Journal of Steroid Biochemistry & Molecular Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Bile acid synthesis precursors in subjects with genetic hypercholesterolemia negative for *LDLR/APOB/PCSK9/APOE* mutations. Association with lipids and carotid atherosclerosis

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 12 August 2016
Accepted 17 October 2016
Available online xxx

Keywords:
Oxysterols
24(*S*)-Hydroxycholesterol
27-Hydroxycholesterol
Bile acid synthesis
Cholesterol metabolism
Hypercholesterolemia
Carotid atherosclerosis

ABSTRACT

Some oxysterols are precursors of bile acid synthesis and play an important role in cholesterol homeostasis. However, if they are involved in the pathogeny of genetic hypercholesterolemia has not been previously explored. We have studied non-cholesterol sterol markers of cholesterol synthesis (lanosterol and desmosterol) and oxysterols (7α-hydroxy-4-cholesten-3-one, 24S-hydroxycholesterol and 27-hydroxycholesterol) in 200 affected subjects with primary hypercholesterolemia of genetic origin, negative for mutations in *LDLR*, *APOB*, *PCSK9* and *APOE* genes (non-FH GH) and 100 normolipemic controls. All studied oxysterols and cholesterol synthesis markers were significantly higher in affected subjects than controls ($P < 0.001$). Ratios of oxysterols to total cholesterol were higher in non-FH GH than in controls, although only 24S-hydroxycholesterol showed statistical significance ($P < 0.001$). Cholesterol synthesis markers had a positive correlation with BMI, triglycerides, cholesterol and apoB in control population. However, these correlations disappeared in non-FH GH with the exception of a weak positive correlation for non-HDL cholesterol and apoB. The same pattern was observed for oxysterols with high positive correlation in controls and absence of correlation for non-FH GH, except non-HDL cholesterol for 24S-hydroxycholesterol and 27-hydroxycholesterol and apoB for 27-hydroxycholesterol. All non-cholesterol sterols had positive correlation among them in patients and in controls. A total of 65 (32.5%) and 35 (17.5%) affected subjects presented values of oxysterols ratios to total cholesterol above the 95th percentile of the normal distribution (24S-hydroxycholesterol and 27-hydroxycholesterol, respectively). Those patients with the highest levels of 24S-hydroxycholesterol associated an increase in the carotid intima media thickness. These results suggest that bile acid metabolism is affected in some patients with primary hypercholesterolemia of genetic origin, negative for mutations in the candidate genes, and may confer a higher cardiovascular risk. Our results confirm that cholesterol synthesis overproduction is a primary defect in non-HF GH and suggest that subjects with non-FH GH show high levels of oxysterols in response to hepatic overproduction of cholesterol.

Contents

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http://dx.doi.org/10.1016/j.jsbmb.2016.10.003
0960-0760/

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 has 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 also been 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 are unknown [7,8], and probably they are a heterogeneous group of diseases including some severe polygenic hypercholesterolemias [9]. For this reason, we named them as non-FH genetic hypercholesterolemias (non-FH GH).

Cholesterol homeostasis is achieved through a highly sophisticated regulation of the uptake, synthesis, esterification and biliary excretion of cholesterol and its derivatives in the body [10]. Oxysterols are oxygenated derivatives of cholesterol that are important as intermediates or end products in cholesterol excretion pathways. The rapid degradation and excretion of oxysterols are facilitated by their physical properties, allowing them to go across lipophilic membranes and to be redistributed in the cell at a much faster rate than cholesterol itself. Important roles with cholesterol turnover, atherosclerosis, apoptosis, necrosis, inflammation, immunosuppression, and the development of gallstones have been described for oxysterols [11–14]. Importantly, oxysterols mediate on cholesterol metabolism to bile acids. The liver nuclear X receptors (LXRs), the liver receptor homologue (LRH) and the hepatocyte nuclear factor (HNF4α) have the ability to bind oxysterols with high affinity [15,16] to produce and secrete bile acids in mice [17]. Important oxysterol ligands for these receptors include: 24S-, 25- and 27- hydroxycholesterol [18] and 3β-hydroxy-5-cholestenoic acid [19].

