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CLINICAL RESEARCH

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.[1,2] 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).[3–5] In contrast to the large heterogeneity of the *LDLR* locus,[6,7] 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.[5,8] 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.[9]

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.[2] 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.[10] Genetic screening of affected families is an efficient way of identifying subjects with ADH[11] and has contributed to reducing cardiovascular morbidity and mortality.[12,13] 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.[7] 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.[9] 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.[14] 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.[15,16] 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.[17–20] 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.[21] 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.[22,23] His DNA was routinely analysed for the presence of mutations in *LDLR, APOB* (amino acids 3441–3615) and *PCSK9* as described before.[7]

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.[24] An ADH phenotype was defined by levels of LDL-C above the 95th percentile for age and gender.[25] 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.[26]

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.[22,23] 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*[7] 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,[11] 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[27] 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 (Botson, USA). First, solution hybrid selection of genomic DNA corresponding to the coding regions of 15 994 genes was performed as described previously.[21,28] 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.[29] 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)[30] and PedCheck.[31] The program Allegro[32] 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[33] and dialysed against PBS overnight. Low-density lipoprotein concentration was determined by the bicinchoninic acid method (Pierce BCA Protein Assay kit, Thermo Scientific #23225).[34] 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.*[35] 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.[7] For *APOB*, we screened for mutations in the vicinity of codon 3527 that is generally considered to be causally related to FDB.[36] 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.[37] 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[38] and PolyPhen-2.[39]

