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No evidence for mevalonate shunting in moderately affected children with Smith-Lemli-Opitz syndrome

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Summary

Smith-Lemli-Opitz syndrome (SLOS) is caused by a genetic deficiency in 7-dehydrocholesterol (7-DHC) reductase (EC 1.3.1.21), the last enzyme of the cholesterol synthetic pathway. In SLOS, plasma cholesterol concentration is reduced and immediate precursor concentration (7-DHC) is elevated. Surprisingly, total sterol synthesis is reduced but HMG-CoA reductase activity, a rate-limiting enzyme in cholesterol synthesis is unaltered as judged by normal urinary excretion of mevalonic acid (MVA) (Pappu, 2002). These findings raise the possibility of increased diversion of MVA into the MVA shunt pathway away from sterol synthesis, by activation of the shunt pathway enzymes. To test this hypothesis, we measured the urinary excretion of 3-methylglutaconic acid (U-3MGC), a by-product of the shunt pathway, in 19 mild-to-moderately severely affected SLOS subjects (10 males, 9 females) receiving either a cholesterol-free or a high cholesterol diet, and in 20 age- and sex-matched controls. U-3MGC was similar in SLOS and controls, and was unaffected by dietary cholesterol intake. Further, no change in U-3MGC was observed in a subset of SLOS subjects (n=9) receiving simvastatin. In contrast, U-MVA was reduced by cholesterol supplementation (~54%, $p<0.05$) and by simvastatin (~50%, $p<0.04$). There was no correlation between U-3MGC and either plasma sterol concentrations, urinary isoprenoids, or the subjects' clinical severity score. However U-3MGC was inversely correlated with age ($p<0.04$) and body weight ($p<0.02$), and higher in females than in males (~65%,

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$p < 0.025$). The data show that *DHCR7* deficiency does not result in 3MGC accumulation in SLOS and suggest that the MVA shunt pathway is not activated in patients with the condition.

INTRODUCTION

Smith-Lemli-Opitz syndrome (SLOS; MIM270400) is a disorder of cholesterol synthesis caused by mutations in the *DHCR7* gene (MIM602858) encoding 7-dehydrocholesterol reductase, the final enzyme in the cholesterol synthetic pathway (Wassif et al 1998; Waterham et al 1998; Fitzky et al 1998; Fig. 1). Affected individuals typically exhibit multiple malformations and mental retardation [Smith et al 1964; Cunniff et al 1997; Kelley and Hennekam 2000; Battaile and Steiner 2000]. The features of SLOS are thought to be primarily related to cholesterol deficiency and accumulation of cholesterol precursors including 7-DHC and 8-dehydrocholesterol (8-DHC) (Tint et al 1994; Engelking et al 2006). However, the clinical phenotype is not fully characterized, the biochemical pathogenesis is incompletely understood, and there is no proven therapy for this condition.

Studies by our group suggest that enzyme deficiency results in cholesterol precursor accumulation and their metabolic diversion toward other branches of the cholesterol pathway. In particular, we have demonstrated that urinary excretion of dolichol and ubiquinone is increased in SLOS (Pappu et al 2006). Although some studies suggest that plasma and urinary dolichols are independently regulated (Humaloja, 1991; Roine, 1992), our urinary findings suggest that in SLOS there is diversion of sterol precursor farnesyl-PP toward long-chain isoprenoids (Fig. 1).

Distal cholesterol precursor accumulation as evidenced by increased long chain isoprenoids suggests that proximal precursors such as mevalonic acid (MVA) may accumulate as well. In a previous study however urinary MVA excretion (U-MVA), a known marker of cholesterol synthesis (Jones et al 1996; Parker et al 1984) was found to be the same in SLOS and controls (Pappu et al 2002), while in another study cholesterol synthesis measured by sterol balance was shown to be reduced by 40% (Steiner et al 2000). To reconcile these findings, we postulated that in SLOS mevalonate is diverted toward other pathways. A logical candidate diversion pathway is the mevalonate shunt pathway originally described by Edmond and Popják (Edmond and Popják 1974). In this pathway, mevalonate, is converted to isopentenyl-PP and dimethylallyl-PP (DMA-PP) and then to 3-methylglutaconyl-CoA via oxidative metabolism instead of being used as building blocks for the synthesis of cholesterol (Fig. 1) (Schroepfer 1981). 3-Methylglutaconyl-CoA is then either excreted in the urine as 3-methylglutaconic acid (3MGC), or further metabolized in the mitochondria to acetyl-CoA and acetoacetyl-CoA. Although 3MGC is also a product of the leucine degradation pathway and can accumulate in the urine when mitochondrial function is impaired (Sweetman 2001), 3MGC is considered a marker of the MVA shunt pathway activity and its increased excretion in the urine may reflect increased MVA diversion through the shunt pathway. One study suggests that such diversion may occur in SLOS (Kelley and Kratz 1995). However in that study, the increase in blood and urine 3MGC was only observed in a few, severely affected SLOS patients and the study findings have not been subsequently confirmed by other investigators. Further, it is possible that that mitochondrial function is altered in severe SLOS as a consequence of altered membrane structure (Tulenکو 2006) or accumulation of toxic 7-DHC-derived oxysterols (Gramajo et al 2010; Xu et al 2010; Korade et al 2010). Impaired mitochondrial function could theoretically result in an increase in urinary 3MGC without any contribution from the MVA shunt pathway. In summary, the increase in urinary 3MGC excretion reported previously in SLOS does not seem to be a universal phenomenon and may not be related to MVA diversion through the shunt pathway.

