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Sterol profiles in plasma and erythrocyte membranes in patients with Smith-Lemli-Opitz syndrome: a six-year experience

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Abstract

Background: This study reports our experience over the last six years in the diagnosis of Smith-Lemli-Opitz syndrome and other inborn errors of cholesterol biosynthesis.

Methods: Gas chromatography/mass spectrometry was used to obtain sterol profiles in plasma and erythrocyte membranes of suspected patients.

Results: Plasma sterol reference values calculated in unaffected subjects (n = 276) were in agreement with those previously reported. Among patients investigated from 2005 to 2010, we report 16 patients affected by Smith-Lemli-Opitz syndrome, three of whom represent new cases and 13 of whom were follow-up patients. In this period we also identified a new case of chondrodysplasia punctata 2 X-linked. The estimated incidence obtained for Smith-Lemli-Opitz syndrome was 1:93 suspected patients (1.08%). We also studied the effect of storage on the dehydrocholesterols/cholesterol ratio in plasma and erythrocyte membranes of patients affected by Smith-Lemli-Opitz syndrome stored at –20°C for up to 22 and 20 months, respectively. A significant negative linear correlation between storage time and the dehydrocholesterols/cholesterol ratio was identified in both plasma and erythrocyte membranes. The decrease in the dehydrocholesterols/cholesterol ratio in erythrocyte membranes was at least two-fold higher than in plasma.

Conclusions: The results of this study may be helpful for diagnosis and interpretation of data in patients with findings suggestive of a cholesterol biosynthesis defect.

Keywords: cholesterol; 7-dehydrocholesterol; multiple malformations; Smith-Lemli-Opitz syndrome.

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Introduction

The defects of cholesterol (CHOL) biosynthesis are inherited disorders caused by different enzyme deficiencies in the CHOL metabolic pathway. Patients affected by these disorders have multiple morphogenic and congenital anomalies including internal organ, skeletal and/or skin abnormalities. They also show increased concentrations of diagnostic metabolites in the blood and carry disease-causing mutations in genes encoding the implicated enzymes (1). The prototype of this group of disorders is the Smith-Lemli-Opitz syndrome (SLOS; MIM 270400) an autosomal recessive disease caused by a defect in 7-dehydrocholesterol reductase (EC 1.3.1.21), the enzyme that catalyzes the last step of CHOL biosynthesis. Typically, patients affected by SLOS show an accumulation of 7-dehydrocholesterol (7-DHC) and its isomer 8-dehydrocholesterol (8-DHC) in blood and tissues, which is also generally associated with a low CHOL concentration (2, 3). The human 7-dehydrocholesterol reductase gene (*DHCR7*) has been cloned and localized to chromosome 11q12-13 by Moebius et al. (4). To date, 80 mutant *DHCR7* alleles have been identified in patients affected by SLOS, and the most frequent is the mutation IVS8-1G > C, which results in abnormal splicing of exon 9 involving a 134 base-pair insertion of intron 8 sequence, a resultant frameshift, and a premature translation stop (5, 6).

SLOS patients show a large and variable spectrum of inherited morphogenic anomalies, including dysmorphic craniofacial features, microcephaly, multiple internal organ, skeletal, and urogenital malformations, growth and mental retardation and behavioral problems. Syndactyly of the second and third toe is the most common physical finding in these patients. The clinical severity score of SLOS is based on the clinical findings in 10 organs: brain, oral, acral, eye, heart, kidney, liver, lung, bowel and genitalia (7, 8). The clinical severity correlates negatively with the CHOL concentration, and positively with the concentrations of 7-DHC and the sum of dehydrocholesterols (DHC; 7-DHC + 8-DHC) expressed as a fraction of total sterols (8, 9). The DHC fraction and the DHC/CHOL ratio better express the systemic sterol abnormality than absolute blood sterol concentrations, which are influenced by individual variability and are only partly dependent on the rate of endogenous CHOL synthesis (8). Two forms of SLOS can be distinguished clinically, type I and type II. Patients with more severe malformations and elevated mortality rate (type II SLOS) generally show lower total CHOL concentrations (CHOL < 10 mg/dL) and higher DHC/sterol ratios than type I SLOS patients, who have mild malformations and a higher probability of survival (9, 10).

