CURRENT OPINION

Impact of rare variants in autosomal dominant hypercholesterolemia causing genes

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Purpose of review
The systematic analysis of the major candidate genes in autosomal dominant hypercholesterolemia (ADH) and the use of next-generation sequencing (NGS) technology have made possible the discovery of several rare gene variants whose pathogenic effect in most cases remains poorly defined.

Recent findings
One major advance in the field has been the adoption of a set of international guidelines for the assignment of pathogenicity to low-density lipoprotein receptor (*LDLR*) gene variants based on the use of softwares, complemented with data available from literature and public databases. The clinical impact of several novel rare variants in *LDLR, APOB, PCSK9, APOE* genes have been reported in large studies describing patients with ADH found to be homozygotes/compound heterozygotes, double heterozygotes, or simple heterozygotes. In-vitro functional studies have been conducted to clarify the effect of some rare ApoB variants on LDL binding to LDLR and the impact of a rare ApoE variant on the uptake of VLDL and LDL by hepatocytes.

Summary
The update of the ADH gene variants database and the classification of variants in categories of pathogenicity is a major advance in the understanding the pathophysiology of ADH and in the management of this disorder. The studies of molecularly characterized patients with ADH have emphasized the impact of a specific variant and the variable clinical expression of different genotypes. The functional studies of some variants have increased our understanding of the molecular bases of some forms of ADH.

Keywords
assignment of pathogenicity, autosomal dominant hypercholesterolemia, rare variants

INTRODUCTION

Autosomal dominant hypercholesterolemia (ADH) is a monogenic disorder characterized phenotypically by isolated high levels of LDL. Rare variants in the low-density lipoprotein receptor (*LDLR*), *APOB*, and *PCSK9* genes are well known causes of the disease. Ex-vivo studies (using fibroblasts or lymphocytes) and in-vitro expression assays are the preferred methods to assess the pathogenic nature of rare gene variants of unknown functionality identified in patients with ADH. However, these studies have been performed only in a small number of variants, as they are costly, time-consuming, and require a specific expertise not usually available in most molecular diagnostic settings. Therefore, web-based tools (also referred to as in-silico analysis) predicting the effect on LDLR activity, ApoB100 binding properties, and PCSK9 functionality are frequently used to determine whether or not a variant is pathogenic. In the following sections we review some recent studies, which focused on the assignment of pathogenicity of rare variants in ADH causing genes.

Classification of pathogenicity of *LDLR* variants in ADH

ADH is, in most cases, because of variants in the *LDLR* gene. The number of these variants has

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KEY POINTS

- Rare genetic variants have been reported in patients with ADH which might impact on plasma LDL through a variety of genetic mechanisms including loss or gain of protein function.
- In most cases, the pathogenic impact of these variants is mostly predicted on the bases of 'in-silico' analysis, complemented with clinical data and co-segregation analysis in families.
- In-vitro functional studies are of paramount importance to properly define the mechanisms of the pathogenic effect of rare variants.

steadily increased and the UCL-LDLR database started in 1997 (http://www.LOVD.nl/LDLR) includes at present more than 1700 variants. One of the major problems concerning these variants is to establish whether they are pathogenic. In 2013, the Association of Clinical Genetic Scientists (ACGS) published guidelines for the classification of novel variants, based on in-silico prediction [1]. ACGS identified five categories for the classification of variants: categories 1 and 2 including variants clearly not or unlikely pathogenic, respectively; category 3 including variants of unknown significance (VUS), and categories 4 and 5 representing variants likely or clearly pathogenic, respectively. Recently, the UCL group updated the UCL variant database and classified the annotated variants according to ACGS guidelines [2▪▪]. Nonsense substitutions, frame-shifting, small and large rearrangements were not subjected to in-silico analyses, as they are accepted to be pathogenic/likely pathogenic (categories 4 and 5). The predicted effects of missense variants were assessed using open access software packages (e.g. PolyPhen-2, SIFT, Mutation Taster); the effect of intronic and synonymous variants on splicing was established using two softwares which give predictive score for splice acceptor and donor sequence for wild-type and variant sequences [2▪▪]. In all these cases, the concordant prediction of *in silico* tools was used to assign pathogenicity. The pathogenic impact of promoter and 5′ UTR variants had been reviewed in a previous study of the UCL group [3▪].

