Atherosclerosis 227 (2013) 342–348

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy

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ARTICLE INFO

Article history:
Received 16 October 2012
Received in revised form
3 January 2013
Accepted 9 January 2013
Available online 19 January 2013

Keywords:
Autosomal dominant hypercholesterolemia
LDL receptor
Apolipoprotein B
PCSK9
Mutations

ABSTRACT

Objective: To determine the spectrum of gene mutations and the genotype–phenotype correlations in patients with Autosomal Dominant Hypercholesterolemia (ADH) identified in Italy.
Methods: The resequencing of *LDLR*, *PCSK9* genes and a selected region of *APOB* gene were conducted in 1018 index subjects clinically heterozygous ADH and in 52 patients clinically homozygous ADH. The analysis was also extended to 1008 family members of mutation positive subjects.
Results: Mutations were detected in 832 individuals: 97.4% with *LDLR* mutations, 2.2% with *APOB* mutations and 0.36% with *PCSK9* mutations. Among the patients with homozygous ADH, 51 were carriers of *LDLR* mutations and one was an *LDLR*/*PCSK9* double heterozygote. We identified 237 *LDLR* mutations (45 not previously reported), 4 *APOB* and 3 *PCSK9* mutations. The phenotypic characterization of 1769 *LDLR* mutation carriers (ADH-1) revealed that in both sexes independent predictors of the presence of tendon xanthomas were age, the quintiles of LDL cholesterol, the presence of coronary heart disease (CHD) and of receptor negative mutations. Independent predictors of CHD were male gender, age, the presence of arterial hypertension, smoking, tendon xanthomas, the scalar increase of LDL cholesterol and the scalar decrease of HDL cholesterol. We identified 13 *LDLR* mutation clusters, which allowed us to compare the phenotypic impact of different mutations. The LDL cholesterol raising potential of these mutations was found to vary over a wide range.
Conclusions: This study confirms the genetic and allelic heterogeneity of ADH and underscores that the variability in phenotypic expression of ADH-1 is greatly affected by the type of *LDLR* mutation.

1. Introduction

The term monogenic hypercholesterolemia is used to indicate a heterogeneous group of Mendelian disorders characterized by the selective increase of plasma low-density lipoprotein cholesterol (LDLc), which causes the accumulation of cholesterol in the arterial wall resulting in accelerated atherosclerosis and premature coronary heart disease (pCHD) [1]. Monogenic hypercholesterolemia includes two genetic subtypes designated Autosomal Dominant Hypercholesterolemia (ADH) and Autosomal Recessive Hypercholesterolemia (ARH), respectively [2].

ADH (OMIM # 143890) is one of the most frequent inherited disorders with an estimated frequency of 1:300/1:500 in most populations [1,2]. Several sets of diagnostic criteria have been adopted for the clinical diagnosis of ADH (MED-PED, Simon Broome Register and Dutch Lipid Clinic Network (DLCN)) [3–5]. ADH is genetically heterogeneous as it can be caused by defects in at least three different genes that encode proteins involved in the hepatic clearance of LDLc mediated by the LDL receptor (LDLR). These defects may be due to mutations in the gene coding for the LDLR (classic Familial Hypercholesterolemia, FH or ADH-1), in the gene coding for the apolipoprotein B (Familial Defective apoB, FDB or ADH-2) and the gene coding for the enzyme PCSK9 (ADH-3) [1–3,6]. Mutations in *LDLR* gene are the most frequent cause of ADH

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http://dx.doi.org/10.1016/j.atherosclerosis.2013.01.007

(from 90 to 95%); mutations in *APOB* account for 3–6% and mutations in *PCSK9* are found in less than 1% of patients [7,8]. In approximately 15–19% of patients with the clinical diagnosis of definite ADH no mutations in the three candidate genes have been detected, suggesting the presence of other yet unknown genes [6,7].

A large number of mutations in *LDLR* gene have been reported in patients with ADH-1 (online FH data base www.ucl.ac.uk/ldlr/LOVD.1.10) [9,10]. With the exception of some populations where few mutations account for most ADH-1 patients (founder effect), in most populations there is a great heterogeneity of *LDLR* mutations [9,11]. By contrast only few mutations of *APOB* and *PCSK9* were found to be responsible for ADH-2 and ADH-3, respectively [6,12].

The aims of this study were the assessment of the molecular bases of ADH in a cohort of patients attending the Italian Lipid Clinics at the University Hospitals of Genova, Modena and Palermo and the systematic analysis of the clinical features of mutation positive ADH patients.

2. Methods

2.1. Subjects

This study includes 1018 unrelated index patients (478 males and 540 females, 43.9 ± 17.3 years of age, range 2–86 years) with the clinical diagnosis of heterozygous ADH, who, over the last two decades, had been investigated at the Lipid Clinics of the University Hospitals of Genova, Modena and Palermo. The clinical diagnosis of ADH was made by adopting a combination of criteria, including: i) untreated plasma low-density lipoprotein cholesterol (LDLc) level above the 95th percentile of the distribution in the population (stratified for gender and age) [13,14] and plasma triglyceride levels below 2.8 mmol/L, after the exclusion of secondary hypercholesterolemias; ii) the presence of tendon xanthomas in the index patient or in at least one family member or the presence of hypercholesterolemia in some prepuberal children of the family; iii) premature coronary heart disease (pCHD), before 55 years of age in males and 65 in females, in the index subject or in one first-degree relative; iv) vertical transmission and bimodal distribution of hypercholesterolemia in the family. The criteria specified in ii) and iii) were fulfilled only in approximately 45% of the index patients. In the remaining 55% of index cases these criteria were not fulfilled for the absence of tendon xanthomas, the lack of reliable family data or the absence of pCHD in the family.

