Molecular Basis of Autosomal Dominant Hypercholesterolemia
Assessment in a Large Cohort of Hypercholesterolemic Children

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Background—Autosomal dominant hypercholesterolemia (ADH) is characterized by elevated low-density lipoprotein cholesterol levels and premature cardiovascular disease. Mutations in the genes encoding for low-density lipoprotein receptor (LDLR), apolipoprotein B (APOB), and proprotein convertase subtilisin/kexin 9 (PCSK9) underlie ADH. Nevertheless, a proportion of individuals who exhibit the ADH phenotype do not carry mutations in any of these 3 genes. Estimates of the percentage of such cases among the ADH phenotype vary widely. We therefore investigated a large pediatric population with an unequivocal ADH phenotype to assess the molecular basis of hereditary hypercholesterolemia and to define the percentage of individuals with unexplained dyslipidemia.

Methods and Results—We enrolled individuals with low-density lipoprotein cholesterol levels above the 95th percentile for age and gender and an autosomal dominant inheritance pattern of hypercholesterolemia from a large referred pediatric cohort of 1430 children. We excluded children with thyroid dysfunction, nephrotic syndrome, autoimmune disease, liver disease, primary biliary cirrhosis, and obesity (body mass index >75th percentile for age and gender), as well as children referred via a cascade screening program and those from families with a known molecular diagnosis. Of the 269 children who remained after the exclusion criteria were applied, 255 (95%) carried a functional mutation (LDLR, 95%; APOB, 5%).

Conclusion—In the vast majority of children with an ADH phenotype, a causative mutation can be identified, strongly suggesting that most of the large-effect genes underlying ADH are known to date. (Circulation. 2011;123:1167-1173.)

Key Words: familial hypercholesterolemia ■ genotype ■ pediatrics ■ phenotype

Autosomal dominant hypercholesterolemia (ADH), commonly referred to as familial hypercholesterolemia, is clinically characterized by severely elevated low-density lipoprotein (LDL) cholesterol (LDL-C) levels from birth on, enhanced atherosclerosis progression, and premature cardiovascular events.1,2 Early diagnosis and treatment of ADH are pivotal because therapy with lipid-lowering agents strongly decreases the risk for such events.3

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The elevated LDL-C levels in ADH are caused by impaired uptake of LDL particles by the LDL receptor (LDLR).4 Currently, mutations in 3 genes can be held responsible for such impaired LDL particle uptake and consequently for the ADH phenotype. In fact, more than a thousand mutations have been identified in the LDLR gene itself, accounting for the majority of ADH cases.5 Mutations in the gene encoding for apolipoprotein B (APOB), the structural protein of the LDL particle that is the ligand for the LDLR, are found in a small minority of cases.6 Recently, even more rare gain-of-function mutations in the gene encoding for proprotein convertase subtilisin kexin type 9 (PCSK9), a protein that promotes LDLR degradation, have also been found to underlie the ADH phenotype.7

Despite these observations, not all patients who exhibit this phenotype carry a functional mutation in any of these genes. In fact, previous publications report widely varying mutation detection rates in patients with a clinical diagnosis of ADH, ranging from only 20% to nearly 90% in some reports.8–12 Therefore, it has been suggested that a plethora of ADH-associated genes have hitherto not been discovered, which consequently might explain this phenotype in a considerable percentage of patients. Conversely, the accuracy of the
clinical phenotype in the patient itself or in his/her family and false-negative results of the molecular techniques involved could also be invoked to explain these large differences in mutation detection rates. These are important issues because any novel gene implicated in the pathogenesis of ADH and cardiovascular disease could become a pharmacological target in itself; hence, it is important to establish whether a large proportion of ADH patients still carry unexplained molecular defects.

To resolve this issue, we took 2 steps. First, we set out to assess the molecular basis of ADH in children and not in adults. Children are subject to fewer environmental influences in terms of their lipoprotein metabolism, and an unequivocal diagnosis of ADH is easier to establish. Second, we used advanced techniques for mutation detection, including sequencing of codons and splice sites, as well as a specific technique for detecting large gene rearrangements. Here, we present our results.