Pathogenicity scores were assigned also by taking into account the family segregation of the variant, the presence of the variant in other patients with ADH and its absence or very low frequency in sequence databases (e.g. the 1000 Genomes 2 and Exome Aggregation Consortium (ExAC), and its functional impact *in vitro*.

The updated UCL-LDLR database (http://www.LOVD.nl/LDLR) [2▪▪] contains 1595 *LDLR* variants which have been assigned to an ACGS pathogenic category. Eighty-two percentage of these ($n = 1317$) have been assigned to category 4 or 5, whereas 7% ($n = 112$) were considered as VUS. At present the number of functionally validated LDLR variants is very low. For example out of 795 missense variants annotated in the database only 75 (9%) had been functionally characterized [2▪▪]. However, a recent survey conducted by Bourbon *et al.* [4] showed that less than 15% of 1891 variants, reported in databases or found in recent literature as the cause of ADH, have any kind of functional evidence for being classified as a disease causing mutations. However, the observation that the in-silico predictions matched in-vitro evidence in 63/73 missense variants [2▪▪] supports the value of the 'in-silico' approach as a starting point for assigning a pathogenicity score in a disorder like ADH caused by a large number of *LDLR* variants, whose in-vitro functional characterization would require an extraordinary effort, difficult to envisage at this stage. Needless to say that development of new in-silico tools and the more information emerging from in-vitro functional studies and genomic surveys will improve the assignment of pathogenicity of those variants presently included in the 'VUS limbo'.

With regard to in-vitro studies, a relevant contribution to the understanding the pathogenic impact of some rare LDLR variants frequently observed in some cohorts of patients with ADH was given by Etxebarria *et al.* [5▪]. These authors investigated seven variants located in various domains of LDLR protein: p.(Cys155Tyr) in ligand binding domain; p.(Arg416Trp), p.(Thr454Asn), p.(Trp577Gly), and p.(Ile624del) in β-propeller domain; p.(Asn825Lys) in FxNPxY motif and p.(Phe800Glyfs∗129) in cytoplasmic tail. Preliminary in-silico analysis had indicated that all variants with the exception of p.(Thr454Asn) were classified as pathogenic. Each variant was expressed in LDLR-deficient Chinese Hamster ovary cells (CHO-*ldlrA7*) and the encoded human LDLR protein was characterized by a variety of methods including Western blot analysis, flow cytometry, and confocal laser scanning microscopy. This combined methodology allowed the assignment of these variants to the following LDLR functional classes: p.(Trp577Gly), p.(Ile624del), and p.(Phe800Glyfs∗129) to class 2 (defective intracellular transport of LDLR); p.(Cys155Tyr) to class 3 (impaired binding of LDL); p.(Asn825Lys) to class 4 (defective LDLR/LDL internalization); p.(Arg416Trp) and p.(Thr454Asn) to class 5 (impaired LDLR recycling), respectively. Against in-silico prediction, in-vitro

studies convincingly demonstrated that the p.(Thr454Asn) originally classified as VUS was indeed pathogenic.

Molecular and clinical features of ADH homozygotes or ADH double heterozygotes identified in large cohorts

