EXPLOITING STEROL PROFILE ANALYSIS: OVER TEN YEARS OF EXPERIENCE. A NARRATIVE REVIEW

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ABSTRACT

The analysis of sterol profile consists in the qualitative and quantitative determination of cholesterol and non-cholesterol sterols. Non-cholesterol sterols include the cholesterol precursors in de novo biosynthesis pathway, cholesterol catabolites and plant sterols absorbed from diet.

The sterol profile analysis can be performed on different biological matrices, i.e., plasma/serum, whole blood, erythrocyte membranes, tissues and cells, depending on the clinical and/or experimental purpose. The reference method for the sterol profile analysis is the gas-chromatography coupled with mass spectrometry, although new methods based on liquid-chromatography or direct mass spectrometry have been developed.

The sterol profile analysis represents a fundamental tool for the biochemical diagnosis of the congenital defects of cholesterol metabolism. In addition, some non-cholesterol sterols have been validated as surrogate markers of de novo synthesis and intestinal absorption of cholesterol. Therefore, the sterol profile analysis has been used for the evaluation of cholesterol homeostasis in different diseases. This review focuses on these applications and our clinical and experimental findings. In the last nine years, we identified 18 new cases of the cholesterol metabolism defects and we found that cholesterol metabolism is impaired in patients with cystic fibrosis.

Key words*: cholesterol, non-cholesterol sterols, gas-chromatography, cholesterol homeostasis, congenital defects of cholesterol synthesis, cystic fibrosis.*

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Cholesterol and non-cholesterol sterols

Cholesterol consists of 27 carbon atoms with a base structure of cyclopentane peridrofenantrene and represents, among steroids, the most abundant in the humans playing fundamental functions, which are already well described in other works[1, 2]. Cholesterol is synthetized in humans by de novo synthesis, mainly in the liver, and obtained from diet by the absorption at intestinal level[3]. These two cholesterol fluxes together with the uptake by peripheral tissues, the synthesis of bile acids, and the liver excretion of both compounds, are finely regulated, achieving cholesterol homeostasis[3].

De novo synthesis of cholesterol consists in a complex pathway including more than 30 enzymatic reactions and starting with the production of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) in the reaction catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-coenzymeA synthase. Subsequently, HMG-CoA is converted to mevalonate by 3-hydroxy-3-methylglutarylcoenzyme A reductase

(HMGCR), the key enzyme that regulates the long pathway of cholesterol biosynthesis, and in the first part the pathway synthesizes the triterpene squalene, a common natural product also produced by plants[3]. In the post-squalene synthesis (Figure 1), two different pathways led to cholesterol formation, starting from lanosterol[4]. The Bloch pathway (Figure 1, on the left) involves 8 enzymes, including a C4-demethylase complex, which is composed by the enzymes NAD(P)H steroid dehydrogenase-like protein (NSDHL, EC 1.1.1.170) and sterol-C4-methyl oxidase (SC4MOL, EC 1.14.18.9).

All the intermediates of this pathway preserve the double bond in C24 of the lanosterol until to the last reaction in which 5α-cholesta-5,24-dien-3β-ol (desmosterol) is converted in cholesterol by the enzyme 24-dehydrocholesterol reductase (DHCR24; Figure 1). The second pathway, discovered by Kandutsch-Russell (Figure 1, on the right), starts with the reduction of the double bond in C24 of the lanosterol by the enzyme DHCR24, proceeds to cholesterol through reactions catalyzed by the same enzymes of the Bloch pathway and concludes with the conversion of 5α-cholesta-5,7-dien-3β-ol (7-dehydrocholesterol, 7-DHC) in cholesterol. However, the two pathways are linked, at all stages, by the reaction catalyzed by DHCR24 (Figure 1), one of the most promiscuous and intriguing enzyme of this segment of the metabolome. In addition, Mitsche et al.[5] found in cultured cells that the Kandutsch-Russell pathway is constitutive, while the Bloch pathway is activated based on the availability of sterols. Furthermore, these authors found in mice a tissue specificity of the two pathways, namely that the Kandutsch-Russell pathway is favored in the muscles, skin and brain, while the Bloch pathway is favored in the testes and adrenal gland[5].