A genetic distinction between type I and type II SLOS is not widely accepted since both types can be caused by mutations at the same genetic locus (7, 8). The clinical manifestations of SLOS may result from CHOL deficiency or from the toxicity of precursor sterols, in particular 7-DHC, which is absent or detected in only trace amounts in plasma of normal subjects. CHOL is essential for cell membrane synthesis, regulation of membrane permeability and fluidity, and as precursor of steroid hormones and bile acids (11). CHOL is also needed for autoprocessing (activation) of sonic hedgehog (Shh), an important protein in early limb patterning and craniofacial development in the embryo (12), and abnormal Shh signaling activation may explain the malformations in SLOS (13). Currently, most SLOS patients are treated with dietary CHOL supplementation and early intervention may be of benefit for the behavioral and autistic aspects of these patients (7, 14).

The identification of the metabolic and molecular bases of SLOS led to the discovery of other defects in post-squalene CHOL biosynthesis, and to date 7 defects have been described in humans (1). The incidence of SLOS is estimated at 1 in 15,000–60,000 births in the European population (6, 15) and at 1 in 60,000 births in the USA (8), while other defects in post-squalene CHOL biosynthesis are extremely rare (16–23).

The biochemical diagnosis of SLOS is generally performed by gas chromatography (GC) or gas chromatography paired to mass spectrometry (GC-MS) for qualitative and quantitative analysis of sterols, and in particular of DHC, following reference methods published previously by Tint et al. and Kelley (3, 24).

In this study we describe our experience over the last six years (2005–2010) in laboratory diagnosis of SLOS and other defects of CHOL biosynthesis by analyzing the sterol profiles in plasma and erythrocyte membranes (EM) by GC-MS. The sterols that we measured were CHOL and its precursors, such as 7-DHC/desmosterol, 8-DHC, lathosterol, and exogenous sterols, such as campesterol, stigmasterol, and sitosterol. Reference values for CHOL and other sterols were calculated in plasma samples of unaffected subjects. Sterols were also analyzed in the EM to confirm the suspect or uncertain results obtained in plasma. In order to ascertain the stability of the DHC, we evaluated storage effects on CHOL and DHC concentrations in plasma and EM samples from SLOS and unaffected subjects, and the correlation between the DHC/CHOL ratio and the storage time of samples.

Materials and methods

Study population

The study population comprised 294 patients (145 males, 149 females) showing clinical findings suggestive of a CHOL biosynthesis defect. All samples were collected during the period from 2005 to 2010. The patients were admitted from the pediatric units of University Hospital Federico II of Naples and pediatric units of other Italian regions. Their ages ranged from a few days to 22 years (mean age 2.7 years).

Specimens

Blood in EDTA (n = 272) and plasma (n = 22) samples were accepted in our laboratory. We recommended that samples be sent in a test tube protected from direct light to prevent 7-DHC photolysis (25) and that they be maintained at 4–8°C and not frozen. Plasma and erythrocytes isolated from blood samples were stored at –20°C until used for analysis.

Reagents

The analytical solvents of HPLC grade, including methanol, dichloromethane, n-hexane, acetone, ethanol and chloroform, were obtained from J.T.Baker (Deventer, Netherlands). Potassium hydroxide was purchased from Merck (Merck KGaA, Darmstadt, Germany). Borate buffer (0.03 mol/L, pH 7.4) was prepared using boric acid (Carlo Erba Reagenti, Italy) and sodium tetraborate (Aldrich-Chemie, Germany). Stock solutions of standard sterols (Sigma, St Louis, MO, USA) were prepared in chloroform/methanol (2:1, v/v) at concentration of 1 mg/mL. The internal standard (IS), 5-α-cholestane (Sigma, St Louis, MO, USA), was prepared as a 50 μg/mL stock solution.

Sample preparation

Plasma and EM sterol profiles were obtained using a GC-MS method previously described (26, 27). Briefly, plasma, buffy coat and erythrocytes were isolated from blood after centrifugation at 1500 *g* for 5 min. Plasma (50 μL) was mixed with 50 μL of IS (50 μg/mL) and hydrolyzed for 60 min at 80°C in 3 mL of 1 N potassium hydroxide in 90% ethanol. After hydrolysis, the sample was diluted with 3 mL of distilled water and extracted three times with 3 mL of hexane. The upper organic phases were pooled and evaporated under a gentle stream of nitrogen at 40°C. Finally, the sterols were derivatized for 20 min at 80°C in 100 μL of a mixture of bis(trimethylsilyl)trifluoro-acetamide and pyridine (7:3) and 1 μL of this solution was injected into the GC-MS. The EM were prepared as follows: erythrocytes were washed twice with physiological saline solution to eliminate residues of plasma. The cells were disrupted with deionized water and with other mechanical techniques (freezing-defrosting, sonication), alternating washings with borate buffer and centrifugations at 3000 *g* for 15 min to remove any trace of hemoglobin. Membranes were homogenized using a mini-potter on ice. Extraction of sterols was performed according to the procedure described above for plasma using an aliquot of homogenate containing at least 1 mg of total proteins measured by Bradford method (28).