Our primary objective was thus to measure U-3MGC in SLOS and confirm Kelley and Kratz 's previous findings in a broader group of patients. We also sought to determine if U-3MGC was affected by cholesterol supplementation, a standard intervention in SLOS, and by simvastatin, a proposed investigational treatment in SLOS patients already receiving cholesterol supplementation. We predicted that if 3MGC was reflecting MVA shunt activity, U-3MGC would decrease with dietary cholesterol supplementation, a regimen known to decrease HMG-CoA reductase activity and down-regulate endogenous cholesterol synthesis, and that simvastatin would further decrease U-3MGC as a consequence of direct HMG-CoA reductase inhibition and reduction in mevalonate and short-chain isoprenoid synthesis.

MATERIALS AND METHODS

Subjects

Subjects were part of an on-going longitudinal study of SLOS which started in 1995. In this study, SLOS patients who agreed to participate are invited to undergo comprehensive clinical, behavioral, cognitive, and metabolic investigations during regular visits to the Oregon Health & Science University (OHSU) Pediatric Clinical and Translational Research Center of the Oregon Clinical and Translational Research Institute. Included in these assessments are blood draws and 24-hr urine collections. All subjects are studied while receiving a high cholesterol diet (SLOS-HI group; Linck et al 2000). However, as part of the study protocol, they are also asked to follow for one month a very low cholesterol (essentially cholesterol-free) diet (SLOS-LO) at the end of which their metabolic phenotype is characterized without the influence of the high cholesterol diet. Not all subjects enrolled in the study agreed to follow the cholesterol-free diet, fearing that it may worsen their condition. For the 3MGC study, we selected subjects whose urine had been collected at least twice, once at the end of their cholesterol-free diet period, and once while receiving the high cholesterol diet.

Altogether, we identified 19 SLOS subjects (9 males, 10 females) with paired samples (i.e. one collected during the cholesterol-free period and another collected while receiving cholesterol). SLOS diagnosis was confirmed in all by plasma sterol and DNA mutation analysis (Table 1). Subjects were on average 6.1 ± 1.5 years-old (mean $\pm$ SE) and weighed 19.6 ± 3.7 kg when studied at the end of the cholesterol-free period. They were 7.3 ± 1.5 years and weighed 22.8 ± 4.6 kg when studied while receiving cholesterol. The difference in daily cholesterol intake between the HI and LO periods was 494.6 ± 113.2 mg/day on average. The disease severity score, assigned according to the method of Kratz and Kelley (Kratz and Kelley 1999) was 15.2 on average (maximum of 100), ranging from 5 to 35 (Table 1). Thus the subjects were mildly to moderately severely affected.

Subjects in the study are also offered to participate in a sub-study of the effects of simvastatin in SLOS. Again, not all subjects agreed to take simvastatin and among the 19 subjects with paired (LO vs. HI cholesterol) urine samples, only 9 had paired simvastatin samples. On average, this subset of SLOS subjects (6 males, 3 females) had a severity score of 13.0 (range 5 to 35), and was 5.4 ± 2.0 years old when studied in the absence of simvastatin (HI cholesterol diet only) and 7.6 ± 2.0 years old when receiving both HI cholesterol and simvastatin (Table 2). At the time of their second urine collection, the patients had been taking simvastatin for 1.2 ± 0.2 years, and the average simvastatin daily dose was approximately 0.23 mg/kg/day.

Controls were also recruited, mostly among siblings of affected children. They included 20 children (10 males, 10 females; 4.9 ± 1.0 years of age; 23.4 ± 6.3 kg body weight). All subjects were consented and the study was approved by the OHSU Institutional Review Board.

Blood and urine sample collection

Urine samples were collected between 1997 and 2008. They were kept at -20°C as part of the OHSU SLOS sample repository, a companion to our longitudinal study. The samples were assayed for 3MGC, MVA and creatinine (to adjust for collection loss and to normalize results). They were also assayed for dolichol and coenzyme Q (CoQ) whenever there was enough sample to perform these assays. In the youngest subjects, urine was extracted from diapers (Steiner et al 2000). Blood draws were performed in SLOS subjects only, not in controls. Samples were collected by venipuncture and assayed for cholesterol, 7-DHC and 8-DHC.

