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Bile acid synthesis precursors in familial combined hyperlipidemia: The oxysterols 24S-hydroxycholesterol and 27-hydroxycholesterol

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ABSTRACT

Familial combined hyperlipidemia (FCHL), the most common inherited disorder of lipid metabolism is characterized by increasing cholesterol synthesis precursors due to hepatic overproduction of cholesterol. The bile acids synthesis pathway has not been previously studied in FCHL. The aim of this work was to study the oxysterol levels which are involved in the bile acids synthesis from cholesterol in FCHL. Clinical parameters and subclinical atherosclerosis were studied in a total of 107 FCHL patients and 126 normolipidemic controls. Non cholesterol sterols (desmosterol and lanosterol) and oxysterols (27-hydroxycholesterol and 24S-hydroxycholesterol) were measured by high performance liquid chromatography tandem mass spectrometry. Desmosterol and lanosterol, markers of cholesterol synthesis, had a positive correlation with BMI and apo B. However, no correlation was found for 24S-hydroxycholesterol and 27-hydroxycholesterol, precursors of bile acids, with these clinical parameters. Only 27-hydroxycholesterol had a positive correlation with apo B, $\rho = 0.204$ ($P = 0.037$). All oxysterol levels were higher in FHCL as compared to normal controls. A total of 59 FCHL subjects (59%) presented values of 24S-hydroxycholesterol above the 95th percentile of this oxysterol in the control population. All oxysterols showed no association with fat mass in contrast with non-cholesterol sterols. FCHL subjects with oxysterol overproduction had less carotid intima media thickness (cIMT), which suggests less atherosclerosis in these subjects. In summary, our data indicate that high oxysterol levels might be good markers of FCHL, unrelated to fat mass, and may exert a protective mechanism for cholesterol accumulation.

1. Introduction

Familial combined hyperlipidemia (FCHL), the most common inherited disorder of lipid metabolism [1,2], is characterized by elevated very low density lipoprotein (VLDL) and/or low-density lipoprotein (LDL) concentrations [3]. Other common features of FCHL are reduced high-density lipoprotein cholesterol (HDL-c), elevated apolipoprotein B (apo B) concentrations, and a preponderance of atherogenic, small dense LDL (sdLDL) subfractions [4]. FCHL is highly atherogenic being frequently found in patients with premature coronary artery disease CAD [5]. A common mechanism underlying FCHL is a hepatic overproduction of apoB-100 containing lipoprotein particles, although FCHL is a complex and heterogeneous disease and other pathogenic mechanisms are also involved [6].

Cholesterol homeostasis is achieved through a highly sophisticated regulation of the uptake, synthesis, esterification and biliary excretion of cholesterol and its derivates in the body [7]. Bile acids synthesis plays a crucial role in the intestinal cholesterol excretion [8]. Hepatic cholesterol induces the enzyme cholesterol 7α-hydroxylase via the nuclear receptors: liver X receptor (LXR), liver receptor homologue (LRH) and the hepatocyte nuclear factor, HNF4α to produce and secrete bile acids in mice [9]. The ligands for LXR are compounds of the pathways from cholesterol to bile acids – 24-, 25- and 27-hydroxycholesterol [10] and 3β-hydroxy-5-cholestenoic acid [11]. However, it is not yet clear what effects can be expected in man from an accumulation of, or deficiency of, these intermediates [10], and they have not been explored so far in subjects with FCHL.

Serum non-cholesterol sterol analysis is an useful method for the evaluation of cholesterol metabolism and a well accepted method for the diagnosis of many inherited disorders in its metabolism in humans [12]. Plasma levels of the cholesterol precursors, as desmosterol or lanosterol, served as markers of cholesterol synthesis [13,14], whereas 24S-hydroxycholesterol and 27-hydroxycholesterol served as markers of bile acids synthesis in the alternative pathway [15].

In order to better characterize the metabolic abnormalities associated with FCHL we have studied non-cholesterol sterol

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markers of cholesterol and bile acid hepatic synthesis in a large group of subjects with FCHL and normolipemic controls, in whom major confounding factors for plasma non-cholesterol sterol were studied. In addition, the non cholesterol sterol profile was analyzed with a new and more sensitive and reliable method by High Performance Liquid Chromatography–Atmospheric Pressure Chemical Ionization–Mass Spectrometry (HPLC–APCI–MS/MS) [16].

