Cardiovascular risk assessment of dyslipidemic children: analysis of biomarkers to identify monogenic dyslipidemia

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Abstract The distinction between a monogenic dyslipidemia and a polygenic/environmental dyslipidemia is important for the cardiovascular risk assessment, counseling, and treatment of these patients. The present work aims to perform the cardiovascular risk assessment of dyslipidemic children to identify useful biomarkers for clinical criteria improvement in clinical settings. Main cardiovascular risk factors were analyzed in a cohort of 237 unrelated children with clinical diagnosis of familial hypercholesterolemia (FH). About 40% carried at least two cardiovascular risk factors and 37.6% had FH, presenting mutations in LDLR and APOB. FH children showed significant elevated atherogenic markers and lower concentration of antiatherogenic particles. Children without a molecular diagnosis of FH had higher levels of TGs, apoC2, apoC3, and higher frequency of BMI and overweight/obesity, suggesting that environmental factors can be the underlying cause of their hypercholesterolemia. An apoB/apoA1 ratio ≥ 0.68 was identified as the best biomarker (area under the curve = 0.835) to differentiate FH from other dyslipemias. The inclusion in clinical criteria of a higher cut-off point for LDL cholesterol or an apoB/apoA1 ratio ≥ 0.68 optimized the criteria sensitivity and specificity. The correct identification, at an early age, of all children at-risk is of great importance so that specific interventions can be implemented. ApoB/apoA1 can improve the identification of FH patients.—Medeiros, A. M., A. C. Alves, P. Aguiar, and M. Bourbon on behalf of the Pediatric Investigators of the Portuguese Familial Hypercholesterolemia Study. Cardiovascular risk assessment of dyslipidemic children: analysis of biomarkers to identify monogenic dyslipidemia. J. Lipid Res. 2014. 55: 947–955.

Dyslipidemia is one of the major cardiovascular risk factors. It can be due to a monogenic condition, but can also be secondary to specific conditions such as obesity, diabetes mellitus, hypothyroidism (1), or even to polygenic or environmental causes (2). Because lipids, and other cardiovascular risk factors, track into adulthood, and the aggregation of classical risk factors such as lipid levels, blood pressure, BMI, diabetes mellitus, and tobacco use is associated with an even higher cardiovascular risk (3, 4), it is important to identify at-risk children at a young age so that therapeutic measures and/or lifestyle modifications can be implemented early in life to decrease their cardiovascular risk (1, 5).

Familial hypercholesterolemia (FH) is the most frequently diagnosed inherited lipid disorder in children and adolescents (6). FH is an autosomal dominant condition resulting in severely elevated LDL cholesterol (LDL-C) concentrations in plasma from birth and has a frequency of about 1:400–500 in most populations (7). FH is mainly due to loss-of-function mutations in the LDL receptor gene (LDLR) (7) or the apolipoprotein B gene (APOB) (8). Gain-of-function mutations in proprotein convertase subtilisin kexin type 9 gene (PCSK9) (9), or even in other genes yet to be described, are a rare cause of FH (10, 11). FH confers lifelong risk of atherosclerosis beginning in childhood and is associated with premature CVD (pCVD), so early screening is justified (6, 12, 13). Evidence from

Supplementary key words cardiovascular risk factor • familial hypercholesterolemia • clinical criteria

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Abbreviations: AUC, area under the curve; FH, familial hypercholesterolemia; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; Lp(a), lipoprotein (a); PCSK9, proprotein convertase subtilisin kexin type 9 gene; pCVD, premature CVD; PFHS, Portuguese Familial Hypercholesterolemia Study; ROC, receiver operating characteristic; sdLDL, small dense LDL cholesterol; TC, total cholesterol.

