



# Advances in genetics show the need for extending screening strategies for autosomal dominant hypercholesterolaemia

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## Aims

Autosomal dominant hypercholesterolaemia (ADH) is a major risk factor for coronary artery disease. This disorder is caused by mutations in the genes coding for the low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*), and proprotein convertase subtilisin/kexin 9 (*PCSK9*). However, in 41% of the cases, we cannot find mutations in these genes. In this study, new genetic approaches were used for the identification and validation of new variants that cause ADH.

## Methods and results

Using exome sequencing, we unexpectedly identified a novel *APOB* mutation, p.R3059C, in a small-sized ADH family. Since this mutation was located outside the regularly screened *APOB* region, we extended our routine sequencing strategy and identified another novel *APOB* mutation (p.K3394N) in a second family. *In vitro* analyses show that both mutations attenuate binding to the LDLR significantly. Despite this, both mutations were not always associated with ADH in both families, which prompted us to validate causality through using a novel genetic approach.

## Conclusion

This study shows that advances in genetics help increasing our understanding of the causes of ADH. We identified two novel functional *APOB* mutations located outside the routinely analysed *APOB* region, suggesting that screening for mutations causing ADH should encompass the entire *APOB* coding sequence involved in LDL binding to help identifying and treating patients at increased cardiovascular risk.

## Keywords

Autosomal dominant hypercholesterolaemia • Familial defective apolipoproteinaemia B • *APOB* • LDL • Exome sequencing • Linkage analysis

## Introduction

Familial hypercholesterolaemia (FH, MIM #143890) is an inherited disorder of lipoprotein metabolism, characterized by elevated levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in the circulation, as well as the presence of tendon xanthomas and premature atherosclerosis, all inherited in an autosomal dominant manner. Mutations in the LDL receptor

gene (*LDLR*, MIM +606945) cause FH due to a lack of functional hepatic receptors for uptake of circulating LDL, leading to increased plasma LDL-C levels.<sup>1,2</sup> The ligand present on LDL for interaction with the LDLR is apolipoprotein B (*APOB*). Mutations in the encoding gene (*APOB*; MIM +107730) therefore also cause the earlier described FH phenotype. This disease is known as familial defective *APOB* (FDB, MIM #144010).<sup>3–5</sup> In contrast to the large heterogeneity of the *LDLR* locus,<sup>6,7</sup> only a few mutations in

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the vicinity of codon 3527 of the *APOB* gene are known to prevent LDL from binding to the LDLR.<sup>5,8</sup> Familial hypercholesterolaemia, FDB, and inherited hypercholesterolaemia of unknown aetiology are commonly referred to as autosomal dominant hypercholesterolaemia (ADH, MIM #143890). Recently, mutations in pro-protein convertase subtilisin kexin type 9 (*PCSK9*, MIM +607786) have also been shown to be a rare cause of ADH.<sup>9</sup>

The prevalence of heterozygous ADH is estimated to be 1 in 500 individuals in most Western countries, and diagnosis is usually made on the basis of clinical symptoms and plasma cholesterol values.<sup>2</sup> However, the physical stigmata usually develop later in life, and thus a molecular (genetic) diagnosis at young age is warranted when striving for maximum health benefit, as recommended by the World Health Organization.<sup>10</sup> Genetic screening of affected families is an efficient way of identifying subjects with ADH<sup>11</sup> and has contributed to reducing cardiovascular morbidity and mortality.<sup>12,13</sup> However, in our index patients (of families with a trait for hypercholesterolaemia), the screening for mutations in the *LDLR*, *APOB* and *PCSK9* genes does in many cases not always result in a molecular diagnosis.<sup>7</sup> This suggests the existence of additional mutations in other (unknown) genes that can cause ADH.

The classical route of identifying novel genes is through linkage analysis studies in large families. This approach proved successful and led to the identification of *PCSK9*.<sup>9</sup> However, genetic heterogeneity, the occurrence of phenocopies (ADH phenotype resulting from other causes) and incomplete penetrance of the mutation hamper the identification of other genes.<sup>14</sup> This is especially true for the (ADH) studies in which a LOD score >3.3 is required (threshold for complex traits), since this means that a large number of individuals is needed for the analysis.<sup>15,16</sup> Another means of identifying novel ADH genes is through genome-wide association studies. This approach received substantial interest in the last few years. For ADH, several new candidate genes were identified.<sup>17–20</sup> However, mutations in most of these genes have thus far not been reported to cause ADH in patients.