The classical and quantitatively most important pathway for bile acid synthesis starts with a 7α-hydroxylation of cholesterol via the rate-limiting hepatic cytochrome P-450 enzyme, cholesterol 7α-hydroxylase (CYP7A1) [20]. In addition to the 7α-hydroxylase pathway, there is a bile acid synthesis alternative pathway, starting with the introduction of a hydroxyl group at the terminal methyl group (C27 position) of the steroid side chain [20]. The first step involves the oxidation of cholesterol to 27-hydroxycholesterol by CYP27A1, which is subsequently hydroxylated by oxysterol 7α-hydroxylase (CYP7B1). It has been calculated that 5%–10% of the total conversion of cholesterol into bile acids starts with an extrahepatic 27-hydroxylation [21,22]. Another alternative pathway for bile acid synthesis is through oxidation of cholesterol to 24 (*S*)-hydroxycholesterol. Cholesterol 24-hydroxylase (CYP46A1) is expressed mainly in the brain. There is no synthesis of 24S-hydroxycholesterol from the human liver, hence is a bile acids precursor not related with the hepatic cholesterol synthesis [23].

Pullinger et al. described a non-FH GH kindred carring a loss-of-function mutation in the *CYP7A1* gene, encoding the cholesterol 7α-hydroxylase enzyme, which catalyzes the initial step in cholesterol catabolism and bile acid synthesis. The mutation led to high levels of LDL cholesterol, markedly deficient rate of bile acid excretion, and upregulation of the alternative bile acid pathway [24]. However, the *CYP7A1* gene has not been further associated with FH neither in linkage analysis [25] nor whole exome sequencing of patients without *LDLR/APOB/PCSK9* mutations [26]. Currently, the effect on lipids that can be expected from the accumulation or deficiency of the intermediate metabolites of bile acid synthesis is not clear [18], and it has not been explored so far in subjects with non-FH GH. Given that bile acid formation is a key metabolic pathway, that the association between oxysterols and cholesterol concentration in humans [27] and that a family with a genetic defect in bile acid formation has been described as a cause of genetic hypercholesterolemia [24], we hypothesized that bile acid precursors could be markers of some forms of non-FH GH. In order to better characterize the metabolic abnormalities associated with non-FH GH, we have studied non-cholesterol sterol markers of cholesterol and bile acid hepatic synthesis in a large group of subjects with non-FH GH and normolipemic controls, in which major confounding factors for plasma non-cholesterol sterols were studied. In addition, the non cholesterol sterol profile was analyzed with a sensitive and reliable method by high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) [28].

2. Methods

2.1. Study population

Selected subjects (n = 200) were unrelated adults 18–79 years of age with the clinical diagnosis of ADH: LDL cholesterol above the 95th percentile of the Spanish population [29], 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 as described below. 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

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<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. 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 dyslipidemia, 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. 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 cholesterol 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).

2.3. Precursors of cholesterol synthesis and cholesterol biliary secretion

Serum oxysterols (7α-hydroxy-4-cholesten-3-one, 24S-hydroxycholesterol and 27-hydroxycholesterol), precursors of different pathways of bile acid synthesis and serum cholesterol synthesis markers (desmosterol and lanosterol) were quantified after 10 h of fasting. Serum concentration of cholesterol, 7α-hydroxy-4-cholesten-3-one, 24S-hydroxycholesterol, 27-hydroxycholesterol, desmosterol and lanosterol were quantified using HPLC–MS/MS according to the method previously described [28], 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-labelled 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-labelled internal standard, [^{2}H7] cholesterol-25,26,26,26,27,27,27, was added to determine cholesterol.

For the hydrolysis of non-cholesterol sterols, the serum samples were first subjected to saponfication. After the addition of 1 ml of freshly prepared 0.71 M ethanolic sodium hydroxide, alkaline hydrolysis was performed for 20 min at 60 °C in an ultrasound bath. Hydrolysis reagents were prepared immediately prior to use. Thereafter, 500 μl of deionized water were added. The sample was extracted twice with 3 ml of hexane. The extract was dried at 30 °C under a stream of nitrogen and reconstituted in 200 μl methanol. This extract was loaded onto the SPE cartridge. The C18 cartridges used for the SPE extraction (100 mg, Discovery DSC-18, Supelco, Spain) were preconditioned with 400 μl of methanol and gravity

Table 1
Clinical and biochemical characteristics in non-FH GH patients and normolipidemic controls.*

