Assessment of the Whole Body Cholesterol Pool Size in Smith-Lemli-Opitz

Syndrome Patients Receiving Cholesterol Supplementation Alone or

Combined with Simvastatin

By

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Abstract

Smith-Lemli-Opitz syndrome (SLOS) is a multiple congenital/mental retardation syndrome, caused by 7-dehydrocholesterol-Δ7-reductase (DHCR7) enzyme deficiency. DHCR7 enzyme is involved in the last step conversion of sterol precursors to cholesterol, thus its deficiency results in low plasma cholesterol, high 7-dehydrocholesterol (7DHC) and 8-dehydrocholesterol (8DHC) levels in tissues and plasma.

Dietary cholesterol supplementation has been used as a standard therapy, aiming to increase plasma and tissue cholesterol concentrations, down regulating 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA R) enzyme activity and perhaps suppressing 7DHC synthesis. HMG CoA R competitive inhibitor (e.g. simvastatin) also has been used in explorative studies in SLOS to decrease toxic build-up of 7DHC and 8DHC.

Since cholesterol supplementation is prescribed routinely for most SLOS patients, it is critical to examine effects of cholesterol supplementation alone (HI) or combined with simvastatin (HI+ST) on maximizing whole body cholesterol pool size (WBCPS) and how other parameters can influence WBCPS in a positive way while down-regulating biosynthesis to decrease the build-up of potentially toxic precursors (7DHC/8DHC).

SLOS patients receiving cholesterol supplementation alone (n=15; mean age: 9.4 ± 1.9 years) or combined with simvastatin (n=4; mean age: 7.3 ± 1.2 years) were administered an intravenous injection of [^{18}O]-cholesterol (1.0-1.4 mg/kg bodyweight) or [$^{2}H_{7}$]-cholesterol (0.9-1.4 mg/kg). Blood samples were

collected at baseline and over a 10 weeks period, cholesterol was extracted from red blood cells, derivatized with piconyl ester and analyzed using liquid chromatography-tandem mass spectrometry (LC/MS/MS). Data were analyzed with SPSS statistical software. General linear regression analysis was used to examine associations between WBCPS and various parameters including body weight, cholesterol intake, and plasma levels of cholesterol, and for comparison of WBCPS values between patients on high cholesterol diet with and without simvastatin. Results are reported as mean ± SEM.

Overall, WBCPS was significantly predicted by body weight (r^2=0.65; $p<0.05$) and age (r^2=0.46; $p<0.05$), but not plasma cholesterol levels (r^2=0.10). WBCPS failed to correlate with cholesterol intake. However, plasma cholesterol concentration correlated with cholesterol intake (r^2=0.42; $p<0.05$). WBCPS was higher in HI+ST compared to HI group (2.76 ± 0.20 vs. 2.53 ± 0.19 mg/kg bodyweight, respectively; p=0.02). Finally, no significant change in WBCPS was seen over time (p=0.06) in SLOS patients (n=6) re-evaluated after 2.0 ± 1.1 years of high cholesterol supplementation with and without simvastatin.

The findings of this current study increased our knowledge about the effects of interventions such as dietary cholesterol supplementation and simvastatin on the WBCPS in children with SLOS.

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List of abbreviations

API	Atmospheric pressure ionization
DHC	dehydrocholesterol
DHCR7	7-dehydrocholesterol-Δ7-reductase
Farnesyl-PP	Farnesyl-diphosphate
GC/MS	Gas chromatography-mass spectrometry
HMG Co A	3-hydroxy-3-methylglutaryl coenzyme A reductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NDSR	Nutrition Data System for Research
NICHD	National Institute of Child Health and Human Development
OHSU	Oregon Health and Science University
RBC	Red blood cells
SEM	Standard error of the mean
Shh	Sonic hedgehog
SLOS	Smith-Lemli-Opitz syndrome
STAIR	Sterol and Isoprenoid Research Consortium
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
WBCPS	Whole body cholesterol pool size
7DHC	7-dehydrocholesterol
8DHC	8- dehydrocholesterol

CHAPTER 1. INTRODUCTION

Background, rationale and objectives

Smith-Lemli-Opitz syndrome is a rare autosomal recessive multiple congenital anomalies disorder discovered in 1964 when Smith, Lemli, and Opitz described in a report three patients with common characteristic facial features, hypospadias, severe feeding problem and global intellectual disability (Smith, Lemli, & Opitz, 1964). Since that first report, many hundreds of clinical cases of SLOS have been reported and remained as clinical diagnosis for up to 30 years before the discovery of the biochemical abnormality in those patients. It is estimated that the incidence of SLOS is 1/20,000 - 1/80,000 live births, depending upon the region. SLOS is more prevalent among individuals of Caucasian descent while infrequent in other ethnic groups (e.g. African, Asian and South American) (Kelley & Hennekam, 2000; Ryan et al., 1998). SLOS is caused by mutations in 7-dehydrocholesterol-reductase gene *(DHCR7)*, resulting in DHCR7 enzyme deficiency, which catalyzes the conversion of 7DHC to cholesterol. DHCR7 enzyme deficiency results in cholesterol deficiency with 7DHC and 8DHC accumulating in tissue and plasma.

Therapeutic interventions for SLOS, including cholesterol supplementation and HMG CoA R competitive inhibitors (e.g. simvastatin), are aimed at enhancing WBCPS while decreasing 7DHC and 8DHC concentrations.

The main objective of this study was to assess WBCPS in SLOS patient's receiving cholesterol supplementation alone or combined with simvastatin. Such assessment should reflect a unique insight into cholesterol metabolism in SLOS patients than plasma cholesterol concentration alone.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Introduction

The following review introduces the reader to Smith-Lemli-Opitz syndrome clinical features, diagnosis, molecular genetics, and prevalence and to share the current knowledge about therapies in SLOS.

2.2 Clinical Description of SLOS

The majority of SLOS patients have minor malformations without affecting major organs (i.e. lung, heart, liver and kidneys), while in more severe forms of SLOS life expectancy is limited by the severity of internal malformation. Some characteristic clinical features of SLOS patients include craniofacial features, limbs, cardio-pulmonary defects, gastro-intestinal anomalies, urogenital malformation, development and behaviour, dermatologic problems, adrenal insufficiency and endocrine problems. These clinical features described herein expanded the known characteristics of this syndrome by many previously published case reports over the past 20 years. The following section below will provide an overview of each of the major clinical characteristic features of SLOS.

2.2.1 Craniofacial

Several distinct craniofacial features are easily recognised in most SLOS patients, such as microcephaly (genetic abnormality in brain causing small head), short nasal bridge, broad nasal tip, bilateral ptosis (dropping of both eyelids), micrognathia, narrow forehead, low set of ears and cleft palate, which contribute to feeding problems and failure to thrive (>50% of patients) (Nowaczyk, Hughes, Costa, & Clarke, 1998; Ryan et al., 1998).

2.2.2 Limbs

The characteristic skeletal anomalies syndactyly (joining) of second and third toes have been present in up to 99% of confirmed cases of SLOS (Cunniff, Kratz, Moser, Natowicz, & Kelley, 1997). Other abnormalities of the digits include bilateral or unilateral polydactyly of hands and/or feet, short thumbs and other lower limb abnormalities such as club foot (Curry et al., 1987).

2.2.3 Cardio-pulmonary

Minor and major congenital cardiac defects have been identified in almost half of all SLOS patients (Kelley & Hennekam, 2000; Lin, Ardinger, Ardinger, Cunniff, & Kelley, 1997), where atrioventricular canal defect was found to be the most common cardiac defect (25%) (Lin et al., 1997).

2.2.4 Gastro-intestinal

Gastro-intestinal anomalies such as pyloric stenosis, intestinal aganglionosis (Hirschprungs' disease) and gastrointestinal reflux, which lead to failure to thrive, are also common in SLOS (Patterson, Toomey, & Chandra, 1983). Growth retardation or failure to thrive because of feeding difficulties in infants due to cleft palate (a gap in the roof of the mouth), swallowing difficulties, poor suckling and gastrointestinal reflux are also seen in SLOS patients; ranging from 10 to 50% of all patients (Cunniff et al., 1997; Kelley & Hennekam, 2000; Ryan et al., 1998).

2.2.5 Urogenital

Genital malformation in SLOS patients range from normal, in mildly affected individuals, to complete sex reversal with a female phenotype 46, XY karyotype in severe SLOS cases (Lin et al., 1997). Hypospadias (shifted opening

of the urethra) and/or cryptorchidism (absence of testes) occur in almost half of all reported SLOS male individuals (A. E. Lin et al., 1997). Bicornate uterus and septate vagina are noted in females 46, XX (Lowry, Miller, & MacLean, 1968).

2.2.6 Development and behaviour

Mental developmental abnormalities and behavioural problems are very common, representing global characteristics of SLOS. Specifically, microcephaly is one of the most common development abnormalities of the central nervous system, affecting up to 95% of all SLOS patients. Mental development abnormalities in SLOS range from normal or borderline to severe intellectual disability (Mueller et al., 2003; Ryan et al., 1998). Behavioural symptoms in individuals with SLOS include irritability, hypersensitivity to visual and auditory stimuli, self-injurious behaviour (up to 35%) and aggression (up to 63%), interrupted sleep cycle and autistic spectrum behaviours (up to 86%) (Kelley & Hennekam, 2000; Nwokoro & Mulvihill, 1997; Ryan et al., 1998; Sikora, Pettit-Kekel, Penfield, Merkens, & Steiner, 2006; Tierney, Nwokoro, & Kelley, 2000; Tint et al., 1995).

2.2.7 Dermatological and ophthalmological

SLOS patients often experience dermatological problems such as skin photosensitivity (Charman et al., 1998) and ophthalmological abnormalities (Atchaneeyasakul, Linck, Connor, Weleber, & Steiner, 1998; Elias, Hansen, Irons, Quinn, & Fulton, 2003).

2.2.8 Adrenal insufficiency

Furthermore, endocrine problems such as adrenal hyperplasia with hypertension can develop in SLOS infants due to cholesterol deficiency, highlighting the importance of cholesterol as a precursor for steroid hormone synthesis (Chemaitilly et al., 2003; Nowaczyk & Waye, 2001).

2.2.9 Other findings

Other findings such as liver disease can vary in severity. Cholestasis can be seen for example in severely affected individuals (Rossi et al., 2005), other clinical features of the syndrome are listed in **Table 1.**

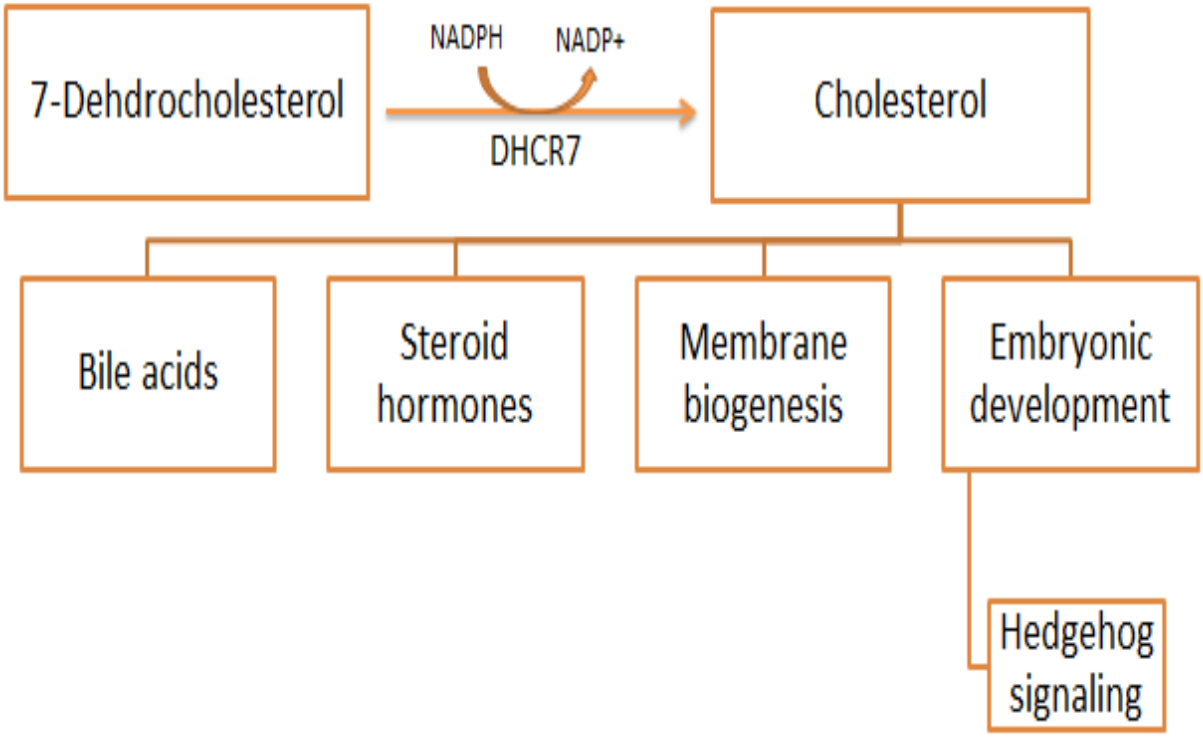
Table 1. Clinical features of Smith-Lemli-Opitz syndrome (DeBarber, Eroglu, Merkens, Pappu, & Steiner, 2011; Porter, 2008).

General	Growth retardation Intellectual disability Failure to thrive Aggressive behaviour or self-injurious Developmental delay Autism
Craniofacial	Cleft palate/bifid uvula Microcephaly Broad nasal bridge Broad aveolar ridges Cataracts bitemporal narrowing ptosis
Cardiac	Atrial septal defect Ventricular septal defect Hypertension Atrio-ventricular canal
Gastrointestinal	Pyloric stenosis Constipation Reflux Malrotation Liver Disease
Limbs	Syndactyly of the second and third toes Polydactyly Short thumbs
Skin	Photosensitivity Eczema/Dry skin
Urogenital	Hypospadias Renal malformation Cryptorchidism

2.3 Molecular genetics

Molecular genetics work discovered many pathogenic mutations in SLOS, including SLOS gene which was identified in 1998 and assigned to chromosome region 11q13 (Fitzky et al., 1998; Wassif et al., 1998). Specifically, SLOS is caused by mutation in *DHCR7* genes encoding DHCR7 enzyme, which impairs the conversion of 7DHC to cholesterol in the last step of cholesterol biosynthesis (**Figure 1**) (Witsch-Baumgartner et al., 2000). There are varying degrees of severity in the *DHCR7* gene mutations, which affect severity of the presentation of the disease as type 1 (mild) or type 2 (severe) SLOS. Individuals with type 1 SLOS have compound heterozygous mutations; a various mutation on each allele of *DHCR7* gene, and are presented with mild dysmorphic features and learning disabilities (Kelley, 1995). Individuals with type 2 SLOS have homozygous mutations, with the more severely affected individuals often dying in first weeks of life due to multiple congenital internal malformations (Correa-Cerro et al., 2005; F. D. Porter, 2000). There are over a hundred mutations discovered to date in SLOS patients population, with IVS8-1G>C identified as the most common mutation, which accounts for nearly one-third of the identified alleles (Correa-Cerro et al., 2005). The IVS8-1G>C mutation represents a splice site acceptor mutation in the last base of intron 8 that leads to an alternative upstream splice acceptor site. This results in the insertion of 134 base pairs of intronic sequence into the *DHCR7* mRNA, resulting in a frameshift and premature stop codon (Mira Irons, 2007). Most SLOS patients are compound heterozygous making it difficult to have strict correlations between clinical and biochemical phenotypes to genotype. Therefore, it is not possible to predict the clinical and biochemical phenotypes based on the genotype, and vice versa (Witsch-Baumgartner et al., 2000).