2. Methods

2.1. Study population

Eligible subjects were unrelated adults 18–79 years of age with the clinical diagnosis of FCHL from the Lipic Clinic at Hospital Universitario Miguel Servet from Zaragoza, Spain. Diagnosis of FCHL was based on the presence of primary combined hyperlipidaemia in untreated patients whose serum cholesterol and triglyceride concentrations were above the sex- and age-specific 90th percentiles for the Spanish population, serum total apolipoprotein B levels $\geqslant$120 mg/dL and at least one first-degree relative with hyperlipidemia (total cholesterol (TC) and/or triglycerides >90th percentile). Subjects disclosing apoE2/E2 genotype or any functional mutation in the *LDLR* or *APOB* genes were not considered for this study. The normolipemic group 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 dyslipidaemia, current acute illness, or use of drugs that might influence glucose or lipid metabolism. 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 dietary assessment

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 registered dietician. In this interview, a Spanish validated 137-item food frequency questionnaire (FFQ) was used [17]. The questionnaire included the consumption frequency of each of the 137 foods by choosing between nine possibilities of frequency (from never or less than once per month to six or more times per day) and the portion size. The total energy and nutrient intakes were calculated based on previously validated Spanish food composition tables [18,19].

2.3. Blood analyses

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-c was measured by a precipitation technique. Apo A1, apo B, lipoprotein (a) and C-reactive protein (CRP) were determined by nephelometry using IMMAGE-Immunochemistry System (Beckman Coulter).

DNA was isolated from EDTA blood samples following standard protocols. *APOE* genotyping was performed in all study subjects as previously described [20]. The DNA of individuals with a clinical diagnosis of FCHL was screened for *LDLR* and *APOB* mutations using Lipochip version 4.0 (Progenika Biopharma S. A., Bilbao, Spain), which is a microarray designed to detect the 204 more prevalent *LDLR* and *APOB* mutations in the Spanish population [21]. Large rearrangements in the *LDLR* gene were analyzed using a method based on quantitative fluorescent multiplex PCR [22]. When no mutations were detected, the *LDLR* gene coding sequences, exon–intron boundaries, and short proximal intronic sequences were sequenced with a GS Junior system (Roche Diagnostics Corporation, Basel, Switzerland).

2.4. Ultrasonographic evaluation

Ultrasonographic evaluation for measurement of carotid intima media thickness (cIMT) was performed according to standardized scan- and image analysis protocols, as has been previously described [23]. In brief, we bilaterally assessed cIMT of the posterior walls of distal centimeter of the arterial segments proximal to the carotid dilatation, the common carotid arterial segment (CC) and the segments between the dilatation and the flow divider, the carotid bulb (CB). Images were collected with an Acuson Sequoia 512 ultrasound scanner equipped with an 8L5 transducer (Siemens AG, Erlangen, Germany) of each arterial segment. Of every carotid arterial segment there was obtained the mean (average) IMT as well as the maximum IMT value of the arterial wall segment. The per subject aggregate of the right and left CC and CB mean and maximum IMT values were used for calculating subject mean and maximum IMT aggregate (mean and maximum cIMT, respectively).

2.5. Measurements of serum sterols

Serum concentration of lanosterol, desmosterol, 24S-hydroxycholesterol and 27-hydroxycholesterol were measured using High Performance Liquid Chromatography tandem Mass Spectrometry (HPLC–MS/MS) according to the method previously described [16] in all subjects, 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 6 μl of deuterium-labeled internal standard, [^{2}H6] cholesterol-26,26,26,27,27,27, (7.9 mM) was added. Alkaline hydrolysis was performed for 20 min at 60 °C in an ultrasound bath. The sample was extracted twice with 3 ml of hexane. The extract was loaded onto the SPE cartridge (1 mg, Discovery DSC-18, Supelco, Spain) which was preconditioned with 400 μl of methanol and gravity eluted with. The non-cholesterol sterols were desorbed with 1.4 ml of 2-propanol by gravity and 40 μl of the final mixture was injected into the HPLC–MS/MS system.

