The Smith-Lemli-Opitz syndrome

A multiple malformation syndrome due to a defect in cholesterol biosynthesis

Petr Jira

Cover: Molecular structure of 7-dehydrocholesterol (upper part) and cholesterol (lower part); gift from Ben Mills, Bedford, United Kingdom.

The Smith-Lemli-Opitz syndrome

A multiple malformation syndrome due to a defect in cholesterol biosynthesis

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op het gebied van de Medische Wetenschappen

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Voor Lia, Bibi, Rens, Milou & Puck

ABBREVIATIONS

5-HT	5-hydroxytryptamin
7DHC	7-dehydrocholesterol
8DHC	8-dehydrocholesterol
BBB	Blood Brain Barrier
DHCR7	7-dehydrocholesterol reductase
GC	Gas Chromatography
GC/MS	Gas Chromatography / Mass Spectrometry
HMG-CoA	Hydroxy Methyl Glutaryl Co-enzyme A
HPLC	High-performance Liquid Chromatography
NMDA	N-methyl-d-aspartate
SLOS	Smith-Lemli-Opitz syndrome
SHH	Sonic Hedgehog
SREBP2	Sterol regulatory element binding protein type 2
SSD	Sterol Sensing Domain
UVA	Ultra Violet A

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General Introduction

GENERAL INTRODUCTION

The subject of this thesis is the Smith-Lemli-Opitz syndrome (SLOS; MIM 270400). This autosomal recessive multiple malformation syndrome was first described in 1964 by David Smith, Luc Lemli and John Opitz. First we will review several clinical, biochemical, pathophysiological, molecular and therapeutical aspects of SLO syndrome whereafter the outline of the thesis is given.

Cholesterol (Figure 1), is an important constituent of the cell membrane of most eukaryotic cells, and secondly, acts as the precursor for steroid hormones, bile acids and myelin formation in the brain, spinal cord and peripheral nervous system. Finally, cholesterol has important interaction with proteins, which control embryonic development.

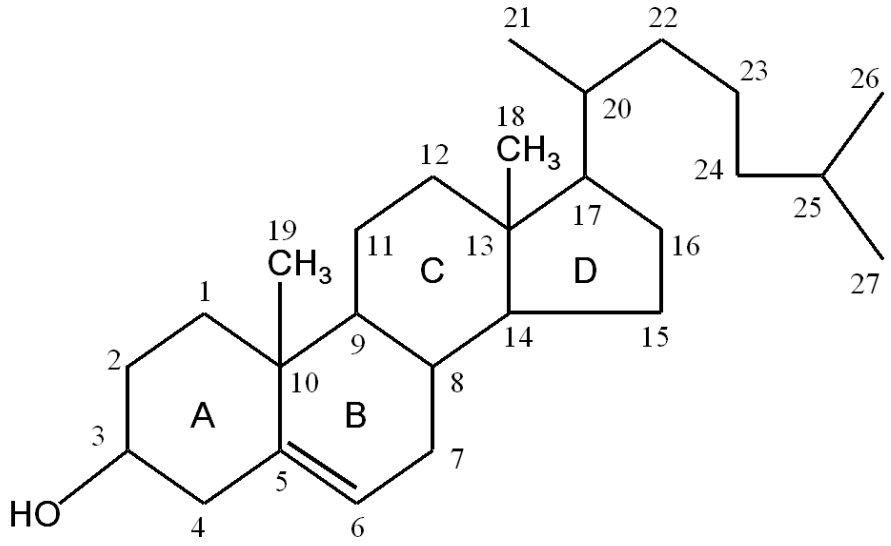

Figure 1. Structure of cholesterol.

SLOS, a severe developmental disorder associated with multiple congenital anomalies, is caused by a defect in cholesterol biosynthesis, i.e. a deficiency of the enzyme 7-dehydrocholesterol reductase, the final enzyme of the cholesterol biosynthetic pathway. Low cholesterol and high concentrations of its direct precursor 7-dehydrocholesterol (7DHC) and its isomer 8-dehydrocholesterol (8DHC) in blood and tissues are the biochemical hallmarks of the syndrome. The plasma sterol concentration generally correlates with severity and outcome. It remains uncertain however whether the clinical symptoms are primarily due to the shortage of cholesterol or to the abundance of the precursors 7DHC and 8DHC, or both.

History

In 1964 David Smith, Luc Lemli and John Opitz described three boys with a similar phenotype. Historically, the syndrome is often also named "RSH"syndrome, formed by the first letter of the original patients' last names (Smith *et al.* 1964). This initial report was soon followed by descriptions of many new clinical cases. Since this original publication, the SLOS has remained a clinical diagnosis for 30 years. The SLOS phenotype described in male and female patients in many case reports disclosed a clinical spectrum with great variability. The estimated incidence of the autosomal recessive SLOS is 1/20,000 – 1/80,000 live births in individuals of European ancestry, but it has a lower incidence in most other ethnic groups (Ryan *et al.* 1998; Kelley & Hennekam 2000; Bzduch *et al.* 2000; Nowaczyk *et al.* 2001; Witsch-Baumgartner *et al.* 2001).

In the past forty years case reports focused on the description of the various organ and dysmorfic anomalies in SLOS. Several experienced reviewers have discussed various clinical and biochemical aspects of SLOS (Cunniff *et al.* 1997; Neklason *et al.* 1999; Opitz 1999a; Fitzky *et al.* 1999; Kelley & Hennekam 2000; Moebius *et al.* 2000; Waterham & Wanders 2000; Kelley & Herman 2001). It is important to note that only the cases described from the mid-90s were biochemically proven after the discovery of the biochemical hallmark underlying the SLOS. Although experienced clinicians claimed to recognise patients with SLOS easily, it became clear that the broad spectrum of anomalies and very variable severity of this syndrome does not make the diagnosis always obvious. Even experienced dysmorphologists admit that making the clinical diagnosis SLOS, especially in mild cases, is not always straightforward and that individuals fulfilling the SLOS criteria in some cases could not be confirmed when biochemical proof became available (Guzetta *et al.* 1996; Nowaczyk *et al.* 2004; own observations).

Clinical features

The past years a delineation of SLOS has been made by many case reports and small series. Although structural defects in many organs may be involved and are described, in the majority of SLOS patients, heart, lungs, liver and kidneys usually are not affected. Conversely, in more severely affected patients, life span is often limited by lethal internal malformations.

Craniofacial

The distinctive craniofacial appearance of SLOS consists of a typical facies with a microcephaly, bilateral ptosis, broad and high forehead, broad nasal bridge, inner epicanthal folds, micrognatia, cleft palate, long philtrum and anteverted nares (as in the patient shown in Figure 2).

Figure 2. Typical facial appearance of a 6-year old boy with SLOS (Photo taken by the parents and printed with parental permission).

Limbs

The characteristic syndactyly of the second and third toe, although sometimes very subtle, is present in about 80-96% of patients with confirmed SLOS. Polydactyly of feet and hands, oligodactyly of hands, short thumbs, hypoplastic thenar eminences and clubfeet are also common in combination with shorter limbs.

Ophthalmologic

Mild to moderate blepharoptosis is common in SLOS, while congenital bilateral cataract and bilateral optic nerve hypoplasia are described in more severe cases (Kretzer *et al.* 1981; Atchaneeyasakul *et al.* 1998). Inherited defects in enzymes of cholesterol metabolism and use of drugs, which inhibit lens cholesterol biosynthesis, are associated with cataracts in animals and man. The basis of this relationship apparently lies in the need of the lens to satisfy its sustained requirements for cholesterol by on-site synthesis. Impairing this synthesis can lead to alteration of lens membrane structure. Lens membrane contains the highest cholesterol content of any known human membrane (Cenedella 1996). SLOS was diagnosed 1.2% of 250 paediatric patients with bilateral cataract and neurological features.

(Cruysberg 1996). Sterol analysis from ocular tissue in SLOS patients and fetuses showed markedly increased 7DHC and 8DHC, whereas the cholesterol concentration in the retinal pigment epithelium, lens, cornea, and sclera was lower (Atchaneeyasakul *et al.* 1998).

Cardio-pulmonary

Minor or major cardiac defects have been described up to 50% in large series of SLOS patients (Lin *et al.* 1997; Kelly & Hennekam 2000). A more specific association with atrioventricular canal defect and patients with an abnormal pulmonary venous return has been described. Striking is the documentation of non- or hypolobated lungs in autopsy studies of severe SLOS patients and animal models (Fitzky *et al.* 2001; Rakheja *et al.* 2003).

Gastrointestinal

Functional and anatomical gastro-intestinal abnormalities such as pyloric stenosis, aganglionosis coli (Hirschprungs' disease), gastrointestinal reflux and failure to thrive, are common.

Urogenital

Genital malformations and its endocrine aspects are very interesting. Hypospadias, under-virilised male sex-differentiation ranging from cryptorchidism to complete sex reversal with a female appearance in 46 XY-individuals is described in SLOS. Further non-specific urogenital and renal abnormalities, as in other syndromes, are described to a lesser extent.

Development and behaviour

In SLOS psychomotor retardation and microcephaly are very common (80-95%). Mental development of SLOS patients ranges from profound mental retardation to borderline normal intelligence (Ryan *et al.* 1998; Langius *et al.* 2003; Mueller *et al.* 2003). The behavioural phenotype includes cognitive delay, sensory hyperreactivity, language impairment, sleep cycle disturbances, self-injury behaviour (up to 35%), syndrome specific motor movements, and autism spectrum behaviour (up to 50%). Some studies report aggression (52-63%), marked irritability from childhood throughout life. Sleeping disorders in the first and self-injurious behaviour in the second decade of live have a great impact on the family and parental acceptance of SLOS. Frequently SLOS patients demonstrate upper-body opisthokinesis; they arch their necks backwards frequently; and stereotypic stretching, accompanied by brief and rapid hand movements, can be observed (Tint *et al.* 1995; Kelley *et al.* 1996; Pauli *et al.* 1997; Nwokoro *et al.* 1997; Ryan *et al.* 1998; Opitz *et al.* 1999b; Tierney *et al.* 2000; Tierney *et al.* 2001).

Growth

SLOS patients have short stature at diagnoses and growth-retardation as documented in several case reports and studies. Some initial attempts have been made with growth-hormone (GH) treatment in SLOS patients. They improved from –4.15 SD at the age of 5.5 years to –1.6 SD at 13 years (Ullrich *et al.* 2002). Larger trials are needed to evaluate the effect of GH on length and neuromotor development. Experience with GH-therapy in Prader-Willi syndrome, Noonan syndrome and Turner syndrome on growth and development may help to introduce this treatment strategy in SLOS.

Dermatologic

7DHC, also known as provitamin D3, is the precursor for Vitamin D (cholecalciferol) biosynthesis. In the presence of hundred to thousand-fold increased levels of 7DHC in serum and tissue one should expect high levels of Vitamin D in SLOS patients. No significant hypercalciaemia or nefrocalcinosis has been documented in SLOS, however.

Conversely, the majority of SLOS patients (60-70%) suffer from mild to severe skin photosensitivity (Charman *et al*. 1998; Anstey *et al.* 1999). Even after several minutes' exposure to bright sunlight, erythema occurs to exposed skin in some SLOS patients and is of permanent clinical concern to a circumscript group of patients. Monochromatic ultraviolet (UV) radiation and visible light testing revealed an immediate and persistent reaction to low-dose UVA at 350 nm and an abnormal erythemal response to visible light at 400 nm (Charman *et al.* 1998). UVA is known to cause peroxidation of membrane lipids with subsequent alterations in the structure and function of the membranes, leading to modifications in transmembrane transport and deregulation of receptors and messenger systems. Dietary cholesterol could improve photosensitivity in SLOS in some patients (Elias *et al.* 1997; Irons *et al.* 1997; Starck *et al.* 2002).

Adrenal insufficiency

Cholesterol is the precursor for corticosteroid production. Adrenal function and stress reaction have been tested in patients, showing conflicting data. Adrenal insufficiency has been suggested in some SLOS patients with low blood sodium, increased blood potassium, high levels of ACTH and renin but normal values of cortisol (Chemaitilly *et al.* 2002). Adrenal insufficiency with persistent hypertension has been documented in a newborn infant with SLOS (Nowaczyk *et al.* 2001).

Endocrine

Precocious puberty, under-virilisation and even complete sex reversal is frequently described in SLOS (Starck *et al.* 1999). Since cholesterol is the precursor of steroid hormones, reports speculated about endocrine abnormalities in SLOS patients. Abnormal steroid production and metabolites have been detected by some researchers but the cause for under-virilisation in SLOS remains speculative. Recent

studies on 40 SLOS patients (age 1 day – 25 years) studied urinary steroids using GC/MS and selected-ion-monitoring. Neonates with SLOS excreted conventional 3β-hydroxy-5-ene steroids, mostly 16α-hydroxylated and 7 and 8-dehydro homologues. Dominant novel steroids excreted by older children and SLOS adults are 7 and 8-dehydro variants of pregnanetriol (Shackleton *et al.* 2002). Recently 17 alpha-hydroxypregna-4,7-diene-3,20-dione and 17 alpha-hydroxy-5 beta-pregn-7-ene-3,20-dione have been tentatively identified as steroid metabolites in SLOS (Guo *et al.* 2003). Abnormal neurosteroidogenesis can influence behaviour and development pre- and postnatally. Delta-7 and/or delta-8-unsaturated neurosteroids may be produced in the brain. Analysis of urinary steroids in post-pubertal SLOS females confirmed the presence of neurosteroid-like compounds in SLOS (Marcos *et al.* 2004).

Brain

Brain imaging and post-mortem examination in SLOS case reports revealed abnormal cerebral gyri, cerebellar hypoplasia, delayed myelinisation, holoprosencephaly, lissencephaly, agenesis or hypoplasia of the corpus callosum, hippocampal malrotation and choroidal cyst. Aspecific migration disorders were documented in up to 37% in smaller series (Kelley *et al.* 1996; Ryan *et al.* 1998; Nowaczyk *et al.* 2001). These observations confirm that SLOS is a multiple malformation/retardation syndrome and patients are at risk for an impaired development from birth. Neuro-imaging in a series of 18 SLOS patients showed four callosal abnormalities, one Dandy Walker variant and one arachnoidal cyst (Caruso *et al.* 2003).

Neurophysiology

Abnormal nerve conduction velocities have been documented in SLOS patients (Starck *et al.* 1999). Some groups studied rod photo responses in children with SLOS and found slow kinetics of photo transduction in all but 3 of 13 patients. Their observation demonstrates altered kinetics of a membrane-bound signaling system. This may be caused by altered sterol composition in the cell membranes of rod photoreceptors (Elias *et al.* 2003). In a rodent-model, the retina revealed anatomical, degenerative and conductive abnormalities when the ratio of 7DHC/Cholesterol was increased (Fliesler *et al.* 2004). Progressive polyneuropathy improved on cholesterol supplementation, whilst stationary forms did not (Starck *et al.* 2002).

Cholesterol biosynthesis

The great breakthrough for SLOS was the discovery of its biochemical characteristics in 1993 by the group of Mira Irons and Richard Kelly (Irons *et al.* 1993; Tint *et al.* 1995). Patients typically show reduced levels of cholesterol and increased levels of 7DHC and its isomer, 8DHC, in plasma and all

tissues. This biochemical abnormality in SLOS is caused by an enzymatic defect in the last step in normal cholesterol biosynthesis (Figure 3).

Cholesterol is a main end product derived from the isoprenoid biosynthetic pathway. This cascade supplies cells with a variety of compounds, collectively called isoprenoids, which function in diverse cellular processes. Among these are Ubiquinone-10 and Heme A, involved in electron transport, dolichol, mediator of protein glycosylation, isopentyl tRNAs, and farnesyl and geranyl groups for prenylation of proteins that are involved in cell signaling and differentiation (Goldstein & Brown 1990). A series of enzyme reactions is required to eventually produce cholesterol. Acetyl-CoA (C2) is converted into the isoprene unit, isopentenyl-PP (C5), by six different serial enzyme reactions. After adding C5 to geranyl-PP (C10) through farnesyl-PP (C15), squalene (C30) is transformed to lanosterol (4,4,14-trimethylcholesta-8(9), 24-dien-3-ol) by cyclization (Figure 3).

Not only humans but the majority of organisms, including animals, plants, fungi and micro-organisms, are equipped with the crucial *de novo* cholesterol biosynthetic pathway, supplying sterols and steroids to cells, membranes and tissues, illustrating the importance of cholesterol biosynthesis for intermediate and end-products.

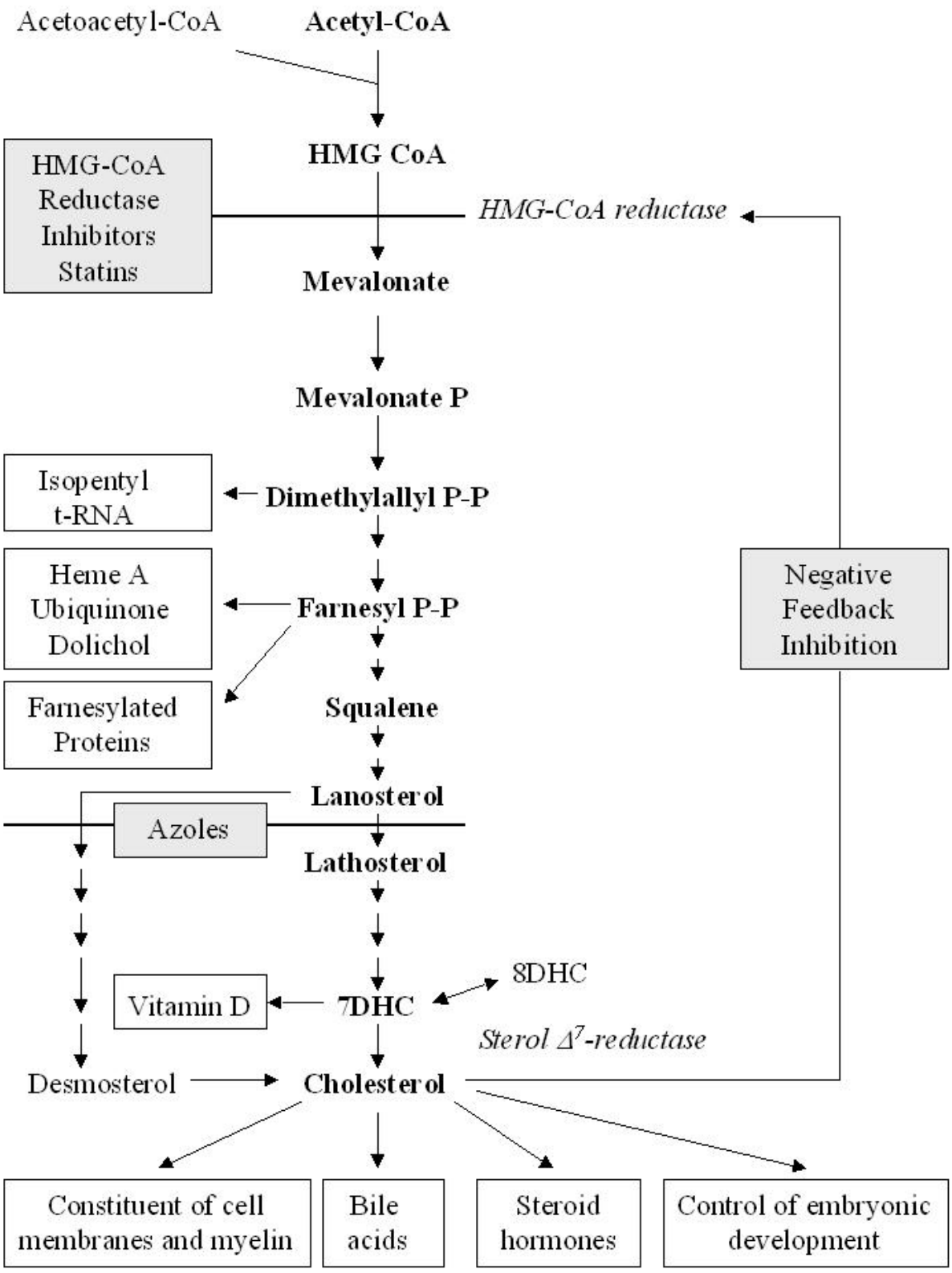

Figure 3. The cholesterol biosynthetic pathway. HMG-CoA reductase is the rate-limiting step for the entire enzymatic pathway. The sterol Δ^7-reductase (DHCR7) catalyses the last step in cholesterol formation. This enzyme is defective in SLOS.

DHCR7 (7-dehydrocholesterol-reductase) deficiency

The SLOS is caused by a deficient enzyme: 7-dehydrocholesterol reductase (DHCR7; E.C. 1.3.1.21; = 3 β-hydroxysterol Δ^7- reductase), localised intracellulary in the membrane of the endoplasmic reticulum (Witsch-Baumgartner *et al.* 2000). The only structural difference between cholesterol and 7DHC is the unsaturated double bond in the B ring at the 7th position in 7DHC (Figure 4.)

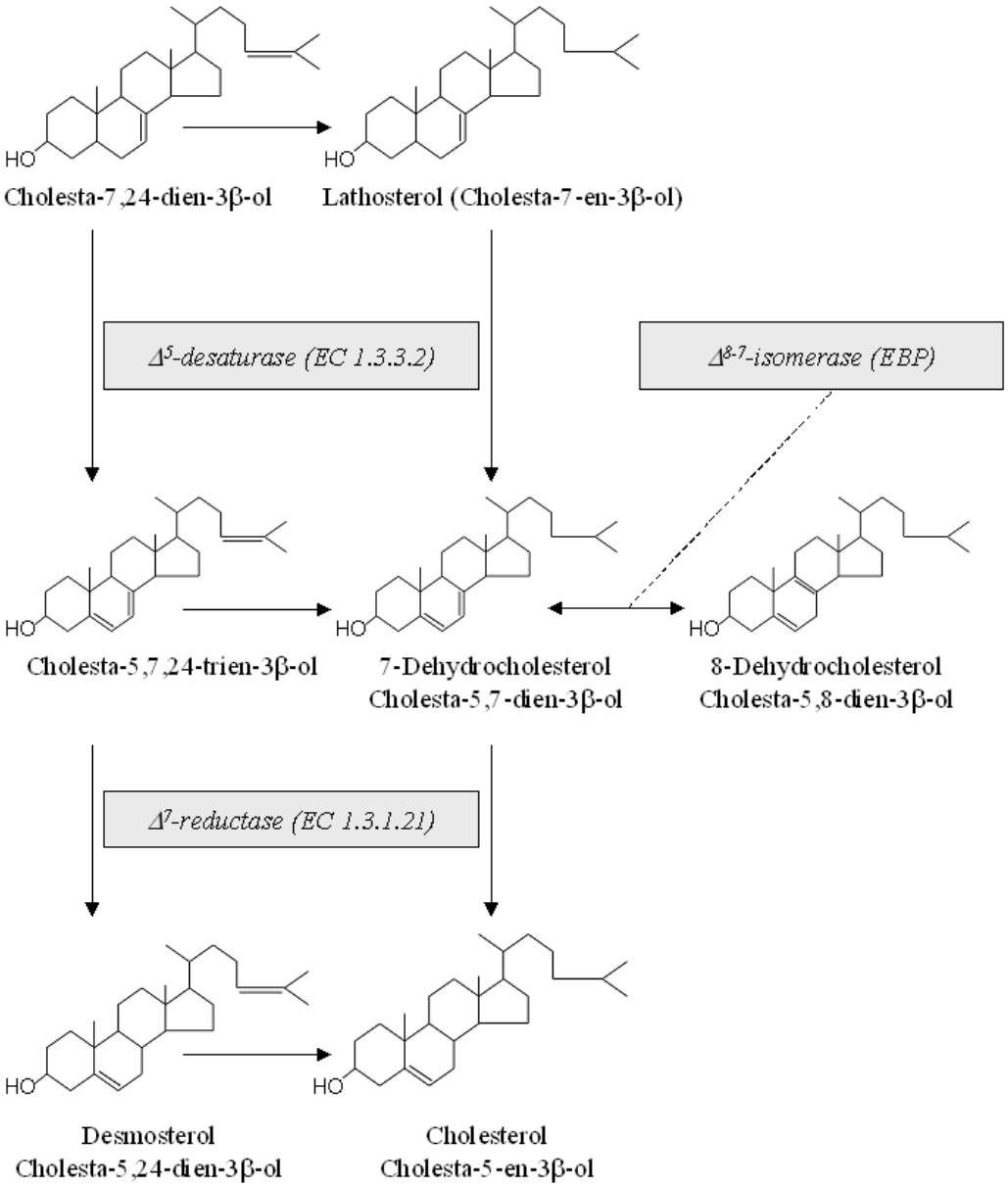

Figure 4. Last steps in the post-squalene part of the cholesterol biosynthesis. Reduction of the Δ^{24} double bond by 3β-hydroxysterol-Δ^{24}-reductase can occur at any point along the pathway. The 3β-hydroxysterol-Δ^5-desaturase (lathosterol-dehydrogenase), 7-dehydrocholesterol-reductase and 3β-hydroxysterol-Δ^{8-7}-isomerase, respectively add, remove and translocate a double bond in the B ring.