GC-MS method

GC-MS analysis was performed by a Fisons apparatus (model GC8000/MD800) controlled by a work station using MassLab 3.4 software. An HP-1MS capillary column (25 m length, 0.2 mm I.D., 0.33 μm film thickness) was used to separate sterols. The carrier gas was helium with a linear velocity of 45 cm/s. Injector and transfer line temperatures were fixed at 280°C. The initial temperature of the oven was 275°C, which was increased to 300°C at a rate of 1°C/min. Qualitative analysis was obtained by scanning the mass range (SCAN mode) from *m/z* 50 to *m/z* 500, and sterol derivatives were identified by comparing their retention times and mass spectra with those of authentic compounds and/or those reported in two libraries (Wiley and National Institute of Standards and Technology). Quantitative analysis was performed by selecting specific ions (SIM mode) for the sterols of interest at *m/z* 217, 357, 372 for 5 α-

cholestane; *m/z* 329, 368, 458 for CHOL; *m/z* 325, 351, 456 for 7-DHC and 8-DHC; *m/z* 255, 353, 458 for lathosterol; *m/z* 343, 382, 472 for campesterol; *m/z* 255, 394, 484 for stigmasterol; and *m/z* 357, 396, 486 for sitosterol (quantification ions are underlined). Sterol concentrations (mg/dL) were obtained by interpolation of analyte/IS peak-area ratio on calibration curves as described previously (26). For determination of 8-DHC concentration, we used the 7-DHC calibration curve, while the concentration of 19-nor-cholestatrienol was calculated from the percentage of the peak area obtained in SCAN mode, compared to the CHOL peak-area value. Since both 7-DHC and desmosterol co-elute at the same retention time, and their spectra show common ions, the quantification of 7-DHC is overestimated due to a contribution from desmosterol, but this is relevant only in samples from subjects without SLOS.

Imprecision and inaccuracy

For each plasma sample, the CHOL concentration (mg/dL) obtained by GC-MS method was compared to the CHOL concentration (mg/dL) obtained using an enzymatic method (Modular; Roche, Switzerland). In order to evaluate imprecision and inaccuracy in the GC-MS method, a positive quality control (Pos QC) was prepared and analyzed as follow: 50 μL of CHOL standard solution (1 g/L) and 5 μL of 7-DHC standard solution (1 g/L) were mixed with 50 μL of IS (50 μg/mL). The solution was evaporated under a gentle stream of nitrogen at 40°C. The dry residue was derivatized and analyzed in duplicate together to the samples. Due to the high instability of 7-DHC, the concentration of this sterol was measured by the UV method (29) in order to adjust the theoretical concentration.

Statistical analysis

Data are reported as average, standard deviation, and percentile (%ile). The statistical significance of differences between groups was evaluated using the appropriate Student's t-test and significance was accepted at the level of $p < 0.05$. Comparison procedures were performed by Passing and Bablok regression, and by linear regression analysis.

Results

From the 294 patients investigated in the 2005–2010 period, we describe 16 SLOS patients, of which 3 were new cases and 13 were follow-up patients. In this period we also identified a new case of chondrodysplasia punctata 2 X-linked (CDPX2; MIM 302960), and performed the follow-up on a lathosterolosis case (MIM 607330). Figure 1 shows typical GC-MS total ion chromatograms obtained using our method, of an unaffected subject (A), and SLOS (B), lathosterolosis (C) and CDPX2 (D) patients. The typical sterol profile in the plasma of unaffected subjects shows two main peaks corresponding to IS and CHOL, while the typical SLOS profile shows additional peaks corresponding to 8-DHC and 7-DHC. Lathosterolosis and CDPX2 profiles are characterized by the presence of lathosterol and 8(9)-cholestenol, respectively. The peaks of diagnostic metabolites and CHOL were well resolved in all chromatograms.