A study on the molecular and clinical characteristics of homozygous ADH (HoADH) has recently been carried out in Spain [6▪]. Data were collected from the Spanish Dyslipidemia Registry of the Spanish Atherosclerosis Society and from all molecular diagnoses performed for ADH in Spain between 1996 and 2015 ($n = 16\,751$). A total of 92 patients were identified as having 'molecular HoADH' – of whom, 42 were true homozygous (1 for *APOB* and 41 for *LDLR*), 45 compound heterozygous for *LDLR*, 3 double heterozygous for *LDLR* and *PSCK9*, and 2 double heterozygous for *LDLR* and *APOB*. Overall, 84 rare variants in *LDLR*, 2 in *APOB*, 1 in *PCSK9* were found in these patients. In addition, five patients were found to suffer from autosomal recessive hypercholesterosterolemia (ARH) due to four mutations in *LDLRAP1* gene. Among all patients, 46.7% did not meet the classic criterion of baseline LDL-C at least 500 mg/dl or 13 mmol/l for the clinical diagnosis of HoADH [7]. The estimated prevalence of molecularly characterized HoADH turned out to be 1 : 450 000; a prevalence higher than expected, but slightly lower than that reported in an extensive molecular survey conducted in the Netherlands [8]. The Spanish survey also confirmed the presence of a more aggressive phenotype (higher LDL-C and more CVD events) in LDLR negative versus LDLR defective mutation carriers, in agreement with previous studies [8,9], and also in true homozygotes versus compound heterozygotes. The latter observation is at variance with that of the Dutch survey [8], which did not show phenotypic difference between homozygous and compound heterozygous *LDLR* mutation carriers.

Although the clinical features of carriers of homozygous and compound heterozygous mutation in one of the ADH causing genes have been described in great detail, little is known about the phenotype of 'double-heterozygous carriers', resulting from a combination of a mutation in *LDLR* and *APOB* or *LDLR* and *PCSK9* or *PCSK9* and *APOB*. In the Netherlands, a large program for ADH identification has allowed the construction of a large database of molecularly characterized patients with ADH. In this context, Sjouke *et al.* [10▪▪] collected the medical data from ADH double heterozygotes and compared these with data from their simple heterozygous and unaffected relatives and homozygous/compound heterozygous *LDLR* mutation carriers identified previously [8]. A total of 28 double heterozygotes (23 *LDLR/APOB* and 5 *LDLR/PCSK9* mutation carriers) were identified. Plasma LDL-C levels were significantly higher in double heterozygotes compared with 28 heterozygous and 18 unaffected relatives but lower compared with homozygous/compound heterozygous *LDLR* mutation carriers. Therefore, double heterozygotes appear to show an 'intermediate phenotype' similar to that occasionally observed in some heterozygous patients with severe hypercholesterolemia [10▪▪]. The Dutch study provides a robust evidence to support the EAS consensus paper on homozygous ADH [7], which states that LDL-C levels were lower in *LDLR/APOB* double heterozygotes than in homozygous/compound heterozygous carriers of *LDLR* mutations. Finally, the Dutch study showed that mean LDL-C level in *LDLR/PCSK9* double heterozygotes tended to be higher than that seen in *LDLR/APOB* double heterozygotes, suggesting a stronger LDL-C raising effect of *PCSK9* mutations with respect to *APOB* mutations.

Only four double heterozygous patients with ADH (14% of the total cohort) [10▪▪] met the clinical criteria for HoADH, defined as an LDL-C level at least 13 mmol/l [7], thus confirming the large heterogeneity of LDL levels among these patients. The proportion of patients suffering from CVD among double heterozygous patients with ADH and homozygous/compound heterozygous *LDLR* mutation carriers was not statistically different. However, the average age of onset of CVD in homozygous/compound heterozygous *LDLR* mutation carriers was significantly lower compared to double heterozygous patients with ADH [10▪▪]. The study emphasizes the point that the number of double heterozygous carriers of ADH mutations is probably underreported [6▪,9] due to selection bias.