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studies with treated and untreated FH children indicates that early identification and treatment is associated with reduced subclinical evidence of atherosclerosis (6, 14). However, in most of the cohorts described, a mutation causing disease can be found in varying percentages, ranging from 20 to 90% of the cases (15–19), which may reflect differences in the clinical criteria applied or in the methodology used in those countries. For patient cardiovascular risk assessment, management, and treatment, it is of great importance to distinguish between a monogenic and a polygenic/environmental dyslipidemia. A monogenic condition is associated with a higher cardiovascular risk and early implementation of pharmacologic treatment is necessary to decrease this increased cardiovascular risk. As for polygenic or environmental dyslipidemia, in the majority of the cases, the risk may be modified just by implementation of a healthy lifestyle (5, 6, 10).

The identification of specific biomarkers that can help to distinguish between monogenic and polygenic/environmental dyslipidemia is important so both groups of children receive the appropriate treatment and/or counseling to reduce their cardiovascular risk. Previous studies in adults have selected plasma levels of apoB and apoA1 as good predictors of cardiovascular risk and the apoB/apoA1 ratio has been considered the best marker of the balance between atherogenic and antiatherogenic particles (2, 20, 21). Small dense LDL-C (sdLDL) has also been associated with CVD independently of established risk factors and represents an emerging cardiovascular risk factor (21–23). Here we present the cardiovascular risk assessment of a cohort of children with a clinical diagnosis of FH in order to identify useful biomarkers for clinical criteria improvement to distinguish between monogenic and polygenic/environmental dyslipidemia in clinical settings.

METHODS

Study population

A total of 237 unrelated children (2–17 years old) were referred as index patients to the Portuguese FH Study (PFHS) (24–27) during 1999–2012, mainly by pediatricians, cardiologists, and clinical geneticists countrywide. Only children with two independent altered fasting lipid profiles were recruited for this study. The recruitment criteria applied was having a clinical diagnosis of FH according to an adaptation of the Simon Broome criteria (24): children were admitted when presenting total cholesterol (TC) >260 mg/dl or LDL-C >155 mg/dl, and a family history of hypercholesterolemia or pCVD. In a few cases, children under the age of 10 were admitted to the study with lower cut-off points for cholesterol values (TC >200 mg/dl or LDL-C >120 mg/dl) when a severe dyslipidemia was present in one of the parents (TC >300 mg/dl or LDL-C >200 mg/dl). Family history of hypercholesterolemia was defined if hypercholesterolemia (TC >290 mg/dl) was present in at least one of the parents and additionally in other members of the family (siblings, grandparents, and/or uncles). According to Simon Broome criteria, pCVD was defined if any of the following events: angina, myocardial infarction, percutaneous transluminal coronary angioplasty, or coronary artery bypass grafting, occurred for the first time before 55 years of age in males and 65 years of age in females.

Additionally 138 children (1–17 years old) were referred to the PFHS cascade screening program as being related to a previously identified FH patient (children, grandchildren, nephews, cousins, or siblings).

Exclusion criteria were thyroid dysfunction and diabetes. Parents and other relatives with and without a clinical diagnosis of FH were also recruited.

Written informed consent was obtained from all participants before their inclusion in the study. The study protocol and database were previously approved by the National Institute of Health (INSA) Ethical Committee and the National Data Protection Commission.

TC, LDL-C, and TGs greater than the 95th percentile, for age and sex, were defined according to the reference values for the Spanish population (28), due to the absence of percentile distributions for fasting serum lipids in the Portuguese population. Because values did not differ greatly between sexes and ages, the following mean values were assumed for the 95th percentile: TC >225 mg/dl, LDL-C >135 mg/dl, and TGs >125 mg/dl.

BMI percentiles were calculated for age and gender according to the Centre for Disease Control growth charts (29) as recommended by the Portuguese Directorate-General for Health. Three BMI percentile cut-offs were defined in this work: 75th, 85th, and 95th. Overweight and obesity were defined as a BMI greater than the 85th and 95th percentile, respectively (29).

Stage 1 and stage 2 hypertension were defined as systolic or diastolic blood pressure greater than the 95th and 99th percentile, respectively, for age and gender according to the “The Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents” tables (30), as recommended by the Portuguese Directorate-General for Health.

