Analysis of Children and Adolescents with Familial Hypercholesterolemia

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Objective To evaluate the effectiveness of criteria based on child-parent assessment in predicting familial hypercholesterolemia (FH)-causative mutations in unselected children with hypercholesterolemia.

Study design *LDLR, APOB*, and *PCSK9* genes were sequenced in 78 children and adolescents (mean age 8.4 ± 3.7 years) with clinically diagnosed FH. The presence of polygenic hypercholesterolemia was further evaluated by genotyping 6 low-density lipoprotein cholesterol (LDL-C)-raising single-nucleotide polymorphisms.

Results Thirty-nine children (50.0%) were found to carry *LDLR* mutant alleles but none with *APOB* or *PCSK9* mutant alleles. Overall, 27 different *LDLR* mutations were identified, and 2 were novel. Children carrying mutations showed higher LDL-C (215.2 ± 52.7 mg/dL vs 181.0 ± 44.6 mg/dL, $P < .001$) and apolipoprotein B levels (131.6 ± 38.3 mg/dL vs 100.3 ± 30.0 mg/dL, $P < .004$), compared with noncarriers. A LDL-C of ~190 mg/dL was the optimal value to discriminate children with and without *LDLR* mutations. When different diagnostic criteria were compared, those proposed by the European Atherosclerosis Society showed a reasonable balance between sensitivity and specificity in the identification of *LDLR* mutations. In children without mutation, the FH phenotype was not caused by the aggregation of LDL-C raising single-nucleotide polymorphisms.

Conclusions In unselected children with hypercholesterolemia, LDL-C levels >190 mg/dL and a positive family history of hypercholesterolemia appeared to be the most reliable criteria for detecting FH. As 50% of children with suspected FH did not carry FH-causing mutations, genetic testing should be considered. *(J Pediatr 2017;183:100-7).*

Familial hypercholesterolemia (FH) (OMIM 143890) is the most frequently diagnosed inherited lipid disorder in children and adolescents.[1] It is an autosomal dominant condition resulting in severely elevated low-density lipoprotein cholesterol (LDL-C) concentrations in plasma from birth and has an estimated frequency of heterozygous FH (HeFH) form between 1 in 200 to 1 in 500 individuals in the general population.[2,3] HeFH is mainly due to loss-of-function mutations in the LDL receptor gene (*LDLR*),[2] but loss-of-function mutations in the apolipoprotein B (ApoB) gene (*APOB*)[4] and gain-of-function mutations in the proprotein convertase subtilisin kexin type 9 gene (*PCSK9*)[5] have been also reported to cause FH, though much less frequently.[2] More recently, a polygenic nature of the FH phenotype has been suggested,[6] and this may explain why approximately 20%-40% of patients with clinically diagnosed FH are negative for mutations in *LDLR*, *APOB*, and *PCSK9* genes.[2]

In adults, HeFH is clinically diagnosed based upon elevated concentration of LDL-C associated with normal triglycerides (TG) in the patient and its relatives, family history of premature cardiovascular complications, and presence of xanthomas and corneal arcus.[2,7] These criteria have been incorporated in a validated set of algorithms, the Make Early Diagnosis to Prevent Early Deaths, the Dutch Lipid Clinic Network (DLCN), or Simone Broome Registry,[2,8-10] which all are currently used for diagnosing HeFH in adulthood. Unfortunately, the diagnosis of HeFH in children and adolescents is much more challenging. Early in life, the disease is asymptomatic; the only characteristic is elevated LDL-C. Physical signs (eg, xanthomas) are uncommon. To overcome these difficulties, various screening strategies have been proposed to identify children with HeFH. Only implementation of general population and selective screening, primarily based on cascade screening, have been reported.[11-15] Recently, the European Atherosclerosis Society (EAS) proposed HeFH diagnostic criteria for children based on LDL-C cut-off values and family history of hypercholesterolemia and/or premature cardiovascular disease.[1] However, their effectiveness in identifying genetically proven HeFH in unselected children with hypercholesterolemia has not been extensively determined.

Following clinical diagnosis of FH, a genetic test may be useful in confirming the disease. Therefore, this study was designed to search genetic confirmation of FH in a cohort of children with elevated LDL-C ascertained in a tertiary pediatric clinic by parent-child screening. The ability of different clinical criteria to predict the results

ApoB	Apolipoprotein B	*LDLR+*	Carrying *LDLR* mutations
ASCVD	Atherosclerotic cardiovascular disease	*LDLR–*	Not carrying *LDLR* mutations
BMI	Body mass index	LDL-C	Low-density lipoprotein cholesterol
DLCN	Dutch Lipid Clinic Network	ROC	Receiver operator characteristic
EAS	European Atherosclerosis Society	SNPs	Single-nucleotide polymorphisms
FH	Familial hypercholesterolemia	TC	Total cholesterol
GRS	Genetic risk score	TG	Total triglycerides
HeFH	Heterozygous FH		

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of mutational analysis has been also evaluated. In addition, we have also investigated whether a form of polygenic hypercholesterolemia was present in children with clinically diagnosed HeFH in whom no mutations in *LDLR*, *APOB*, or *PCSK9* were identified.