The non-cholesterol sterols are all sterols that have a chemical structure similar to cholesterol. They include all precursors of post-squalenic biosynthesis of cholesterol (Figure 1), phytosterols (Figure 2) and the catabolites that maintain the core of cholesterol chemical structure. Phytosterols are mainly represented by sitosterol, campesterol, stigmasterol and Δ^5-avenasterol (Figure 2). Humans are not able to synthesize the phytosterols, which are up-taken from diet, specifically from vegetables and seeds[6].

Other important non-cholesterol sterols are some intermediates of bile acid synthesis, such as cholestanol[7]. This non-cholesterol sterol is an intermediate of the alternative (or acidic) pathway of bile acid synthesis. This alternative pathway contributes in minor extent to bile acids synthesis under physiological conditions, compared to the classical (or neutral) pathway[8]. It has been observed that the acidic pathway becomes predominant in the case of liver disease or injury with increased levels of circulating bile acids intermediates, such as the cholestanol in plasma from subjects with cholestasis[9].

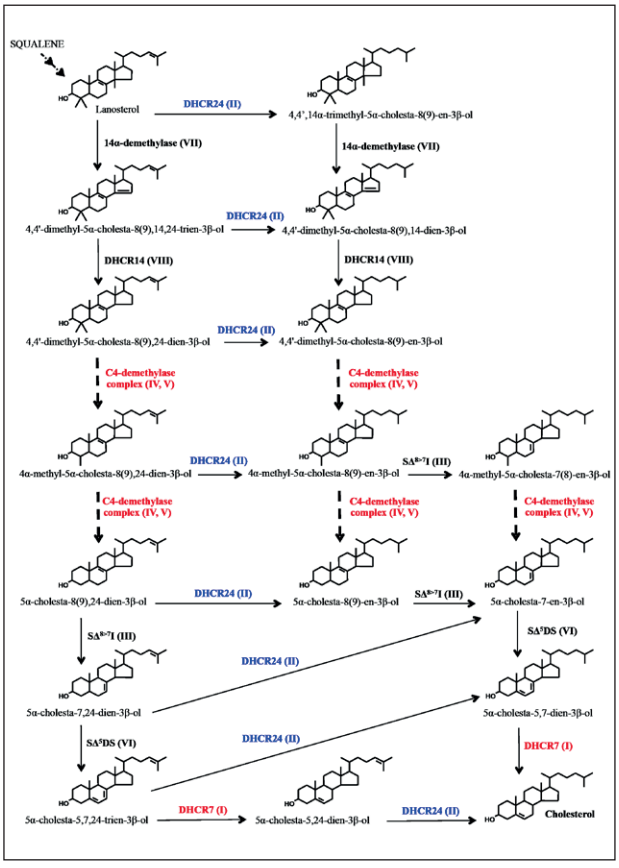

Figure 1: Post-squalenic cholesterol synthesis. The Bloch pathway (on the left) starts with the enzyme 14α-demethylase reaction. The Kandutsch-Russell pathway (on the right) starts with the enzyme DHCR24 reaction. The Roman numeral indicates the disorder caused by the enzyme defect. I: Smith-Lemli-Opitz syndrome (SLOS, OMIM #270400); II: Desmosterolosis (OMIM #602398); III: X-linked dominant disorder chondrodysplasia punctata-2 (CDPX2, OMIM #302960); IV: The *NSDHL*-related disorders, i.e., congenital hemidysplasia with ichthyosiformerythroderma and limb defects syndrome (CHILD, OMIM #308050), an X-linked syndrome that is lethal in men and observed only in females, and CK syndrome (OMIM #300831), an X-linked disorder affecting males; V: sterol-C4-methyl oxidase deficiency (SC4MOL, OMIM #616834); VI: Lathosterolosis (OMIM #607330); VII: Antley-Bixler syndrome (OMIM #201750); VIII: Greenberg dysplasia (OMIM #215140). DHCR7: 7-dehydrocholesterol reductase; DHCR14: sterol-Δ14-reductase; DHCR24: 24-dehydrocholesterol reductase; SΔ5DS: 3β-hydroxysteroid-Δ5-desaturase; SΔ8>7I: 3β-hydroxysteroid-Δ8, Δ7-sterol isomerase.