	Non-FH GH (n = 200)	Control (n = 100)	*P*#
Female, n (%)	100 (50)	55 (55)	0.408
Age, years	45.2 ± 11.7	46.1 ± 16.6	0.606
Current Smokers, n (%)	50 (25)	27 (27)	0.532
Former Smokers, n (%)	58 (29)	24 (24)	
Non Smokers, n (%)	92 (46)	49 (49)	
Apolipoprotein E, n (%)			0.001
E3/3	133 (66.5)	70 (70.0)	
E3/4	54 (71.8)	17 (17.0)	
E3/2	3 (1.5)	9 (9.0)	
E4/4	9 (4.5)	1 (1.0)	
E2/4	1 (0.5)	3 (3.0)	
Systolic Blood Pressure, mm Hg	127 ± 17	126 ± 22	0.588
Diastolic Blood Pressure, mm Hg	80 (71–90)	80 (72–87)	0.955
Body Mass Index, kg/m^2	25.3 ± 3.5	25.8 ± 5.1	0.407
Weight, kg	70.0 ± 12.6	72.0 ± 15.0	0.232
Waist Circumference, cm	86.8 ± 10.7	88.7 ± 13.5	0.240
Cholesterol enzymatic method, mg/dL	295 ± 44	198 ± 31	<0.001
Cholesterol HPLC–MS/MS, mg/dL	282 ± 62	192 ± 46	<0.001
Triglycerides, mg/dL	109 ± 38	83.3 ± 37	<0.001
HDL Cholesterol, mg/dL	59 ± 16	54 ± 13	0.004
Non HDL cholesterol, mg/dL	236 ± 43	142 ± 36	<0.001
LDL Cholesterol, mg/dL	213 ± 42	128 ± 26	<0.001
Apolipoprotein A1, mg/dL	162 ± 32	159 ± 29	0.404
Apolipoprotein B, mg/dL	150 ± 29	99 ± 222	<0.001
Lipoprotein (a), mg/dL	24 (11–56)	12 (5–22)	<0.001
C-Reactive Protein, mg/dL	1.6 (0.5–2.7)	1.7 (0.7–4.2)	0.202
Glucose, mg/dL	88 (82–96)	87 (79–96)	0.270
Gamma-glutamyl transpeptidase, U/L	22.0 (14.5–29.5)	18.5 (15.0–25.5)	0.088
Glutamic-pyruvic transaminase, U/L	21.0 (17.0–40.0)	20.0 (15.2–25.2)	0.111
Mean common carotid IMT, mm	0.696 ± 0.153	0.693 ± 0.144	0.881
Mean carotid IMT, mm	0.749 ± 0.156	0.705 ± 0.147	0.048

*Values are mean ± SD or median (interquartile range). #*P* refers to differences calculated by Student's *t*-test for data normally distributed and Mann–Whitney *U* test for skewed data.
HDL denotes high density lipoprotein; LDL, low density lipoprotein; HPLC, high-performance liquid chromatography; MS, mass spectrometry

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eluted. The non-cholesterol sterols were desorbed with 1.4 ml of 2-propanol by gravity and 40 μl of the final mixture were injected into the HPLC–MS/MS system.

2.4. Genetic analysis

DNA was isolated from EDTA blood samples following standard protocols. *APOE* sequencing was performed in all study subjects as previously described [30]. Large rearrangements in the *LDLR* gene were analyzed using a method based on quantitative fluorescent multiplex PCR. Brieftly, the method involves two PCR reactions; the first amplifies the selected exons using primer sequences tagged with universal primers, while the second amplifies the first amplicons using the universal primers. One of the second PCR reaction universal primers is labelled with a fluorescent dye which is incorporated into the PCR products which are then electrophoresed on the DNA sequencer. The relative amounts of the amplified peak areas are determined and compared to ratios obtained for DNA from normal controls [31]. 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 LIPOchip® 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 LIPOchip® 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) [32].

2.5. Ultrasonographic evaluation

Ultrasonographic evaluation for measurement of carotid intima media thickness (IMT) was performed according to standardized scan- and image analysis protocols, as has been previously described [33]. In brief, we bilaterally assessed carotid IMT of the posterior walls of distal centimeter of the arterial segments proximal to the carotid dilatation, the common carotid arterial segment (CC), the segment, between the dilatation and the flow divider, the carotid bulb (CB) and the internal carotid (IC). 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, the mean (average) IMT value of the arterial wall segment was obtained. The per subject aggregate of the right and left CC (mean common carotid IMT), CB and IC mean IMT values were used for calculating subject mean IMT aggregate (mean carotid IMT).