Biochemical diagnosis in SLOS

When a patient is clinically suspected of SLOS, the diagnosis can be confirmed by the detection of 7DHC (and or 8DHC) in various tissues. The presence of increased concentrations of 7DHC in plasma, CSF, amniotic fluid, or tissues is diagnostic for SLOS (Irons *et al.* 1994, Tint *et al.* 1995, Shefer *et al.* 1995; Mills *et al.* 1996; van Rooij *et al.* 1997). Routine hospital laboratories use cholesterol-oxidase methods to measure serum cholesterol. This test however is not useful in diagnosing SLOS while it does not detect abnormal sterols. 7DHC-detection can be performed with different techniques such as Gas Chromatography (GC), Gas Chromatography-mass spectrometry (GC-MS), HPLC, electrospray ionization tandem mass spectrometry, time-of-flight secondary ion mass spectrometry and Tandem MS (Irons *et al.* 1993; Tint *et al.* 1995; Guzzetta *et al.* 1996, Zimmerman *et al.* 1997; Starck *et al.* 2000; Rizzo *et al.* 2000; Johnson *et al.* 2001; Scalco *et al.* 2003). Most European countries have one or more laboratories that can biochemically confirm the clinical diagnosis of SLOS.

DHCR7-enzyme activity has been studied in fibroblasts, liver microsomes and chorionic villus samples (Salen *et al.* 1996; Bae *et al.* 1997; Shefer *et al.* 1997). Total body sterol balance studies using stable isotopes are time consuming and expensive (Shefer *et al.* 1995; Honda *et al.* 1996; Lund *et al.* 1996; Steiner *et al.* 2000; Linck *et al.* 2000). Neither are primary diagnostic tools but can be important in research setting and may give important answers to experimental questions concerning cholesterol biosynthesis and metabolism in patients.

Prenatal Diagnosis

Prenatal diagnosis in the late 90's was available through measurement of 7DHC levels in chorionic villi at 11-12 weeks and amniotic fluid at 15-16 weeks of gestation. Currently, more accurate diagnosis is possible by molecular genetic studies of mutations in the DHCR7 gene of the unborn child. Abnormal synthesis of cholesterol in the adrenal foetal glands with SLOS results in equine-type estrogens in the maternal plasma and urine detected by GC-MS and may serve as a potentially non-invasive alternative screening (Shackleton *et al*. 1999). Prenatal screening for SLOS by measuring maternal plasma levels of non-conjugated estriol (uE3), as performed at 15-20 weeks of pregnancy to determine the risk of Down's syndrome, trisomy 18 and open neural tube defects, is suggested, since uE3 synthesis is dependent on cholesterol produced by the fetal tissue (McKeever *et al.* 1990).

Molecular Genetics

Although initial evidence suggested that the human SLOS gene was located at 7q32.1, the human DHCR7 (Gen Bank accession number: AF034544) was identified in 1998 and assigned to chromosome region 11q13 (Fitzky *et al.* 1998; Wassif *et al.* 1998; Waterham *et al.* 1998). The cDNA has an open reading frame of 1,425 base-pair coding for a polypeptide of 475 amino acids. Mutations,

expression and genotype/phenotype studies have been performed on SLOS patients. Molecular genetic work elucidated many pathogenic mutation and enabled accurate prenatal diagnosis.

Cholesterol and the brain

Cholesterol in the human brain, developing sheep brain, and rat pup brain is made locally from glucose, acetate, or polyunsaturated fatty acids (Jurevics *et al*. 1995; Likhodii *et al.* 1995; Snipes *et al.* 1997). Sterols formed in the brain by the mevalonate pathway have an active and independently regulated biosynthesis. Cholesterol is not imported from peripheral blood across the Blood Brain Barrier (BBB) by lipoprotein uptake (Andersson *et al.* 1990; Björkhem *et al.* 1997). Even during fetal brain development, including the time before closure of the BBB, lipoproteins circulating through the central nervous system are not used as a source of cholesterol, but are synthesized locally (Serougne *et al.* 1976; Andersson *et al.* 1990; Edmond *et al*. 1991; Saheki *et al.* 1994; Turley *et al.* 1996; Björkhem *et al.* 1997).

Chronic treatment with cholesterol synthesis-blocking agents impaired brain sterols and associative learning in rats. Exogenous cholesterol, however, failed to correct for the learning impairment produced by the chemical DHCR7-inhibition (O'Brien *et al*. 2002). Neurons from both control and mutant pups exhibited similar reversible, tetrodotoxin-sensitive sodium currents in response to a voltage step to –30 mV from a holding potential of –90 mV. Mean peak amplitudes of the control and the mutant sodium currents were not significantly different and both were able to generate a single action potential upon injection of a depolarising current from a potential of –55 mV. However, an impaired glutamate response was detected in mutated pups. How the NMDA receptor function could be influenced is not known in SLOS. Neurosteroids are thought to be endogenous ligands for sigma 1(σ_1) receptors and to modulate NMDA receptor function. Whether elevations of sterol biosynthetic intermediates might competitively inhibit neurosteroid synthesis or affect neurosteroid interaction with sigma 1(σ_1) receptors should be studied in future (Wassif *et al.* 2001). Behavioural phenotypes and autistic behaviour is studied with mouse models and linked to abnormal and increased 5-HT (serotonin) neurons and fibres (Waage-Baudet *et al.* 2003). In the hindbrains of mutant (Dhcr7-/-) mice an impressive increase in 5-HT immunoreactivity could be observed. This increase in 5-HT immunoreactivity was suggested to represent an increase in total number of 5-HT neurons and fibres and may help to explain the behavioural phenotype seen in SLOS (Waage-Baudet *et al.* 2003). SLOS and other disorders in cholesterol biosynthesis can provide new insights in development, brain growth and brain function.

Other disorders of cholesterol biosynthesis

Eight distinct inherited disorders have been linked to different defects in cholesterol biosynthesis (Table 1 and Figure 5). Two disorders are known to result from an enzyme defect in the pre-squalene segment of the pathway: the classical form of mevalonic aciduria and the hyperimmunoglobulinemia D/Periodic fever syndrome, also known as Dutch-type periodic fever.

All six enzyme defects in the post-squalene segment of the pathway, have been resolved on the basis of elevated levels of specific sterol intermediates in tissues of affected patients, followed by demonstrating disease-causing mutations in the encoding genes. These include the two X-linked dominant inherited and male-lethal disorders Conradi-Hünermann-Happle syndrome and Congenital Hemidysplasia with Ichthyosiform erythroderma and Limb Defects: CHILD syndrome, caused by deficiencies of sterol Δ^{8-7} isomerase and sterol C-4 demethylase, respectively. The three extremely rare autosomal recessive disorders, Greenberg skeletal dysplasia (=HEM, Hydrops Ectopic calcification Moth-eaten skeletal dysplasia), lathosterolosis and desmosterolosis, characterized by multiple congenital anomalies, have clinical overlap with the most common inborn error of cholesterol biosynthesis: SLOS. Table 1 gives a summary of disorders in cholesterol biosynthesis, genes, enzymes, most important features and their references.

Table 1. Disorders of cholesterol biosynthesis

Disorder	**MIM**	**Enzyme**	**Gene**	**Chromosomal localisation**	**Features**	**Reference**
Mevalonic aciduria	251170	Mevalonate kinase 2.7.1.36	MVK	12q24	Dysmorfic facies Developmental delay Anaemia, hepato-splenomegaly	Hoffmann *et al.* 1986 Houten *et al.* 2000 Haas *et al*. 2001 Simon *et al*. 2004
Hyper immuno-globulinemia D Periodic Fever	260920	Mevalonate kinase 2.7.1.36	MVK	12q24	Episodic fever, lymfadenopathy, arthralgia, skin rash	Drenth *et al*. 1999 Houten *et al*. 1999 Simon *et al.* 2004
Greenberg Skeletal Dysplasia HEM	215140	Sterol Δ^{14}-reductase	LBR 600024	1q24.1	Short-limb dwarfism, severe hydrops fetalis, moth-eaten bone appearance	Greenberg *et al*. 1988 Offiah *et al.* 2003 Waterham *et al.* 2003
Congenital Hemidysplasia with Ichtyosiform Eryhtoderma and Limb Defects (CHILD)	308050	Sterol C4-demethylase	NSDHL	Xq28	Ichtyosis, limb malformation, asymmetric hypoplasia, male lethality	Happle *et al.* 1980 Konig *et al.* 2000 Bittar *et al.* 2004
Lathosterolosis	607330	Sterol Δ^{5}-desaturase	SC5DL	11q23.3	Multiple congenital anomalies, cleft palate, limb patterning defects, micrognatia, mental retardation and liver disease.	Brunetti-Pierri *et al.* 2002 Krakowiak *et al*. 2003
Conradi-Hünermann-Happle Syndrome CDPX2	302960	Sterol $\Delta^{8\text{-}7}$-isomerase	EBP 300205	Xp11.22-23	Striated hyperkeratosis, cataracts, rhizomelic shortening of limbs	Happle *et al.* 1979 Derry *et al*. 1999 Herman *et al*. 2002 Bittar *et al.* 2004
Smith-Lemli-Opitz syndrome SLOS	270400	Sterol Δ^{7}-reductase	DHCR7	11q13	Multiple congenital anomalies, 2/3 toe syn/polydactyly, cleft palate, intersex and retardation.	Smith *et al.* 1964 Irons *et al.* 1993 Kelley & Herman 2001
Desmosterolosis	602398	Sterol Δ^{24}-reductase	DHCR24 606418	1p31.1-p33	Multiple lethal congenital malformations, osteosclerosis, short limbs, ambiguous genitalia, cleft palate and macrocephaly	FitzPatrick *et al.* 1998 Waterham *et al.* 2001 Andersson *et al*. 2002

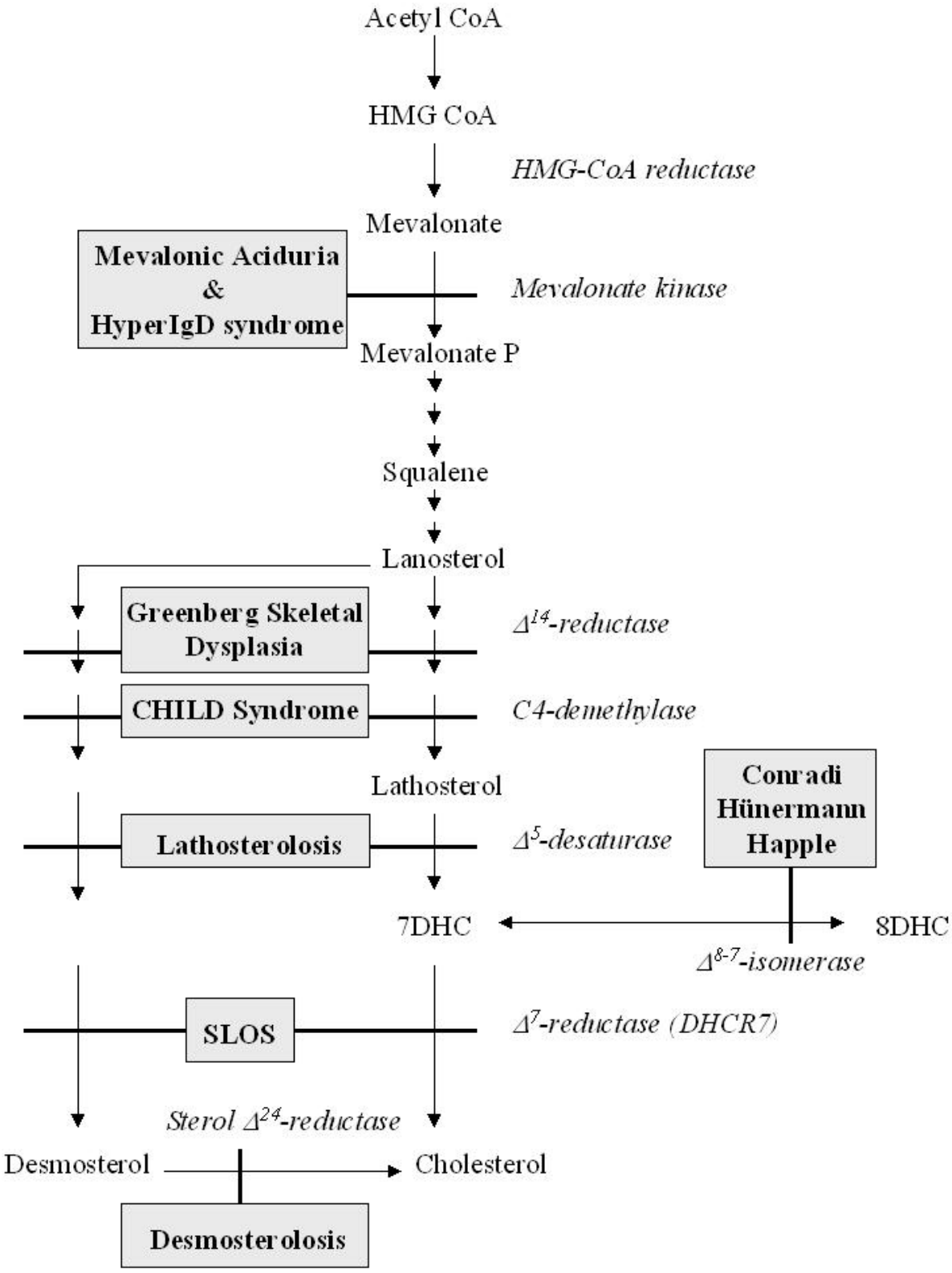

Figure 5. Pre and post-squalene cholesterol biosynthesis. The eight human disorders are shown in grey boxes.

Cholesterol deficiency in teratology and embryogenesis

Shortly after the discovery of cholesterol impairment in SLOS in the mid 90's, researchers focused on its role in morphogenesis. Cholesterol has a pivotal role during embryogenesis where it functions as a transporter molecule for the Sonic hedgehog (Shh) signaling protein, which is essential for normal morphogenesis. Without cholesterol, Shh transport and/or function is impaired (Hall *et al.* 1995; Porter *et al.* 1996; Hall *et al.* 1997; Lanoue *et al.* 1997; Cooper *et al.* 1998). The Cholesterol modification of Shh activates signaling molecules such as Patched (Ptc), Smoothened (Smo) and Gli, which function in the development of numerous tissues by regulating cellular differentiation and proliferation (Theil *et al.* 1999; Wallis and Muenke 1999; Ogden *et al.* 2004).

Teratology

Some of the major malformations seen in SLOS can be mimicked in animal models utilising specific cholesterol-lowering drugs: BM 15.766, AY-9944 and YM 9429 inhibiting the Δ^7-reductase enzyme in cholesterol biosynthesis (Honda *et al.* 1996; Kolf-Clauw *et al.* 1996; Dehart *et al.* 1997; Roux *et al.* 2000). Treatment of pregnant rats with inhibitors of DHCR7, either AY9944 or BM15.766, has provided a valuable model to study the pathogenesis in SLOS (Gofflot *et al.* 1999). In brain tissue a 7DHC/Cholesterol ratio as high as 3 easily can be detected after treating rats with AY9944 (Keller *et al.* 2004). The most characteristic malformations in this animal "holoprosencephalic syndrome" include cyclocephaly, cyclopia, monorhinia, palatoschizis and agenesis of the pituitary gland in up to 80 % of subjects (Honda *et al.* 1996; Kolf-Clauw *et al.* 1996; Incardona *et al.* 2000). Teratogenic compounds/inhibitors used to study SLOS interfere with the morphogenetic Shh-Ptc-Smo-Gli signaling pathway. In animal studies, reduced or absent expression of Shh has been proven to influence expression of several other important morphogenetic genes in brain, heart, limb and mid-face development (Gofflot *et al.* 2003; Digilio *et al.* 2003; Hill *et al*. 2003; Cox 2004). Indeed, in a large molecular genetic study of 200 holoprosencephaly patients, not only SHH mutations but also mutations in ZIC2, SIX3, and TGIF genes could be disclosed (Dubourg *et al.* 2004). This provides further evidence that malformations in several organs may originate directly from cholesterol disruption or impaired Shh signaling activity.

SLOS knockout-mice model: Dhcr7-/-

A targeted mutation of the Dhcr7 gene eliminates enzyme activity, making it virtually undetectable (1 ± 1 pmol/mg protein/min) in mice (Wassif *et al.* 2001). As in human patients, the SLOS/RSH mouse model showed marked reduction of circulating and tissue cholesterol and marked increase of 7DHC. Serum 7DHC levels were increased one hundred to one thousand fold and the percentage of 7DHC to total sterols of 50-80% in tissues of Dhcr7-/- mice (Wassif *et al.* 2001).

Dhcr7-/- pups weighed significantly less: 1.11-1.14 g compared with 1.42-1.44 g for Dhcr7 +/+ pups (Wassif *et al.* 2001 ; Fitzky *et al.* 2001). Homozygotes do not die prenatally but within 18

hours, presumably from respiratory failure or dehydration. Dhcr7-/- pups are easily identified shortly after birth by their laboured breathing, blue coloration, and lack of movement. Homozygous Dhcr7-/- mutant pups had craniofacial malformations, demonstrated decreased movement and none of the pups suckled and therefore failed to feed (Wassif *et al*. 2001). In 30 % of the Dhcr7-/- pups a nasal plug was noted and in 9% even the absence of an nasal opening. Mutant pups, rarely vocalised alarm, had a hypotonic appearance and had less vigorous movements, compared with control groups. In Dhcr7-/- mice isolated cleft palate was noted in 8-12 % (Wassif *et al.* 2001; Fitzky *et al*. 2001).

Surprisingly, histological analysis showed no gross abnormalities of brain, heart, intestine, adrenal gland or kidney, as in severe human cases of SLOS. However, microscopic examination of fixed and stained sections of lung sections from Dhcr7-/- mice demonstrated compact lungs with sparse, unconnected air spaces that were similar in appearance to normal 15 to 16-gestational day mice. Diffuse alveolar atelectasis, however, was often detected but no limb or skeletal malformations (Wassif *et al.* 2001; Fitzky *et al*. 2001). Mutant pups lack a stereotypic rhythmic sucking/swallowing reflex leading to aspiration in trachea, bronchi and lung parenchyma. Gross and histological examination of the pharynx and trachea revealed no malformation (Wassif *et al.* 2001).

Although smaller, mutant brain weights were proportional to body weight; there was no increased apoptosis in brains of Dhcr7-/- pups. Cholesterol deficits in homozygous mutant mice were most profound in the brain with dehydrocholesterols making up 80% of total sterols. In Dhcr7-/- mice sterol biosynthesis is suppressed as in the average child with SLOS, illustrated as a percentage of reductions in liver total sterols, HMG-CoA reductase protein levels, and HMG-CoA reductase activities (68%, 64%, and 83%, respectively) (Fitzky *et al.* 2001). In the central nervous system, 3β-hydroxysterol Δ^7-reductase (DHCR7) reduces 7DHC to form desmosterol and, as expected, desmosterol levels were markedly reduced in cortex and midbrain from Dhcr7-/- pups, compared with levels found in samples from either Dhcr7+/+ or Dhcr7+/- pups. Neuronal impairment might be the result of replacement of cholesterol and desmosterol by cholesterol-precursors: 7DHC and 8DHC in neuronal membranes.

Pathophysiology

Hedgehog-signaling proteins are involved in numerous developmental processes. They are modified by the addition and presence of cholesterol that appears to be required for the proper action and transport of hedgehog morphogens in the cell and tissues (Hall *et al.* 1997; Ingham 2001; Jeong *et al.* 2002). The Sonic hedgehog must be covalently linked to cholesterol to be activated and is essential for the development of the brain, limbs and face (Roux *et al.* 2000). Cooper and co-workers could demonstrate a compromised Hedgehog signal in mutant cells from mouse models of SLOS and in normal cells pharmacologically depleted of sterols (Cooper *et al.* 2003). Molecular mechanisms underlying limb- (syn/polydactyly) and (non-lobulated) lung anomalies are studied to understand

imbalance of hedgehog expression or disruption of cascades as seen in SLOS (Gofflot *et al.* 2003; Yu *et al.* 2004). Different chemo- and phenotypes in SLOS patients with the same genotype suggest that there are more factors that have a pre- and postnatal effect on cholesterol biosynthesis and its important signaling cascades (Witsch-Baumgartner *et al.* 2004). Alternative pathways, maternal apo E genotype, LDL receptor gene variations, maternal diet during pregnancy and other factors must be studied in relation to modulation of embryonic development and SLOS malformations.

Therapy

Soon after the discovery of reduced cholesterol synthesis in SLOS, baseline cholesterol and precursor levels were associated with severity and developmental impairment. Several trials with dietary supplementation were initiated with the aim of increasing cholesterol and reducing the *de novo* synthesis and accumulation of 7DHC and 8DHC. Therapeutic trials in SLOS patients used dietary supplementation of cholesterol powder or egg yolk, with or without bile acids. (Xu *et al.* 1995a; Xu *et al.* 1995b; Irons *et al.* 1995; Ulrich *et al.* 1996; Elias *et al.* 1997; Irons *et al.* 1997; Nwokoro *et al.* 1997; Linck *et al.* 2000). The concentration of plasma cholesterol could be increased to subnormal levels in some SLOS patients. However, dietary cholesterol could not decrease levels of 7DHC and 8DHC in plasma or tissues.

Overall clinical improvement with cholesterol supplementation in SLOS is disappointing (Xu *et al.* 1995a; Xu *et al.* 1995b; Irons *et al.* 1995; Ulrich *et al.* 1996, Elias *et al.* 1997; Irons *et al.* 1997; Nwokoro *et al.* 1997; Linck *et al.* 2000). Cholesterol and bile acid supplementation could improve photosensitivity in SLOS in some patients (Elias *et al.* 1997; Azurdia *et al.* 2001; Starck *et al.* 2002) Progressive polyneuropathy improved on cholesterol supplementation, whilst stationary forms did not (Starck *et al.* 2002). In a study of 14 SLOS children receiving cholesterol supplementation, developmental quotients did not improve over time (Sikora *et al.* 2004).

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Chapter 2

Aim of the Study

AIM OF THE STUDY

Following the first clinical description in 1964 and the elucidation of the biochemical defect in 1993, a number of questions related to Smith-Lemli-Opitz syndrome remained to be answered. Among these were:

Is there a laboratory test to confirm the clinical diagnosis SLOS and are there any pitfalls ?

Is substitution of cholesterol or reduction of precursors more effective in the treatment of SLOS ?

Can we design new treatment stategies to improve neuro-motor development in SLOS patients ?

What is the gene responsible for SLOS and can we detect mutations in our patients ?

Could the broad clinical spectrum be explained by a genotype-phenotype correlation in SLOS and what is the real incidence of this disease and its carrier frequency in different parts of the world ?

Is it the deficient cholesterol or the excess of precursors in plasma, membrane and tissue that contribute to the pathophysiology of SLOS ?

Do we understand how an inborn error of metabolism can lead to a multiple malformation syndrome ?

What is the mechanism of a disturbed cholesterol biosynthesis leading to a human malformation syndrome ?

The aim of our study was to

- Introduce the reader in the Smith-Lemli-Opitz syndrome and 7-dehydrocholesterol reductase (DHCR7) deficiency and to share our current knowledge about diagnosis, clinical manifestations, molecular genetic aspects, therapy and pathophysiology in SLOS (Chapter 3).
- Investigate plasma of 8 patients, clinically diagnosed with SLOS and furthermore, describe pitfalls in measuring plasma cholesterol in SLOS with common oxidase methods used routinely in laboratories (Chapter 4).
- Explore new treatment strategies in SLOS. To describe and discuss sterol-exchange kinetics between plasma and erythrocyte-membranes, the effect of plasma exchange transfusions and longterm effect of HMG-CoA reductase inhibition with simvastatin in patients (Chapter 5 and 6). Explain the beneficial effect of simvastatin on plasma precursor / cholesterol ratio as observed during long-term therapy in SLOS. Understand this effect by studying the molecular effects of simvastatin on DHCR7 enzyme activity and DHCR7 mRNA levels in SLOS fibroblasts and finally correlate biochemical, molecular and clinical effect in SLOS patients (Chapter 7).
- Search for new and known mutations in the DHCR7-gene in 13 patients with SLOS and study possible genotype-phenotype correlation (Chapter 8).