Blood and urine chemistry

3MGC was measured using a method recently established in our laboratory as modified from Kelley [Kelley, 1993]. In brief, the samples were spiked with 0.5 μmoles of 2,2-dimethylglutaric acid as internal standard, acidified, extracted into ethyl acetate, dried and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Analyses were performed using a gas chromatograph equipped with a mass spectrometry detector and a splitless injection into an HP5 column (Agilent GCMS 5975, Agilent Technologies, Wilmington, DE). Authentic 3MGC (cis- β -methylglutaconic acid) was used during the validation of the assay. It was prepared by the alkaline hydrolysis (10 % aqueous KOH, at ambient temperature, overnight) of ethyl isodehydroacetate (5 g, SIGMA-ALDRICH, St. Louis, MO). The product was isolated by repeated ether extraction of the acidified cold mixture and prolonged removal of ether under vacuum which eventually produced a solid, 2.6 g, melting from $74 - 110^{\circ}\text{C}$. When dissolved in 40 ml of ether a crystalline precipitate (2.6 g, melting from $115^{\circ}\text{C} - 125^{\circ}\text{C}$) was produced at -12°C very slowly. Two more crystallizations from ether resulted in 0.31 g of a beige solid with a melting point of 149°C , similar to authentic 3MGC (Feist 1906; Jackman and Wiley 1958). Identity was confirmed by $^1\text{H-NMR}$ analysis ($^1\text{H-n.m.r.}$ spectrum 90 MHz, D_6 -acetone + CDCl_3 , $\text{Si}(\text{CH}_3)_4 = 0$: $\delta_2 = 5.89$ ppm, T-Quart., $J_{2\text{CH}_3} = 1.4$ Hz, $J_{24} = 0.7$ Hz, 1H; $\delta_4 = 3.75$, D, br., $J \approx 0.7$, 2H; $\delta_{\text{CH}_3} = 2.01$, D, 3H. The assay was linear between 1 and 65 picomoles of 3MGC injected (Fig. 2A). The target ions were 273 for 3MGC (qualifying ion 198; Fig. 2B) and 289 (qualifying ion 204) for the internal standard (*not shown*). 3MGC and the internal standard had the same retention time (~ 12.4 min; Fig. 2C). Within-run variation (coefficient of variation) was 7% (for an average of 3.6 picomoles injected, $n = 10$) and 5% (for an average of 32.8 pmoles injected, $n = 10$). Within-subject day-to-day variation was 9% (subject 1, average U-3MGC = 4.0 ± 0.2 nmol/ μmol creatinine; $n = 5$ consecutive days) and 13% (subject 2, average U-3MGC = 11.4 ± 0.7 nmol/mmol creatinine; $n = 5$ consecutive days).

Dolichol and CoQ measurements were performed by reverse-phase high performance liquid chromatography (Pappu et al 2006). Intra- and interassay variations for dolichol were $<8\%$, and $<5\%$ for CoQ. Mevalonate was determined using the radioenzymatic isotope dilution method we reported previously (Pappu et al 1989). Plasma cholesterol (pl-Chol), plasma 7-DHC and plasma 8-DHC concentrations were determined by gas chromatography (Merkens et al 2004). A subset of the dolichol, CoQ, MVA and sterol data presented in this report have been reported in part in a previous publication (Pappu et al 2006). New measurements from samples not analyzed at the time of the previous publication, were, however performed to match the current 3MGC data set, thus increasing the power of correlative analyses between U-3MGC and other metabolites of the cholesterol pathway.

Statistics

Statistical analyses were performed using Statgraphics 5.1 Plus (Manugistics Inc., Rockville, Md., U.S.A.). Group data are expressed as mean $\pm$ SE. Unpaired (Control vs. SLOS) and paired (Lo vs. HI cholesterol or $\pm$ simvastatin) t tests (two-tailed) were used to compare

groups, with p values > 0.05 indicating a significant difference. Correlations were examined using simple linear regression, general linear regression models that included categorical and continuous variables, or non-parametric rank correlation analysis when appropriate.

RESULTS

The results of the 3MGC analyses are presented in Tables 2 and 3 together with other biochemical parameters. Table 2 shows group data for the SLOS subjects on LO and HI cholesterol, and for control subjects, whereas Table 3 shows group data for the SLOS subgroup with pre and post simvastatin samples.

On average, U-3MGC was similar in Controls and SLOS, whether SLOS subjects were studied on a cholesterol-free diet or on a cholesterol-supplemented diet. U-3MGC decreased from 12.2 ± 1.9 nmol/ μ mol creatinine (SLOS-LO) to 9.9 ± 1.2 nmol/ μ mol creatinine (SLOS-HI), but the change was not statistically significant. The urinary excretion of mevalonate was also similar in Controls and SLOS. However, unlike U-3MGC, U-MVA was significantly lower (by approximately 54%) in SLOS samples collected during the cholesterol supplementation phase than in those collected during the cholesterol-free phase ($p < 0.05$). In contrast, the urinary excretion of dolichol (U-dolichol) and CoQ (U-CoQ) was significantly elevated in SLOS-LO as compared to Controls (~340% and 420% increase, $p < 0.035$ and $p < 0.007$ for U-dolichol and U-CoQ respectively as compared to controls), but was not significantly affected by dietary cholesterol intake (Table 2).

Interestingly, we noted a difference in dolichol chain length between SLOS patients on a cholesterol-free diet and controls (Fig. 3). Dolichols elute in 9 distinct peaks which we identified as fractions 1 to 9. These fractions include dolichols with chain lengths varying from 80 to 115 carbons (16 to 23 isoprene units) for fractions 1 to 8, and dolichols of chain lengths higher than 115 carbons (fraction 9). The data show a significant difference in chain length profile between Controls and the SLOS-LO group ($\chi^2 = 48.4$, $p < 0.001$), with the SLOS group having on average longer dolichols than controls (fraction 8 is approximately 2.5 fold higher in SLOS than in Controls, $p < 0.001$) whereas fractions 5 and 7 are respectively decreased by 97% ($p < 0.02$) and 92% ($p = 0.015$) in SLOS-LO as compared to controls. The data also shows a decrease in fractions 1–4 and 6, and a slight increase in fraction 9, but these differences were not statistically significant. No change in dolichol chain length profile was observed in SLOS patients when treated either with cholesterol supplementation or with simvastatin (*data not shown*).

