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# Cosegregation of serum cholesterol with cholesterol intestinal absorption markers in families with primary hypercholesterolemia without mutations in *LDLR*, *APOB*, *PCSK9* and *APOE* genes

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ABSTRACT

*Background and aim:* The genetic cause and pathogenic mechanism of approximately 20–40% of autosomal dominant hypercholesterolemias (ADH) are unknown. Increased cholesterol intestinal absorption has been associated to ADH. If this variation contributes to their pathogenesis is unknown.
*Methods and results:* We studied cholesterol absorption (phytosterols and cholestanol serum concentrations) and cholesterol synthesis (desmosterol serum concentration) in 20 families with ADH without causal mutations in *LDLR*, *APOB*, *PCSK9* or *APOE* genes (non-FH ADH) selected from 54 non-FH ADH probands with (non-cholesterol sterol concentrations above 75th percentile) and without (under 75th percentile) hyperabsorption. The concentrations of cholestanol, sitosterol, campesterol and stigmasterol were higher in affected than in non-affected subjects ($p = 0.003$, <0.001.<0.001, 0.002, respectively). There was a strong cosegregation of hyperabsorption with high LDL cholesterol within hyperabsorber families with odds ratio 6.80 (confidence interval 1.656–27.9), $p = 0.008$. In hyperabsorber families, 60.5% of subjects were hyperabsorbers and 76% of them had high LDL cholesterol versus 38.3% and 63% in non-hyperabsorber families, respectively.
*Conclusion:* Most hypercholesterolemic family members with a hyperabsorber proband are hyperabsorbers. These absorption markers are significantly and positively associated with LDL cholesterol, and predispose to high LDL cholesterol in family members. Our data suggest that complex interindividual variation in cholesterol absorption is involved in many non-FH ADH.


## 1. Introduction

Autosomal dominant hypercholesterolemias (ADH) are characterized by high levels of low-density lipoprotein (LDL) cholesterol, familial presentation and high risk of premature cardiovascular disease [1]. Most ADH have familial hypercholesterolemia (FH) due to mutations in the *LDLR* gene that encodes for the LDL receptor [2]. Approximately 2–15% of ADH subjects have familial defective apolipoprotein B-100 (FDB) due to mutations in the LDL receptor-binding domain coding region of the *APOB* gene, which encodes for apolipoprotein B-100 [3], or mutations in proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*), a protein involved in the LDL receptor recycling [4]. Recently, a mutation in *APOE* (p.Leu167del) has been also associated with ADH [5,6]. Patients with mutations in these genes present an indistinguishable phenotype and are now included in the FH definition [2]. The genetic cause and pathogenic mechanism of approximately 20–40% of ADH, named in short as non-FH ADH, are unknown [7,8], and probably they are a heterogeneous group of diseases including some severe polygenic hypercholesterolemias [9].

Cholesterol concentration in plasma depends on the amount of cholesterol from the diet and its intestinal absorption, on *de novo*

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synthesis, and on its biliary excretion [10]. Previous studies have reported increased intestinal cholesterol absorption in non-FH ADH subjects that may partially explain plasma hypercholesterolemia in these subjects [11,12]. However, no familial cosegregation studies have been performed to study the linkage between hyperabsorption and high LDL cholesterol in non-FH ADH families.

Normal serum contains small but detectable amounts of non-cholesterol sterols, including plant sterols, also named phytosterols, and cholestanol, and their ratios to cholesterol are accepted surrogate markers for the efficiency of cholesterol intestinal absorption [13,14].

Efficiency of cholesterol intestinal absorption is a partly inherited phenomenon. Heredity of cholesterol absorption has been demonstrated in siblings of hypercholesterolemic probands with low and high serum cholestanol to cholesterol ratio [15].

Considering that some cases of non-FH ADH are associated with cholesterol intestinal hyperabsorption, the aim of our work was to determine if the efficiency of intestinal cholesterol absorption, measured by non-cholesterol sterol surrogate markers, cosegregates with LDL cholesterol concentration in non-FH ADH families.