Figure 1. Final step of cholesterol biosynthesis pathway of Smith-Lemli-Opitz syndrome. 7-dehydrocholesterol-Δ7-reductase (DHCR7) mutations affect the reduction of 7-dehydrocholesterol (7DHC) to cholesterol in the final step of cholesterol biosynthesis pathway. Cholesterol deficiency as a result will affect different biological functions such as bile acids synthesis, Steroid hormones synthesis, membrane biogenesis and embryonic development. NADPH, Nicotinamide adenine dinucleotide phosphate and it is a reduced form of NADP+ (Porter, 2008).



2.4 Prevalence

It is estimated that the prevalence of SLOS ranges from 1/20,000 to 1/70,000 in Caucasians. However, it is difficult to accurately estimate the true prevalence because of the high variability in the forms of mutations (Porter, 2000), which can result in neonatal death in severely affected individuals (Cunniff et al., 1997) or missing mildly affected patients (Kelley & Hennekam, 2000). The IVS8-1G>C mutation has a carrier rate of approximately 1% for the Caucasian population in North America and 2 to 3.3% in Central European populations (F. D. Porter, 2000; Tint et al., 1994).

2.5 Biochemical diagnosis in SLOS

The elevated levels of 7DHC and its isomer 8DHC in SLOS are present as a result of reduced activity of the enzyme DHCR7, which converts 7DHC to cholesterol (Irons, Elias, Salen, Tint, & Batta, 1993; Irons et al., 1994). Currently, clinical suspicion of SLOS can be confirmed by the detection of increased serum concentrations of 7DHC and 8DHC in blood, plasma, cerebrospinal fluid (CSF), amniotic fluid and various tissues (Irons et al., 1994; Mills et al., 1996; Shefer et al., 1995). Routine measurement of total cholesterol (cholesterol plus precursors) by enzymatic method is not useful in diagnosing SLOS because it does not differentiate between cholesterol and 7DHC. The detected cholesterol levels may fall within the normal levels due to the presence of high amounts of 7DHC, therefore normal cholesterol values do not rule out SLOS. The cholesterol precursors, 7DHC and 8DHC, can only be properly detected with mass spectrometry analytical techniques such as gas chromatography-mass spectrometry (GC-MS) (Irons et al., 1993; Shefer et al., 1995) and liquid chromatography mass spectrometry (LC-MS) (Sattler, Leis, Kostner, & Malle, 1995; William J. Griffiths, 2008). Analysis by GC-MS

allows also for recognition of other sterol metabolic disorders such as desmosterolosis and lathosterolosis, where cholesterol precursors desmosterol and lathesterol accumulate in rare 'SLOS-like' syndromes, respectively. Alternatively, mutation analysis can be used to confirm the diagnosis of SLOS if blood or tissue is not available, or to elucidate equivocal biochemical results. In rare cases, sterol analysis of cultured cells (fibroblast) in cholesterol depleted medium can be used to confirm or exclude the diagnosis of SLOS (**http://www.nature.com/ejhg/journal/v16/n5/fig_tab/ejhg200810f1.html**).

2.6 Therapy

2.6.1 Cholesterol supplementation

Many uncontrolled, non-randomized trials of cholesterol supplementation in SLOS had emerged soon after discovery of the sterol biochemical defect. These cholesterol supplementation studies were aimed at increasing cholesterol in serum and tissues and reducing accumulation of 7DHC and 8DHC. Currently dietary cholesterol supplementation has become the main treatment strategy for individuals with SLOS because it has been shown to increase plasma and tissues cholesterol concentrations (Elias, Irons, Hurley, Tint, & Salen, 1997; M. Irons et al., 1994; Nwokoro & Mulvihill, 1997), and reduce levels of 7DHC and 8DHC by suppression of 7DHC/8DHC synthesis through feedback inhibition of HMG CoA R enzyme activity (Linck, Lin, Flavell, Connor, & Steiner, 2000).

The most common type of dietary cholesterol supplementation is either as food-based in natural forms (eggs, liver, meat, and cream) or crystalline cholesterol delivered by various vehicles, in oil-based or aqueous solution. In addition, there is an encapsulated cholesterol powder, which is a new cholesterol formulation developed by Solace Nutrition (Solace Nutrition company, Pawcatuck, CT, USA) as a potential medical food for SLOS patients. The dose of cholesterol given in previously reported trials ranged from 30 to 40 mg cholesterol/kg/day for egg yolk versus higher doses of crystalline cholesterol at 150 to 300 mg/kg/day due to lower intestinal absorption in SLOS patients (DeBarber, Eroglu, Merkens, Pappu, & Steiner, 2011; Lin, Steiner, Flavell, & Connor, 2005). In general, dietary cholesterol supplementation has become routinely prescribed to SLOS individuals even though there are no controlled, randomized, clinical trials to justify their efficacy. Some trials of cholesterol supplementations have yielded various

responses to therapy between patients and protocols suggested (Irons et al., 1997; Linck, Lin, Flavell, Connor, & Steiner, 2000; Nwokoro & Mulvihill, 1997).

In a multicentre trial of fourteen SLOS patients, Irons et al., (1997) reported an increase in plasma cholesterol levels of 164%, but no significant decreases in plasma 7DHC and 8DHC levels after a treatment protocol of cholesterol supplementation for 6 to 15 months, which was supplied either as crystalline cholesterol in soy oil, or as food in the form of egg yolk, the dose ranged from 60 to 120 mg/kg/day (Irons et al., 1997). However, a parallel report by Nwokoro and Mulvihill (1997) showed an overall increase in plasma cholesterol levels (up to ~50%) in three SLOS children but identical changes were not seen in three SLOS adults. In addition, two of the three children also had decreased in plasma 7DHC levels (20-50% decrease). Those patients were re-evaluated over 8 to 27 months of cholesterol replacement therapy of 77 to 1000 mg/kg/day alone or with bile acid treatment (Nwokoro & Mulvihill, 1997). Correspondingly, Linck et al. (2000) evaluated the effect of cholesterol supplementation with egg yolk in four SLOS children and found a 116% increase in mean plasma cholesterol level from 53 to 114 mg/dl ($p<0.05$) after 35 to 90 weeks of egg yolk feeding. Additionally there was a 67% decrease in mean plasma 7DHC level; from 35 to 11.7 mg/dl ($p<0.05$) (Linck et al., 2000). A likely explanation for the dissimilarities in the results of the above mentioned studies may be related to the source of cholesterol used and the length of the therapy. In this regards Lin et al., (2005) observed that crystalline cholesterol absorption was lower, at 20.5% absorption rate, compared to egg yolk cholesterol absorption rate, which was 27.3% in eleven SLOS patients, but the difference was not statistically significant ($p<0.21$) (Lin, Steiner, Flavell, & Connor, 2005).

Several observational studies suggest that cholesterol supplementation improves some of the features related to central nervous system in SLOS patients such as behaviour and development (Elias et al., 1997; Nwokoro & Mulvihill, 1997). Nwokoro et al., (1997) reported improvement in the developmental psychosocial status in three SLOS adults and children, and controlled aggressive behaviour in adults only, after receiving 77 to 1000 mg/kg/day of cholesterol therapy over 8-27 months (Nwokoro & Mulvihill, 1997). Likewise, the overall results from anecdotal study by Elias et al., (1997) proposed clinical benefits from cholesterol supplementation (40-125 mg/kg/day) over a two year period (±2 yrs.). Results included increased developmental progress (cognitive skills and language), decreased behavioural problems like hyperactivity, self-injurious behaviour and irritability in six SLOS children after cholesterol supplementation (Elias, Irons, Hurley, Tint, & Salen, 1997). Martin et al., (2001) showed marked improvement in aggression, self-injurious behaviour and hyperactivity within 48 hours of starting cholesterol supplementation (approximately 500 mg) in a case report (Martin et al., 2001). Sikora et al., (2004) conducted a study wherein continuous high cholesterol supplementation at approximately 18 to 60 mg/kg/day in the form of egg yolk were given to fourteen SLOS patients and their development quotients were assessed in areas of motor, cognitive and adaptive skills every 6 to 12 months using a standardized, reliable assessment instrument. Results of the study found no improvement in developmental quotients in these SLOS patients receiving high cholesterol supplementation (Sikora et al., 2004). A recent clinical trial by Tierney et al., (2010) measured hyperactivity through aberrant behaviour checklist subscale in ten SLOS patients receiving dietary cholesterol

supplementation treatment and placebo for 8 weeks. The results of this study showed no differences between treatment ($p=1.0$) and placebo ($p=0.97$) phases.

The result of the last two mentioned clinical trials do not agree with those early published reports (Elias et al., 1997; Martin et al., 2001; Nwokoro & Mulvihill, 1997) that are reviewed earlier in the cholesterol supplementation section, which suggested a beneficial role of cholesterol therapy on improving behaviour in SLOS patients. One possible reason for disagreement in the results is that circulating plasma cholesterol from cholesterol supplementation cannot cross the blood brain barrier (Sikora et al., 2004), which might have beneficial effects on brain 7DHC synthesis and the content of brain cholesterol, which is important for developmental progress.

Beyond the effects of cholesterol supplementation on brain function, there are documented observational positive outcomes of cholesterol supplementation on various features of SLOS including decreased photosensitivity, increase appetite, improved direct personal contact and sociability, improvement in morphometric measurements such as weight, height and head circumference, diminished skin rashes, subsided gastrointestinal symptoms and diminution in severity and number of infections (Azurdia, Anstey, & Rhodes, 2001; Elias et al., 1997; Irons et al., 1997; Nwokoro & Mulvihill, 1997).

2.6.2 Statin therapy and other treatments

Dietary cholesterol supplementation has been prescribed routinely for SLOS patients as a standard therapy; but its limitation in improving features of SLOS present at the level of central nervous system due to its inability to cross the blood-brain barrier (Björkhem & Meaney, 2004) led to the proposed use of statin drug in SLOS (Jira, Wevers, de Jong, Rubio-Gozalbo, & Smeitink, 1997; Jira et al., 2000). Statins are a class

of drugs used routinely to lower cholesterol levels by inhibiting HMG CoA R, which catalyzes the conversion of 3-hydroxy-3-methylglutaryl coenzyme (HMG CoA) to mevalonate; the rate limiting step in endogenous cholesterol synthesis. More specifically, simvastatin, a class of statins drugs, has been suggested to be used in explorative studies in SLOS. Simvastatin has the ability to cross the blood brain-barrier (Saheki, Terasaki, Tamai, & Tsuji, 1994), and therefore might have a beneficial effect in treating the biochemical defect related to the central nervous system in SLOS patients by blocking cholesterol *de novo* synthesis pathway to prevent the build-up of toxic levels of precursor sterols such as 7DHC and 8DHC (Haas et al., 2007; P. E. Jira et al., 2000; Starck, Lovgren-Sandblom, & Bjorkhem, 2002).

Skin fibroblasts from SLOS patients, with residual DHCR7 enzymatic activity, that were treated with simvastatin showed a decrease in 7DHC concentration and increased residual cholesterol synthesis due to increased *DHCR7* gene expression in cell lines when they were cultured in either cholesterol contacting or cholesterol-deficient medium. These outcomes can be beneficial if such up-regulation can be accomplished *in vivo* (Wassif et al., 2005).

Despite the potential benefits, statin also exhibit undesirable outcome in SLOS by decreasing cholesterol levels as well. However, beneficial effect of statin therapy can outweigh its side-effect of decrease plasma cholesterol levels as demonstrated by Jira et al., (2000). Jira et al., (2000) showed that use of simvastatin in two mildly affected SLOS children resulted in a significant decrease in 7DHC and 8DHC levels to 33% of the pre-treatment level in plasma, erythrocyte membranes, and CSF. In this study, patient A had eight exchange transfusions while patient B had only five exchange transfusions,

followed by treatment with simvastatin (0.2 to 1.0 mg/kg/day) for 23 months in patient A and 14 months in patient B without dietary cholesterol supplementation. Additionally, improvements in morphometric measurements were also documented in these patients. The simvastatin therapy was well tolerated in this study and no unwanted clinical side effects occurred (Jira et al., 2000). Another study, using a retro-elective design, evaluated the effectiveness of simvastatin in combination with cholesterol supplementation on plasma sterols in 39 SLOS subjects and on anthropometric measurement in 20 SLOS subjects. The results showed that dietary cholesterol supplementation (60-150 mg/kg/day) with simvastatin therapy (0.5-1.0 mg/kg/day) decreased plasma 7+8DHC to cholesterol ratio (which reflects the severity of the disease) significantly from 0.23 to 0.18, but without any improvement in morphometric measurements or behaviour (Haas et al., 2007). Conversely, two 15 years old severely affected SLOS patients were given simvastatin (0.5-0.7 mg/kg/day) for two months (±3) with dietary cholesterol supplementation (90-100 mg/day) and bile acids to evaluate the effectiveness of simvastatin (Starck, Lovgren-Sandblom, & Bjorkhem, 2002). As a result the ratio of DHC to sterols was reduced from 0.25 to 0.15 ($p>0.05$), but on the other hand there were serious side-effects including elevation in liver enzyme (transaminases), which might indicate inflammation or damage to liver cells. Other adverse effects included aggravation of photosensitivity and hypocholesterolemia, which resulted in discontinuation of statin treatment in one patient; the other patient showed a moderate increase in creatine kinase (muscle enzyme) as well (Starck et al., 2002). Likewise, Haas et al., (2007) reported that six of 39 SLOS patients had reversible side-effects such as severe sleeping problem, increase aggression and behaviour problems from additional

simvastatin therapy, which led to reduction from 0.5 to 0.25 mg/kg/day or discontinuation of the simvastatin treatment (Haas et al., 2007).