SLOS subjects on cholesterol-free diet had low plasma cholesterol concentrations, on average about half of those expected in a healthy, age-matched population (Kwiterovich 1991), and had detectable and measurable concentrations of 7-DHC and 8-DHC in their plasma (Table 2). The high cholesterol diet significantly increased pl-Chol by ~20% ($p < 0.001$) but did not affect either 7-DHC or 8-DHC concentrations. However, it reduced the [7-DHC+8-DHC]-to-Chol ratio (~30%, $p < 0.02$), a ratio which has been used as a metabolic index of disease severity (Wassif et al 1998) (Table 2).

Simvastatin significantly lowered urinary mevalonate excretion by approximately 50% on average (from 415 ± 70 nmol/ μ mol creatinine to 261 ± 61 nmol/ μ mol creatinine, $p < 0.008$, Fig. 4A), but did not change the urinary excretion of 3MGC (9.7 ± 2.0 nmol/ μ mol creatinine vs. 10.5 ± 2.1 nmol/ μ mol creatinine for no simvastatin and simvastatin respectively; NS, Fig. 4B), dolichol (301 ± 175 ng/mg creatinine vs. 84 ± 35 ng/mg creatinine; $p = 0.242$, paired t test), and Co-Q (179 ± 68 ng/mg creatinine vs. 707 ± 404 ng/mg creatinine; $p = 0.151$, paired t test). Simvastatin did not change the urinary dolichol chain length profile

(*data not shown*) and did not change plasma cholesterol, 7-DHC and 8-DHC concentrations (Table 3).

We next examined whether U-3MGC was correlated with markers of disease severity. We found no significant relationship between U-3MGC (SLOS-LO and SLOS-HI) and the clinical severity score (not shown). Similarly, there was no relationship between U-3MGC and either plasma cholesterol, 7-DHC or the ratio of [7-DHC+8-DHC] to cholesterol (*data not shown*). We also examined whether 3MGC excretion was correlated with the urinary excretion of long-chain isoprenoids in SLOS, and found no significant relationship between U-3MGC and either U-dolichol or U-CoQ (*data not shown*).

Last, we sought to determine if there was a correlation between 3MGC and MVA, 3MGC's closely related precursor within the mevalonate shunt pathway. No significant relationship was found, whether this relationship was examined in SLOS (SLOS-LO and SLOS-HI), controls, or the combined SLOS + Control group (Fig. 5).

Because there was no indication that U-3MGC was altered in SLOS, Control and SLOS (SLOS-LO) 3MGC data were pooled and examined for possible relationship with subject characteristics. Overall, U-3MGC was inversely related to age (Pearson rank correlation coefficient = -0.34 , $p < 0.036$) and body weight (Pearson rank correlation coefficient = -0.37 , $p < 0.02$). As illustrated in Fig. 6, these were not direct relationships: the data showed a sharp drop of U-3MGC in the first 2 years of life (Fig. 6A) or when body weight increased to approximately 10 kg (Fig. 6B) followed by a slow decline as subjects were older or heavier. A gender difference was also observed, with males having lower U-3MGC (8.1 ± 1.6 nmol/ μ mol creatinine; $n = 19$) than females (13.4 ± 1.5 nmol/ μ mol creatinine; $n = 20$) ($p = 0.025$; Fig. 7).

We last postulated that subtle differences in gender and age (or body weight) between SLOS and control subjects might have masked group difference. We thus further analyzed our data using the following general linear regression model: $[U-3MGC] = (a * \text{LOG}(\text{body weight or age})) + (b * \text{group}) + (c * \text{gender}) + d$ (with a , b , c , and d being constants). This analysis did not reveal any significant group difference and thus confirmed our univariate 3MGC data analysis.

DISCUSSION

U-3MGC is not elevated in SLOS at least in mildly or moderately severely affected patients. An increase in 3MGC plasma concentration and urine excretion was reported earlier by Kelley and Kratz (Kelley and Kratz 1995) in the “younger, more severely affected SLOS patients with lower cholesterol levels” suggesting that more severe enzyme defects leads to more shunting of mevalonate and short-chain isoprenoids. The results of the present study do not support this hypothesis. Although we did not measure plasma 3MGC concentration in our study, we found no evidence for increased 3MGC urine excretion, even in the most severely affected subjects. The three SLOS subjects with the highest clinical severity scores in our study (25, 30 and 35) had U-3MGC of 11.4, 11.8 and 6.0 nmol/ μ mol creatinine respectively, i.e. well within the range of U-3MGC values in control subjects (9.5 ± 2.8 nmol/ μ mol creatinine, mean $\pm$ 2SE). Since Kelley had previously reported that U-3MGC decreased with age (Kelley 1993), we attempted to adjust our comparison between control and SLOS subjects for age (and body weight) on the premise that the differences between the results of Kelley's study and ours might be related to age difference. This analysis yielded no evidence for a significant U-3MGC difference between SLOS and control subjects. It could be that our study did not include enough severely affected subjects. Kelley's report however indicates that only 5 of the 17 patients with severe type II SLOS had elevated plasma and

urine 3MGC. Thus, the accumulation of 3MGC that these investigators reported may not have been related to disease severity. Further, the patients with the highest 3MGC concentrations had the lowest plasma cholesterol concentrations but not the highest 7-DHC concentration, hence they may not have had the most severe DHCR7 deficiency. In brief, the model whereby DHCR7 block would lead to MVA accumulation and overflow into the shunt pathway to be excreted as 3MGC seems to be flawed.