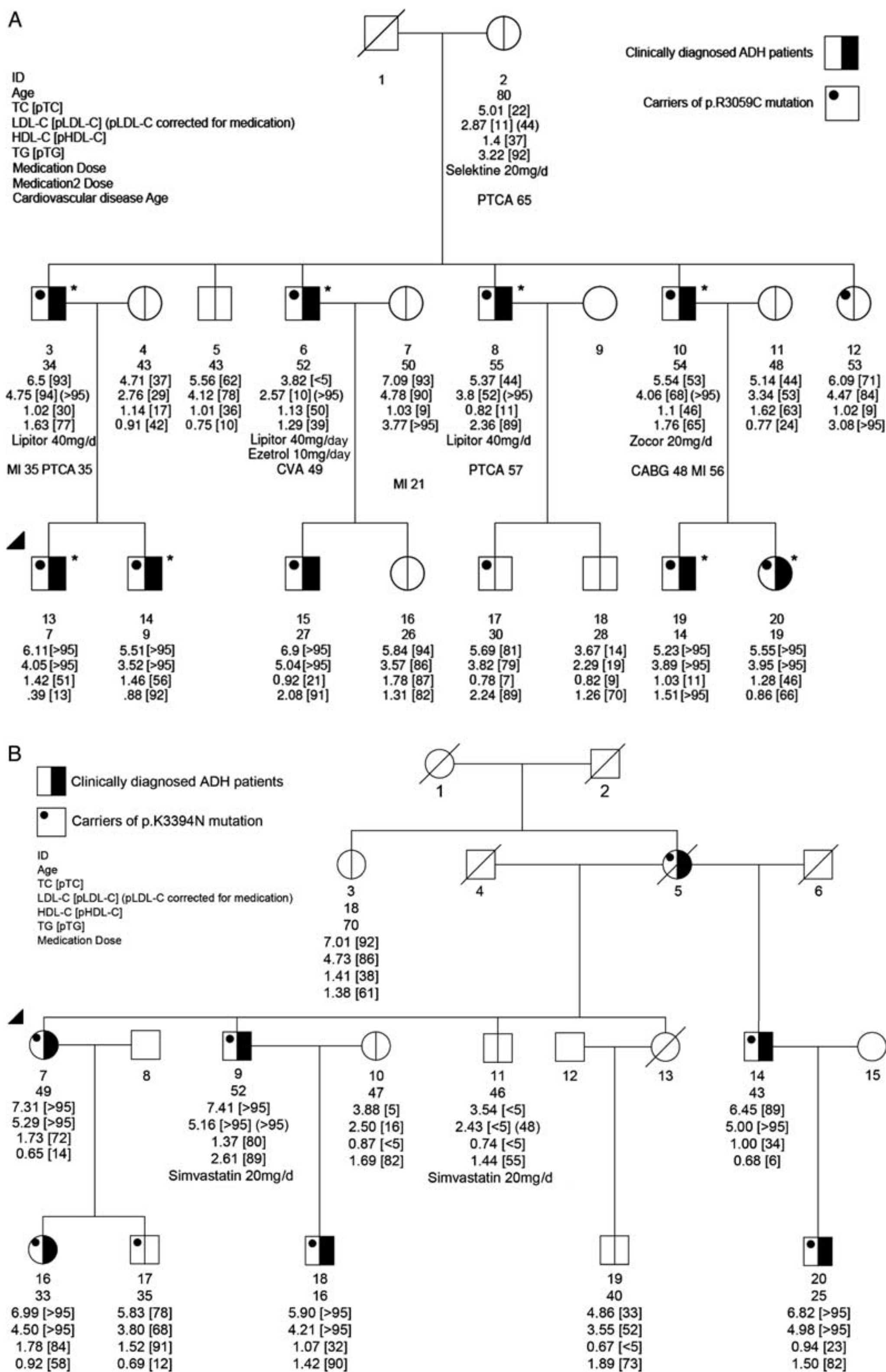

Figure 1 Pedigrees of the families with autosomal dominant hypercholesterolaemia. (*A*) Pedigree of the proband with p.R3059C mutation: asterisk shows individuals that have been included in exclusion linkage analysis; (*B*) pedigree of the proband with p.K3394N mutation. Age, age when lipid profile was 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[a]	25
Weakly conserved amino acid and predicted 'tolerated' by SIFT[37] and 'benign' by Polyphen-2[38a]	28
Variants with validated rs number[a]	43
Weakly conserved amino acid (nOrthod/cOrthos <=0.5)	65
Predicted to be tolerated by SIFT[37] and benign by PolyPhen-2[38]	73
Weakly conserved nucleotide (phastcons <0.33)	81
Variants with rs number	117
Predicted to be benign by PolyPhen-2[38]	124
Moderately conserved amino acid (nOrthod/cOrthos 0.5–0.8)	182
Predicted to be tolerated by SIFT[37]	213
Small physicochemical difference between amino acids (Grantham score ≤70)	349
Moderate physicochemical difference between amino acids (Grantham score 70–140)	425

[a]These criteria were used as a prioritization filter in this study.

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[a]	4614
3. Not excluded by linkage[b]	391
4. Frequency <2.5% in in-house database[c]	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[37] and PolyPhen[38]	
Not having a validated rs number	
Not present in control cohort	
Co-segregation	
Co-segregation with phenotype	1

[a]Possible splicing effects were evaluated by SpliceSiteFinder[40] and MaxEnt.[41]
[b]Variants residing in linkage intervals with parametric multipoint LOD score < −2.
[c]Control exome database containing exome data from 60 control DNA samples of European descent, sequenced with the same methods.

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,[40,41] 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 ∼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 (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[38] and PolyPhen-2,[39] 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

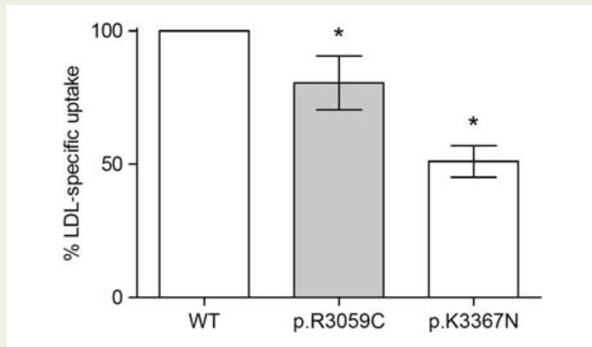

Figure 2 Low-density lipoprotein-specific uptake by HepG2 cells. Data represent mean percentage ± SD of three independent experiments relative to the value of the wild-type, in which the wild-type was used as reference and set to 100%. *$P = 0.038$ (Mann–Whitney test, one-tailed).