In order to obtain a probability score of having ADH we retrospectively classified the patients according to the clinical criteria of the Dutch Lipid Clinic Network (DLCN) [15]. According to DLCN score, 473 subjects (46.5%) were classified as “definite ADH”, 257 (25.2%) as “probable ADH” and 288 (28.3%) as “possible ADH”, respectively. Data on current and previous smoking (SMO), arterial hypertension (AH), diabetes or other diseases with impact on cardiovascular risk were recorded. The few patients with diabetes (6 patients with type II diabetes) and those carrying the β-thalassemia trait [16], were excluded from the clinical survey as both conditions are known to affect plasma lipoprotein levels.

In addition 50 patients were referred to the Lipid Clinics with the clinical diagnosis of probable homozygous ADH (HO-ADH) on the basis of the following criteria: i) plasma LDLc ≥13 mmol/L; ii) the presence of tendon and cutaneous xanthomas in infancy and iii) history of hypercholesterolemia in both parents. In addition, two children were assumed to have homozygous ADH in view of the presence of extensive cutaneous xanthomatosis, despite a LDLc level (10.21 and 12.88 mmol/L, respectively) below the cut-off of 13 mmol/L.

All subjects found to have molecularly defined ADH underwent cardiovascular examination, including exercise ECG, thallium test or stress echocardiography and ultrasound evaluation of the carotid arteries. In some cases coronary angiography was also performed. The subjects were considered positive for CHD (CHD+) as reported previously [17] and described in the supplemental methods.

Informed written consent was obtained from the index subjects and their family members or, in the case of children, from their parents. The study protocol was approved by the institutional human investigation committee of each participating institution.

2.2. Biochemical analyses

Plasma concentrations of total cholesterol (Tc), triglycerides (Tg) and high-density lipoprotein cholesterol (HDLc) were measured by standard methods [18]. LDLc was calculated by the Friedewald’s formula.

2.3. LDL receptor activity

The assay of LDL receptor activity in cultured skin fibroblasts was performed as previously reported [19]. This assay was performed in all patients found to carry two *LDLR* mutant alleles and in a few other patients either simple heterozygous for *LDLR* mutations or double heterozygous for *LDLR* and *PCSK9* mutations [19,20].

2.4. Sequence analysis of candidate genes for ADH

Genomic DNA was extracted from peripheral blood by a standard procedure. *LDLR* gene was analyzed by direct re-sequencing [20]. The search for major gene rearrangements was performed by Southern blot analysis [19] or by multiple ligation-dependent probe amplification (MLPA) (MRC Holland, Amsterdam, The Netherlands) [21]. The re-sequencing of the whole *PCSK9* gene and of the 3′ end of exon 26 of *APOB* gene (from c.9216 to c.11788 +152 nt of intron 26) were performed in: i) all patients negative for *LDLR* mutations; ii) in all patients with the clinical features consistent with the diagnosis of homozygous ADH in whom only one *LDLR* mutant allele had been found [20].

The mutations were designated according to the Human Genome Variation Society, 2012 version (http://www.hgvs.org/mutnomen/recs-DNA.html). LDLR, APOB and PCSK9 protein sequence variants were designated according to http://www.hgvs.org/mutnomen/recs-prot.html.

2.5. Northern blot analysis and reverse transcription-PCR amplification (RT-PCR)

The analysis of LDLR mRNA, was performed in ADH-1 subjects carrying major gene rearrangements or intronic mutations suspected to affect splicing, whose skin fibroblasts were available in our cell bank [19].

2.6. In silico analysis

The *in silico* prediction of the effect of the missense mutations of *LDLR*, *APOB* and *PCSK9* genes was performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT Human Protein (http://sift.jcvi.org/), refined SIFT [9] and Mutation Taster (http://neurocore.charite.de/MutationTaster/).

The *in silico* prediction of the effect of intronic variants was performed using NetGene2 (http://www.cbs.dtu.dk/services/NetGene2), Human Splicing Finder (http://www.umd.be/HSF/HSF.html) and Automated Splice Site Analysis (https://splice.uwo.ca).

2.7. Statistical analysis

Statistical analyses were performed using SPSS (PASW Statistics 18, Release Version 18.0; SPSS, Inc., 2009, Chicago, IL, www.spss.com). Details of the statistical analysis are given in supplemental methods.

3. Results

3.1. Mutation detection

In the group of 1018 index subjects, suspected to have heterozygous ADH (HE-ADH), mutations in the candidate genes were detected in 832 individuals (809 Italians and 23 of different ethnicities) with a mutation detection rate of ~82%. Among these subjects 811 (97.4%) were carriers of *LDLR* mutations (ADH-1), 18 (2.2%) of *APOB* mutations (ADH-2) and 3 (0.36%) of *PCSK9* mutations (ADH-3), respectively. The mutation detection rate in the three groups of subjects stratified according to DLCN score [15] was the following: "definite ADH" 435/473 (91.9%); "probable ADH" 197/257 (76.6%) and "possible ADH" 200/288 (69.4%).