Data collection

Demographic and clinical data on cardiovascular risk factors, such as blood pressure, weight and height, physical activity, personal medical history, lipid profile, and family history of pCVD, as well as lipid-lowering measures, were obtained by the assistant clinician for all index patients in a form adapted from the Simon Broome Registry at the time of referral to the study. For relatives, a simpler form was fulfilled with only demographic data, personal medical history of pCVD, lipid profile, and lipid-lowering measures.

Biochemical characterization of lipids and lipoproteins

Fasting blood samples were collected from individuals at the time of referral to the study. TC, direct LDL-C, HDL cholesterol (HDL-C), TGs, apoA1, apoB, and lipoprotein (a) [Lp(a)] were determined for all individuals in a Cobas Integra 400 plus system (Roche) by enzymatic colorimetric and immunoturbidimetric methods. Additionally, in all children included as index patients and received after 2010, serum levels of apoA2, apoC2, apoC3, apoE, and sdLDL (sdLDL-EX “SEIKEN” kit) were measured by direct quantification in an RX Daytona analyzer (Randox Laboratories), mostly by enzymatic colorimetric and immunoturbidimetric methods.

Molecular analysis

The genetic diagnosis of FH was performed by the molecular analysis of APOB (two fragments of exons 26 and 29), LDLR (including the study of splice regions and large rearrangements), and PCSK9 genes as reported previously (26). Mutations were considered to be pathogenic if cosegregation of the mutation with the phenotype was observed and if mutations were previously described in other populations. Pathogenicity of novel variants was assessed according to Cotton and Scriver criteria (31):
cosegregation analysis (in at least 80% of the relatives), absence in a panel of a minimum of 50 normolipidemic individuals, amino acid nature and conservation in different species, and, when possible, by functional assays as reported before (32, 33).

Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0 for Windows; SPSS, Chicago, IL). Comparison of frequencies between qualitative variables was carried out using the chi-squared test. Mean values of quantitative variables were compared with the Student’s t test or ANOVA for independent data while median values were compared with the nonparametric Mann-Whitney or Kruskal Wallis median tests. Pearson correlation was conducted to determine associations between variables. \( P < 0.05 \) was considered to be statistically significant.

Biomarker cut-off values were determined from receiver operating characteristic (ROC) curves with the area under the curve (AUC) >0.7 using pretreatment values for FH and non-FH children in order to find biomarkers to distinguish these two groups. For biomarker selection, criteria were sensitivity and specificity values above 50% and sensitivity higher than specificity. The value that maximized the sum of sensitivity and specificity was selected as the optimal cut-off point for each biomarker.

Different criteria for the clinical diagnosis of FH were established using novel cut-off points and were compared with the genetic diagnosis using cross-tables. Sensitivity, specificity, and kappa statistic were calculated to evaluate the inter-diagnostic agreement. Kappa statistic ranges between negative values and 1, indicating no agreement and perfect agreement, respectively, among raters.

RESULTS

A total of 237 unrelated children (131 girls and 106 boys) were referred to PFHS. Mean age at inclusion was 10.0 ± 3.6 years (2–17 years).

Cardiovascular risk factors

All children were reported to be nonsmokers. Physical symptoms such as xanthoma were absent and, therefore, all children were classified as “possible FH” according to Simon Broome criteria. Clinical and biochemical characteristics are shown in Table 1.

Besides the dyslipidemia present in all the children referred to PFHS as index patients, other cardiovascular risk factors such as obesity/overweight, hypertension, physical inactivity, and family history of pCVD (1st/2nd degree relative) were also evaluated (Table 1). TC and/or LDL-C above the 95th percentile were the most frequent cardiovascular risk factors in the study population (89.5%), followed by overweight/obesity (41.7%), family history of pCVD (24.5%), TGs >95th percentile (16%), and hypertension (16%). About 40% carried at least two cardiovascular risk factors.