also result in a functional defect in LDL uptake via the LDLR pathway. To test this, we isolated LDL particles from carriers and non-carries, 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 ± 13%; p.K3394N: −49 ± 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.[42] 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.[43] However, most studies are restricted to specific mutation screening of the most commonly known *APOB* mutations.[44–53] In other studies, only the region around codon 3527[54–56] 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*.[57,58] 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,[59] 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.[60–62] 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.[63] 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.[64] These positively charged areas are thought to interact with negatively charged cysteine-rich domains of LDLR (R1-R7) at neutral pH[65] since changing any of the basic amino acids in this region to neutral amino acids results in defective receptor binding.[36,64] 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.

References

1. Huijgen R, Vissers MN, Defesche JC, Lansberg PJ, Kastelein JJ, Hutten BA. Familial hypercholesterolemia: current treatment and advances in management. *Expert Rev Cardiovasc Ther* 2008;**6**:567–581.
2. Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Basis of Inherited Disease*. New York: McGraw-Hill; 2001, p2863–2913.
3. Defesche JC, Pricker KL, Hayden MR, van der Ende BE, Kastelein JJ. Familial defective apolipoprotein B-100 is clinically indistinguishable from familial hypercholesterolemia. *Arch Intern Med* 1993;**153**:2349–2356.
4. Innerarity TL, Weisgraber KH, Arnold KS, Mahley RW, Krauss RM, Vega GL, Grundy SM. Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. *Proc Natl Acad Sci USA* 1987;**84**:6919–6923.
5. Whitfield AJ, Barrett PH, van Bockxmeer FM, Burnett JR. Lipid disorders and mutations in the APOB gene. *Clin Chem* 2004;**50**:1725–1732.
6. Fouchier SW, Defesche JC, Umans-Eckenhausen MA, Kastelein JJP. The molecular basis of familial hypercholesterolemia in The Netherlands. *Hum Genet* 2001;**109**: 602–615.
7. Fouchier SW, Kastelein JJ, Defesche JC. Update of the molecular basis of familial hypercholesterolemia in The Netherlands. *Hum Mutat* 2005;**26**:550–556.
8. Boren J, Ekstrom U, Agren B, Nilsson-Ehle P, Innerarity TL. The molecular mechanism for the genetic disorder familial defective apolipoprotein B100. *J Biol Chem* 2001;**276**:9214–9218.
9. Abifadel M, Varret M, Rabes JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derre A, Villeger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG, Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet* 2003;**34**:154–156.
10. World Health Organisation. Human Genetics Programme: Familial hypercholesterolemia: report of a second WHO consultation. Geneva; 1999. Report No.: WHO/HGN/FH/Cons/98.7.