Describe a patient with a “Smith-Lemli-Opitz-like” phenotype caused by a distal chromosome 7q36 deletion disrupting the human Sonic Hedgehog (SHH) gene and discuss remarkable finding in the perspective of SLOS pathogenesis (Chapter 9).

Finally, summarize and discus all findings and highlight some specific future perspectives in SLOS and cholesterol biosynthesis in general (Chapter 10).

Chapter 3

Smith-Lemli-Opitz syndrome and the *DHCR7* gene

A Review

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SUMMARY

Smith-Lemli-Opitz syndrome, a severe developmental disorder associated with multiple congenital anomalies, is caused by a defect of cholesterol biosynthesis. Low cholesterol and high concentrations of its direct precursor 7-dehydrocholesterol in plasma and tissues are the diagnostic biochemical hallmarks of the syndrome. The plasma sterol concentrations correlate with severity and disease outcome.

Mutations in the *DHCR7* gene lead to a deficient activity of 7-dehydrocholesterol reductase (DHCR7), the final enzyme of the cholesterol biosynthetic pathway. The human *DHCR7* gene is localised on chromosome 11q13 and its structure has been characterized. Ninety different mutations in the *DHCR7* gene to date have been published. This paper is a review on clinical, biochemical and molecular genetic aspects.

KEY WORDS: Smith-Lemli-Opitz Syndrome, 7-dehydrocholesterol reductase, mutations, cholesterol.

Smith-Lemli-Opitz syndrome (SLOS MIM 270400), a severe developmental disorder associated with multiple congenital anomalies, is caused by a defect of cholesterol biosynthesis, i.e. a deficiency of the enzyme 7-dehydrocholesterol reductase (DHCR7; EC 1.3.1.21), the final enzyme of the cholesterol biosynthetic pathway. Low cholesterol and high concentrations of its direct precursor 7-dehydrocholesterol (7DHC) and its isomer 8-dehydrocholesterol (8DHC) in blood and tissues are the biochemical hallmarks of the syndrome (Smith *et al*. 1964; Irons *et al*. 1993; Tint *et al*. 1995). The plasma sterol concentration generally correlates with severity and outcome (Tint *et al*. 1995; Witsch-Baumgartner *et al*. 2000). That a single metabolic (enzymatic) defect in humans could lead to a multiple malformation syndrome was new and unexpected. Cholesterol, an important constituent of the cell membrane of most eukaryotic cells, has important interaction with proteins, which control embryonic development. In addition, cholesterol acts as the precursor for steroid hormones, bile acids and myelin formation in the brain, spinal cord and peripheral nervous system (Figure 1).

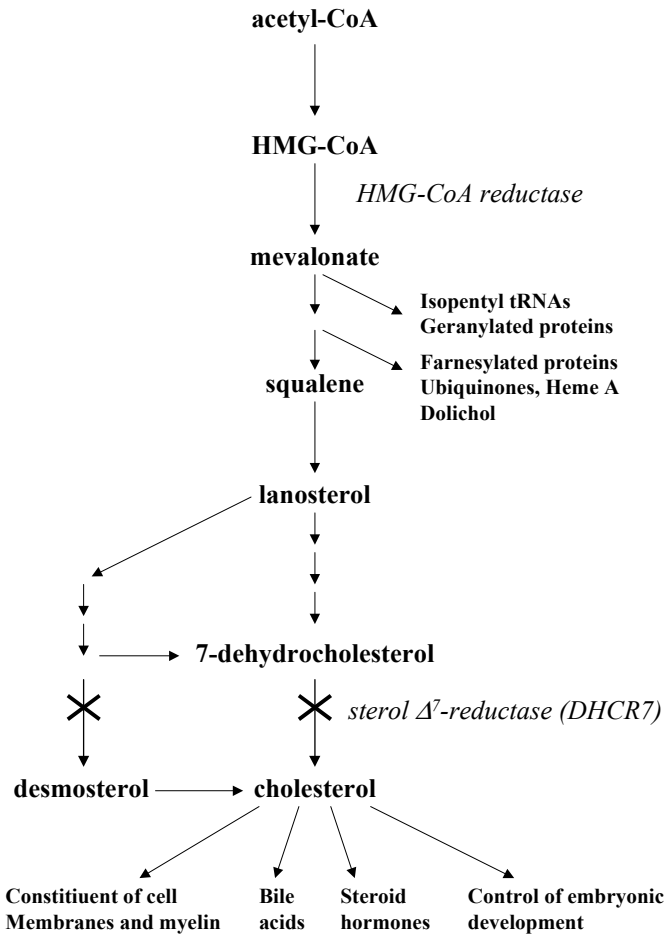

Figure 1. *Cholesterol biosynthetic pathway.*

Recently, several other reviewers have discussed various aspects of SLOS and other defects of cholesterol biosynthesis (Opitz 1999; Fitzky *et al*. 1999; Kelley & Hennekam 2000; Moebius *et al*. 2000; Waterham & Wanders 2000; Battaile & Steiner, 2000; Haas *et al.* 2001; Nwokoro *et al.* 2001; Kelley & Herman 2001). Here we discuss the clinical, biochemical and molecular genetic aspects of SLOS.

CLINICAL ASPECTS OF SLOS

Since the first description of SLOS as a clinical entity in 1964 (Smith *et al*. 1964) many papers have appeared describing a variety of common and less common clinical findings in patients with this multiple malformation/retardation syndrome (e.g. Opitz 1999; Kelley & Hennekam 2000; Cunnif *et al. 1*997; Ryan *et al*. 1998). The clinical spectrum includes different morphogenic abnormalities such as craniofacial, internal organ, limb/skeletal and urogenital anomalies besides (intrauterine) growth and mental retardation, failure to thrive and behavioral problems. The frequently occurring clinical symptoms apparent in 164 biochemically confirmed SLOS cases (Kelley & Hennekam 2000) are summarized in Table 1. While many of the signs on itself are not disease-specific for SLOS, the combination of several may point to this disorder. Indeed the occurrence of second/third toe syndactyly, polydactyly, microcephaly, ptosis, long philtrum, (congenital) cataract, photosensitivity, Hirschprungs' disease (colonic aganglionosis), pyloric stenosis, genital developmental anomalies (ranging from hypospadias to a complete sex reversal) in combination with neuro-developmental delay, should raise the clinical suspicion of SLOS (Figure 2). Through the years attempts have been made to distinguish a severe (lethal) type II form from the relatively mild type I form. The identification of the biochemical and molecular basis of SLOS however, has made clear that SLOS forms a clinical and biochemical continuum ranging from hardly recognizably mild to severe lethal forms (Kelley 1998; Kelley 2000; Waterham *et al*. 1998; Cunnif *et al*. 1997; Tint *et al*. 1995; Jira *et al.* 2001). The clinical description in the literature on SLOS probably is biased towards more severely affected and thus readily recognizable patients. Reviewing published SLOS families, the incidence of fetal death and spontaneous abortions is twice as high as in the general population suggesting the occurrence of severe cases leading to early death. With the introduction of selective screening and the availability of biochemical and genetic testing following the recent elucidation of the underlying cause of SLOS, mildly affected cases or even patients without clinical suspicion of SLOS can be readily diagnosed.

Table 1. *Clinical symptoms in SLOS.* Findings in 164 biochemically confirmed cases (Kelly & Hennekam 2000)

Finding	(%)
2/3 toe syndactyly	97
Mental retardation	95
Microcephaly	84
Postnatal growth retardation	82
Anteverted nares	78
Ptosis	70
Genital anomalies	65
Congenital heart defects	54
Polydactyly†	48
Cleft palate*)	47
Abnormal lung lobation	45
Renal anomalies	43
Structural brain anomalies	37
Cataract	22
Colonic aganglionosis	16
Pyloric stenosis	14

*) Includes cleft soft palate, submucous cleft, and cleft uvula.
† Includes postaxial polydactyly of hand(s)/foot.

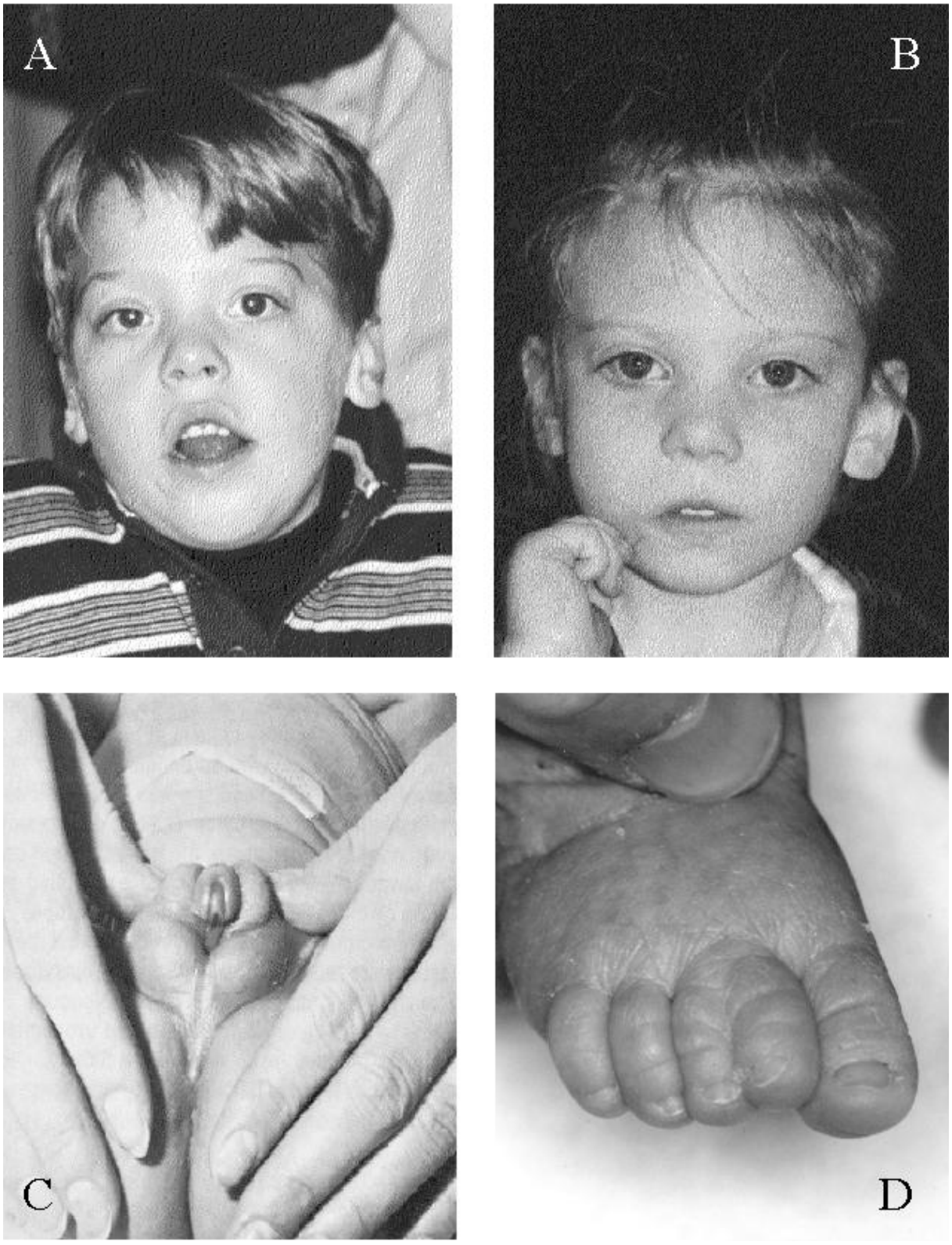

Figure 2. *Clinical characteristics in SLOS.* Facial dysmorphias including: mild ptosis, a long philtrum, anteverted nares in a 5 year old boy (A) and a 3 year old girl (B). Genital anomalies in boys (C) and 2/3 toe syndactyly (D) present in respectively 65 % and 97 % of SLOS cases.

Therapeutic trials using dietary supplementation of cholesterol with or without bile acids have shown that plasma cholesterol levels can be increased in some patients. Concentrations of the precursors 7DHC and 8DHC, however, were only marginally altered and clinical effects of the treatment so far have been rather disappointing (Irons *et al.* 1995; Ullrich *et al.* 1996; Elias *et al.* 1997). An alternative therapeutic strategy has been developed, treating 5 SLOS patients with Simvastatin (an oral HMG-CoA reductase inhibitor) for a median period of 2 years. The overall biochemical effect was impressive with a decrease of 7DHC+8DHC and increase of cholesterol in plasma to respectively 28 % and 162 % of the initial concentration with promissing clinical improvement (Jira *et al*. 1997; Jira *et al*. 2000; and unpublished data). This therapeutic statin approach is currently being tested in a multicenter European Trial.

BIOCHEMICAL ASPECTS

SLOS is caused by a deficiency of the enzyme 7-dehydrocholesterol reductase (DHCR7; EC 1.3.1.21), the final enzyme of the cholesterol biosynthetic pathway. Molecular cloning of the cDNA showed that the human enzyme is a protein with a calculated molecular weight of 54.5 kDa and nine putative/predicted transmembrane segments. It is microsomal membrane-bound and in humans the mRNA is expressed ubiquitously with the highest expression in adrenal gland, liver, testis, and brain (Moebius *et al*. 1998).

Cholesterol is a main endproduct derived from the isoprenoid biosynthetic pathway (Goldstein and Brown 1990). This pathway supplies cells with a variety of compounds, collectively called isoprenoids, which function in diverse cellular processes. Among these are *ubiquinone-10* and *heme A*, involved in electron transport, *dolichol*, mediator of protein glycosylation, *isopentyl tRNAs*, and *farnesyl and geranyl groups* for prenylation of proteins that are involved in cell signalling and differentiation (Goldstein & Brown 1990). Acetyl-CoA (C2) is converted into the isoprene unit: isopentenyl-PP (C5) by six different serial enzyme reactions. After adding C5 to geranyl-PP (C10) through farnesyl-PP (C15), squalene (C30) is transformed to lanosterol (4,4,14-trimethylcholesta-8(9), 24-dien-3-ol) by cyclization. A series of enzyme reactions is required to eventually produce cholesterol (Figure 1). Not only humans but the majority of organisms including animals, plants, fungi and micro-organisms are equipped with this important *de novo* (chole)sterol biosynthetic pathway, supplying sterols and steroids to cells, membranes and tissues. Cholesterol has a pivotal role during embryogenesis where it functions as a transporter-molecule for the Sonic hedgehog (Shh) signalling protein, which is essential for normal morphogenesis. Without cholesterol, Shh-transport and/or function is impaired (Hall *et al.* 1995; Porter *et al*. 1996; Hall *et al.* 1997; Lanoue *et al*. 1997; Cooper *et al*. 1998). The sterol derangement in SLOS (accumulation of 7DHC/8DHC and shortage of available cholesterol) undoubtedly influences the proper activation of the Shh-receptor. These findings

may explain the phenotypic consequences of the DHCR7 deficiency as observed in this syndrome: microcephaly, a distinctive facies, cataract, syn/polydactyly, and a variety of organ malformations including genital abnormalities ranging from intersex to complete sex reversal in boys. Although the mechanism of Shh-induced signaling pathway in vertebrates is not completely defined, it is known to regulate dorso-ventral patterning within the neural tube, limb, lung, genital, ocular, and retinal development as well as craniofacial morphogenesis (Krishnan *et al.* 1997; Hayes *et al.* 1998; Imokawa *et al.* 1997; Levine *et al*. 1997; Helms *et al.* 1997; Marigo *et al.* 1995; Hall *et al.* 1995; Kumar *et al*. 1996). In a recent *null mutation* mouse model without DHCR7 activity, accumulated 7-dehydrocholesterol was found to suppres sterol biosynthesis posttranslationally in line with earlier observations in skin fibroblasts from SLOS patients (Shefer *et al.* 1997). This effect might exacerbate abnormal development in SLOS by increasing the fetal cholesterol deficiency (Fitzky *et al.* 2001).
The most predictive biochemical value in SLOS is the 7DHC/cholesterol ratio in plasma (Tint *et al*. 1995; Witsch-Baumgartner *et al*. 2000). In general, patients with plasma 7DHC/cholesterol ratio between 0.5 – 1.0 have moderate SLOS. Patients with a plasma ratio <0.5 have a mild presentation and course while plasma 7DHC/cholesterol ratio >1.0 is associated with severe SLOS (Krakowiak.*et al.* 2000; Jira *et al*. 2001). This biochemical ratio could be a usefull tool for prognosis and treatment in SLOS but cannot predict severity accurately.

To date, seven distinct inherited disorders have been linked to different defects in cholesterol biosynthesis. Two disorders are known to result from an enzyme defect in the pre-squalene segment of the pathway: the classical form of mevalonic aciduria (MIM 251170: Hoffmann *et al*. 1986; Houten *et al.* 2000; Haas *et al.* 2001) and the hyperimmunoglobulinemia D and periodic fever syndrome also known as Dutch-type periodic fever (MIM 260920: Houten *et al*. 1999; Drenth *et al*. 1999; Houten *et al*. 2000). Of the remaining five disorders, all due to different enzyme defects in the post-squalene segment of the pathway, four have been resolved at the molecular level recently by the demonstration of disease-causing mutations in the encoding genes. These include SLOS and the two X-linked dominant inherited and male-lethal disorders Conradi-Hünermann-Happle syndrome (CDPX2; MIM 302960: Happle 1979 and CHILD syndrome; MIM 308050: Happle *et al*. 1980) caused by deficiencies of sterol Δ^{8-7}-isomerase (*EBP* gene at Xp11.22-23) or a sterol C-4 demethylase (*NSDHL* gene at Xq28) respectively. In patients with Desmosterolosis, a rare autosomal recessive disorder, characterized by multiple congenital anomalies, elevated levels of the cholesterol precursor desmosterol, in plasma, tissue, and cultured cells suggested a deficiency of the enzyme 3beta-hydroxysterol Δ^{24}-reductase (MIM 602398: FitzPatrick *et al*. 1998). Four mutations in two patients, only recently, gave the molecular confirmation of this defect (*DHCR24* gene at 1p31.1-p33: Waterham *et al*. 2001). The last, extremely rare and probably autosomal recessively inherited disorder of sterol synthesis, namely Greenberg skeletal dysplasia (MIM 215140: Greenberg *et al*. 1988), presumably due to a deficiency of sterol Δ^{14}-reductase, is detected by elevated levels of specific sterol

intermediates in conjunction with decreased levels of cholesterol in tissues and cells of affected patients. Confirmation at the molecular level of this disorder awaits the identification of the corresponding gene and/or molecular analysis in affected patients.

MOLECULAR GENETICS OF THE *DHCR7* GENE

Organisation and conservation

In 1998, the human 7-dehydrocholesterol reductase gene (DHCR7 Gen Bank accession number: AF034544) was identified and assigned to chromosome region 11q13 (Fitzky *et al*. 1998: Wassif *et al*. 1998: Waterham *et al*. 1998). Ninety different mutations in the DHCR7 gene of patients with Smith-Lemli-Opitz syndrome have been described to date (Fitzky *et al*. 1998; Wassif *et al*. 1998; Waterham *et al*. 1998; Yu *et al*. 2000; De Brasi *et al*. 1999; Witsch-Baumgartner *et al*. 2000; Patrone *et al*. 2000; Waterham & Wanders 2000; Krakowiak *et al*. 2000; Jira *et al*. 2001; Witsch-Baumgertner *et al*. 2001; Evans *et al*. 2001; Prasad *et al*. 2002). The cDNA has an open reading frame of 1,425 base-pairs coding for a polypeptide of 475 amino acids (Figure 3). Alternative splicing has not been described. Structurally, the protein is strongly related to plant and yeast sterol reductases (Rahier *et al*. 1996: Waterham *et al*. 1998). The percentage of identity of the human *DHCR7* with the *A. thaliana* sterol Δ7-reductase and the *S. cerevisiae* sterol Δ14-reductase is 38 and 34 respectively (Waterham *et al*. 1998).

Frequency and ethnic aspects

SLOS is the most frequently occurring defect of cholesterol biosynthesis known to date. Estimated incidences of SLOS have historically been based on clinical diagnosis and initially ranged from 1:40,000-50,000 births (Opitz *et al.* 1994; Cunniff *et al.* 1997). Now that biochemical and genetic testing for this trait has become available the incidence turns out to be significantly lower due to formerly incorrect inclusions of cases with SLOS-like phenotypes. Based on biochemically confirmed cases an incidence of approximately 1:60,000 births has now been reported in the UK and the USA (Ryan *et al.* 1998; Kelley & Hennekam 2000). From the number of biochemically and genetically confirmed Dutch SLOS cases we have estimated an incidence of approximately 1:80,000 births in The Netherlands (Jira *et al*. 2001; Waterham & Wanders 2000). On the other hand, an incidence of 1: 20,000-26,500 of biochemically confirmed SLOS cases has been reported for Slovakia and Canada, respectively (Bzduch *et al.* 2000; Nowaczyk *et al.* 2001).

The reason for the rather high incidence of SLOS is unclear but may be a consequence of both founder effects and heterozygote advantage. A founder effect has been suggested to explain the fact that SLOS is most common in Caucasian populations from east-European descent. As possible advantage for heterozygotes a relative protection from atherosclerosis due to lowered blood

cholesterol levels as well as a lower risk for children to acquire rickets due to increased vitamin D production from elevated 7-DHC levels has been suggested (Kelley 1998; Kelley & Hennekam 2000).

Pathogenic mutations and polymorphisms

We performed a survey of the literature on mutations described in patients diagnosed with SLOS. Molecular data are presented in Table 2. The position of the mutations related to the position of the transmembrane segments and loops of the DHCR7 protein is illustrated in Figure 3.

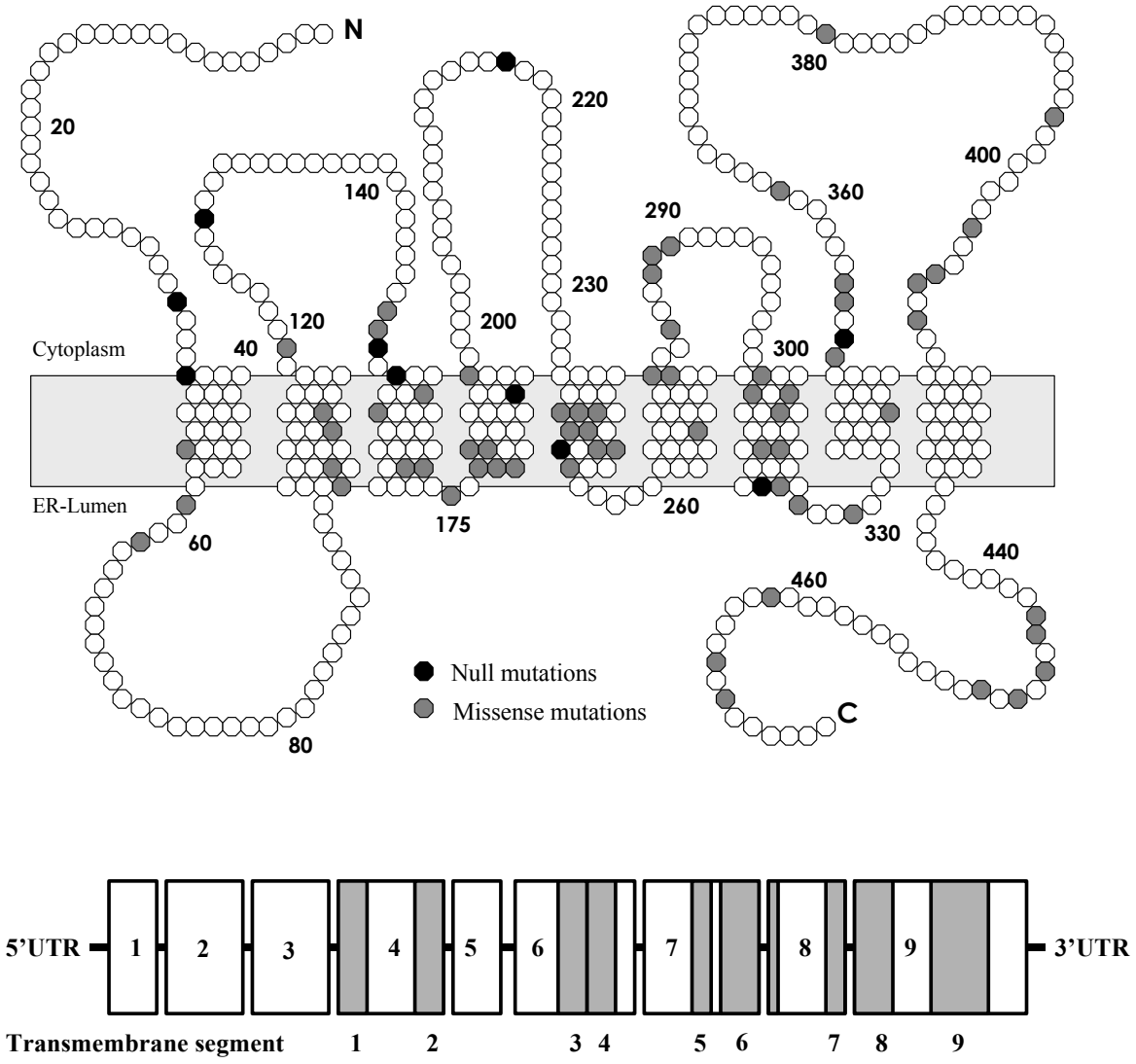

Figure 3. *Predicted membrane topology (upper part) and intron-exon organisation (lower part) of the 7-dehydrocholesterol reductase, based on the data of Fitzky et al.1998.* Transmembrane segments 4 to 8 represent the, highly conserved, sterol-sensing domain. The coding sequences of the transmembrane segments are given as grey areas in the lower part of the figure. Position of null mutations (black) and missense mutations (grey) are indicated.