Over the last few years, advances in DNA enrichment and next-generation sequencing technology have made it possible to quickly and cost-effectively sequence the 'exome', i.e. the protein-coding portion of the genome. The use of such exome sequencing data sets has helped in the identification of the causes of Mendelian diseases with Familial Combined Hypolipidemia being one example.<sup>21</sup> The current study shows for the first time that combining exome sequence data with linkage analysis can be used to identify disease-causing alleles in small-sized families with ADH.

## Methods

### Sample and diagnostic procedure

The proband was clinically diagnosed with FH using a uniform protocol and internationally accepted criteria by cardiologists and internists.<sup>22,23</sup> His DNA was routinely analysed for the presence of mutations in *LDLR*, *APOB* (amino acids 3441–3615) and *PCSK9* as described before.<sup>7</sup>

The family of the proband was expanded and clinically assessed. All participants gave written informed consent. After an overnight fast, blood was sampled and plasma concentrations of TC, high-density lipoprotein cholesterol (HDL-C), and triglycerides were measured by commercially available kits (Boehringer Mannheim, Mannheim,

Germany). Low-density lipoprotein cholesterol concentrations were calculated by the Friedewald formula only when the triglyceride concentration was <4.5 mmol/L.<sup>24</sup> An ADH phenotype was defined by levels of LDL-C above the 95th percentile for age and gender.<sup>25</sup> When untreated lipid values were not available, estimated baseline LDL-C values were calculated based on the potency of cholesterol-lowering therapy that was used.<sup>26</sup>

Genomic DNA was extracted from 10 mL whole blood on an AutopureLS apparatus according to a protocol provided by the manufacturer (Gentra Systems, Minneapolis, MI, USA).

### Cohorts

Through the participation of 64 Lipid Clinics, evenly distributed throughout The Netherlands since 1994, a representative group of over ~17 000 Dutch clinically diagnosed ADH patients has been collected using a uniform protocol and internationally accepted criteria by cardiologists and internists.<sup>22,23</sup> All participants gave written informed consent. From this cohort, 600 undefined and unrelated ADH cases were selected after routine analysis for the presence of mutations in *LDLR*, *APOB* (amino acids 3441–3615) and *PCSK9*<sup>7</sup> was negative and baseline TC and LDL-C levels were above the 95th percentile for age and gender.

The control cohort consisted of 500 unrelated and untreated individuals, recruited via the national genetic cascade screening programme for FH,<sup>11</sup> with baseline LDL-C levels below the 20th percentile for age and gender and tested negative for the familial ADH mutation.

The *APOB* domains, including amino acids 2955–3092 and amino acids 3303–3492, were analysed in the 600 molecularly undefined ADH cases by direct Sanger sequencing. The newly identified variants were screened by a PCR digestion protocol in the cohort of 500 individuals with low LDL-C levels.

For mutation nomenclature, numbering was based on the cDNA with nucleotide c.1 being A of the ATG initiation codon p.1<sup>27</sup> The used nomenclature differs by adding up 27 amino acids compared with the former regularly used nomenclature of the *APOB* gene. The *APOB* reference sequence NM\_000384.2 was used.

### Exome sequencing

Exome sequencing was performed at the Broad Institute (Boston, USA). First, solution hybrid selection of genomic DNA corresponding to the coding regions of 15 994 genes was performed as described previously.<sup>21,28</sup> Sequencing of the exome was performed on an Illumina GA-II sequencer using 76-base-pair paired-end reads. Unaligned reads were aligned to the human genome (HG18) using Maq (<http://maq.sourceforge.net/>). Reads not corresponding to the 28 646 006 targeted bases of the exome were discarded. Variants were then called using the UnifiedGenotyper module of the Genome Analysis Toolkit.<sup>29</sup> Variants were retained for downstream analysis if they met the following quality-control criteria: (i) depth of coverage is >7; (ii) ratio of the variant confidence score divided by the depth of coverage is >5; and (iii) non-reference allele is present in at least 35% of reads. A total of 10 255 heterozygous variants that passed quality-control criteria were identified in the proband.

### Linkage analysis

For selected family members, genotyping was performed with the HumanCytoSNP-12 SNP array (Illumina). In order to verify the relationship between individuals, the data were subjected to standard quality control routines, including graphical representation of relationship errors (GRR)<sup>30</sup> and PedCheck.<sup>31</sup> The program Allegro<sup>32</sup> was applied for parametric multipoint linkage analysis with the assumption of autosomal-dominant mode of inheritance of the disease.