11. Umans-Eckenhausen MA, Defesche JC, Sijbrands EJ, Scheerder RL, Kastelein JJ. Review of first 5 years of screening for familial hypercholesterolaemia in the Netherlands. *Lancet* 2001;**357**:165–168.
12. Huijgen R, Kindt I, Verhoeven SB, Sijbrands EJ, Vissers MN, Kastelein JJ, Hutten BA. Two years after molecular diagnosis of familial hypercholesterolemia: majority on cholesterol-lowering treatment but a minority reaches treatment goal. *PLoS One* 2010;**5**:e9220.
13. Versmissen J, Oosterveer DM, Yazdanpanah M, Defesche JC, Basart DC, Liem AH, Heeringa J, Witteman JC, Lansberg PJ, Kastelein JJ, Sijbrands EJ. Efficacy of statins in familial hypercholesterolaemia: a long term cohort study. *BMJ* 2008; **337**:a2423.
14. Altmuller J, Palmer LJ, Fischer G, Scherb H, Wjst M. Genomewide scans of complex human diseases: true linkage is hard to find. *Am J Hum Genet* 2001;**69**: 936–950.
15. Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 1995;**11**:241–247.
16. Lander ES, Schork NJ. Genetic dissection of complex traits. *Science* 1994;**265**: 2037–2048.
17. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, Pirruccello JP, Ripatti S, Chasman DI, Willer CJ, Johansen CT, Fouchier SW, Isaacs A, Peloso GM, Barbalic M, Ricketts SL, Bis JC, Aulchenko YS, Thorleifsson G, Feitosa MF, Chambers J, Orho-Melander M, Melander O, Johnson T, Li X, Guo X, Li M, Shin CY, Jin GM, Jin KY, Lee JY, Park T, Kim K, Sim X, Twee-Hee OR, Croteau-Chonka DC, Lange LA, Smith JD, Song K, Hua ZJ, Yuan X, Luan J, Lamina C, Ziegler A, Zhang W, Zee RY, Wright AF, Witteman JC, Wilson JF, Willemsen G, Wichmann HE, Whitfield JB, Waterworth DM, Wareham NJ, Waeber G, Vollenweider P, Voight BF, Vitart V, Uitterlinden AG, Uda M, Tuomilehto J, Thompson JR, Tanaka T, Surakka I, Stringham HM, Spector TD, Soranzo N, Smit JH, Sinisalo J, Silander K, Sijbrands EJ, Scuteri A, Scott J, Schlessinger D, Sanna S, Salomaa V, Saharinen J, Sabatti C, Ruokonen A, Rudan I, Rose LM, Roberts R, Rieder M, Psaty BM, Pramstaller PP, Pichler I, Perola M, Penninx BW, Pedersen NL, Pattaro C, Parker AN, Pare G, Oostra BA, O'Donnell CJ, Nieminen MS, Nickerson DA, Montgomery GW, Meitinger T, McPherson R, McCarthy MI, McArdle W, Masson D, Martin NG, Marroni F, Mangino M, Magnusson PK, Lucas G, Luben R, Loos RJ, Lokki ML, Lettre G, Langenberg C, Launer LJ, Lakatta EG, Laaksonen R, Kyvik KO, Kronenberg F, Konig IR, Khaw KT, Kaprio J, Kaplan LM, Johansson A, Jarvelin MR, Cecile JWJ, Ingelsson E, Igl W, Kees HG, Hottenga JJ, Hofman A, Hicks AA, Hengstenberg C, Heid IM, Hayward C, Havulinna AS, Hastie ND, Harris TB, Haritunians T, Hall AS, Gyllensten U, Guiducci C, Groop LC, Gonzalez E, Gieger C, Freimer NB, Ferrucci L, Erdmann J, Elliott P, Ejebe KG, Doring A, Dominiczak AF, Demissie S, Deloukas P, de Geus EJ, de FU, Crawford G, Collins FS, Chen YD, Caulfield MJ, Campbell H, Burtt NP, Bonnycastle LL, Boomsma DI, Boekholdt SM, Bergman RN, Barroso I, Bandinelli S, Ballantyne CM, Assimes