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Lipoprotein profile assessed by 2D-1H-NMR and subclinical atherosclerosis in children with familial hypercholesterolaemia

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ABSTRACT

Background and aims: Familial hypercholesterolaemia (FH) is underdiagnosed in children. In addition to lipid concentrations, lipoprotein particle quantity and quality could influence cardiovascular risk. We aimed to perform a comprehensive plasma lipid study, including lipoprotein particle number and size assessment by two-dimensional nuclear magnetic resonance (2D-1H-NMR), in children with FH compared to non-affected children and to evaluate the clinical value of these factors as subclinical atherosclerosis biomarkers.

Methods: One hundred eighty-three children participating in the broad "Hypercholesterolemia Early Detection Programme" (Decopin Project) were recruited. They were categorized as FH, if they had either a positive genetic test or clinical certainty, or as control children (CCh). Medical history, anthropometry and clinical variables were recorded. Standard biochemical measurements were performed. The lipoprotein profile was studied by 2D-1H-NMR. Carotid intima-media thickness (cIMT) was assessed by sonography in 177 children.

Results: FH children had a significant 36% increase in LDL particles. The small LDL fraction was increased by 33% compared to CCh. The relative relationship between large, medium and small LDL and the mean LDL particle size was similar between FH children and CCh. The total and small LDL particle numbers were directly associated with and contributed to the determination of the mean cIMT according to bivariate and multivariate analyses in FH children.

Conclusions: The higher cholesterol levels of FH children are due to an overall increased number of all LDL particle subclasses, including a notable 33% increase in small LDL. Total and small LDL particle number shows a good correlation with cIMT in FH children.

1. Introduction

One out of 250 children has familial hypercholesterolaemia (FH), but this clinical diagnosis is largely undetected and untreated [1]. FH is an autosomal-dominant genetic disease that is present in all racial and ethnic groups and has long been recognized as a cause of premature atherosclerotic coronary heart disease [1,2]. Elevated low-density lipoprotein cholesterol (LDL-C) is observed at an early age. In most cases (93%), this disease is caused by mutations in the low-density lipoprotein receptor (*LDLR*) gene [3], but it can also be

Abbreviations: 2D-1H-NMR, two-dimensional nuclear magnetic resonance; CG, Control group; cIMT, carotid intima-media thickness; CCh, control children; DLCN, Dutch Lipid Clinic Network scale; HeFH, heterozygous familial hypercholesterolaemia; HoFH, homozygous familial hypercholesterolaemia; IQR, interquartile range; PCSK9, proprotein convertase subtilisin-like kexin type 9; VLDL, very low-density lipoprotein.

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caused by mutations in the apolipoprotein B100 (*APOB*) (5%) [4] and proprotein convertase subtilisin-like kexin type 9 (*PCSK9*) (1%) genes [5].

Two types of this disease are known, including the heterozygous form (HeFH), which is the most common inherited disorder with a prevalence of 1/217 children [6], and the homozygous form (HoFH), which has a prevalence of 1/450,000 individuals according to the latest data published for the Spanish population [7].

In Europe, the estimated number of affected individuals is approximately 4.5 million, with children and adolescents accounting for 20–25% of this population [8]. Clinical criteria for FH diagnosis, such as the Dutch Lipid Clinic Network scale (DLCN), cannot be applied to individuals under 18 years of age. Therefore, diagnoses in children should preferably be established by genetic testing. If the genetic test is not available, the diagnosis can be determined by the presence of an increased LDL-C concentration in the context of a family history of FH [9].

In addition to elevated LDL-C levels, the quality of LDL particles is also an important risk determinant, given that smaller LDL particles are associated with a higher cardiovascular risk [10].

NMR methods assess lipoprotein size and then, lipoproteins are arbitrary distributed in different subgroups according particle size. Physiologically the small LDL subclass accounts for 30–50% of total LDL particle number. Some pathological states like hypertriglyceridemia lead to increased number of smaller LDL.

Smaller LDL particles are denser, more atherogenic and more easily oxidized [11]; because they tend to have a lower affinity for the LDL receptor, a harmful increase in the proportion of this LDL subclass in FH children could be anticipated. Although an increase of smaller LDL particles in FH children has been reported [12], information regarding LDL subclass proportion, quantity and quality beyond cholesterol concentrations alone is scarce.