The genetic analysis revealed that 14 subjects assumed to be ADH heterozygotes (with LDLc level ranging from 6.90 to 12.40 mmol/L) and classified as "definite ADH" were in fact: i) homozygotes (HO) or compound heterozygotes (CHE) for mutations in *LDLR* gene (8 HO and 4 CHE, respectively) or ii) double heterozygotes for mutations in *APOB* and *LDLR* genes (one subject) or for mutations in *PCSK9* and *LDLR* genes (one subject).

The candidate gene analysis in the 52 patients assumed to have HO-ADH revealed that 51 were carriers of *LDLR* mutations. More specifically, 32 were HO, 17 were CHE, while 2 were found to carry only one mutant *LDLR* allele, despite the clinical features consistent with HO-ADH. In these two patients no mutations in *APOB* and *PCSK9* were found. The last HO-ADH patient was found to be a *PCSK9*/*LDLR* double heterozygote.

3.2. Mutations in the ADH candidate genes

LDLR gene. In our cohort we found 237 different mutations distributed as follows: 25 major rearrangements (10.5%), 26 min deletions/insertions (11.0%), 160 single nucleotide mutations in the coding sequence (118 missense, 27 nonsense and 15 frameshift) (67.5%) and 26 splice site mutations (11.0%), all listed in Supplementary Tables S.1, S.2, S.3A, S.4, S.5 and S.6. We found 45 novel mutations which are shown, together with their predicted effect on LDLR protein, in Supplementary Tables S.1, S.2, S.3B, S.4, S.5, S.6 and S.7.

APOB gene. The four *APOB* gene missense mutations found in our index cases are reported in Supplementary Table S.8. Three of them were known mutations, while the other [c.9639 C>A, p.(N3213K)], found in an Asian-Indian patient with LDLc level of 8.75 mmol/L was novel.

PCSK9 gene. The three missense mutations found in index cases had been previously described by us [20] and other groups [12] (Supplementary Tables S.9).

3.3. In silico analysis of the LDLR, APOB and PCSK9 missense mutations

The *in silico* analysis of the missense mutations of the three ADH genes is shown in Supplementary Tables S.3A, S.8 and S.9. In the case of the LDLR, 102 mutations resulted to be pathogenic and 6 non-pathogenic, based on the consensus of five algorithms. The other 10 mutations, whose pathogenic effect *in silico* was uncertain, were considered to be possibly damaging on the basis of several criteria, such as the amino acid conservation during evolution [9] and the changes in polarity, molecular weight and hydropathy index (Supplementary Table S.3B). Some of these mutations were classified as pathogenic also by other groups (see References indicated in Supplementary Table S.3A).

The *in silico* analysis of APOB mutations confirmed the pathogenic effect of the three known mutations [p.(R3527W), p.(R3527Q) and p.(R3558C)], while the new mutation p.(N3186K) was defined as benign by PolyPhen-2 and pathogenic by SIFT. *In silico* analysis of this mutation for effect on splicing gave negative results. In the absence of familial segregation analysis or *in vitro* functional studies the effect of this mutation remains to be defined.

The *in silico* analysis of PCSK9 mutations indicated that these mutations could affect the function of the PCSK9 protein. The three mutations we found in our cohort [p.(S127R), p.(N425S) and p.(R496W)] can be regarded as gain of function (GOF) mutations in view of the results of *in vitro* studies [12,22]. The p.(R496W) and p.(N425S) mutations were found to exacerbate the hypercholesterolemic phenotype when associated with a *LDLR* mutation [20], while the p.(S127R) mutation was reported in patients with monogenic hypercholesterolemia not linked to *APOB* or *LDLR* genes [12].

3.4. In silico analysis of the intronic mutations of LDLR

The *in silico* analysis of the intronic *LDLR* mutations and their presumed or documented effect on LDLR protein is shown in Supplementary Table S.6. The *in silico* analysis as well as previous observations (see supplementary references reported in Table S.6), showed that 18 mutations were pathogenic, 1 possibly pathogenic and 6 non-pathogenic.

3.4.1. In silico prediction of pathogenicity

The combined *in silico* techniques enabled us to assign with confidence pathogenic predictions to 88% of all missense variants (to 16 out of 18 novel missense variants) and to 76% of all intronic variants (to 3 out of 3 novel intronic variants).

3.5. Screening for the ADH mutations in family members of the ADH index subjects

By extending the genomic analysis to family members of mutation positive index cases we were able to recruit additional mutation carriers (984 with *LDLR* mutations, 22 with *APOB* mutations and 2 with *PCSK9* mutations).

3.6. Phenotypic characterization of ADH-1 heterozygotes

We conducted the clinical characterization of all subjects heterozygous for *LDLR* mutations (811 index cases and 984 family members). We excluded from this analysis 19 index cases and 7 family members carrying non-pathogenic mutations. As a whole we included 1769 ADH-1 heterozygotes in the clinical study.