Methods

Study Population and Procedures

All children 4 to 18 years of age who visited the pediatric lipid clinic at the Academic Medical Center in Amsterdam between July 1989 and January 2008 whose relevant laboratory and DNA data were complete were potentially eligible. These children, from various regions in the Netherlands, were referred to the outpatient lipid clinic because of dyslipidemia, and we were asked to establish the underlying cause of this disorder. We collected the medical history of the patient and went to great lengths to meticulously list the presence of dyslipidemia and cardiovascular disease in as many family members as possible. Furthermore, we performed a physical examination and gathered anthropomorphic data. In addition, we analyzed fasting blood samples for lipid profile and thyroid, liver, and renal function. From this population, we selected only patients with a clear clinical phenotype of ADH. This was defined as an LDL-C level >95th percentile for age and gender and autosomal dominant inheritance pattern of hypercholesterolemia, ie, at least 1 biological parent on treatment for hypercholesterolemia and a family history of hypercholesterolemia and cardiovascular disease. Because physical symptoms are rare in children, we did not consider this an inclusion criterion. Exclusion criteria were thyroid dysfunction, nephrotic syndrome, autoimmune disease, liver disease, primary biliary cirrhosis, and obesity. For this study, we chose a body mass index (BMI) cutoff at the 75th percentile for age and gender because it has recently been shown that at a BMI at or above the 80th percentile, significant abnormalities in cholesterol levels begin to occur in children.

Furthermore, we excluded children who were referred to the lipid clinic through the national cascade screening program and children from families with a known molecular diagnosis. If >1 child from the same family was eligible, we selected the first child in alphabetical order by first name.

Lipids and Lipoproteins

Blood samples were collected after at least a 12-hour overnight fast. Total plasma cholesterol and triglycerides were determined by an enzymatic colorimetric procedure (CHOD-PAP, Roche Diagnostics, Basel, Switzerland); high-density lipoprotein cholesterol was measured as cholesterol remaining after precipitation of apolipoprotein (Apo) B–containing lipoproteins by MnCl₂. LDL-C was calculated with the Friedewald formula. ApoA1 and Apo B100 (ApoB) concentrations were determined by immunonephelometry.

DNA Analysis

Genomic DNA was prepared from 5 mL whole blood on an AutopureLS apparatus according to a protocol provided by the manufacturer (Gentra Systems, Minneapolis, MN). Mutation identification in the LDLR and APOB genes was performed by direct Sanger sequencing; identification of large rearrangements in the LDLR gene was done by multiplex ligation-dependent probe technique as described previously in more detail. Sequence analysis was performed by direct sequencing with the Big Dye Terminator ABI Prism Kit, version 1.1 (Applied Biosystems, Foster City, CA). Products of sequence reactions were run on a Genetic Analyzer 3730 (Applied Biosystems), and sequence data were analyzed by the use of the Sequencer package (GeneCodes Co, Ann Arbor, MI).

Mutations were described according to the nomenclature as proposed by den Dunnen and Antonarakis. Thus, for all genes, the numbering was based on the cDNA with nucleotide +1 being A of the ATG initiation codon and codon 1 as the ATG initiation codon. The reference sequences NM_000527.3 and NM_000384.2 were used for LDLR and APOB, respectively. For all mutations, functionality has been established by cosegregation analysis of pedigree data.

Statistical Analysis

Differences between individuals with and without a molecular diagnosis of ADH for continuous and binary variables were assessed by independent-sample t tests and chi² tests, respectively. The association between LDL-C and the different groups was adjusted for potential confounders by means of linear regression analysis. Variables with a skewed distribution were log-transformed before analysis. A value of P<0.05 was considered significant. The analyses were performed with SPSS 16.0 software (SPSS Inc, Chicago, IL).