Methods

Children with suspected FH were identified within a cohort of 719 children (2-19 years old) referred to a tertiary pediatric clinic for the presence of abnormal lipid values detected during an occasional laboratory test. At their first visit, lipids and lipoprotein were measured in index children, in all available siblings, and in their parents. Only children with complete biochemical and family data were considered. Based upon the presence of at least 1 parent with hypercholesterolemia (LDL-C > 95th and TG < 75th) and/or with premature atherosclerotic cardiovascular disease (ASCVD), 78 children with hypercholesterolemia (31.1%) were clinically classified as FH (**Figure 1**; available at www.jpeds.com).

Demographic and clinical information (including history of hypertension, diabetes mellitus, premature ASCVD, and current lipid-lowering treatment) were collected in all family members. Premature ASCVD was defined if any of the following events occurred for the first time before age 55 years (in male family member) and age 65 years (in female family members): angina, myocardial infarction, percutaneous transluminal coronary angioplasty, or coronary artery bypass grafting. Physical examination was also carried out in all family members with special reference to the presence of tendon xanthomas. In children, weight was measured using an electronic scale (Soehnle, Murrhardt, Germany), and the standing height was measured with the Harpenden Stadiometer (Holtain, Crymych, Great Britain). Body mass index (BMI) was determined as weight/height^2 (kg/m^2). The pubertal status was assessed according to Tanner. The degree of obesity was quantified using Cole's least mean-square method, which normalizes the skewed distribution of BMI and expresses BMI as SD score.[16] Children with concomitant endocrine disorders were excluded. The Ethical Committee of Sapienza University of Rome approved the study protocol, and written informed consent was obtained from all parents or legal guardians.

Blood samples were collected early in the morning after an overnight fast in ethylenediaminetetraacetic acid-containing tubes. Plasma samples were obtained by centrifugation at 4°C and immediately used for lipid determinations. Plasma total cholesterol (TC), TG, and high-density lipoprotein cholesterol were determined as previously described.[17] LDL-C was calculated by using the Friedewald equation. Plasma glucose and insulin levels were determined as reported elsewhere.[18] Age- and sex-specific percentiles for TC and TG reported by the Lipid Research Clinics were used as reference values in children.[19] Example levels of 90th percentile for TC in children aged 5 and 10 years were 4.7 mmol/L and 4.9 mmol/L (for boys) and 4.9 mmol/L and 4.9 mmol/L (for girls), respectively; those for 90th TG percentile were 0.8 mmol/L and 1.1 mmol/L (for boys) and 1.2 mmol/L and 1.2 mmol/L (for girls). Percentiles reported by Menotti et al[20] in a large random sample of the general Italian population were used for adults (age >20 years).

Mutational Analyses

DNA from peripheral blood was extracted as described.[17] Coding region ad intron-exon boundaries of *LDL*R (18 exons), *PCSK9* (12 exons), and exons 26 of *APOB* were polymerase chain reaction amplified, purified, and then directly sequenced in 5′- and 3′-directions in ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, California) as described.[5,17,21] Exon 26 of *APOB* was selected because it was where the vast majority of mutations coding ApoB with defective binding capacity have been located.[4] All the sequence variants identified were verified by manual inspection of chromatograms, and changes were confirmed by an independent resequencing reaction. To detect rearrangements within the coding sequence of *LDLR*, the multiplex ligation-dependent probe amplification was performed according to the manufacturer's protocol (MRC-Holland, Amsterdam, The Netherlands) in all samples without *LDLR* mutation at resequencing.

To determine the functional effects of sequence variants, we used PANTHER, (www.pantherdb.org), Polyphen-2 (www.genetics.bwh.harvard.edu/pph2), SIFT (www.sift.jcvi.org), Mutation T@ster Prediction (http://www.mutationtaster.org/), and Human Splice Finder (http://www.umd.be/HSF3/HSF.html) algorithms. The score from each program were grouped into three categories: "probably deleterious," "possibly deleterious," and "benign." The majority of biochemically proven functional mutations have scores of either "probably" or "possibly" deleterious for all programs. Mutations were also considered to be pathogenic if co-segregation of the mutation with the phenotype was observed, and if mutations were previously described in other populations. The amino acid nature and conservation in different species as well as the availability, when possible, of results of functional assay were also used as additional criteria for pathogenicity.

To further investigate whether a form of polygenic hypercholesterolemia was present in the group of children with clinically diagnosed FH,[6] we genotyped children without FH-causing mutations for 6 LDL-C raising single-nucleotide polymorphisms (SNPs) to derive a genetic risk score (GRS), as reported.[22] In brief, TaqMan assays (Life Technologies, Carlsbad, California), and genotype calling for all assays was carried out using an automated system, the results of which were checked manually by study personnel using SNP viewer software. The GRS was calculated using weighted sums for 6 SNPs of the highest effect.[22] A group of 47 children with normocholesterolemia was used for comparison. Their clinical and biochemical characteristics are shown in **Table I** (available at www.jpeds.com).

Statistical Analyses

All statistical analyses were performed with SPSS/WIN program v 20.0 (SPSS Inc, Chicago, Illinois). Descriptive statistics such as means, SD, and ranges were undertaken for all variables. Continuous variables were compared by the Mann-Whitney U test, whereas the categorical variables were compared by χ^2 or Fisher