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Effects of Dietary Cholesterol and Simvastatin on Cholesterol Synthesis in Smith-Lemli-Opitz Syndrome

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ABSTRACT: Deficient cholesterol and/or excessive 7-dehydrocholesterol (7-DHC) may be responsible for the pathology of Smith-Lemli-Opitz syndrome (SLOS). Both high-cholesterol diets given to ameliorate cholesterol deficiency while decreasing 7-DHC and cholesterol-enriched diets plus simvastatin to further decrease sterol synthesis have been used as potential therapies. However, the effect of dietary cholesterol and simvastatin on cholesterol synthesis in SLOS has not been reported. Twelve subjects with SLOS enrolled in the study: Nine had received a high cholesterol diet (HI) for 3 y and three were studied after 4 wk on a low cholesterol diet (LO). Cholesterol fractional synthesis rate (FSR) was measured after oral administration of deuterium oxide, using gas chromatography isotope ratio mass spectrometry. FSR was lower in HI compared with LO (HI: 1.46 ± 0.62%/d; LO: 4.77 ± 0.95%/d; $p < 0.001$). Three HI subjects were retested after 0.8 y taking simvastatin (HI + ST). Simvastatin tended to reduce FSR and significantly decreased ($p < 0.01$) plasma 7-DHC compared with cholesterol supplementation alone. The study demonstrates the utility of the deuterium incorporation method to understand the effect of therapeutic interventions in SLOS. The data suggest that dietary cholesterol supplementation reduces cholesterol synthesis in SLOS and further support the rationale for the combined treatment of SLOS with a cholesterol-enriched diet and simvastatin. **(*Pediatr Res* 65: 681–685, 2009)**

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive disorder, which is characterized by multiple anomalies and mental retardation (1–5). Patients with SLOS typically exhibit low-plasma cholesterol concentrations along with high concentrations of 7-dehydrocholesterol (7-DHC) (6), which is the immediate precursor of cholesterol, as well as its isomer 8-DHC (7). The abnormal cholesterol- and noncholesterol-sterol profiles in SLOS are present as a result of deficient activity of 7-DHC Δ7-reductase (DHCR7) (8), which converts 7-DHC to cholesterol in the last step of the cholesterol biosynthetic pathway. Because both cholesterol deficiency and excess 7-DHC, likely, contribute to the pathogenesis of SLOS, the therapeutic goal for the treatment of SLOS has been to enhance cholesterol accretion while decreasing accumulation of potentially toxic cholesterol precursors such as 7-DHC.

Cholesterol-enriched diets can ameliorate cholesterol deficiency in SLOS *via* cholesterol absorption from the diet. At the same time, synthesis of sterols including cholesterol and its precursors, such as 7-DHC, is inhibited *via* feedback inhibition on 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA R) by cholesterol supplementation (4,9). Therefore, dietary cholesterol supplementation has been the most commonly proposed potential treatment for SLOS (10–13). However, the effects of dietary cholesterol consumption on cholesterol synthesis in the SLOS population have not been widely studied. More recently, statins, HMG CoA R inhibitors, have been suggested as a potential treatment (14). Statins have been postulated to be beneficial in SLOS *via* reduction of 7-DHC synthesis by inhibiting HMG CoA R (14) and paradoxically increasing cholesterol synthesis by enhancing the residual activity of mutant *DHCR7*, as noted in *in vitro* human studies and *in vivo* animal studies (15,16). Simvastatin has been chosen for SLOS studies because it can cross the blood-brain barrier and potentially ameliorate the sterol derangements in both plasma and brain. Indeed, circulating 7-DHC and 8-DHC concentrations were observed to be reduced in subjects with SLOS (14,17) and increased cholesterol levels were obtained (14) as a result of simvastatin administration. Cerebrospinal fluid sterol ratios were improved as well (14). Because the use of simvastatin as potential treatment for SLOS yielded promising results in ameliorating sterol profiles in SLOS (14,17), it is crucial to evaluate the effects of a high cholesterol diet, with and without simvastatin, on whole-body cholesterol synthesis and circulating sterol levels in subjects with SLOS. The objective of this study was, therefore, to evaluate cholesterol synthesis and sterol concentrations in

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Abbreviations: 7-DHC, 7-dehydrocholesterol; **8-DHC,** 8-dehydrocholesterol; **DHCR7,** 7-dehydrocholesterol reductase; **D_2O,** deuterium oxide; **FSR,** cholesterol fractional synthesis rate; **GC,** gas chromatography; **HMG CoA R,** 3-hydroxy-3-methylglutaryl coenzyme A reductase; **HI,** high cholesterol diet group; **HI + ST,** high cholesterol diet with simvastatin group; **LO,** low cholesterol diet group; **RBC,** red blood cells; **SLOS,** Smith-Lemli-Opitz syndrome

SLOS to determine the effect of diet and simvastatin to help establish the optimal therapeutic strategy for SLOS.

