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5	Corresponding Author	Family Name	**Oláh**
6		Particle	
7		Given Name	**Anna V.**
8		Suffix	
9		Organization	University of Debrecen
10		Division	Department of Laboratory Medicine, Medical and Health Science Center
11		Address	98 Nagyerdei krt., Debrecen 4032, Hungary
12		e-mail	olaha@med.unideb.hu
13	Author	Family Name	**Szabó**
14		Particle	
15		Given Name	**Gabriella P.**
16		Suffix	
17		Organization	University of Debrecen
18		Division	Department of Pediatrics
19		Address	Debrecen, Hungary
20		e-mail	
21	Author	Family Name	**Varga**
22		Particle	
23		Given Name	**József**
24		Suffix	
25		Organization	University of Debrecen
26		Division	Department of Nuclear Medicine, Medical and Health Science Center
27		Address	Debrecen, Hungary
28		e-mail	
29	Author	Family Name	**Balogh**

30		Particle	
31		Given Name	**Lídia**
32		Suffix	
33		Organization	Semmelweis University
34		Division	1st Department of Pediatrics
35		Address	Budapest, Hungary
36		e-mail	
37	Author	Family Name	**Csábi**
38		Particle	
39		Given Name	**Györgyi**
40		Suffix	
41		Organization	University of Pécs
42		Division	Department of Pediatrics
43		Address	Pecs, Hungary
44		e-mail	
45	Author	Family Name	**Csákváry**
46		Particle	
47		Given Name	**Violetta**
48		Suffix	
49		Organization	Markusovszky County Hospital
50		Division	
51		Address	Szombathely, Hungary
52		e-mail	
53	Author	Family Name	**Erwa**
54		Particle	
55		Given Name	**Wolfgang**
56		Suffix	
57		Organization	LKH Medical University
58		Division	
59		Address	Graz, Austria
60		e-mail	
61	Author	Family Name	**Balogh**
62		Particle	
63		Given Name	**István**
64		Suffix	
65		Organization	University of Debrecen
66		Division	Department of Laboratory Medicine, Medical and Health Science Center

Address	98 Nagyerdei krt., Debrecen 4032, Hungary	
e-mail		

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Abstract

Smith–Lemli–Opitz syndrome (SLOS), a multiple congenital anomaly with severe mental retardation, is caused by decreased activity of 7-dehydrocholesterol reductase. Fifteen Hungarian patients were diagnosed with SLOS on the basis of clinical symptoms, serum cholesterol, 7-dehydrocholesterol, and molecular genetic testing. Their age at the time of diagnosis in mild SLOS (n =4, clinical score <20) was 0.5–18 years, cholesterol was 2.37 ± 0.8 mmol/L, and 7DHC was 0.38 ±0.14 mmol/L. In the group of typical SLOS (n =7, score 20–50), the diagnosis was set up earlier (age of 0.1–7 years); t-cholesterol was 1.47 ±0.7 mmol/L, and 7DHC was 0.53 ±0.20 mmol/L. Patients with severe SLOS (n =4, clinical score >50) died as newborns and had the lowest t-cholesterol (0.66 ± 0.27 mmol/L), and 7DHC was 0.47 ±0.14 mmol/L. Correlation coefficient with clinical severity was 0.74 for initial t-cholesterol and 0.669 for Cho/7DHC. Statistically significant difference was between the initial t-cholesterol of mild and severe SLOS (p =0.01), and between the Cho/7DHC ratios of groups (p =0.004). In severe SLOS, the percentage of α-lipoprotein was significantly lower than in typical (p =0.003) and mild SLOS (p =0.004). Although serum albumin, total bilirubin, and hemostasis parameters remained in the reference range during cholesterol supplementation (n =10) combined with statin therapy (n =9), increase of aspartate aminotransferase and alanine aminotransferase in 50 % of the patients probably refers to a reversible alteration of liver function; therefore, statin therapy was suspended. *Conclusion*: life expectancy is fundamentally determined by the initial t-cholesterol, but dehydrocholesterol and α-lipoprotein have prognostic value. Accumulation of hepatotoxic DHC may inhibit the synthesis of α-lipoproteins, decreasing the reverse cholesterol transport. During statin therapy, we suggest monitoring of lipid parameters and liver function.