Supplementary Fig. S.1 shows the distribution of LDLc level in ADH-1 heterozygotes, highlighting the large inter-individual variability of this parameter. More specifically 3.8% of individuals had LDLc levels below the 95th percentile of the general population level and 2.8% had LDLc levels above 10 mmol/L, a value occasionally observed in ADH-1 homozygotes (see below). Supplementary Fig. S.2 shows the age-dependent variations of plasma LDLc in both sexes.

Table 1 shows the clinical features in the index subjects and in the whole HE-ADH-1 group (index cases plus family members) carrying pathogenic mutations of *LDLR* gene. There was no gender difference with regard to LDLc level, prevalence of tendon xanthomas and carotid atherosclerosis. Males had lower levels of HDLc

Table 1
Clinical features and plasma lipids in ADH-1 heterozygotes with pathogenic mutations in the *LDLR* gene.

	Index cases		*P*	All subjects		*P*
	Males	Females		Males	Females	
No.	371	421		818	951	
Age (years)	42.3 ± 16.8	45.4 ± 17.8	0.02	35.2 ± 19.0	38.1 ± 20.0	0.003
BMI (kg/m^2)	24.7 ± 3.2	24.0 ± 4.4	0.03	23.3 ± 3.9	22.5 ± 4.5	0.003
Tc	9.52 ± 1.75	9.75 ± 1.82	NS	9.03 ± 1.74	9.21 ± 1.80	NS
	9.30 ± 1.67[a]	9.40 ± 1.59[a]		9.00 ± 1.47[b]	9.15 ± 1.50[b]	
LDLc	7.68 ± 1.70	7.75 ± 1.74	NS	7.22 ± 1.69	7.27 ± 1.70	NS
	7.48 ± 1.64[a]	7.44 ± 1.53[a]		7.15 ± 1.42[b]	7.23 ± 1.41[b]	
HDLc	1.19 ± 0.33	1.41 ± 0.35	0.0001	1.20 ± 0.32	1.39 ± 0.34	0.0001
	1.20 ± 0.33[a]	1.41 ± 0.35[a]		1.24 ± 0.26[b]	1.33 ± 0.27[b]	
Tg	1.44 ± 0.65	1.32 ± 0.62	0.02 0.001	1.37 ± 0.68	1.22 ± 0.61	0.0001
	1.38 ± 1.62[a]	1.23 ± 0.59[a]		1.27 ± 0.48[b]	1.21 ± 0.48[b]	0.03
Tx	43.6%	44.3%	NS	29.9%	31.3%	NS
AH	16.2%	23.8%	0.03	10.3%	15.7%	0.005
Previous SMO	31.8%	11.3%	0.0001	19.5%	6.2%	0.0001
Current SMO	18.1%	12.6%	NS	15.1%	8.4%	0.0004
CA-ATS	52.7%	57.3%	NS	43.7%	42.4%	NS
CHD	42.9%	23.3%	0.0001	27.4%	14.4%	0.0001

Values are mean ± SD; lipid values are reported in mmol/L.
Tx: tendon xanthomatosis; AH: arterial hypertension; SMO: smoking habit; CA-ATS: carotid atherosclerosis with >25% stenosis; CHD: coronary heart disease; NS: no significant difference.
[a] Adjusted for age.
[b] Adjusted for age and familial relations.

and higher levels of Tg than females. Smoking habit was prevalent in males, while arterial hypertension was prevalent in females. The prevalence of CHD was significantly higher in males.

Supplementary Table S.10 shows the clinical features in subjects with and without tendon xanthomas (Tx). Subjects with Tx were older, had higher levels of LDLc and Tg and a higher prevalence of arterial hypertension, carotid atherosclerosis and CHD. Supplementary Fig. S.3 shows the prevalence of Tx as a function of age.

In order to assess the impact of the *LDLR* mutations on the phenotype, the mutation carriers were divided into three groups designated receptor-negative (RN), receptor-defective (RD) and receptor-unclassified (RU) group, respectively. The RN group included carriers of exon deletions, exon duplications, nonsense mutations, nucleotide deletions and insertions in the coding sequence and splicing mutations (predicted to result in null alleles or premature truncations of the receptor protein), as well as carriers of some missense mutations resulting in LDL receptor activity (measured in cultured skin fibroblasts) <5% in homozygotes and <55% in heterozygotes of the activity found in control fibroblasts. The RD group included the carriers of some missense mutations resulting in a residual receptor activity in cultured fibroblasts between 5% and 35% in homozygotes and >55% in heterozygotes [18,19]. The RU group included carriers of unclassified missense mutations, some exon duplications and in frame amino acid deletions, duplications or deletions/insertions.

Table 2 shows that, as compared to RD subjects, the RN subjects had significantly higher levels of Tc and LDLc and lower levels of HDLc; they also had a higher prevalence of Tx, carotid atherosclerosis and CHD.

Table 2
Clinical features and plasma lipids in ADH-1 heterozygotes carrying LDL receptor-defective (RD), receptor-negative (RN) and receptor unclassified (RU) mutations.