Table 2 summarizes mutations described in SLOS patients by: nucleotide change, effect on coding sequence (for numbering of nucleotides and amino acids see: Waterham *et al*. 1998), affected exon, localisation in DHCR7 protein, incidence in study cohorts, their references and overall incidence.
DNA sequencing of almost 400 SLO-alleles identified 90 different mutations. The *DHCR7* gene mutations identified in SLOS patients were shown to have deleterious effects on the function of the DHCR7 protein (Fitzky *et al*. 1998; Wassif *et al*. 1998; Waterham *et al*. 1998).

Apart from the majority of missense mutations, four nonsense mutations (W37X, Q149X, W151X, Y217X), eight frameshift mutations leading to a premature stop; four deletions: IVS3-1-195 del, 384-IVS5+4del, and 720-735del, and 1057Gdel), two splice site mutations (IVS8-1G>C and IVS8-1G>T), and two insertions (682insC; and 762insT) have been reported. Recently a 3 bp deletion has been detected resulting in the in-frame removal of a single histidine residue from position 356 of the DHCR7 protein. This deletion of one codon does not lead to a premature truncation of the protein and some residual activity may be expected although expression has not been determined.
The seven most frequent mutations described to date are: IVS8-1G>C, T93M, R404C, W151X, V326L, R352W and E448K with a frequency of: 31.5, 11.2, 10.7, 6.4, 6.3, 3.2 and 3.2 percent, respectively, representing two third of the mutations in analysed alleles. Eighty mutations detected in SLOS alleles are single amino acid substitutions.

Two splice site mutations have been described at the same position (The frequent IVS8-1 G>C mutation and a rare variant, namely an IVS8-1 G>T ; Jira *et al.* 2001). The G>C mutation causes aberrant splicing producing a mRNA with 134 base-pairs of retained intron 8 sequence at nucleotide position 963, which upon translation will lead to a frame shift and a stopcodon at nucleotide 1235 (TGA) predicted to produce an inactive, truncated protein lacking 154 amino acids of its original C-terminal sequence (Waterham *et al*. 1998; Waterham *et al*. 2000). This was confirmed by enzyme activity measurements in patients homozygous for this mutation (Moebius *et al*.1998; Waterham *et al.*1998; Witsch-Baumgartner *et al.* 2000; Waterham & Wanders 2000; Yu *et al*. 2000; Bataille *et al*. 1999) which revealed no activity. Severely affected patients with extremely short life span have been identified who were homozygous for this mutation (Waterham *et al.* 1998; Witsch-Baumgartner *et al*. 2000) with plasma cholesterol levels as low as 20 μmol/l/ (Jira *et al*. 2001). The number of homozygotes for this mutation, however, is lower than expected on the basis of the Hardy Weinberg equation. Hence, homozygosity for this *null* allele have been predicted to lead to spontaneous termination of pregnancy in many cases and consequently to an underestimation of the true incidence of SLOS (Kelley & Hennekam 2000; Waterham & Wanders 2000).
So far fifteen polymorphic silent mutations are found in the *DHCR7* gene (Table 2).

Table 2. *90 Mutations in the DHCR7 gene causing SLOS (A) and 15 polymorphic mutations found in SLOS patients and controls (B).*

A

Nucleotide Change	Effect on coding sequence	Exon	Localisation in DHCR7	Percentage of published mutations	Reference	Overall Incidence
3G>A	M1	3	N-terminus	2/64	2	
98-194del	W33-S65del	3	-	1/6	5	
99G>A	W37X	4	-	1/60	3	
151C>T	P51S	4	MAH1	2/168, 2/60	1,3	
176G>T	M59R	4	MAH1 (loop1-2)	1/64	2	
185A>T	D62V	4	Loop1-2	1/64	2	
278C>T	T93M	4	Loop1-2	14/168, 6/64, 7/60, 7/18, 4/30	1,2,3,4,7	11.2 %
296T>C	L99P	4	MAH2 (loop1-2)	2/168, 1/60	1,3	
321G>C	Q107H	4	MAH2	1/68, 1/30	1,7	
326T>C	L109P	5	MAH2	1/168, 1/64, 1/30	1,2,7	
356A>T	H119L	5	MAH2	1/64	2	
384-IVS5+4del	Frameshift		-	1/18	4	
385-IVS5+5del	Frameshift		-	1/168	1	
440G>A	G147D	6	Loop2-3	1/168, 1/30	1,7	
443T>G	L148R	6	Loop2-3	1/60	3	
445C>T	Q149X	6	-	1/64	2	
452G>A	W151X	6	-	14/168, 2/64, 1/18, 1/30	1,2,4,7	6.4 %
461C>T	T154M	6	MAH3	3/168, 2/64, 1/30	1,2,7	
470T>C	L157P	6	MAH3	1/168	1	
502T>A	F168I	6	MAH3	1/60	3	
506C>T	S169L	6	MAH3	4/168, 1/64, 1/60	1,2,3	
523G>C	D175H	6	Loop3-4	1/60	3	
529T>C	W177R	6	MAH4	1/30	7	
533T>A	I178N	6	MAH4		8	
536C>T	P179L	6	MAH4	1/60	3	
545G>T	W182L	6	MAH4	1/64	2	
546G>C	W182C	6	MAH4	1/168	1	
548G>A	C183Y	6	MAH4	1/64	2	

592A>G	K198E	6	MAH4	1/64	2	
651C>A	Y217X	7	-	1/64	2	
682Cins	Frameshift	7	-	1/6	5	
720-735del	Frameshift	7	-	1/168	1	
724C>T	R242C	7	MAH5	3/168, 1/30	1,7	
725G>A	R242H	7	MAH5	1/64, 1/30	2,7	
728C>G	P243R	7	MAH5	2/60	3	
730G>A	G244R	7	MAH5	2/64	2	
740C>T	A247V	7	MAH5	1/168, 1/30	1,7	
744G>T	W248C	7	MAH5	1/64	2	
753C>G	I251M	7	MAH5	1/64	2	
755A>G	N252S	7	MAH5	2/64	2	
762Tins	Frameshift	7	-	1/6	5	
765C>A	F255L	7	MAH5	1/64	2	
808A>G	M270V	7	MAH6	1/64	2	
839A>G	Y280C	8	MAH6	1/2	10	
841G>A	V281A	8	MAH6	1/168	1	
852C>A	F284L	8	MAH6	2/64, 1/60	1,3	
861C>A	N287K	8	Loop6-7	3/60	3	
862G>A	E288K	8	Loop6-7		8	
866C>T	T289I	8	Loop6-7	2/168, 2/30	1,7	
906C>G	F302L	8	Loop6-7	1/64, 1/60	1,3	
920G>A	G307D	8	MAH7		8	
925G>A	G309S	8	MAH7		8	
931T>G	C311G	8	MAH7		1	
932G>A	C311Y	8	MAH7		1	
952T>C	Y318N	8	MAH7	1/30	7	
956C>T	T319M	8	MAH7	1/64	2	
957G>A	T319A	8	MAH7		8	
IVS8-1G>C	Frameshift		-	49/168,19/64,20/60, 1/6,1/2,11/30,5/8,1/2	1,2,3,5,6,7,9,10	31.5%
IVS8-1G>T	Frameshift		-	1/64	2	
970T>C	Y324H	9	MAH7	3/168, 1/60	1,3	
976G>T	V326L	9	MAH7	12/168, 1/64, 5/60, 1/8	1,2,3,9	6.3%
986C>T	P329L	9	Loop7-8	1/ 2	6	

1022T>C	L341P	9	MAH8	1/30	7	
1054C>T	R352W	9	Loop8-9	5/168, 1/64, 2/60, 2/18	1,2,3,4	3.2 %
1055G>A	R352Q	9	Loop8-9	3/168	3	
1057Gdel	Frameshift	9	-	1/64	2	
1058T>C	V353A	9	Loop8-9	1/168	1	
1063A>G	N355D	9	Loop8-9	1/64	2	
1068-1070del	356delH	9	Loop8-9	1/8	9	
1084C>T	R362C	9	Loop8-9	1/168	1	
1138T>A	C380S	9	Loop8-9	1/168	1	
1138T>C	C380R	9	Loop8-9	1/168	1	
1139G>A	C380Y	9	Loop8-9	2/168, 1/64	1,2	
1190C>T	S397L	9	Loop8-9	1/168	1	
1210C>T	R404C	9	Loop8-9	18/168	1	10.7 %
1210C>A	R404S	9	Loop8-9	2/168, 1/60	1,2	
1219A>T	N407Y	9	Loop8-9	1/18	4	
1222T>C	Y408H	9	Loop8-9	1/168, 1/30	1,7	
1228G>A	G410S	9	Loop8-9	2/168, 1/60	1,3	
1228G>C	G410R	9	Loop8-9	1/168	1	
1327C>T	R443C	9	C-terminus	1/168, 1/64	1,2	
1328G>A	R443H	9	C-terminus		8	
1331G>A	C444Y	9	C-terminus	1/30	7	
1337G>A	R446Q	9	C-terminus	2/168	1	
1342G>C	E448Q	9	C-terminus	1/168	1	
1342G>A	E448K	9	C-terminus	3/168,2/64,2/60, 2/18, 1/30,1/8	1,2,3,4,7	2.9 %
1349G>T	R450L	9	C-terminus	1/168	1	
1384T>C	Y462H	9	C-terminus	1/60	3	
1400C>T	P467L	9	C-terminus		8	
1406G>C	R469P	9	C-terminus	2/60	3	

*) MAH=Membrane associated helix (see figure 3). Total of 358 SLOS-alleles.

1) Fitzky *et al*. 1998 ; Witsch-Baumgartner *et al*. 2000. 2) Waterham *et al*. 1998; de Die-Smulders *et al*. 1999; Waterham & Wanders 2000; Jira *et al*. 2001. 3) Yu *et al*. 2000. 4) De Brasi *et al*. 1999. 5) Wassif *et al*. 1998. 6) Patrono *et al*. 2000. 7) Krakowiak *et al*. 2000. 8) Witsch-Baumgartner *et al*. 2001 (this study is not included in the calculation of the overall incidence of mutations). 9) Evans *et al*. 2001. 10) Prasad *et al*. 2002.

B

15 Polymorphisms (silent mutations)

Nucleotide Change	Effect on coding sequence	Exon	Localisation in DHCR7	Percentage of published mutations	Reference	Overall Incidence
-223T>C	Non coding	1	Polymorphism		1	
-23T>C	Non coding	2	Polymorphism		8	
139C>T	L47	4	Polymorphism		8	
189A>G	Q63Q	4	Polymorphism		1,2,3,4	
207C>T	T69T	4	Polymorphism		2,3	
231C>T	T77T	4	Polymorphism		1,2,3,4	
285A>G	K95K	4	Polymorphism		1	
IVS4-60T>G	Non coding	-	Polymorphism		8	
438C>T	N146N	6	Polymorphism		1,2,3	
969G>T	L323L	9	Polymorphism		1	
1020C>T	V340V	9	Polymorphism		9	
1158C>T	D386D	9	Polymorphism		1,2,3	
1272T>C	G424G	9	Polymorphism		1,2,3,4	
1341C>T	D447D	9	Polymorphism		2	
1350C>G	R450R	9	Polymorphism		1	

Localization of mutations in the protein

Mutations occur further throughout the whole gene/protein without evident hotspots. A substantial number of mutations, however, are found in the so called "sterol-sensing domain" encompassing five transmembrane segments 4 – 8 as proposed by Fitzky *et al*. 1999 and Bae *et al*. 1999 (Figure 3). The specific DHCR7 membrane-spanning segments show strong homology to segments found in five other human proteins. These proteins in nature all have crucial interaction with sterols: 1] HMG-CoA reductase (Olender *et al*. 1992), 2] Niemann-Pick C1 gene product (Loftus *et al*. 1997), 3] Sterol regulatory element-binding protein-SCAP (Nohturfft *et al*. 1998), 4] the morphogene receptor PATCHED (Loftus *et al*. 1997) and 5] the DISPATCHED protein (Burke *et al*. 1999). According to the hydrophobicity in the DHCR7 protein, membrane-spanning segments 4 to 8 are closely spaced and connected by relatively short hydrophilic amino acid loops. In contrast to DHCR7, SCAP and HMG-CoA reductase have a long COOH-terminal domain that projects into the cytosol (Nohturfft *et al*. 1998).

The majority of SLOS patients are compound heterozygous for two different mutations in *DHCR7*. Sibs with the same *DHCR7*-mutations or genotypically identical patients, reported by different groups, may display similar or rather different plasma sterol values and/or different phenotypes (Krakowiak *et al*. 2000; Jira *et al*. 2001). The mutational site and effect on the coding sequence seemed to be only partially predictive for clinical and biochemical severity.

Expression studies and Genotype-Phenotype

Enzymatic activities of DHCR7 can be determined by assaying the conversion of either isotope-labeled 7DHC or its precursors such acetate or lathosterol (cholesta-7-en-3β-ol) to cholesterol using thin layer chromatography or HPLC (Lund *et al*. 1996; Shefer *et al*. 1997; Necklason *et al*. 1999). An alternative is the use of non-radiolabeled ergosterol (ergo-5,7,22-trien-3β-ol) as substrate for DHCR7. Incubation of microsomal preparations or cell homogenates from rat and human with ergosterol results in the reduction of the Δ7 double bond which produces brassicasterol (ergo-sta-5,22-dien-3β-ol). The ergosterol conversion followed by GC-MS detection of produced sterols, although less sensitive than the isotope-based assays, avoids the problems caused by the instability and availability of the radiolabeled precursors (Honda *et al*. 1996; Shefer *et al*. 1998). Functional analysis, by expression of DHCR7 mutations in mammalian cells, demonstrated that all but one of the missense (R450L; C-terminus) mutations result in unstable protein (Waterham *et al*. 1998; Witsch-Baumgartner *et al*. 2000; Jira *et al*. 2001). Residual enzyme activity may explain some of the phenotypic variability seen in SLOS. Enzyme kinetic studies support this theory (Neklason *et al*. 1999).

Mutations that alter, interfere with or truncate the sterol-sensing domain of the DHCR7 protein are likely to cause a more severe clinical and biochemical SLOS-phenotype. Making predictions of the SLOS phenotype from genotype, however, continues to be difficult since there is

significant clinical and biochemical variability among genetically identical infants. A higher incidence of severe null mutations (IVS8-1C>G insertion and W151X) in a population could explain a lower incidence of SLOS occurring. The 50 % incidence of the IVS8-1G>C mutations observed in our European SLOS-study (Jira *et al*. 2001) exceeds the findings of others who identified the IVS8-1G>C mutation in 21 of 66 (32%) and 18 of 52 (35%) SLOS-alleles, respectively (Yu *et al*. 2000; Bataille *et al*. 2000). The development of a simple PCR-RFLP can be used as a screening-method for detecting this frequent SLO-mutation. Data on population screening for the IVS8-1G>C mutation indicated the high carrier frequency of about 1 in 100 and 1 in 30 for U.S. Caucasians and European Caucasians, respectively (Yu *et al*. 2000, Nowaczyk *et al.* 2001). A study of DHCR7 mutations from three European areas (Poland, Germany/Austria and Great Britain) revealed extreme frequency gradients for this mutation. The frequency gradient of the W151X mutation is in a direction opposite to the gradient of the IVS8-1G>C mutation (Witsch-Baumgartner *et al.* 2001).

In conclusion, SLOS is a multiple malformation/retardation syndrome with a clinical and biochemical spectrum ranging from a lethal to a difficult to diagnose very mild presentation. Among the 90 different mutations observed and reviewed, the majority are missense mutations (80). There is a clustering in three domains of the DHCR7 protein; 1] in the transmembrane domain, 2] in the fourth cytoplasmatic loop, 3] and at the C–terminus.

Twelve *null* mutations (4 nonsense, 4 deletions , 2 splice site mutation and 2 single nucleotide insertions) lead to absent enzyme activity. As far as studied, missense mutations, on the other hand, have demonstrated only decreased protein stability in expression studies.

Severe clinical phenotype in SLOS patients is due to *null* allele and fourth loop mutations whereas C-terminal and transmembrane mutations cause a mild to moderate clinical phenotype. Further molecular genetic studies will enable insight in carrier frequency of specific *DHCR7* mutations in various populations. Additional clues for prenatal, maternal, environmental, other genetic and compensatory biochemical determinants that can modify the phenotypical consequences of the functional DHCR7 deficiency in SLOS is needed.

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Chapter 4

Pitfalls in measuring plasma cholesterol in the Smith–Lemli–Opitz syndrome

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ABSTRACT

Correct quantitative results for plasma cholesterol, 7-dehydrocholesterol (7-DHC), and 8-dehydrocholesterol (8-DHC) are invaluable for making the correct diagnosis in patients with the Smith–Lemli–Opitz syndrome (SLO) and for biochemical monitoring of these patients during therapy. The enzymatic method for cholesterol measurement based on cholesterol oxidase gives falsely high values for plasma cholesterol in samples from patients with SLO. Both 7-DHC and 8-DHC contribute substantially to the test result, given that they are accepted substrates of cholesterol oxidase. All cholesterol methods making use of this enzyme are expected to give unreliable results with plasma samples from SLO patients. Cholesterol values found with these methods may be low-normal in individual cases with SLO. Therefore, other techniques for measuring cholesterol, 7-DHC, and 8-DHC, e.g., gas chromatography, should be used for diagnosing these patients and for follow-up during therapy. However, a normal value for plasma cholesterol, as obtained by gas chromatography, does not exclude SLO. The diagnosis should always be confirmed or excluded by testing for the presence of high concentrations of 7-DHC and 8-DHC in plasma. We found that one patient with a severe form of the disease had a plasma cholesterol concentration of 20 μmol/L, to our knowledge, the lowest value ever recorded in a human being.

KEY WORDS: cholesterol oxidase, inborn errors of metabolism, gas chromatography, dehydrocholesterol, enzymatic assays, analytical error

INTRODUCTION

In 1964, Smith et al. (Smith *et al.* 1964) described a syndrome, now generally referred to as the Smith–Lemli–Opitz syndrome (SLO), characterized by a number of birth defects affecting nearly every organ system.1 The patients are mentally retarded, have a growth disorder, and show failure to thrive. Both dysmorphic facial signs (microcephaly, palatoschizis, cataracts, ptosis, micrognathia) and limb abnormalities (syndactyly of the second and third toe, polydactyly) occur in the patients. Genital disorders, hypospadias, and cryptorchidism have been described.

The syndrome is estimated to be among the most common autosomal recessive disorders among Caucasians, its prevalence being ~1:20 000 births (Opitz 1994). A possible abnormality of steroid secretion was first postulated by Chesalow et al. (Chesalow *et al.* 1985). Since then, Irons et al. have shown that SLO is caused by a defect in the cholesterol biosynthesis pathway (Irons *et al.* 1993; Tint *et al.* 1994). Patients have decreased concentrations of cholesterol in plasma and increased concentrations of the precursor 7-dehydrocholesterol (cholesta-5,7-dien-3ß-ol; 7-DHC) and its isomer 8-dehydrocholesterol (cholesta-5,8-dien-3ß-ol; 8-DHC) in plasma, erythrocytes, cultured skin fibroblasts, amniotic fluid, and various tissues (Irons *et al.* 1993; Tint *et al.* 1994; McGaugran *et al* 1994; Johnson *et al.* 1994; Batta *et al.* 1995), findings that suggest a block in reduction of the C-7 double bond (Irons *et al.* 1993; Tint *et al.* 1994). Recently, those researchers confirmed that the enzyme defect involved the 7-dehydrocholesterol-7-reductase in liver microsomes (Shefer *et al.* 1995).

The aim of our study was to evaluate the reliability of the standard enzymatic assay (based on cholesterol oxidase) for measuring plasma cholesterol in samples from SLO patients. Because the technique for measuring 7-DHC in plasma is not widely available, clinicians may wish to use the presence of low concentrations of plasma cholesterol in patients with SLO as a first step towards confirming the diagnosis. Furthermore, measurement of plasma cholesterol will play a role in the follow-up of therapy strategies with high-cholesterol diets. We also compared the plasma cholesterol results obtained with the cholesterol oxidase method with those of a gas-chromatographic (GC) technique.

MATERIALS AND METHODS

Patients

We analyzed plasma from eight patients (ages 2 weeks to 33 years) who had the characteristic clinical signs and symptoms of SLO. Forty-eight plasma samples, included as a diseased control group, had been sent to our laboratory for metabolic screening; however, the patients in this control group did not show clinical signs and symptoms characteristic for SLO, and some were under special clinical

conditions (e.g., feeding problems, metabolic crises, special dietary formulas). This group provides the background against which metabolic screening laboratories have to diagnose patients with SLO. Because this study was not devised to obtain reference ranges for cholesterol in various age groups, the data presented later (in Table 1) should not be considered as such.

Enzymatic assays

Plasma cholesterol was measured at 30 °C with an enzymatic test on a Hitachi 747 analyzer (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer and with use of Boehringer reagents (CHOD-PAP test; SYS-3:1127578/1489704). In this test, cholesterol and cholesterol esters are converted by the sequential action of cholesterol esterase and cholesterol oxidase. The H2O2 formed in the latter reaction is determined quantitatively in the last step by using a peroxidase that converts phenol and 4-aminophenazone into 4-(p-benzoquinone-monoimino)-phenazone.

GC assays

For GC determination of cholesterol and its precursors (7-DHC and 8-DHC), we combined 60 μL of plasma with 7.58 nmol of 5ß-cholestane-3-ol as internal standard (no. C5050; Steraloids, Wilton, NH) in 1 mL of a solution of 0.32 mol/L KOH in 95% ethanol. The CFAS-calibrator for cholesterol was used (no. 759350; Boehringer Mannheim) in combination with two control sera (Precinorm and Precipath; also from Boehringer Mannheim). After an incubation of 15 min at 55 °C, 1 mL of H2O and 4 mL of pentane were added and mixed for 5 min. The steroids, which were extracted into the pentane layer, were taken up by pipetting after centrifugation (5000g • min). The pentane was then evaporated with nitrogen at ~50 °C. To derivatize the steroids, we added 100 μL of an equivolume solution of N,O-bis(trimethylsilyl)trifluoroacetamide (no. 15238; Fluka, Buchs, Switzerland) and pyridine and incubated at 60 °C for 30 min. GC analysis was performed with a Hewlett-Packard (Amstelveen, The Netherlands) Model 5890 GC and a 25 m x 0.25 mm (i.d.) CP-Sil-19 CB column (film thickness 0.2 μm; Chrompack, Bergen op Zoom, The Netherlands). The temperature program was started at 240 °C, increased to 300 °C at 5 °C/min, and held for 3 min at 300 °C. Temperatures of the injector and detector were 280 and 300 °C, respectively. Pure 7-DHC for calibration purposes was purchased from Sigma Chemical Co., St. Louis, MO; no. D-3625); to quantify 8-DHC, we used the calibration curve for 7-DHC because 8-DHC for calibration was not available commercially.

Table 1. Sterol concentrations (μmol/L[a]) in plasma from patients with Smith-Lemli-Opitz syndrome and from disease controls.