Keywords separated by ' - '	7-Dehydrocholesterol - Cholesterol - Lipoprotein electrophoresis - Liver function - Smith–Lemli–Opitz syndrome - Statin
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ORIGINAL ARTICLE

Relation between biomarkers and clinical severity in patients with Smith–Lemli–Opitz syndrome

Anna V. Oláh · Gabriella P. Szabó · József Varga · Lídia Balogh · Györgyi Csábi · Violetta Csákváry · Wolfgang Erwa · István Balogh

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Abstract Smith–Lemli–Opitz syndrome (SLOS), a multiple congenital anomaly with severe mental retardation, is caused by decreased activity of 7-dehydrocholesterol reductase. Fifteen Hungarian patients were diagnosed with SLOS on the basis of clinical symptoms, serum cholesterol, 7-dehydrocholesterol, and molecular genetic testing. Their age at the time of diagnosis in mild SLOS (n=4, clinical score <20) was 0.5–18 years, cholesterol was 2.37±0.8 mmol/L, and 7DHC was 0.38±0.14 mmol/L. In the group of typical SLOS (n=7, score 20–50), the diagnosis was set up earlier (age of 0.1–7 years); t-cholesterol was 1.47±0.7 mmol/L, and 7DHC was 0.53±0.20 mmol/L. Patients with severe SLOS (n=4, clinical score>50) died as newborns and had the lowest t-cholesterol (0.66±0.27 mmol/L), and 7DHC was 0.47±0.14 mmol/L. Correlation coefficient with clinical severity was 0.74 for initial t-cholesterol and 0.669 for Cho/7DHC. Statistically significant difference was between the initial t-cholesterol of mild and severe SLOS (p=0.01), and between the Cho/7DHC ratios of groups (p=0.004). In severe SLOS, the percentage of α-lipoprotein was significantly lower than in typical (p=0.003) and mild SLOS (p=0.004). Although serum albumin, total bilirubin, and hemostasis parameters remained in the reference range during cholesterol supplementation (n=10) combined with statin therapy (n=9), increase of aspartate aminotransferase and alanine aminotransferase in 50 % of the patients probably refers to a reversible alteration of liver function; therefore, statin therapy was suspended. *Conclusion*: life expectancy is fundamentally determined by the initial t-cholesterol, but dehydrocholesterol and α-lipoprotein have prognostic value. Accumulation of hepatotoxic DHC may inhibit the synthesis of α-lipoproteins, decreasing the reverse cholesterol transport. During statin therapy, we suggest monitoring of lipid parameters and liver function.

Keywords 7-Dehydrocholesterol · Cholesterol · Lipoprotein electrophoresis · Liver function · Smith–Lemli–Opitz syndrome · Statin

A. V. Oláh (✉) · I. Balogh
Department of Laboratory Medicine, Medical and Health Science Center, University of Debrecen, 98 Nagyerdei krt., Debrecen 4032, Hungary
e-mail: olaha@med.unideb.hu

G. P. Szabó
Department of Pediatrics, University of Debrecen, Debrecen, Hungary

J. Varga
Department of Nuclear Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

L. Balogh
1st Department of Pediatrics, Semmelweis University, Budapest, Hungary

G. Csábi
Department of Pediatrics, University of Pécs, Pecs, Hungary

V. Csákváry
Markusovszky County Hospital, Szombathely, Hungary

W. Erwa
LKH Medical University, Graz, Austria

List of abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
t-Cho	Total cholesterol
CK	Creatine kinase
7-DHC	7-Dehydrocholesterol

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DHCR7	7-Dehydrocholesterol reductase
HDL-C	High-density cholesterol
KM	Kilomicron
GGT	gamma-Glutamyltransferase
LDH	Lactate dehydrogenase
SLOS	Smith–Lemli–Opitz syndrome