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. When significant differences were detected, multiple comparisons were made by using the Bonferroni correction for normally distributed variables. The significance was set at $P < 0.05$ for the variables. Non-cholesterol sterol to cholesterol ratios were log transformed to achieve variance homogeneity. Data are presented as mean and standard deviation (SD) for continuous variables. All statistical analyses were performed with SPSS software (version 15.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Main clinical and biochemical characteristics

The main clinical and biochemical characteristics of 200 non-FH GH patients and 100 normolipemic controls are presented in Table 1. Non-FH GH participants had statistically significant higher values of total cholesterol, HDL cholesterol, LDL cholesterol, Lp(a), apoB and mean carotid IMT than normolipemic controls. No differences in age, BMI, weight and waist circumference were found between patients and controls. Oxysterols (24S-hydroxycholesterol and 27-hydroxycholesterol) and cholesterol synthesis precursors (lanosterol and desmosterol) were significantly higher in non-FH GH than in controls ($P < 0.001$). Ratios of oxysterols to total cholesterol were higher in non-FH GH than in normolipemic controls, although only 24S-hydroxycholesterol showed statistical significance ($P < 0.001$) (Table 2).

3.2. Spearman's rank correlations in non-FH GH patients and in normolipemic controls

Spearman's rank correlations in non-FH GH patients and in normolipemic controls are reported in Table 3 and supplemental figure. As expected, desmosterol and lanosterol, markers of cholesterol synthesis, had a positive correlation with BMI, triglycerides, cholesterol and apoB in control population. However, these positive correlations disappeared in non-FH GH with the exception of a weak positive correlation for non-HDL cholesterol and apoB. The same pattern was observed for bile acid precursors with high positive correlation in controls and absence of correlation for non-FH GH, except non-HDL cholesterol for 24S-hydroxycholesterol and 27-hydroxycholesterol and apoB for 27-hydroxycholesterol.

Spearman's rank correlations of non-cholesterol sterols in non-FH GH patients and in normolipemic controls are reported in

Table 2
Non-cholesterol sterols in non-FH GH patients and normolipidemic controls.*

	Non-FH GH (n = 200)	Control (n = 100)	P#
Desmosterol mg/dL	0.70 (0.54–0.90)	0.45 (0.32–0.61)	<0.001
Lanosterol mg/dL × 10^2	0.033 (0.025–0.045)	0.023 (0.014–0.033)	<0.001
27-hydroxycholesterol mg/dL × 10^2	1.54 (1.12–2.04)	0.93 (0.66–1.19)	<0.001
24S-hydroxycholesterol mg/dL × 10^2	0.98 (0.71–1.17)	0.47 (0.34–0.60)	<0.001
7α-hydroxycholestenona mg/dL × 10^2	0.26 (0.17–0.40)	0.14 (0.076–0.22)	<0.001
Desmosterol −to-TC × 10^3	2.44 (1.79–3.60)	2.63 (1.66–3.48)	0.951
Lanosterol −to-TC × 10^3	0.111 (0.081–0.186)	0.133 (0.075–0.177)	0.646
24S-hydroxycholesterol −to-TC × 10^3	0.033 (0.024–0.048)	0.026 (0.020–0.033)	<0.001
27-hydroxycholesterol −to-TC × 10^3	0.054 (0.037–0.080)	0.054 (0.032–0.069)	0.059
7α-hydroxycholestenona −to-TC × 10^3	0.0092 (0.0059–0.016)	0.0071 (0.0043–0.014)	0.069

*Values are mean ± SD or median (interquartile range). #*P* refers to differences calculated by Student's *t*-test for data normally distributed and Mann–Whitney *U* test for skewed data.

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Table 3
Spearman's correlation coefficients of non-cholesterol sterols and lipids in non-FH GH patients and normolipidemic controls.* denotes stadistical significance.