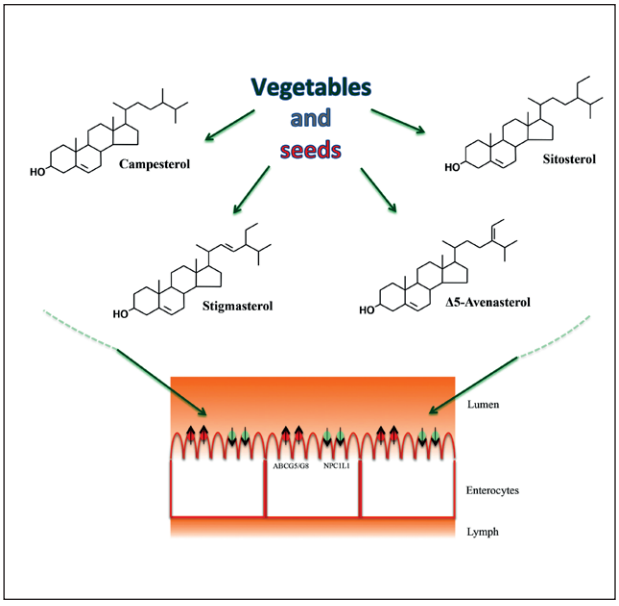

Figure 2: Chemical structures of phytosterols. These sterols, as well as cholesterol, are absorbed from intestinal lumen by Niemann-Pick C1-Like 1 (NPC1L1) transporter and excreted by ATP-binding cassette (ABC) G5/G8.

Finally, non-cholesterol sterols also include oxysterols, a fascinating group of steroids that can be produced by enzymatic[10] or auto-oxidation[11] processes. They have many important functions as cellular activity regulators, regulators of the immune system and brain homeostasis[10], and some of them represent specific disease biomarkers, i.e., 24-hydroxycholesterol is considered a biomarker for neurodegenerative diseases[12] as well as 7α-hydroxycholestenone represent a biomarker of the bile acid diarrhea[13].

Methods for sterol profile analysis

The sterol profile analysis can be performed on different biological matrices, i.e., plasma/serum, whole blood, erythrocyte membranes, tissues and cells, depending on the clinical and/or experimental purposes[14]. The reference method for analyzing plasma sterols for diagnostic purposes is based on sterol extraction and GC-MS analysis[15]. Based on the organic solvent used for the extraction procedure, it is possible to direct the extraction to less polar (neutral) or more polar sterols. In fact, hexane favors the extraction of cholesterol, its synthetic precursors and neutral catabolites[14], while dichloromethane is more efficient to extract the oxysterols[16].

Over the years, various methods have been developed to reduce the invasiveness of the sampling technique and reduce analytical time and costs. For this purpose, methods for the analysis of the sterol profile on dried blood spots (DBS) by GC coupled with flame ionization detector (FID) or MS[17] as well as by LC-MS/MS systems[18] have been developed. The analysis of sterols on DBS is challenging for different reasons[19]. Firstly, some sterols such as 7-DHC have a high auto-oxidation rate[20] and therefore a very low stability on filter paper[21]. We stabilized the levels of 7-DHC and its isomer, 8-DHC, by adding an antioxidant to filter paper before of blood deposition[17, 21]. Another critical point is the limited sample volume available in DBS.

Therefore, sensitivity could represent a limitation in the development of a method on DBS[19]. Finally, the step of sterol extraction from filter paper represents another disadvantage that lengthens the analysis run time. Hence, very innovative MS techniques, such as the atmospheric pressure thermal desorption chemical ionization mass spectrometry (APTDCI-MS), have been developed to analyze sterols on DBS without sample preparation that is necessary for traditional procedures[22].

Diagnosis of congenital defects of cholesterol metabolism

To date, it has been discovered nine disorders in humans caused by inherited defects of enzymes involved in post-squalenic cholesterol synthesis (Figure 1). The clinical as well as biochemical and genetic characteristics of these inherited defects have been well described in previous works[23, 24]. To note, these disorders are characterized by overlapping clinical phenotypes often with facial and/or somatic pluri-malformations. Therefore, it is of fundamental importance the differential diagnosis of these defects achievable through the analysis of plasma sterol profiles by chromatographic methods. In fact, each disorder is characterized by the accumulation of a specific precursor/substrate (biomarker) of the defective enzyme in plasma and/or tissues from affected patients[23]. In particular, the patients with Smith-Lemli-Opitz syndrome (SLOS; OMIM # 270400), which is the most frequent defect of cholesterol synthesis, present with increased plasma concentrations of 7-DHC and its isomer, i.e., 8-DHC, together with reduced or normal cholesterol levels[14, 23].