SUBJECTS AND METHODS

Subjects. Twelve subjects were recruited from the clinics at Oregon Health & Science University (OHSU) and from outside referrals to the OHSU SLOS research program. The OHSU Institutional Review Board approved these studies and written informed consent was obtained in all cases. In addition to noting the presence of characteristic clinical features (5), diagnosis of patients with SLOS was confirmed by blood-sterol analysis documenting diagnostic elevation of plasma 7-DHC, and/or documented mutations in *DHCR7*, the gene encoding the DHCR7 enzyme deficient in SLOS. Severity score was reported using a previously established scoring system (5,18).

Experimental design, protocol, and diets. This study evaluated the effect of a high cholesterol diet on cholesterol synthesis relative to a low cholesterol diet, and the effects of simvastatin treatment combined with dietary cholesterol supplementation. Three subjects (4.4 to 11.3 y) were evaluated after consuming a low cholesterol diet (0.5 to 5 mg cholesterol/kg/d) for 4 wks and nine subjects (1.1 to 15.7 y) were evaluated while treated with a high cholesterol diet (HI; average treatment: 3 y). Three of the HI subjects (1.8 to 3 y of age) were reevaluated after treatment with the HI and simvastatin (HI + ST) for ~0.8 y.

Food sources for infants or tube-fed children consisted of infant or pediatric formula. Toddlers and children who ate orally and were consuming the low cholesterol diet were fed regular table foods that were very low in cholesterol, such as fruits, vegetables, breads, soy products, and fat-free dairy products. During the high cholesterol diet period, dietary cholesterol was given either in a food-based or crystalline form. Food-based cholesterol was given at a mean of 34.5 mg/kg/d, whereas crystalline cholesterol was given at 47 mg/kg/d. Cholesterol-containing foods used in the study included egg yolk and other foods high in cholesterol including butter, cheese, and meat. Egg yolk was incorporated into food in high cholesterol diet in a variety of ways. Dried or mashed hard-boiled egg yolk was added to formula, liquids, baby food, or solid foods. Raw egg yolks were cooked in an eggnog-type drink or raw pasteurized egg yolks were incorporated into beverages. In a few cases, subjects refused to eat eggs and were provided crystalline cholesterol. The crystalline cholesterol was suspended in a commercial aqueous emulsifier (OraPlus, Paddock Laboratories, Inc., Minneapolis, MN) and was administered as medication.

Subjects were given simvastatin orally or *via* feeding tube. Parents of subjects with SLOS were properly instructed in drug administration and in monitoring potential side effects before the initiation of simvastatin treatment. The dose of simvastatin was gradually increased from 0.2 mg/kg, to a maximum of 0.4 mg/kg, based on effects of the medication on plasma sterols in patients with SLOS.

Stable isotope administration protocol and blood sample processing. Subjects were admitted to the General Clinical Research Center (now the Pediatric Clinical and Translational Research Center of the Oregon Clinical and Translational Research Institute-National Institutes of Health CTSA grant) at OHSU. A baseline blood sample (10 mL) was taken in the morning before breakfast was consumed. Then, deuterium oxide (D_2O; 99.8 atom percent excess; CDN isotopes, Montreal, Canada) was given at a dose of 500 mg/kg of body weight. A second blood sample (10 mL) was collected 24 h after D_2O administration. Samples were centrifuged at 2000 rpm for 10 min to separate red blood cells (RBC) and plasma. Buffy coat was discarded and the RBC and plasma were immediately stored at −80°C until further analysis.

Subjects treated with simvastatin were monitored for potential adverse effect of the drug by measuring creatine kinase and transaminase serum concentration. Simvastatin treatment was discontinued if creatine kinase was above normal and/or serum transaminases were greater than three times the upper limit of normal.