Introduction

The Smith–Lemli–Opitz syndrome (OMIM 270400), an autosomal recessive, severe developmental disorder with multiple congenital anomalies, is caused by a defect of cholesterol biosynthesis [3, 12, 20–22]. The syndrome was first reported by Smith et al. in 1964 and was characterized by a dysmorphic face, microcephaly, hypospadiasis, and severe growth retardation [26]. The cause of Smith–Lemli–Opitz syndrome (SLOS) is the defective function of the 7-dehydrocholesterol reductase (DHCR7) enzyme which catalyzes the last step of cholesterol biosynthesis [7, 32]. This enzyme is responsible for the transformation of 7-dehydrocholesterol to cholesterol. Cholesterol is an important component of the cell membrane, mitochondrial membrane, and myelin formation in the brain, spinal cord, and peripheral nervous system. Cholesterol acts also as a precursor for bile acids and steroid hormones, and plays an important role in the embryonic hedgehog signaling mechanism during embryogenesis as well [13]. For phenotypic characterization, the modified Bialer scoring system of Kelley and Hennekam has been used which weight embryologically separate organ systems equally [12, 13].

Calculation of clinical severity scores is based on evaluation of anatomical abnormalities in ten embryologically separated organs (brain, oral region, eye, heart, kidney, liver, lung, bowel, and genitals) [13]. In 1998, the human *DHCR7* gene was cloned by three different groups [4, 35, 36]. Over 140 different mutations in the *DHCR7* gene have been published to date [39, 40 and Human Gene Mutation Database]. For the treatment of the disease, during the past 20 years, different therapeutic approaches were applied such as cholesterol substitution, with or without bile acids, and simvastatin that decreases the level of 7DHC and enhances the residual activity of the DHCR7, reportedly with improvement in both biochemical parameters and clinical symptoms [7, 8, 18, 32]. Recently, the efficiency of cholesterol supplementation has been debated [5, 25], and statins cannot be considered as a safety approach in each SLO patient because of potential side effects [28].

In the past, the diagnosis of SLO syndrome was established mainly on the basis of characteristic phenotypic features, including severe mental and somatic retardation. The wide range of gene mutations in SLO syndrome and alteration of biochemical parameters have been described in different populations [2, 27]. A new mutation of SLO syndrome in our first patient was identified by L. Kozak and his coworkers [31]. Very recently, we published a paper [1] on the genetic background of the Hungarian patients with SLOS. Similar to the finding of Porter [21], we did not find a strong connection between the genotype and phenotype. Therefore, the relation between biomarkers and clinical feature was investigated in diagnostic and therapeutic aspects.

Patients and methods

During the last decade, 15 patients (age of 0.1–18 years, eight males and seven females) were diagnosed with SLO syndrome in Hungary, the first case in 2002 [30]. After observation of clinical symptoms, their diagnosis was proved by serum 7DHC level. Anatomical abnormalities of ten embryologically separated organs (brain, oral region, acral, eye, heart, kidney, liver, lung, bowel, and agenitals) have been scored [13]. On the base of clinical severity scores, patients were assigned to three groups: patients with mild SLOS were defined by a score below 20, typical SLOS means 20–50 scores, and a score above 50 means a severe type of SLO syndrome [12, 13]. The patients enrolled into the mild group are still alive (n=4; age when the diagnosis was set up, 0.5–18 years). In the typical SLOS group (n=7; age at diagnosis, 0.1–7 years), two children lived less than 2 years of age; five children are still alive. All patients with severe SLOS died in the newborn period (n=4, age<2 months). After setting up the diagnosis, all patients received cholesterol substitution (n=10, Cholesterol Module, 50–250 mg/kg/day, Nutricia; no 18.012). It was completed with statin therapy in nine patients. Dosage of the statin was 0.2–0.4 mg/kg/day, and clinical state and efficiency of therapy were monitored by regular clinical checkup in 3- to 6-month periods including the evaluation of clinical condition, anthropometric parameters, serum cholesterol, 7DHC level, liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase, gamma-glutamyltransferase (GGT)), and creatine kinase (CK) activity. Statin therapy was suspended in five patients because of the side effects.