The analysis of plasma sterol profile allows to perform the biochemical diagnosis of all congenital defect of post-squalenic cholesterol synthesis. However, three disorders, i.e., CHILD, CK and Antley-Bixler syndromes, present with precursor accumulation only in tissues and/or cell cultures[23]

and a negative result of sterol profile analysis of plasma does not allow to exclude the clinical suspicion of these defects and further analysis on cells collected from the patient are required.

Beyond the defects of cholesterol synthesis, the analysis of plasma sterol profile allows to perform differential diagnosis of Cerebrotendinous xanthomatosis (CTX, OMIM #213700) and Sitosterolemia (OMIM #210250), two dyslipidemias with some common signs, e.g. hypercholesterolemia and xanthomas, as in familial hypercholesterolemia[25]. CTX represents the most frequent defect of bile acids synthesis and is caused by mutations in the CYP27A1 gene leading to a defect of sterol-27-hydroxylase with a reduced production of bile acids from cholesterol[26].

A biochemical feature of CTX consists in the increased plasma concentrations of cholestanol and some cholesterol precursors, i.e., 7-DHC, 5α-cholesta-8(9)-en-3β-ol (zymostenol), 5α-cholesta-7-en-3β-ol (lathosterol), and bile-alcohols in urine[27]. While, sitosterolemia is a lipid storage disease caused by mutations in ABCG5/G8 genes causing a defect of sterols excretion from cells, in particular intestine and liver. Patients with sitosterolemia show increased plasma levels of cholesterol and phytosterols[28]. Therefore, the measurement of plasma cholestanol and phytosterols allows to distinguish CTX and sitosterolemia, respectively, as well as to monitor pharmacological treatments of patients[26-28].

We previously described our six-year experience (from 2005 to 2010) in the diagnosis of SLOS and other defects of cholesterol biosynthesis by the sterol profile analysis[14]. Herein, we update this report describing the experience of the last nine years (from 2011 to 2019) in the diagnosis and follow-up of cholesterol metabolism disorders (Table 1). We screened newborns, children and adult patients with a clinical suspicious of a cholesterol metabolism defects coming from clinical units of Federico II University Hospital, from other hospitals in Naples (Vanvitelli University Hospital, Monaldi Hospital, Santobono Hospital) and other Italian Hospitals, e.g., Meyer (Florence), Vittorio Emanuele (Catania) and SS. Giovanni di Dio and Ruggi d'Aragona (Salerno) University Hospitals.

In table 1, we reported the number of screened patients and the number of the tested plasma specimens for year. For some patients, both diagnosis and follow-up have been performed in the same year. When sterol profile showed the presence of interfering compounds from parenteral nutrition and/or other treatments, a further analysis has been carried out on a second plasma sample. In the last nine years, we screened 439 subjects and identified 18 new cases (4.3%) of defects of cholesterol metabolism. The new diagnoses have been confirmed by molecular analysis of the involved gene. Among these new diagnoses, we identified a case of SC4MOL deficiency[29] that was the fifth case described in the literature, after the four cases described by He et al.[30, 31].

In agreement with these previous cases, the sterol profile of our patient was characterized by the presence of C4-monomethyl and C4-dimethyl sterols (Figure 1). In addition, we identified 4 new cases of CTX, among which we found an atypical case with spinal cord involvement and without tendon xanthomas[32]. Finally, we identified 12 new cases of SLOS and a new case of X-linked dominant disorder chondrodysplasia punctata-2 (CDPX2, OMIM #302960) showing increased level of zymostenol, in agreement with an our previous case[14]. In all cases with positive or borderline results, we performed the sterol profile also on red blood cell membranes (RBCM) to validate the results of the analysis on plasma[14, 27, 29].