Cholesterol fractional synthesis rate methodology. The rate of deuterium from body water incorporated into RBC membrane free cholesterol during 0 h to 24 h of each visit was taken as an indicator of cholesterol synthesis. Therefore, deuterium enrichments were measured in both RBCs and plasma water. The deuterium enrichment of free cholesterol extracted from RBCs was analyzed in samples obtained at 0 h and 24 h of each visit using a gas chromatography/pyrolysis/isotope ratio mass spectrometry (GC/P/IRMS) approach. The free cholesterol was extracted from RBCs through the following procedures. Methanol was added to RBCs and samples were heated at 55°C in a shaking water bath for 15 min, before the addition of hexane:chloroform (4:1, by volume) and double-distilled water. Thereafter, samples were centrifuged for 15 min at 1500 rpm at 4°C. Supernatants of the samples were dried down under nitrogen and redissolved with hexanes and subsequently transferred in injection vials. Samples were injected into an Agilent 6890N GC. A 30-m capillary column (SAC-5; Supelco, Bellefonte, CA) was installed in the GC and programmed to separate the sterols: the starting temperature was 160°C and was increased by 15°C/min to 245°C and isothermal for 4 min; increased by 15°C/min to 280°C and isothermal for 4 min; increased by 25°C/min to 305°C and isothermal for 10 min; and returned to 160°C by 48°C/min. The GC was connected to a Delta V plus IRMS through pyrolysis furnace (alumina tubing, reactor temperature at 1450°C). Under these conditions, the organic hydrogen and deuterium from newly synthesized free cholesterol was converted to hydrogen and deuterium gas. The deuterium enrichment of this gas was then detected by the Delta V plus IRMS system. Isotopic ratios were expressed as δ D/H in per mil against Vienna Standard Mean Ocean Water. The deuterium enrichment of plasma water was measured using differential IRMS coupled with a manually operated dual-inlet system with electrical H^{3+} compensation (VG Isomass 930D) as reported previously (19). An aliquot of plasma water sample was analyzed by temperature conversion elemental analyzer (TC/EA) IRMS. After correction for free cholesterol pool, fractional synthesis rate (FSR) was taken as the indicator of the fraction of the cholesterol pool that is synthesized during the 24-h period and was calculated as previously reported (20). It is anticipated that 7-DHC and 8-DHC as well as cholesterol are synthesized from the orally administered D_2O. Although 7-DHC could be separated from cholesterol, 8-DHC coelutes with cholesterol using our current method. Accordingly, the FSR measure of cholesterol synthesis also includes some synthesis of 8-DHC.

Plasma sterol analysis. Plasma samples were saponified and extracted in hexane. The concentration of the trimethylsilyl ether derivatives of individual plasma sterols was measured by capillary-column GC with a CP-Wax 57 column (25 M, 0.32-mm ID; Chrompack-Varian, Walnut Creek, CA). Internal standard 5α-cholestane and authentic standard of cholesterol were used for calibration.

Statistical analyses. Data were analyzed using the SAS software (version 8.0; SAS 214 Institute Inc, Cary, NC). Group data are presented as means and standard deviations. A *t* test was used to compare LO and HI, and a paired *t* test was used to compare HI and HI + ST. Statistical difference was set at $p < 0.05$ for all comparisons.

RESULTS

Twelve subjects with SLOS were recruited for the study. Nine subjects were provided with a high cholesterol diet; in one case, crystalline cholesterol was provided instead of egg yolk. A high cholesterol diet with simvastatin, which provided cholesterol in the form of egg yolk, was given to three subjects with SLOS. Three subjects with SLOS were fed the low cholesterol diet. Liver transaminases and creatine kinase were not elevated in any of the three subjects who received simvastatin with a high cholesterol diet (data not shown). On the basis of Severity Score system, six subjects were classified as mild (<20); five subjects were typical (20 to 35); and one subject was severe (>35) (15).

Cholesterol fractional synthesis rate, lipid profiles, and their responses to cholesterol feeding with and without statin. Nine subjects underwent FSR and sterol profile analysis while consuming a high cholesterol diet. Table 1 presents the individual FSR values, cholesterol, 7-DHC concentrations, cholesterol/(7-DHC + cholesterol) ratios, and Severity Score in subjects with SLOS treated with high cholesterol diet and low cholesterol diet. In comparing LO with HI, FSR was reduced ($p < 0.001$) as a result of high cholesterol feeding (1.46 ± 0.62%/d and 4.77 ± 0.95%/d for HI and LO, respectively). The effects of high cholesterol diet and high cholesterol diet with simvastatin on FSR, cholesterol, and 7-DHC concentrations are displayed in Figure 1. There was a tendency toward additional FSR reduction in HI + ST compared with HI, but this effect was not statistically significant (FSR = 1.61 ± 0.91%/d and 0.85 ± 0.44%/d for HI and HI + ST, respectively; $p = 0.175$; Fig. 1*A*). There was, however, a significant decrease in 7-DHC from high cholesterol diet to high choles-