Imprecision and inaccuracy of the GC-MS method were <10%, as evaluated by analyzing 7-DHC in Pos QC. The CHOL levels in plasma samples of unaffected subjects measured by GC-MS were compared to those obtained using the enzymatic method. In this comparison, enzymatic CHOL values of SLOS patients were excluded because CHOL oxidase shows low specificity in discriminating CHOL from DHC and gives falsely high values (30). The results obtained with the GC-MS CHOL method were in good agreement with those obtained using the enzymatic method ($n = 276$; slope: 1.08, CI 95%: 0.978–1.182; intercept: –0.42, CI 95%: –7.818–6.500).

The clinical findings in the study population, divided on the basis of affected organ, are listed in Table 1. The most common disease features were brain malformations (53%), typical SLOS craniofacial features (41.3%) and acral deformities (39.2%). The clinical features were present equally in both males and females, and the most of individuals presented multiple SLOS clinical signs.

Plasma sterol concentrations in the 16 SLOS patients and 276 unaffected subjects are reported in Table 2. The reference values calculated in unaffected subjects, aged from 1 week to 11 years, are reported as the range from 2.5%ile to 97.5%ile for CHOL values, and up to 95%ile for other sterol values. SLOS patients showed abnormally higher concentrations of 7-DHC and 8-DHC compared to unaffected subjects. The DHC concentrations in SLOS samples range from 5% to 86% (average 33%) relative to total sterols, and were at least 100-fold higher than the upper limit value (95%ile) of unaffected subjects. Similarly, the median DHC/CHOL ratio in SLOS patients was 0.35 and at least 150-fold higher than the upper limit value (95%ile) of unaffected subjects. In addition to DHC, a sterol artifact from GC analysis of 8-DHC (31) was detected in only 5 of 16 samples from SLOS patients (31%), it was identified as 19-nor-cholestatrienol by mass spectrum, and its concentration ranged from 0.2 to 6.7 mg/dL. Forty-four percent of the SLOS patients (7/16) had CHOL concentrations lower than 2.5%ile (37.1 mg/dL) of the reference values, while the remaining 56% (9/16) had concentrations ranging from 39.9 to 194.6 mg/dL, which was within the reference values of unaffected subjects. Even though CHOL concentrations show wide individual variability, the average values for SLOS and unaffected subjects were significantly different ($p = 0.002$). In contrast, the concentrations of lathosterol and plant sterols in plasma samples of SLOS and unaffected subjects were not statistically different.

EM samples ($n = 15$) of SLOS patients stored at –20°C for a variable time, ranging from 1 to 20 months, were analyzed. The EM samples were isolated from different blood samples of nine SLOS patients collected at different times during the period of this study (three samples from patient #6, two samples from patients #2, #3, #8, #9 and one sample from patients #4, #10, #11, #14) (Table 3). In order to obtain a normal EM sterol profile, we analyzed EM samples of unaffected subjects accepted in 2010 ($n = 20$). As shown in Table 3, CHOL concentrations in EM of SLOS samples are on average 3-fold lower than unaffected subjects ($p < 0.001$), and the DHC concentrations are on average 55-fold higher in SLOS patients than in unaffected subjects ($p < 0.01$). The average of DHC/CHOL ratio in EM of SLOS patients was 0.16 and was at least 200-fold higher than unaffected