	Index cases			*P*	All subjects			*P*
	RD	RN	RU		RD	RN	RU	
Gender M/F	150/156	169/203	51/63	NS	273/285	443/544	121/122	NS
Age (years)	42.7 ± 18.1	45.1 ± 16.7	43.2 ± 18.3	NS	36.5 ± 19.8	36.7 ± 19.4	37.5 ± 20.3	NS
BMI (kg/m^2)	23.9 ± 3.9	24.8 ± 4.0[c]	23.6 ± 3.9	0.02	22.9 ± 4.2	22.9 ± 4.3	22.5 ± 4.0	NS
Tc	8.96 ± 1.58	10.19 ± 1.75[e]	9.47 ± 1.74	0.0001	8.45 ± 1.60	9.58 ± 1.76[e]	9.04 ± 1.69	0.0001
	8.72 ± 1.41[a]	9.86 ± 1.62[a,e]	9.22 ± 1.51[a]		8.42 ± 1.37[b]	9.53 ± 1.41[b,e]	8.99 ± 1.44[b]	
LDLc	6.98 ± 1.52	8.30 ± 1.65[e]	7.56 ± 1.67	0.0001	6.55 ± 1.52	7.72 ± 1.66[e]	7.14 ± 1.63	0.0001
	6.77 ± 1.37[a]	8.00 ± 1.54[a,e]	7.33 ± 1.48[a]		6.51 ± 1.29[b]	7.65 ± 1.32[b,e]	7.08 ± 1.38[b]	
HDLc	1.37 ± 0.36[d]	1.25 ± 0.33[d]	1.33 ± 0.39	0.0001	1.34 ± 0.34	1.27 ± 0.34[d]	1.30 ± 0.37	0.001
	1.38 ± 0.35[a]	1.26 ± 0.31[a,d]	1.33 ± 0.37[a]		1.33 ± 0.29[b]	1.26 ± 0.26[b,d]	1.31 ± 0.31[b]	
Tg	1.35 ± 0.66	1.42 ± 0.63	1.30 ± 0.62	NS	1.26 ± 0.64	1.31 ± 0.66	1.30 ± 0.63	NS
	1.28 ± 0.61[a]	1.33 ± 0.60[a]	1.22 ± 0.58[a]		1.26 ± 0.50[b]	1.32 ± 0.50[b]	1.30 ± 0.49[b]	
Tx	28.1%	58.3%[e]	34.8%	0.0001	18.4%	39.7%[e]	25.3%	0.0001
AH	18.0%	23.2%	16.2%	NS	13.4%	14.0%	9.2%	NS
Previous SMO	20.2%	19.0%	27.9%	NS	12.4%	11.2%	15.7%	NS
Current SMO	15.5%	12.9%	22.0%	NS	13.7%	9.7%	13.6%	NS
CA-ATS	45.9%	60.9%[d]	56.0%	0.01	36.6%	45.9%[d]	43.9%	NS
CHD	25.8%	40.7%[e]	20.9%	0.0001	16.8%	23.7%[e]	16.1%	0.003

Values are mean ± SD; lipid values are reported in mmol/L.
Tx: tendon xanthomatosis; AH: arterial hypertension; SMO: smoking habit; CA-ATS: carotid atherosclerosis with >25% stenosis; CHD: coronary heart disease; NS: no significant difference.
[a] Adjusted for gender and age.
[b] Adjusted for gender, age and familial relations.
[c] Significantly different from RU.
[d] Significantly different from RD.
[e] Significantly different from RD and RU.

Multiple logistic regression analysis showed that independent predictors of the presence of Tx were, in addition to age, the quintiles of LDLc, the presence of CHD and RN mutations (Supplementary Table S.11).

Since CHD was not detected in subjects below 30 years of age, we compared the clinical features and plasma lipids in subjects over 30 with (CHD+) or without (CHD−) coronary heart disease. In CHD+ group we found a higher prevalence of males, Tx, arterial hypertension, previous smoking habit, carotid atherosclerosis, and of carriers of RN mutations. Moreover, as compared to CHD−, the CHD+ subjects were older, had higher BMI, higher levels of Tc, LDLc and Tg, and lower levels of HDLc (Table 3). Supplementary Fig. S.4 shows the prevalence of CHD as a function of age and the prevalence of premature CHD (pCHD). Multiple logistic regression analysis (Table 4) showed that independent predictors of CHD were male gender, age, the presence of arterial hypertension, current and previous smoking habit, the presence of Tx, the scalar increase of LDLc and the scalar decrease of HDLc.

3.7. Clusters of unrelated families carrying the more frequent mutations of LDLR gene

The survey of ADH-1 heterozygotes has led to the identification of several unrelated families carrying the same *LDLR* mutation. The list of mutation clusters consisting of ten or more unrelated families sharing the same mutation is shown in Table 5. Taking advantage of the relatively high number of subjects in each cluster, we calculated the "severity score" for each mutation (see supplemental methods for calculation of the mutation severity score) and the percentage of subjects over 30 years of age with Tx, CHD and pCHD. This analysis suggested that the mutations found in these clusters can be stratified in three categories of severity: "mild" (LDLc 6.38 ± 1.19 mmol/L, mutation score ≤0.300), "intermediate" (LDLc 7.28 ± 1.29 mmol/L, mutation score >0.300 and ≤0.700) and "severe" (LDLc 8.23 ± 1.20 mmol/L, mutation score >0.700). Each group of mutations significantly differed from the others ($P = 0.001$). The correlations between mutation score and percent prevalence of tendon xanthomas (Tx), CHD and pCHD (Spearman rank correlation) were the following: mutation score vs %Tx ($r = 0.824$, $P = 0.001$), mutation score vs %CHD ($r = 0.790$, $P = 0.001$), mutation score vs % pCHD ($r = 0.739$, $P = 0.004$).