Table 1. *Cholesterol fractional synthesis rate (FSR) in subjects with SLOS treated with low cholesterol diet (*n = *3) and high cholesterol diet (*n = *9)*

Subject	Age (y)	Gender	Formulation	Cholesterol intake (mg/kg/d)	FSR (%/d)	Plasma sterols: Cholesterol (mmol/L)	Plasma sterols: 7-DHC (mmol/L)	Plasma sterols: Cholesterol/ (7-DHC + cholesterol)	SS
Low cholesterol diet									
1	4.4	M		0.5	5.7	3.68	0.01	0.998	5
2	10.4	F		4.5	4.8	2.46	0.12	0.953	10
3	11.3	F		4.6	3.8	2.18	0.13	0.944	10
Mean				3.2	4.77	2.77	0.09	0.965	8.3
SD				2.3	0.95	0.80	0.07	0.029	2.9
High cholesterol diet									
4	1.1	F	Egg yolk	38.7	1.6	0.81	0.36	0.696	40
5	1.6	M	Egg yolk	46.0	0.9	2.61	0.25	0.914	20
6	2.2	F	Egg yolk	37.4	2.6	3.22	0.16	0.954	5
7	2.4	M	Egg yolk	27.7	1.4	1.87	0.20	0.904	30
8	3.3	M	Egg yolk	32.9	0.8	2.30	0.33	0.876	35
9	5.2	F	Egg yolk	32.3	0.6	1.04	0.30	0.775	25
10	5.9	M	Egg yolk	29.5	1.8	4.15	0.12	0.972	6
11	10.3	F	Crystalline cholesterol*	46.9	1.7	3.24	0.08	0.977	17
12	15.7	F	Egg yolk	31.4	1.7	1.34	0.45	0.748	33
Mean				35.9	1.46	2.29	0.25	0.868	23.4
SD				6.9	0.62	1.13	0.12	0.104	12.5

* Crystalline cholesterol suspended in OraPlus.

FSR, cholesterol fractional synthesis rate; 7-DHC, 7-dehydrocholesterol; SS, Severity Score.

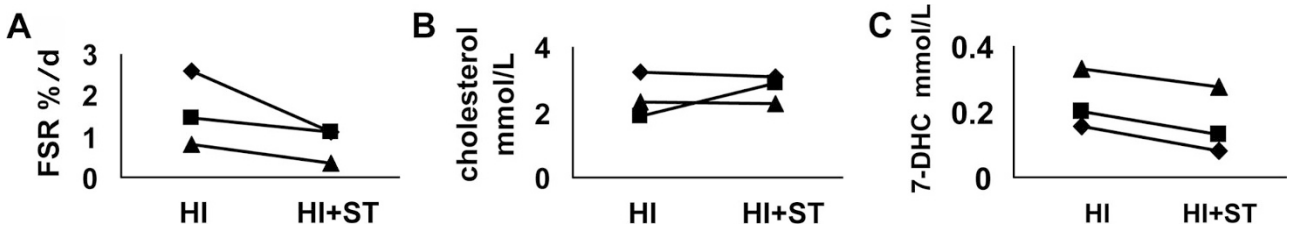

Figure 1. Comparing HI with HI + ST. There is tendency toward reduction of (*A*) cholesterol fractional synthesis rate (FSR) but no change in (*B*) cholesterol concentrations. There is a significant decrease in (*C*) plasma 7-dehydrocholesterol (7-DHC) concentrations; $p = 0.01$, obtained by paired *t* test; ◆, subject 6; ■, subject 7; ▲, subject 8.

terol diet with simvastatin ($p = 0.01$; Fig. 1*C*). Cholesterol concentrations were unchanged in HI + ST, compared with HI (cholesterol = 2.46 ± 0.69 mM and 2.77 ± 0.43 mM for HI and HI + ST, respectively; $p = 0.5$; Fig. 1*B*).