SLO patient	Sex, age	Enzymatic cholesterol	Gas chromatography				
			Cholesterol	7-DHC	8-DHC	Total[b]	Ratio[c]
1	F, 11 d	700	20	430	266	716	34.8
2	M, 7 m	1000	260	300	385	945	2.6
3	M, 7 y	1820	975	280	264	1519	0.6
4	M, 26 y	1560	1118	182	127	1427	0.3
5	M, 27 y	1300	884	143	107	1134	0.3
6	M, 28 y	1700	1170	220	284	1674	0.4
7	F, 29 y	2080	1261	442	308	2011	0.6
8	F, 33 y	3800	2030	848	555	3433	0.7
Range, disease control group	<1 y (12)[d]	2400-4900	2523-4805	<7	<5		<0.004
	1-5 y (12)	2100-5300	2139-5447	<5	<5		<0.003
	6-15 y (10)	3200-5700	3138-5775	<5	<5		<0.002
	>15 y (14)	2900-8600	3174-8226	<8	<10		<0.003
Reference intervals for healthy children[e]	1-3 y	1150-4700					
	4-6 y	2800-4800					
	7-9 y	2900-6400					

[a] To convert cholesterol from μmol/L to mg/dL: 1000 μmol/L = 38.67 mg/dL.

[b] Cholesterol (GC) + 7-DHC (GC) + 8-DHC (GC).

[c] [7-DHC (GC) + 8-DHC (GC)]/cholesterol (GC).

[d] n for each group is listed in parentheses.

[e] 0.025-0.975 fractiles, according to Lockitch *et al.* 1988

GC-MS

Mass spectra from peaks separated by GC were obtained by using a VG Trio 2 quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, UK) coupled to the Hewlett-Packard 5890 GC. The cholesterol peak was characterized by fragments at m/z 329, 353, and 458. 7-DHC and 8-DHC gave identical spectra, with characteristic fragments at m/z 351 and 456.

Recovery studies

To test whether 7-DHC contributes to the result of the enzymatic cholesterol assay, we performed recovery experiments for cholesterol and 7-DHC in a human serum matrix and in human serum albumin solution (Sigma, no. A 16535; 50 g/L in 0.15 mol/L NaCl). The sterols, which had been

dissolved to 33 mmol/L solution in ethanol, were added to both matrices to yield final concentrations of 1, 2, and 3 mmol/L. Complete solubilization was obtained by adding Nonidet P-40 (no. N6507; Sigma) to a final concentration of 100 mL/L.

RESULTS

Enzymatic assay for plasma cholesterol

Table 1 shows the plasma cholesterol concentrations obtained with the cholesterol oxidase method for our diseased control group and the SLO patients. For the group of SLO patients as a whole, the cholesterol concentrations generally were below the concentration range found in the control group. However, there was an overlap between values for the two groups. One SLO patient (case 8) had a plasma cholesterol concentration of 3.8 mmol/L with this test, which would be interpreted as a low but normal value for an adult. The values found for the SLO children (cases 1–3) were close to the range found in our diseased control group; their diagnosis could easily have been missed if no further data had been available.

GC analysis of cholesterol, 7-DHC, and 8-DHC

The gas chromatograms of the plasma samples from a patient in the diseased control group and from two patients with SLO all showed peaks for the internal standard, cholesterol, 8-DHC, and 7-DHC (Figure 1). Identification of the compounds was based on their retention times and on mass spectra (not shown). In agreement with the data from Axelson (Axelson *et al.* 1991), we observed that 7-DHC occurs in trace amounts in normal human plasma. Using calibration curves for cholesterol, 7-DHC, and the internal standard, we confirmed the linearity of the method for the concentration range used in this study (data not shown).

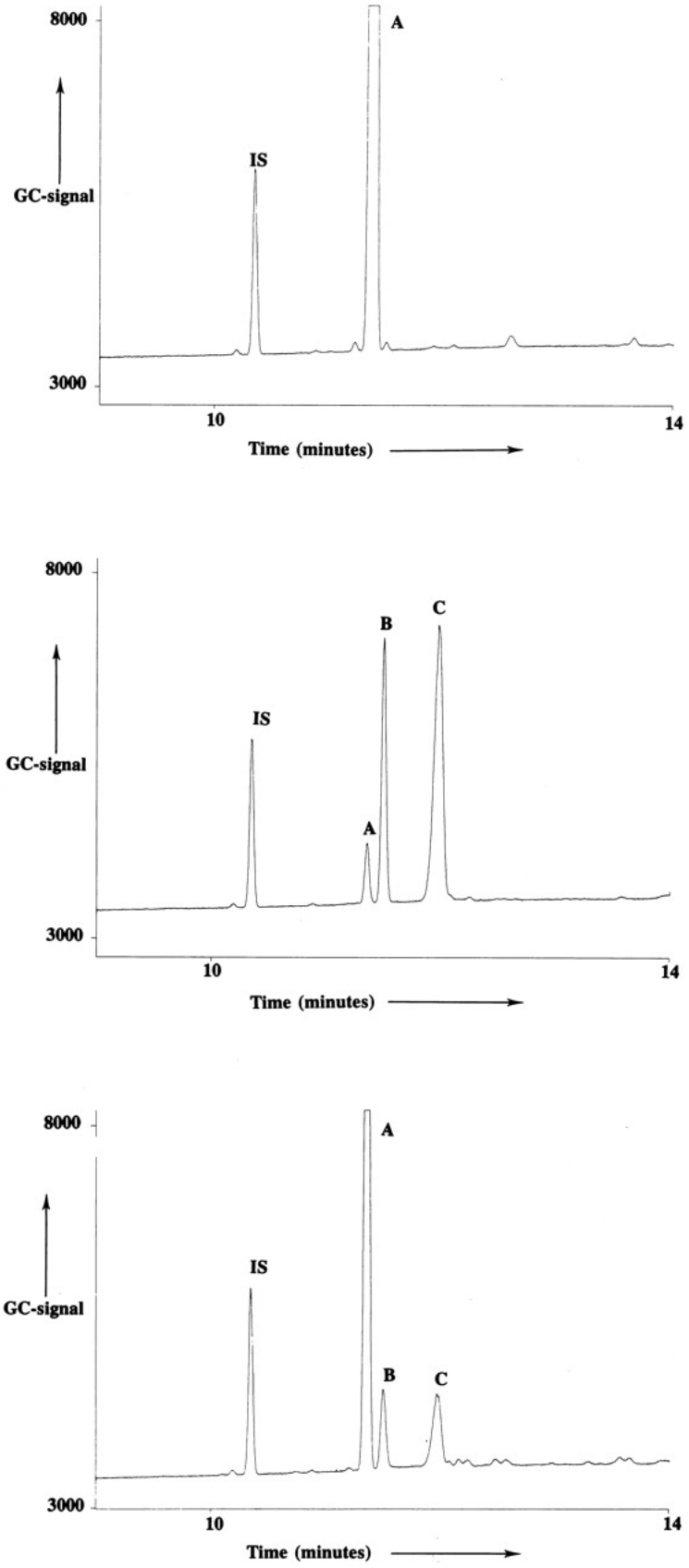

Figure 1. Capillary-column gas chromatograms of control plasma (top panel) and plasma from two SLO patients (the middle panel shows plasma from patient 1 and the bottom panel plasma from patient 5, Table 1). Peaks: IS, internal standard (5ß-cholestane-3-ol); A, cholesterol; B, 8-DHC; and C, 7-DHC.

Capillary-column gas chromatograms of control plasma (top panel) and plasma from two SLO patients (the middle panel shows plasma from patient 1 and the bottom panel plasma from patient 5, Table 1). Peaks: IS, internal standard (5ß-cholestane-3-ol); A, cholesterol; B, 8-DHC; and C, 7-DHC.

Plasma concentrations of cholesterol measured with GC vary considerably among SLO patients (20–2030 µmol/L) and also show an overlap in concentrations between the SLO group and the diseased control group, similar to the overlap observed in the enzymatic cholesterol assay. In all patients with SLO, the concentrations of 7-DHC and 8-DHC were clearly increased (GC results, Table 1). These data lead to a straightforward diagnosis for all SLO cases in this study. The ratio of (7-DHC + 8-DHC)/cholesterol may correlate with the severity of the disease. Patients 1 and 2, who had a severe form of the disease (type 1), gave higher values for this ratio than did the rest of the SLO group.

The correlation between the enzymatic assay and the GC assay results for plasma cholesterol was good in the diseased control group (Figure 2), the Passing and Bablok (Passing *et al.* 1983) regression line (and 95% confidence intervals) being y = 1.02 (0.95–1.08) x - 0.080 (-0.31 to 0.20). In the SLO group, the results of both methods for the plasma samples all deviated from that correlation line, to yield y = 0.68 (0.37–1.15) x - 0.211 (-0.84 to 0.44). Because the cholesterol concentrations found for the SLO group were systematically higher by the enzymatic test than by GC (Table 1), we hypothesized that 7-DHC and 8-DHC might contribute to the results of the enzymatic cholesterol assay; consequently, we performed recovery experiments with cholesterol and 7-DHC.

Recovery experiments

Cholesterol recovery in the enzymatic assay was complete, the mean recovery in the serum matrix being 108% (n = 9, range 100–120%) and the mean in the albumin matrix being 94% (n = 9, range 90–100%). Most of the added 7-DHC was also measured: mean in serum matrix, 61% (n = 6, range 5–70%); mean in albumin matrix, 71% (n = 6, range 70–75%). Possibly, the known instability of 7-DHC contributed to the partial recovery of this compound.

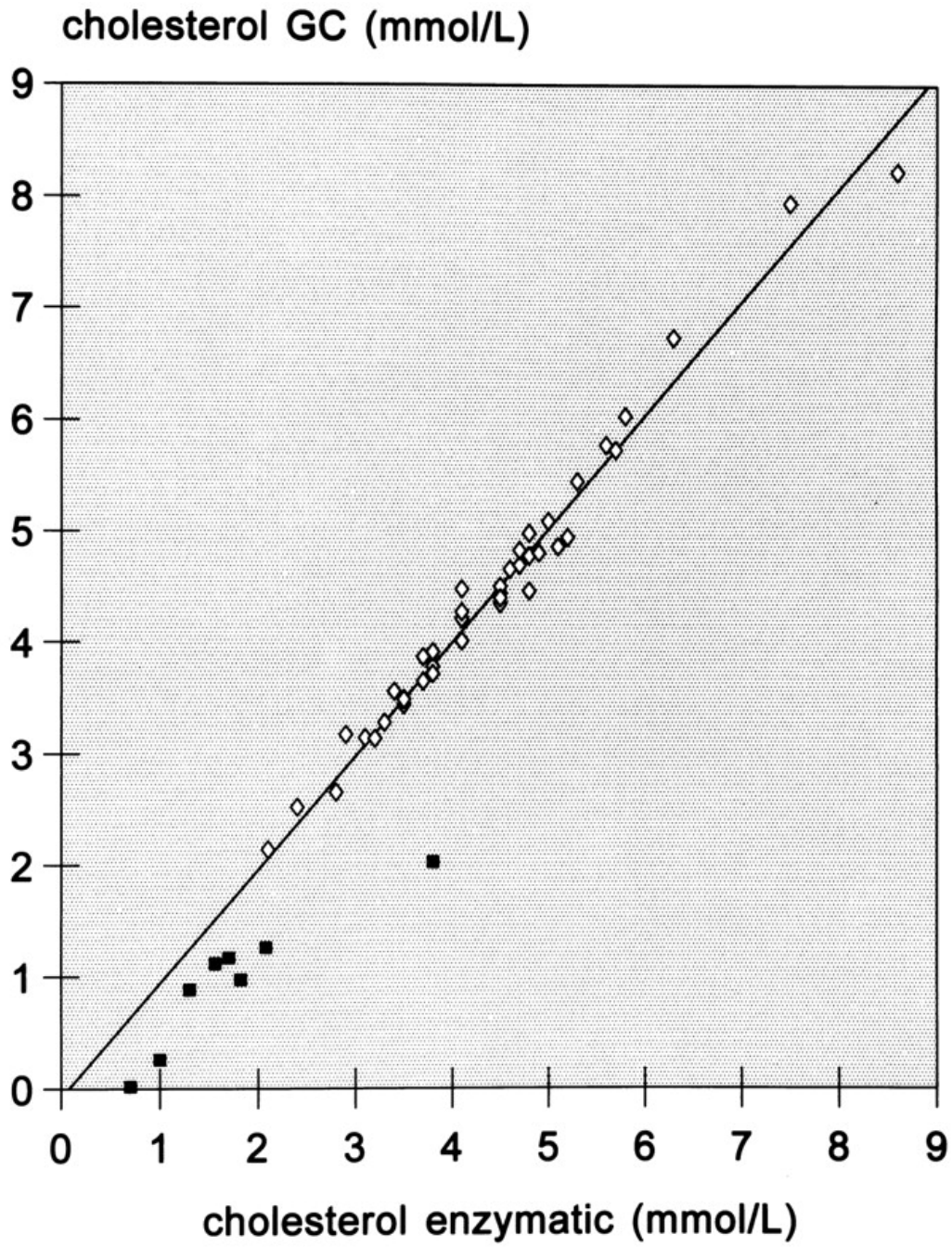

Figure 2. Correlation between enzymatic cholesterol oxidase results and GC results for plasma cholesterol in SLO patients (■) and diseased control patients (◇).

DISCUSSION

Reliable quantitative results for cholesterol, 7-DHC, and 8-DHC are invaluable to confirm the diagnosis in patients clinically suspected to have SLO, and for follow-up of SLO patients being treated with high-cholesterol diets. Until now, studies on reference values for plasma cholesterol have focused on the upper limit of the reference range: Very few data are available for the lower limit of the cholesterol reference range in plasma from children of various age groups. Lane and McConathy have studied changes in serum cholesterol in the first month of life using GC (Lane *et al.* 1986). Lockitch (Lockitch *et al.* 1988) determined age-related reference values for serum cholesterol in 450 healthy children, ages 1 to 19 years. Plasma cholesterol data in our diseased control group are in line with the data of Lockitch et al. (Lockitch *et al.* 1988). The 0.025 fractile those authors found for the age groups older than 6 years is similar to the lowest value found in our diseased control group. For the age group between 1 and 5 years, Lockitch et al. found a 0.025 fractile value of 1.15 mmol/L, whereas the lowest value in our diseased control group was 1.67 mmol/L. Because patients with SLO are often severely affected at birth, reference values for plasma cholesterol in the first weeks of life are of special interest. Lane and McConathy (Lane *et al.* 1986) observed that cholesterol at day 28 postpartum ordinarily is in the lower 5th percentile of the adult population values; at day 3 postpartum, values were even lower (Lane *et al.* 1986). Age-related reference values are thus invaluable for the correct interpretation of plasma cholesterol concentrations in children.

As Table 1 makes clear, SLO patients measured with the enzymatic assay may give plasma cholesterol values close to the lower limit of the range of the diseased control group or even within this range. In one of our adult patients (case 8), the plasma cholesterol concentration of 3.8 mmol/L would be interpreted as normal. We were able to better discriminate between both groups by assessing the cholesterol values measured by GC. However, even with this assay, the result for cholesterol could easily be misinterpreted. We conclude that the diagnosis of SLO cannot be excluded definitely on the basis of the plasma cholesterol results. The control samples gave results by both the enzymatic and the GC method for cholesterol that correlated well (Table 1 and Figure 1). For SLO patients, however, obvious differences between the techniques were apparent, the cholesterol concentrations measured with the enzymatic assay being invariably higher than the GC results. As Table 1 shows, the summed GC data for cholesterol, 7-DHC, and 8-DHC correlated well with the enzymatically determined cholesterol concentration. This suggests that 7-DHC and 8-DHC may contribute to the plasma cholesterol results measured by the enzymatic cholesterol assay. We partly confirmed this by adding 7-DHC to solutions of albumin and to plasma samples. The majority (61–71%) of 7-DHC added was measured as cholesterol by the enzymatic cholesterol test, in both the serum matrix and the human albumin matrix. The results for cholesterol measured by GC were not influenced by these additions (data not shown). Apparently the enzymatic cholesterol test cannot discriminate between the various steroids. This may be due to aspecific conversion of 7-DHC and 8-

DHC by cholesterol oxidase. Using a sterol monolayer system, Slotte has described that cholesterol oxidase from Streptomyces cinnamomeus oxidizes 7-DHC at a rate 5.1-fold slower than it oxidizes cholesterol (Slotte *et al.* 1992). We expect that similar results will be found for other commercially available reagents for plasma cholesterol determinations that make use of cholesterol oxidase. Our results indicate that the enzymatic test for measuring plasma cholesterol gives falsely high results for SLO plasma samples. The plasma cholesterol concentration in our youngest patient with SLO was 20 μmol/L, whereas the (7-DHC + 8-DHC)/cholesterol ratio for this patient was by far the greatest we saw. This is, to our knowledge, the lowest plasma cholesterol ever reported in humans. Clinically, the affected girl had a very severe form of the disease (type I), with major malformations; the patient died at age 5 weeks. As also described by Tint (Tint *et al.* 1995), we observed that the plasma cholesterol concentration in older SLO patients is generally higher than in young, more severely affected patients. In our study the lowest and the highest cholesterol values we saw differed by 20-fold. Those for 7-DHC differ by only 6-fold, and the concentrations of 7-DHC and 8-DHC are not significantly different between younger and older patients in our study. The (7-DHC + 8-DHC)/cholesterol ratio, however, is clearly higher in the young patients (cases 1 and 2 in Table 1). The differences in plasma cholesterol concentrations of SLO patients at different ages might result from a higher dietary intake of cholesterol in older, clinically more mildly affected patients. Kelley (Kelley *et al.* 1994) suggested that higher cholesterol concentrations correlate more with the length of survival and the amount of dietary cholesterol than with clinical severity. Another explanation for higher cholesterol concentrations in older SLO patients could be the residual activity of the cholesterol biosynthesis pathway in older patients, as was suggested by Tint (Tint *et al.* 1995). This would also explain the milder course of the disease in this group. At present, it is unknown whether the shortage of cholesterol and the abundance of cholesterol precursors both contribute to the development of the clinical signs and symptoms of SLO. As with cholesterol oxidase, other enzymes involved in cholesterol-converting pathways may also accept a 7-dehydro or 8-dehydro variant of their normal substrates. This would give rise to unexpected intermediates with unpredictable functional characteristics. This concept may give further impetus to attempts to understand the importance of cholesterol precursors in the development of the clinical picture.

Summarizing, we conclude that the enzymatic test for measuring plasma cholesterol gives unreliable results in SLO patients. For diagnosis of patients clinically suspected to have the disease, measurements of plasma cholesterol (by any method) should not be used. Obviously, quantification of plasma 7-DHC and 8-DHC is the method of choice here (Irons *et al.* 1993; Tint *et al.* 1994; Tint *et al.* 1995). For follow-up studies of SLO patients receiving high-cholesterol dietary treatment, the enzymatic test for measuring plasma cholesterol is also not suitable. Instead, the method of choice for this purpose may be GC, which provides reliable quantitative data for plasma cholesterol, 7-DHC, and 8-DHC.

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Chapter 5

New treatment strategy for Smith-Lemli-Opitz syndrome

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The Smith-Lemli-Opitz syndrome is caused by deficient activity of Δ^7-dehydrocholesterol reductase, the final enzyme of the cholesterol biosynthetic pathway, resulting in low cholesterol and high concentrations of its direct precursors, 7-dehydrocholesterol (7DHC) and 8DHC in blood and tissues (Irons *et al.* 1993; Tint *et al.* 1995). Cholesterol fulfils an essential role during embryogenesis where it functions as a transporter-molecule for hedgehog signalling proteins required for normal morphogenesis (Porter *et al.* 1996). Without cholesterol their transport is impaired (Porter *et al.* 1996). These findings may explain the phenotypic consequences of Δ^7-reductase deficiency as observed in Smith-lemli-Opitz syndrome: microcephaly, distinctive facies, organ malformations, syndactyly, and genital abnormalities. Once morphogenesis is complete, it is not known whether the low cholesterol or the increased concentration of precursors is more harmful. In abetalipoproteinaemia, cholesterol concentrations are similar to those in Smith-Lemli-Opitz syndrome without clinical side-effects; we thus postulated that 7DHC, 8DHC, or both may be the toxic substances. Therapeutical trials of dietary supplementation of cholesterol with or without bile acids have shown that plasma cholesterol concentrations can be increased in some patients. Concentrations of the precursors 7DHC and 8DHC, however, were only marginally altered and clinical results so far have been disappointing (Irons *et al.* 1995; Ullrich *et al.* 1996).

We performed repeated exchange transfusions in combination with inhibition of de-novo cholesterol synthesis with a HMG CoA reductase-inhibitor in a 3-month old girl with this disorder, after having obtained informed parental consent. This strategy aimed for simultaneously to remove precurors while supplying extra cholesterol from the donor blood and inhibit renewed de-novo production of precursors at a higher level in the cholesterol pathway. The girl underwent eight whole-blood exchange transfusions during a period of 5 months. Total exchanged volume accounted approximately for eight times her circulating blood volume. Oral simvastatin treatment was begun on day 20. No complications or drugrelated adverse effects were documented. Sterol plasma and erythrocyte-membrane concentrations during the treatment period of 190 days showed a substantial decrease of 7DHC (and 8DHC), as well as an increase in and finally a normal cholesterol (table).

Table. Gas chromatograph analysis of sterols in plasma (μmol/L) and erythrocyte membrane (μmol/60μL) isolated erythrocytes during therapy and percentage of initial concentration

Day	**Plasma**			**Erythrocyte membrane**		
	Cholesterol	7DHC	7DHC/CH Ratio	Cholesterol	7DHC	7DHC/CH Ratio
0*	1338 (100)	362 (100)	0.27	1273 (100)	1087 (100)	0.85
38*	1196 (89)	272 (75)	0.23	1454 (114)	830 (76)	0.57
93	1608 (120)	149 (41)	0.09	1762 (138)	388 (36)	0.22
147*	2312 (173)	191 (53)	0.08	2621 (206)	470 (43)	0.18
190	2594 (194)	160 (44)	0.06	2700 (212)	400 (37)	0.15

Exchange transfusions were on day 1, 4, 11, 39, 40, 148, 150, and 152. Simvastatin was begun on day 20, daily dose 0.2 mg/kg increasing to 0.4 mg/kg at day 30, and 0.6 mg/kg from day 40. * Immediately before exchange transfusions. CH=Cholesterol; percentages shown in parentheses.

After the first three exchange transfusions, plasma 7DHC increased from 151 to 332 μmol/L over 5 days. After exchange transfusions four and five (days 39 and 40) plasma 7DHC concentrations remained stable. Mental, motor, and social development improved. At age 8 months, the child's neuromotor development corresponded to a child of 5 months on the Bayley scales of infant development. Measurements of head circumference, height, and weight followed the same percentage as before the start of treatment.

Repeated exchange transfusions in combination with a HMG CoA-inhibitor reduced plasma and erythrocyte membrane precursor concentrations and improved the plasma 7DHC/cholesterol ratio greatly in this child. We are encouraged to explore the long-term effects of this treatment strategy as a potentially useful therapeutic option in the treatment of young patients with Smith-Lemli-Opitz syndrome.

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Chapter 6

Simvastatin: a new therapeutic approach for Smith-Lemli-Opitz syndrome

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ABSTRACT

The Smith-Lemli-Opitz syndrome (SLOS) is caused by deficient 7-dehydrocholesterol reductase, which catalyzes the final step of the cholesterol biosynthetic pathway, resulting in low cholesterol and high concentrations of its direct precursors 7-dehydrocholesterol (7DHC) and 8DHC. We hypothesized that i) 7DHC and 8DHC accumulation contributes to the poor outcome of SLOS patients and ii) blood exchange transfusions with hydroxymethylglutaryl (HMG)-CoA reductase inhibition would improve the precursor-to-cholesterol ratio and may improve the clinical outcome of SLO patients. First, an in vitro study was performed to study sterol exchange between plasma and erythrocyte membranes. Second, several exchange transfusions were carried out in vivo in two SLOS patients. Third, simvastatin was given for 23 and 14 months to two patients. The in vitro results illustrated rapid sterol exchange between plasma and erythrocyte membranes. The effect of exchange transfusion was impressive and prompt but the effect on plasma sterol levels lasted only for 3 days. In contrast, simvastatin treatment for several months demonstrated a lasting improvement of the precursor-to-cholesterol ratio in plasma, erythrocyte membranes, and cerebrospinal fluid (CSF). Plasma precursor concentrations decreased to 28 and 33% of the initial level, respectively, whereas the cholesterol concentration normalized by a more than twofold increase. During the follow-up period all morphometric parameters improved. The therapy was well tolerated and no unwanted clinical side effects occurred.

This is the first study in which the blood cholesterol level in SLOS patients is normalized with a simultaneous significant decrease in precursor levels. There was a lasting biochemical improvement with encouraging clinical improvement. Statin therapy is a promising novel approach in SLOS that deserves further studies in larger series of patients.