		Body mass Index		Triglycerides		HDL-cholesterol		HDL non-cholesterol		Apolipoprotein B	
		ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*
Desmosterol	Controls	0.295*	0.004	0.545*	<0.001	−0.162	0.116	0.526*	<0.001	0.611*	<0.001
	Non-FH GH	0.012	0.862	0.072	0.307	−0.043	0.540	0.217*	0.002	0.115	0.108
Lanosterol	Controls	0.295*	0.004	0.446*	<0.001	−0.132	0.201	0.460*	<0.001	0.571*	<0.001
	Non-FH GH	0.055	0.440	0.050	0.478	−0.001	0.989	0.200*	0.004	0.163*	0.022
24S-hydroxycholesterol	Controls	0.162	0.119	0.524*	<0.001	−0115	0.266	0.358*	<0.001	0.482*	<0.001
	Non-FH GH	−0.101	0.153	−0.002	0.981	−0.060	0.396	0.188*	0.007	0.130	0.067
27-hydroxycholesterol	Controls	0.347*	0.001	0.579*	<0.001	−0.234*	0.022	0.461*	<0.001	0.588*	<0.001
	Non-FH GH	0.013	0.855	0.1122	0.083	−0.129	0.066	0.291*	<0.001	0.310*	<0.001
7α-hydroxycholestenona	Controls	0.259*	0.012	0.339*	0.001	−0.162	0.117	0.095	0.353	0.069	0.513
	Non-FH GH	−0.011	0.873	0.096	0.177	−0.120	0.090	−0.026	0.712	0.043	0.545

Table 4
Spearman's correlation coefficients of non-cholesterol sterols in non-FH GH patients and normolipidemic controls.* denotes stadistical significance.

		Lanosterol		24S-hydroxycholesterol		27-hydroxycholesterol		7α-hydroxycholestenona	
		p	*p*	*p*	*p*	*p*	*p*	*p*	*p*
Desmosterol	Controls	0.884*	<0.001	0.699*	0.001	0.809*	<0.001	0.209*	0.050
	Non-FH GH	0.789*	<0.001	0.757*	<0.001	0.756*	<0.001	0.417*	<0.001
Lanosterol	Controls	–	–	0.689*	<0.001	0.785*	<0.001	0.236*	<0.001
	Non-FH GH	–	–	0.669*	<0.001	0.634*	<0.001	0.342*	<0.001
24S-hydroxycholesterol	Controls	–	–	–	–	0.738*	<0.001	0.135	0.181
	Non-FH GH	–	–	–	–	0.743*	<0.001	0.450*	<0.001
27-hydroxycholesterol	Controls	–	–	–	–	–	–	0.262*	<0.001
	Non-FH GH	–	–	–	–	–	–	0.431*	<0.001

Table 4. All non-cholesterol sterols had positive correlation among them in patients and in controls. This correlation was high between cholesterol synthesis markers and 24S-hydroxycholesterol and 27-hydroxycholesterol, and weak, although significant, with 7α-hydroxy-3-cholesten-4-one except for 24S-hydroxycholesterol.

3.3. Population distribution depending on oxysterols and cholesterol synthesis markers

Fig. 1 shows the distribution of 24S-hydroxycholesterol, 27-hydroxycholesterol, desmosterol and lanosterol in non-FH GH and in controls. Distribution of these oxysterols in non-FH GH indicated a group of subjects with higher values of both oxysterols compared with the control population. The distribution of 7α-hydroxy-3-cholesten-4-one did not show differences between patients and controls.

3.4. Clinical characteristics and subclinical atherosclerosis in non-FH GH patients according to the 24S-hydroxycholesterol and 27-hydroxycholesterol ratios to TC

Clinical characteristics and subclinical atherosclerosis in non-FH GH patients according to the 24S-hydroxycholesterol and 27-hydroxycholesterol ratios to TC are presented in Table 5. There was a trend to higher carotid IMT and statistical significance was reached for mean common carotid IMT in subjects with 24S-hydrocholesterol ratio to TC over the 95th percentile of the control population. The clinical characteristics, BMI and apoB were significantly lower in cases with 24S-hydroxycholesterol ratio to TC over the 95th percentile of the control population. Cholesterol synthesis precursors and oxysterols showed higher concentrations when 24S-hydroxycholesterol and 27-hydroxycholesterol were over the 95th percentile, $P < 0.001$.

4. Discussion

Serum non-cholesterol sterol analysis is a 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 [34]. Plasma levels of the cholesterol precursors, as desmosterol or lanosterol, served as markers of cholesterol synthesis [34,35], whereas 7α-hydroxy-3-cholesten-4-one and 24S-hydroxycholesterol and 27-hydroxycholesterol served as precursors of bile acid synthesis in the classic and in the alternative pathway, respectively [36].