Based on these and other studies care should be taken in the administration of statin to SLOS patients because adverse side-effects in severely affected SLOS patients can occur from statin therapy. To avoid some adverse reactions related to statin therapy a close monitoring is recommended; specifically assessment of transaminases and creatine kinase activities should be done after the first dosing of simvastatin therapy, then every 3 to 4 months during treatment (Starck et al., 2002).

Overall, more controlled and blinded studies with larger counts of patients are needed to evaluate beneficial effects and safety of statins in SLOS patients. Moreover, the use of standardized instruments to record behavioural patterns during various treatment stages will aid in evaluating statin treatment.

In addition to cholesterol supplementation and statin therapy there are other treatment strategies used to alleviate the symptoms of SLOS, including the use of steroids therapy in children with congenital adrenal hyperplasia (Masturzo et al., 2001), and bile acid replacement therapy to treat neonatal cholestatic liver disease (Hofmann, 1999; Nwokoro & Mulvihill, 1997).

2.7 Cholesterol biosynthesis, absorption, excretion and function

2.7.1 Cholesterol biosynthesis

In 1993 the featured biochemical characteristics of SLOS were discovered (M. Irons et al., 1993). Typical SLOS patients show reduced levels of cholesterol and increased levels of 7DHC and 8DHC in all tissues and plasma, and the main cause of this

biochemical abnormality is DHCR7 enzyme deficiency. This enzyme is involved in the last step of conversion of 7DHC to cholesterol (Fitzky et al., 1998; Wassif et al., 1998).

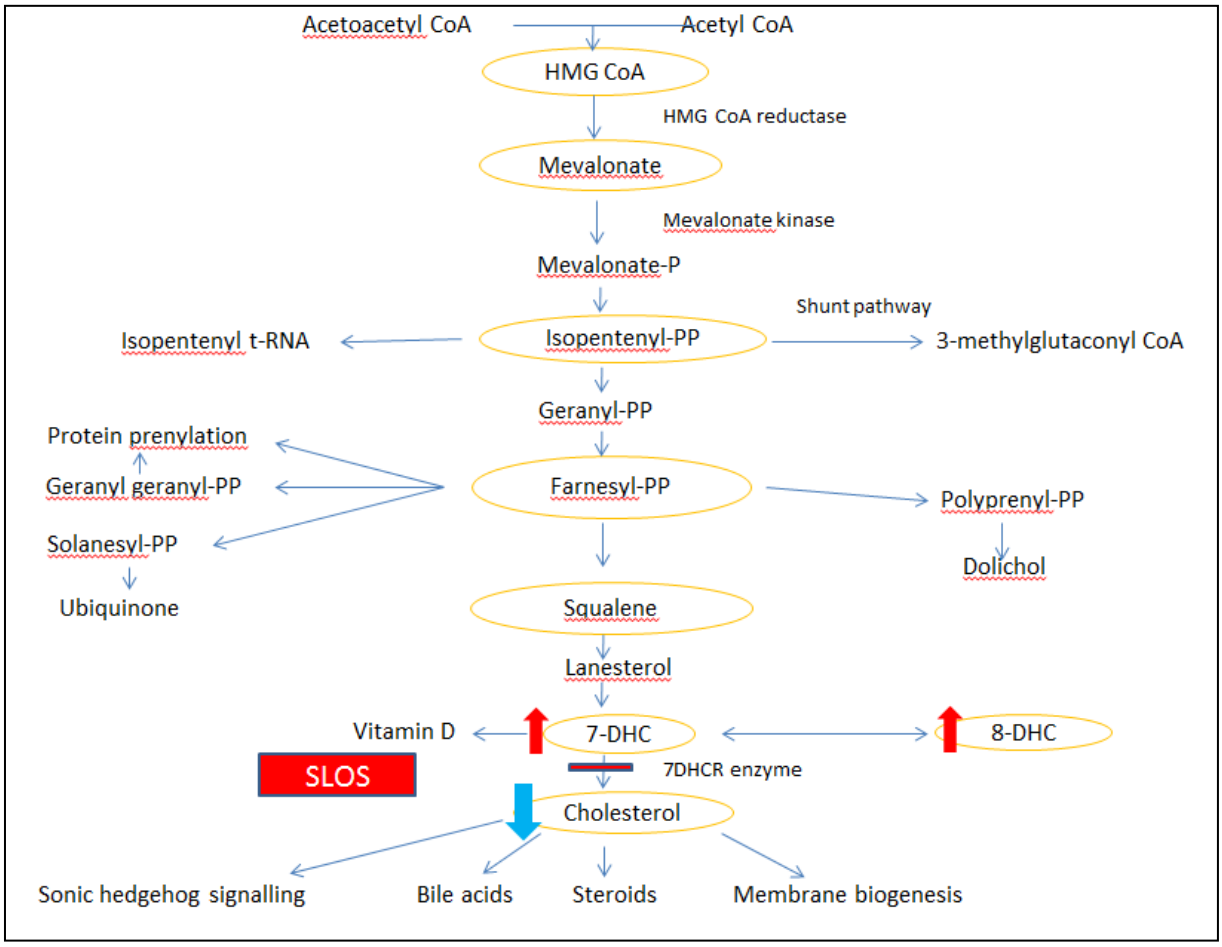
Cholesterol plays a vital role in the maintenance of cell membrane structure and function, essential for synthesis of bile acids, steroids hormones production, and is a major component of myelin sheath formation in the brain. Cholesterol deficiency can affect transportation of sonic hedgehog (Shh) signalling protein, an important protein for craniofacial features and limbs development in human embryo (Porter, Ekker, et al., 1996; Porter et al., 1995; Porter, Young, & Beachy, 1996).

Cholesterol synthesis in mammalian tissues goes through a multistep process in, which cholesterol is synthesized from acetyl-CoA with the regulation of four key enzymes HMG CoA synthase, HMG CoA R, farnesyl diphosphate synthase and squalene synthase (Esser, Limbird, Brown, Goldstein, & Russell, 1988). The isoprenoid pathway plays critical roles in cellular function by providing various hydrocarbon compounds, that are collectively called isoprenoids such as, Heme A and ubiquinone-10, (Goldstein & Brown, 1990).

Figure provides an overview of cholesterol biosynthetic pathway, where sterols are synthesized from acetyl-CoA and acetoacetyl-CoA, which are then condensed by HMG CoA synthase to form HMG CoA. HMG CoA is reduced to mevalonate by HMG CoA R, utilizing two molecules of nicotinamide adenine dinucleotide phosphate (NADPH, a coenzyme used in anabolic reactions). A series of enzymes then metabolizes mevalonate to farnesyl-diphosphate (farnesyl-PP). Two molecules of farnesyl-PP are condensed to form squalene by squalene synthase. The squalene epoxidase and oxidosqualene:lanosterol cyclase converts squalene to lanosterol. A series of oxidations, reductions, and demethylations will convert lanosterol to cholesterol (Vance, 2008)

Figure 2. Overview of cholesterol biosynthetic pathway. 3-hydroxy-3-methylglutaryl coenzyme A reductase HMG CoA R is the rate limiting step for the whole enzymatic pathway. 7-dehydrocholesterol-Δ7-reductase (DHCR7) enzyme catalyses the last step in cholesterol biosynthesis. In SLOS this enzyme is defective. NADPH, nicotinamide adenine dinucleotide phosphate; farnesyl-PP, farnesyl-diphosphate; 7-DHC, 7-dehdrocholesterol; 8-DHC, 8-dehydrocholesterol. SLOS, smith-lemli-opitz-syndrome; 7-

DHC, 7-dehydrocholesterol; 8-DHC, 8-dehydrocholesterol (Adapted from sterol and isoprenoid research consortium website).



2.7.2 Cholesterol absorption in SLOS

The two main sources of cholesterol entering the intestinal tract are endogenous hepatic cholesterol synthesis or dietary cholesterol and bile. Dietary cholesterol can be free or esterified with fatty acids, while biliary cholesterol remains un-esterified. Once esterified cholesterol reaches the intestine, it will be hydrolysed by pancreatic cholesterol esterase (McIntyre, 1976). In order for free cholesterol to be absorbed, cholesterol should be solubilized in mixed micelles containing sufficient amount of bile, fatty acids, monoglycerides and lysolecithin (Hofmann & Borgstrom, 1962). Following intestinal absorption cholesterol will enter the lymphatic system, travel through thoracic duct and eventually blend with the subclavian vein in the neck and undergo disposal into the general circulation of pools of cholesterol at that point.

As discussed earlier in the literature review, providing dietary cholesterol supplementation for SLOS patients has received special attention because it is thought to increase plasma concentration of cholesterol, which in turn may suggest a substantial intestinal absorption of dietary cholesterol by these patients. Despite such postulation, the potentiality of absorption has not been fully explored. A 2005 study by Lin et al., examined the absorption of cholesterol in twelve SLOS subjects who received dietary cholesterol in the form of egg yolk or crystalline cholesterol labelled with radioactive ^{14}C-cholesterol. Their results demonstrated that absorption in SLOS patients, especially those who received it in the form of crystalline cholesterol, was lower (20.5 ± 10.3%) than in those who received it in the form of egg yolk (27.3 ± 6.7%) but was not significantly different ($p < 0.13$) (Lin et al., 2005). Cholesterol absorption capacity in SLOS children can be confounded by many factors such as bile acid production and

impaired cholesterol metabolism. For example, low productivity of bile acids could decrease cholesterol absorption generally (Grundy, 1983) and specifically in SLOS children (M. Irons et al., 1994). However, Steiner et al., (2000) have reported that bile acids synthesis in SLOS subjects (3.5 mg/kg/day) did not differ significantly from control subjects (4.6 mg/kg/day, $p<0.2$) (Steiner, Linck, Flavell, Lin, & Connor, 2000). Another factor that can affect cholesterol absorption in SLOS patients is accumulation of unesterified cholesterol due to a defect in its esterification process, which was found in cultured SLOS fibroblast (McIntyre, 1976; Wassif et al., 2002). Further, 7DHC can compete with cholesterol for transportation and absorption, according to an animal study done in 1999 (Gaoua, Chevy, Roux, & Wolf, 1999). Moreover, many other factors such as the amount and type of fat content in the diet can also contribute to the capability for intestinal absorption of cholesterol by SLOS patients.

2.7.3 Cholesterol metabolism in SLOS

Total body cholesterol is a harmonized mixture of anabolic and catabolic reaction, in addition to the movement of cholesterol between various compartments, or pool to pool in the mammalian body. Studying cholesterol metabolism in human can help us measure the changes in whole body cholesterol pool sizes, synthesis and turnover in normal and diseased subjects under the experience of different nutritional supplement and physiological body state, and whether such changes has an impact on sustaining human health.

2.8 Turnover, production rate, and mean size of exchangeable body cholesterol

Cholesterol turnover kinetic studies have provided sufficient amount of quantitative information about cholesterol metabolism in man that are of interest to

researchers (Goodman & Noble, 1968; Goodman, Noble, & Dell, 1973). Indeed, most of the early cholesterol kinetic studies in human allowed for assessment of whole body cholesterol metabolism by analysing the turnover of plasma cholesterol following injection of radio-isotope labelled cholesterol such as ^{14}C-cholesterol as the isotopic tracer. In these isotopic studies an open mammillary two compartments system is used to resemble the main cholesterol pool A (into which tracer is injected) and an interconnected peripheral pool B. Each component of the system is believed to be in a homogenous state (Bassingthwaighte & Raymond, 2012). The tracer methods in these studies were used to estimate cholesterol body masses, various kinetic parameters such as the cholesterol production rate, cholesterol removal rate, and turnover of exchangeable cholesterol in humans (Goodman & Noble, 1968). After an injection of radioactively labelled cholesterol, the specific radioactivity of plasma cholesterol was then obtained over several weeks. Analysing the plasma cholesterol specific activity-time curves revealed values for many unique model parameters; the primary parameters include the size of the fast turning cholesterol pool A, the maximum and minimum values of the slowly turning cholesterol pool B, production rate in the first pool, rate of removal from each pool and the rate constants for transfer between the pools (Goodman & Noble, 1968; Goodman et al., 1973; Goodman et al., 1980; Samuel & Lieberman, 1973; Samuel, Lieberman, & Ahrens, 1978). Chobanian et al., (1962) performed a postmortem analysis of tissues of nine terminally ill patients, who received intravenous tracer doses of ^{14}C-cholesterol from 1 to 226 days before death. They demonstrated that rates of equilibration between serum and tissue cholesterol varied among different tissues. These studies helped to establish that cholesterol in several viscera (e.g., spleen, erythrocytes, lungs,

intestine and liver) equilibrate rapidly with plasma cholesterol (within 20 days), followed by cholesterol in peripheral tissue (e.g., skeletal muscle), which equilibrate at a slower rate with plasma cholesterol (within 30 days), and a much slower rate (>30 days) for intimal cholesterol in arteries (Chobanian, Burrows, & Hollander, 1962). However, the brain and other nervous system tissues showed almost no exchange with serum cholesterol (Chobanian et al., 1962; Field, Swell, Schools, & Treadwell, 1960). Goodman and Noble also investigated the turnover of plasma cholesterol in normal men and treated/untreated hyperlipidemic patients (n=10) after receiving an intravenous injection of ^{14}C-cholesterol. The radioactivity of plasma cholesterol versus time was measured over a 10 week period. Kinetic analysis of turnover curves of plasma cholesterol conformed to a two-pool compartments (pools A and B). The rapidly turning over pool A comprises cholesterol in plasma, red cell, spleen, intestine and liver, while the slowly turning over pool B comprises cholesterol in skeletal muscle, skin and adipose tissue. Analysing the turnover of plasma cholesterol by a two-pool model enabled Goodman and his colleague to determine the size of the first pool A (M_A = 25 g) and the mean value for the production rate in pool A (PR_A = 1.35 g/day) in normal subjects, while the size of pool B could not be determined as this can only occur if cholesterol biosynthesis in the major organs is equal to zero, which is highly unlikely (Goodman & Noble, 1968). Five years later, Goodman et al., (1973) reported that a three-pool model gave a better explanation of the long-term turnover curve than did the two-pool model. In this study six men were injected intravenously with ^{14}C-cholesterol followed by measurement of the radioactivity of plasma cholesterol versus time over a 32-41 week period. A third pool of cholesterol was proposed beside the cholesterol in the first and second pools, which were

described in his first study in 1968. Cholesterol in the third pool was found to equilibrate at a very slow rate with plasma cholesterol and peripheral tissues cholesterol, such as skeletal muscle. The mean size of the first pool A was (M_A = 23.4 g) and the mean value for the production rate in pool A was (PR_A = 1.13 g/day), estimation of the upper and lower values for second and third pools were calculated, as well as the total exchangeable body cholesterol, pool B values ranged from 11.3 to 20.2 g, pool C values ranged from 35.7 to 72.1 g and the value for the total exchangeable body cholesterol ranged from 70.4 to 106.8 g (Goodman et al., 1973). Additionally, Nestel et al., (1969) studied cholesterol metabolism in 22 subjects, where calculation of cholesterol turnover was based on the two-pool model described previously by Goodman and Noble (1968). The mean value for pool A was 17.9 g, the estimation for upper and lower values of pool B were on average, 35 and 60 g, while the daily production rate of cholesterol was PR_A =1.10 g/day for ideal body weight and increases by 0.0220 g/day for every excess kilogram of body weight (Nestel, Whyte, & Goodman, 1969).