KEY WORDS: cholesterol biosynthesis, Smith-Lemli-Opitz syndrome, simvastatin, exchange transfusion

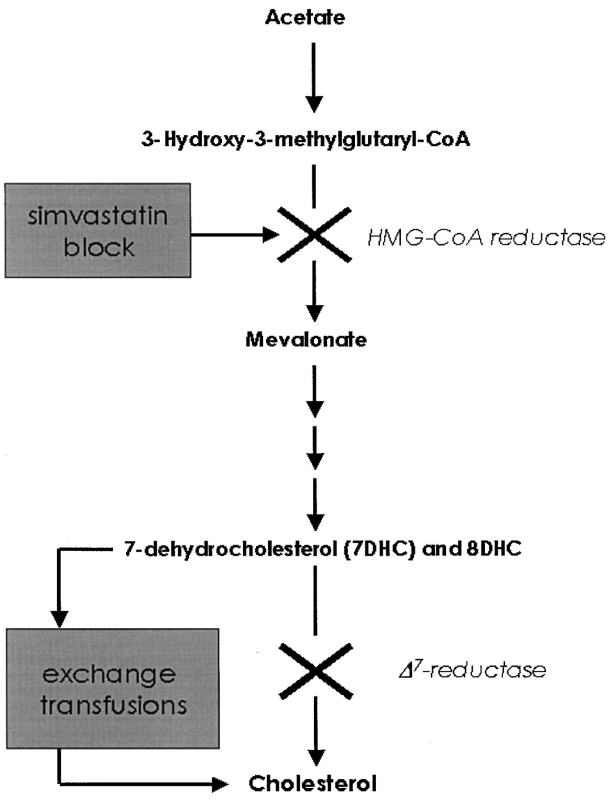
INTRODUCTION

The Smith-Lemli-Opitz syndrome (SLOS) is caused by a deficient 7-dehydrocholesterol reductase activity, the final enzyme of the cholesterol biosynthetic pathway. Low cholesterol and high concentrations of its direct precursors 7-dehydrocholesterol (7DHC) and its isomer, 8-dehydrocholesterol (8DHC), in blood and tissues are the biochemical hallmarks of the syndrome (Smith *et al.* 1964; Irons *et al.* 1993; Tint *et al.* 1995). Plasma sterol concentrations generally correlate with syndrome severity and outcome (Tint *et al.* 1995). 7-Sterol reductase activity in the liver of SLOS patients is markedly decreased (Shefer *et al.* 1995). The human 7-sterol reductase gene has been characterized and assigned to chromosome 11q12-13. Mutations in this gene cause SLOS (Fitzky *et al.* 1998; Waterham *et al.* 1998; Wassif *et al.* 1998). Cholesterol fulfills an essential role in embryogenesis, during which it functions as a transporter molecule for sonic hedgehog signaling proteins required for correct morphogenesis. Without sufficient cholesterol their transport and function are impaired (Porter *et al.* 1996; Cooper *et al.* 1998). These findings may explain the phenotypic consequences of the 7-reductase deficiency as observed in SLOS: microcephaly, a distinctive facies, cleft palate, various organ malformations, syn/polydactyly, and genital abnormalities.

It is still a matter of debate whether the low cholesterol or the increased concentration of precursors or both is the most harmful component in growth and development of these patients. On the basis of experience with familial hypobetalipoproteinemia (Linton *et al.* 1993), in which plasma cholesterol in heterozygotes is as low as in some SLOS cases, without any clinical effect, we hypothesized that the cholesterol precursors 7DHC and 8DHC may be toxic. Both precursors are structurally similar to cholesterol (differing only in having an extra double bond in the cholesterol B-ring) and therefore may interfere with the important role of cholesterol. Cholesterol is the precursor of steroid hormones; therefore, a reduction of the availability of cholesterol or incorporation of precursors by adrenal and testicular cells may reduce or interfere with normal synthesis of corticosteroids and androgens. Steroid hormones also affect a wide variety of behavioral and psychological states. In SLOS abnormal bile acid profiles already have been documented (Natowicz *et al.* 1994). Furthermore, some enzyme systems accepting cholesterol as substrate have also been shown to accept 7DHC and 8DHC as a substrate (Jira *et al.* 1997).

So far, therapeutic trials in SLOS patients used dietary supplementation of cholesterol with or without bile acids. The concentration of plasma cholesterol could be increased to subnormal levels in some patients. The concentrations of the precursors 7DHC and 8DHC, however, were only marginally influenced in patients and animal experiments, and clinical improvement until now was in general disappointing (Xu *et al.* 1995a; Irons *et al.* 1995; Ullrich *et al.* 1996; Elias *et al.* 1997; Irons *et al.* 1997; Nwokoro *et al.* 1997; Xu *et al.* 1995b). We here report the results of a study performed to investigate i) the in vitro exchange kinetics of cholesterol and precursors between plasma and erythrocyte membranes, ii) the in vivo effect of exchange transfusions in SLOS patients, and iii) the

effect of simvastatin (hydroxymethylglutaryl [HMG]-CoA reductase inhibitor) on cholesterol and precursor levels in two young unrelated SLOS patients. The exchange transfusions aimed simultaneously to remove precursors while supplying additional cholesterol from the donor blood. Simvastatin inhibits de novo production of precursors at the level of HMG-CoA reductase in the cholesterol biosynthetic pathway (Figure 1).



CLINICAL AND LABORATORY INVESTIGATIONS

Subjects

In the Pediatric Clinic (University Hospital Nijmegen, Nijmegen, The Netherlands) two patients with SLOS were treated with exchange transfusions and simvastatin. Parents were informed about the aim of the study, study protocol, and potential side effects. Informed parental consent was obtained on behalf of both patients for the application of repeated exchange transfusions and for the use of simvastatin as an investigational drug, based on previously published evidence of the safety and efficacy of statins in children. The consent to participate in the study was strictly voluntary and could be renounced at any time by the parents without disadvantage for further medical care of their child.

Patient A

Patient A, a girl, first child of healthy unrelated parents, was born after an uneventful pregnancy. A cesarean section was performed at 38+4 weeks gestational age because of breech position. Apgar scores were 8 and 10 after 1 and 5 min, respectively. Birth weight was 3,520 g (97th percentile), length was 50 cm (75th percentile) and head circumference was 33 cm (50th percentile). The observed facial dysmorphias, ptosis, syndactyly of second and third toes, and failure to thrive gave rise to the suspicion of SLOS. At the age of 2 months she was admitted to our clinic, where the diagnosis was confirmed biochemically. Organ malformations were not present. Brain magnetic resonance imaging (MRI) was normal. Ophthalmological and neurophysiological (electroencephalogram [EEG], brainstem auditory evoked potential [BAEP]) examinations revealed no abnormalities.

Patient B

Patient B, a boy, was born after the second pregnancy of unrelated parents. At 37 weeks gestational age he underwent an external cephalic version due to a breech position and was born at 40 weeks. Apgar scores were 10 and 10 after 1 and 5 min, respectively. Birth weight was 2,570 g (5th percentile), length was 51 cm (90th percentile), and head circumference was 31.5 cm (25th percentile). He had mild facial dysmorphias, syndactyly of the second and third toe, and failure to thrive. Diagnosis of SLOS was confirmed biochemically in plasma at the age of 5 months. No organ malformations could be detected. MRI of the brain showed normal structures and myelinization and the EEG was normal. Ophthalmological examination showed no cataract or other abnormalities.

Biochemical studies

The initial diagnosis and the biochemical effect of our therapeutic approaches on cholesterol and precursors in plasma, erythrocyte membranes, and cerebrospinal fluid (CSF) were investigated by gas chromatography and gas chromatography/mass spectrometry as described previously (Jira *et al.* 1997a; Van Rooij *et al.* 1997).

In vitro sterol exchange study

To study sterol exchange kinetics between plasma and erythrocyte membranes two in vitro experiments were designed: *i)* Erythrocytes from patient A were isolated, washed three times with saline, and incubated at 37°C in normal donor plasma. The patient's erythrocytes again were isolated after an incubation time of 0, 20, 40, 60, 120, and 240 min, washed three times with saline, and analyzed; *ii)* similarly, donor erythrocytes were incubated with plasma from patient A and studied after 0, 20, 60, 120, and 360 min of incubation.

In vivo exchange transfusions

After surgical insertion of a subclavian venous catheter eight blood exchange transfusions with 800 mL of donor blood were performed in patient A. The 2-h procedures took place on days 1, 4, 11, 39, 40, 148, 150, and 152, in total accounting for eight times her circulating blood volume. In patient B three exchange transfusions were performed, on days 1, 4, and 7 with 800 mL each, accounting for five times his blood volume.

HMG-CoA reductase inhibition by simvastatin therapy

Simvastatin was started on day 20 and gradually increased from 0.2 to 1.0 mg/kg per day (two daily doses) during 23 months in patient A. In patient B the simvastatin dosage was not altered and was maintained at 0.6 mg/kg per day for 14 months. Study methods consisted of baseline hematological investigations and blood chemistry profiles. These were repeated at 2- to 6-week intervals from the start of therapy. Both children received standard pediatric formula without dietary supplementation of cholesterol or bile acids. Patient B was, in addition, treated during the last 3 months with oral cholesterol supplementation (100 mg/kg per day; cholesterol-module available from Nutricia (product code number 18,012; Special Product Service, Zoetermeer, The Netherlands) in combination with simvastatin therapy. Sterol analysis in plasma, erythrocytes and CSF was carried out before and during therapy. Clinical course, neuromotor development, neuroimaging by MRI, and growth were monitored, scored, and compared with normative data from the Dutch population (Gerver *et al.* 1996). Informed parental consent was obtained for the application of repeated exchange transfusions and for the use of simvastatin as investigational drugs based on previously published evidence of the safety and efficacy of statins in children (Stein 1989; Ducobu *et al.* 1992; Sinziger *et al.* 1992; Patterson *et al.* 1993; Knipscheer *et al.* 1996; Lambert *et al.* 1996; Coleman *et al.* 1996; Sanjad *et al.* 1997;).

RESULTS

Both patients had total cholesterol concentrations below the age-related reference values and 100-fold higher concentrations than the upper reference range limits for 7DHC and 8DHC in plasma, erythrocytes, and CSF before treatment, confirming the diagnosis of SLOS as shown in Table 1. Mutation analysis will be reported elsewhere.

Table 1. Sterol effect of simvastatin during a 23- and 14 month period in two young infants with SLO syndrome.

	Patient A					**Patient B**				Controls
	Months of Therapy					Months of Therapy				
	0	3	6	14	23	0	3	6	14	
Age (Months)	3	6	9	17	26	10	13	16	24	
Biochemistry										
Plasma										
Cholesterol (mmol/L)	1.338	1.608	2.549	2.916	2.815	1.281	1.567	2.050	3.312	2.600-5.200
7DHC+8DHC (mmol/L)	0.625	0.289	0.289	0.212	0.172	0.407	0.147	0.178	0.136	< 0.01
Ratio[a]	0.47	0.18	0.11	0.07	0.06	0.32	0.09	0.09	0.04	< 0.01
Erythrocytes										
Cholesterol (mmol/L)	1.273	1.762	2.700	2.368	2.499	1.846	2.230	2.706	2.997	2.400-3.200
7DHC+8DHC (mmol/L)	1.547	0.583	0.577	0.372	0.299	1.137	0.388	0.451	0.212	< 0.01
Ratio[a]	1.22	0.33	0.21	0.16	0.12	0.62	0.17	0.17	0.07	< 0.01
CSF										
Cholesterol (nmol/L)	3.217	2.564	1.938	2.776	-	1.972	-	2.656	2.902	3.500-5.100[d]
7DHC (mmol/L)	610	340	168	160	-	404	-	276	280	< 20
Ratio[b]	0.19	0.13	0.09	0.06	-	0.20	-	0.10	0.10	< 0.01
Morphometrics										
Length (cm)	55	62	70	78	-	65	68	76	83	
SD	-3.2	-1.8	-0.5	-1.2	-	-6.6	-5.0	-1.9	-1.3	
Weight (kg)	4.11	5.95	7.25	9.75	-	5.15	6.55	7.20	8.60	
SD	-3.5	-1.9	-1.5	-0.7	-	-5.6	-3.7	-2.7	-2.4	
Head Circumference (cm)	34.0	38.0	40.5	43.2	-	40.4	42.3	43.2	44.0	
SD[c]	-6.8	-4.7	-3.3	-2.6	-	-5.2	-4.4	-3.5	-3.5	

a (7DHC+8DHC)/cholesterol
b 7DHC/cholesterol ratio
c Standard deviation below 50th percentile
d Range of values from van Rooij *et al.* 1997

Sterol exchange study

SLO erythrocytes were incubated in donor plasma with cholesterol, 7DHC, and 8DHC concentrations of 5,038, 6, and 16 μmol/L, respectively. A rapid increase in cholesterol in membranes of SLO erythrocytes in 240 min was observed, from 1,070 to 2,019 μmol/L. Simultaneously, the 7DHC + 8DHC concentration in SLO erythrocytes decreased from 1,180 to 613 μmol/L, improving the (7DHC + 8DHC)/cholesterol ratio from 1.10 to 0.30 (Figure 2, experiment 1).

In the second incubation normal human donor erythrocytes were incubated in SLO plasma with concentrations of cholesterol, 7DHC, and 8DHC of 1,228, 326, and 274 μmol/L, respectively. A significant and rapid increase in the (7DHC + 8DHC)/cholesterol ratio from 0.01 to 0.32 occurred over 6 h (Figure 2, experiment 2). These experiments show that cholesterol, 7DHC, and 8DHC exchange easily and rapidly between plasma and membrane compartments, which encouraged us to proceed in performing exchange transfusions in our two patients. Cholesterol exchange between red cell membrane and serum lipoproteins has been studied previously. In accordance with our observations, these investigators documented a rate constant for movement of cholesterol from erythrocytes to plasma and from plasma to erythrocytes with the half-time for efflux of 4 to 6 h (Gold *et al.* 1990; Gottlieb 1980).

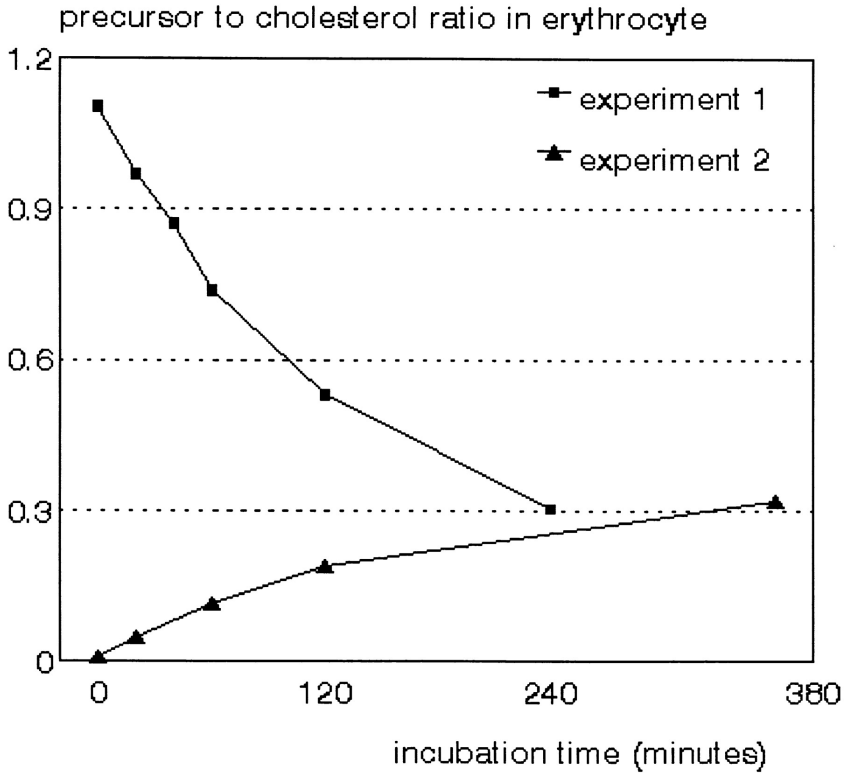


Figure 2. In vitro sterol exchange kinetics between erythrocyte membranes and plasma. Sterol composition of SLOS erythrocytes incubated in normal plasma (experiment 1) and sterol composition of normal erythrocytes incubated in plasma of an SLOS patient (experiment 2).

Exchange transfusions

The effect of exchange transfusion on correcting plasma cholesterol, 7DHC, and 8DHC is prompt as illustrated by the plasma sterol concentrations in the period of three exchange transfusions on days 1, 4, and 11 in patient A (Figure 3). A significant amount of cholesterol could be delivered to the patient. Also, a substantial quantity of precursors could be removed from the patient. The plasma precursor-to-cholesterol ratio improved significantly. The beneficial effect on the plasma levels lasted for only 3 days (Figure 3).

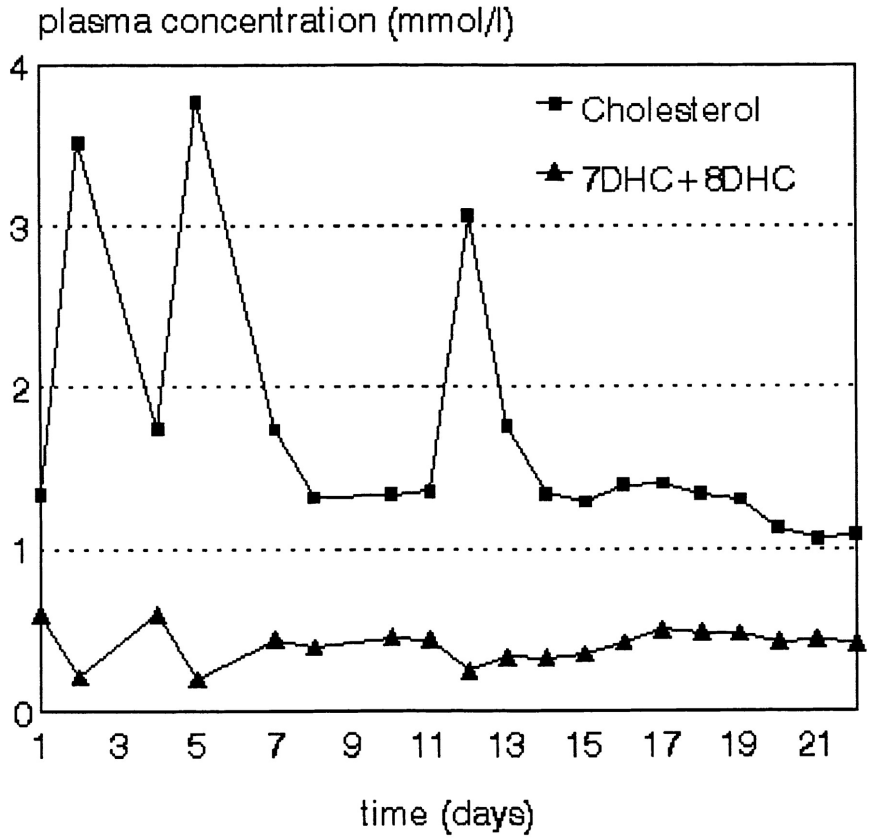


Figure 3. Effect of the first three exchange transfusions (days 1, 4, and 11) on plasma cholesterol (solid squares) and precursor (solid triangles) concentration in time in patient A.

The patient total body/tissue cholesterol uptake from donor blood, calculated from initial donor blood concentrations and the concentrations in the remaining exchanged blood in patient A during the exchange transfusions on days 148, 150, and 152, was 1.7 g (0.4 + 0.6 + 0.7 g, respectively). For patient B, cholesterol uptake was 1.9 g (0.6 + 0.6 + 0.7 g, respectively) during his three exchange transfusions. This body cholesterol delivery of 300–400 mg/kg, achieved by donor cholesterol uptake through three exchange transfusions in both patients, is substantial when seen from the perspective of a normal daily cholesterol synthesis of 8.3;–14.5 mg/kg documented in healthy children (Illingworth *et*

al. 1997; Illingworth *et al.* 1980). The mean amount of plasma precursors (7DHC + 8DHC) for both patients removed by one exchange transfusion is 53 mg (variation, 32–92 mg).

Unfortunately, the effect of a single exchange transfusion on the plasma sterol levels was limited to 2–3 days. Also, repeated exchange transfusions did not result in a lasting change in either the plasma cholesterol or the plasma precursor concentrations (Figure 4). This limited effect motivated us to evaluate the effect of HMG-CoA reductase inhibition by simvastatin therapy for several months.

Simvastatin effect

Patients A and B were treated with simvastatin for 23 and 14 months, respectively. Precursor levels decreased significantly to 28 and 33% of the initial (pretreatment) level in plasma, in erythrocyte membranes, and CSF (Table 1 and Figure 4). Surprisingly, an increase and finally a normalization of the plasma cholesterol concentration (>2.6 mmol/L) was observed after several months. As mentioned earlier, patients did not receive cholesterol supplementation during the simvastatin treatment period (except for the last 3 months for patient B). The plasma (7DHC + 8DHC)/cholesterol ratio finally decreased from 0.47 to 0.06 in patient A and from 0.32 to 0.04 in patient B. Although promising, it is important to note that despite the significant reduction in precursor plasma level and the increase in cholesterol plasma level with this therapy, the (7DHC + 8DHC)/cholesterol ratio was still above normal. The 7DHC/8DHC ratio in the plasma of both patients remained unchanged during treatment (0.82–1.42), uninfluenced by the decrease in total precursor values. In erythrocyte membranes the 7DHC/8DHC ratio was significantly higher (1.66–2.78) compared with plasma. These data suggest that erythrocyte membranes incorporate 7DHC more readily than 8DHC.

During the months of treatment cholesterol precursor concentrations decreased in the CSF of both patients, improving the precursor-to-cholesterol ratio as illustrated in Table 1. During treatment, brain-specific proteins (neuron-specific enolase, S-100, and myelin basic protein) and neurotransmitter metabolites (homovanillic acid [HVA], 5-hydroxyindoleacetic acid [5-HIAA], and 3-methoxy-4-hydroxy-phenylethyleneglycol [MHPG]) in the CSF of both patients remained in the normal range (results not shown). The blood–brain barrier (BBB) function was intact both before and during the therapy period. Simvastatin supplementation therapy was well tolerated. Neuromuscular complications were not observed. Plasma enzymatic activity of aminotransferases and creatine kinase remained in the normal range. No cataract developed.

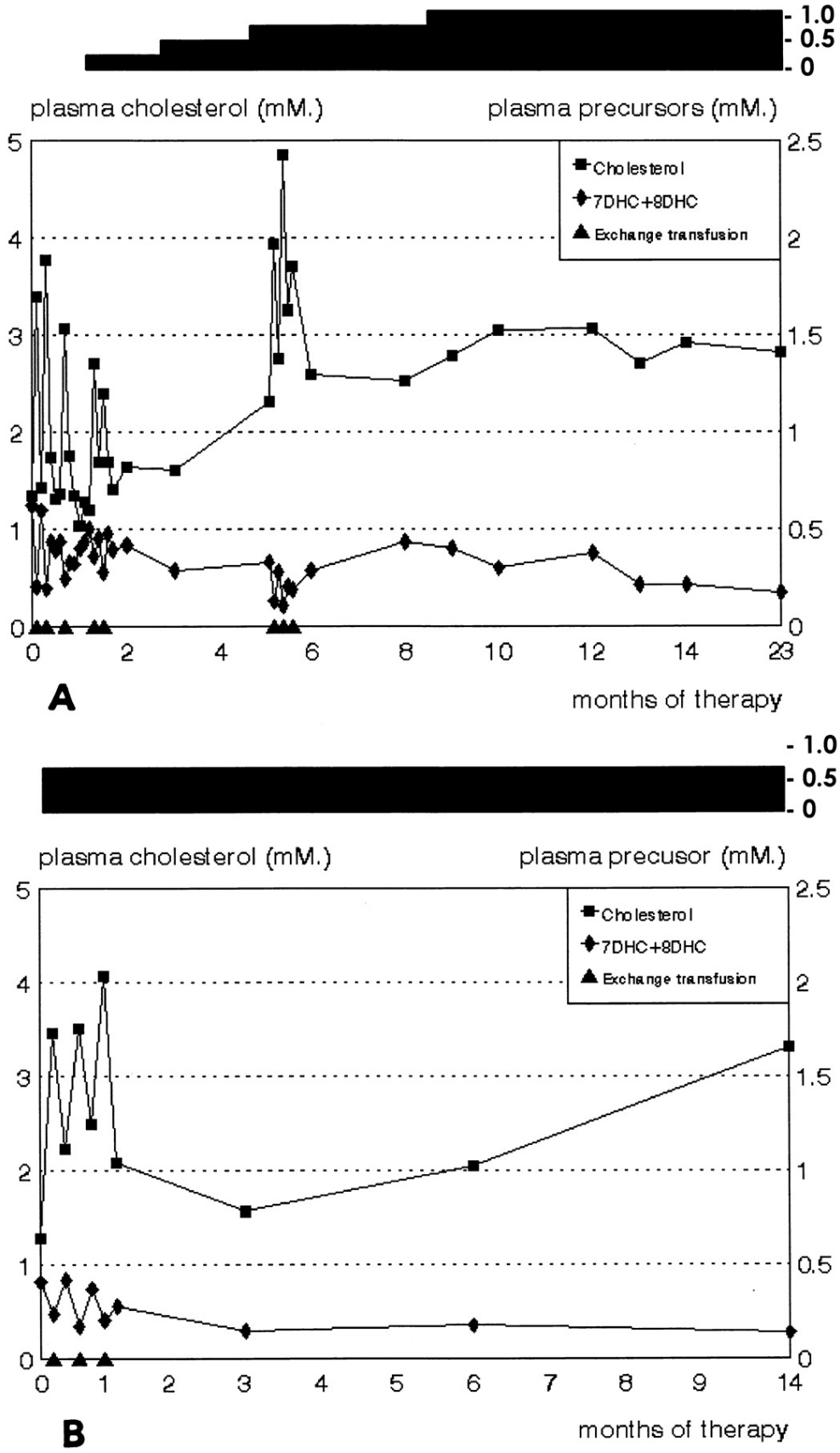


Figure 4. Effect of exchange transfusions (solid triangles) and simvastatin on plasma cholesterol (solid squares) and precursor (solid diamonds) levels (mmol/L) in patient A (A) and patient B (B). Simvastatin dosage is illustrated by the black bars, which represent oral simvastatin dosage in mg per kilogram per day.