TL, Quertermous T, Altshuler D, Seielstad M, Wong TY, Tai ES, Feranil AB, Kuzawa CW, Adair LS, Taylor HA Jr, Borecki IB, Gabriel SB, Wilson JG, Holm H, Thorsteinsdottir U, Gudnason V, Krauss RM, Mohlke KL, Ordovas JM, Munroe PB, Kooner JS, Tall AR, Hegele RA, Kastelein JJ, Schadt EE, Rotter JI, Boerwinkle E, Strachan DP, Mooser V, Stefansson K, Reilly MP, Samani NJ, Schunkert H, Cupples LA, Sandhu MS, Ridker PM, Rader DJ, van Duijn CM, Peltonen L, Abecasis GR, Boehnke M, Kathiresan S. Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* 2010; **466**:707–713.
18. Chasman DI, Pare G, Zee RY, Parker AN, Cook NR, Buring JE, Kwiatkowski DJ, Rose LM, Smith JD, Williams PT, Rieder MJ, Rotter JI, Nickerson DA, Krauss RM, Miletich JP, Ridker PM. Genetic loci associated with plasma concentration of low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, apolipoprotein A1, and Apolipoprotein B among 6382 white women in genome-wide analysis with replication. *Circ Cardiovasc Genet* 2008;**1**:21–30.
19. Waterworth DM, Ricketts SL, Song K, Chen L, Zhao JH, Ripatti S, Aulchenko YS, Zhang W, Yuan X, Lim N, Luan J, Ashford S, Wheeler E, Young EH, Hadley D, Thompson JR, Braund PS, Johnson T, Struchalin M, Surakka I, Luben R, Khaw KT, Rodwell SA, Loos RJ, Boekholdt SM, Inouye M, Deloukas P, Elliott P, Schlessinger D, Sanna S, Scuteri A, Jackson A, Mohlke KL, Tuomilehto J, Roberts R, Stewart A, Kesaniemi YA, Mahley RW, Grundy SM, McArdle W, Cardon L, Waeber G, Vollenweider P, Chambers JC, Boehnke M, Abecasis GR, Salomaa V, Jarvelin MR, Ruokonen A, Barroso I, Epstein SE, Hakonarson HH, Rader DJ, Reilly MP, Witteman JC, Hall AS, Samani NJ, Strachan DP, Barter P, van Duijn CM, Kooner JS, Peltonen L, Wareham NJ, McPherson R, Mooser V, Sandhu MS. Genetic variants influencing circulating lipid levels and risk of coronary artery disease. *Arterioscler Thromb Vasc Biol* 2010;**30**:2264–2276.
20. Willer CJ, Sanna S, Jackson AU, Scuteri A, Bonnycastle LL, Clarke R, Heath SC, Timpson NJ, Najjar SS, Stringham HM, Strait J, Duren WL, Maschio A, Busonero F, Mulas A, Albai G, Swift AJ, Morken MA, Narisu N, Bennett D, Parish S, Shen H, Galan P, Meneton P, Hercberg S, Zelenika D, Chen WM, Li Y, Scott LJ, Scheet PA, Sundvall J, Watanabe RM, Nagaraja R, Ebrahim S, Lawlor DA, Ben-Shlomo Y, Davey-Smith G, Shuldiner AR, Collins R, Bergman RN, Uda M, Tuomilehto J, Cao A, Collins FS, Lakatta E, Lathrop GM, Boehnke M, Schlessinger D, Mohlke KL, Abecasis GR. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat Genet* 2008;**40**:161–169.
21. Musunuru K, Pirruccello JP, Do R, Peloso GM, Guiducci C, Sougnez C, Garimella KV, Fisher S, Abreu J, Barry AJ, Fennell T, Banks E, Ambrogio L, Cibulskis K, Kernytsky A, Gonzalez E, Rudzicz N, Engert JC, DePristo MA, Daly MJ, Cohen JC, Hobbs HH, Altshuler D, Schonfeld G, Gabriel SB, Yue P, Kathiresan S. Exome sequencing, ANGPTL3 mutations, and familial combined hypolipidemia. *N Engl J Med* 2010;**363**:2220–2227.