2.6. Statistical analysis

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. Non-cholesterol sterol to cholesterol ratios were log transformed to achieve variance homogeneity. To detect group differences in non-cholesterol sterol ratios, an analysis of covariance was performed considering log-transformed values as dependent variables, and sex, age, and body mass index (BMI) as confounding variables (covariates). The homogeneity of regression coefficients was verified for each covariate. When the omnibus test gave a significant result, the Tukey's least-significant difference *post hoc* test was used to detect significant differences in pair wise group comparisons. *APOE* genotype influence in non-cholesterol sterol ratios was specifically investigated with similar statistical tests. Analyses were performed with SPSS software (version 11.0; SPSS, Inc., Chicago, IL), with statistical significance set at $P < 0.05$.

3. Results

The characteristics of the 107 patients with FCHL and 126 normolipidemic controls are presented in Table 1. As compared with controls, patients with FCHL showed higher BMI, waist circumference, LDL cholesterol, triglycerides, apo B and lower values of HDL cholesterol and apo A1. Mean values of desmosterol and lanosterol showed statistically significant higher values in FCHL compared to controls. 24S-hydroxycholesterol and 27-hydroxycholesterol values were also significantly higher in FCHL than in normolipidemic controls.

Spearman's rank correlations for FCHL are reported in Table 2. Desmosterol and lanosterol, markers of cholesterol synthesis, had a positive correlation with BMI and apo B. However, no correlation was found for 24S-hydroxycholesterol and 27-hydroxycholesterol, precursors of bile acids, with these clinical parameters. Only 27-hydroxycholesterol had a positive correlation with apo B, $\rho = 0.204$ ($P = 0.037$).

There was positive correlation between desmosterol and lanosterol, $\rho = 0.402$ ($P = 0.001$) and between 24S-hydroxycholesterol and 27-hydroxycholesterol, $\rho = 0.768$ ($P = 0.001$). There were also positive correlations between all non-cholesterol sterols studied, but non between lanosterol and 24S-hydroxycholesterol.

Fig. 1 shows the distribution of 24S-hydroxycholesterol, 27-hydroxycholesterol desmosterol and lanosterol in FCHL and controls. Distributions of both oxysterols in FCHL were displaced to the right, indicating higher values of both oxysterols in FCHL. A total of 59 FCHL subjects (55%) presented values of 24S-hydroxycholesterol above the 95th percentile of this oxysterol in the control population. The same pattern was found for the concentration of 27-hydroxycholesterol with 37 FCHL subjects (35%) above the 95th percentile of controls. We studied clinical characteristics and subclinical atherosclerosis according to the concentration of both oxysterols (Table 3). There was a trend to lower triglycerides, non-HDL cholesterol and cIMT in subjects in the high oxysterols group. However, statistical significance only was reached for mean and maximum values of common cIMT for 27-hydroxycholesterol.

Table 1
Clinical characteristics and non-cholesterol sterols of patients with familial combined hyperlipidemia (FCHL) and normolipidemic controls.