Rapid determination of 7DHC in serum was performed by the modified UV spectrophotometric method of Honda et al. [6] and was compared with the gas chromatography–mass spectrometry (GC/MS) method described by Kelly [10]. Serum samples of the patients were stored frozen at −20 °C until use (~2 weeks). A total of 200 μL serum was extracted with 1.6 mL of c-hexane/i-propanol mixture (3:1). Humatrol normal serum (Human, Magdeburg, Germany) was used as negative control. For calibration, 200 μL aliquot of negative control serum and 100 μL of 400 mg/L

7DHC stock solution (c-hexane/i-propanol, 3:1) were mixed, and then 1.5 mL extracting solution was added to it. All samples were covered and centrifuged at 400×*g* for 5 min. The absorbance of clear supernatant was measured at 285 nm. For reagent, blank c-hexane/i-propanol, 3:1, was used. The between-run and within-run coefficients of variation were <10 %. The detection limit of this method is about 10 mg/L (or 5 mg/L when using 400 μL of serum), and it is linear in the range of 10–400 mg/L. The t-cholesterol/7-dehydrocholesterol (7-DHC) ratio was calculated in the same unit (in milligram per liter). These 7-DHC concentrations were compared to those obtained by a published GC/MS method [10]. When we have compared the UV method with GC in ten samples (LKH Graz, Austria), the same samples proved to be positive although the UV method resulted in lower 7-DHC values. Serum total cholesterol level was determined by a routinely used enzymatic colorimetric assay (Modular, Roche Ltd, Mannheim, Germany). Therefore, we compared total cholesterol (cholesterol oxidase (CHOD)–peroxidase (POD)) results with LC-MS, and UV method of 7DHC with LC-MS (7+8DHC) in eight patients. The correlation coefficient was 0.962 between the two cholesterol methods and 0.9477 between (7+8DHC) and 7DHC results. The proportion of alpha-, beta-lipoproteins, and kilomicron fractions was analyzed by agarose gel electrophoresis (Hydragel 15, Sebia, AL Instruments, Lisses, France). Enzyme activities were determined in serum by IFCC (AST, ALT, GGT, CK) or optimized UV kinetic method (LDH) on a Modular P800 analyzer (Roche Ltd, Mannheim). Serum total and conjugated bilirubin was determined by colorimetric assay and cholesterol by enzymatic colorimetric method (CHOD–POD) on the same analyzer.

Statistical comparison of cholesterol, 7DHC, and α-lipoprotein levels among the three groups was carried out by Kruskal–Wallis test. For the cholesterol and α-lipoprotein levels that showed Gaussian distribution, the Bonferroni test was applied for pairwise comparisons.

Results

The age of patients when the diagnosis was set up was in wide range (0.5–18 years); in the mild-type SLOS group (n=4, clinical score <20), the mean level of serum cholesterol was 2.37±0.8 mmol/L and the mean of 7DHC was 0.38±0.14 mmol/L (147±55 mg/L). In the group of typical SLOS, diagnosis was set up earlier (age of 0.1–7 years) (n=7; clinical score, 20–50); the mean of serum cholesterol level was 1.47±0.7 mmol/L, and 7DHC was 0.53±0.20 mmol/L (202±77 mg/L).

Those patients who died as newborns (at the age of less than 2 months) were enrolled into the severe SLOS group (n=4, clinical score >50). Their 7DHC level (0.47±0.14 mmol/L; 181±52 mg/L) was similar to the typical SLO group with great scatter, but their cholesterol level (0.66±0.27 mmol/L) was significantly lower than in mild SLOS (2.37±0.8 mmol/L). In spite of the limited number of patients, our data refer to the prognostic value of initial cholesterol level regarding the life expectance.

Clinical severity scores, genotypes, and initial lipid parameters are listed in Table 1. Correlation between initial serum cholesterol and clinical scores is shown in Fig. 1 (n=15; regression line, r=0.74). The initial Cho/7DHC ratio showed a similar weak inverse relationship with clinical scores (r=0.669). Statistical evaluation of the three SLOS groups showed significant difference in the initial cholesterol levels of the mild and severe SLOS groups (Bonferroni test, p=0.01; Fig. 2a). A significant difference could be observed between the Cho/7DHC ratios of the groups as well (Kruskal–Wallis test, p=0.004; Fig. 2b).