22. Defesche JC. Familial hypercholesterolemia. In Betteridge DJ, Dunitz M, eds. *Lipids and Vascular Disease*. London: Ltd. 2000. p65–76.
23. Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Basis of Inherited Disease*. New York: McGraw-Hill; 1995. p1981–2030.
24. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;**18**:499–502.
25. Gotto AM Jr, Bierman EL, Connor WE, Ford CH, Frantz ID Jr, Glueck CJ, Grundy SM, Little JA. Recommendations for treatment of hyperlipidemia in adults. A joint statement of the Nutrition Committee and the Council on Arteriocslerosis. *Circulation* 1984;**69**:1065A–1090A.
26. Walma EP, Visseren FL, Jukema JW, Kastelein JJ, Hoes AW, Stalenhoef AF. [The practice guideline 'Diagnosis and treatment of familial hypercholesterolaemia' of the Dutch Health Care Insurance Board]. *Ned Tijdschr Geneeskd* 2006;**150**:18–23.
27. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat* 2000;**15**:7–12.
28. Gnirke A, Melnikov A, Maguire J, Rogov P, LeProust EM, Brockman W, Fennell T, Giannoukos G, Fisher S, Russ C, Gabriel S, Jaffe DB, Lander ES, Nusbaum C. Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat Biotechnol* 2009;**27**:182–189.
29. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 2010;**20**:1297–1303.
30. Abecasis GR, Cherny SS, Cookson WO, Cardon LR. GRR: graphical representation of relationship errors. *Bioinformatics* 2001;**17**:742–743.
31. O'Connell JR, Weeks DE. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 1998;**63**:259–266.
32. Gudbjartsson DF, Jonasson K, Frigge ML, Kong A. Allegro, a new computer program for multipoint linkage analysis. *Nat Genet* 2000;**25**:12–13.
33. Redgrave TG, Roberts DC, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal Biochem* 1975;**65**:42–49.
34. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;**150**:76–85.
35. Zelcer N, Hong C, Boyadjian R, Tontonoz P. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science* 2009;**325**: 100–104.
36. Boren J, Olin K, Lee I, Chait A, Wight TN, Innerarity TL. Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding. *J Clin Invest* 1998;**101**:2658–2664.
37. Boren J, Lee I, Zhu W, Arnold K, Taylor S, Innerarity TL. Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. *J Clin Invest* 1998;**101**:1084–1093.
38. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009;**4**: 1073–1081.
39. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat Methods* 2010;**7**:248–249.
40. Zhang MQ. Statistical features of human exons and their flanking regions. *Hum Mol Genet* 1998;**7**:919–932.
41. Yeo G, Burge CB. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. *J Comput Biol* 2004;**11**:377–394.
42. Soria LF, Ludwig EH, Clarke HR, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci USA* 1989;**86**:587–591.
43. Marcel YL, Innerarity TL, Spilman C, Mahley RW, Protter AA, Milne RW. Mapping of human apolipoprotein B antigenic determinants. *Arteriosclerosis* 1987;**7**: 166–175.
44. Nelken J, Meshkani R, Chahal N, McCrindle B, Adeli K. Detection of familial defective apoB (FDB) mutations in hypercholesterolemic children and adolescents by denaturing high performance liquid chromatography (DHPLC). *Clin Biochem* 2008;**41**:395–399.
45. Marduel M, Carrie A, Sassolas A, Devillers M, Carreau V, Di FM, Erlich D, Abifadel M, Marques-Pinheiro A, Munnich A, Junien C, Boileau C, Varret M, Rabes JP, Farnier M, Luc G, Lecerf JM, Bruckert E, Bonnefont-Rousselot D, Giral P, Kalopissis A, Girardet JP, Polak M, Chanu B, Moulin P, Perrot L, Krempf M, Zair Y, Bonnet J, Ferrieres J, Ferrieres D, Bongard V, Cournot M, Reznick Y, Schlienger JL, Fredenrich A, Durlach V. Molecular spectrum of autosomal dominant hypercholesterolemia in France. *Hum Mutat* 2010;**31**: E1811–E1824.
46. Taylor A, Bayly G, Patel K, Yarram L, Williams M, Hamilton-Shield J, Humphries SE, Norbury G. A double heterozygote for familial hypercholesterolaemia and familial defective apolipoprotein B-100. *Ann Clin Biochem* 2010;**47**(Pt 5): 487–490.