Genetic analysis and cascade screening

A molecular defect was identified in 89 children referred as index patients. A total of 85 children had a \( \text{LDLR} \) mutation and 4 had an \( \text{APOB} \) mutation (37.6%). In the \( \text{APOB} \), two different mutations were found that have been reported before as a cause of FH (supplementary Table 1).

In the \( \text{LDLR} \), 63 different mutations were found and considered pathogenic: 42 were null alleles or had been proven to be pathogenic by functional studies; 14 did not have functional studies but had been reported in FH patients from other populations and in general fulfilled Cotton and Scriver criteria (31) for a mutation causing disease, with exception for 4 mutations that showed low penetrance in children (2 functional mutations and 2 without functional studies), and another 3 mutations that showed weak cosegregation but had been described before as mutations causing disease. The remaining seven mutations were novel and predicted to be pathogenic according to Cotton and Scriver criteria (31) (supplementary Table 1). Homozygous or compound heterozygous were not identified in the present cohort. No mutations were found in the PCSK9 gene.

In the remaining 148 children (62.4%), no mutations considered pathogenic were identified using the current molecular biology techniques previously published (26, 27).

The cascade screening program performed in the FH families of the PFHS led to the additional identification of 82 children, making a total of 171 children identified with FH. Index patients presented a more severe phenotype than affected relatives, with higher mean values in almost all lipids and lipoproteins, including significantly higher mean TC, LDL-C, and sdLDL values (\( P < 0.001 \), \( P = 0.003 \), \( P = 0.006 \), respectively) (supplementary Table II). The distribution of mutations (null, defective, splicing, and \( \text{APOB} \)) was similar in both groups (index patients versus relatives).

All children carrying null mutations in \( \text{LDLR} \) presented a more severe phenotype with significantly higher mean TC and LDL-C values compared with children carrying defective mutations (\( P = 0.011 \), \( P = 0.007 \), respectively) (supplementary Table III). Children with \( \text{APOB} \) mutations presented lower values, but not significantly lower, than the carriers of defective mutations (supplementary Table III).

Lipids, lipoproteins, and genetic findings: FH versus non-FH children

The cohort was divided in two groups according to the molecular diagnosis of FH in order to assess children’s cardiovascular risk (Table 2). No statistically significant differences were found in the frequency of children above the 95th percentile for TC or LDL-C values between the two groups, however hypercholesterolemia parameters were statistically higher (\( P < 0.001 \) for TC and LDL-C) in the group of children with a molecular diagnosis of FH. Mean apoB and sdLDL levels were also significantly higher in FH children (\( P < 0.001 \) for both). Additionally mean HDL-C, apoA1, and apoA2 levels were significantly lower in those with a molecular diagnosis (\( P < 0.001 \) for HDL-C and apoA1, \( P = 0.013 \) for apoA2). Consequently, children with FH had higher non-HDL-C/HDLC and apoB/apoA1 ratios (\( P < 0.001 \) for both). Lp(a) was not significantly different between groups (Table 2). TGs were slighty, but not significantly, higher in non-FH children; however, mean apoC2 and apoC3 values were statistically higher in those without a genetic defect (\( P = 0.019 \), \( P = 0.002 \), respectively) (Table 2). Mean apoE level was significantly higher in the group with an established gene mutation (\( P = 0.037 \) (Table 2).
TABLE 1. Clinical and biochemical characteristics of all the children included in the study