A normal 3MGC urinary excretion also seem to exclude the possibility of impaired mitochondrial oxidative function. One could propose that in SLOS, diversion toward 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), and then to ketone bodies, fatty acids or CO₂ (pathway 2 in Fig. 1) offsets a potential excess of 3-methylglutaconyl-CoA coming from MVA, and maintains U-3MGC within normal range. However, without evidence that either ketone bodies (e.g. β -hydroxybutyrate), MVA-derived CO₂ production or fatty acid synthesis are elevated, one must conclude that steady-state 3-MGC level is normal in SLOS.

Quite interestingly, U-3MGC varies by gender, being lower in males than in females. The significant gender difference persists after adjustment for age and cannot be explained by differences in muscle mass since U-3MGC is normalized by creatinine excretion. This finding bears further exploration which may benefit our understanding of metabolic diseases that show established 3-methylglutaconic aciduria.

The present study confirms with additional data our previous report of increased urinary excretion of dolichol and CoQ in SLOS (Pappu et al 2006). Taken together with normal MVA and 3MGC excretion, the long-chain isoprenoid data suggest that the enzymatic block in SLOS leads to increased synthesis of long-chain isoprenoids. It is tempting to speculate that the increased in isoprenoid urinary excretion is a consequence of an overflow of cholesterol precursors into the isoprenoid synthesis pathway. However, this scenario seems unlikely. Studies have shown that isoprenoid hepatic concentrations and rates of synthesis are not modified under conditions that greatly alter cholesterologenesis (Keller and Vilsaint 1993). These studies were performed in the rat though and the consequences of cholesterol synthesis deficiency may have a different impact on branched pathways in humans. Interestingly, the Keller and Vilsaint study showed that isoprenoid chain length increases with tissue mevalonate concentration. In our study, dolichol chain length was significantly increased in SLOS subjects on a cholesterol-free diet as compared to controls, suggesting that mevalonate accumulation and diversion into longer-chain isoprenoids occur in SLOS. However, neither supplementation with dietary cholesterol nor treatment with simvastatin which both significantly decreased urinary mevalonate excretion, changed urinary dolichol chain length or dolichol urinary excretion. Thus, it seems that in SLOS, increased urinary dolichol excretion may be secondary to a constitutive increase in the activity of the enzymes of the dolichol synthesis pathway rather than to an increased diversion of mevalonate and sterol precursors into this pathway. It is important to also note that urinary dolichol excretion is not necessarily related to plasma dolichols and may only reflect local renal synthesis (Humaloja et al 1991, Roine et al 1992). Thus, it is possible that whole body isoprenoid pool size is not increased in SLOS. Unfortunately we did not collect blood in control subjects and did not have enough blood sample left in the SLOS group to further explore this possibility. Whether or not increased urinary isoprenoid excretion is an adaptive response to impaired DHCR7 deficiency will need to be explored in future studies using patients' cells in culture or animal models of the disease.

The significant decrease in U-MVA observed in SLOS subjects given a high cholesterol diet or the combination simvastatin and high cholesterol diet indicates that HMG-CoA reductase activity is normally regulated in SLOS by exogenous cholesterol (down-regulation) and by specific inhibitors (simvastatin). The data thus suggest unaltered feed-back regulation of

HMG-CoA reductase (HMGR) activity in SLOS as anticipated from the results of several animal and in vitro studies (Chambers et al 1997; Xu et al 1995; Fitzky et al. 2001). To have a functional, “responsive” HMG-CoA reductase amenable to feedback regulation is important because it may prove useful in developing treatments for SLOS. Indeed, it has been suggested that in SLOS, inhibition of HMGR with dietary cholesterol, simvastatin or a combination of both may reduce the tissue accumulation of toxic cholesterol precursors (7-DHC and 8-DHC) and ameliorate the clinical phenotype (Jira et al 2000). Surprisingly, we did not observe any significant decrease in 7-DHC (or 8-DHC) after cholesterol supplementation or treatment with simvastatin. It is possible though that the massive dietary cholesterol intake so profoundly down-regulated the expression of the LDL receptor that LDL clearance was reduced. Because 7-DHC, like cholesterol, is transported by LDL and other apo-B containing lipoproteins, its plasma clearance may have been reduced as well, thus resulting in unchanged steady-state plasma 7-DHC concentrations.

In conclusion, the data show that U-3MGC is not elevated in SLOS at least in mildly or moderately severely affected patients. The fact that U-3MGC is not correlated with the clinical or biochemical phenotype or affected by dietary cholesterol supplementation or treatment with simvastatin is further evidence that active mevalonate shunting is unlikely to be involved in the pathogenesis of SLOS, and is not a likely future target for therapy development.

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List of abbreviations

3MGC	3-methylglutaconic acid
7-DHC	7-dehydrocholesterol
8-DHC	8-dehydrocholesterol
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
DHCR7	7-dehydrocholesterol reductase (Δ 7-sterol reductase)
HMG-CoA	3-methyl-3-glutarylcoenzyme A
CoQ	coenzyme Q
GCMS	gas chromatography-mass spectrometry
MVA	mevalonic acid or mevalonate
SLOS	Smith-Lemli-Opitz syndrome
SLOS-HI	group of SLOS subjects receiving a high cholesterol diet