This is the first study, to our knowledge, to analyze the bile acid synthesis precursors in a large number of well-clinically defined subjects with non-FH GH. Our study shows that many subjects with non-FH GH show an increase in their concentrations of bile acid synthesis precursors and cholesterol synthesis markers. When oxysterols concentrations were adjusted by total cholesterol levels, this increase was only observed for 24S-hydroxycholesterol. Although there is a large variation and heterogeneity in oxysterols concentration, a very high percentage of subjects had a 27-hydroxycholesterol and 24S-hydroxycholesterol concentration above the 95th percentile of the values of a normolipemic population, suggesting that non-FH GH is heterogeneous and that bile acid metabolism could be involved in the pathogenesis of some of these patients.

The serum concentration of oxysterols depends on the balance between their production and hepatic metabolism. 24S-hydroxycholesterol is mainly produced in cerebral tissue in humans and this cerebral production remains fairly constant in adults. Hence, its plasma concentrations are mainly modified by hepatic metabolism. The higher concentration of 24S-hydroxycholesterol in our study suggests that 24S-hydroxycholesterol could be related to a lower efficiency of hepatic catabolism, given that metabolic

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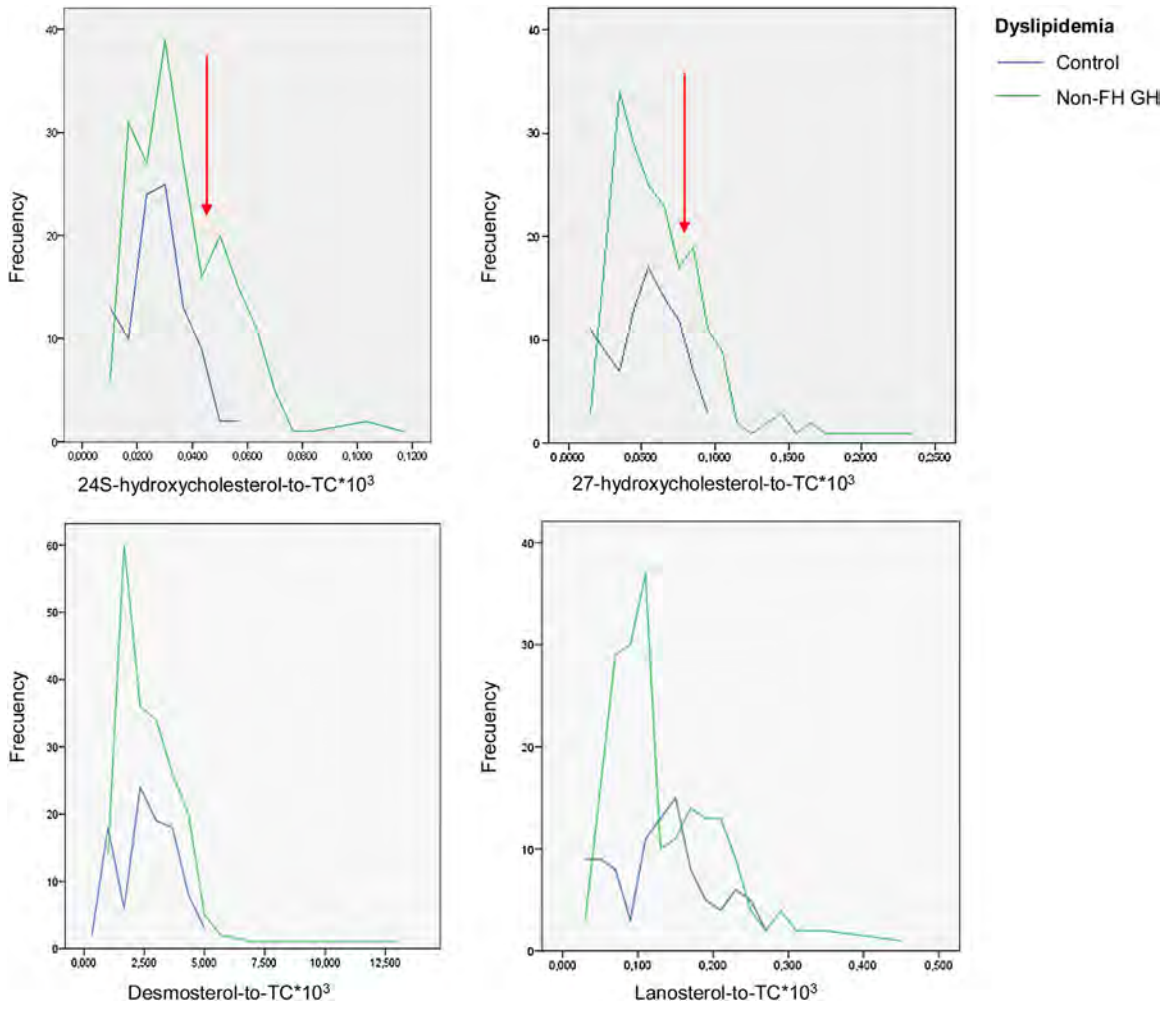

Fig. 1. Distribution of 24S-hydroxycholesterol, 27-hydroxycholesterol, desmosterol and lanosterol in non-HF GH subjects and in control population.