| Demographic and Clinical Data* | Cardiovascular Risk Factors* | Lipids (mg/dl) |
|-------------------------------|-----------------------------|----------------|
| **Demographic**               |                             |                |
| Index patients                | 237                         | n = 237        |
| Age (years)                   | 10.0 ± 3.6                  |                |
| Male gender                   | 44.7%                       |                |
| Family history                | >95th percentile            |                |
| Physical symptoms             | Physical activity           |                |
| Xanthomas                     | 0.0%                        |                |
| Therapeutic measures          | On diet                     |                |
| On medication                 | 32.3%                       |                |
| Blood pressure                | n = 150                     | apoA2          |
| Prehypertension               | 64.7%                       | apoC2          |
| Stage 1 and 2 hypertension    | 16.0%                       | apoE           |
| Smoking habits                | 0.0%                        | sdLDL          |
| Lipid biomarkers and clinical criteria from the basic and advanced lipid profile (Table 2) that were statistically different between the two groups were selected for further investigation to identify biomarkers that better distinguish FH children from other dyslipidemic children. A total of 155 index cases (50 FH and 105 non-FH) with pretreatment lipid values were included in this analysis. Optimal cut-off points were obtained for the best six biomarkers using TC, LDL-C, apoB/apoA1 ratio, non-HDL-C/HDL-C ratio, apoA1, and apoB pretreatment values. Cut-off points and their sensitivity and specificity are shown in Table 3. Figure 2 illustrates the ROC curves obtained for three biomarkers that better discriminate between FH and non-FH children. The apoB/apoA1 ratio was identified as the best biomarker (AUC = 0.835).

Analysis of the Simon Broome criteria regarding specificity, sensitivity and modifications to these criteria are presented in Table 4. Pretreatment lipid values were available for a total of 261 children (100 FH and 161 non-FH), including index cases and relatives that were included in this analysis. Adjustments made to Simon Broome criteria (criteria 1) included the newly determined biomarker apoB/apoA1 ratio (criteria 3 and 5) and the novel cut-off value for LDL-C (criteria 4 and 5). A set of criteria with only the apoB/apoA1 ratio was also evaluated (criteria 2). The proposed criteria were analyzed in three groups of children (index cases, relatives, and the entire cohort) using only pretreatment values. Simon Broome criteria showed a reasonable balance between sensitivity and specificity in the identification of index cases with FH (76.0 and 68.6%; k = 0.402), but revealed a very low sensitivity in the identification of relatives with FH (36.0 and 100.0%; k = 0.402), presenting an elevated number of false negatives. The same was observed in the whole cohort (Table 4; supplementary Table IV). The use of the biomarker apoB/apoA1 ratio ≥0.68 (criteria 2) as the sole clinical criteria increased the number of FH children correctly assessed and decreased the number of false negatives identified in the entire cohort, but decreased the number of true positives in index patients. This novel biomarker combined with no significant differences were observed between both groups, even when a larger group including index patients and relatives was considered.

**Other cardiovascular risk factors: FH versus non-FH children**

BMI was statistically higher in the group of children without a molecular diagnosis than in those with FH (*P* = 0.010) (Table 2), as was the frequency of overweight/obesity (BMI >85th percentile) (*P* = 0.017). When BMI >85th percentile was used as an exclusion criterion for the clinical diagnosis of FH, 59 out of 127 children (46.5%) had a mutation causing disease, but would fail to detect 26 FH children. Considering BMI >75th percentile, the same statistical difference is seen (*P* = 0.038). However, when BMI >75th percentile was used as an exclusion criterion, only 48 out of 104 children (46.2%) had a mutation causing disease and this would fail to detect 42 FH children.

Frequency of hypertension (stage 1 or stage 2) was slightly, but not significantly, higher in children without an identified mutation (15.1% versus 10.5%). About 8.1% of the children without FH had simultaneous overweight/obesity and hypertension; a weak association was found between these two cardiovascular risk factors (*P* = 0.047, Pearson correlation).

The frequency of children with a family history of pCVD was similar in both groups.

Most children in both groups engaged in physical activity for less than 2 h/week presenting physical inactivity, with no statistical differences between the groups (Table 2).

There were no statistical differences between FH and non-FH children concerning the number of accumulative cardiovascular risk factors, except for having four cardiovascular risk factors (*P* = 0.008) (**Fig. 1**).

**Lipid biomarkers and clinical criteria**

After analyzing the lipid profiles of the children according to the molecular diagnosis of FH, the 12 parameters

Data are expressed as mean ± SD unless otherwise noted.

*At the time of referral to PFHS.

*Highest values reported without treatment.