## Prioritization strategy

To set up a prioritization strategy, 1000 disease-related mutations were evaluated *in silico* using different filtering criteria. A Swissvar (<http://www.expasy.org/swissvar>) database 'disease query' of 96 cholesterol-related #MIM numbers (<http://www.ncbi.nlm.nih.gov/Omim>) was run to retrieve and select 1000 mutations, classified as 'Disease', in 48 different genes. All mutations were evaluated based on presence in dbSNP, conservation and prediction models for functionality via Alamut version 2.0 (Interactive Biosoftware, Rouen, France).

## Low-density lipoprotein-specific uptake assay

Human LDL (d 1.019–1.063 g/mL) was isolated from plasma by gradient ultracentrifugation<sup>33</sup> and dialysed against PBS overnight. Low-density lipoprotein concentration was determined by the bicinchoninic acid method (Pierce BCA Protein Assay kit, Thermo Scientific #23225).<sup>34</sup> Low-density lipoprotein was incubated with DyLight 488 NHS-Ester (Thermo Scientific #46402) for 1 h followed by overnight dialysis against PBS and stored at 4°C in the dark.

Low-density lipoprotein uptake assays were done essentially as described in Zelcer *et al.*<sup>35</sup> Briefly, HepG2 cells were plated at a density of 200 000 cells per well and cultured for 24 h in DMEM containing 10% FCS after which they were washed twice with pre-warmed PBS and incubated for an additional 16 h in lipoprotein-deficient medium (10% lipoprotein-deficient FCS, 5 µM simvastatin, and 10 µM mevalonic acid) to induce expression of *LDLR*.

Low-density lipoprotein uptake was started by incubating cells in culture medium supplemented with 5 µg/mL of DyLight-labelled LDL at 37°C for 30 min. Assays were stopped by washing cells twice with ice-cold PBS containing 0.2% BSA (W/V), and lysed in cold RIPA buffer. An aliquot of the cell lysate was transferred to a 384-well plate and fluorescence was measured on a Typhoon imager (GE) at 488 nm.

## Statistical analysis

The GraphPad Prism software package (version 5.01, GraphPad Software, Inc., La Jolla, CA, USA) was used for data analysis. The differences in uptake of LDL particles between carriers of both mutations and controls were tested with the non-parametric one-tailed Mann–Whitney test. A *P*-value <0.05 was considered statistically significant.

## Results

A group of over ~17 000 Dutch clinically diagnosed ADH patients was collected over a period of 17 years. As a referral centre for the molecular ADH diagnosis in The Netherlands, our laboratory performs routine sequencing of the the *LDLR*, *APOB* and *PCSK9* genes as described.<sup>7</sup> For *APOB*, we screened for mutations in the vicinity of codon 3527 that is generally considered to be causally related to FDB.<sup>36</sup> By this method, we were able to identify the cause of ADH in only 41% of the cases. Of these, mutations in *LDLR* or *APOB* were found in 87.6 and 12.3%, respectively. Only five mutations (<0.1%) were identified in *PCSK9*.

In a proband of a small ADH family (Figure 1A), we did not identify mutations in the *LDLR*, *APOB*, and *PCSK9* genes following the routine diagnostics of our laboratory. To identify the molecular basis for the ADH phenotype in this family, we first sequenced the exome of the proband. Among 10 255 heterozygous variants identified, we noted a novel *APOB* variant (c.9175C>T, p.R3059C). This variant was identified in all family members with

*LDL-C* levels above the 95th percentile for age and gender, while it was not found in any family members with *LDL-C* levels below the 50th percentile for age and gender. However, the segregation of this new variant with high *LDL-C* was incomplete. While two individuals (ID:17 and ID:12) with only mildly elevated *LDL-C* levels (3.80 and 4.47 mmol/L, respectively) carried the mutation, two other family members (ID:5 and ID:16) with similar *LDL-C* levels (4.12 and 3.57 mmol/L, respectively) did not, indicating the need for in-depth studies. In addition to p.R3059C, six additional *APOB* variants were also identified in the exome of the proband. All these variants were present with a >2.5% frequency in the control pool and were therefore considered to be unlikely the cause of the ADH phenotype in this patient. To investigate the possible causality of the newly identified p.R3059C variant, we used the following approaches.