Lipoprotein gel electrophoresis detected decreased percentage of α-lipoprotein in severe SLOS (7±5 %) compared to the age-matched control group 25.4±1.6 % (n=5; age, 0–3 years) without lipid disorder or to the typical (31.6±9 %) and mild SLOS (33±6 %). Bonferroni test proved that the ratio of α-lipoprotein in the severe SLOS group was significantly lower than in the typical (p=0.003) and mild SLOS group (p=0.004); see Fig. 2c. It might be clinically relevant that alpha lipoproteins are hardly detectable by gel electrophoresis in severe SLOS (Fig. 3a), while the distribution of lipoproteins is generally normal in mild cases (Fig. 3b).

Our findings suggest that the initial level of serum cholesterol fundamentally determines the severity and life expectancy in SLOS, and the ratio of Cho/7DHC and α-lipoprotein has additional prognostic value.

Liver function was monitored in those patients who survived the age of 1 year (n=10). LDH activity was elevated in one patient, and creatine kinase was high in another patient—both of them were treated by simvastatin. We have observed more cases of elevated AST and ALT activities in typical SLOS (n=4/5; age, 6±5.1 years; score, >20) than in mild cases (n=1/5; age, 5.1±1 years; score, <20) during cholesterol supplementation (n=10) combined with statin therapy (n=9). The transaminase activities were twice as much in typical SLOS (AST, 50±29 U/L; ALT, 47±25 U/L) than in mild type (AST, 23±7 U/L; ALT, 21±21 U/L). Besides the similar age in the two groups, we have to notice that the duration of therapy was longer (2.9±2.6 years) in the typical group compared to the mild SLOS (0.8±1 year). Although serum albumin (36.8–47.3 g/L), total bilirubin, and the hemostasis parameters remained in the reference range, the increase of AST and ALT in 50 % of the patients probably refers to a reversible alteration of liver function. Increased sensibility of liver in SLOS may be the consequence of higher DHC level. When statin treatment (simvastatin, atorvastatin) was suspended in the affected five patients, their liver enzyme activities returned to the

Table 1 Initial individual clinical data, genotypes, and lipid levels in patients with SLOS (n=15)

Patient	Sex	Age at diagnosis (years)	Age (years)	Genotype	Severity score	Clinical severity type	7DHC (mmol/L)	7DHC (mg/L)	Cho (mmol/L)	Cho (mg/L)	Cho/7DHC (mg/mg)	Lipoprotein		KM (%)
												Alpha (%)	Beta (%)	
				Reference ranges→	Mild<20, severe>50		<0.00038	<0.15	<1 year, 1.3–4.9; >1 year, 2.8–5.2	–[a]	>10,000	24–27[b]	73–76[b]	0[b]
1	F	6	10	c.452G>A, c.740 C>T	10	M	0.34	130	3.47	1343	10.30	32	68	
2	F	18	18	c.452 G>A, ?	15	M	0.22	87	2.44	944	10.90	42	58	0
3	F	0.5	2	c.1295A>G; c.1328G>A	15	M	0.56	217	2.00	774	3.56	30	70	0
4	M	0.6	1	c.976G>T, c.452G>A	20	M	0.41	156	1.57	608	3.89	28	72	0
5	M	7	15	c.964-1G>C, c.1097G>T	25	T	0.26	102	2.77	1072	10.50	38	62	0
6[c]	F	0.1	1[c]	c.976G>T, c.374 A>G	40	T	0.53	205	2.10	813	3.96	25	75	0
7	M	0.1	1	c.452G>A, c.1295 A>G	20	T	0.71	274	1.47	569	2.07	41	59	0
8[d]	M	0.1	3	IVS8-1G>C, c.1190C>T	40	T	0.66	253	1.08	418	1.65	32	64	4
9[d]	M	0.2	8	IVS8-1G>C, c.1190C>T	50	T	0.78	302	1.40	542	1.79	28	72	0
10	M	0.1	2	c.730G>A, c.976G>T	40	T	0.40	155	0.72	279	1.80	16	84	0
11[c]	M	0.1	1[c]	c.326T>C, c.452G>A	30	T	0.33	126	0.77	298	2.36	41	59	0
12[c]	F	0.1	Newborn[c]	IVS8-1G>C homozygote	55	S	0.28	109	0.31	120	1.10	12	88	
13[c]	F	0.1	<0.2[c]	IVS8-1G>C, c.1190 C>T	55	S	0.45	174	0.58	224	1.29	2	98	
14[c]	F	0.1	Newborn[c]	c.725G>A; c.452 G>A	55	S	0.56	215	0.89	344	1.60	8	92	
15[c]	M	0.1	Newborn[c]	n.d.	55	S	0.58	224	0.86	333	1.49			