*Advanced lipid profile performed at INSA recorded only for children without treatment.

*Specific lipid profile performed at INSA (only available after 2010) recorded only for children without treatment.
with Simon Broome criteria (criteria 3), improved sensitivity in the three groups with a decrease in the number of false negatives, but also increased the number of false positives (Table 4; supplementary Table IV). The use of the novel cut-off value for LDL-C (≥190 mg/dl) (criteria 4) in the Simon Broome criteria improved the number of true negatives (specificity) for the identification of index cases, but also increased the number of false negatives and decreased the number of true positives (sensitivity). A combination of Simon Broome criteria with a LDL-C cut-off point at 190 mg/dl and an apoB/apoA1 ratio ≥0.68 (criteria 5) was found to represent the optimal balance between sensitivity and specificity for the identification of index cases (86.0 and 68.6%; k = 0.480), relatives (84.0 and 75.0%; k = 0.586), and both index cases and relatives (85.0 and 70.8%; k = 0.526) (Table 4); the kappa statistic value also indicates that criteria 5 has the best degree of agreement between specificity and sensitivity. This was the criteria that improved the number of children with FH and also the non-FH children correctly assessed (true positives and true negatives) with a low number of false negatives in the three groups analyzed (Table 4; supplementary Table IV).

![Fig. 1. Number of cumulative cardiovascular risk factors presented in this cohort of dyslipidemic children. The differences between groups are only statistically different for having four cardiovascular risk factors (*P = 0.008).](image)

**DISCUSSION**

In a group of 237 unrelated children with a clinical diagnosis of a monogenic dyslipidemia, FH, a molecular defect was identified in 37.6% of the children. A total of 65 different mutations were found in the LDLR and APOB genes in our cohort. From these, 66% were null alleles or had been proven to be pathogenic by functional assays and 23% had been described before in other populations and fulfilled Cotton and Scriver criteria for a mutation causing disease (31). Patients carrying the four mutations that showed low penetrance were included in this analysis, because it is well-known that cases of low penetrance in children are often observed (34, 35), even in families with functional mutations (two out of four are functional mutations). Carriers of three other mutations that showed weak cosegregation were also included, because these alterations were described before as mutations causing disease by experienced groups in the FH field (36–43). Pathogenicity of the remaining 11% was assessed by segregation analysis of the mutations with the phenotype in those families, amino acid nature and conservation in different species, and absence in the normolipidemic Portuguese control panel [Cotton and Scriver criteria (31), and therefore have been accepted as pathogenic in the present cohort. Functional assays will be carried on in the future to prove the pathogenicity of these mutations.

The remaining 62.4% of children without a molecular diagnosis of FH had less pronounced hypercholesterolemia and higher prevalence of obesity when compared with those with FH. These findings together with elevated levels of TGs and significantly higher levels of lipoproteins (apoC2, apoC3) associated with TGs suggest that these children were misclassified as having a monogenic condition and
lipid profiles. Children with null LDLR mutations revealed a more severe phenotype when compared with children with missense mutations, regarding parameters such as TC and LDL-C. Although children with splicing mutations also revealed a severe phenotype, similar to null mutations, the correct assessment of splicing mutations, classified as null or defective, can only be performed by functional assays of transcript quantification; so these patients were not statistically compared. These results suggest that molecular characterization of FH patients could provide additional information for the correct management of these patients; patients with null mutations should be even more aggressively treated.

Current clinical criteria that include specific cholesterol levels for children, family history of pCVD, and/or severe hypercholesterolemia (Simon Broome criteria), revealed good sensitivity (76%) but low specificity (68.6%) for the identification of Portuguese index FH children and low sensitivity (36%) and high specificity (100%) for the identification of young relatives with FH. These results illustrate that using these criteria, a high number of false positives (index cases) have to be studied and the criteria are not adequate to be used for cascade screening because they present a low sensitivity that leads to a high number of false negatives. Other clinical criteria used worldwide, the MED-PED (Make Early Diagnosis to Prevent Early Deaths) criteria (45) or the Dutch Lipid Network criteria (46), were not analyzed because they only apply to adult patients and do not present specific values to identify children as index cases. However, based in our previous studies, both clinical criteria do not differ greatly (47), and so probably would not have a better discriminative power. To make the genetic diagnosis more cost effective, the improvement of these clinical criteria is imperative.