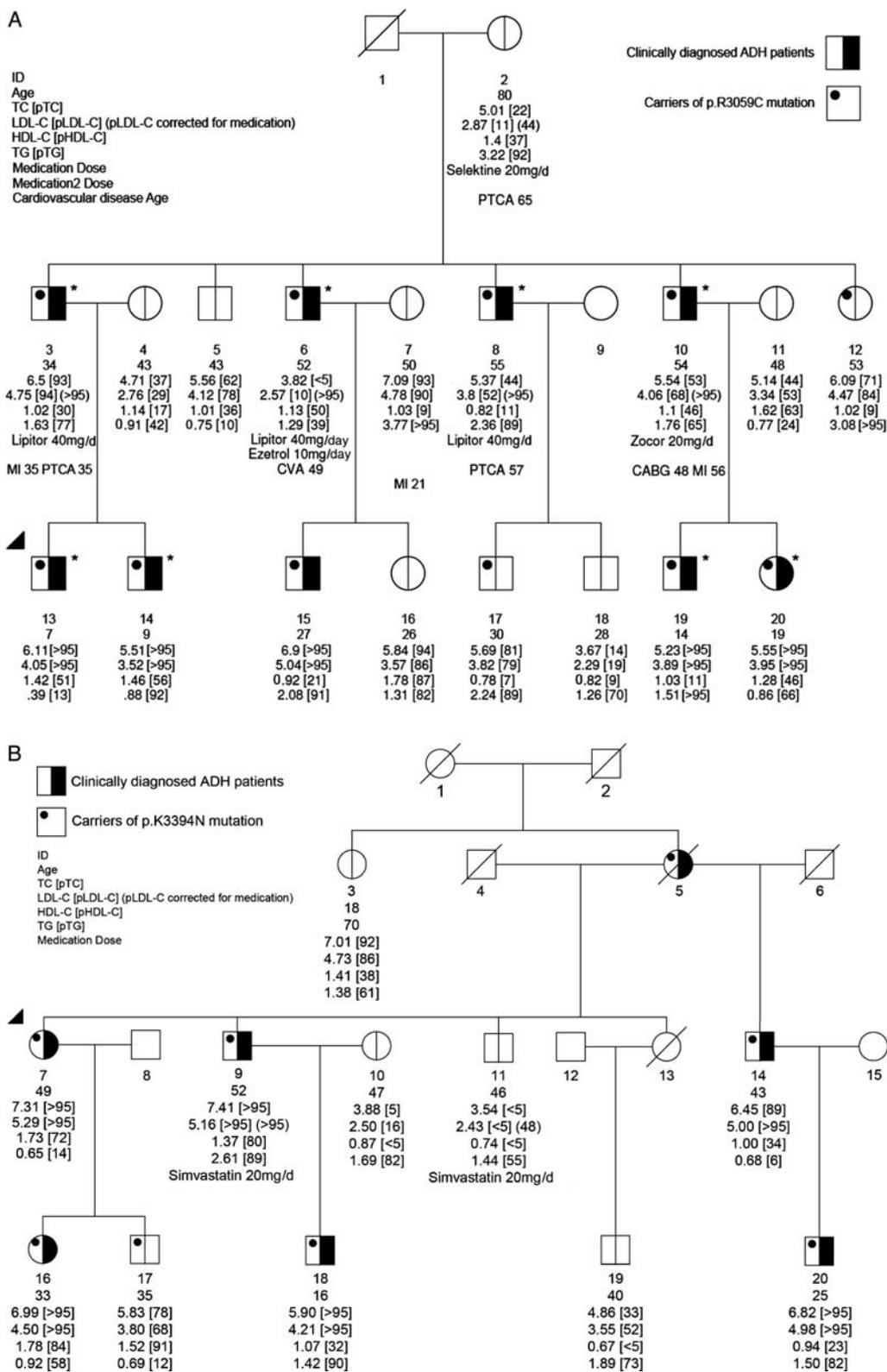
## Screening for additional novel *APOB* mutations