In the majority of cases, families sharing the same mutation were living in or came from the same geographical district of Italy. The geographical distribution of the mutation clusters across the country is shown in Supplementary Fig. S.5.

3.8. Phenotypic characterization of ADH-2 heterozygotes

We found the APOB p.(R3527Q) mutation in 13 families (32 HE), the p.(R3527W) in 1 family (3 HE) and p.(R3558C) in 3 families (4 HE). As compared to ADH-1 heterozygotes, the ADH-2 heterozygotes had significantly lower levels of Tc (7.71 ± 0.86 vs 9.08 ± 1.49, $P < 0.001$) and LDLc (5.78 ± 0.83 vs 7.19 ± 1.42, $P < 0.001$) and a delayed clinical onset of CHD (61.5 ± 8.7 vs 53.4 ± 11.6 years of age).

3.9. Phenotypic characterization of ADH-3 heterozygotes

We found only three carriers of gain of function mutations of PCSK9. The p.(R496W) mutation was found in two subjects from the same family: the index case (a 62 year-old female with severe carotid stenosis and LDLc of 5.58 mmol/L) and her granddaughter (a 2 year-old female with LDLc level of 3.62 mmol/L). The p.(S127R) mutation was found in a 69 year-old obese female, with carotid atherosclerosis, arterial hypertension and LDLc level of 6.05 mmol/L.

3.10. Phenotypic characterization of ADH homozygotes

We have characterized 40 HO-ADH-1 (from 33 families) and 23 CHE-ADH-1 (from 21 families). A detailed description of these patients is reported in Supplementary Table S.12. The comparison between ADH-1 patients carrying RN mutations and those carrying RD mutations is reported in Supplementary Table S.13. RN mutation carriers were younger, had higher levels of Tc and LDLc, and lower levels of HDLc, and a higher prevalence of cutaneous xanthomas

Table 3
Clinical features and plasma lipids in ADH-1 heterozygotes over 30 years of age with and without coronary heart disease (CHD).

	Index cases		*P*	All subjects		*P*
	CHD (−)	CHD (+)		CHD (−)	CHD (+)	
Gender M/F	147/231	154/94	0.0001	264/438	212/130	0.0001
Age (years)	49.1 ± 11.8	53.0 ± 10.9	0.0001	47.7 ± 12.2	53.4 ± 11.6	0.0001
BMI (kg/m^2)	24.4 ± 3.7	25.7 ± 3.5	0.0001	24.3 ± 3.7	25.6 ± 3.3	0.0001
Tc	9.58 ± 1.57	10.57 ± 1.68	0.0001	9.39 ± 1.57	10.42 ± 1.64	0.0001
	9.58 ± 1.54[a]	10.50 ± 1.69[a]		9.43 ± 1.34[b]	10.21 ± 1.40[b]	
LDLc	7.61 ± 1.53	8.57 ± 1.62	0.0001	7.43 ± 1.53	8.51 ± 1.58	0.0001
	7.62 ± 1.51[a]	8.57 ± 1.62[a]		7.52 ± 1.32[b]	8.33 ± 1.37[b]	
HDLc	1.36 ± 0.36	1.20 ± 0.32	0.0001	1.35 ± 0.36	1.19 ± 0.31	0.0001
	1.35 ± 0.35[a]	1.24 ± 0.31[a]	0.001	1.33 ± 0.31[b]	1.20 ± 0.28[b]	
Tg	1.34 ± 0.61	1.63 ± 0.64	0.0001	1.34 ± 0.66	1.65 ± 0.68	0.0001
	1.36 ± 0.59[a]	1.56 ± 0.64[a]		1.30 ± 0.48[b]	1.58 ± 0.57[b]	
Tx	41.3%	69.2%	0.0001	36.7%	67.4%	0.0001
AH	13.8%	36.7%	0.0001	12.0%	36.7%	0.0001
Previous SMO	14.6%	35.8%	0.0001	10.9%	33.1%	0.0001
Current SMO	18.4%	11.9%	NS	15.7%	13.6%	NS
CA-ATS	45.3%	80.1%	0.0001	35.5%	79.6%	0.0001
RD/RN[c]	152/163	75/153	0.0002	264/346	98/213	0.0008

Values are mean ± SD; lipid values are reported in mmol/L.
Tx: tendon xanthomatosis; AH: arterial hypertension; SMO: smoking habit; CA-ATS: carotid atherosclerosis with >25% stenosis; CHD: coronary heart disease; p.CHD: premature CHD (before 55 years in males and 65 years in females).
NS: no significant difference.
[a] Adjusted for gender and age.
[b] Adjusted for gender, age and familial relations.
[c] Number of patients carrying receptor-defective (RD) and receptor-negative (RN) mutations.

Table 4
Parameters associated with CHD in ADH-1 heterozygotes over 30 years of age by multiple logistic regression analysis.