Among all these disorders, CTX is a treatable disease, generally with chenodeoxycholic acid, and the follow-up of plasma cholestanol levels represents a biochemical approach to monitor the pharmacological treatment of affected patients[27]. Herein we show the follow-up of the 4 CTX patients diagnosed in the last nine years, by sterol profile analysis (Figure 3). After 1 year of treatment, plasma cholestanol levels decreased on average of 55%, except for one patient that showed a slight increase after 1 year and a reduction of 58% after two years (Figure 3).

Finally, in Table 1, we reported, as inconclusive diagnoses, the cases that had an altered sterol profile, but without alterations in the corresponding gene. Furthermore, in this group, we included the patients whom we have not received a second sample after a first one that was not suitable for analysis (e.g. hemolyzed plasma) or containing compounds interfering with the gas chromatographic analysis.

Evaluation of cholesterol homeostasis

The direct evaluation of the main cholesterol fluxes, i.e., synthesis, absorption and excretion, is carried out by the sterol balance technique that represents the gold standard method[33]. Overall, this method is complex, time-consuming and requires the administration of radiolabelled isotopes (14C or 3H) of cholesterol.

Year	Total specimens (n)	Total patients (n)	Males (%) [a]	Females (%) [a]	Negative patients (n,%) [a]	Inconclusive diagnoses (n,%) [a]	Positive patients (n,%) [a]	SLOS (n)	CDPX2 (n)	SC4MOL (n)	CTX (n)	Sitosterolemia (n)	Specimens from positive patients (n)	Diagnosis (n)	Follow-up (n)
2011	63	59	67.8	32.2	55 (93.2)	-	4 (6.8)	3	1	-	-	-	5	1	4
2012	54	49	61.2	38.8	41 (83.7)	4 (8.2)	4 (8.2)	3	-	-	1	-	5	4	1
2013	47	47	64.4	35.6	41 (87.2)	3 (6.4)	3 (6.7)	1	-	-	2	-	3	2	1
2014	59	54	67.9	32.1	48 (88.9)	5 (9.3)	1 (1.9)	-	-	-	1	-	1	-	1
2015	53	52	67.3	32.7	42 (80.8)	4 (7.7)	6 (11.5)	3	-	1	2	-	6	3	3
2016	64	57	58.9	41.1	45 (78.9)	2 (3.5)	10 (17.9)	7	-	1	2	-	13	4	9
2017	41	38	76.3	23.7	33 (86.8)	1 (2.6)	4 (13.5)	1	1	-	2	-	5	2	3
2018	54	46	62.5	37.5	30 (65.2)	5 (10.9)	11 (25.0)	9	-	-	2	-	12	2	10
2019	42	37	58.5	41.5	26 (70.3)	3 (8.1)	8 (19.5)	4	-	-	3	1	8	-	8
Total	477	439	64.9	35.1	361 (82.2)	27 (6.2)	51 (11.6)	31	2	2	15	1	58	18	40

Table 1:Report of our diagnostic activity of cholesterol metabolism disorders by sterol profile analysis from 2011 to 2019.

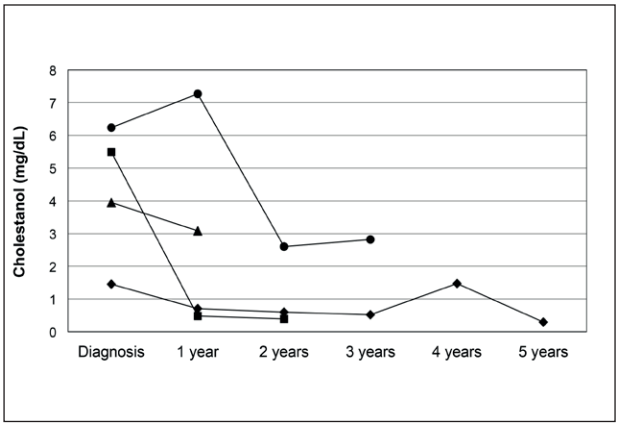

Figure 3: Follow-up of 4 patients affected by Cerebrotendinous xanthomatosis (CTX).