We first analysed whether the p.R3059C mutation might account for FDB in 600 unrelated index cases with molecularly undefined ADH. This effort revealed two additional probands. Furthermore, the variant was not identified in a cohort of 500 individuals with *LDL-C* levels below the 20th percentile for age and gender. In view of our finding that FDB-causing mutations are potentially not restricted to the vicinity of codon 3527, we extended our *APOB* screening protocol and sequenced the region encompassing amino acids 3386–3396. This region forms the *APOB* region responsible for binding to the *LDLR*.<sup>37</sup> This extended screen of 600 unrelated index cases with molecularly undefined ADH revealed an additional novel *APOB* mutation (10182G>T, p.K3394N) in one case, which was found in five additional family members with *LDL-C* levels above the 95th percentile (Figure 1B). However, as for p.R3059C, one carrier of p.K3394N had normal *LDL-C* levels (ID:17; 3.80 mmol/L). The p.K3394N mutation was not found in 500 individuals with low *LDL-C* levels. Direct sequencing of the complete coding sequence of the *APOB* gene in the proband carrying the p.K3394N mutation revealed the presence of three additional *APOB* variants. Two out of three were found with a >2.5% frequency in the control pool, and one variant (rs72653095) has previously been identified in various populations with a minor allele frequency of 0.37% in European American population [*n* = 1339, NHLBI Exome Sequencing Project (ESP), Seattle, WA, USA]. Therefore, these variants were unlikely the cause of the ADH phenotype in the proband.

## Studying all gene variants

The earlier mentioned data suggest that the new *APOB* variants may cause ADH. However, due to incomplete penetrance of these novel mutations, it might still be possible that other gene variants could have caused ADH in these families. We tested this possibility in the p.R3059C-carrying family.

Through exome sequencing, we identified 33 021 variants in the proband carrying the p.R3059C variant. A general approach widely used to down-size the candidate variant list is to discard all variants present in public SNP databases and variants predicted to be non-functional by prediction models such as SIFT<sup>38</sup> and PolyPhen-2.<sup>39</sup>



**Figure 1** Pedigrees of the families with autosomal dominant hypercholesterolaemia. (A) Pedigree of the proband with p.R3059C mutation: asterisk shows individuals that have been included in exclusion linkage analysis; (B) pedigree of the proband with p.K3394N mutation. Age, age when lipid profile was measured; TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglycerides; p, percentile calculated for age and gender; MI, myocardial infarction; CVA, cerebrovascular accident; PTCA, percutaneous trans-luminal coronary angioplasty; CABG, coronary artery bypass graft.

**Table 1** *In silico* evaluation of the effect of applying different filtering strategies on removing functional variants involved in cholesterol metabolism

Criteria	# Functional variants filtered
Weakly conserved amino acid and small physicochemical difference between amino acids <sup>a</sup>	25
Weakly conserved amino acid and predicted 'tolerated' by SIFT <sup>37</sup> and 'benign' by Polyphen-2 <sup>38a</sup>	28
Variants with validated rs number <sup>a</sup>	43
Weakly conserved amino acid (nOrthod/cOrthos $\leq 0.5$ )	65
Predicted to be tolerated by SIFT <sup>37</sup> and benign by PolyPhen-2 <sup>38</sup>	73
Weakly conserved nucleotide (phastcons $< 0.33$ )	81
Variants with rs number	117
Predicted to be benign by PolyPhen-2 <sup>38</sup>	124
Moderately conserved amino acid (nOrthod/cOrthos 0.5–0.8)	182
Predicted to be tolerated by SIFT <sup>37</sup>	213
Small physicochemical difference between amino acids (Grantham score $\leq 70$ )	349
Moderate physicochemical difference between amino acids (Grantham score 70–140)	425

<sup>a</sup>These criteria were used as a prioritization filter in this study.

However, *in silico* analysis of these filters suggest that this may result in discarding a large fraction of potential functional mutations involved in cholesterol metabolism (Table 1). In order to minimize the chance of filtering out relevant functional variants, we followed an adapted filtering strategy for evaluating the variants found in the proband (Table 2). This involved exclusion of (i) homozygous variants, (ii) heterozygous intergenic, intronic and synonymous variations predicted not to have an effect on mRNA splicing,<sup>40,41</sup> and (iii) variants located in genomic intervals not linked to the ADH phenotype (i.e. those with an LOD score  $< -2$ ; Supplementary material online, Figure S1, and Table S1). To assess this last criterion, we combined whole genome SNP data from eight family members with LDL-C above the 95th percentile, and one clearly unaffected relative with LDL-C below the 50th percentile for age and gender with the exome data of the proband. This approach resulted in a  $\sim 92\%$  reduction in the total number of variants found in the proband. Finally, all variants present with a minor allele frequency equal or  $> 2.5\%$  in our control exome database (i.e. exome data from 60 control DNA samples of European descent, obtained using the exact same methodology) were excluded. The number of variants possibly responsible for ADH in the index case was reduced to 31 (Table 2).