In summary, assessment of plasma cholesterol turnover in humans conformed to a two–pool model whenever the curves of plasma cholesterol specific activity vs. time are analysed after an isotope bolus is given. In the two-pool model the first pool A, includes cholesterol in liver, erythrocytes, plasma and probably some of the cholesterol in viscera, and the second pool B represents all other tissue (Goodman & Noble, 1968; Nestel, Whyte, & Goodman, 1969). Cholesterol turnover as represented by the two-pool model means that the various tissue pools of cholesterol can be categorized into two groups in terms of the rates at which they equilibrate with cholesterol in plasma. The first group of pools is apparently in reasonably rapid equilibrium (in terms of hours to days) with

plasma cholesterol, whereas the second group of pools is in reasonable slow equilibrium (in terms of days to weeks) with plasma cholesterol. Within each group, the rates of equilibration of the distinct pools with plasma cholesterol are probably adequately similar so that the group act as a single pool, when evaluated in terms of the turnover curve of plasma total cholesterol (Goodman & Noble, 1968). So the conformation of the turnover of plasma cholesterol to a two-pool model after injecting the radioactive isotope helps to collect data from the tracer curve of cholesterol specific activity vs. time, and it would be interesting if we applied the same principles to measure the cholesterol pool size in SLOS patient consuming high cholesterol diet with and without simvastatin using stable labelled isotopes, which are preferred over radiolabeled isotope because they are non-radioactive and safe to use in humans at all ages (Jones & Leatherdale, 1991).

Stable isotopes are naturally abundant in humans, and using stable isotopic labelled compounds (the tracer) that are chemically and functionally identical to the naturally abundant stable isotope (the tracee) will enable us to measure the enrichment of stable isotope in the blood or tissues. This can be achieved by mass spectrometry, which will detect the mass difference between the tracer and the tracee.

CHAPTER 3. STUDY OBJECTIVES

The objectives of the present study are:

1. To measure whole body cholesterol pool size in SLOS patients receiving high cholesterol supplementation, with and without simvastatin.
2. To delineate relationships that may exist between whole body cholesterol pool size and various biochemical parameters; including plasma cholesterol, 7DHC and 8DHC levels in SLOS patients and other parameters such as age, weight, and cholesterol intake.

CHAPTER 4. MANUSCRIPT

Assessment of the Whole Body Cholesterol Pool Size in Smith-Lemli-Opitz Syndrome Patients Receiving Cholesterol Supplementation Alone or Combined with Simvastatin

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4.1 Abstract

Smith-Lemli-Opitz syndrome (SLOS) is a rare disease caused by mutations in 7-dehydrocholesterol-reductase (*DHCR7*) gene, resulting in DHCR7 enzyme deficiency, which catalyzes conversion of 7-dehydrocholesterol (7DHC) to cholesterol. DHCR7 enzyme deficiency results in hypocholesterolemia with 7DHC and 8-dehydrocholesterol (8DHC) accumulating in tissues and plasma. Therapeutic interventions for SLOS, including cholesterol supplementation and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA R) competitive inhibitors (e.g. simvastatin), are aimed at enhancing whole body cholesterol pool size (WBCPS) while decreasing pool sizes of 7DHC and 8DHC. Simvastatin is believed to function, in part, by up-regulating residual DHCR7 enzymatic activity. The present study aimed to assess the mass of rapid turnover of WBCPS in SLOS patients receiving cholesterol supplementation alone (HI) or combined with simvastatin (HI+ST).

SLOS patients receiving cholesterol supplementation alone (n=15; mean age: 9.4 ± 1.9 years) or combined with simvastatin (n=4; mean age: 7.3 ± 1.2 years) were administered an intravenous dose of [^{18}O]-cholesterol (1.0-1.4 mg/kg bodyweight) or [$^{2}H_7$]-cholesterol (0.9-1.4 mg/kg). Blood samples were collected at baseline and at repeated time intervals over 10 weeks. Cholesterol was extracted from red blood cells, derivatized with piconyl ester, and analyzed using liquid chromatography-tandem mass spectrometry. Results are reported as mean ± SEM.

Overall, WBCPS was significantly predicted by body weight (r^2=0.49; $p<0.05$) and age (r^2=0.46; $p<0.05$), but not plasma cholesterol levels (r^2=0.01; $p>0.05$). WBCPS failed to correlate with cholesterol intake (r^2=0.02; $p>0.05$). However, plasma cholesterol

concentration correlated positively with cholesterol intake ($r^2=0.42$; $p<0.05$). Subjects on HI+ST showed higher WBCPS values compared those on HI only (2.76 ± 0.20 vs. 2.53 ± 0.19 mg/kg bodyweight, respectively; $p<0.05$). Furthermore, 7DHC level was lower in subjects on HI+ST compared with HI (3.40 ± 0.66 vs. 10.72 ± 1.97 mg/dL, respectively; $p<0.05$), and similar reduction in 8DHC level (3.75 ± 0.36 vs. 10.17 ± 1.70 mg/dL, respectively; $p<0.05$) was also observed, with no obvious parallel decrease in cholesterol levels in subjects on HI+ST vs. HI (111.50 ± 7.57 vs. 82 ± 11.70 mg/dL, respectively; $p=0.10$). Finally, no significant change in WBCPS was seen over time ($p>0.05$) in SLOS patients (n=6) re-evaluated after 2.0 ± 1.1 years of high cholesterol supplementation with and without simvastatin.

In conclusion, our findings demonstrate that WBCPS was significantly predicted by body weight and age, so as one grows and his/her body weight increases, so does their WBCPS. Furthermore, a HI+ST therapy resulted in the desired increase of WBCPS in SLOS children, which can meet the therapeutic goals of this diet in SLOS children.

The findings of this current study increased our knowledge of the effects of interventions such as dietary cholesterol supplementation and statin on whole body cholesterol pool size in children with SLOS. Assessment of WBCPS is a good indicator of cholesterol metabolism and trafficking in SLOS patients.

4.2 Introduction

Smith-Lemli-Opitz syndrome (SLOS) is a rare autosomal recessive disorder associated with multiple congenital anomalies and intellectual deficits (Smith et al., 1964). The typical biochemical features of SLOS are hypocholesterolemia and elevated levels of cholesterol precursor's 7DHC and its isomer 8-dehydrocholesterol (8DHC) in tissues and plasma (Irons et al., 1994; Salen et al., 1995; Shefer et al., 1995; Tint et al., 1994). The main cause of this distinctive biochemical abnormality in the sterol profile is due to mutations in 7-dehydrocholesterol-reductase (*DHCR7*) gene, resulting in DHCR7 enzyme deficiency, which is involved in the last step conversion of 7DHC and 8DHC to cholesterol (Fitzky et al., 1998; Wassif et al., 1998).

The discovery of the biochemical characteristics of SLOS contributed significantly towards the use of several treatment strategies such as cholesterol supplementation (Elias et al., 1997; Irons et al., 1997; Linck, Hayflick, et al., 2000; Nwokoro & Mulvihill, 1997) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors e.g. simvastatin (Jira et al., 2000). Simvastatin is believed to be beneficial in SLOS via down-regulating synthesis of 7DHC by inhibiting HMG CoA reductase (Jira et al., 2000) and believed also to function, in part, by up-regulating residual DHCR7 enzymatic activity, which results in up-regulating cholesterol synthesis (Wassif et al., 2005). These treatment strategies are targeting SLOS therapeutically and clinically. Therapeutic goals are aimed at enhancing whole body cholesterol content while decreasing levels of potentially toxic precursors 7DHC and 8DHC. Clinical goals for SLOS patients focus mainly on improving: 1) mental developmental abnormalities, e.g. IQ (Mueller et al., 2003; Ryan et al., 1998), 2) behavioural problems, such as irritability, hypersensitivity to visual and auditory stimuli, self-injurious behaviour,

aggression, interrupted sleep cycle and autistic spectrum behaviours (Kelley & Hennekam, 2000; Nwokoro & Mulvihill, 1997; Sikora, Pettit-Kekel, Penfield, Merkens, & Steiner, 2006), 3) morphometric measurements such as length, weight and head circumference (Haas et al., 2007; Irons et al., 1997).

Whole body cholesterol pool size (WBCPS) represents the body cholesterol content/plasma cholesterol turnover. The assessment of plasma cholesterol turnover in humans conformed to a two–pool model whenever the curves of plasma cholesterol specific activity vs. time are analysed after an isotope bolus is given. In the two-pool model the first pool A, is in reasonable rapid equilibrium (in terms of hours to days) with plasma cholesterol and includes cholesterol in liver, erythrocytes, plasma and probably some of the cholesterol in viscera, and the second pool B, is in reasonable slow equilibrium (in terms of days to weeks) with plasma cholesterol and represents cholesterol in all other tissue (Goodman & Noble, 1968; Nestel et al., 1969). So the conformation of the turnover of plasma cholesterol to a two-pool model after injecting the radioactive isotope helps to collect data from the tracer curve of cholesterol specific activity vs. time, and it would be interesting if we applied the same principles to measure the WBCPS in SLOS patient consuming high cholesterol diet with and without simvastatin using stable labelled isotopes because they are safer and non-radioactive (Jones & Leatherdale, 1991).

The aim of the present study was to assess the WBCPS in SLOS patients receiving cholesterol supplementation alone or combined with simvastatin. Such assessment should provide a significant insight about whole body cholesterol content, which would be more informative than plasma cholesterol concentration alone. Thus, we

hypothesized that WBCPS will serve as a more efficient biomarker of treatment efficacy in SLOS patients.

4.3 Materials and methods

4.3.1 Subjects

As a part of an on-going prospective interventional longitudinal study, SLOS participants were recruited from several institutes that are members of the Sterol and Isoprenoid Research Consortium (STAIR) Rare Disease Network: Oregon Health and Science University (OHSU), Portland, OR; National Institute of Child Health and Human Development (NICHD), National Institute of Health, Bethesda, MD and Cincinnati Children's Hospital Medical Centre, Cincinnati, OH are sites that actively recruited study participants. SLOS patients of all ages and health statues, with confirmed diagnosis were recruited (i.e. no strict inclusion/exclusion criteria except for confirmed diagnosis of SLOS for inclusion and patients inability to travel to a STAIR site for exclusion). The study was approved by the institutional review board at each site, and all SLOS participants were consented prior to the commencement of the study.

Nineteen (19) SLOS patients (8 female, 11 male) are included in this study. Diagnosis of SLOS was confirmed by blood-sterol analysis, documented by high concentrations of serum 7-DHC (>0.11 mg/dl), and molecular analysis to document mutations in *DHCR7* gene. All subjects underwent a complete assessment for clinical measurements, including growth and total cholesterol intake before treatment intervention and after at least 3 weeks of stable cholesterol intake. The Kelly and Hennekam scoring system was used to classify the severity of SLOS as mild (<20,);

moderate (20 to 35) or severe (<35) (Kelley & Hennekam, 2000; Kratz & Kelley, 1999). This assessment was done before treatment and repeated after two years.

4.3.2 Study design

19 SLOS patients were recruited in this study to examine the relationship between dietary cholesterol supplementation and WBCPS, as well as the effects of statin treatment, combined with dietary cholesterol supplementation on WBCPS in 4 of the 19 SLOS subjects only. Four of the 19 subjects were re-evaluated after receiving a high cholesterol diet (HI) alone for ~ 1 year and two subjects were re-evaluated after receiving a high cholesterol diet, combined with simvastatin (HI+ ST) for ~ 1 year.

4.3.3 Dietary cholesterol supplementation and statin administration

SLOS patients receiving a high cholesterol diet alone (n=15; mean age: 9.4 ± 1.9 years) or combined with simvastatin (n=4; mean age: 7.3 ± 1.2 years) were evaluated after taking a stable high cholesterol diet that matches the study goals. Subjects were supplemented with food-based cholesterol at a mean of 35 mg/kg bodyweight/day, or encapsulated cholesterol preparation at 47 mg/kg/day. Food-based cholesterol used in this study included egg yolk (dried egg yolk powder, fresh, pasteurized eggs, or unpasteurized cooked eggs) from hard-boiled eggs mixed with formula, breast milk, or solid food or any other forms of alternative cholesterol such as heavy cream, butter, meat, cheese, and milk. SLOesterol (Solace nutrition company, Pawcatuck, CT, USA), a new encapsulated cholesterol powder preparation was used in cases of intolerance to egg yolk.

Dietary cholesterol intake was assessed three times yearly using 24-hour food recall data collected from the parents and/or caregivers via telephone interviews conducted by a

registered dietician. Food recalls were analysed using Nutrition Data System for Research (NDSR) (University of Minnesota, SE, USA).

Simvastatin therapy was started for four SLOS subjects either orally or via feeding tube, depending on patients feeding abilities. Simvastatin dose was gradually increased from 0.2 to 0.4 mg/kg/day; according to routine check-up (every 6 months) of plasma sterols levels and other biochemical parameters including aminotransferases, alkaline phosphatase, and creatine kinase (CK) to monitor any potential reversible side effects of simvastatin (Starck et al., 2002).

4.3.4 Assessment of whole body cholesterol pool size by stable isotope method

4.3.4.1 Stable isotope application protocol and blood sample collection

Following overnight admission of the patient to a STAIR site, twelve-hour fasting baseline venous blood samples (10 ml) were collected before breakfast on day 0. [^{18}O]-cholesterol (1.0-1.4 mg/kg body weight) or [$^{2}H_7$]-cholesterol (0.9-1.4 mg/kg body weight) was administered in an intravenous solution of intra-lipid. Two isotopes were used in this study due to the market availability of [^{18}O]-cholesterol or [$^{2}H_7$]-cholesterol at the time of assessment. Additional blood samples (10 ml) were collected at 12 h, 24 h, 48 h, and 72 h, and at approximately bi-weekly intervals thereafter for a total of 10 weeks for 18 subjects and for 26 weeks in the 19th subject.