Patient outcome

Mental, motor, and social development of both patients shows constant improvement. At the age of 17 months (patient A) and 24 months (patient B), the neuromotor assessment of both patients, as determined by the Hoskins–Squires test (Hoskins *et al.* 1993), and cognitive skills corresponded to 11 and 14 months, respectively. Weight, length, and head circumference during treatment are shown in Table 1. Eating behavior, however, was unchanged. Patient A received a percutaneous gastrostomy after prolonged nasogastric tube feeding while patient B is eating orally with substantial effort of his parents. By the age of 2 years both patients walked with help and started to communicate by word expression.

Our results suggest that simvastatin therapy may represent a simple, effective, and safe way to reduce accumulated cholesterol precursors while improving cholesterol plasma levels in patients with SLOS.

DISCUSSION

Our therapeutic approach aimed at a fast supply of cholesterol and removal of a substantial amount of precursors by blood exchange therapy. Thereafter use of simvastatin was aimed at blocking the cholesterol biosynthesis pathway as a way to avoid the formation of large amounts of the cholesterol precursors 7DHC and 8DHC, which may be potentially harmful to patients. Both aims were met in our first patient, where we accomplished a significant reduction in the plasma levels of 7DHC and 8DHC as published earlier (Jira *et al.* 1997b). We were encouraged by this biochemical response and decided to continue therapy, using simvastatin as the only medication. Also, a second patient was included to confirm our findings. In this second patient three exchange transfusions were carried out for rapid supplementation of cholesterol and removal of part of the precursor load. The patient received simvastatin without dietary cholesterol for 11 months. As in our first patient the precursor levels in plasma, erythrocytes, and CSF declined significantly, fully confirming our findings in the first patient.

This is the first time that a therapeutic approach has succeeded in normalizing plasma cholesterol levels with simultaneous significant reduction of cholesterol precursor levels in plasma. We believe that our therapeutic approach is superior to the approach of dietary cholesterol with or without bile acid supplementation (Irons *et al.* 1995; Ullrich *et al.* 1996; Elias *et al.* 1997; Irons *et al.* 1997; Nwokoro *et al.* 1997). In 11 SLOS patients, treated with cholesterol and bile acids, the mean cholesterol-to-total sterol ratio in plasma only increased from 55% to 72% (Irons *et al.* 1997). In 6 other SLOS patients treated with cholesterol and bile acids this ratio did not exceed 60% (Nwokoro *et al.* 1997). These data are comparable to our own experience with 2 other SLOS-patients treated with

cholesterol and bile acid supplementation for more than 1 year. In these patients there was no significant decline in plasma precursor levels and only a temporary and limited increase in plasma cholesterol that never reached normal levels. The cholesterol-to-total sterol ratios were unchanged during therapy and remained below 79 and 68%. In contrast, the two patients in this study reached cholesterol-to-total sterol ratios of 94 and 96%.

The gradual disappearance of cholesterol precursors 7DHC and 8DHC under statin therapy is relatively easy to understand. The statin blocks the de novo synthesis of the precursors at the level of HMG-CoA reductase. The simultaneous rise in plasma cholesterol is unexpected. It is difficult to understand because our patients did not receive extra cholesterol supplementation. Simvastatin may influence the expression level of the deficient 7-reductase. Such an effect was described for the combination of cholestyramine and lovastatin in rats (Bae *et al.* 1999). Moreover, Shefer (Shefer *et al.* 1997) showed an upregulation of the 7-reductase in human fibroblasts in cholesterol-deficient medium supplemented with lovastatin.

We observed in both patients an impressive improvement in all morphometric parameters (Table 1). In most of the described SLOS patients the head circumference, height, and weight stay below the third percentile, even during conventional therapy (Ullrich *et al.* 1996; Elias *et al.* 1997; Irons *et al.* 1997; Nwokoro *et al.* 1997). Whether growth and developmental progress are, in fact, the result of therapy and relate to the biochemical corrections, or might have occurred otherwise, is difficult to prove. Data derived from rats, however, support the view that oxidized 7DHC derivates play a role in embryo toxicity and growth retardation (Gaoua *et al.* 1999). Confirmation with a larger group of patients over a longer period of time is needed. Studies of cholesterol metabolism ultimately will result in more understanding of the origin and metabolism of cholesterol that is used in growth, development, and myelin in the CNS in children.

A small number of studies demonstrated the efficacy and safety of statin therapy in childhood. Pediatric studies showed that statins were well tolerated by children with familial ypercholesterolemia (Knipscheer *et al.* 1996; Lambert *et al.* 1996; Ducobu *et al.* 1992; Sinziger *et al.* 1992; Stein 1989;), nephrotic syndrome (Sanjad *et al.* 1997; Coleman *et al.* 1996), and Niemann;–Pick disease type C (Patterson *et al.* 1993). One study, however, in which an attempt was made to reduce mevalonate accumulation by administering simvastatin to two children with mevalonate kinase deficiency (Hoffmann *et al.* 1993), documented impressive acute adverse effects. In adults the use of simvastatin did not show any significant adverse effects on brain activity measured by EEG, evoked potentials, mood, sleep, or cognitive performance (Harrison *et al.* 1994). The beneficial effects of simvastatin used in our study without any unwanted clinical side effects encouraged us to proceed to use this therapy in our patients. Of course, a careful clinical follow-up of the patients is required to prevent any complications in liver function or other unwanted side effects of the drug. It remains to be established whether simvastatin use will also work to the same extent in other, perhaps more severely affected SLOS patients. Further studies will also be required in new SLOS cases to find out whether the initial

exchange transfusions really are required. The transfusions have a relatively high clinical risk. When a similar effect of statin use on plasma cholesterol and precursor levels can be found without exchange transfusions this approach would of course be preferred. Also, it remains to be established in further studies whether dietary cholesterol supplementation therapy with simultaneous statin use can add to the success of statin use.

Brain is the most cholesterol-rich organ in the body. Cholesterol in the human brain, developing sheep brain, and rat pup brain is made locally from glucose, acetate, or polyunsaturated fatty acids (Jurevics *et al.* 1995; Likhodii *et al.* 1995; Snipes *et al.* 1997). Sterols formed in the brain by the mevalonate pathway have an active and independently regulated biosynthesis. Cholesterol is not imported from peripheral blood across the BBB by lipoprotein uptake (Turley *et al.* 1996; Edmond *et al.* 1991). Even during fetal brain development, including the time before closure of the BBB, lipoproteins circulating through the central nervous system are not used as a source of cholesterol, but are synthesized locally (Edmond *et al.* 1991; Andersson *et al.* 1990; Bjorkheim *et al.* 1997; Serougne *et al.* 1976). In our two treated patients we demonstrated that precursor concentrations are highly increased and that cholesterol concentrations in CSF are decreased in comparison with controls. Dietary supplementation of cholesterol alone, in SLOS, will not influence an impaired (7DHC + 8DHC)/cholesterol ratio in the central nervous system. In line with previously described evidence (Jurevics *et al.* 1995; Likhodii *et al.* 1995; Snipes *et al.* 1997; Turley *et al.* 1996; Edmond *et al.* 1991; Andersson et al. 1990; Bjorkheim *et al.* 1997; Serougne *et al.* 1976) the only way to reduce cerebral accumulation of cholesterol precursors in SLOS individuals is by means of local inhibition of brain cholesterol biosynthesis. Statins with lipophilic properties (simvastatin and lovastatin) cross the BBB (Saheki *et al.* 1994) and are potential inhibitors of cerebral cholesterol precursor accumulation in SLOS. The half-life for cholesterol calculated in rat brain studies was found to be about 5;–6 months (Snipes *et al.* 1997; Andersson et al. 1990; Bjorkheim *et al.* 1997). Elimination of brain cholesterol precursors is therefore not expected to be a rapid process. This could explain why in our patients the sterol improvement in plasma and erythrocyte membranes was faster and superior to the correction observed in CSF.

In conclusion, simvastatin therapy reduced 7DHC and 8DHC and normalized cholesterol concentrations in two children with SLOS and was well tolerated without side effects. Blocking the de novo synthesis of cholesterol precursors reduced accumulation of these intermediates in plasma, membranes, and CSF. Biochemical and clinical follow-up was 23 and 14 months, respectively, and we are encouraged to explore the long-term beneficial effects of this new treatment strategy in these and other SLOS patients.

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Chapter 7

Mechanism and efficacy of simvastatin treatment in patients with Smith-Lemli-Opitz syndrome

Submitted

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SUMMARY

Background

Smith-Lemli-Opitz syndrome (SLOS) is a common autosomal recessive developmental disorder characterized by facial dysmorphisms, mental and developmental retardation, and multiple congenital anomalies. The disorder is caused by a reduced activity of the cholesterol biosynthetic enzyme 7-dehydrocholesterol reductase (7DHCR), due to mutations in the encoding *DHCR7* gene. As a consequence, patients have low cholesterol and elevated 7-dehydrocholesterol levels in plasma and tissues, correlating rather well with clinical severity.

Methods

We treated 3 relatively mild SLOS patients for 80, 49 and 31 months, respectively, with simvastatin, an oral HMG-CoA reductase inhibitor and determined the effect and efficacy of the treatment on development and general well being of the patients. In addition, we studied in vitro the effect of simvastatin on 7DHCR expression and activity in primary skin fibroblast cells of the patients.

Findings

All patients tolerated the simvastatin therapy well without apparent clinical side effects. During treatment, we observed a marked decrease of plasma 7-dehydrocholesterol levels in all three and a concomitant increase of the plasma cholesterol concentration in two patients. Moreover, two patients showed a good and one patient a moderate clinical improvement of growth and development.

The i*n vitro* studies with cultured primary skin fibroblast cells of the patients showed that simvastatin induces an increase in gene transcription of the *DHCR7* gene, which results in an increase of 7DHCR protein and, consequently, an increase in (residual) 7DHCR activity. Since 7DHCR catalyses the rate-limiting step in SLOS, this increase in residual 7DHCR activity leads to an increased flux through the cholesterol biosynthetic pathway resulting in lower 7-dehydrocholesterol and increased cholesterol levels, as observed in the treated patients.

Interpretation

Simvastatine treatment in SLOS patients with a mild phenotype and residual activity of 7DHCR in cells results in increased 7DHCR expression and activity levels leading to a lowering of 7-dehydrocholesterol and an increase of cholesterol levels.

Relevance

The data provide an explanation for the unexpected rise of plasma cholesterol in SLOS patients upon simvastatin treatment. Simvastatin treatment may be a good long-term therapeutic option in SLOS patients with residual 7DHCR activity and a mild clinical presentation.

INTRODUCTION

Smith-Lemli-Opitz syndrome (SLOS; MIM 270400) is an autosomal recessive inborn error of cholesterol biosynthesis with an incidence of 1:20,000-60,000 dependent on the geographic region. Patients with SLOS may display a variety of morphogenic and congenital anomalies, including dysmorphic craniofacial features, microcephaly, multiple internal organ, limb/skeletal, and urigenital malformations, (intrauterine) growth and mental retardation, and behavioural problems (Smith *et al.* 1964; Cunniff *et al.* 1997; Kelley *et al.* 2000; Waterham *et al.* 2000) After the identification of the biochemical and molecular basis of SLOS (see below) it has become clear that patients with SLOS constitute a clinical and biochemical continuum ranging from hardly recognizably mild to a severe, lethal form.

SLOS is caused by a deficiency of the enzyme 3β-hydroxysterol Δ^7-reductase (7DHCR; E.C.1.3.1.21 Tint *et al.* 1994; Irons *et al.* 1993) due to mutations in the encoding *DHCR7* gene located at chromosome 11q13 (Fitzky *et al.* 1998; Waterham *et al.* 1998; Wassif *et al.* 1998). 7DHCR catalyses the predominant final step in cholesterol biosynthesis, which is the reduction of the C_7-C_8 (Δ7) double bond of 7-dehydrocholesterol (7DHC; cholesta-5,7-dien-3β-ol) to produce cholesterol (cholest-5-en-3β-ol). As a consequence of the 7DHCR deficiency, patients with SLOS typically have reduced plasma and tissue cholesterol concentrations and elevated levels of 7DHC, constituting the major biochemical hallmark used to confirm the clinical diagnosis of the syndrome. In addition, elevated 8-dehydrocholesterol (8DHC; cholesta-5,8(9)-dien-3β-ol) levels are observed, presumably synthesized from the accumulating 7DHC by the enzyme sterol Δ^8-Δ^7 isomerase functioning in the reverse direction.

Several studies have shown that overall clinical severity in SLOS correlates best either with the absolute cholesterol levels or with the sum of 7DHC plus 8DHC expressed as the fraction of total sterol (Tint *et al.* 1995; Cunniff *et al.*1997; Witsch-Baumgartner *et al.* 2000) Indeed, patients with only very minimal symptoms have been identified with (near) normal cholesterol levels and barely elevated levels of 7DHC and 8DHC reflecting partially reduced 7DHCR activities ranging from ~20 to 50% of the activities in controls (Kelley *et al.* 2001; Langius *et al.* 2003).

It is generally considered that the availability of cholesterol during development of the foetus is one of the major determinants of the phenotypic expression in SLOS. Since most anomalies occurring in SLOS are of early-embryonic origin, it will not be feasible to develop a postnatal therapy to entirely cure the patients. The therapy currently mostly employed aims to replenish the lowered cholesterol levels in the patients through dietary supplementation of cholesterol with or without bile acids. This treatment indeed leads to a substantial elevation of plasma cholesterol concentrations in patients, but plasma concentrations of 7DHC and 8DHC are only marginally altered. Moreover, this treatment does not significantly change the sterol levels in brain, which are dependent on *de novo*

cholesterol synthesis due to the limited ability of cholesterol to cross the blood-brain barrier (Jurevics & Morell 1995). Nevertheless, several reports have indicated that dietary cholesterol supplementation may improve behaviour, growth and general well being in children with SLOS (Nwokoro & Mulvihill 1997; Elias *et al.* 1997; Irons *et al.* 1997). However, a recent standardized study with 14 patients indicated that cholesterol supplementation has hardly any effect on developmental progress Sikora *et al.* 2004).

We recently reported promising results of an alternative therapeutic strategy aimed primarily at lowering of the elevated 7DHC and 8DHC levels through treatment with simvastatin, an oral HMG-CoA reductase inhibitor. The observed biochemical effects in two treated SLOS patients were a marked decrease of 7DHC and 8DHC levels and, somewhat unexpectedly, a concomitant increase of cholesterol in both plasma and cerebrospinal fluid in conjunction with promising clinical improvement (Jira *et al.* 1997; Jira *et al.* 2000). We now report the results of a long-term biochemical and clinical follow-up of three SLOS patients treated with simvastatin. In addition, we studied the effect of simvastatin on the regulation of cholesterol biosynthesis in cultured primary skin fibroblast cells of these patients to provide an explanation for the effect on the patients' sterol levels upon the simvastatin treatment. Our results show that the simvastatin treatment leads to an increased expression of the rate-limiting 7DHCR in SLOS patients, and thus results in an increased flux through the cholesterol biosynthesis pathway. The efficacy of the simvastatin treatment appears to correlate well with the residual activity of 7DHCR and clinical severity in patients.

METHODS

Patients

Parents were informed about the aim of the study, study protocol, and potential side effects. Informed parental consent was obtained on behalf of the patients for the use of simvastatin as an investigational drug, based on previously published evidence of the safety and efficacy of statins in children (de Jongh *et al.* 2002). The consent to participate in the study was strictly voluntary and could be renounced at any time by the parents without disadvantage for further medical care of their child. Three young patients, diagnosed with SLOS based on clinical signs and confirmed at the biochemical and molecular level (Table 1), were treated for 80, 49 and 31 months, respectively with oral simvastatin (Zocor®; Merck Sharp & Dome) at a daily dose of 0.6 – 1.0 mg / kg body weight. During the first year of simvastatin therapy, all three patients received cholesterol supplementation at a dosage of 40 mg/kg/day. After one year the cholesterol supplementation was stopped without any noticeable effects.

Procedures

To study the effect of simvastatin *in vitro* we used primary skin fibroblasts of 4 patients with SLOS, one carrier of SLOS and one control subject. These included fibroblasts of the three patients included in the simvastatin therapy and fibroblasts of a severe patient reported previously (Waterham *et al.* 1998; Jira *et al.* 2000). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Foetal Calf Serum (FCS) and 1% penicillin/streptomycin in a temperature and humidity controlled incubator (95% air, 5% CO_2 as the gas phase) at 37°C. At day 1, the cells were trypsinized and seeded in duplo in T75 culture flasks (Costar) at 60% confluency in DMEM containing 10% FCS. At day 2, the medium was substituted for DMEM containing 10% lipoprotein (cholesterol)-depleted FCS to induce *de novo* cholesterol synthesis. At day 3, simvastatin (1 µM final concentration; for preparation of stock solution see (Houten *et al.* 2003) was added to one set of cells while a second set received the solvent without simvastatin. At day 8, all cells were harvested by trypsinization, washed twice with PBS, aliquoted, snap-frozen as pellets in liquid nitrogen and stored at –80^{o}C until further use.

For sterol analysis, fibroblasts were saponified for 2 h at 70°C in alkaline ethanol after which sterols were extracted with hexane, converted to trimethylsilyl derivatives using bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane and analysed by gas chromatography/mass spectrometry as described previously (Waterham *et al.* 1998). Sterol analysis in plasma was performed as described previously (Jira *et al.* 2000).

The enzyme activity of 7DHCR in homogenates of cultured fibroblasts was determined by measuring the time-dependent conversion of ergosterol into brassicasterol by sterol analysis using gas chromatography/mass spectrometry similar as described previously by (Langius *et al.* 2003; Honda *et al.* 1996). Protein concentrations of the lysates were determined with the bicinchoninic acid protein assay (Sigma).

Mutations in the *DHCR7* gene of the patients were identified after PCR amplification of coding exons 3-9 plus flanking intron sequences followed by sequencing of the DNA fragments on an ABI 377A automated DNA sequencer as described previously (Jira *et al.* 2001).

Antibodies were produced in a rabbit against a fusion protein composed of amino acid residues D359 until K393 of 7DHCR (corresponding to a large cytosolic loop of the protein) fused to the C terminus of glutathione-S-transferase (GST) and expressed in *Eschericha coli*. The crude antiserum was affinity-purified on a column with an immobilized fusion protein composed of the same 7DHCR peptide but then fused to the carboxyl terminus of maltose binding protein (MBP) expressed in *E. coli* and coupled to cyanogen bromide sepharose (Pharmacia) as described previously (Hogenboom *et al.* 2002). For the generation of the GST-7DHCR and MBP-7DHCR fusion proteins, a cDNA fragment comprising bp 1075 until 1179 of the *DHCR7* coding region was amplified by PCR from human control cDNA using as forward primer

DHCR7$_{1075\text{-}1092}$ 5'-TAATA*GGATCC*GACCTGTTCCGCCGCACG -'3 (introduces a *Bam*HI site) and as reverse primer DHCR7$_{1179\text{-}1160}$ 5'-TATAT*GAATTC*TCACTTGCTGTGGTGCCTCTGC-3' (introduces an in-frame stop codon and an *Eco*RI site). After amplification, the DNA fragment was subcloned into the pGEM-T vector (Promega) followed by sequencing to verify the absence of PCR-induced errors. Subsequently, the *DHCR7* insert was released from pGEM-T as a *Bam*HI-*Eco*RI fragment and ligated into the *Bam*HI and *Eco*RI sites of pGEX-4T (Pharmacia; for GST-7DHCR expression) or as an *Bam*HI-*Sal*I fragment into the *Bam*HI and *Sal*I sites pMALC2 (New England Biolabs; for MBP-7DHCR expression).

For immunoblot analysis, equal amounts of proteins were separated on a 10 % SDS-polyacrylamide + 8M urea gel and transferred onto nitrocellulose by wet blotting. After blotting, the blots were stained reversibly with Ponceau-S to verify the equal transfer of proteins. Immunoblot analysis was performed using the western-light detection kit of Applied Biosystems. The affinity-purified anti-7DHCR antibody was used in a 1:1000 dilution.

The expression levels of *DHCR7* mRNA in the various cell lines were determined and related to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA using the LightCycler system (Roche). Total RNA, free from genomic DNA, was isolated with the SV RNA total isolation system (Promega) and used to prepare first strand cDNA as described previously (26). Quantitative real-time PCR analysis was performed with the LightCycler FastStart DNA Master SYBR green I kit (Roche) using the following primers: DHCR7-Fw 5'-TCGGGAAGTGGTTTGACTTC-'3; DHCR7-Rev 5'-TGTGGTTCATGTCTGGGACG-'3; GAPDH-Fw 5'-ACCACCATGGAGAAGGCTGG-'3; and GAPDH-Rev 5'-CTCAGTGTAGCCCAGGATGC-'3. Expression levels in each sample were determined in triplicate.

Role of the funding source

No external funding was obtained for this study.

RESULTS

We treated three SLOS patients with 0.6-1.0 mg simvastatin per kg body weight per day, following our previously described protocol (Jira *et al.* 2000). Patients SLOS-1, SLOS-2 and SLOS-3 were treated for 80, 49 and 31 months, respectively (Table 1). In this period, the combined plasma 7DHC

Table 1. Clinical, molecular and biochemical data of three SLOS patients treated with simvastatin.

Patient	SLOS-1		SLOS-2		SLOS-3	
Gender	Female		Male		Male	
SLOS genotype	IVS8-1G>C		IVS8-1G>C		IVS8-1G>C	
	T154M		K198E		W182L	
SLOS phenotype	Mild		Moderate		Mild	
Clinical severity score*)	1		4		1	
Plasma sterols	At start	After	At start	After	At start	After
	(3 mo)	($6^{11/12}$y)	($5^{1/12}$y)	($9^{2/12}$y)	(10 mo)	($3^{4/12}$y)
• 7DHC + 8DHC (µmol/l)	625	172	1072	343	407	88
• Cholesterol (µmol/l)	1338	2815	1534	1488	1281	2540
• Ratio **)	0.467	0.046	0.699	0.231	0.318	0.032
Duration of simvastatin treatment		80 months		49 months		31 months
Morphometrics (SD)	Before	After	Before	After	Before	After
	-3.2	0.0	-1.3	-2.0	-4.5	-1.9
• Length	-3.5	+0.7	-1.1	+4.3	-4.8	-2.9
• Weight	-6.8	-1.5	-2.8	-1.5	-4.1	-3.8
• Head Circumference						
Clinical effect of simvastatin	Good		Moderate		Good	

*) Clinical severity of patients was determined on the basis of a scoring system in which malformations in each of 10 embryologically distinct areas were scored to weigh embryologically separate organ systems equally (Kelley & Hennekam 2000)

**) Plasma ratio = 7DHC + 8DHC / Cholesterol. Initial data of patients SLOS-1 and SLOS-3 have been described previously (Jira *et al.* 2000)

and 8DHC levels showed a marked decrease to 28, 32 and 22% of the levels at the start of the simvastatin therapy. Moreover, a concomitant increase of the plasma cholesterol concentration was observed in patients SLOS-1 and SLOS-3 eventually leading to normalization (i.e. $\geq$2.5 mmol/L). This effect on sterol levels was already apparent after two months on simvastatin and was independent of the supplementation of cholesterol. Although in all three patients a clear decrease in precursor to cholesterol ratio was observed, this ratio remained relatively high in patient SLOS-2, primarily due to the fact that his plasma cholesterol levels did not increase during the treatment.