Table 5
Clinical characteristics of non-FH GH subjects with values of oxysterols above and below the 95th percentile of the control population.*

	24S-OHC to TC < 95th percentile	24S-OHC to TC > 95th percentile	*p*#	27-OHC to TC <95th percentile	27-OHC to TC >95th percentile	*p*#
N	135	65		165	35	
Female, n (%)	47 (52.2)	56 (50.9)	0.958	92 (57.5)	15 (37.5)	0.001
Age, years	46.1 ± 11.6	42.8 ± 11.6	0.098	45.6 ± 11.7	40.5 ± 11.2	0.056
Body Mass Index, kg/m^2	25.8 ± 3.5	24.2 ± 3.3	0.025	25.3 ± 3.5	24.8 ± 3.3	0.123
LDL Cholesterol, mg/dL	216 ± 42	209 ± 42	0.216	214 ± 41	212 ± 48	0.392
Triglycerides, mg/dL	110 ± 41	106 ± 32	0.865	108 ± 39	120 ± 31	0.263
HDL Cholesterol, mg/dL	59 ± 16	57 ± 17	0.276	59 ± 16	56 ± 18	0.348
HDL Non cholesterol, mg/dL	239 ± 43	231 ± 43	0.251	236 ± 42	235 ± 48	0.381
Apolipoprotein A1, mg/dL	162 ± 32	162 ± 35	0.614	162 ± 33	158 ± 33	0.541
Apolipoprotein B, mg/dL	153 ± 26	143 ± 25	0.032	150 ± 31	151 ± 22	0.238
Mean common carotid IMT, mm	0.684 ± 0.115	0.726 ± 0.235	0.026	0.691 ± 0.159	0.701 ± 0.099	0.909
Mean carotid IMT, mm	0.745 ± 0.139	0.762 ± 0.203	0.243	0.751 ± 0.163	0.722 ± 0.103	0.201
Desmosterol −to-TC × 10^3	1.95 (1.67–2.48)	3.89 (3.27–4.45)	<0.001	2.11 (1.77–3.12)	4.38 (3.84–5.20)	<0.001
Lanosterol-to-TC × 10^4	0.962 (0.732–1.16)	1.98 (1.60–2.35)	<0.001	1.05 (0.78–1.56)	2.31 (1.74–2.83)	<0.001
27-hydroxycholesterol −to-TC × 10^4	0.433 (0.331–0.571)	0.830 (0.682–1.02)	<0.001	–	–	
24S-hydroxycholesterol −to-TC × 10^4	–	–		0.502 (0.218−0.429)	0.594 (0.509−0.677)	<0.001
7α-hydroxycholestenona −to-TC × 10^4	0.072 (0.045−0.110)	0.163 (0.107−0.221)	<0.001	0.082 (0.053−0.14)	0.176 (0.121−0.239)	<0.001

*Values are mean ± SD or median (interquartile range). #*P* refers to differences calculated by Student's *t*-test for data normally distributed and Mann–Whitney *U* test for skewed data.

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capacity of the liver is a critical factor for the 24S-hydroxycholesterol levels in human serum [37].

Increased cholesterol synthesis is frequently seen in subjects with obesity and type 2 diabetes [38]. Actually, hepatic overproduction of cholesterol is one of the mechanisms responsible for dyslipidemia in these conditions [39,40]. The association between body mass index and cholesterol synthesis was clearly observed in our control population, hence confirming previous reports. Interestingly, patients with non-FH GH have higher cholesterol synthesis markers. However, this association was unrelated to BMI, in contrast with the control population (supplemental figure). This confirms that cholesterol synthesis overproduction is a primary defect in non-FH GH [41]. Oxysterols correlate with cholesterol synthesis markers, and therefore we speculate that subjects with non-FH GH show high levels of oxysterols in response to hepatic overproduction of cholesterol. The enzymes involved in hepatic metabolism of 24S-hydroxycholesterol have not been fully defined and it has been suggested that they are less effective than those involved in the metabolism of other oxysterols [42], and this could explain the higher 24S-hydroxycholesterol levels observed in our study.