DISCUSSION

Our results suggest that a high cholesterol diet reduces whole body *in vivo* cholesterol synthesis in children with SLOS as measured by FSR, compared with a low cholesterol diet. The decrease is likely due to inhibition of HMG CoA R, thereby reducing sterol (including cholesterol) synthesis, as well as presumably 7-DHC and 8-DHC synthesis. Moreover, simvastatin added to the high cholesterol diet further decreased circulating 7-DHC concentrations. Therefore, the rationale for considering a high cholesterol diet, with or without simvastatin, as potential therapy is supported. Individuals with SLOS, in whom excess 7-DHC likely contributes to the pathogenesis, may benefit, by reduction of potentially toxic cholesterol precursors: 7-DHC and its isomer 8-DHC.

It has been consistently observed that circulating cholesterol concentrations are increased in SLOS patients after cholesterol supplementation (10–13). It has also been shown that dietary cholesterol can be absorbed by subjects with SLOS, although absorption rates may tend to be lower than normal (21). Cholesterol homeostasis is defined by cholesterol absorption, synthesis, and excretion, which together determine blood cholesterol concentration. Supplementing cholesterol in the diet increases circulating cholesterol concentrations (22) directly by absorption of cholesterol from the diet. At the same time, dietary cholesterol depresses sterol (including cholesterol) synthesis *via* feedback inhibition (23,24). The effects of interventions such as dietary cholesterol supplementation cannot always be reliably predicted in advance, because of the delicate balance between cholesterol absorption, synthesis, excretion, and uptake from the circulation. This is especially true in disease states affecting cholesterol homeostasis such as SLOS, and this prompted this study, with the goal of increasing our knowledge of the effects of interventions on sterol homeostasis in SLOS and evaluation of the deuterium incorporation stable isotope methodology for measuring cholesterol synthesis in SLOS.

To our knowledge, this study is the first to apply the D_2O method to measure cholesterol synthesis from D_2O in SLOS, a relatively noninvasive approach for determining cholesterol FSR and the first to directly measure the effect of simvastatin on cholesterol synthesis in SLOS in humans *in vivo*. We

previously measured whole-body cholesterol synthesis using the sterol balance technique in SLOS children on a low cholesterol diet (25). However, in children consuming a high cholesterol diet, the large mass of cholesterol excreted renders detection of the small differences between intake and excretion unreliable by that method. Our current results using a different methodology validate the findings demonstrated in our previous study that dietary cholesterol-induced feedback inhibition is intact in subjects with SLOS (9).

FSR in children has been measured by the D_2O method previously. Indeed, it had been shown previously that FSR ranged from 2 to 7%/d and was correlated inversely to dietary cholesterol intake in normal healthy 4-mo-old infants who did not have SLOS (26). It is apparent that mean FSRs in this study are lower than those reported earlier, but the subjects in the earlier published study were younger. Measuring cholesterol synthesis by sterol balance showed that subjects with SLOS (aged 0.5 to 13 y) had reduced cholesterol synthesis compared with healthy controls (aged 1–5 y) (25). Future studies with more closely age-matched subjects will clarify if there is a difference in cholesterol synthesis between children with SLOS and healthy children.

There was a tendency toward further FSR reduction when high cholesterol diet with simvastatin was compared with a high cholesterol diet alone, but this did not reach statistical significance. However, 7-DHC concentrations fell significantly with simvastatin administration compared with the high cholesterol diet without a decrease in plasma cholesterol concentrations. Taken together, it is very likely that simvastatin effectively reduced 7-DHC accumulation, by inhibiting HMG CoA R and decreasing flux through the cholesterol synthetic pathway. In addition, expression of *DHCR7* was induced by simvastatin in a previous *in vitro* study (15). In a subsequent animal study, it was further confirmed that expression of *DHCR7* increased in response to simvastatin (16). Therefore, it may be that simvastatin up-regulates residual DHCR7 activity. In the literature, effects of simvastatin on cholesterol concentrations in SLOS *in vivo* are inconsistent. Jira *et al.* (14) reported that circulating cholesterol concentrations were increased almost 3-fold after simvastatin was administered to two subjects with SLOS for at least 14 mo, even without a high cholesterol diet for a part of the study duration. In a larger retrospective study, Haas *et al.* (17) observed a decrease of cholesterol concentrations after simvastatin added to a high cholesterol diet was compared with a high cholesterol diet alone. In our current study, although simvastatin with a high cholesterol diet resulted in no further significant change in FSR, plasma cholesterol concentrations were also unchanged compared with a high cholesterol diet alone. A study with more subject numbers will be necessary to confirm the effect of administration of simvastatin with a high cholesterol diet on cholesterol concentrations and cholesterol synthesis in children with SLOS.