Results

Patient Selection and Mutation Detection Rate

Of 1430 children who visited the pediatric lipid clinic, 1161 were not included in our study cohort because of the predefined criteria (the Figure). In total, our study cohort comprised 269 subjects. In only 14 of these individuals (5%), no mutation could be identified in LDLR, APOB, or PCSK9 (Figure). In 2 of these 14 children, mutations were identified previously, but they were nonpathogenic. Of the remaining 255 subjects (95%), 242 carried a mutation in LDLR (95%) and 13 (5%) carried a mutation in APOB; no mutations were discovered in PCSK9. In total, 77 different types of mutations were found, of which 5 have not been reported before in the
Table 1. Nomenclature and Characteristics of the Identified Mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>cDNA Protein</th>
<th>n (%)</th>
<th>Reference</th>
<th>Old Name</th>
<th>Type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR</td>
<td>5’-UTR</td>
<td>c.-156C&gt;T</td>
<td>p.?</td>
<td>1 (0.4)</td>
<td>Novel NL, 19</td>
<td>2</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 1</td>
<td>c.1?_G7&gt;?,del</td>
<td>p.?</td>
<td>3 (1.1)</td>
<td>c.1?_68?-del</td>
<td>3</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 1–4</td>
<td>c.1?_1186 del</td>
<td>p.?</td>
<td>1 (0.4)</td>
<td>c.1?_1187-del</td>
<td>3</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 1</td>
<td>c.1A&gt;T</td>
<td>p.Met1</td>
<td>1 (0.4)</td>
<td>17</td>
<td>M-21L 1</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 1</td>
<td>c.1A&gt;G</td>
<td>p.Met1</td>
<td>4 (1.5)</td>
<td>20</td>
<td>M-21V 1</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 2</td>
<td>c.131G&gt;A</td>
<td>p.Trp44X</td>
<td>13 (4.8)</td>
<td>20</td>
<td>W23X 2</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 1–8</td>
<td>c.1-?_1186 del</td>
<td>p.?</td>
<td>5 (1.9)</td>
<td>Novel 3</td>
<td></td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 1–14</td>
<td>c.1-?_2140 del</td>
<td>p.?</td>
<td>1 (0.4)</td>
<td>Novel 3</td>
<td></td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 1–8</td>
<td>c.1-?_1186 del</td>
<td>p.?</td>
<td>5 (1.9)</td>
<td>Insertion of 10 kb, exon 3–8 (intron 8) (FH-Alkmaar-1)</td>
<td>3</td>
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<tr>
<td>LDLR</td>
<td>Exon 3</td>
<td>c.241C&gt;A</td>
<td>p.Arg81Ser</td>
<td>1 (0.4)</td>
<td>20</td>
<td>R60C 1</td>
</tr>
<tr>
<td>LDLR</td>
<td>Intron 3</td>
<td>c.313+1G&gt;A</td>
<td>p.Trp44X</td>
<td>17 (6.3)</td>
<td>20</td>
<td>Insertion of 8.5 kb, exon 3–6</td>
</tr>
<tr>
<td>LDLR</td>
<td>Intron 3</td>
<td>c.313+1G&gt;A</td>
<td>p.Trp44X</td>
<td>17 (6.3)</td>
<td>20</td>
<td>Insertion of 8.5 kb, exon 3–6</td>
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<tr>
<td>LDLR</td>
<td>Exon 3–8</td>
<td>c.191-?_1186 del</td>
<td>p.?</td>
<td>16 (6.0)</td>
<td>Insertion of 2.5 kb, exon 7–8 (FH-Cape Town 2)</td>
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<tr>
<td>LDLR</td>
<td>Exon 7–8</td>
<td>c.941-?_1186 del</td>
<td>p.?</td>
<td>16 (6.0)</td>
<td>Insertion of 4.4 kb (in intron 12) (FH-Leiden-3)</td>
<td>3</td>
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<tr>
<td>LDLR</td>
<td>Exon 7–15</td>
<td>c.941-?_2311 del</td>
<td>p.?</td>
<td>1 (0.4)</td>
<td>c.940?-?_2312 del</td>
<td>3</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 7</td>
<td>c.1004G&gt;T</td>
<td>p.Gly335Val</td>
<td>2 (0.7)</td>
<td>20</td>
<td>G314V 1</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 7</td>
<td>c.1012T&gt;G</td>
<td>p.Gly338Gly</td>
<td>1 (0.4)</td>
<td>20</td>
<td>C317G 1</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 7</td>
<td>c.1027G&gt;A</td>
<td>p.Gly343Ser</td>
<td>1 (0.4)</td>
<td>20</td>
<td>G322S 1</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 7</td>
<td>c.1048C&gt;T</td>
<td>p.Arg348X</td>
<td>3 (1.1)</td>
<td>20</td>
<td>R329X 2</td>
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<tr>
<td>LDLR</td>
<td>Exon 8</td>
<td>c.1069G&gt;A</td>
<td>p.Glu357Lys</td>
<td>1 (0.4)</td>
<td>20</td>
<td>E336K 1</td>
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<tr>
<td>LDLR</td>
<td>Exon 8</td>
<td>c.1118G&gt;A</td>
<td>p.Gly373Asp</td>
<td>2 (0.7)</td>
<td>20</td>
<td>G352D 1</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 8</td>
<td>c.1176C&gt;A</td>
<td>p.Cys392X</td>
<td>2 (0.7)</td>
<td>20</td>
<td>C371X 2</td>
</tr>
<tr>
<td>LDLR</td>
<td>Exon 9–12</td>
<td>c.1187-?_1845 del</td>
<td>p.?</td>
<td>1 (0.4)</td>
<td>Insertion of 4.4 kb (in intron 12) (FH-Leiden-3)</td>
<td>3</td>
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<tr>
<td>LDLR</td>
<td>Exon 9</td>
<td>c.1196C&gt;A</td>
<td>p.Ala399Asp</td>
<td>1 (0.4)</td>
<td>20</td>
<td>A378D 1</td>
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<tr>
<td>LDLR</td>
<td>Exon 9</td>
<td>c.1216C&gt;A</td>
<td>p.Ala399Asp</td>
<td>1 (0.4)</td>
<td>20</td>
<td>R385R 2</td>
</tr>
</tbody>
</table>

