriers than in noncarriers (Table 7) and the previous report of an increased tumor necrosis factor-α in β^0-thalassemia homozygous patients[59] indicate that the overproduction of some cytokines by macrophages is a plausible mechanism for the LDL-lowering effect of β^0-thalassemia.

The LDL-lowering effect of β^0-thalassemia trait in heterozygous FH raises the question as to whether this effect might slow down the progression of coronary atherosclerosis and delay the occurrence of myocardial infarction. The answer to this question requires a well-designed prospective study of a large number of molecularly defined FH patients with and without the β^0-thalassemia trait.

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