SLOS-LO group of SLOS subjects receiving a cholesterol-free diet

REFERENCES

- Bataille KP, Steiner RD. Smith-Lemli-Opitz syndrome: the first malformation syndrome associated with defective cholesterol synthesis. *Mol Genet Metab.* 2000; 71:154–162. [PubMed: 11001806]
- Chambers CM, McLean MP, Ness GC. Smith-Lemli-Opitz syndrome produced in rats with AY 9944 treated by intravenous injection of lipoprotein cholesterol. *Am J Med Genet.* 1997; 68:322–327. [PubMed: 9024567]
- Cunniff C, Kratz LE, Moser A, Natowicz MR, Kelley RI. Clinical and biochemical spectrum of patients with RSH/Smith-Lemli-Opitz syndrome and abnormal cholesterol metabolism. *Am J Med Genet.* 1997; 68:263–269. [PubMed: 9024557]
- Edmond J, Popják G. Transfer of carbon atoms from mevalonate to n-fatty acids. *J Biol Chem.* 1974; 249:66–71. [PubMed: 4809631]
- Engelking LJ, Evers BM, Richardson JA, Goldstein JL, Brown MS, Liang G. Severe facial clefting in Insig-deficient mouse embryos caused sterol accumulation and reversed by lovastatin. *J Clin Invest.* 2006; 116:2356–2365. [PubMed: 16955138]
- Feist F. Carbacetessigester und isodehydracetsäureester. *Annalen.* 1906; 345:60.
- Fitzky BU, Witsch-Baumgartner M, Erdel M, et al. Mutation in the delta7-sterol reductase gene in patients with Smith-Lemli-Opitz syndrome. *Proc Natl Acad Sci USA.* 1998; 95:8181–8186. [PubMed: 9653161]
- Fitzky BU, Moebius FF, Asaoka H, et al. 7-Dehydrocholesterol-dependent proteolysis of HMG- CoA reductase suppresses sterol biosynthesis in a mouse model of Smith-Lemli- Opitz/RSH syndrome. *J Clin Invest.* 2001; 108:905–915. [PubMed: 11560960]
- Gramajo AL, Zacharias LC, Neekhra A, et al. Mitochondrial DNA damage induced by 7-ketocholesterol in human retinal pigment epithelial cells in vitro. *Invest Ophthalmol Vis Sci.* 2010; 51:1164–1170. [PubMed: 19834037]
- Humaloja K, Roine RP, Salmela K, et al. Serum dolichols in different clinical conditions. *Scand J Clin Lab Invest.* 1991; 51:705–709. [PubMed: 1806986]
- Jackman L, Wiley RH. Nuclear magnetic resonance and the stereochemistry of cis-trans-isomers, the isolation of pure trans- β -methylglutaconic acid. *Proc Chem Soc.* 1958; 196
- Jira PE, Wevers RA, de Jong J, et al. Simvastatin. A new therapeutic approach for the Smith-Lemli-Opitz syndrome. *J Lipid Res.* 2000; 41:1339–1346. [PubMed: 10946022]
- Jones PJ, Pappu AS, Hatcher L, Li ZC, Illingworth DR, Connor WE. Dietary cholesterol feeding suppresses human cholesterol synthesis measured by deuterium incorporation and urinary mevalonic acid levels. *Arterioscler Thromb Vasc Biol.* 1996; 16:1222–1228. [PubMed: 8857917]
- Keller RK, Vilsaint F. Regulation of isoprenoid metabolism in rat liver: near constant chain lengths of dolichyl phosphate and ubiquinone are maintained during greatly altered rates of cholesterolgenesis. *Biochim Biophys Acta.* 1993; 1170:204–210. [PubMed: 8399346]
- Keller RK, Arnold TP, Fliesler SJ. Formation of 7-dehydrocholesterol-containing membrane rafts in vitro and in vivo, with relevance to the Smith-Lemli-Opitz syndrome. *J Lipid Res.* 2004; 45:347–355. [PubMed: 14594996]
- Kelley RI. Quantification of 3-methylglutaconic acid in urine, plasma, and amniotic fluid by isotope-dilution gas chromatography/mass spectrometry. *Clin Chim Acta.* 1993; 220:157–164. [PubMed: 8111960]
- Kelley RI, Kratz L. 3-Methylglutaconic acidemia in Smith-Lemli-Opitz syndrome. *Pediatr Res.* 1995; 37:671–674. [PubMed: 7603789]
- Kelley RI, Hennekam RC. The Smith-Lemli-Opitz syndrome. *J Med Genet.* 2000; 37:321–335. [PubMed: 10807690]
- Korade Z, Xu L, Shelton R, Porter NA. Biological activities of 7-dehydrocholesterol-derived oxysterols: implications for Smith-Lemli-Opitz syndrome. *J Lipid Res.* 2010; 51:3259–3269. [PubMed: 20702862]