KM kilomicrons

[a] Reference ranges for Cho <1 year, 503–1,896 mg/L; >1 year, 1,084–2,012 mg/L

[b] Percent of lipoproteins in the age-matched healthy control group (0–3 years, n=5)

[c] Died as newborn or toddler

[d] Patients 8 and 9 are siblings

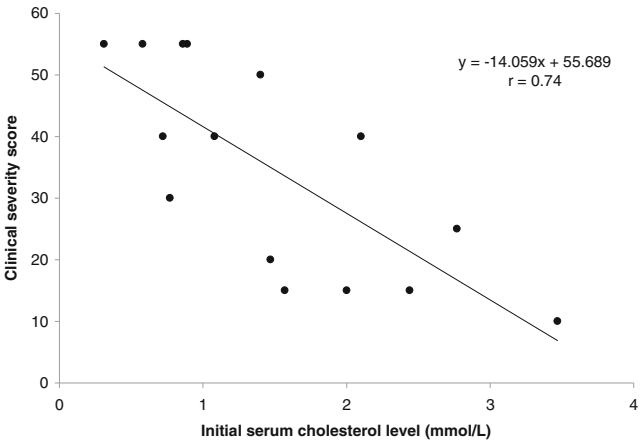

Fig. 1 Relation between initial cholesterol level and clinical severity in SLO syndrome (n=15). Initial cholesterol fundamentally determines the severity of SLOS and life expectancy

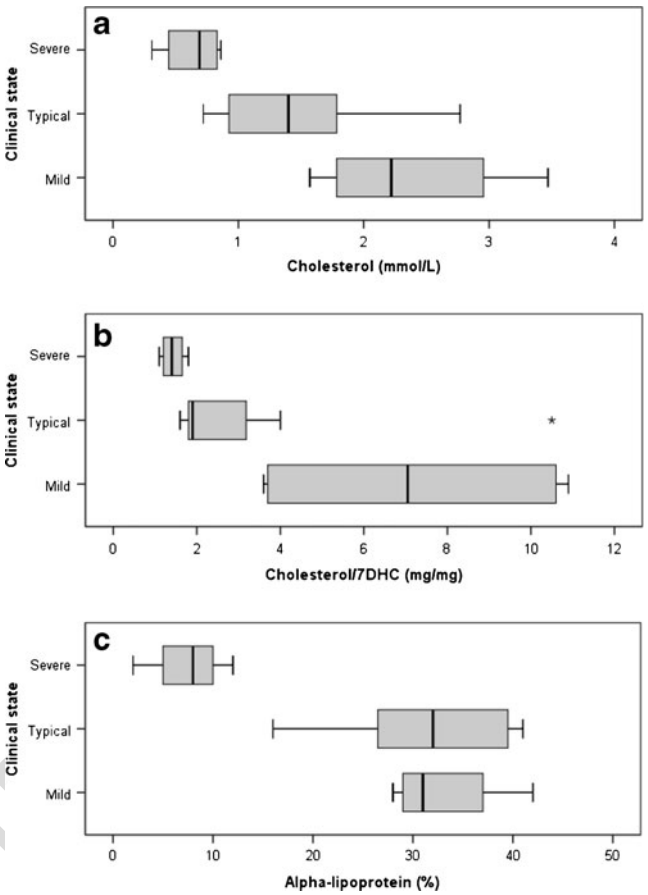

Fig. 2 **a** Distribution of initial serum cholesterol level in severe, typical, and mild clinical types of SLOS (*box* and *whiskers*). Bonferroni test showed significant difference between the initial cholesterol levels of mild and severe SLOS groups (p=0.01). **b** Distribution of initial serum cholesterol/7DHC ratio in severe, typical, and mild clinical types of SLOS. There was significant difference between the Cho/7DHC ratios of the patient groups (Kruskal–Wallis test: p=0.004). **c** Ratio of initial serum α-lipoprotein in severe, typical, and mild types of SLOS. Bonferroni test proved that the ratio of α-lipoprotein in severe SLOS group was significantly lower than in the typical (p=0.003) and mild SLOS group (p=0.004)