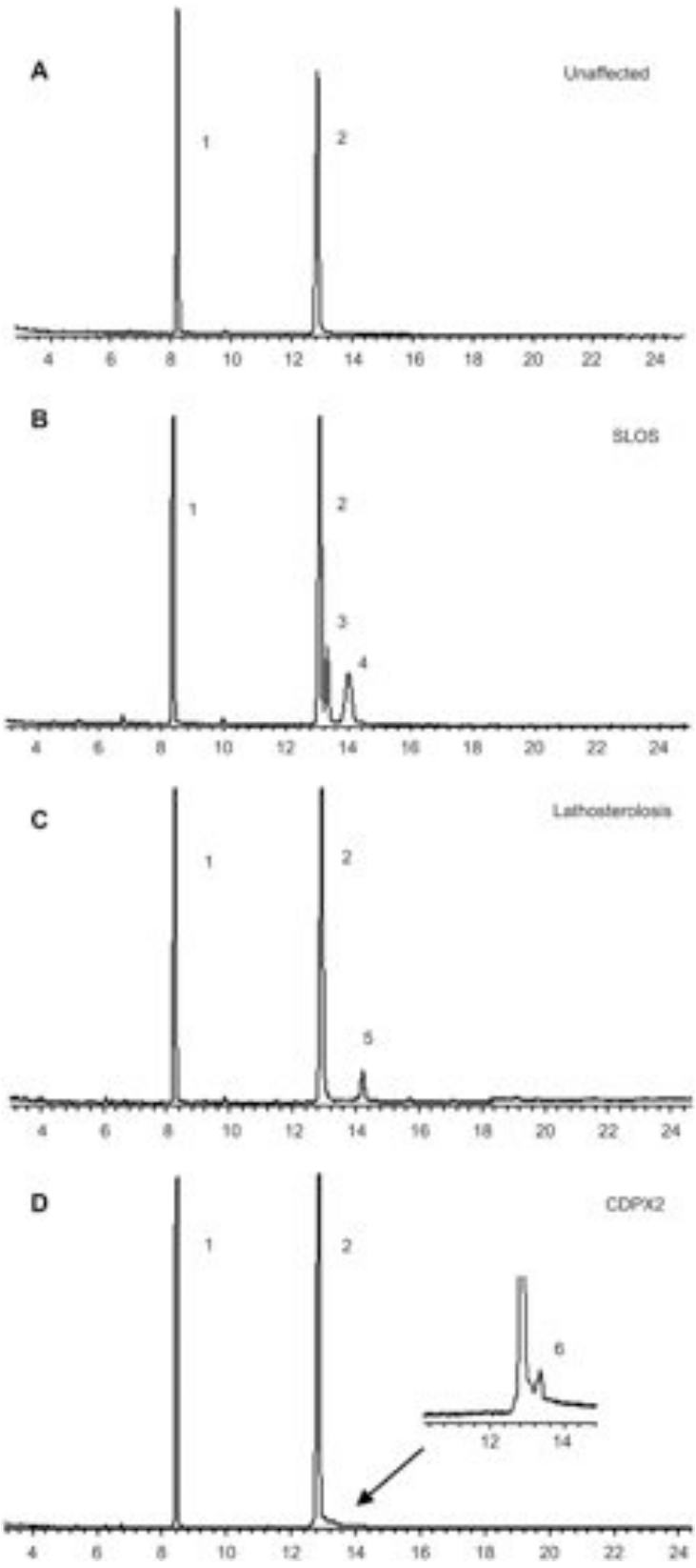

Figure 1 Typical GC-MS total ion chromatograms of sterols in plasma of an unaffected subject (A), and SLOS (B), lathosterolosis (C) and CDPX2 (D) patients.
1: IS; 2: CHOL; 3: 8-DHC; 4: 7-DHC; 5: lathosterol; 6: 8(9)-cholestenol.

Table 1 Main clinical findings of the study population.

Organ	%	Clinical findings
Brain	53	Holoprosencephaly, microcephaly, agenesis of the corpus callosum, convulsion, psycomotor retardation
Craniofacial	41.3	Ptosis, anteverted nares, micrognathia, broad nasal bridge, hypertelorism
Acral	39.2	Syndactyly, hypotonia, hypertonia, hypoplasia
Liver	13	Liver disease, icterus
Eye	13	Cataracts, microphthalmia
Heart	10.8	Heart arrhythmia, hypertrophic cardiomyopthy, atrial septal defect
Genitalia	4.4	Genital malformations, precocious puberty
Bowel	4.4	Short bowel syndrome, inguinal hernia
Oral	4.3	Cleft palate
Lung	2.2	Bronchopulmonary dysplasia
Kidney	2.2	Ectopic kidney

subjects. In SLOS samples, the DHC/CHOL ratio decreased during storage. As depicted in Figure 2A, the regression analysis performed between the DHC/CHOL ratio and storage time depicts a significant linear negative correlation (slope = –0.0223; r = 0.598; $p < 0.01$). Furthermore, the DHC/CHOL ratio in plasma of SLOS samples (Figure 2B; n = 12) analyzed within 24 h and after a variable period of storage, ranging from 4 to 22 months, also showed a significant linear negative correlation with the storage time (slope = –0.0098; r = 0.449; $p < 0.025$). Based on these results in SLOS samples stored at –20°C, the rate at which the DHC/CHOL ratio in EM decreases during storage is at least 2-fold higher than the decrease observed in plasma samples.