To prioritize these 31 candidate variants, we applied very careful criteria based on the *in silico* evaluation of disease-causing mutations (Table 1). In short, low-priority variants were variants that

**Table 2** Workflow for exclusion and prioritization of exome sequencing variants

Criteria	# Exome variations
Exclusion	
Variants found by exome sequencing index case	33 021
1. All heterozygous variants (coding + non-coding)	10 255
2. After excluding intergenic, intronic and synonymous variants with no predicted effect on splicing <sup>a</sup>	4614
3. Not excluded by linkage <sup>b</sup>	391
4. Frequency $< 2.5\%$ in in-house database <sup>c</sup>	31
Prioritization	
Not involving a weak amino acid conservation with small physicochemical difference between wild-type and variant amino acid	13
Not involving a weak amino acid conservation and predicted not to affect protein function by both SIFT <sup>37</sup> and PolyPhen <sup>38</sup>	
Not having a validated rs number	
Not present in control cohort	
Co-segregation	
Co-segregation with phenotype	1

<sup>a</sup>Possible splicing effects were evaluated by SpliceSiteFinder<sup>40</sup> and MaxEnt.<sup>41</sup>

<sup>b</sup>Variants residing in linkage intervals with parametric multipoint LOD score  $< -2$ .

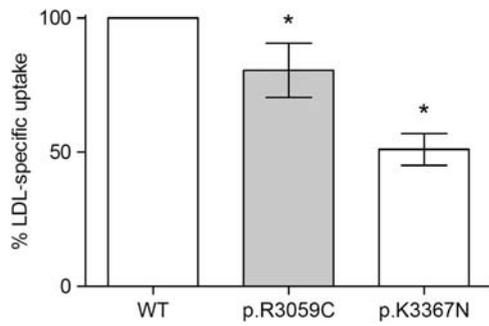
<sup>c</sup>Control exome database containing exome data from 60 control DNA samples of European descent, sequenced with the same methods.

(i) resulted in a change of a weakly conserved amino acid with a small physicochemical difference between wild-type and variant amino acid, (ii) resulted in a change of a weakly conserved amino acid and predicted to be non-functional by both SIFT<sup>38</sup> and PolyPhen-2,<sup>39</sup> or (iii) had a validated rs number. Additionally, all variants that were identified in our 60 control samples with low frequency were set to low priority. This prioritization scheme retained 13 high potential variants putatively causing ADH in the proband, including seven variants located in a 53 MB interval on chromosome 2, three variants on chromosome 11, two on chromosome 16, and one variant on chromosome 19.

Co-segregation analysis of these 13 remaining variants in additional strictly selected affected (LDL-C above the 95th percentile for age and gender) and unaffected relatives (LDL-C below the 50th percentile for age and gender) (Figure 1A, ID:15 and ID:18, respectively) showed that the novel variant in the *APOB* gene (c.9175C>T, p.R3059C) was the only variant showing complete co-segregation with the ADH phenotype. To validate our prioritization step, we also performed co-segregation studies (including haplotype analysis and direct Sanger sequencing) and frequency analyses of the 18 low-priority variants and found no data indicating segregation or functionality of these variants (data not shown).

### **In vitro analysis of the novel mutations**

The newly identified *APOB* mutations map to the region that binds the LDLR. We therefore set out to test whether these mutations



**Figure 2** Low-density lipoprotein-specific uptake by HepG2 cells. Data represent mean percentage  $\pm$  SD of three independent experiments relative to the value of the wild-type, in which the wild-type was used as reference and set to 100%. \* $P = 0.038$  (Mann–Whitney test, one-tailed).

also result in a functional defect in LDL uptake via the LDLR pathway. To test this, we isolated LDL particles from carriers and non-carriers, and performed *in vitro* LDL uptake assays in hepatocytes-like HepG2 cells. Uptake of LDL particles was significantly reduced when using LDL particles from carriers of both mutations compared with controls (p.R3059C:  $-28 \pm 13\%$ ; p.K3394N:  $-49 \pm 6\%$ ,  $P = 0.038$ , Mann–Whitney test, Figure 2).

## Discussion

This study shows that exome sequencing can increase our understanding of the aetiology of common complex disorders such as ADH. Specifically, two novel mutations in *APOB*, located outside the commonly screened regions, were shown to cause FDB.