Parameter	β ± SE(β)	OR	95% CI (OR)	*P*
Gender (M vs F)	1.36 ± 0.21	3.91	2.59–5.89	0.0001
Age (5 years)	0.20 ± 0.04	1.22	1.13–1.33	0.0001
AH	1.42 ± 0.21	4.14	2.71–6.33	0.0001
Current and previous smoking	0.64 ± 0.19	1.90	1.31–2.75	0.001
Tendon xanthomas	0.90 ± 0.19	2.46	1.68–3.60	0.0001
LDLc (quintiles)	0.34 ± 0.07	1.41	1.23–1.63	0.0001
HDLc (quintiles)	−0.17 ± 0.07	0.84	0.74–0.96	0.02

and CHD. The age-related prevalence of CHD in the two groups is reported in Supplementary Fig. S.6.

We have also identified two related subjects found to be double heterozygotes for a LDLR mutation (p.Y419*) and an APOB mutation (p.R3558C) (Supplementary Table S.12). Surprisingly, the LDLc level of these subjects (7.12 ± 0.31 mmol/L) was similar to that observed in the heterozygous carriers of the p.(Y419*) mutation (7.89 ± 0.98 mmol/L).

The three double heterozygotes for LDLR and PCSK9 mutations (Supplementary S.12) had been previously reported by our groups [20].

4. Discussion

We conducted a clinical and molecular survey of a cohort of ADH patients, who entered the process of molecular analysis following stringent diagnostic criteria. The use of these criteria accounts for the relatively high mutation detection rate in the three major ADH candidate genes (overall ∼ 82%), which is similar to that obtained by other investigators [7,23,24]. We have retrospectively classified patients according to the clinical criteria proposed by Dutch Lipid Clinic Network. The high mutation detection rate we found in "definite", "probable" and "possible" ADH, is probably related to: i) a patient selection bias, as we analyzed patients referred to tertiary lipid clinics after an accurate exclusion of secondary hypercholesterolemias and repeated measurements of plasma lipid values; ii) the presence in the categories of "probable" or "possible" ADH of patients with the highest scores of the corresponding categories (i.e. patients with "probable" ADH had a mean score close to 8 and those with "possible" ADH had a mean score close to 5).

The analysis of the distribution of the mutations among the three candidate genes shows that 97.4% of mutation positive HE-ADH index cases had mutations in *LDLR*, 2.2% in *APOB* and 0.36% in *PCSK9*. This distribution is comparable with that reported in the Spanish ADH Cohort (96.4% *LDLR*, 3.5% *APOB* and 0.036% *PCSK9*) [8] but slightly different from that found in a French cohort (91.2% *LDLR*, 8.1% *APOB* and 0.8% *PCSK9*) [7] and the large Dutch Cohort (88% *LDLR*, 12% *APOB* and no *PCSK9* mutations) [25]. The main difference with respect to the French and Dutch studies is the percentage of individuals with ADH-2 due to APOB p.(R3527Q) mutation, which in Italy, as well as in other Southern European countries like Spain [8] and Portugal [26], is low as compared to the countries of Northern/Central Europe. This finding probably reflects the different migration of the descendants of the putative Celtic Ancestor carrier of the mutation from Central Europe to the other European regions [11].

Among index patients with the clinical diagnosis of HE-ADH, we found 12 patients who, at the molecular level, turned out to be true homozygotes or compound heterozygotes for *LDLR* mutations, which were assumed to be pathogenic since they satisfied the accepted criteria for functional mutations [27]. This unexpected finding underscores the large variability of the clinical expression of ADH-1 which is partly due to the presence of mutations with different functional impact (see below). With one exception, all patients with the clinical diagnosis of homozygous ADH had mutations in the *LDLR* gene, in good agreement with the distribution of candidate gene mutations found in HE-ADH index cases.

As expected, we found a large number of *LDLR* mutations, 19% of which were novel molecular events. Of the 192 known mutations previously reported in some large surveys of European populations, 40% had been found in the Netherlands, 31% in Spain, 28% in the UK and 17% in France [7,8,28,29]. In our survey we firmly established the presence of 13 clusters of mutations which accounted for almost 51% of *LDLR* mutation positive index cases. More specifically five of these mutations [p.(D221G), p.(G549D), p.(V523M), p.(G592E) and p.(Q474Hfs*63)] accounted for 37% of mutation positive index cases.

The identification of mutations clusters, allowed us to compare the phenotype among carriers of different mutations. To this purpose we introduced the "mutation severity score" as a parameter to assess the LDLc raising potential of each mutation. This score showed a positive correlation with the prevalence of tendon xanthomatosis and premature coronary heart disease. These results suggest that the mutation score derived from the analysis of

Table 5
Major Italian ADH-1 clusters of unrelated families carrying the same LDLR gene mutation.