Because the exclusion of children with other metabolic conditions did not improve our patient identification, newly determined cut-off points for lipid biomarkers (apoB/apoA1 ≥0.68, AUC = 0.835 and LDL-C ≥190 mg/dl, AUC = 0.743) were conjugated with the Simon Broome criteria to improve patient identification. This combination showed an improvement in clinical criteria, especially for relatives that usually present a milder phenotype and therefore are not correctly identified by clinical criteria. In fact, if TC above 260 mg/dl or LDL-C above 190 mg/dl were considered as cut-off points, along with a family history of hypercholesterolemia or pCVD, and also including...
patients that do not fulfill these criteria or for whom personal data, as pretreatment values or family data, are not available but have an apoB/apoA1 ratio $\geq 0.68$ (criteria 5), a low rate of false positives and false negatives is achieved and sensitivity reaches 84% in the identification of relatives. In order to find universal clinical criteria for the identification of index patients and relatives with FH, our novel criteria were tested in the entire cohort and a sensitivity of 85% and a specificity of 70.8% were observed, making these the best criteria for clinical identification of FH patients. This way the novel criteria (criteria 5) can be applied for index cases and relatives because it improves the sensitivity of Simon Broome criteria (56% versus 85%) with only a slight decrease in specificity (79.5% versus 70.8%). In the future, this criteria (criteria 5) will be implemented in our population, for the correct identification of FH children, both index patients and relatives, to allow a better discrimination between monogenic and polygenic/environmental dyslipidemia in clinical settings. Genetic diagnosis will consequently be more cost-effective as the rate of true positives will increase without a significant increase in false positives and a clear reduction of false negatives. The novel lipid cut-off points and criteria presented here could be used in other populations, but validation in the different countries is recommended.

In a Dutch cohort, it has been shown that if children with secondary causes of dyslipidemia (thyroid dysfunction, nephrotic syndrome, autoimmune disease, liver disease, primary biliary cirrhosis) and also those with LDL $<95^{th}$ percentile and BMI $>75^{th}$ percentile are not included in the group of clinical FH children, and so excluded from molecular testing, the patient identification can be improved to 95%. They applied these exclusion criteria to a cohort of hypercholesterolemic children and only 269/1,430 children remained in their cohort and 255 (95%) carried a functional mutation (48). Using the same criteria in our cohort, a molecular defect was present in 47 out of 93 children (50.5%) and 42 FH children were not detected, so about half of our FH children would not be identified using these exclusion criteria. Van der Graaf et al. (48) did not include the false negative rate in their study, so we cannot compare their results with our findings. Thus in our cohort, the use of an exclusion criteria of BMI $>75^{th}$ percentile does not add benefits to the differentiation between FH and polygenic/environmental dyslipidemia, and the same is possibly true for other populations. Although our studies present different outcomes, we agree with the Dutch researchers in that only a very small portion of clinical FH patients will have another unknown gene defect causing FH; in our cohort, we believe that the hypercholesterolemia in the majority of the non-FH patients has a polygenic/environmental cause.