Collected blood samples were centrifuged at 2000 rpm for 10 min for separation of red blood cell (RBC) and plasma. The white blood cell layer was removed and the RBC and plasma samples were stored at -80^{o}C until analyzed.

4.3.5 Analytical methods

4.3.5.1 Blood sample analysis/cholesterol extraction and derivatization

The enrichment of [^{18}O]-cholesterol or [$^{2}H_7$]-cholesterol in RBC samples were measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) adapted from Honda et al., (Honda et al., 2009) with some modification. Briefly; the free cholesterol was extracted from RBCs by methanol, which was added to RBCs and samples heated at 55^{o} C in a shaking water bath for 15 minutes, before the addition of hexane: chloroform (4:1, by volume) and double distilled water. Thereafter, samples were centrifuged for 15 minutes at 1500 rpm at 4^{o} C. Supernatants of the samples were dried down under nitrogen and redissolved with hexanes and then transferred into vials. Further, the extracted cholesterol was derivatized to picolinyl ester. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (100 mg), dimethylaminopyridine (30 mg), picolinic acid (80 mg), and triethylamine (200 µl). The freshly prepared reagent mixture (170 µl) and triethylamine (200 µl) were added to the dried RBC, and the reaction mixture was allowed to stand at room temperature for 1-1.5 hour. Then samples were centrifuged for 3 minutes at 12000 rpm. After evaporation of the supernatant under nitrogen, the residue was redissolved in 1 ml of acetonitrile of formic acid and then filtered into a liquid chromatography vial to be injected into a Waters Acquity UPLC system. A Kinetix XB-C18 column (2.1 x 100mm, particle size 1.7 µm; Phenomenex, Torrance, CA, USA) with an in line filter (KrudKatcher ULTRA HPLC In-Line Filter, 0.5 µm Depth Filter x 0.004in; Phenomenex, Torrance, CA, USA) was installed in the UPLC system and programmed to separate the sterols: the column temperature was maintained at 35°C. The Waters Acquity Ultra Performance LC-MS/MS

system was equipped with Atmospheric pressure ionization (API) probe in positive ion mode (ESI+) (Waters Micromass Quattro Micro API, Waters Corporation, Milford, MA, USA).

4.3.5.2 Plasma sterol analysis

Plasma concentrations of cholesterol, 7DHC and 8DHC in SLOS patients were assessed by gas chromatography mass spectrometry (GC/MS) at OHSU, using established methods (Merkens et al., 2004).

4.3.6 Pharmacokinetics and statistical analysis (calculation of cholesterol pool size and turnover)

An open mammillary compartmental system was applied resembling two cholesterol pools, pool A and pool B, where pool A was the pool into, which tracer was injected (**Figure 4**). This two-pool model was used to calculate the turnover of plasma cholesterol, cholesterol pool size, number of kinetics and different parameters from plasma cholesterol turnover curve based on the assumption and calculation methods established in previous study (Goodman & Noble, 1968). A graphical representation of the general two-pool model can be seen in **Figure 3**, where S_A is the rate of entry of cholesterol from the diet and synthesis in pool A while S_B represents the rate of endogenously synthesised cholesterol in pool B.

The constants K_{AB} and K_{BA} represent the rate of flux of cholesterol between the two pools. According to the assumption reported from other laboratories (Grundy & Ahrens, 1970; Nestel et al., 1969) we will assume firstly that no cholesterol was excreted from pool B so that K_B approximately equals zero. Secondly there was no cholesterol synthesis in either pools so that $S_B = 0$ and S_A stand for the amount of cholesterol absorbed from the diet. Thirdly, to determine the cholesterol turnover rate and to calculate the lower range

of pool B, both K_B and S_B were assumed to equal zero (Grundy & Ahrens, 1970; Nestel et al., 1969).

According to the model shown in **Figure 3**, the time dependent disappearance of the stable isotope [^{18}O]-cholesterol (tracer) enrichment in RBC in pool A, following its initial introduction to pool A, can be expressed by the sum of two exponential curves in **Figure 4**. And the equation:

Equation 1. $\mathbf{C = C_A e^{kAt} + C_B e^{kBt} + B}$

In, which C is the enrichment of the stable isotope in pool A; C_A, C_B, alpha (K_A), beta (K_B), and B are constants; e is the base of the natural logarithm; and t= time.

The determination of the pharmacokinetic parameters C_A, C_B, alpha (k_A) and beta (k_B) was through computer software.

Figure 3. General two pool model (A and B) for compartmental analysis of cholesterol turnover. The squares represent the two pools A and B; S_A is the rate of entry of cholesterol from the diet and the amount of cholesterol synthesis in pool A; S_B represents the rate of endogenously synthesised cholesterol in pool B. K_A and K_B is the amount of cholesterol excreted from each pool A and B respectively. The constants K_{AB} and K_{BA} represent the rate of flux of cholesterol between the two pools.

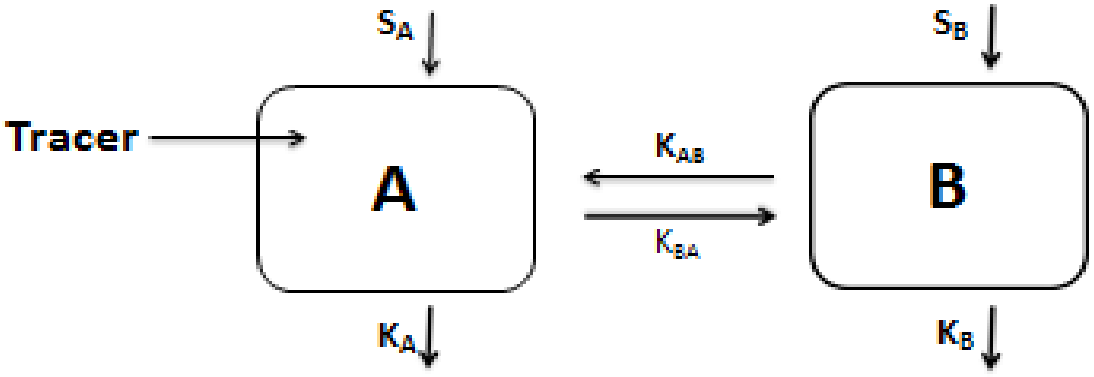
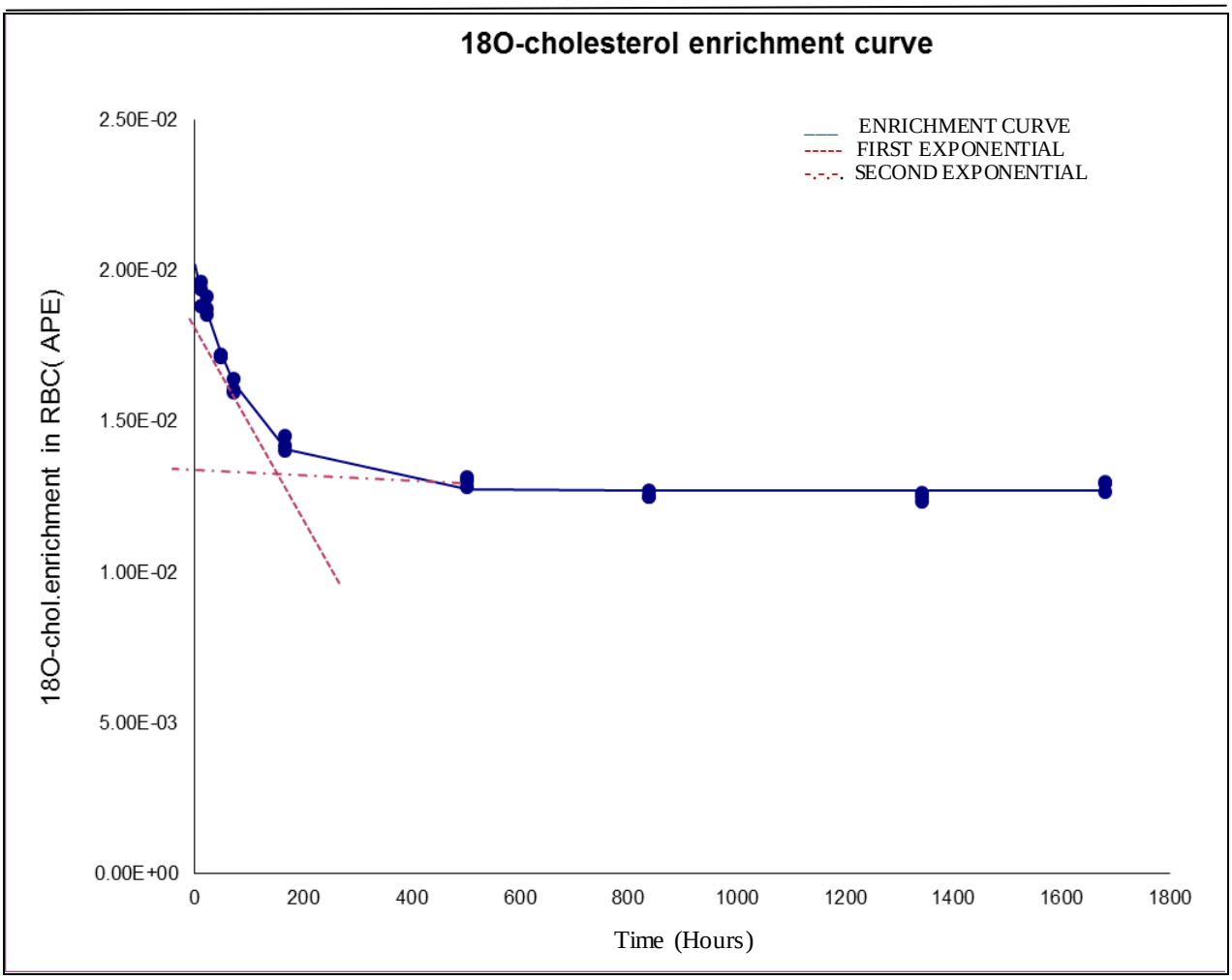


Figure 4. Determination of pharmacokinetic parameters, C_A, C_B, alpha (k_A) and beta (k_B) from a semi logarithmic plot of cholesterol concentration from red blood cells against time, following an intravenous injection of [^{18}O]-cholesterol. This patient was studied for up to ten weeks. APE is atom percent excess, which is an alternative unit for tracer/tracee ratio and it is analogue to specific activity of the radio labelled isotope.



4.3.7 Statistical analysis

Statistical analyses of data were conducted using SPSS software (version 2012, IBM Company, NY, USA). Multiple regression analysis was performed to assess the relationship between whole body cholesterol pool size and various parameters including plasma cholesterol, 7DHC and 8DHC levels. Independent t-tests or Pearson correlations were used to compare the effects of cholesterol supplementation alone versus statin and cholesterol supplementation on whole body cholesterol pool size in SLOS patients. Results were expressed as mean ± standard error of the mean (SEM). Statistical significance was set at p-value of 0.05 for all analyses.

4.4 Results

4.4.1 Subject characteristics

In this study, 19 subjects with SLOS (8 female, 11 male) were recruited. Characteristics of the 19 SLOS subjects who received HI or HI+ST are shown in **Table 2**. All subjects were provided with a high cholesterol diet in the form of egg yolk or encapsulated cholesterol powder. Four of the 19 subjects (mean age: 9.1 ± 1.4 years) were provided with simvastatin in addition to cholesterol (HI+ST), with biochemical analyses indicating no elevations in liver transaminases and creatine kinase levels in any of the four subjects (data not shown). Six of the 19 SLOS subjects (mean age: 9.4 ± 1.9 years) were re-evaluated after two years of high cholesterol supplementation; in two cases, simvastatin was provided in addition to the high cholesterol diet (**Table 3**).

According to the severity scoring system, seven subjects were classified as mild (<20); seven subjects moderate (20-35); one subject was severe (>35); and four subjects were unclassified (Kelley & Hennekam, 2000).

Table 2. Characteristics of 19 SLOS subjects who received high cholesterol diet alone (HI) or combined with simvastatin (HI+ST).

Patient[a]	Study Duration	Gender	Age	Body Weight	Severity Score[b]	Cholesterol Intake	Plasma Sterols			Treatment	WBCPS[f]
							Cholesterol	7DHC[c]	8DHC[c]		
	week		year	kg		mg/day	mg/dL	mg/dL	mg/dL		mg/kg body weight
1	10	F[g]	18.2	29.8	33	170	53	16.0	21.3	HI[d]	1070.3
2	10	M[g]	10.8	24.8	11	523	133	1.6	2.8	HI+ST[d]	352.2
3	10	M	3.7	14.3	38	644	95	15.0	10.0	HI	406.5
4	12	M	5.3	17.8	35	766	116	9.1	7.8	HI	444.0
5	10	F	8.3	17.0	25	1473	95	16.7	13.1	HI	466.1
6	12	M	7.4	17.0	30	920	98	3.4	3.6	HI+ST	912.6
7	10	F	16.5	33.8	10	363	78	3.1	3.6	HI	364.3
8	10	F	17.4	38.7	10	139	91	3.8	4.3	HI	353.4
9	10	F	5.9	13.5	5	665	105	3.8	4.5	HI+ST	812.9
10	72hr	M	7.2	22.3	5	2924	200	0.3	0.4	HI	362.2
11	72 hr	M	5.6	17.5	20	1017	78	7.9	7.2	HI	387.1
12	12	F	1.1	8.6	15	320	51	13.6	19.4	HI	355.9
13	10	M	2.0	9.1	25	175	55	8.2	9.2	HI	361.7
14	10	F	5.2	18.5	11	973	110	4.8	4.1	HI+ST	433.2

15	10	M	0.5	5.4	33	98	16	26.1	17.6	HI	142.8
16	8	M	1.9	7.4	n.d[e]	209	111	1.5	3.8	HI	205.9
17	10	M	17.2	34.5	n.d.	997	64	9.8	9.2	HI	289.1
18	10	M	20.6	36.4	n.d.	1161	45	19.0	15.5	HI	211.8
19	5	F	16.7	54.5	n.d.	1704	104	9.1	5.2	HI	331.8
Mean	-		9.0	22.2	20.4	800.2	89.4	9.1	8.6	-	399.4
SEM[h]	-		6.6	12.9	11.6	692.8	39.8	7.0	6.1	-	200.5
Normal Range	-	-		-	-	-	66±10.3 to 200	<0.02	<0.02	-	

[a] patients 1, 2, 3, 4, 6, and 13 were re-evaluated after two years. See **Table 4** below.
[b] Severity score classification: mild (<20,); moderate (20 to 35) or severe (<35). See reference (Kelley & Hennekam, 2000).
[c] 7DHC, 7-dehydrocholesterol; 8DHC, 8-dehydrocholesterol.
[d] HI, high cholesterol diet; HI+ST, high cholesterol diet +simvastatin.
[e] not done.
[f] WBCPS, whole body cholesterol pool size.
[g] F, female; M, male.
[h] SEM, standard error of the mean.