(Continued)

van der Graaf et al Phenotype and Genotype in Children With ADH

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Dutch population and 2 have not been reported before at all (Table 1).17-22 Two patients were compound heterozygotes and carried 2 different mutations of LDLR.

**Group Characteristics**

Characteristics of the groups with and without a molecular diagnosis of ADH are shown in Table 2. The 14 children who exhibited the ADH phenotype but in whom a molecular diagnosis could not be established were slightly older (non-significant) than those carrying an established mutation (mean±SD age, 12.2±3.4 versus 10.9±3.5 years, respectively; P=0.425). BMI and gender were not significantly different between groups, nor was the proportion of subjects with a first-degree relative who suffered a cardiovascular event.

**Lipids and Lipoproteins**

Although in both groups the mean total cholesterol and LDL-C levels were well above the 95th percentiles for age and gender, hypercholesterolemia was less pronounced in the group without an established gene mutation compared with those with a molecular diagnosis, with an LDL-C of 4.40±0.48 versus 6.13±1.53 mmol/L (P<0.001), respectively (Table 2). This difference remained significant after adjustment for age, gender, and BMI (P<0.001). In addition, ApoB levels were lower in the group without a molecular diagnosis: 1.19±0.19 versus 1.68±0.42 g/L (P=0.014). Mean high-density lipoprotein cholesterol and ApoA1 levels were slightly but not significantly higher in those without an identified mutation (P=0.275 and P=0.835, respectively).
Triglycerides were similar and within the normal range in both groups. Overall, lipid profiles of the nonmutation carriers were more similar to the subjects with an APOB mutation (familial defective ApoB) compared with subjects with an LDLR mutation (familial hypercholesterolemia).

Of the children who carried an LDLR mutation, 11 (4%) exhibited physical symptoms; of those 11, 8 subjects had tendon xanthomas, 2 showed an arcus cornealis, and 1 had xanthelasmas. Physical symptoms were absent in the children with an APOB mutation and in the nonmutation carriers.

### Discussion

In a large group of referred children with a strictly defined phenotype of ADH, we were able to identify 77 different functional gene mutations in 95% of cases. We therefore demonstrate that in almost all children the ADH phenotype can be explained by mutations in genes that are currently known to underlie this syndrome.

Our results have a number of important implications. First, detection rates in our patients are much higher than reported by our colleagues. They assessed mutation detection rates to establish the clinical utility of diagnostic tools and found them to vary widely from 20% to 90%. On the basis of these data, claims were made that a large proportion of ADH is still unexplained. We strongly disagree with this contention on the basis of the present study. The discrepancies between our data sets and the others could be explained by the rigorous criteria we used to select for ADH. In fact, earlier studies also demonstrated better detection rates when a more stringent definition of phenotype was applied.