	FCHL	Control	*p*
N	107	126	
Men, *n* (%)	63.2	38.1	<0.001
Age, years	45.7 ± 11.8	39.6 ± 16	0.001
Current smokers	36.2	18.9	<0.001
Former smokers	28.6	15.6	
Non smokers	35.2	65.6	
Systolic blood pressure (mm Hg)	130 ± 16.5	114 ± 14.3	<0.001
Diastolic blood pressure (mm Hg)	82.7 ± 10.6	73.7 ± 11.1	<0.001
Body mass index (kg/m^2)	26.3 ± 2.29	23.6 ± 3.84	<0.001
Waist circumference (cm)	93.7 ± 7.50	83.7 ± 11.57	<0.001
Total cholesterol (mg/dL)	288 (265–323)	181 (165–201)	<0.001
Triglycerides (mg/dL)	248 (198–379)	59.0 (44.0–81.0)	<0.001
HDL cholesterol (mg/dL)	44.6 ± 11.5	53.5 ± 13.0	<0.001
HDL non cholesterol (mg/dL)	245 (223–275)	130 (111–144)	<0.001
LDL cholesterol (mg/dL)	204 (177–227)	116 (101–131)	<0.001
Apolipoprotein A1 (mg/dL)	142 ± 26.8	150 ± 30.6	<0.001
Apolipoprotein B (mg/dL)	166 (147–184)	89 (78–101)	<0.001
Lipoprotein (a) (mg/dL)	25.7 (7.01–60.7)	15.5 (7.12–40.6)	0.132
C-reactive protein (mg/dL)	2.65 (1.10–4.07)	1.15 (0.50–2.40)	<0.001
Glucose (mg/dL)	92.7 ± 12.0	86.5 ± 9.8	<0.001
Apolipoprotein E, *n* (%)			0.445
E3/3	68	70.5	
E3/4	51.1	18.9	
E3/2	7.2	10.7	
Desmosterol (mg/dL)	1.9 (1.5–2.4)	0.73 (0.63–0.90)	<0.001
Lanosterol (mg/dL)	0.15 (0.10–0.23)	0.059 (0.040–0.086)	<0.001
24S-hydroxycholesterol (mg/dL)	0.034 (0.022–0.043)	0.011 (0.0091–0.014)	<0.001
27-Hydroxycholesterol (mg/dL)	0.039 (0.030–0.059)	0.017 (0.015–1.021)	<0.001
Desmosterol*10^3/mg/dL cholesterol	6.5 (5.1–8.3)	4.0 (3.5–4.7)	<0.001
Lanosterol*10^3/mg/dL cholesterol	0.54 (0.36–0.78)	0.33 (0.23–0.48)	<0.001
24S-hydroxycholesterol*10^4/mg/dL cholesterol	1.2 (0.73–1.6)	0.62 (0.51–0.76)	<0.001
27-Hydroxycholesterol*10^4/mg/dL cholesterol	1.3 (1.0–1.8)	0.96 (0.80–1.1)	<0.001

Values are mean (SD) or median (interquartile range).

4. Discussion

Our findings strongly indicate that patients with FCHL are prone to use an alternative pathway of bile acids synthesis from cholesterol via 27-hydroxycholesterol and 24S-hydroxycholesterol. This is an exploration of bile acid synthesis precursors in a large number of subjects with well clinically defined FCHL. In our study we also confirm the increased levels of desmosterol and lanosterol in FCHL [24]. Interestingly, cholesterol synthesis precursors were correlated with plasma apo B and BMI, however bile acid precursors were unrelated to BMI and positively associated with apo B. Elevated levels of oxysterols could be a good marker of the primary lipid abnormality associated to FCHL and suggests that FCHL could be, at least in part, the result of an imbalance in the cholesterol excretion and bile acid synthesis pathways.

Fecal elimination of bile acids and their metabolites represent a key event in cholesterol homeostasis, because it is the main mechanism of cholesterol excretion in humans [25]. Increased bile acid synthesis significantly affect circulating cholesterol levels [26,27]. The main bile acid biosynthesis pathway, also named neutral pathway, takes place exclusively in the liver, resulting in the production of 7α-hydroxycholestrol, and cholesterol 7α-hydroxylase (CYP7A1) is the limiting enzyme of this mechanism [28]. The alternative pathway of bile acid synthesis is also likely to play a relevant role in cholesterol metabolism even if its quantitative contribution has not been defined so far neither its physiological role in humans [29,30]. In this study we showed that, compared to normolipidemic controls, FCHL patients exhibit large increases in serum levels of both oxysterols involved in the alternative pathway of bile acid synthesis, 24S-hydroxycholesterol and 27-hydroxycholesterol. If

Table 2
Spearman's correlation coefficients in FCHL patients.

	Desmosterol		Lanosterol		24S-hydroxycholesterol		27-Hydroxycholesterol	
	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*
Total cholesterol	−0.142	0.147	−0.015	0.878	−0.139	0.162	−0.058	0.559
Body mass index	0.219	0.025	0.203	0.038	0.014	0.892	0.078	0.427
Triglycerides	−0.130	0.188	0.039	0.696	−0.080	0.420	−0.101	0.304
HDL-cholesterol	0.084	0.394	−0.130	0.185	−0.164	0.099	−0.152	0.121
Non HDL-cholesterol	−0.160	0.102	0.029	0.769	−0.125	0.207	−0.032	0.745
Apolipoprotein B	0.196	0.045	0.134	0.173	0.098	0.326	0.204	0.037