- Kratz LE, Kelley RI. Prenatal diagnosis of the RSH/Smith–Lemli–Opitz syndrome. *Am J Med Genet.* 1999; 82:376–381. [PubMed: 10069707]
- Kwiterovich PO. Plasma lipid and lipoprotein levels in childhood. *Ann NY Acad Sci.* 1991; 623:90–107. [PubMed: 2042879]
- Linck LM, Lin DS, Flavell D, Connor WE, Steiner RD. Cholesterol supplementation with egg yolk increases plasma cholesterol and decreases plasma 7-dehydrocholesterol in Smith-Lemli-Opitz syndrome. *Am J Med Genet.* 2000; 93:360–365. [PubMed: 10951458]
- Merkens LS, Connor WE, Linck LM, Lin DS, Flavell DP, Steiner RD. Effects of dietary cholesterol on plasma lipoproteins in Smith-Lemli-Opitz syndrome. *Pediatr Res.* 2004; 56:726–732. [PubMed: 15319461]
- Pappu AS, Illingworth DR, Bacon S. Reduction in plasma low-density lipoprotein cholesterol and urinary mevalonic acid by lovastatin in patients with heterozygous familial hypercholesterolemia. *Metab Clin Exper.* 1989; 38:542–549. [PubMed: 2725293]
- Pappu AS, Steiner RD, Connor SL, et al. Feedback inhibition of the cholesterol biosynthetic pathway in patients with Smith-Lemli-Opitz syndrome as demonstrated by urinary mevalonate excretion. *J Lipid Res.* 2002; 43:1661–1669. [PubMed: 12364550]
- Pappu AS, Connor WE, Merckens LS, et al. Increased nonsterol isoprenoids, dolichol and ubiquinone, in the Smith-Lemli-Opitz syndrome: effects of dietary cholesterol. *J Lipid Res.* 2006; 47:2789–2798. [PubMed: 16983147]
- Parker TS, McNamara DJ, Brown CD, et al. Plasma mevalonate as a measure of cholesterol synthesis in man. *J Clin Invest.* 1984; 74:795–804. [PubMed: 6565710]
- Roine R, Nykänen I, Salaspuro M. Effects of alcohol on urinary and blood dolichols. *Biochem Cell Biol.* 1992; 70:404–407. [PubMed: 1280442]
- Schroepfer GJ Jr. Sterol biosynthesis. *Ann Rev Biochem.* 1981; 50:585–621. [PubMed: 7023367]
- Smith DW, Lemli L, Opitz JM. A newly recognized syndrome of multiple congenital abnormalities. *J Pediatr.* 1964; 64:210–217. [PubMed: 14119520]
- Steiner RD, Linck LM, Flavell DP, Lin DS, Connor WE. Sterol balance in the Smith-Lemli-Opitz syndrome. Reduction in whole body cholesterol synthesis and normal bile acid production. *J Lipid Res.* 2000; 41:1437–1447. [PubMed: 10974051]
- Sweetman, L.; Williams, JC. Branched chain organic acidurias. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. *The Metabolic and Molecular Bases of Inherited Disease.* New York: McGraw-Hill; 2001. p. 2125-2163.
- Taylor MM, Macdonald K, Morris AJ, McMaster CR. Enhanced apoptosis through farnesol inhibition of phospholipase D signal transduction. *FEBS J.* 2005; 272:5056–5063. [PubMed: 16176276]
- Tint GS, Irons M, Elias ER, et al. Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N Engl J Med.* 1994; 330:107–113. [PubMed: 8259166]
- Tulenko TN, Boeze-Battaglia K, Mason RP, et al. A membrane defect in the pathogenesis of the Smith-Lemli-Opitz syndrome. *J Lipid Res.* 2006; 47:134–143. [PubMed: 16258167]
- Wassif CA, Maslen C, Kachilele-Linjewile S, et al. Mutations in the human sterol delta7-reductase gene at 11q12-13 cause Smith-Lemli-Opitz syndrome. *Am J Hum Genet.* 1998; 63:55–62. [PubMed: 9634533]
- Waterham HR, Wijburg FA, Hennekam RC, et al. Smith-Lemli-Opitz syndrome is caused by mutations in the 7-dehydrocholesterol reductase gene. *Am J Hum Genet.* 1998; 63:329–338. [PubMed: 9683613]
- Xu G, Salen G, Shefer S, Ness GC, et al. Treatment of the cholesterol biosynthetic defect in Smith-Lemli-Opitz syndrome reproduced in rats by BM 15.766. *Gastroenterology.* 1995; 109:1301–1307. [PubMed: 7557099]
- Xu L, Korade Z, Porter NA. Oxysterols from free radical chain oxidation of 7-dehydrocholesterol: product and mechanistic studies. *J Am Chem Soc.* 2010; 132:2222–2232. [PubMed: 20121089]

Figure 1.

Possible origins of urinary excretion of 3MGC (U-3MGC) in SLOS. Dotted lines represent multiple metabolic steps. Red arrows indicate reported changes in metabolite concentration or excretion. When in parenthesis, the arrows indicate putative changes. In SLOS, 7-dehydrocholesterol reductase (7-DHC reductase) deficiency causes low cholesterol, 7-DHC accumulation and diversion of 7-DHC precursors toward dolichol and ubiquinone, whereas mevalonate (MVA) is normal. Short-chain isoprenoid precursor 3,3'-dimethylallyl pyrophosphate (DMA-PP) may also accumulate as a consequence of 7-DHC reductase deficiency. DMA-PP may be metabolized into 3-methylglutaconyl-CoA via the shunt pathway and excreted in the urine as 3MGC (pathway 1). Alternatively, 3-methylglutaconyl-CoA may be converted to HMG-CoA by mitochondrial 3MGC-CoA hydratase and further converted to CO₂ or fatty acids (pathway 2). 3-Methylglutaconic acid is also a byproduct of the mitochondrial leucine catabolic pathway (pathway 3). Hence, in SLOS, increased U-3MGC may be caused by 1) increased diversion of MVA through the shunt pathway

secondary to either MVA accumulation and overflow or activation of the enzymes of the MVA shunt pathway, or 2) impaired mitochondrial function, a potential consequence of altered cell membrane structure (Tulenko 2006).

Figure 2. 3MGC assay by GCMS. (A) assay linearity (area under the curve vs. amount injected); (B) example of the characteristic fragmentation pattern obtained for synthetic 3MGC prepared as described in *Materials & Methods* showing the target ion used for quantitation (m/z 273) and the qualifying ion used to verify identity (m/z 198). The Agilent -5975 detector was operated in the electron impact mode at 70eV; (C) typical GC profile (see assay conditions and instrument setting in *Materials & Methods*) of a urine sample spiked with 2,2-dimethylglutaric acid as internal standard (IS). 3MGC and IS co-elute at ~ 12.4 min. The IS target ion was 289 and the qualifying ion was 204.