Furthermore, the thorough molecular analyses of the LDLR, APOB, and PCSK9 genes, including splice-site sequencing and detection of large deletions and insertions, might provide additional reasons for the higher detection rate in our patients.

Another consideration might be that we ruled out secondary and environmental dyslipidemia not only by using stringent criteria but also by investigating children. Hypercholesterolemia resulting from causes other than ADH is much less prevalent in children compared with adults. For example, familial combined hyperlipidemia, another common lipid disorder, often does not exhibit its phenotype until early adulthood. In a recent study, LDLR mutations were identified in 20% of adults patients with a familial combined hyperlipidemia phenotype, which underscores the overlap between the ADH and familial combined hyperlipidemia phenotypes in adults. We therefore feel that a pediatric population is better suited for the definition of the molecular basis of a dyslipidemic phenotype, given that the specific syndrome is of course expressed in childhood. This contention is in disagreement with findings from others; Assouline et al stated that it is difficult to suspect and confirm the diagnosis of ADH in a pediatric population because children usually exhibit a normal physical examination and clinical manifestations of atherosclerosis in children are very rare.

They found a mutation in 71% of French Canadian children with a clinical diagnosis of ADH, even though molecular heterogeneity in this population is very small. However, obese children were not excluded, the same LDL cutoff (3.6 mmol/L) was applied for all ages, and only the 5 common French Canadian molecular defects were tested. A similar study in an Italian pediatric population reported a mutation in the LDLR gene in only 14 of 27 children (52%) with a clinical diagnosis of ADH. In this study, total cholesterol cutoffs (>90th percentile) instead of LDL cutoffs were applied, obese children were not excluded, and other loci (APOB and PCSK9) and intronic regions of LDLR were not analyzed. Again, the discrepancies between these and our finding can be explained by different use of inclusion and exclusion criteria and thorough molecular analytic methodology.

However, we also established that other as-yet unknown factors must underlie ADH in children. It is likely that these...
factors are hereditary on the basis of the autosomal dominant pattern of inheritance of hypercholesterolemia and cardiovascular events in relatives of many of these individuals. Although recent genome-wide association studies have identified several single-nucleotide proteins and candidate genes associated with LDL-C levels and cardiovascular disease, their role in ADH remains to be established. Indeed, the discovery of novel genes with a major effect on LDL-C would enable researchers to identify new targets for cholesterol-lowering drugs.

Conclusions

In the vast majority of children with an ADH phenotype, a causative mutation can be identified, strongly suggesting that most of the large-effect genes underlying ADH have been found. Efforts to elucidate the molecular basis of the ADH phenotype in the children with unexplained dyslipidemia are consequently underway, but our data show that expectations for gene identification in this phenotype need to be toned down considerably. Previous estimates concerning the percentage of unexplained ADH cases were incorrect, most likely because of insufficient clinical assessment of index patients and their kindreds.

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Autosomal dominant hypercholesterolemia (ADH) is characterized by severely elevated low-density lipoprotein cholesterol levels from birth on, enhanced atherosclerosis progression, and premature cardiovascular events. Functional and morphological changes of the arterial wall are observed in children with ADH, which indicates that the atherosclerotic process has already been initiated. Early diagnosis and treatment of ADH are pivotal because therapy with lipid-lowering agents strongly decreases the risk for cardiovascular events. If possible, a clinical diagnosis of FH should preferably be confirmed by molecular genetic testing. Furthermore, the imprecision of any current clinical screening strategy for ADH emphasizes the relevance of genetic testing for definite diagnosis of ADH and screening purposes in affected families. This study shows that, if stringent criteria are used, a functional mutation can be found in 95% of children. We therefore feel that children are better suited for the definition of the molecular basis of a dyslipidemic phenotype. This knowledge can help clinicians establish the definite diagnosis of ADH in families. Once a child is identified as having ADH, cascade screening can be performed to screen more distant relatives using the inheritance pattern across the pedigree. Furthermore, these data strongly suggest that most of the large-effect genes underlying ADH have been found, at least in the Netherlands. This is of importance because any novel gene implicated in the pathogenesis of ADH and cardiovascular disease could become a pharmacological target in itself; hence, it is important to establish whether a large proportion of ADH patients still carry unexplained molecular defects.
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