Other biomarkers have shown to be promising for the clinical differentiation between monogenic and polygenic dyslipidemia, but need further investigation because they have not been extensively studied yet, namely: apoE, mean levels were significantly higher in the group of children with a molecular diagnosis of FH compared with those without FH, which can be explained by the absence of functional LDL receptors that delays the catabolism of apoE in FH patients (49); and sdLDL, apoC2, and apoC3 levels were also statistically different between FH and non-FH children and are associated with TG-rich particles not characteristic of FH. Probably due to the small number of children analyzed (n = 100) these biomarkers presented a weak discrimination (AUC 0.599–0.694), but we

### Table 4. Simon Broome criteria and the best criteria analyzed for the clinical diagnosis of FH using novel cut-off points

| Criteria       | Sensitivity (%) | Specificity (%) | Kappa Statistic |
|----------------|----------------|----------------|-----------------|
| Index cases    |                |                |                 |
| Criteria 1     | 76.0           | 68.6           | 0.402           |
| Criteria 2     | 70.0           | 76.2           | 0.439           |
| Criteria 3     | 88.0           | 55.2           | 0.356           |
| Criteria 4     | 82.9           | 66.0           | 0.486           |
| Criteria 5     | 86.0           | 68.6           | 0.480           |
| Relatives     |                |                |                 |
| Criteria 1     | 36.0           | 100.0          | 0.373           |
| Criteria 2     | 82.9           | 75.0           | 0.567           |
| Criteria 3     | 86.0           | 75.0           | 0.605           |
| Criteria 4     | 28.0           | 100.0          | 0.291           |
| Criteria 5     | 84.0           | 75.0           | 0.586           |
| Index cases + relatives |    |                |                 |
| Criteria 1     | 56.0           | 79.5           | 0.363           |
| Criteria 2     | 76.0           | 75.8           | 0.503           |
| Criteria 3     | 87.0           | 62.1           | 0.450           |
| Criteria 4     | 47.0           | 88.8           | 0.384           |
| Criteria 5     | 85.0           | 70.8           | 0.526           |

Results obtained for sensitivity, specificity, and kappa statistic to evaluate the inter-diagnostic agreement are also presented. Criteria were determined using pretreatment values (n = 155). Bold indicates the best criteria representing optimal balance between sensitivity and specificity. Criteria 1 (Simon Broome Criteria): (TC $\geq 260$ mg/dl or LDL-C $\geq 155$ mg/dl) and (family history of pCVD or family history of hypercholesterolemia); criteria 2: apoB/apoA1 ratio $\geq 0.68$; criteria 3: [(TC $\geq 260$ mg/dl or LDL-C $\geq 155$ mg/dl) and (family history of pCVD OR family history of hypercholesterolemia)] or apoB/apoA1 ratio $\geq 0.68$; criteria 4: (TC $\geq 260$ mg/dl or LDL-C $\geq 190$ mg/dl) and (family history of pCVD OR family history of hypercholesterolemia); criteria 5: [(TC $\geq 260$ mg/dl or LDL-C $\geq 190$ mg/dl) and (family history of pCVD OR family history of hypercholesterolemia)] or apoB/apoA1 ratio $\geq 0.68$. 

Analysis of biomarkers for monogenic dyslipidemia 953
believe this could be improved by increasing the sample size. Therefore, we will continue this investigation concerning the use of these potential biomarkers for diagnostic proposes, as they can now be easily determined in an autoanalyzer within a lipid clinic laboratory, the cost being only slightly higher than apoB or apoA1.

Children with a clinical diagnosis of FH, regardless of the origin of their dyslipidemia, presented several cardiovascular risk factors in addition to elevated plasma cholesterol above the 95th percentile; 40% of the dyslipidemic children already have two cardiovascular risk factors. This indicates that dyslipidemia and other cardiovascular risk factors need to be addressed in childhood, so preventive and corrective measures can be implemented at early ages to reduce CVD in adulthood (6). Encouraging an increase in physical activity should be a priority, because in this study it was found that the majority of the children did not fulfill the daily recommendations. The correct identification and stratification of those at risk for CVD in childhood, along with an increase in educational initiatives, followed by implementation of healthy lifestyle habits, and early implementation of lipid-lowering therapy for children with FH (10) will slow the burden of CVD at the population level.

Finally, our results suggest that determination of apoB and apoA1 in routine practice, as mentioned in the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS) guidelines (13) for severe dyslipidemia, can improve at-risk patient identification and consequently patient stratification, management, and prognosis.

APPENDIX

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