47. Ejarque I, Real JT, Martinez-Hervas S, Chaves FJ, Blesa S, Garcia-Garcia AB, Millan E, Ascaso JF, Carmena R. Evaluation of clinical diagnosis criteria of familial ligand defective apoB 100 and lipoprotein phenotype comparison between LDL receptor gene mutations affecting ligand-binding domain and the R3500Q mutation of the apoB gene in patients from a South European population. *Transl Res* 2008;**151**:162–167.
48. Gasparovic J, Basistova Z, Fabryova L, Wsolova L, Vohnout B, Raslova K. Familial defective apolipoprotein B-100 in Slovakia: are differences in prevalence of familial defective apolipoprotein B-100 explained by ethnicity? *Atherosclerosis* 2007;**194**: e95–e107.
49. Merino-Ibarra E, Castillo S, Mozas P, Cenarro A, Martorell E, Diaz JL, Suarez-Tembra M, Alonso R, Civeira F, Mata P, Pocovi M. Screening of APOB gene mutations in subjects with clinical diagnosis of familial hypercholesterolemia. *Hum Biol* 2005;**77**:663–673.
50. Castillo S, Tejedor D, Mozas P, Reyes G, Civeira F, Alonso R, Ros E, Pocovi M, Mata P. The apolipoprotein B R3500Q gene mutation in Spanish subjects with a clinical diagnosis of familial hypercholesterolemia. *Atherosclerosis* 2002;**165**: 127–135.
51. Heath KE, Humphries SE, Middleton-Price H, Boxer M. A molecular genetic service for diagnosing individuals with familial hypercholesterolaemia (FH) in the United Kingdom. *Eur J Hum Genet* 2001;**9**:244–252.
52. Ceska R, Vrablik M, Horinek A. Familial defective apolipoprotein B-100: a lesson from homozygous and heterozygous patients. *Physiol Res* 2000;**49**(Suppl. 1): S125–S130.
53. Alberto FL, Figueiredo MS, Zago MA, Araujo AG, Dos-Santos JE. The Lebanese mutation as an important cause of familial hypercholesterolemia in Brazil. *Braz J Med Biol Res* 1999;**32**:739–745.
54. Soufi M, Sattler AM, Maerz W, Starke A, Herzum M, Maisch B, Schaefer JR. A new but frequent mutation of apoB-100-apoB His3543Tyr. *Atherosclerosis* 2004;**174**: 11–16.
55. Yu W, Nohara A, Higashikata T, Lu H, Inazu A, Mabuchi H. Molecular genetic analysis of familial hypercholesterolemia: spectrum and regional difference of LDL receptor gene mutations in Japanese population. *Atherosclerosis* 2002;**165**:335–342.
56. Bednarska-Makaruk M, Bisko M, Pulawska MF, Hoffman-Zacharska D, Rodo M, Roszczynko M, Solik-Tomassi A, Broda G, Polakowska M, Pytlak A, Wehr H. Familial defective apolipoprotein B-100 in a group of hypercholesterolaemic patients in Poland. Identification of a new mutation Thr3492Ile in the apolipoprotein B gene. *Eur J Hum Genet* 2001;**9**:836–842.
57. Gaffney D, Hoffs MS, Cameron IM, Stewart G, O'Reilly DS, Packard CJ. Influence of polymorphism Q3405E and mutation A3371V in the apolipoprotein B gene on LDL receptor binding. *Atherosclerosis* 1998;**137**:167–174.
58. Pullinger CR, Hennessy LK, Chatterton JE, Liu W, Love JA, Mendel CM, Frost PH, Malloy MJ, Schumaker VN, Kane JP. Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. *J Clin Invest* 1995;**95**:1225–1234.
59. Henderson BG, Wenham PR, Ashby JP, Blundell G. Detecting familial defective apolipoprotein B-100: three molecular scanning methods compared. *Clin Chem* 1997;**43**:1630–1634.
60. Mahley RW, Innerarity TL, Pitas RE, Weisgraber KH, Brown JH, Gross E. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. *J Biol Chem* 1977;**252**:7279–7287.
61. Weisgraber KH, Innerarity TL, Mahley RW. Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J Biol Chem* 1978;**253**:9053–9062.
62. Mahley RW, Weisgraber KH, Innerarity TL. Interaction of plasma lipoproteins containing apolipoproteins B and E with heparin and cell surface receptors. *Biochim Biophys Acta* 1979;**575**:81–91.
63. Krisko A, Etchebest C. Theoretical model of human apolipoprotein B100 tertiary structure. *Proteins* 2007;**66**:342–358.
64. Li A, Sadasivam M, Ding JL. Receptor-ligand interaction between vitellogenin receptor (VtgR) and vitellogenin (Vtg), implications on low density lipoprotein receptor and apolipoprotein B/E. The first three ligand-binding repeats of VtgR interact with the amino-terminal region of Vtg. *J Biol Chem* 2003;**278**:2799–2806.
65. Rudenko G, Henry L, Henderson K, Ichtchenko K, Brown MS, Goldstein JL, Deisenhofer J. Structure of the LDL receptor extracellular domain at endosomal pH. *Science* 2002;**298**:2353–2358.