An increased synthesis of bile acids could be a compensatory mechanism in presence of cholesterol accumulation in the liver [43]. However, in our study there was a weak correlation between cholesterol and oxysterols in non-FH GH subjects. Therefore, the increase in oxysterol is not simply explained for hepatic cholesterol accumulation.

Involvement of bile acid synthesis in the pathogenesis of non-FH GH is supported by genetic studies revealing significant associations between human blood sterols and variants in genes encoding enzymes or proteins that are known to be involved in the homeostasis of cholesterol. Genetic variants in *CYP27A1*, which encodes for sterol 27-hydroxylase [44], in *CYP39A1*, which encodes 7α-hydroxylase [45], and in *EPHX2* [46], which encodes for epoxide hydrolase, have been associated with both oxysterol and cholesterol concentrations. Furthermore, as mentioned ahead, a kindred with three homozygous subjects for a loss-of-function mutation in CYP7A1 express a phenotype compatible with FH, "in vivo" demonstration of the importance in cholesterol homeostasis and raising the possibility that other genetic defects in bile acid metabolism could predispose to non-FH GH. Although bile acid synthesis precursors were higher in cases than in controls, their distribution do not support a severe monogenic defect because no extreme values of oxysterol were found in non-FH GH subjects. Therefore, if genetic variation is responsible of the high oxysterol levels observed in our study, it could be more easily explained by polygenic variations with minor effects than by monogenic major effects.

24S-hydroxycholesterol has been associated with carotid atherosclerosis in our non-FH GH population, even after adjusting for other major risk factors of atherosclerosis. In our previous publication, we described that in familial combined hyperlipidemia, a genetic hypercholesterolemia with high cardiovascular risk, subjects with low 27-hydroxycholesterol concentrations have increased maximum cIMT and mean cIMT [47]. If oxysterols are involved directly in the production of atherosclerosis, or if they are simply a passive outstand would need a further investigation. However, there are some evidences linking oxysterols with atherosclerosis: increased oxysterol concentrations in plasma and arterial wall are associated with elevated superoxide anion production in aortic tissue [48]; oxysterols promote inflammatory response in the arterial wall [49]; the oxysterol concentration in atherosclerotic vascular plaques relates with the severity of the lesions [50]; and oxysterols are elevated in plasma and arteries from patients with severe peripheral artery disease [51]. However no association between plasma 27-hydroxycholesterol levels and coronary disease was observed in a large prospective study [52]. Babiker et al. found that in subjects with advanced atherosclerosis increased 27-oxygenated steroids were found and may reflect an activated antiatherogenic defense mechanism, although other explanation cannot be excluded [53].

Our study has several limitations. First, we have analyzed a small number of oxysterols in the complex metabolic pathways of bile acid synthesis, and these concentrations do not reflect the full spectrum of their metabolism. Second, this study had a cross-sectional design, and causality can not be inferred from our atherosclerosis results due to the lack of prospective follow-up. Third, ultrasonographic evaluation of carotid intima media thickness is a debatable atherosclerosis subrogate in clinical practice.

5. Conclusion

In this large cohort of patients with primary hypercholesterolemia of genetic origin, negative for mutations in candidate genes for FH, plasma concentrations of oxysterols are increased in a large proportion of cases, associated with increased cholesterol synthesis. After adjusting for total cholesterol, only 24S-hydroxycholesterol showed increased concentration, suggesting a hepatic catabolic defect in some non-FH GH subjects. Those patients with the highest levels of 24S-hydroxycholesterol associated more carotid intima media thickness. These results suggest that bile acid metabolism is involved in the pathogenesis of some patients with non-FH GH, and may confer a higher cardiovascular risk.

Acknowledgments

This study was supported by grants from the Spanish Ministry of Economy and Competitiveness PI12/01087 and RIC Red de Investigación Cardiovascular (RIC) RD12/0042/0055. RIC is an initiative of Instituto de Salud Carlos III (ISCIII), Spain. Authors thank Proteomic Unit, Instituto de Investigación Sanitaria Aragón (IIS Aragón), ProteoRed member, for technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2016.10.003.

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