Discussion

In this study, we report our experience over the last six years in the diagnosis of CHOL biosynthesis defects. Over this period, we investigated the sterol profile in plasma samples from 294 patients using GC-MS. Among these patients we reported 16 affected by SLOS, of which 13 had been previously identified (from 1995 to 2004), and three were new SLOS cases. In addition, we identified a new case of CDPX2 in 2009. The plasma sterol profile of the CDPX2 patient, consistent with previous studies, showed an abnormal presence of 8(9)-cholestenol, and because of the variability of CDPX2 clinical manifestations, the biochemical determina-

Table 2 Plasma sterol profiles by GC-MS in samples from Smith-Lemli-Opitz patients (n = 16) compared to unaffected subjects (n = 276).

Patients	Age, years	Sex	CHOL	8-DHC	7-DHC[a] ($mg \cdot dL^{-1}$)[b]	Lathosterol	Campesterol	Stigmasterol	Sitosterol	DHC/CHOL
SLOS patients										
#1	6	M	28.9	2.1	4.9	0.25	0.31	0.07	0.16	0.24
#2	1	M	80.4	14.2	14.3	0	0	0	0	0.35
#3	22	F	11.2	3.0	7.2	0.69	0.04	0.07	0.06	0.91
#4	2	F	194.6	7.3	3.8	0.16	0.22	0.02	0.16	0.06
#5	0.1	M	9.0	5.1	6.9	0	0	0	0	1.33
#6	14	F	134.1	21.0	34.1	0.30	0.10	0.09	0.10	0.41
#7	9	M	24.3	2.4	2.3	0	0	0	0	0.19
#8	7	M	39.9	4.3	6.2	0.20	0.51	0.10	0.39	0.26
#9	15	M	160.6	18.8	28.9	0.84	0.14	0.02	0.02	0.30
#10	15.1	M	57.2	7.7	11.5	0	0	0	0	0.34
#11	10	F	123.2	9.5	12.9	0.32	0.12	0.14	0.10	0.18
#12	0.6	F	8.8	21.4	34.2	0	0	0	0	6.32
#13	3.8	F	89.7	11.6	11.7	0	0	0	0	0.26
#14	0.1	F	45.2	10.0	10.2	0.22	0.10	0.05	0.07	0.45
#15	0.2	M	26.8	8.8	11.2	0.32	0.15	0.14	0.13	0.75
#16	0.04	F	9.6	9.9	15.7	0	0	0	0	2.67
Average (SD)	6.6 (6.9)		65.2 (59.3)[c]	9.8 (6.3)	13.5 (10.2)[d]	0.21 (0.25)	0.11 (0.14)	0.04 (0.05)	0.07 (0.10)	0.94 (1.17)[e]
Unaffected subjects										
Range[f]	0.02–11		37.1–217.4	<0.10	<0.19	<0.25	<0.33	<0.15	<0.39	<0.002

0: less than the detection limit (LOD = 0.01). [a]7-DHC + desmosterol contribution to ion *m/z* 325 abundance. [b]SLOS sterol concentrations correspond to the first determination. [c]$p = 0.0002$ vs. unaffected subjects. [d]$p < 0.0001$ vs. unaffected subjects. [e]$p < 0.05$ vs. unaffected subjects. [f]range is reported as 2.5%ile–97.5%ile for age and CHOL, and <95%ile for the other sterols and DHC/CHOL ratio.
Conversion factors to SI units (mg/dL × Factor = mmol/L) are: 0.0259 for Cholesterol and Lathosterol; 0.0260 for 7- and 8-DHC; 0.0250 for Campesterol; 0.242 for Stigmasterol; 0.241 for Sitosterol.

Table 3 CHOL and DHC concentrations in EM samples (n = 15) from SLOS and unaffected subjects (n = 20) analyzed after different storage times.