## 2. Materials and methods

### 2.1. Probands

Selected subjects (n = 54) were unrelated adults 18–79 years of age with the clinical diagnosis of ADH: LDL cholesterol above the 95th percentile of the Spanish population [16], triglycerides below 200 mg/dL, primary cause, and familial presentation (at least one first-degree relative with the same phenotype) from the Lipid Clinic at Hospital Universitario Miguel Servet, Zaragoza, Spain. In all subjects, the presence of functional mutations in *LDLR*, *APOB* and *PCSK9*, and p.Leu167del in *APOE* were ruled out by DNA sequencing as previously described [5,7]. Secondary causes of hypercholesterolemia including: obesity (body mass index > 30 kg/m$^2$), poorly controlled type 2 diabetes (HbA1c > 8%), renal disease with glomerular filtration rate <30 mL/min and/or macroalbuminuria, liver diseases (ALT > 3 times upper normal limit), hypothyroidism (TSH > 6 mIU/L), pregnancy, autoimmune diseases and protease inhibitors were exclusion criteria. Subjects disclosing *APOE* ε2/ε2 genotype were not considered for 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. 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. Dietary intake was determined by interview with one single nutritionist dietitian. In this interview, a Spanish validated 137-item food frequency questionnaire (FFQ) was used [17].

### 2.2. Biochemistry determinations

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 triglycerides were determined by standard enzymatic methods. HDL cholesterol was measured by a precipitation technique. Apo A1, apo B and lipoprotein (a) were determined by nephelometry using IMMAGE-Immunochemistry System (Beckman Coulter). LDL cholesterol was calculated using Friedewald's formula.

### 2.3. Intestinal absorption and synthesis markers

Serum phytosterols and cholestanol, all of them markers of cholesterol absorption, 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 concentration of cholesterol, sitosterol, campesterol, stigmasterol, cholestanol and desmosterol were quantified using HPLC-MS/MS according to the method previously described [18], and were expressed as mg/dL as well as normalized to mg/dL of total cholesterol. Briefly, 100 μl of serum were 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 non-cholesterol sterols. Another 100 μl of serum were transferred to a screw-capped vial, 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. The extracts were loaded onto the SPE cartridge (1 mg, Discovery DSC-18, Supelco, Spain) which was preconditioned with 400 μl of methanol and gravity eluted. The non-cholesterol sterols and cholesterol were desorbed with 1.4 ml of 2-propanol by gravity and 40 μl of the final mixtures were injected into the HPLC-MS/MS system.

### 2.4. Definition of cholesterol intestinal hyperabsorption

We defined as hyperabsorber those subjects that showed ≥3 intestinal non-cholesterol sterols >75th percentile of the distribution in normolipidemic population. Subjects with serum phytosterol and cholestanol concentrations under 75th percentile were considered as non-hyperabsorbers. One hundred normolipidemic subjects (LDL cholesterol under the 75th percentile and triglycerides <200 mg/dL) were used to determine the normal non-cholesterol sterol distribution in our population. This group consisted of healthy, unrelated men and women volunteers aged 18–79 years, who underwent a medical examination at the Hospital Universitario Miguel Servet of Zaragoza. Exclusion criteria for normolipemic 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.

### 2.5. Family studies

Available family members of all hyperabsorber probands and the same number of families with a non-hyperabsorber proband were studied for cosegregation analysis. Clinical, biochemical, and non-cholesterol sterol analyses were performed in family members as in probands, except for genetic studies that were not made in family members. The same exclusion criteria and hyperabsorption definition were used in family members as were used in probands. Family members were considered affected if LDL cholesterol was above 90$^{th}$ percentile of the Spanish distribution in absence of secondary causes.

All subjects: non-FH ADH probands, normolipemic controls, and family members signed informed consent to a protocol previously approved by our local ethical committee (Comité Ético de Investigación Clínica de Aragón, Zaragoza, Spain).

### 2.6. Statistical analyses

Comparison of lipid variables among groups was performed using the Student's t test for data normally distributed and Mann–Whitney U test for skewed data. When significant differences were detected, multiple comparisons were made by using the