Table 3. Characteristics of 6 re-evaluated SLOS subjects on high cholesterol diet alone (HI) or combined with simvastatin (HI+ST).

Patient[a]	Study Duration	Gender	Age	Body Weight	Severity Score[b]	Cholesterol Intake	Plasma Sterols			Treatment	WBCPS[c]
							Cholesterol	7DHC[c]	8DHC[c]		
	week		year	kg		mg/day	mg/dL	mg/dL	mg/dL		mg/kg body weight
1	10	F[g]	18.2	29.8	33	170	53	16.0	21.3	HI[d]	1070.3
1	10		20.7	34.0	33	725	51	19.0	14.0	HI	194.8
2	10	M[g]	10.8	24.8	11	523	133	1.6	2.8	HI+ST[d]	352.2
2	12		13.3	32.9	11	1358	102	2.6	2.9	HI+ST	364.9
3	10	M	3.7	14.3	38	644	95	15.0	10.0	HI	406.5
3	10		5.7	18.3	38	745	67	14.6	8.2	HI	285.4
4	10	M	5.3	17.8	35	766	116	9.1	7.8	HI	444.0
4	10		7.3	21.1	35	832	97	11.0	10.0	HI	281.7
4	10		9.3	26.9	35	669	71	11.1	8.1	HI	307.3
5	12	M	7.4	17.0	30	920	98	3.4	3.6	HI+ST	912.6
5	26		10.0	22.9	30	924	122	6.6	6.2	HI+ST	211.1
6	10	M	2.0	9.1	25	175	55	8.2	9.2	HI	361.7
6	10		2.9	10.8	25	487	69	9.3	7.5	HI	296.9

[a] patient 4 was re-evaluated 3 times.
[b] Severity score classification: mild (<20,); moderate (20 to 35) or severe (<35). See reference (Kelley & Hennekam, 2000).

[c] 7DHC, 7-dehydrocholesterol; 8DHC, 8-dehydrocholesterol; WBCPS, whole body cholesterol pool size.
[d] HI, high cholesterol diet; HI+ST, high cholesterol diet +simvastatin.

4.4.2 Analysis of cholesterol turnover curves and distribution

Figure 5 shows the cholesterol enrichment curve following intravenous [^{18}O]-cholesterol administration in one of the SLOS patients who was studied for up to 10 weeks (1680 hours). The pattern of the [^{18}O]-cholesterol or [$^{2}H_7$]-cholesterol enrichment curve was similar for each SLOS patient. The semilogarithmic plot of [^{18}O]-cholesterol enrichment or [$^{2}H_7$]-cholesterol versus time described a curve during the first 4 weeks, whereas from 5 weeks onward the plot followed a straight line. Each turnover curve was analysed for the determination of kinetically distinguishable pools of cholesterol, involved in the turnover of plasma cholesterol in SLOS subjects. In every instance the results of the analysis conformed to a two-pool model (Gurpide, Mann, & Sandberg, 1964). A detailed review of the kinetic analysis of the two-pool systems was discussed in the pharmacokinetics and statistical analysis section.

Table 4 displays correlations between WBCPS, plasma concentrations of cholesterol, 7DHC and 8DHC, and independent variables such as age, body weight, and cholesterol intake. WBCPS was significantly predicted by body weight (r^2=0.49; $p<0.05$) and age (r^2=0.46; $p<0.05$), but not plasma cholesterol level (r^2=0.01). WBCPS failed to correlate with 7DHC levels (r^2= - 0.06; $p<0.05$) and 8DHC levels (r^2= - 0.00; $p<0.05$). Also, WBCPS failed to correlate with cholesterol intake ($p>0.05$). However, cholesterol intake directly correlated with plasma cholesterol concentration (r^2=0.32; $p<0.05$), and inversely correlated with 7DHC (r^2= - 0.51; $p<0.05$) and 8DHC levels (r^2= - 0.56; $p<0.05$).

Figure 5. Cholesterol enrichment curve following intravenous [^{18}O] cholesterol administration in a SLOS, the patient was studied for up to 10 weeks (1680 hours). The circles connected by a solid line (A) represent the experimental values analysed during the 10 week study. (B) Represent the first exponential and (C) represent the second exponential. APE is atom percent excess, which is an alternative unit for tracer/tracee ratio and it is analogue to specific activity of the radio labelled isotope.

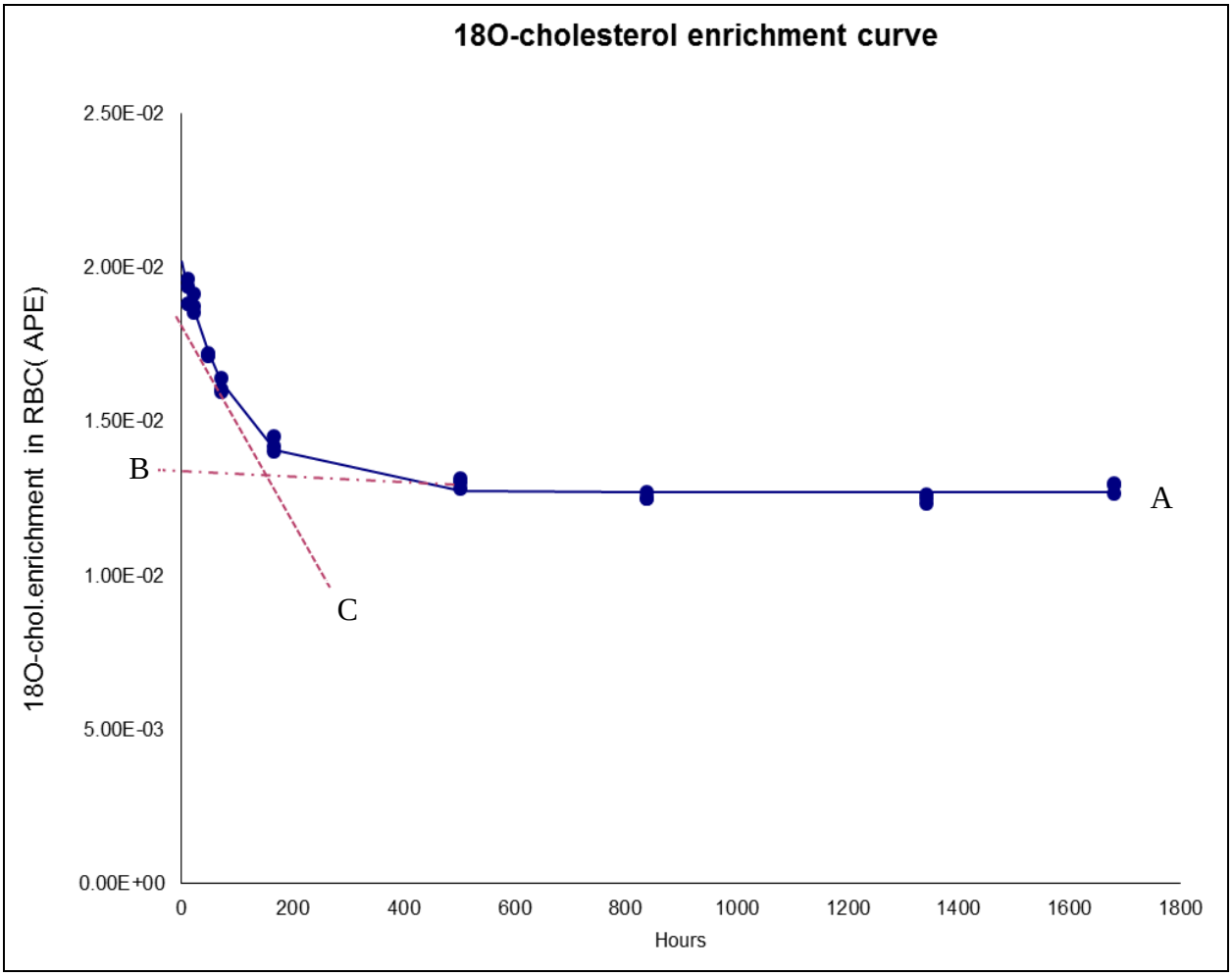


Table 4. Correlations between WBCPS, plasma cholesterol and independent variables. The first part of this table shows the correlation between WBCPS and independent variables while second part of the table shows the correlation between plasma cholesterol level and independent variables.

Dependant variable	Independent variable	Pearson r	*P*-value	N
WBCPS (mg)	Age (years)	0.67	0.002*	19
	Weight (kg)	0.65	0.002*	19
	Plasma cholesterol (mg/dL)	0.10	0.69	18
	Cholesterol intake (mg/day)	0.15	0.54	19
	Plasma 7DHC (mg/dL)	-0.18	0.46	18
	Plasma 8DHC (mg/dL)	-0.00	0.99	18
Plasma cholesterol (mg/dl)	Age (years)	-0.09	0.71	18
	Weight (kg)	0.03	0.88	18
	Plasma cholesterol (mg/dL)	n/a	n/a	18
	Cholesterol intake (mg/day)	0.65	0.003*	18
	Plasma 7DHC (mg/dL)	-0.72	0.001*	18
	Plasma 8DHC (mg/dL)	-0.75	0.001*	18

* Significant p-value ($p<0.05$).

Subjects on HI+ST therapy showed tendency towards higher WBCPS values compared to those given only cholesterol (2.76 ± 0.20 vs. 2.53 ± 0.19 mg/kg body weight, respectively; p=0.02; **Fig.6**). Furthermore, subjects on HI+ST resulted in a desired significant reduction of 7DHC levels compared to those on HI (3.40 ± 0.66 vs. 10.72 ± 1.97 mg/dL, respectively; p=0.001; **Fig. 8**) and a similar significant reduction in 8DHC (3.75 ± 0.36 vs. 10.17 ± 1.70 mg/dL, respectively; p=0.001; **Fig. 9**) with no obvious parallel reduction in cholesterol levels in patients given HI+ST versus patients given HI (111.50 ± 7.57 vs. 82 ± 11.70 mg/dL, respectively; p=0.10; **Fig. 7**).

Finally, there was no significant change in WBCPS over time (p>0.05) in the six re-evaluated SLOS patients after 2.0 ± 1.1 years of high cholesterol supplementation with and without statin. Two of the six subjects were treated with high cholesterol diet with simvastatin (**Figures 10&11**).

Figure 6. Comparing HI with HI+ST. SLOS patients receiving high cholesterol diet combined with simvastatin (HI+ST) had a higher WBCPS than patients who received high cholesterol diet alone (HI) (2.76 ± 0.20 vs. 2.53 ± 0.19 mg/kg body weight, respectively; p=0.02); n= number of participants; obtained by independent t-test.

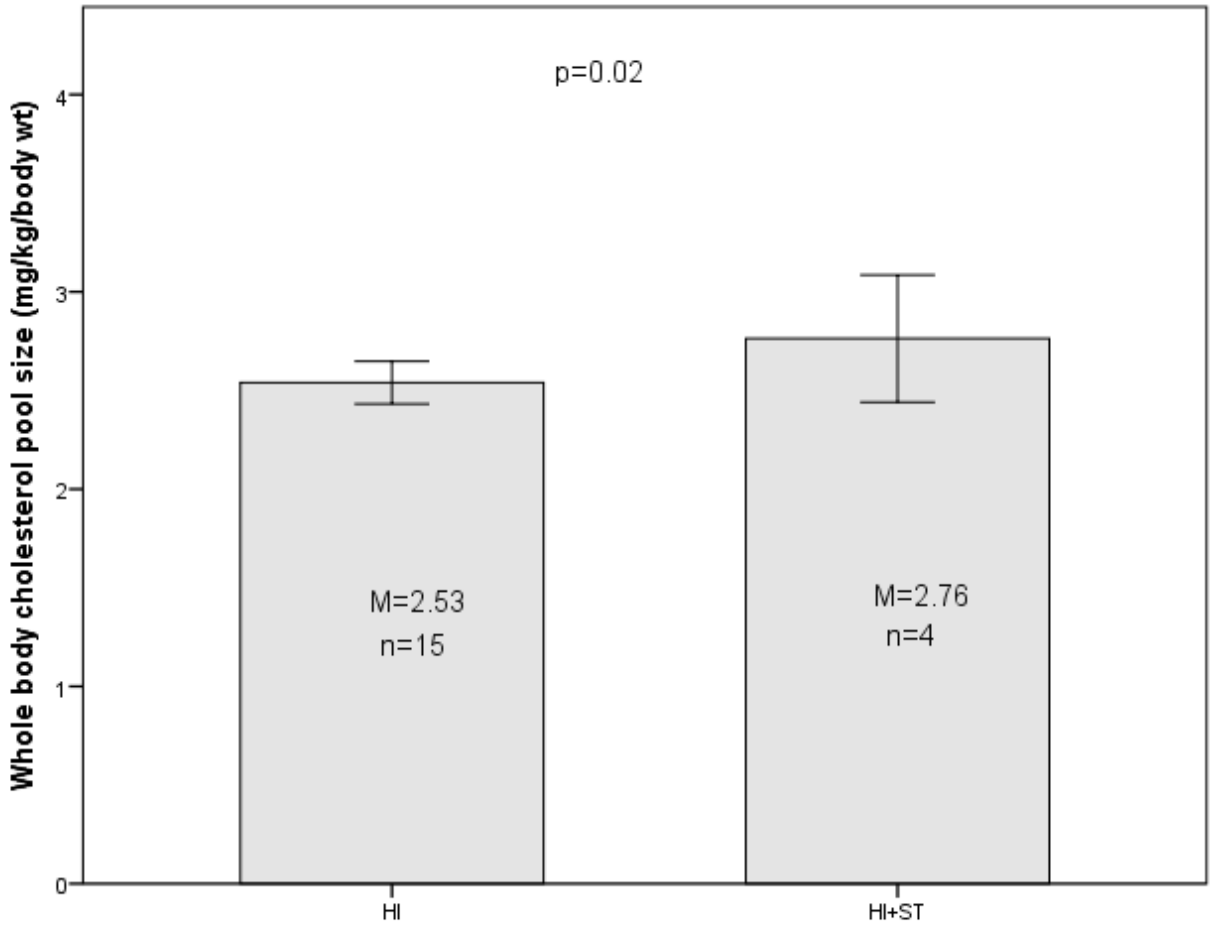


Figure 7. Comparing HI with HI+ST. SLOS patients receiving high cholesterol diet combined with simvastatin (HI+ST) had no significant drop in plasma cholesterol than patients who received high cholesterol diet alone (HI) (111.50 ± 7.57 vs. 82 ± 11.70 mg/dL, respectively; p=0.10); n= number of participants; obtained by independent t-test.