Patients	CHOL, mg·g prot^{-1}	DHC, mg·g prot^{-1}	DHC/CHOL	Storage time, months
SLOS patients				
#2 (Sample 1)	53.3	6.49	0.12	11
#2 (Sample 2)	43.9	4.45	0.10	4
#3 (Sample 1)	21.6	0.73	0.03	19
#3 (Sample 2)	13.7	0.84	0.06	13
#4 (Sample 1)	124.4	2.05	0.02	14
#6 (Sample 1)	17.1	0.63	0.04	20
#6 (Sample 2)	17.0	3.61	0.21	11
#6 (Sample 3)	17.3	15.63	0.90	1
#8 (Sample 1)	36.0	0.45	0.01	18
#8 (Sample 2)	56.0	4.07	0.07	10
#9 (Sample 1)	26.5	5.23	0.20	14
#9 (Sample 2)	28.9	2.89	0.10	4
#10 (Sample 1)	59.2	22.88	0.39	5
#11 (Sample 1)	19.6	1.24	0.06	19
#14 (Sample 1)	89.6	2.88	0.03	7
Average (SD)	41.6 (31.3)[a]	4.94 (6.24)[b]	0.16 (0.23)[c]	11 (6)
Unaffected subjects				
Average (SD)	123.6 (39.2)	0.09 (0.11)	0.7· 10^{-3} ($0.8 \cdot 10^{-3}$)	6 (3)

[a]$p < 0.001$ vs. unaffected subjects. [b]$p < 0.01$ vs. unaffected subjects. [c]$p < 0.025$ vs. unaffected subjects.

tion of 8(9)-cholestenol in plasma samples of suspected patients is required to confirm the diagnosis (17).

In this study, we identified three new SLOS cases from 280 suspected patients (1.08%). Kelley et al. reported results from the two laboratories that perform at least 80% of the biochemical testing for SLOS in the USA; they identified 40 new cases per year between 1995 and 1998, estimating an incidence of <1 in 60,000 births (8). Although our laboratory is recognized for the diagnosis of CHOL biosynthesis defects, the SLOS incidence cannot be evaluated accurately from our data, since the study population consisted of a limited number of suspected patients coming from Italian pediatric units. Besides, as previously reported, the SLOS incidence biochemically confirmed is lower than the estimates based on known carrier rates of *DHCR7* mutations in the general population. This discrepancy is due to the difficulty in diagnosing of SLOS cases at either extreme of the clinical spectrum, such as cases resulting in prenatal and neonatal deaths and cases of mild/atypical SLOS in surviving children, and prenatal mortality for the most severely affected cases of SLOS may be as high as 80% (15, 32). The clinical signs in diseases caused by defects of CHOL biosynthesis are multiple and variable. These signs are generally present at birth but may only be revealed some years after birth. The 280 patients investigated for the first time in this study showed the typical SLOS clinical findings described by Kelley et al. (8), although SLOS was only confirmed in 1.08% of these subjects and CDPX2 in only 0.4% of the subjects. The remaining patients were unaffected by CHOL biosynthesis defects, and this result underlines the importance of sterol profile analysis for the diagnosis of SLOS and other defects of CHOL biosynthesis.

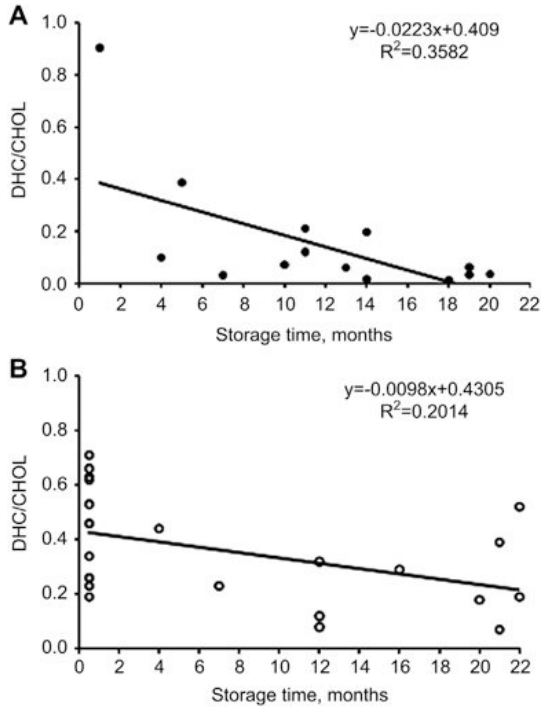

Figure 2 Correlation between the storage time and DHC/CHOL ratio in EM (panel A: $y = -0.0223x + 0.409$; $r = 0.598$; $p < 0.01$; $n = 15$) and plasma samples of SLOS (panel B: $y = -0.0098x + 0.430$; $r = 0.449$; $p < 0.025$; $n = 12$).