Old and new gain of function variants of *PCSK9* gene

PCSK9 gain of function (GOF) variants are a rare cause of ADH. A list of these rare GOF variants reported since 2003 in patients with ADH is shown in Supplemental Table 1, http://links.lww.com/COL/A16 [11–24,25▪▪,26▪,27–30]. To gain insight on the impact of some of these variants on plasma lipids and ADH phenotype, data of 164 patients with ADH heterozygous for *PCSK9* mutations were collected from 12 lipid centers in eight countries throughout the world [25▪▪]. Patients carried 16 different *PCSK9* variants, six of which p.(Val4Ile), p.(Glu48Lys), p.(Pro71Leu), p.(Arg96Cys), p.(Asp129Asn), and p.(Ser465Leu)

were novel [Supplemental Table 1, http://links.lww.com/COL/A16]. The study demonstrated that *PCSK9* GOF variants exhibited significant phenotypic variability. Carriers of p.(Asp374Tyr) and p.(Ser127Arg) variants (Supplemental Table 1, http://links.lww.com/COL/A16) had severe dyslipidemia, whereas p.(Glu32Lys), p.(Arg215His), and p.(Ser465Leu) carriers had a relatively mild phenotype, although substantial variation was present in patients carrying the same mutation. Despite variability in disease severity of individual mutations, pooled analyses revealed significantly greater LDL-C levels in *PCSK9* GOF mutation patients compared with patients with *LDLR* or *APOB* mutations drawn from the Dutch Hypercholesterolemia Registry [25▪▪].

Novel putative *PCSK9* variants p.(Ala53Gly) and p.(Arg476Cys) were reported by Siouke *et al.* [10▪▪] in two double heterozygous patients with ADH and p.(Ala62Asp)/p.(Pro467Ala) by Alves *et al.* [26▪] in an ADH compound heterozygote. Through a series of in-vitro studies, Alves *et al.* [26▪] demonstrated that p.(Ala62Asp) and p.(Pro467Ala) variants reduced the expression of LDLR by approximately 50% as the positive control variant p.(Asp374Tyr) and markedly decreased the cell uptake of LDL, thus indicating that these variants are 'bona fide' gain of function variants.

Rare *APOB* gene variants as the cause of ADH

The most common mutation found in *APOB* as the cause of ADH is a single amino acid substitution of arginine for glutamine at position 3527 [p.(Arg3527Gln)] previously designated Arg3500Gln (referred to the mature protein), which markedly reduces the affinity of ApoB for the LDLR (Familial Defective ApoB-100, FDB) [31,32▪]. Other extremely rare pathogenic ApoB variants have been described such as p.(Arg3527Trp) and p.(Arg3558Cys) [32▪,33,34] and, more recently, other variants p.(Arg3059Cys), p.(Lys3394Asn), p.(Arg50Trp), p.(Arg1164Thr), and p.(Gln4494del) have been added to the list [35–37] (Supplemental Table 2, http://links.lww.com/COL/A16). Interestingly, the latter three variants do not reside in the consensus region of the LDLR binding domain. Fernández-Higuero *et al.* [38▪] investigated the biological and physico-chemical properties of LDL particles containing the two novel ApoB variants: p.(Arg1164Thr) and p.(Gln4494del). They demonstrated a defective binding of LDL/p.(Arg1164Thr) and LDL/p.(Gln4494del) to human lymphocytes and HepG2 cells, which was comparable to that of LDL/p.(Arg3527Gln). In addition, they showed that the capacity of U937 cells of growing in the presence of LDL/p.(Arg3527Gln), LDL/p.(Arg1164Thr), or LDL/p.(Gln4494del) was reduced to a similar extent (by 60%) compared with wild-type LDL. Secondary structure of the human ApoB100 was investigated by infrared spectroscopy and LDL particle size was assessed by dynamic light scattering and electron microscopy. The results showed differences in secondary structure and/or in particle size of LDL/p.(Arg1164Thr) and LDL/p.(Gln4494del) variants compared with wild-type LDL. It is suggested that these changes underlie the defective binding and uptake of p.(Arg1164Thr) and p.(Gln4494del) ApoB variants to LDLR.

The other rare *APOB* gene variant p.(Arg50Trp), predicted *in silico* to be deleterious, was reported in an ADH family by Thomas *et al.* [35] who found that LDL/p.(Arg50Trp) accumulate in the circulation (as compared to wild-type LDL), most likely as a result of a defective hepatic uptake and clearance. However, cell binding studies of this ApoB mutant have not been performed yet. Taken together these findings suggest that in patients with ADH the analysis of *APOB* gene should not be confined to exon 26 (containing the region encoding the LDLR binding domain) but must include the whole gene to identify rare variants along the ApoB molecule, which may disrupt the binding capacity of ApoB to the LDLR.