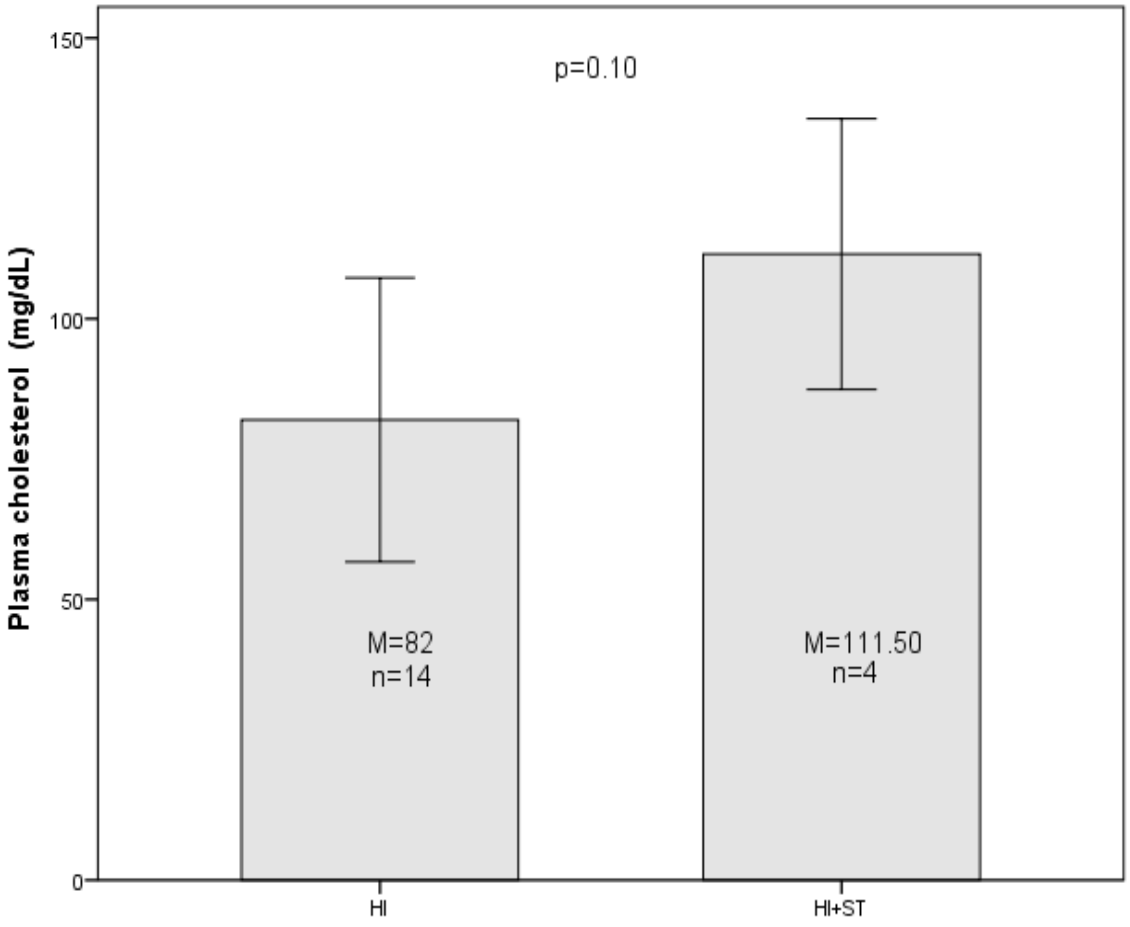


Figure 8. Comparing HI with HI+ST. SLOS patients receiving high cholesterol diet combined with simvastatin (HI+ST) had a significant drop in plasma 7DHC than patients who received high cholesterol diet alone (HI) (3.40 ± 0.66 vs. 10.72 ± 1.97 mg/dL, respectively; p=0.001); n= number of participants; obtained by independent t-test.

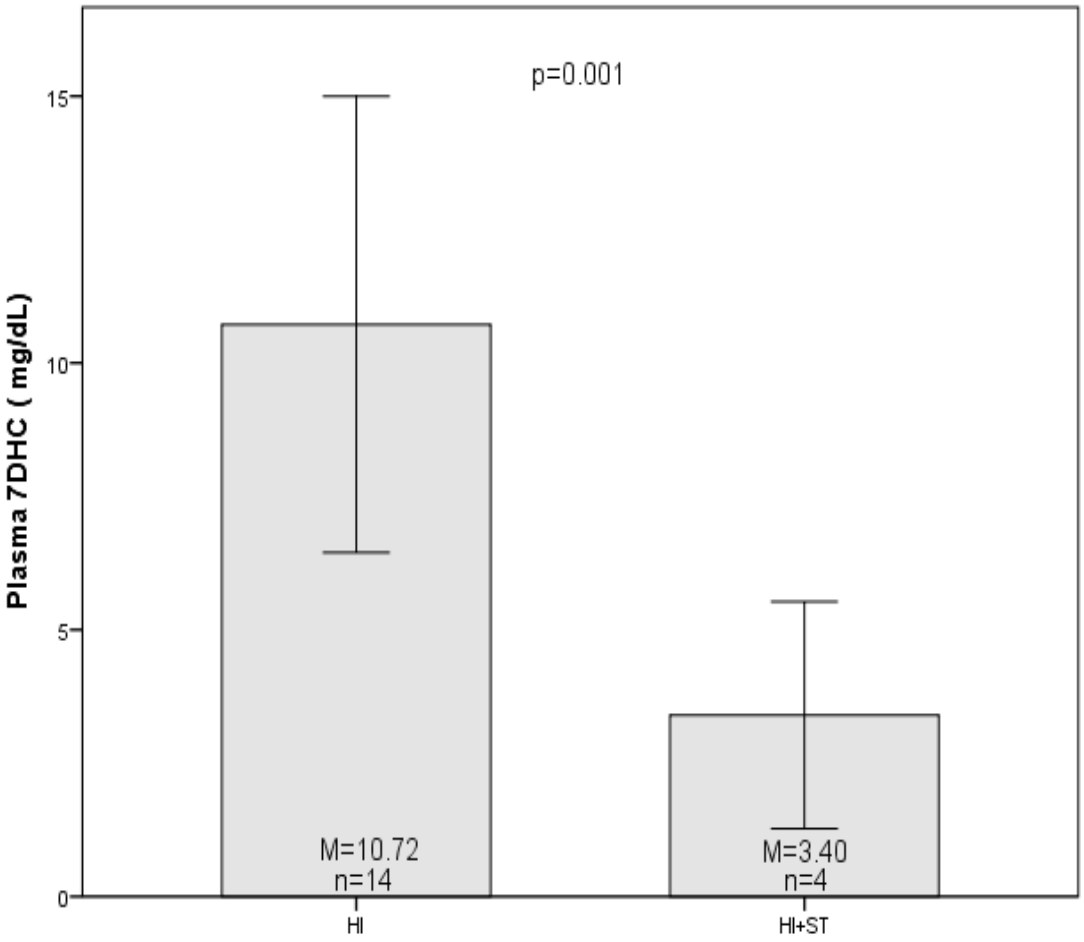


Figure 9. Comparing HI with HI+ST. SLOS patients receiving high cholesterol diet combined with simvastatin (HI+ST) had a significant drop in plasma 8DHC than patients who received high cholesterol diet alone (HI) (3.75 ± 0.36 vs. 10.17 ± 1.70 mg/dL, respectively; p=0.001); n= number of participants; obtained by independent t-test.

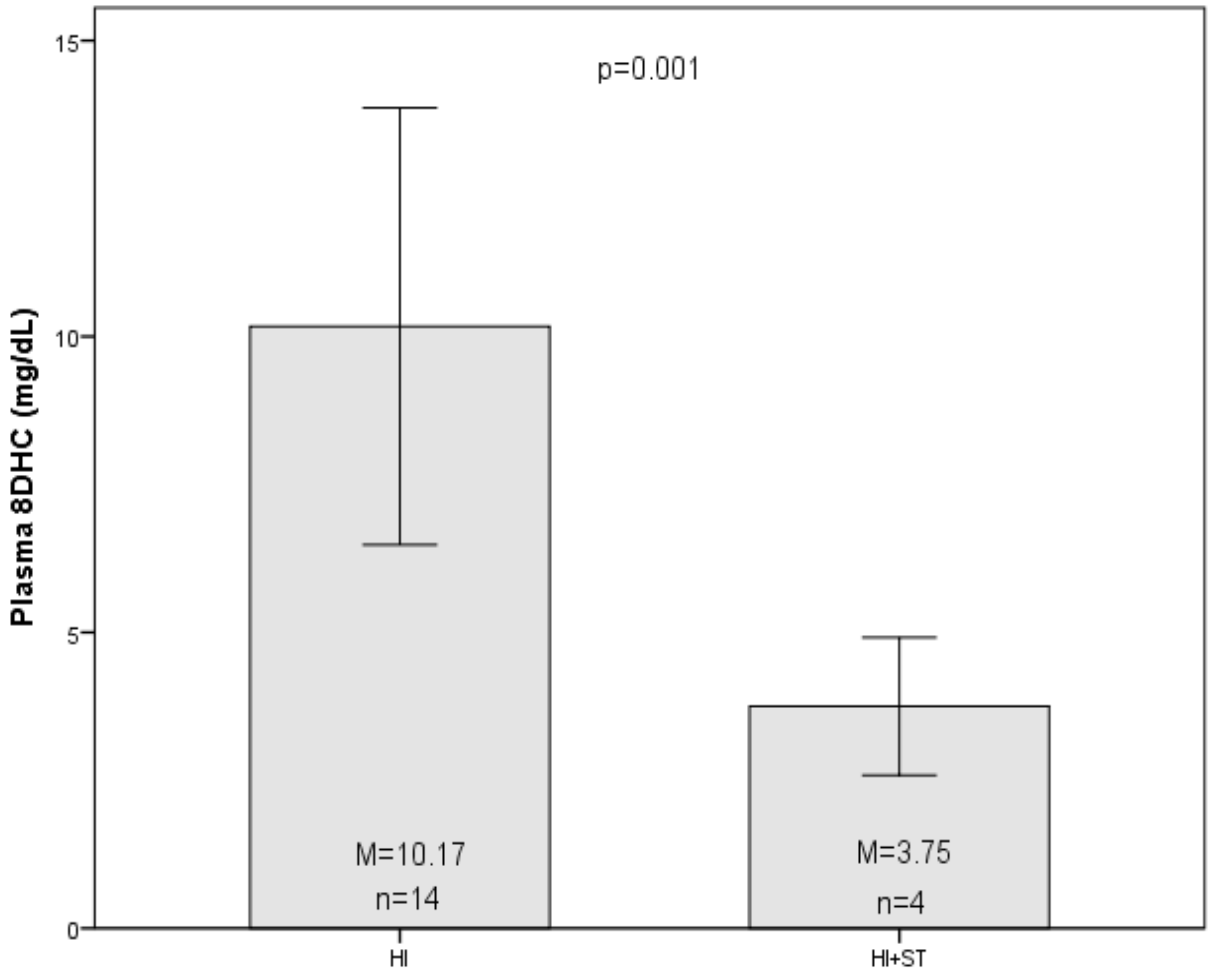


Figure 10. WBCPS in four re-evaluated patients after two (± 1.1 year) of high cholesterol supplementation. There was no significant change in WBCPS over time from (A) (mean=570.6±334.8) to WBCPS (B) (mean=264.7±47.0), p=0.20.