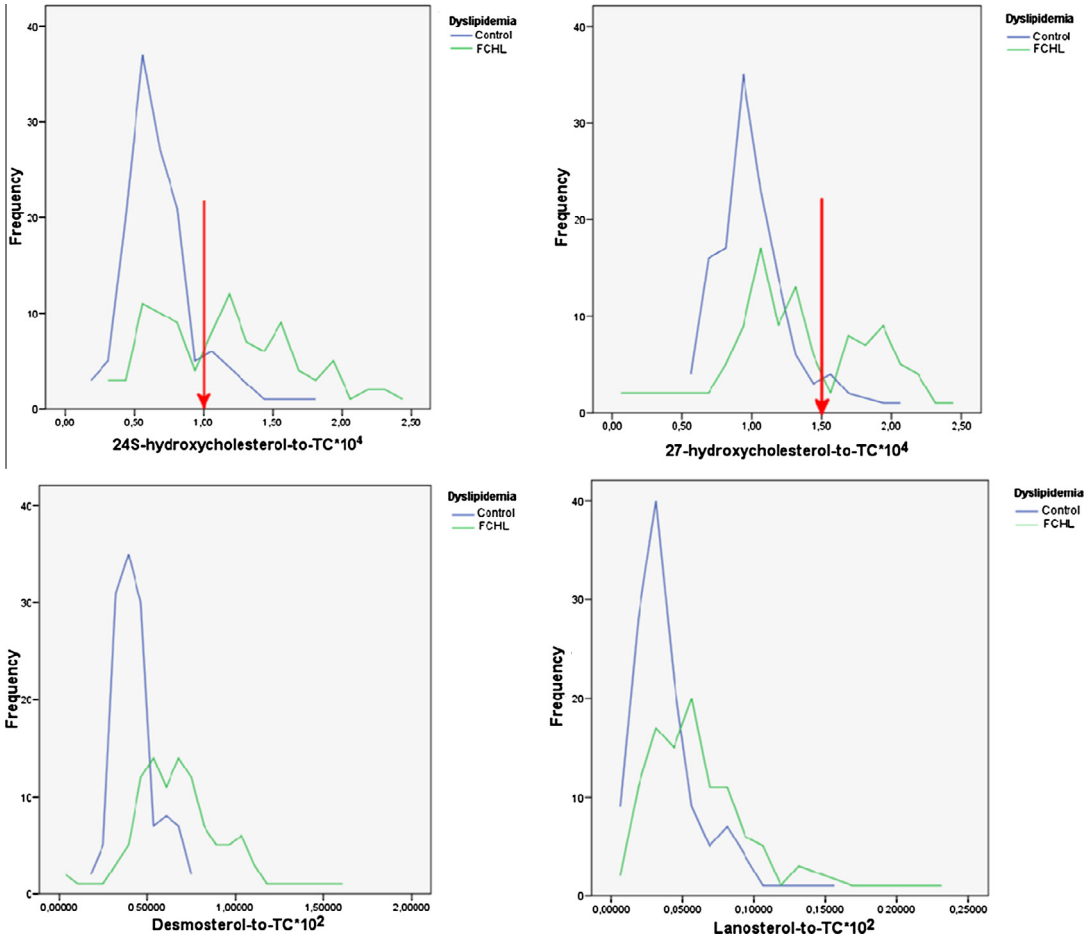

Fig. 1. 24S-hydroxycholesterol-to-TC, 27-hydroxycholesterol-to-TC, desmosterol-to-TC and lanosterol-to-TC distribution in FCHL and normolipidemic subjects. The red arrows indicate 95th percentile mean value of oxysterol in normolipidemic subjects.

Table 3
Clinical characteristics of FCHL subjects with values of oxysterols above and below the 95th percentile in the control population.