Mutation	no. families/subjects	LDLc	HDLc	Tg	Mutation score[c]	Tx[d]	CHD[d]	pCHD[d]
c.1567 G>A, p.(V523M)[a]	57/90	6.09 ± 0.98	1.29 ± 0.25	1.29 ± 0.60	0.222	13%	20%	18%
c.1775 G>A, p.(G592E)[a]	40/64	6.51 ± 1.35	1.31 ± 0.28	1.40 ± 0.51	0.343	24%	18%	14%
c.662 A>G, p.(D221G)[a]	79/165	6.49 ± 1.26	1.37 ± 0.31	1.27 ± 0.50	0.266	31%	26%	20%
c.2054 C>T, p.(P685L)[a]	28/40	6.57 ± 1.27	1.25 ± 0.17	1.34 ± 0.42	0.300	40%	24%	16%
c.313+1 G>A, p.(S65_P105del)[b]	15/31	7.19 ± 1.12	1.27 ± 0.18	1.35 ± 0.48	0.484	33%	22%	16%
c.68-?_1845+?del, p.(V23Gfs*29)[b]	12/61	7.04 ± 1.11	1.22 ± 0.24	1.25 ± 0.55	0.459	63%	37%	20%
c.1646 G>A, p.(G549D)[b]	73/152	7.39 ± 1.41	1.19 ± 0.27	1.30 ± 0.54	0.612	64%	38%	28%
c.1415_1418dup, p.(Q474Hfs*63)[b]	48/188	7.50 ± 1.16	1.34 ± 0.24	1.32 ± 0.53	0.686	59%	35%	20%
c.1735 G>T, p.(D579Y)[b]	15/55	7.68 ± 1.08	1.37 ± 0.28	1.15 ± 0.50	0.673	61%	48%	48%
c.1257 C>G, p.(Y419*)[b]	10/38	7.90 ± 0.98	1.20 ± 0.13	1.52 ± 0.40	0.763	48%	40%	24%
c.1778 delG, p.(G593Afs*72)[b]	12/47	8.30 ± 1.33	1.24 ± 0.23	1.21 ± 0.42	0.851	76%	48%	33%
c.682 G>A, p.(E228K)[b]	16/32	8.28 ± 1.32	1.18 ± 0.34	1.22 ± 0.55	0.875	73%	44%	44%
c.1846-?_2140+?del, p.(E615fs*16)[b]	10/28	8.64 ± 1.11	1.27 ± 0.18	1.44 ± 0.30	0.857	77%	46%	32%

Plasma lipid concentrations (mmol/L, mean ± SD) are adjusted for age, gender and familial relations.
[a] LDL-RD mutations.
[b] LDL-RN mutations.
[c] Ratio between the number of subjects with adjusted LDLc values above the median value of the whole sample and the total number of subjects carrying the same mutation.
[d] Percent prevalence of Tx, CHD and pCHD (before 55 years in males and 65 years in females) refer to subjects over 30 years of age.

mutation clusters provides a tool to identify those patients who require an early and a more aggressive treatment to prevent or delay the progression of atherosclerotic lesions.

We have also been able to locate the mutation clusters in some specific areas of the country, an observation that suggests the presence of a founder effect, as opposed to the hypothesis of a recurrent mutational event occurring in a relatively restricted geographical area.

In one cluster p.(G579E) we identified two subsets of patients living in two fairly distant areas (the North-West and the South districts respectively) (Supplementary Fig. S.5). Interestingly, in the patients of the North-West district the level of LDLc (adjusted for gender, age and familial relations) was higher than that found in the patients of the Southern districts (LDLc 7.12 ± 1.13 mmol/L vs 5.64 ± 1.16 mmol/L, $P < 0.001$). Whether this difference is related to environmental/dietary factors or to the presence of different modifying genes is an open question.

This study indicates that LDLR mutations have a different impact on the phenotypic expression of ADH-1. We extended and confirmed the previous observations [18] that carriers of mutations assumed to result in a complete obliteration of LDLR activity (Receptor Negative mutations, RN) had a more severe phenotype not only in terms of higher LDLc but also of higher prevalence, in both genders, of tendon xanthomatosis, carotid atherosclerosis, and coronary heart disease, with respect to carriers of Receptor Defective mutations (RD). This concept was reinforced by two observations: i) patients with the clinical diagnosis of homozygous ADH-1 carrying receptor negative mutations were found to have a more severe phenotype than carriers of receptor defective mutations; ii) among the HE-ADH-1 patients with coronary heart disease (CHD+) there was a higher prevalence of RN mutation carriers. Collectively these observations emphasize the idea that the functional impact of the *LDLR* mutations plays a major role in determining the ADH-1 phenotype, including carotid atherosclerosis and premature CHD [18,30,31].

In conclusion, this survey provides a link between mutational events in the three major ADH candidate genes (specifically the *LDLR* gene) and the key clinical features found in ADH patients. More specifically the study emphasizes that: i) there is broad spectrum of severity in the clinical expression of ADH-1, from moderate to extremely severe, depending on the number and the type of the mutant alleles; ii) even in a country with a heterogeneous genetic background there are clusters of mutations in specific geographical districts suggesting a founder effect; iii) the study of individuals belonging to a specific cluster provides an opportunity to compare the phenotype in patients with the same mutation and who most likely share other genetic and environmental/dietary factors.

Acknowledgment

This study was supported by grants of the University of Genova (to S.B.), the University of Modena and Reggio Emilia and Emilia Romagna Region (to S.C.) and grants of the University of Palermo (to M.A., A.B.C.) and the Italian Ministry of Research (to M.A.)

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.01.007.

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