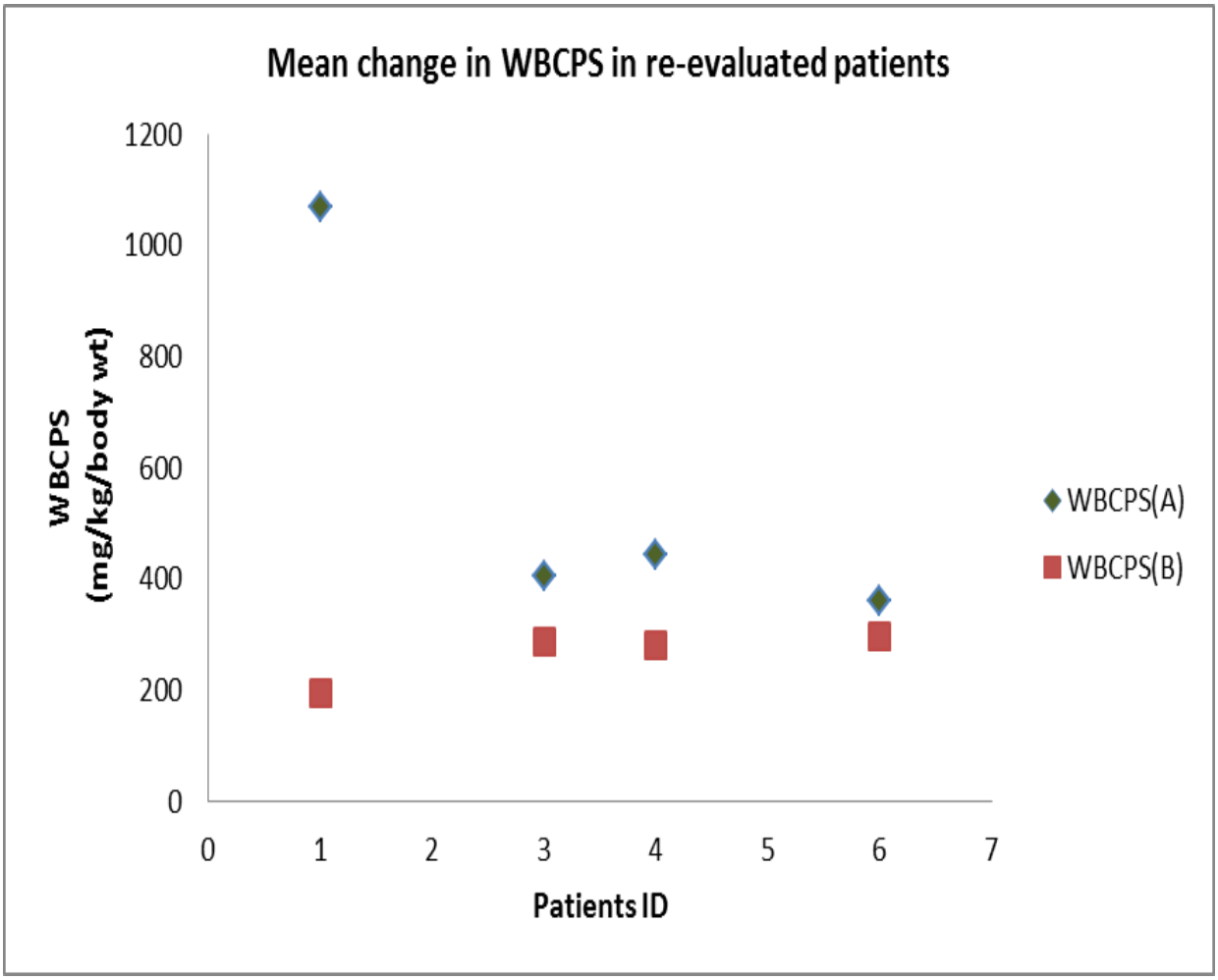
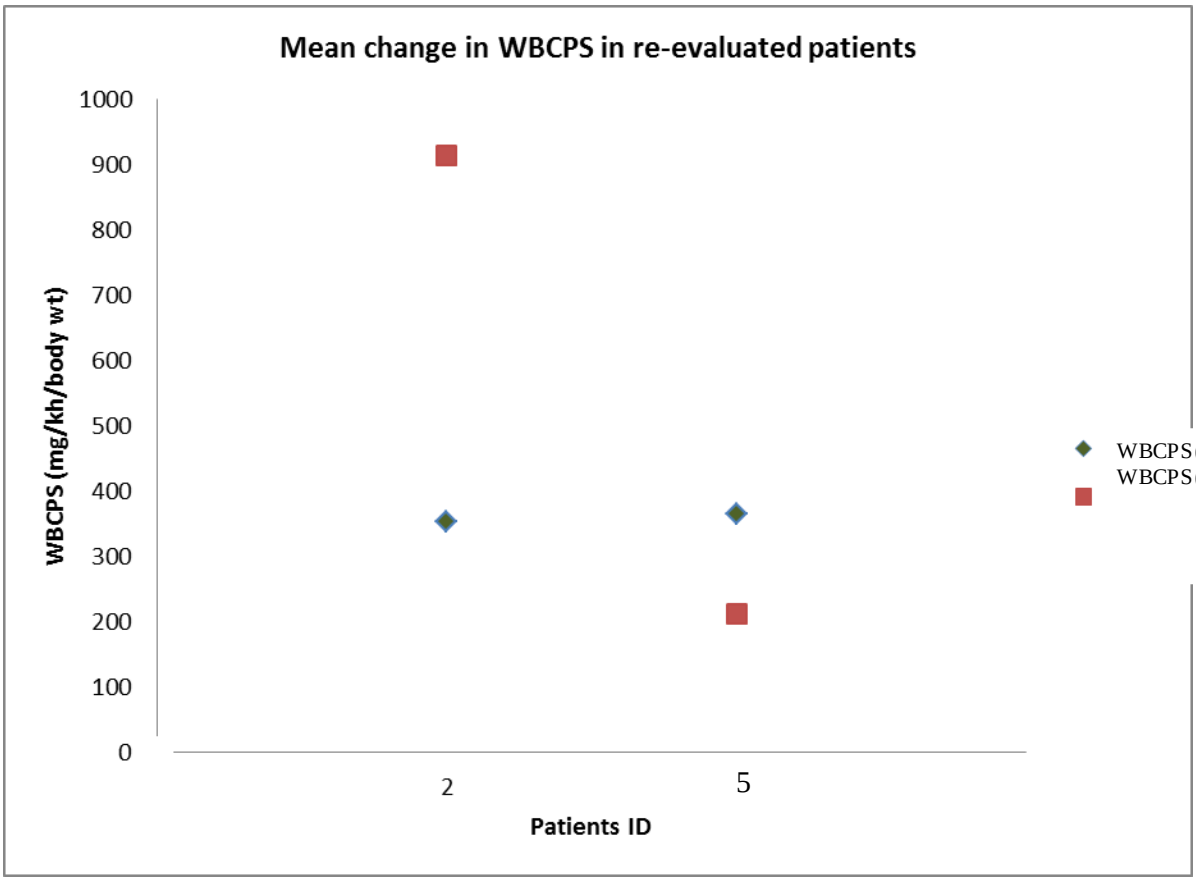


Figure 11. WBCPS in two re-evaluated patients after two (± 1.1 year) of high cholesterol supplementation combined with simvastatin. There was no significant change in WBCPS over time from (mean=632±280.2) to WBCPS (B) (mean=288±108.75), p=0.51. WBCPS, Whole body cholesterol pool size.



4.5 Discussion

In the present study WBCPS was significantly predicted by body weight and age, suggesting that as one grows and his/her body weight increase, so does the WBCPS. This finding is consistent with those of Goodman and co-workers (Goodman, Smith, Seplowitz, Ramakrishnan, & Dell, 1980; Nestel et al., 1969; Smith, Dell, Noble, & Goodman, 1976).

The outcome of the present results showed an absence of an association between WBCPS and plasma cholesterol concentration. Likewise, Nestle et al., (1969) failed to find an association between plasma cholesterol concentrations (ranged from 181 to 407 mg/dL) and cholesterol pool sizes in 22 subjects who received intravenous injections of ^{14}C-cholesterol where calculation of cholesterol turnover was based on the two-pool model analysis of medium-term decay curves described previously by Goodman and Noble (1968) (Nestel et al., 1969). In contrast, Goodman et al., (1980) detected positive association between plasma cholesterol concentrations (ranged from 134 to 560 mg/dL) and the cholesterol pool sizes in 54 subjects (>6 with familial hypercholesterolemia) who received intravenous injections of ^{14}C-cholesterol, where calculation of cholesterol turnover was based on the three-pool model analysis of long-term decay curves (Goodman et al., 1980). Also, Smith et al., (1976) reported in a study of 24 subjects (>3 with familial hypercholesterolemia) the positive relationship between plasma cholesterol concentration and the plasma cholesterol pool size calculated from a three-pool model of a long-term decay curves, the range of plasma cholesterol was from 167 to 551 mg/dl (Smith et al., 1976). The discrepancy between the results can probably be explained by the different number of cholesterol pool models used, as we can see that a three-model can reflect the association between WBCPS and plasma cholesterol concentration better

than a two-pool model. Also the low concentration of plasma cholesterol in our SLOS subjects, which ranged from 16 to 200 mg/dL compared to the higher range of plasma cholesterol concentration in the other studies (Goodman et al., 1980; Smith et al., 1976) can play a role in reflecting the association between WBCPS and plasma cholesterol concentration. Moreover, plasma cholesterol concentration represents only a small fraction of total body cholesterol and it may be independent of cholesterol turnover in the body as a whole. Also cholesterol metabolism and degradation play a critical role in maintaining plasma cholesterol concentration and overall accretion. Thus we were unable to report a significant relationship between plasma cholesterol concentration and the WBCPS in our SLOS patients.

In our current study, plasma cholesterol concentrations correlated positively with cholesterol intake and negatively with 7DHC and 8DHC levels; This finding is in agreement with what has been consistently observed that cholesterol supplementation would increase circulating levels of cholesterol and reduce the levels of 7DHC and its isomer 8DHC by suppression of 7DHC/8DHC synthesis through feedback inhibition of HMG CoA R enzyme activity (Irons et al., 1997; Linck et al., 2000). Correspondingly, Linck et al. (2000) evaluated the effect of cholesterol supplementation with egg yolk in four SLOS children and found a 116% increase in mean plasma cholesterol level from 53 to 114 mg/dL ($p<0.05$). Additionally there was a 67% decrease in mean plasma 7DHC level; from 35 to 11.7 mg/dL ($p<0.05$) after receiving a high cholesterol diet (Linck et al., 2000). Also, a parallel report by Nwokoro and Mulvihill (1997) showed an overall increase in plasma cholesterol (up to 50%) in three SLOS children after receiving a high cholesterol diet. However, two of the three children had decreased plasma 7DHC levels

(50-20% decrease) (Nwokoro & Mulvihill, 1997). One multicentre trial of fourteen SLOS patients, Irons et al., (1997) reported also an increase in plasma cholesterol of 164%, but no significance decreases in plasma 7DHC and 8DHC after receiving a high cholesterol diet (Irons et al., 1997). Furthermore, our results indicated that cholesterol intake can increase plasma cholesterol concentration but not WBCPS, suggesting that cholesterol metabolism (absorption, synthesis and elimination) is auto-regulated to maintain cholesterol homeostasis. Such a homeostatic mechanism especially in disease state like SLOS would mitigate potentially positive effects of cholesterol supplementation. High cholesterol diets may not necessarily be reflected in great shifts in the size of WBCP just as changes in plasma cholesterol concentration are not necessarily reflected in changes in overall cholesterol homeostasis.

Our results suggest that a high cholesterol diet with simvastatin increases significantly WBCPS in children with SLOS, compared with a high cholesterol diet only. Furthermore, a high cholesterol diet with simvastatin resulted in the desired reduction of 7DHC/8DHC levels with no obvious corresponding decrease in cholesterol levels. It has been shown that cholesterol supplementation increases cholesterol concentration in SLOS patients and suppresses the levels of 7DHC/8DHC as well (Irons et al., 1997; Linck et al., 2000). Moreover, simvastatin added to the high cholesterol diet causing further reduction in circulating 7DHC and 8DHC concentrations (Haas et al., 2007; Jira et al., 2000) or it might be that simvastatin up-regulates residual *DHCR7* activity by inducing *DHCR7* expression as discussed previously in the literature (Wassif et al., 2005). Another possible reason for the increase in the WBCPS in the high cholesterol diet with simvastatin group is that when simvastatin up-regulates residual *DHCR7* activity all the accumulating

7DHC and 8DHC are converted to cholesterol, which result in an indirect increase in plasma cholesterol concentration, thus increasing WBCPS. Larger sample sizes may be required to discern percentage changes in WBCPS due to long term cholesterol supplementation with or without simvastatin.

In our present study, we had the opportunity to measure WBCPS in the same patients after two years from the initial assessment, with the results showing no improvement in follow-up WBCPS measurements. That is, WBCPS was not significantly changed in the four re-evaluated SLOS patients given high cholesterol diet or in the two re-evaluated SLOS patients on simvastatin with a high cholesterol diet. High severity scores in the six re-evaluated SLOS patients may have contributed to the inability of high cholesterol diet, with and without simvastatin, to provide discernible changes in WBCPS due to low levels of plasma cholesterol in SLSO patients with high severity score, so recruiting SLOS patients with low severity score for such evaluation is advised in future studies. Moreover, a study with larger groups of patients over a longer period of time will be necessary to evaluate the effect of administration high cholesterol diet with and without simvastatin on WBCPS in SLOS children.

Several limitations of the present study are worthy of discussion. First, the low enrolment of subjects studied, especially when comparing the HI and the HI+ST groups. Second, a more controlled dietary cholesterol intake assessment is needed to ensure that we have a faultless diet history from the parents and/or caregivers and to monitor dietary adherence on regular basis. The third limitation of the current study was the lack of use of appropriate kinetic analysis and modelling software, which enable us to obtain a surer and easier parameters value along with the cholesterol pool size information and

statistics. Furthermore, the source of cholesterol used and the length of the therapy may have affected plasma cholesterol concentrations in our patients (Lin et al., 2005). So a more appropriate treatment design is needed for SLOS patients. Moreover, supplementing different doses of high cholesterol diet for a longer term would be helpful in future studies to design more effective treatment strategy for SLOS.

Lastly, according to what have been discussed previously (Goodman & Noble, 1968), it must be realized that the two pools in the model represent mathematical constructs and do not have any conclusive physical meaning. The finding that the short-term turnover of plasma cholesterol conforms to a two-pool model in human means that the various tissue pools of cholesterol categorized into two groups according to the rate at which they equilibrate with plasma cholesterol. As discussed previously (Goodman & Noble, 1968), pool A, which mainly consist of cholesterol in several viscera (e.g., spleen, erythrocytes, lungs, intestine and liver) equilibrate rapidly (in terms of hours to days) with plasma cholesterol. Pool B, which consists of cholesterol in peripheral tissue (e.g., skeletal muscle) equilibrates at a much slower rate (in terms of days to weeks) with plasma cholesterol. It is necessary to differentiate between short term and long term studies when it comes to study body cholesterol metabolism. Short term studies of cholesterol kinetics in various compartments (Goodman, 1964; Nestel & Couzens, 1966) usually deals with cholesterol pool A, and unfortunately such quick, short-studies cannot deal with deeper pool movement or total cholesterol production. Thus, a long-term kinetic study is needed to provide quantitative information of the cholesterol production rate, of the sizes of various pools involved, and of the rate constants for transfer between the various pools.

4.6 Significance of Research

To our knowledge, this is the first study to measure WBCPS in SLOS patients through the use of stable isotope techniques and the first to directly determine the effect of simvastatin on WBCPS in SLOS patients.

These data are the first to add to a limited literature and to support the ability of the combination of simvastatin and high cholesterol supplementation on maximizing the WBCPS in SLOS patients through the application of stable isotope techniques in *in vivo* studies. And this study might enable us to consider the potentials of dietary cholesterol supplementation with or without statin as a valid treatment strategy for SLOS patients.

4.7 Conclusion

In conclusion, our findings demonstrate that WBCPS was significantly predicted by body weight and age, so as one grows and his body weight increase, so does the WBCPS in SLOS children. Furthermore, a high cholesterol diet with simvastatin resulted in the desired increase of WBCPS in SLOS children, which can meet the therapeutic goals of high cholesterol diet with simvastatin in SLOS because simvastatin decreased precursors pools 7DHC and 8DHC without causing a parallel decrease in plasma cholesterol levels. Detailed understanding of the effect of dietary cholesterol and simvastatin used independently and together at various durations will be required to show significant changes in WBCPS over time.

4.8 Acknowledgments

We thank the staff of the OHSU, the children and their families for participation in this study. We also thank all health care providers for caring for the subjects who participated in this study and for referring them for this research study

CHAPTER 5. RECOMMONDATION FOR FUTURE RESEARCH

There are several suggestions for future studies to increase our knowledge on the effect of interventions on whole body cholesterol homeostasis in SLOS patients. Assessing the balance between cholesterol absorption, synthesis and excretion using triple isotope methods gives a better reflection of tissue cholesterol content. Also, using modelling software for kinetic analysis will enables us to create systems of differential equations from a specific compartmental model, and obtains solution along with parameters value and input information. In addition, such modelling will allow us to carry out various analyses to explore relationships that may exist between the model parameters whole body cholesterol metabolism and the various physiological independent variables that were assessed in each patient. Also, future studies carried out for longer periods will enable us to use a three-pool model for whole body cholesterol pool size measurements (includes M1, M2 and M3). The three-pool model will provide the best fit to the intermediate–term data in each patient. Thus, the three-pool model will validate its use in SLOS patients to study the body cholesterol turnover and for future comparison of normal subjects with SLOS patients.

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