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Cholesterol oversynthesis markers define familial combined hyperlipidemia *versus* other genetic hypercholesterolemias independently of body weight☆

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Abstract

Primary hypercholesterolemia of genetic origin, negative for mutations in *LDLR, APOB, PCSK9* and *APOE* genes (non-FH GH), and familial combined hyperlipidemia (FCHL) are polygenic genetic diseases that occur with hypercholesterolemia, and both share a very high cardiovascular risk. In order to better characterize the metabolic abnormalities associated with these primary hypercholesterolemias, we used noncholesterol sterols, as markers of cholesterol metabolism, to determine their potential differences. Hepatic cholesterol synthesis markers (desmosterol and lanosterol) and intestinal cholesterol absorption markers (sitosterol and campesterol) were determined in non-FH GH (n=200), FCHL (n=100) and genetically defined heterozygous familial hypercholesterolemia subjects (FH) (n=100) and in normolipidemic controls (n=100). FCHL subjects had lower cholesterol absorption and higher cholesterol synthesis than non-FH GH, FH and controls (P<.001). When noncholesterol sterols were adjusted by body mass index (BMI), FCHL subjects had higher cholesterol synthesis than non-FG GH, FH and controls (P<.001). An increase in BMI was accompanied by increased cholesterol synthesis and decreased cholesterol absorption in non-FH GH, FH and controls. However, this association between BMI and cholesterol synthesis was not observed in FCHL. Non-high-density-lipoprotein cholesterol showed a positive correlation with cholesterol synthesis markers similar to that of BMI in non-FH GH, FH and normolipemic controls, but there was no correlation in FCHL. These results suggest that FCHL and non-FH GH have different mechanisms of production. Cholesterol synthesis and absorption are dependent of BMI in non-FH GH, but cholesterol synthesis is increased as a pathogenic mechanism in FCHL independently of age, gender, *APOE* and BMI.

Keywords: Cholesterol synthesis; Noncholesterol sterols; Genetic hypercholesterolemias; Familial combined hyperlipidemia; Body mass index

1. Introduction

Many primary hypercholesterolemias of genetic origin, negative for mutations in *LDLR, APOB, PCSK9* and *APOE* genes, have a clear familial component mimicking a monogenic defect [1–3]. These types of hypercholesterolemias without genetic defects causing familial hypercholesterolemia (FH) can be divided into non-FH autosomal dominant hypercholesterolemias, when triglycerides (TGs) are within the normal range [4], and familial combined hyperlipidemia (FCHL), when the proband or one family member presents mixed hyperlipidemia [5,6]. However, extensive studies in these two phenotypes have clearly demonstrated that most of these cases are not due to a monogenic defect; in fact, familial segregation analysis and single nucleotide variations associated with low-density lipoprotein (LDL) cholesterol and exome analyses show that the genetic component is mainly polygenic with important interaction with environmental factors [7–10]. Absence of monogenic defects led us to denominate the former phenotype as non-FH genetic hypercholesterolemia (non-FH GH) [9]. Environmental interactions are especially relevant for FCHL, and it has been suggested that this disease is the simple association of a polygenic hypercholesterolemia with overweight or obesity [11]. Actually, some single nucleotide variations associated with LDL cholesterol in the population have been found in a higher frequency in FCHL [10]. Therefore, at the present time, it is not known if non-FH GH and FCHL are the same genetic entity only differentiated by the type of diet and the body weight.

Hypercholesterolemia mechanisms in individual patients and atherosclerosis risk can be studied throughout the cholesterol synthesis and cholesterol absorption measurement. Furthermore, cholesterol is also catabolized to produce steroid hormones and bile acids. Bile acid formation is quantitatively the most important. Bile acids serve to keep biliary cholesterol dissolved in the bile and to

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support transit of dietary cholesterol and fat through the small intestine [12]. Bile acids are also involved in the regulation of their own synthesis and that of glucose and lipid metabolism [13,14]. The direct assessment of cholesterol absorption, synthesis and catabolism is very laborious and unpractical to be used in clinical studies. Therefore, alternative procedures have been developed based on the measurement of surrogate markers in fasting serum that reflect cholesterol absorption, cholesterol synthesis and bile acid synthesis. Plant sterols have been shown to use similar intestinal transport system for absorption as cholesterol [15]. For over 25 years, the fasting serum levels of campesterol and sitosterol corrected for total cholesterol levels have been used to monitor the daily cholesterol absorption rate [16–20]. Also, serum cholestanol, the 5α-saturated derivative of cholesterol, acts as a surrogate marker for cholesterol absorption [21]. The cholesterol precursor desmosterol is an established marker of cholesterol synthesis [17]. Serum 7α-hydroxycholesterol or its follow-up product 7α-hydroxy-4-cholesten-3-one (C4) has been established as surrogate markers for bile acid synthesis [22–24] and has been shown to correlate to bile acid synthesis measurement performed with stable isotopes [25]. Bile acid synthesis is known to contain a neutral and major pathway based on cholesterol 7α-hydroxylation as the first and rate-limiting step as well as an acidic and minor pathway based on cholesterol 27-hydroxylation as the initial step. Serum 27-hydroxycholesterol has not been investigated as a marker to date.

Previous studies have shown that non-FH GH is characterized by higher cholesterol intestinal absorption [26,27] and FCHL by increased hepatic cholesterol synthesis [28,29]. However, there is a strong association between hepatic cholesterol synthesis and obesity, and cholesterol oversynthesis is one of the mechanisms found in the dyslipidemia of the metabolic syndrome [30] and type 2 diabetes [31], which is frequently associated to FCHL [32]. Due to the fact that cholesterol synthesis and cholesterol absorption are inversely correlated [19], it could be possible that previous data were influenced by this inversed association.