Clear evidence indicates that high levels of LDL-C in a child accelerate the formation and development of atheromatous lesions during the first years of life, as assessed by carotid intima-media thickness (cIMT) and imaging techniques [13]. Different studies have shown faster cIMT progression in children with FH from 7 years of age [8].

The purpose of the present study was to compare lipoprotein subclass particle number and sizes beyond lipid concentrations alone between FH children and control children (CCh) to attain a more comprehensive metabolic characterization of FH children and assess the putative role of this lipid profile as cardiovascular risk marker according to its association to subclinical atherosclerosis.

2. Materials and methods

2.1. Study design and patients

This is a cross-sectional study. One hundred eighty-three children and adolescents aged 4–18 years were enrolled in this study between March 2013 and June 2016. They were participating in the "Early Familial Hypercholesterolemia Detection Project" (DECOPIN Project), which focuses on the implementation of opportunistic, direct and reverse cascade FH screening. Children were classified as FH ($n = 82$) if they had a positive genetic test, or LDL-C >150 mg/dL and one of the parents had a DLCN score >8 in the case of no available or negative genetic test result. The CCh group included children attending our unit because of FH suspicion but not fulfilling FH criteria, and non-affected siblings of FH children studied because of family screening ($n = 101$). At the time of inclusion, no patients were receiving lipid-lowering therapy. The exclusion criteria were chronic renal, hepatic or thyroid disease and type 1 diabetes mellitus, hypercalciuria, eating disorders, autoimmune disease, homozygous FH and other chronic diseases.

An exhaustive medical history, including family cardiovascular and dyslipidaemia history, a complete physical examination and anthropometry data was recorded. To calculate body mass index (BMI) in children, we used the BMI score, which was calculated by the following equation: [(BMI children − BMI 50th percentile of Orbegozo's growth curves)/standard deviation (SD) 50th percentile of Orbegozo's growth curves] [14].

The Hospital's Ethics Committee approved the study protocol. All participants or participants' tutors provided written consent, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. Blood sample collection and storage

Blood samples were obtained after overnight fasting. Plasma and serum aliquots were prepared and stored at −80 °C in the BioBanc of our centre until further use.

2.3. Standard biochemical analysis

Serum cholesterol and triglyceride levels were measured using enzymatic colorimetric tests (CHOD-POD for cholesterol and GPO-POD for triglycerides), and apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB) were measured using immunoturbidimetric assays. HDL cholesterol was measured using a direct enzymatic colorimetric method that is dependent on detergents that solubilise only the HDL. The lipid profile was analysed according to Spintrol "H" CAL GC-MS reference methods. Spintrol "H" Normal was used as a quality control. All reagents were from Spinreact SA (Spain) and were performed in a Cobas Mira Plus autoanalyser (Roche Diagnostics, Spain). LDL-C levels were calculated by the Friedewald equation: LDL cholesterol = total cholesterol − (high-density lipoprotein (HDL) cholesterol + [triglycerides ÷ 5]).

2.4. 2D-1H-NMR lipid profile evaluation

The 2D-1H-NMR Liposcale is a new generation 1H-NMR test developed with the collaboration of our group (15). A 500-μl aliquot of plasma was shipped on dry ice to Biosfer Teslab (Reus, Spain) for lipoprotein analysis. The particle sizes and numbers of nine subtypes of lipoproteins [large, medium and small very low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL)] were determined as previously reported. Briefly, particle concentrations and diffusion coefficients were obtained from the measured amplitudes and attenuation of their spectroscopically distinct lipid methyl group NMR signals using the 2D diffusion-ordered NMR spectroscopy pulse. By this method, the hydrodynamic characteristics of the molecules can be measured as is the case of the diffusion coefficient associated with each subclass of lipoprotein. From the diffusion coefficients, the sizes of different subclasses of lipoproteins are directly calculated through the Stokes-Einstein equation. The direct measurement of the size, as in this method, is of particular importance since it is used to calculate the number of lipoprotein particles. The methyl signal was surface-fitted with 9 Lorentzian functions associated with each lipoprotein subtype. The area of each Lorentzian function reflected the lipid concentration of each lipoprotein subtype, and the size of each subtype was calculated from the diffusion coefficient. The particle number of each lipoprotein subtype was calculated by dividing the lipid volume by the particle volume of a given class. Lipid volumes were determined using common conversion factors to convert concentration units into volume units. The variation coefficients for particle number were between 2% and 4%. The variation coefficients for particle size were lower than 0.3% [15].