	24S-hydroxycholesterol <95th percentile	24S-hydroxycholesterol >95th percentile	*p*	27-Hydroxycholesterol <95th percentile	27-Hydroxycholesterol >95th percentile	*p*
N	41	59		65	37	
Men, *n* (%)	68	58	0.284	65	57	0.437
Age, years	46.6 ± 12	45.2 ± 11.7	0.548	45.9 ± 12.2	45.5 ± 11.1	0.864
Body mass index (kg/m$^{2)}$	26.7 ± 1.76	26.3 ± 2.61	0.397	26.4 ± 1.76	26.3 ± 2.64	0.808
Waist circumference (cm)	95.2 ± 6.29	92.9 ± 7.99	0.121	92.1 ± 7.86	93.1 ± 6.79	0.573
Triglycerides (mg/dL)	250 (202–381)	239 (192–377)	0.486	259 (198–383)	221 (195–360)	0.404
HDL cholesterol (mg/dL)	46.0 ± 8.88	44.2 ± 11.5	0.456	45.0 ± 10.9	44.4 ± 12.9	0.790
HDL non cholesterol (mg/dL)	257 (228–274)	239 (221–273)	0.264	247 (224–276)	235 (220–272)	0.337
Apolipoprotein B (mg/dL)	165 (148–181)	166 (147–188)	0.594	163 (149–177)	176 (147–196)	0.103
Glucose (mg/dL)	91.8 ± 13.2	93.9 ± 11.1	0.394	91.3 ± 11.4	95.8 ± 12.2	0.065
Gamma-glutamyl transpeptidase (U/L)	29 (19–45)	29 (19–46)	0.925	27 (18–43)	31 (19–51)	0.392
Glutamic-pyruvic transaminase (U/L)	25 (20–30)	25 (20–32)	0.861	25 (19–36)	25 (21–32)	0.775
Maximum cIMT (mm)	0.86 ± 0.12	0.82 ± 0.15	0.198	0.87 ± 0.14	0.80 ± 0.13	0.032
Mean cIMT (mm)	0.72 ± 0.11	0.70 ± 0.13	0.415	0.73 ± 0.13	0.68 ± 0.10	0.048
Desmosterol$*10^3$/mg/dL cholesterol	5.89 (4.83–6.67)	7.58 (5.85–10.0)	<0.001	6.09 (4.89–7.00)	8.56 (7.18–10.2)	<0.001
Lanosterol$*10^3$/mg/dL cholesterol	0.524 (0.323–0.765)	0.545 (0.356–0.805)	0.651	0.497 (0.306–0.723)	0.58 (0.381–0.903)	0.044

Values are mean (SD) or median (interquartile range).

this increase is a primary defect or a compensatory mechanism associated to overproduction of cholesterol cannot be concluded from our study.

The neutral pathway for bile acid synthesis is confined to the liver, but the alternative pathway can also occur in other tissues. Indeed, the formation of 24S-hydroxycholesterol in the brain, and

27-hydroxycholesterol in the lung and in macrophages, represent a mechanism for cholesterol removal from these tissues back to the liver [31,32], and has been associated with reverse cholesterol transport and protection from atherosclerosis [33]. It could be hypothesized that the alternative pathway in hepatic and extrahepatic cells is increased in the presence of cholesterol overload to eliminate cholesterol into bile acids and blood respectively. Our carotid atherosclerosis data clearly show a tendency of lower subclinical vascular lesions in the present of high 24S-hydroxycholesterol and 27-hydroxycholesterol levels, what supports this concept. Obviously, much more research needs to be done to confirm this preliminary data.

Our results confirm the high cholesterol synthesis in FCHL patients previously reported by Garcia-Otin et al. [34]. Another study by van Himbergen et al. [35] demonstrated that FCHL patients have an increased synthesis of cholesterol when compared to their normolipemic relatives, being this effect more prominent in women than in men. In our study, we also evidence a high correlation between cholesterol synthesis precursors and bile acid synthesis precursors not previously reported in FCHL.

Fat mass has a central role in the pathogenesis of FCHL [36]. In agreement with this concept, we found an interaction between serum cholesterol synthesis precursors and BMI. We did not find this relationship between bile acid synthesis precursors and BMI or obesity. Relation of oxysterols with fat mass has not been previously explored. We observed that a substantial proportion of FCHL subjects had an increased concentration of oxysterols with respect to normal controls. Whether oxysterols can be used as diagnostic marker in FCHL should be further explored.

Our study is an exploratory analysis and it has several limitations. Firstly, this is a preliminary study with a limited number of subjects. Secondly, the presence of scattered data makes them difficult to interpret in some subjects, since there is some overlap between cases and controls. Thirdly, we do not have a mechanistic explanation for the high levels of oxysterols. Lastly, FCHL is a heterogeneous disease and probably overproduction of oxysterols is not an universal phenomenon in these subjects.

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