In this work, the sterol concentrations in plasma samples of SLOS and unaffected subjects were in agreement with the literature, even though, compared to that reported by Kelley, our 7-DHC reference values were overestimated due to desmosterol co-eluting with 7-DHC (24, 30). Studies on reference values for plasma CHOL are generally focused on the upper limit of the reference range, and very few data are

available for the lower limit of CHOL reference values in plasma of children stratified by age. Lockitch et al. determined age-related reference value for serum CHOL in 450 healthy children aged from 1 to 19 years (33), and in the age group from 1 to 3 years they found a value of 44.5 mg/dL (1.15 mmol/L) at the 2.5%ile. In contrast, the 2.5%ile in our unaffected subjects was 37.1 mg/dL, and the difference could be due to differences in age, health status and ethnic origin between the two study populations.

In plasma samples from five of SLOS patients we detected a peak corresponding to 19-nor-cholestatrienol, and its level, as percentage of total sterols, ranged from 0.3% to 3.4%. The presence of this metabolite is in agreement with other studies (31, 34), and as demonstrated by Ruan et al. this sterol is a GC artifact of samples containing 8-DHC and not a component of normal or SLOS blood (31). Elevated DHC concentrations in plasma and tissues are considered diagnostic markers of SLOS, and CHOL levels lower than reference values are considered a marker of SLOS or of other CHOL biosynthesis defects. Only 44% of the SLOS patients in our study had CHOL concentrations lower than 2.5%ile of the reference values obtained in our study or those reported by Lockitch et al. (33). Furthermore, previous studies have found that plasma of SLOS patients measured with the enzymatic assay may give total CHOL values close to the lower limit or within the reference ranges of healthy subjects (30). These observations suggest that some SLOS patients might be undiagnosed if diagnosis is based only on the enzymatic measurement of CHOL rather than the sterol profile.

Our method also allows the detection of plant sterols and definition of their reference values. Plant sterols are present in small and similar amounts in unaffected subjects and SLOS patients. The determination of plant sterol reference values is important because the concentration of these sterols is increased in sitosterolemia, an inherited defect of lipid metabolism, characterized by abnormal intestinal absorption of CHOL and plant sterols (35).

In order to evaluate the effects of storage on the stability of the CHOL metabolites, we analyzed CHOL and DHC concentrations in plasma and EM samples of SLOS patients and unaffected subjects at different times after storage at –20°C. DHC concentrations and DHC/CHOL ratios in the plasma of SLOS patients were still significantly elevated after storage at –20°C, although they showed on average a 40% reduction after 22 months. After a similar period of storage (20 months), the DHC/CHOL ratios in EM decreased on average by 88%. The average DHC level in EM, as percentage of the total sterols, was 5-fold lower than that reported by Tint et al. (3), using fresh EM samples from SLOS patients. In addition, the DHC/CHOL ratio reported in our study is 4- to 8-fold lower than the values reported in EM samples from two SLOS patients analyzed by Jira et al. (36). The regression analysis between the DHC/CHOL ratio and storage time showed a significant negative linear correlation ($p < 0.01$), which confirms the high instability of DHC in EM samples during storage, and is in agreement with a previous study performed using dried blood spots (26). In addition, as recently reported by Xu et al. the instability of 7-DHC in solution and in liposomes is due to its susceptibility to peroxidation, which is at least 10-fold more reactive than arachidonic acid, a polyunsaturated fatty acid that is normally considered to be a highly oxidizable lipid, and at least 200-fold more reactive than cholesterol (37). Based on these results, the instability of DHC should be prevented by adding antioxidants in plasma and EM to stabilize lipids during the storage and the analytical procedure. The study of antioxidant effects, added to blood, plasma, and other biological fluids, on DHC peroxidation merit further research. Our study highlights the need among obstetricians, neonatologist, and other healthcare providers for increased awareness of the various presentations of SLOS in the fetal and newborn periods in order to diagnose this condition effectively. The analysis of sterol profiles should be required for all patients showing suspect clinical features suggestive of CHOL biosynthesis defects. In addition, the effect of storage time on DHC concentrations in plasma and EM, and the resultant possibility of false-negative results, should be taken into account when interpreting the results.

Finally, considering that SLOS is the most frequent of the CHOL biosynthesis defects and its prevalence has not yet been accurately determined, further work should be directed to implement new high-throughput methods for the measurement of DHC in dried blood spots. These methods, based on tandem mass spectrometry or other approaches, could represent useful tools for newborn screening of this syndrome (38).

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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