reference range—a typical case is demonstrated on Fig. 4. Cholesterol monotherapy was enough to improve total cholesterol with 0.5–1 mmol/L in three patients.

Discussion

The incidence of Smith–Lemli–Opitz syndrome is ranging from 1:20,000 to 1:60,000 [11, 17]. Our northern neighboring countries, in the Czech Republic and in the Slovak population, the incidence is even higher: from 1:10,000 to 1:20,000 [2]. We could correctly identify 15 patients by the rapid determination of 7DHC in serum which was performed with UV spectrophotometry that showed good correlation with the GC/MS method [6, 10]. It is important to mention that UV spectrophotometry is an easy-to-use method but less sensitive compared to GC–MS, and there is a difference between the two methods (2–39 %) as Honda and Batta also described [5]. Our findings are in accordance with the recently emerged and accepted opinion that the initial value of serum cholesterol fundamentally determines the severity, development, and life expectancy of SLOS [25]. The cholesterol/7DHC ratio has additional prognostic value in the classification of SLOS.

The decreased ratio of α-lipoproteins detected in severe SLO compared to the other types of SLO or to the age-matched control group may be the consequence of cholesterol biosynthesis disorder. On the other hand, the impaired liver function which can be observed in typical and severe SLO because of accumulated toxic dehydrocholesterol and other sterol metabolites may further inhibit the synthesis of lipoproteins. As the reactivity of 7DHC with oxygene radicals increased [42], a high blood level of 7DHC in severe SLO phenotype tends to accelerate lipid peroxidation causing further damage of proteins and antioxidant enzymes on the surface of high-density cholesterol. Because of an extremely low α-lipoprotein level in severe SLOS, we suppose that cholesterol reverse transport will slow down causing a greater extent of cholesterol deficiency. Although the number Q9 of our patients is limited and results cannot be evaluated properly statistically, our long-run clinical experiences, e.g., poor life expectancy, seem to be in accordance with this hypothesis.

Treatment with cholesterol with or without bile acids can improve the sterol abnormalities observed in patients with SLO syndrome [18, 34]. Introduction of statins in the treatment of SLO patients is based on the fact that inhibition of HMG-CoA reductase results in a decrease in the precursors

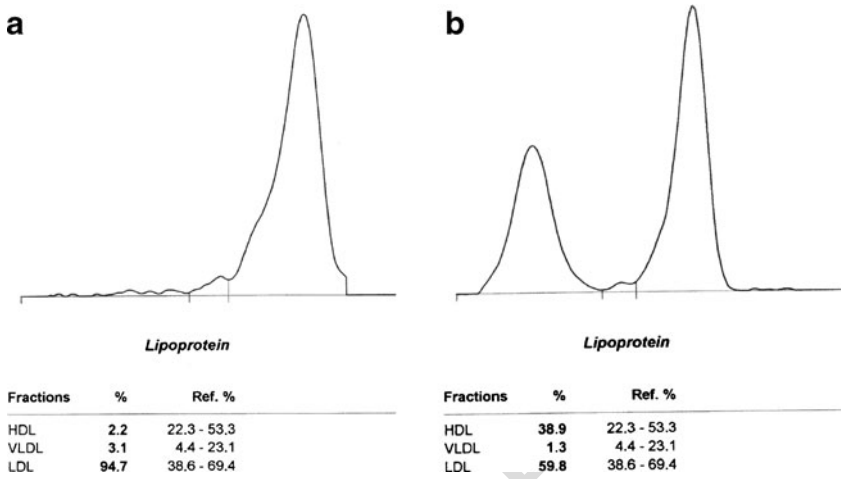

Fig. 3 **a** Alpha lipoproteins are hardly detectable in severe SLOS (patient 13, Sebia gel electrophoresis). **b** Percentage of lipoprotein fractions may be normal in mild-type SLOS (patient 1, Sebia gel electrophoresis)