A valid alternative is represented by the measurement of surrogate biomarkers of liver cholesterol synthesis and intestinal absorption[34]. It has been observed that serum/plasma phytosterols and cholestanol levels, particularly their ratios to cholesterol, were positively associated to absolute cholesterol absorption in randomly selected males[34, 35]. In addition, positive correlations between absolute synthesis and serum levels of cholestenol, desmosterol, and lathosterol were found[36].

Mashnafi et al.[37] carried out a meta-analysis of different studies regarding the evaluation of the surrogate biomarkers in some metabolic diseases. Interestingly, these authors noted that specific patterns of cholesterol absorption/synthesis could be detected suggesting that the metabolic disorders could be classified as "cholesterol absorbers" or "cholesterol synthesizers". For example, in overweight and obese subjects reduced cholesterol absorption and increased synthesis were found, while this relation is reversed after slimming diet. In addition, patients with type 1 diabetes mellitus have higher cholesterol absorption and lower cholesterol synthesis than controls. On the other hand, in patients with type 2 diabetes an opposite trend was observed[37].

We applied this approach to evaluate liver cholesterol synthesis and intestinal absorption in patients with cystic fibrosis (CF)[38]. CF is a congenital disorder caused by mutations into the gene encoding for cystic fibrosis transmembrane conductance regulator (CFTR). Mutated CFTR lead to a defective transport of chloride and other ions through epithelial cells and a secretion of thick mucus[39]. In particular, patients with CF suffer of pancreatic insufficiency (PI) that cause malabsorption of fat and fat-soluble vitamins[40]. Consequently, patients with CF show reduced plasma cholesterol levels, although free cholesterol accumulation has been observed in tissues of CF animal models and cell cultures[41]. Hence, we analyzed the sterol profile in plasma from CF patients that showed reduced plasma phytosterols and increased lathosterol levels, indicating a reduced intestinal absorption and an enhanced de novo synthesis of cholesterol, respectively, compared to healthy subjects[38].

Furthermore, we found that CF patients with PI had plasma cholesterol and phytosterols concen-

trations significantly lower than CF patients with pancreatic sufficiency (PS) and healthy subjects, suggesting a relation between the reduced intestinal absorption of sterols and PI[42]. These findings suggest that sterol profile analysis could be useful to follow-up the pancreatic status in CF patients and to monitor the pancreatic supplementation therapy.

Finally, we applied the sterol profile analysis to evaluate the effect of lumacaftor/ivacaftor, a CFTR protein modulator therapy, on cholesterol absorption and synthesis by measuring the surrogate biomarkers[43]. This study showed that lumacaftor/ivacaftor improved the cholesterol metabolism and enterohepatic flux, but it did not increase cholesterol, which instead decreased by unknown mechanisms[43].

Discussion and conclusions

This review displays the clinical applications of the sterol profile analysis. It represents the gold-standard method for the biochemical diagnosis of congenital defects of cholesterol metabolism that are characterized by plasma accumulation of one or more biosynthetic precursor of cholesterol. Therefore, these molecules represent specific biomarkers and their detection allows them to perform differential diagnosis of these disorders. Moreover, the same analysis allows us to evaluate indirectly the rate of hepatic synthesis and intestinal absorption of cholesterol through the plasma levels of the surrogate markers. In particular, we reported the studies of cholesterol metabolism in patients with CF and the effects of lumacaftor/ivacaftor. To date, new therapies with different CFTR protein modulators have been proposed[44] that together with a Mediterranean diet and physical activity[45, 46] have extended the average life span of CF patients. Further studies, on cholesterol metabolism in these patients, should take into account the cellular senescence[47, 48]) that is caused by the chronic inflammation in CF patients and triggered by intracellular accumulation of cholesterol[49]. Finally, ex vivo model of nasal epithelial cells from CF patients has emerged as a very useful tool for assessing the residual function of CFTR protein in patients bearing rare variants[50, 51] as well as for testing new drugs going to a personalized medicine of CF[52]. The evaluation of cholesterol metabolism by sterol profile analysis in treated CF patients can give new information about the drug effects, at the metabolic level, that could enrich the findings from the nasal epithelial cell model. However, it should take into account that surrogate biomarkers, in some clinical conditions, may lose its potency and therefore, for a correct evaluation, it should be considered several markers of absorption and synthesis[34] simultaneously.

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