Safety of statin use in SLOS is of paramount importance. Although, there is a paucity of data on safety of statin in children with SLOS, there is a fair amount of published data on safety of statins in children who do not have SLOS. Indeed, statins have been one of the standard medications prescribed to children or adolescents with dyslipedemia (27). A recent comprehensive United States Preventive Services Task Force review of published studies of statins in children revealed a low prevalence of transaminase and creatine kinase elevation and no evidence for significant adverse health effects (28). Specifically, elevations of transaminases and creatine kinase levels with statins have been rarely noted in some studies with children and adolescents, with several studies finding no elevations (29–32). Numerous clinical trials attest to the safety of statins in children and adolescents (28). Furthermore, the US Food and Drug Administration has approved the use of some statins in children aged 8 y or older, with familial hypercholesterolemia (27). However, the safety of simvastatin specifically in subjects with SLOS was raised in a two-patient study where significant increase in alanine aminotransferase was observed in one subject (33). Furthermore, simvastain treatment may possess potential adverse effects in severely affected individuals with SLOS. An *in vitro* study revealed death of SLOS cell lines with two null mutations in *DHCR7* in response to simvastatin treatment (15). In consideration of the potential risks, it is recommended that patients who receive statins undergo routine liver function and creatine kinase tests (27). In the retrospective study of statins in SLOS by Haas *et al.,* six of 39 patients who received additional simvastatin reported side effects, the most consistent of which was sleep problems, which necessitated a reduction or discontinuation of the medication. Many of those subjects had simvastatin reintroduced without difficulty. In this study, subjects taking simvastatin did not have elevated serum transaminases or creatine kinase levels over the course of the study. This may be due to the fact that none of them had Severity Score >35, which was classified to be severe (15). In summary, statins have an excellent safety profile in children who do not have SLOS, but they do have potential toxicity, and the long-term safety in children and in SLOS has not yet been determined, suggesting that widespread use of statins in SLOS outside of the research arena is unwarranted and close monitoring is required.

There were limitations to this study. First, the number of subjects studied was small, so caution is advised in generalizing the results of this study. Second, the mean Severity Score of the HI group was higher than the mean score in the LO group. We used the severity scoring system that was developed on the basis of anatomical abnormalities (34) and subsequently modified (5,18). In some studies, the Severity Score was shown to be correlated with biochemical markers (2,3). Therefore, the differences in Severity Score between HI and LO could partially account for the lower FSR in HI, as a higher Severity Score is likely to indicate a more severe enzyme defect. The third limitation of this study was that 8-DHC could not be separated from cholesterol using our current methodology. Therefore, the FSR presented in this study may represent both cholesterol and 8-DHC synthesis. However, our earlier sterol balance results showed 7-DHC synthesis represented $\sim$10% of cholesterol synthesis (25). The concentrations of 7-DHC and 8-DHC in plasma are usually similar. Assuming that the synthesis of 7-DHC and 8-DHC are also similar, it is believed that the contribution of 8-DHC to the FSR is likely to be small. More importantly, FSR in this

study represents a measure of flux through the cholesterol synthetic pathway. Therefore, even if our current FSR include 8-DHC synthesis, the overall interpretation of the results is valid, as we are most interested in the effects of interventions on flux through the pathway; we showed decreased flux through the pathway with a high cholesterol diet. Finally, it would be advantageous to measure the FSR of 7-DHC and 8-DHC directly, but technical challenges have so far prevented us from accomplishing that aim.

In conclusion, our findings demonstrate as expected in this cholesterol deficiency syndrome that cholesterol synthesis in children with SLOS is lower than the published values in normal healthy infants, although a direct comparison of FSR in age-matched children using the same methodology in a single study is needed. Furthermore, a high cholesterol diet resulted in the desired reduction of FSR in children with SLOS. A high cholesterol diet with simvastatin may be therapeutic in SLOS because simvastatin decreased plasma 7-DHC with no apparent corresponding drop in cholesterol. A comprehensive understanding of the effects of dietary cholesterol and statins used separately and together, at different doses and for varying durations, will be required to optimize future therapeutic strategies in SLOS to determine efficacy. The stable isotope D_2O method of determination of FSR can be applied in this patient population and should prove invaluable in determining the effects of these and other therapies in SLOS for testing proposed mechanisms as well as for safety and efficacy monitoring.

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