such as 7DHC or 8DHC [8]. Moreover, in in vitro human fibroblast culturing in a cholesterol-deficient medium supplemented with statin, an upregulation of the DHCR7 activity was detected [24]. The phenomenon that cholesterol substitution in combination with simvastatin treatment decreases the level of the abnormally high 7DHC as well as increases the cholesterol level recently is debated. In accordance with Starck, during statin therapy, we observed significant liver function impairment in SLOS which emphasizes the vulnerability of patients with limited liver detoxication capacity, and that needs special attention in therapeutic approach [28]. We agree with Starck et al. that simvastatin treatment in SLOS cannot be considered as a safe approach in each case. When hepatotoxic effect is detected, modification of therapy (e.g., cholesterol supplementation without statin) may be considered which can increase the cholesterol and reestablish the liver function [28]. According to Haas' opinion, the mechanism of these therapeutic approaches is different: the level of cholesterol can be elevated by supplementation, and statins may reduce the DHC level which increases the cholesterol/7DHC ratio [5].

Determination of the lipid parameters in different categories of SLO syndrome is essential, because initial lipid levels have prognostic value. Monitoring of lipids and liver function helps to evaluate the efficiency of cholesterol supplementation and detects the side effect of statin therapy.

Although it is generally accepted that SLO syndrome has wide genetic variability, and genotype and phenotype are not in close connection, the blood level of cholesterol precursor 7DHC and clinical severity depend on mutation types [3, 5, 12, 41]. Therefore, biochemical markers still have a significant role besides the phenotype in setting up the diagnosis, prognosis, and later in the follow-up.

The early diagnosis is the precondition of the effective therapy although the individual results are different with strong limitation. Prenatal diagnosis with biochemical methods and molecular genetic test are available if the disease-causing mutation(s) in the family is known [14, 15, 20, 23]. Traditional biochemical screening of the substrates (7DHC and 8DHC) in serum is not reliable for detection of carrier status because there is an overlap between the ranges of serum concentrations of cholesterol and 7DHC in carriers and noncarriers; therefore, the biochemical testing of fibroblasts (7DHC) or the molecular genetic analysis of the disease-causing mutations in the family is recommended [24].

Based on the identification of family-specific gene mutations in the affected families, the prenatal genetic examination was introduced in Hungary in 2009. A web page has been set up to provide detailed information about the diagnostic and therapeutic possibilities (www.smithlemliopitz.hu).

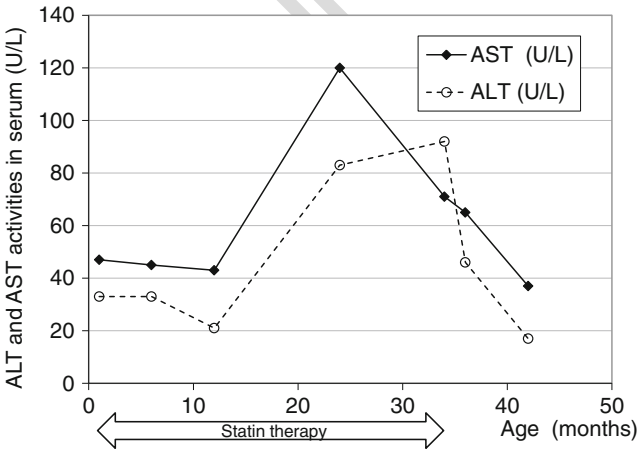

Fig. 4 A typical case of statin intolerance in patient 8 during cholesterol supplementation combined with simvastatin therapy. When the liver function and clinical condition were impaired, statin therapy was finished, and transaminase activities returned to the normal range. The *arrow* shows the duration of statin therapy

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Q5. The acronym "CHOD-POD" was expanded as "cholesterol oxidase–peroxidase". Please check if appropriate.
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Q7. Please check if the changes in the sentence "Our findings suggest that the initial level of serum cholesterol...." are appropriate.
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