A rare *APOE* gene variant in ADH

In 2013, Mardue *et al.* [39] described a large French family including 14 members with ADH, in whom no mutations in *LDLR, APOB*, and *PCSK9* genes had been found. These patients were found to be heterozygous carriers of a rare variant of *APOE* gene: an in-frame 3 base pairs deletion [c.500_502delTCC] resulting in the elimination of a leucine residue at position 167, p.(Leu167del), formerly designated Δ149Leu in mature protein. A similar observation was reported by Awan *et al.* [40], who described a patient with ADH phenotype who was also heterozygous for the p.(Leu167del) variant. This variant had been previously reported in some patients with hypertriglyceridemia to be associated with sea-blue histiocytosis (OMIM #26960), a condition characterized by splenomegaly, mild thrombocytopenia, and, in the bone marrow, numerous histiocytes containing cytoplasmic granules, which stained bright blue [41–43]. The ApoE p.(Leu167del) had also been reported in patients with familial combined hyperlipidemia (FCHL) and with type III dyslipoproteinemia, with or without splenomegaly [44].

Recently, Cenarro *et al.* [45▪▪] performed a systematic study to determine the frequency of

p.(Leu167del) ApoE variant in patients with ADH, in whom *LDLR, APOB*, and *PCSK9* mutations had been excluded. They sequenced *APOE* gene in 288 patients with ADH and 220 patients with normolipidemia. Nine patients with ADH (3.1%) were heterozygous carriers of the p.(Leu167del) variant. All available family members of probands with p.(Leu167del) variant (30 patients from eight pedigrees) were investigated. Ten family members carrying the variant were identified; among them, six showed isolated hypercholesterolemia, three presented a mixed hyperlipidemia, and only one had plasma cholesterol level less than 90th percentile. The LDL cholesterol concentration was found to be approximately 50 mg/dl lower in p.(Leu167del) carriers than that reported for heterozygous ADH because of *LDLR* mutations in the Spanish population. This milder hypercholesterolemia was associated with absence of tendon xanthomas and lower prevalence of cardiovascular disease.

The study also demonstrated that in VLDL of the mutation carriers the wild-type ApoE3 was almost five fold the level of ApoE p.(Leu167del). Furthermore, in-vitro studies indicated that VLDL of the mutation carriers showed a significantly higher uptake by HepG2 and TPH1 cells compared to VLDL of patients with E3/E3 or E2/E3 genotype. This increased cell uptake of carriers' VLDL was associated with a reduction of the transcription of *LDLR* gene, suggesting that reduced LDLR protein on cell surface would be responsible for the raised LDL in the circulation. In view of these findings, Cenarro *et al.* [45▪▪] suggest that p.(Leu167del) of *APOE* gene is a 'gain of function' variant for the lipoprotein uptake by LDLR or other receptor of LDLR family involved in VLDL catabolism. However, the molecular mechanism whereby p.(Leu167del) increases VLDL uptake by the cells remains poorly understood. In conclusion, these studies show that screening of the *APOE* gene is warranted in the setting of molecular diagnosis of ADH (in patients with type IIa and type IIb phenotype) along with the *LDLR*, *APOB*, and *PCSK9* genes [46].

CONCLUSION

Systematic sequencing of the major ADH candidate genes (*LDLR, APOB*, and *PCSK9*) has revealed a large number of rare variants, whose pathogenic impact in most cases is not clearly defined. A set of criteria for assignment of pathogenicity (as an alternative to in-vitro assays) has been adopted in the updating of the public database on ADH and in the survey of patients with homozygous ADH (true homozygous/compound heterozygous and double heterozygotes). In addition, several rare variants of *LDLR*, *APOB*, *PCSK9*, and *APOE* have been reported in patients with ADH and their biological impact documented *in vitro*.

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Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
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