Mutations in the vicinity of codon 3527 of *APOB* are long known to cause FDB.<sup>42</sup> It has also been shown that the carboxy-terminal domain of *APOB* located downstream of amino acid 2153 (in exon 26) mediates binding of LDL to the LDLR.<sup>43</sup> However, most studies are restricted to specific mutation screening of the most commonly known *APOB* mutations.<sup>44–53</sup> In other studies, only the region around codon 3527<sup>54–56</sup> is considered in attempts to explain FDB in patients, including the setup of routine sequencing in our own referral centre for molecular ADH diagnostics. This is likely related to the cost and time-consuming nature of Sanger sequencing for extensive DNA analysis, especially confounded in the case of *APOB*, given the size of the gene: the *APOB* gene consists of 29 exons encoding for one of the largest proteins known in man (4563 amino acids), with exon 26 being the largest coding exon in the human genome (7572 bp).

To our knowledge, only two other papers describe the analysis of additional regions within the LDLR-binding domain of *APOB*.<sup>57,58</sup> In one, the region (amino acids 3377–3493) containing the actual LDLR-binding site was considered and in the second paper, a region containing amino acids 3029–3732 was analysed. These studies may be hampered by techniques used such as SSCP and DGGE,<sup>59</sup> the criteria used for sample selection and sample size, which may explain why in both studies only one novel *APOB*

variant was identified. However, these studies do support our findings and suggestion that additional regions of the *APOB* gene should be assessed in ADH.

The current study shows that both p.R3059C and p.K3394N can cause FDB in two different families, but that this is not a *sine qua non* despite clear *in vitro* evidence that both mutations attenuate cellular LDL uptake. It has previously been shown that selective chemical modification of arginine or lysine residues of *APOB* decrease LDLR-binding affinity.<sup>60–62</sup> Recently, a tertiary structure model of human *APOB* is postulated, dividing the protein into eight domains, of which domains 4 and 5 (amino acids 2820–3202 and 3243–3498, respectively) represent the LDLR-binding site.<sup>63</sup> This model suggests that domain 4, in which p.R3059C is located, can indeed interact with the LDLR. The second mutation, p.K3394N, is located in *APOB* Site B of domain 5, which has been shown to be functionally important for LDLR binding due to richness in positively charged amino acid residues (e.g. R3386, R3389, K3390, and R3391). These residues are evolutionary conserved and share a high level of conservation with other similar receptor-binding regions as seen in, for example, *APOE* and vitellogenin.<sup>64</sup> These positively charged areas are thought to interact with negatively charged cysteine-rich domains of LDLR (R1–R7) at neutral pH<sup>65</sup> since changing any of the basic amino acids in this region to neutral amino acids results in defective receptor binding.<sup>36,64</sup> These data also support our results proposing the importance of arginine at position 3059 and lysine at position 3394 for *APOB*–LDLR interactions.

Our initial finding led us to extend our routine *APOB* sequencing. This resulted in the identification of additional carriers of novel variants, suggesting that the prevalence of *APOB* mutations may be higher than expected in ADH.

From a genetic standpoint, it is important to note the absence of a high LDL-C phenotype in some carriers of either of the new functional mutations and the presence of non-carriers without clinically evident ADH, but rather elevated LDL-C levels. This emphasizes that classical linkage approaches to identify new causal gene variants (not located in the established candidate genes) in similar families are unlikely to be successful. We, therefore, used the identification of the novel *APOB* mutation in the first family to test the feasibility of combining linkage analysis data and exome sequencing data (exclusion linkage analysis) to help identify disease-causing variant in small-sized families (lacking power for classical linkage analysis). Although the extent of variants excluded by this approach directly depends on the number of individuals included in the linkage study and on recombination events, we show that this approach can be used to exclude variants that are unlikely to cause disease. Considering recent advances in sequencing technology, it is conceivable that DNA diagnosis will soon no longer be limited to the *LDLR*, *PCSK9* genes and a small portion of the *APOB* gene when trying to reveal the origin of ADH in patients with elevated LDL-cholesterol.

## Conclusion

This study suggests that the routine ADH analysis in patients at increased cardiovascular risk could be improved by technological advances in sequencing. In addition, it shows that exclusion linkage analysis can be useful in identifying causal gene variants in small-sized families.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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**Conflict of interest:** none declared.

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