Figure 3.

Dolichol chain length profile in controls and in SLOS patients on a cholesterol-free diet. Data are expressed as mean ($\pm$ SE) percent of total dolichols. Fractions 1 to 8 correspond to dolichols with chain lengths ranging from 80 to 115 carbons and fraction 9 corresponds to dolichols with chain lengths higher than 115 carbons. * indicates $p < 0.05$. (*): $p = 0.07$

Figure 4.
Effect of simvastatin treatment in SLOS patients receiving cholesterol supplementation, on urinary MVA (3A) and 3MGC (3B) excretion. (-): no simvastatin, (+) simvastatin.

Figure 5. Relationship between urinary excretion of 3-MGC and U-MVA in controls (◇), SLOS-LO (▲), SLOS-HI (▲) and SLOS-HI treated with simvastatin (●). There was no significant correlation between U-3MGC and U-MVA either within each group or when all groups were pooled.

Figure 6. Relationship between urinary excretion of 3MGC (U-3MGC) and age (A) or body weight (BW; (B)). Open symbols (○) represent SLOS subjects; closed symbols (●) represent Controls. To illustrate trends, trend lines were generated by fitting experimental data to the following equation: $U-3MGC = A * (age \text{ or } BW)^B$ where “A” and “B” are constants.

Figure 7.
Urinary excretion of 3MGC (U-3MGC) in males (n = 19) and females (n = 20) (mean +1 SE).

Table 1

SLOS subjects' severity scores and mutations

Subject #	Severity Score	Mutation 1	Mutation 2
1	5	R242C	V466M
2	6	P51S	IVS8-1G>C
3	6	C380Y	IVS8-1G>C
4	10	R450L	IVS8-1G>C
5	10	R450L	IVS8-1G>C
6	10	T154M	IVS8-1G>C
7	11	A247V	IVS8-1G>C
8	11	F302L	L470Q
9	11	A247V	IVS8-1G>C
10	15	W151X	S169L
11	17	F302L	L470Q
12	17	F284L	V325L
13	17	F302L	L470Q
14	20	T93M	V326L
15	20	M1V*	Q98X
16	22	R352W	IVS8-1G>C
17	25	R404C	G410S
18	30	Y462H	IVS8-1G>C
19	35	T93M	IVS8-1G>C

* loss of a start codon.

Table 2

Plasma (pl-) and urine (U-) chemistry in control (n = 20) and SLOS (n = 19) subjects. SLOS were studied on a cholesterol-free diet (SLOS-LO) and after 1 year (on average) of dietary cholesterol supplementation (SLOS-HI).

	U-3MGC mmol/μmol creatinine	U-MVA mmol/mmol creatinine	U-Dolichol ng/mg creatinine	U-CoQ ng/mg creatinine	pl-Chol mM	pl-7-DHC mM	pl-8-DHC mM	[7-DHC+8- DHC]/Chol ratio
Control	9.5±1.4	532±174	69.2±26.5 ^a	171±73 ^b	ND ^{**}	ND ^{***}	ND	ND
*	NS	NS	↑ <i>p</i> <0.035	↑ <i>p</i> <0.02	-	-	-	-
SLOS-LO	12.2±1.9	735±195	320±117 (314±135) ^c	886±261 (773±231) ^d	2.02±0.19	0.22±0.03	0.18±0.02	0.28±0.05
**	NS	↓ <i>p</i> <0.05	NS	NS	↑ <i>p</i> <0.001	NS	NS	↓ <i>p</i> <0.02
SLOS-HI	9.9±1.2	338±96	292±147 ^c	546±199 ^d	2.68±0.22	0.19±0.03	0.17±0.02	0.18±0.04

Results are mean ± SE

^a
n = 14;

^b
n = 14;

^c
n = 15 paired samples;

^d
n = 16 paired samples

ND = not determined; NS = not significant

* p values, unpaired *t* test (Controls vs. SLOS-LO);

** p values, paired *t* test (SLOS-LO vs. SLOS-HI)

*** reference range in children (age 5 to 9): 3.1 to 5.3 mM (5th – 95th percentile) (Kwiterovich, 1991)

pl-7-DHC in healthy children: 0.03 ± 0.01 mM (Cunniff, 1997)

Table 3

Effect of simvastatin on urinary (U-) and plasma (pl-) cholesterol metabolism parameters in SLOS subjects (n = 9) treated with dietary cholesterol supplementation (SLOS-HI). Cholesterol intake was 444 ± 55 mg/day and 389 ± 44 mg/day for the “simvastatin” and “no simvastatin” groups respectively)

	pl-Chol mM	pl-7-DHC mM	pl-8-DHC mM	[7-DHC+8- DHC]/Chol ratio
<i>No simvastatin</i>	3.04 ± 0.32	0.16 ± 0.03	0.15 ± 0.03	0.12 ± 0.03
<i>**</i>	<i>NS</i>	<i>NS</i>	<i>NS</i>	<i>NS</i>
<i>Simvastatin</i>	2.95 ± 0.37	0.13 ± 0.03	0.12 ± 0.02	0.11 ± 0.03

NS = not significant

*
p values (SLOS-No simvastatin vs. SLOS-Simvastatin)