To establish if non-FH GH and FCHL have different production mechanisms, we have studied in a large group of well-characterized non-FH GH and FCHL subjects their hepatic cholesterol synthesis and intestinal cholesterol absorption, and we have analyzed their interaction with body weight. For comparison, we have also studied two control groups composed by normolipidemic subjects and subjects with genetically defined heterozygous FH.

2. Methods

2.1. Study population

Hypercholesterolemic subjects (n=400) from the Lipid Clinic at Hospital Universitario Miguel Servet, Zaragoza, Spain, were selected. They were unrelated adults 18–79 years old with the clinical and genetic diagnosis of FH (n=100), non-FH GH (n=200) and FCHL (n=100). FH was defined as the presence of LDL cholesterol levels above the 95th percentile of the Spanish population [33], TGs below 200 mg/dl, familial presentation (at least one first-degree relative with the same phenotype) and heterozygous for a functional mutation in *LDLR, APOB, PCSK9 or APOE* genes; non-FH GH was defined with the same criteria as FH but in the absence of causative mutation; FCHL was defined as the presence of total cholesterol and TG concentrations above the sex- and age-specific 90th percentiles, apolipoprotein (apo) B levels ≥120 mg/dl and at least one first-degree relative with hyperlipidemia (total cholesterol and/or TGs >90th percentile). Exclusion criteria for these three dyslipidemias were as follows: secondary causes of hypercholesterolemia including severe obesity [body mass index (BMI) >35 kg/m^2], poorly controlled type 2 diabetes (hemoglobin A1c >8%), renal disease with glomerular filtration rate <30 ml/min and/or macroalbuminuria, liver diseases [alanine transferase (ALT) >3 times upper normal limit], hypothyroidism (thyroid-stimulating hormone >6 mIU/L), pregnancy, autoimmune diseases and protease inhibitors consumption. Subjects disclosing *APOE* ε2/ε2 genotype were also excluded from this study. Subjects with previous cardiovascular disease or high risk for cardiovascular disease (>20% in the next 10 years) were excluded except if they were not on lipid-lowering drugs. In all subjects with non-FH GH and FCHL, the presence of functional mutations in *LDLR*, *APOB* and *PCSK9* and p.(Leu167del) in *APOE* genes was ruled out by DNA sequencing as previously described [34].

The normolipemic group (n=100) consisted of healthy, unrelated men and women volunteers aged 18–79 years who underwent a medical examination at the Hospital Miguel Servet of Zaragoza. Exclusion criteria for control subjects were personal or parental history of premature cardiovascular disease or dyslipidemia, current acute illness, or use of drugs that might influence glucose or lipid metabolism.

Cardiovascular risk factors assessment, personal and family history of cardiovascular disease, consumption of drugs affecting intestinal or lipid metabolism and anthropometric measurements were performed in all participants. All subjects signed informed consent to a protocol previously approved by our local ethical committee (Comité Ético de Investigación Clínica de Aragón, Zaragoza, Spain).

2.2. Clinical and laboratory parameters

Fasting blood for biochemical profiles was drawn after at least 5–6 weeks without hypolipidemic drug treatment, plant sterols or fish oil supplements. Cholesterol and TGs were determined by standard enzymatic methods. High-density lipoprotein (HDL) cholesterol was measured by a direct method. Apo A1, apo B, lipoprotein(a) and C-reactive protein were determined by immunonephelometry using IMMAGE-Immunochemistry System (Beckman Coulter).

DNA was isolated from EDTA blood samples following standard protocols. *APOE* sequencing was performed in all study subjects as previously described [35]. The screening for *LDLR* and *APOB* mutations was carried out using Lipochip Platform (Progenika Biopharma S.A., Bilbao, Spain). The platform consists of two consecutive steps: the first one is the LIPOchip1 microarray analysis for the detection of the most frequent Spanish point mutations in the *LDLR* gene and in the *APOB* exon 26, as well as CNVs in *LDLR*. When the LIPOchip1 microarray gives a negative result (no mutation is found), the *LDLR*, *APOB* (binding domain) and *PCSK9* gene coding sequences; exon–intron boundaries; and short proximal intronic sequences were sequenced with a GS Junior system (Roche Diagnostics Corporation, Basel, Switzerland) [36].

2.3. Serum sterol determinations

Serum concentrations of phytosterols and cholestanol, some surrogate markers of cholesterol absorption, cholesterol synthesis precursors, desmosterol and lanosterol, the cholesterol metabolite 27-hydroxycholesterol and cholesterol were quantified after 10 h of fasting. Subjects were without lipid-lowering drugs or phytosterol supplements at least 5 weeks before blood extraction. Serum concentrations of all sterols were quantified using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) according to the method previously described [37] and were expressed as mg/dl, as well as normalized to mg/dl of total cholesterol. Briefly, 100 μl of serum was transferred to a screw-capped vial, and deuterium-labeled internal standard (^{2}H6) cholesterol-26,26,26,27,27,27 (7.9 mM) was added to determine noncholesterol sterols. Another 100 μl of serum was transferred to a screw-capped vial, and deuterium-labeled internal standard (^{2}H7) cholesterol-25,26,26,26,27,27,27 was added to determine cholesterol. Alkaline hydrolysis was performed for 20 min at 60°C in an ultrasound bath and extracted twice with 3 ml of hexane. Extracts were loaded onto the SPE cartridge (1 mg, Discovery DSC-18, Supelco, Spain),