All patients tolerated the simvastatin therapy well and no clinical side effects were evident. No neuromuscular complications or development of cataracts were observed and the enzyme activities of aminotransferases and creatine kinase in plasma remained within the normal range.

During the course of the simvastatin treatment, all three patients showed clinical improvement of growth and development with the exception of length in patient SLOS-2 (Table 1). Accordingly, the improvement was judged as good in patients SLOS-1 and SLOS-3 and moderate in patient SLOS-2.
Also the neuro-motor development improved during therapy in all three patients. Patient SLOS-1, who started simvastatin therapy at 3 months of age, was able to rotate at 11 months and could stand with some help at 22 months. At 36 months, she could speak a few words and understand simple instructions. She walked with help at 3.5 years and without help at 4 years. She currently enjoys horse-riding and swimming.

Patient SLOS-2 was 5 years old at the start of the simvastatin therapy and at that time could only walk with extensive help. He currently walks independently with help of a rollator walker. Although his awareness and social behaviour improved during simvastatin therapy, his language perception and speech performance has only marginally improved. During therapy he learned to eat. His photosensitivity was not influenced by the statin treatment.

Patient SLOS-3, who started simvastatin therapy at 10 months, was able to crawl at 15 months, to sit stable at 18 months, and to walk independently at 2 years and 1 month. He is currently able to climb stairs and to eat with a spoon.

To evaluate the effect of simvastatin on the cholesterol biosynthesis in SLOS, we incubated primary skin fibroblasts of four SLOS patients, one obligate heterozygote and one unaffected control subject with 1 μm simvastatin for six days in lipoprotein (cholesterol)-depleted medium. As a control, we treated in parallel cells in the same manner except for the addition of simvastatin. Sterol analysis after the six days incubation period showed that the cells of the SLOS patients treated with simvastatin did no longer contain detectable levels of 7DHC, except for the IVS8-1G>C homozygous cell line SLOS-4, which still contained a moderate 7DHC level. In contrast, all untreated SLOS cells contained significantly elevated 7DHC levels (Figure 1A; Table 2).

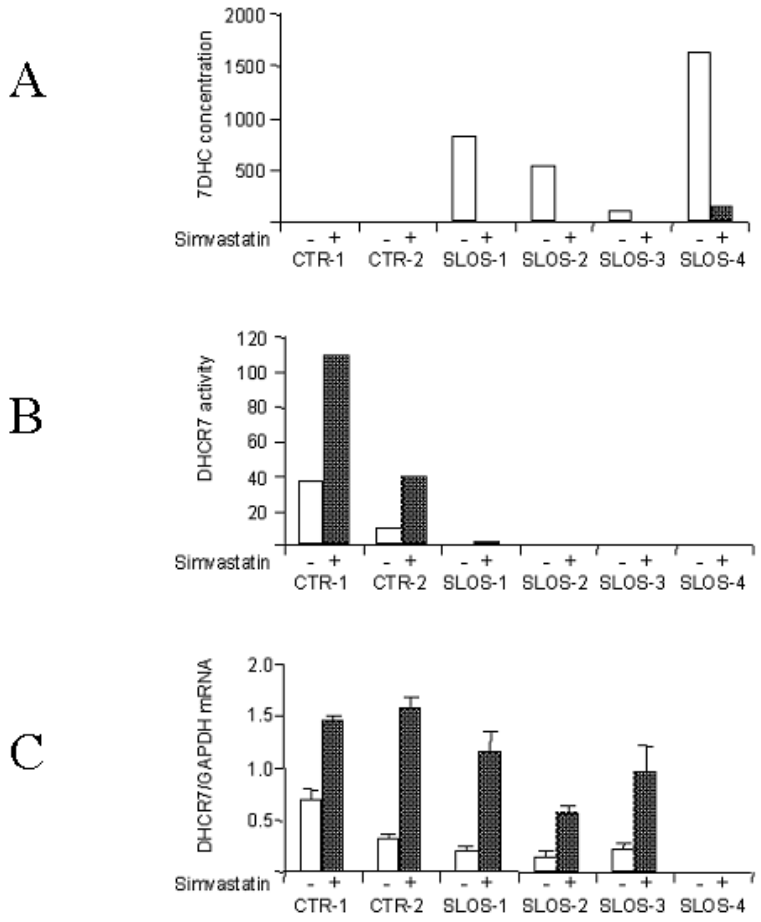

Figure 1. In vitro biochemical and molecular analysis of cells incubated with simvastatin. Primary skin fibroblasts of a control subject (CTR-1), an obligate heterozygote (CTR-2), the three SLOS patients treated with simvastatin (SLOS-1-3) and a severe SLOS patient (SLOS-4) were incubated for six days with (+) or without (-) 1 µm simvastatin in lipoprotein (cholesterol)-depleted medium. After harvesting, the cells were analysed for 7-dehydrocholesterol concentrations (A; expressed as µmol/mg protein), 7DHCR activity (B; expressed as pmol/min.mg protein) and DHCR7/GAPDH mRNA ratios (C).

Table 2. Biochemical and molecular data of 6 different cell lines incubated with or without simvastatin.

Cell line	CTR-1	CTR-2	SLOS-1	SLOS-2	SLOS-3	SLOS-4
DHCR7 genotype	Normal Normal	IVS8-1G>C Normal	IVS8-1G>C T154M	IVS8-1G>C K198E	IVS8-1G>C W182L	IVS8-1G>C IVS8-1G>C
Patient phenotype	Normal	Normal	Mild SLOS	Moderate SLOS	Mild SLOS	Severe SLOS[3]
7DHC concentration[1]						
– simvastatin	Nd	Nd	842	542	777	1648
+ simvastatin	Nd	Nd	Nd	Nd	Nd	147
7DHCR activity[2]						
	35.4	9.29	0.17	Nd	Nd	Nd
– simvastatin	108.3	38.1	1.12	Nd	0.65	Nd
+ simvastatin	3.06	4.10	6.59	NA	NA	NA
ratio +/-						
DHCR7/GAPDH mRNA						
	0.70	0.33	0.19	0.16	0.23	Nd
– simvastatin	1.45	1.58	1.16	0.58	0.97	Nd
+ simvastatin	2.07	4.79	6.11	3.63	4.22	NA
ratio +/-						

[1] in µmol/mg protein
[2] in pmol/min.mg protein
[3] Patient died in early neonatal period (Waterham *et al.*1998)
Nd, not detectable; NA not applicable.

Enzyme activity measurements in homogenates prepared from the cells after the six days incubation showed a 3-4-fold increase in 7DHCR activity in the simvastatin-treated control and heterozygote cells when compared to the same cells without treatment. An increase in 7DHCR activity was also observed in homogenates of the simvastatin-treated SLOS-1 and SLOS-3 cells, while the activity in the SLOS-2 and SLOS-4 cells remained below detection levels (Figure 1B; Table 2).

The increase in 7DHCR enzyme activity in the simvastatin-treated cells was paralleled by an increase of 7DHCR protein as demonstrated by immunoblot analysis of homogenates using affinity-purified antibodies generated against a portion of 7DHCR (Figure 2).

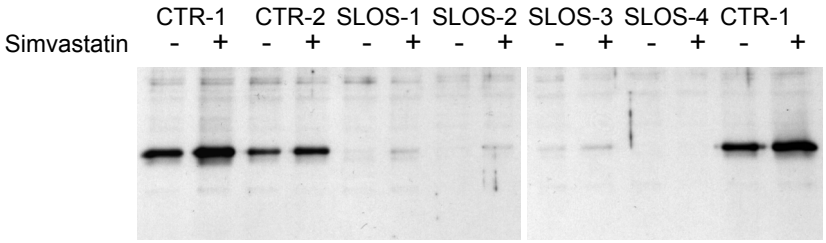

Figure 2. Immunoblot analysis of 7DHCR in cells incubated with simvastatin. Primary skin fibroblasts of a control subject (CTR-1), an obligate heterozygote (CTR-2), the three SLOS patients treated with simvastatin (SLOS-1-3) and a severe SLOS patient (SLOS-4) were incubated for six days with (+) or without (-) 1 μm simvastatin in lipoprotein (cholesterol)-depleted medium. After harvesting, equal amounts of cell homogenates were analysed by immunoblot analysis for the presence of 7DHCR protein using affinity-purified antibodies raised against 7DHCR.

We observed a significant increase in 7DHCR protein levels in the control cells and the SLOS1-3 cells, but not in the SLOS-4 cells. It should be noted here, however, that our antiserum would not be able to detect any 7DHCR protein in the SLOS-4 cells, since it was raised against a portion of 7DHCR that is not synthesized due to the shift in reading frame resulting from the IVS8-1G>C mutation for which this cell line is homozygous. In accordance with this, the observed increase of 7DHCR protein observed in the SLOS1-3 cells and the CTR-2 cell line (which are all heterozygous for IVS8-1G>C) is solely due to the expression of the non-IVS8-1G>C allele, while in the control line both *DHCR7* alleles are responsible for the increase.

Quantitative real-time RT-PCR using primers that only identify and amplify the *DHCR7* mRNA derived from the non-IVS8-1G>C allele (amplification of nt 686-979 of *DHCR7* cDNA) revealed that the increase in 7DHCR protein upon simvastatin treatment can be explained by an increased transcription rate of the *DHCR7* gene (Figure 1C; Table 2).

DISCUSSION

SLOS is the most common defect among the various inborn errors of cholesterol biosynthesis known to date (Kelley & Hennekam 2000; Waterham & Wanders 2000; Kelley & Herman 2001; Waterham 2002; Porter 2002). Cholesterol is one of the end products of the isoprenoid biosynthesis pathway, which, in addition to cholesterol, provides the cell with a variety of other important non-sterol isoprenoids involved in various cellular processes. The flux through the isoprenoid biosynthesis pathway is tightly regulated by the levels of its end products to prevent shortage of these or over-accumulation of intermediates (Goldstein & Brown 1990). Under normal conditions, the rate-limiting step of the pathway is catalysed by the enzyme HMG-CoA reductase, which is subject to different regulatory mechanisms. For example, the translation efficiency of HMG-CoA reductase mRNA is dependent on the cell's requirement of non-sterol isoprenoids, while the degradation rate of the protein is dependent on the cell's requirement of both sterol and non-sterol isoprenoids (Goldstein & Brown 1990). In addition to these post-transcriptional regulatory mechanisms involving primarily HMG-CoA reductase, all genes encoding the enzymes involved in cholesterol biosynthesis are subject to a coordinate transcriptional feedback regulation via the so-called sterol regulatory element binding protein type 2 (SREBP2; Brown & Goldstein 1997; Kim *et al.* 2001; Sakakura *et al.* 2001). SREBP2 is a conditional positive transcription factor that enhances transcription when sterols are absent, but is not required for basal transcription when sterols are present.

Statins, including simvastatin, are potent competitive inhibitors of HMG-CoA reductase and are widely used to treat atherosclerosis and familial hypercholesterolemia. These drugs reduce the synthesis of mevalonate by HMG-CoA reductase, which results in a decreased synthesis of isoprenoid end products, including sterols. This reduction in end products leads to activation of the SREBP regulatory pathway resulting in an increased transcription of the genes encoding the various enzymes involved in isoprenoid/cholesterol biosynthesis and, consequently, in elevated levels of proteins.

While under normal conditions the rate-limiting step in cholesterol biosynthesis is catalysed by HMG-CoA reductase, in SLOS patients the conversion of 7DHC into cholesterol has become rate-limiting due to the reduced activity of 7DHCR, the enzyme catalysing this conversion. Remarkably, however, despite the lower levels of the end product cholesterol in SLOS patients, there appeared no elevation of HMG-CoA reductase activity in SLOS cells and liver tissue, although two other enzymes of the pathway, HMG-CoA synthase and squalene synthase, were elevated (Honda *et al.* 1998; Honda *et al.* 2000). These findings point to a defective coordinate regulation between HMG-CoA reductase and the other enzymes in the cholesterol biosynthetic pathway in SLOS patients. Indeed, it appeared that 7DHC is a much more potent feedback inhibitor of HMG-CoA reductase than cholesterol, suggesting that 7DHC is responsible for the uncoupled regulation in SLOS (Honda *et al.* 1998; Honda *et al.* 2000). This was confirmed in more recent studies with a mouse model of SLOS in

which it was shown that 7DHC accelerates the proteolysis of HMG-CoA reductase protein (Fitzky *et al.* 2001), which results in a reduced flux through the cholesterol biosynthetic pathway independent of the feedback regulation by SREBP2 (Steiner *et al.* 2000).

Our results show that one direct effect of simvastatin treatment in SLOS is the lowering of the 7DHC levels, most probably due to a further inhibition of HMG-CoA reductase activity as can be deduced from the decreased 7DHC levels observed in the SLOS-4 cells, which completely lack 7DHCR activity. In line with the feedback regulation mechanism discussed above, this induces an increase in gene transcription of the genes encoding cholesterol biosynthetic enzymes including *DHCR7*, which results in an increase of 7DHCR protein and, consequently, an increase in residual 7DHCR activity. Since in SLOS, 7DHCR catalyses the rate-limiting step, this increase in residual 7DHCR activity will lead to an increased flux through the cholesterol biosynthetic pathway causing a further lowering of 7DHC levels and an increase in cholesterol levels, as observed in the patients. The advantage of using simvastatin is that the drug passes the brain-blood barrier and thus also has this effect in the brain, as was confirmed by the previously reported decrease in 7DHC and increase in cholesterol levels in cerebrospinal fluids of patients SLOS-1 and SLOS-3 (Jira *et al.* 2000). This concept predicts that milder patients with relatively high residual 7DHCR activity should show the best response to simvastatin treatment. Indeed, in our study we found a good correlation between the clinical severity of the patient, the residual activity of 7DHCR in cells of the patient and the efficacy of the simvastatin treatment. Patients SLOS-1 and SLOS-3 displayed higher residual 7DHCR activities and showed a better response, biochemically as well as clinically, than patient SLOS-2. This is also supported by the reported outcome of simvastatin treatment in two more severely affected SLOS patients, who did not clinically benefit from the treatment and of whom the most severely affected patient showed hepatoxic side effects (Starck *et al.* 2002).

In conclusion, our combined results show that simvastatin treatment may provide a good long-term therapeutic option in SLOS patients with a mild presentation, but not in severe SLOS cases. Based on our promising results, the simvastatin treatment is currently tested in a larger cohort of SLOS patients in a multicenter European trial.

Conflict of interest statements

None declared.

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Chapter 8

Novel mutations in the 7-dehydrocholesterol reductase gene of 13 patients with Smith-Lemli-Opitz syndrome

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SUMMARY

Smith-Lemli-Opitz syndrome (SLOS) is caused by mutations in the DHCR7 gene leading to a deficient activity of 7-dehydrocholesterol reductase (DHCR7; EC 1.3.1.21), the final enzyme of the cholesterol biosynthetic pathway resulting in low cholesterol and high concentrations of its direct precursor 7-dehydrocholesterol in plasma and tissues. We here report mutations identified in the DHCR7 gene of 13 children diagnosed with SLOS by clinical and biochemical criteria. We found a high frequency of the previously described IVS8-1G>C splice acceptor site mutation (two homozygotes, eight compound heterozygotes). In addition, thirteen missense mutations and one splice acceptor mutation were detected in eleven patients with a mild to moderate SLO-phenotype. The mutations include three novel missense mutations (W182L, C183Y, F255L) and one novel splice acceptor site mutation (IVSG-1G>T).

Two patients, homozygous for the IVS8-1 G>C mutation, presented with a severe clinical phenotype and died shortly after birth. Seven patients with a mild to moderate SLO-phenotype disclosed compound heterozygosity of the IVS8-1 G>C mutation in combination with different novel and known missense mutations.

KEY WORDS: Smith-Lemli-Opitz Syndrome, 7-DHCR, 7-dehydrocholesterol-7-reductase, mutations, cholesterol

INTRODUCTION

Smith-Lemli-Opitz syndrome (SLOS MIM 270400) is an inherited disorder of variable severity caused by a deficient activity of 7-dehydrocholesterol reductase (DHCR7; EC 1.3.1.21), the final enzyme of the cholesterol (Kandutsch-Russell) biosynthetic pathway. Low cholesterol and high concentrations of its direct precursor 7-dehydrocholesterol (7DHC) and its isomer 8-dehydrocholesterol (8-DHC) in blood and tissues are the biochemical hallmarks of the syndrome (Smith *et al*. 1964; Irons *et al*. 1993; Tint *et al*. 1995). The plasma sterol concentration generally correlates with severity and outcome (Tint *et al*. 1995; Witsch-Baumgartner *et al*. 2000). Cholesterol has a pivotal role during embryogenesis where it functions as a transporter-molecule for the sonic hedgehog (Shh) signalling protein, which is essential for normal morphogenesis. Without cholesterol, Shh-transport and/or function is impaired (Porter *et al*. 1996; Lanoue *et al*. 1997; Cooper *et al*. 1998). The sterol derangement in SLOS (accumulation of 7DHC/8DHC) may influence the activation of the Shh-receptor. These findings may explain the phenotypic consequences of the DHCR7 deficiency as observed in this syndrome: microcephaly, a distinctive facies, cataract, syn-/polydac-tyly, and a variety of organ malformations including genital abnormalities ranging from intersex to complete sex reversal in boys.

The human 7-dehydrocholesterol reductase gene (DHCR7) was identified recently and assigned to chromosome region 11q13 (Fitzky *et al*. 1998; Wassif *et al*. 1998; Waterham *et al*. 1998). Over 60 different mutations in the DHCR7 gene of patients with Smith-Lemli-Opitz syndrome have been described (Fitzky *et al*. 1998; Wassif *et al*. 1998; Waterham *et al*. 1998; Yu *et al*. 2000; De Brasi *et al*. 1999; Witsch-Baumgartner *et al*. 2000; Patrone *et al*. 2000; Krakowiak *et al*. 2000). Molecular cloning of the cDNA showed that the human enzyme is a microsomal membrane-bound protein with a calculated molecular weight of 54.5 kDa and nine putative transmembrane segments (Moebius *et al.* 1998). The cDNA has an open reading frame of 1,425 bp coding for a polypeptide of 475 amino acids. Structurally, the protein is strongly related to plant and yeast sterol reductases (Rahier *et al*. 1996; Waterham *et al*. 1998). In humans the mRNA is expressed ubiquitously with the highest expression in adrenal gland, liver, testis, and brain (Moebius *et al*. 1998).

We performed sequence analysis of the DHCR7 gene of 13 children diagnosed with SLOS. Clinical, biochemical and molecular data are presented. This paper is a further delineation of DHCR7-mutations in man.

MATERIALS AND METHODS

Patients

The clinical diagnosis of SLOS in patients included in this study was based on clinical manifestations as previously described (Smith *et al*. 1964; Irons *et al.* 1993. Tint *et al.* 1995). Patients were characterized by a revised scoring system wherein malformations in each of ten embryologically distinct areas were scored to weigh embryologically separate organ systems equally (Kelley *et al*. 2000). The clinical diagnosis was biochemically confirmed by analysis of sterols in plasma and/or tissue by gas chromatography as described earlier (Jira *et al.* 1997). Patient 2, 4 and 13 are Dutch SLOS-patients reported previously (Waterham *et al*. 1998).

Mutation analysis of DHCR7 gene

Genomic DNA was extracted from skin fibroblasts or lymphocytes from patients using the Wizard Genomic DNA Purification Kit according to the instructions of the supplier (Promega). Four sets of DHCR7-specific primers with either -21M13 (5'-TGTAAAACGACGGCCAGT-3') or M13-Rev (5'-CAGGAAACAGCTATGACC-3') extensions were used to amplify by PCR the coding exons 3-9 of the DHCR7 gene. Sequences of these primers are as follows: primer set A for amplification of exons 3+4: DHCR7-IVS2-fw (5'-[-21M13]-GGTGGATGCAACAGGGAAAG-3') and DHCR7-IVS4-rev (5'-[M13-Rev]-GCTCCCCACCTGCTGTGTC-3'); primer set B for amplification of exons 5+6: DHCR7-IVS4-fw (5'-[-21M13]-GTGATCAGGCTGCTTGTGTG-3') and DHCR7-IVS6-rev (5'-[M13-Rev]-TTCTACATCAGGCTGGACCC-3'); primer set C for amplification of exons 7+8: DHCR7-IVS6-fw (5'-[-21M13]-TGGGCTCTCGCTAAGTAAGG-3') and DHCR7-IVS8-rev (5'-[M13-Rev]-TAGCATGTGTCTGCCAAATGC-3'); primer set D for amplification of coding part of exon 9: DHCR7-IVS8-fw (5'-[-21M13]-CGTGTGTCAGAGGCAGAGC-3') and DHCR71510-1490 (5'-[M13-Rev]-AGTTGGAGCTGGGATGCCAG-3'). PCR reactions contained 0.4 m of each primer, 10 mM Tris/HCl pH8.4, 50 mM KCl, 1.5 mM (primer set A) or 2 mM MgCl2 (primer sets C-D), 0.01% w/v BSA, 0.2 mM dNTPs and 1.5 U Taq DNA polymerase (Promega). PCR amplification involved 2 min of denaturation at 96C followed by 17 cycles during which the annealing temperature was lowered with 1C per cycle from 72C to 55C. Every cycle started with 30 s of denaturation at 94C followed by 30 s of annealing and 1.5 min of extension at 72C. These 'touchdown' cycles were followed by 12 cycles composed of 30 s of denaturation at 94C and 1.5 min of annealing/extension at 72C followed by a final extension step of 15 min at 72C. PCR fragments were sequenced by means of -21M13 or M13-Rev fluorescent primers on an Applied Biosystems 377A automated DNA sequencer according to the manufacturer's protocol (Perkin-Elmer).

RESULTS

Thirteen patients (8 Dutch, 4 German and 1 Scottish) with biochemically confirmed SLOS were studied. All had mental retardation, facial anomalies, bilateral second-third toe syndactyly, and failure to thrive. Three patients had a cleft palate and two Hirschsprungs' disease.

Table 1 summarises the patients: karyotyping, plasma (or tissue) sterol concentrations, 7DHC/cholesterol ratio, ancestry, clinical presentation, clinical severity score and age at biochemical diagnosis (age of death in patients 1, 2 and 5). Plasma sterol concentrations are initial values on standard diet without supplemental cholesterol. The patients in both tables are ordered based on the mutational findings shown in Table 2. Clinical severity and outcome in our cohort was strongly correlated with the plasma sterol abnormality. The most predictive biochemical value is the 7DHC/cholesterol ratio in plasma. In general patients with plasma 7DHC/Cholesterol ratio between 0.5 - 1.0 had moderate SLOS. Patients with a plasma ratio <0.5 had a mild presentation and course while plasma 7DHC/Cholesterol ratio >1.0 was associated with severe SLOS. This chemical ratio is a usefull tool considering prognosis and treatment in SLOS but cannot predict severity accurately.

Table 2 shows the results of mutation analysis in the 13 patients by: affected exon and transmembrane domain, nucleotide position of the mutation, amino acid substitution and their effect on the coding sequence (for numbering of nucleotides and amino acids see: Waterham *et al*. 1998) and clinical phenotype. DNA sequencing of 26 SLO-alleles identified 13 different mutations. Among these mutations we detected 4 novel mutations; 3 missense (W182L, C183Y and F255L) and one IVS8-1 G>T splice acceptor site mutation.

Patients 1-11 were either homozygotes or compound heterozygotes for a IVS8-1 splice acceptor mutation. This mutation causes aberrant mRNA splicing which leads to the introduction of 134 basepairs and a frameshift resulting in a truncated protein lacking 154 amino acids of its original C-terminal sequence (Waterham *et al*. 1998). Patient 6 contained a new variant of the IVS8-1 G>C, namely an IVS8-1 G>T resulting in the same truncated protein. In this patient, with a mild SLOS-phenotype, the mutation was combined with a missense 765 C>A (Phe255Leu) mutation at the other allele.

Eleven of the 13 patients (patients 3 - 13) were compound heterozygous for two different mutations altering the sequence of the 7-sterol reductase. The mutational site and effect on the coding sequence seemed to be only partially predictive for clinical and biochemical severity. Although unrelated, patients 7 and 8 had the same genotype with, a mild phenotype, however, the girl was diagnosed earlier in life and had a significantly different plasma sterol level and clinical course. Two sibs (patients 9 and 10) with the same DHCR7-mutations but differing in age, had different plasma sterol values with a similar clinical presentation.

Table 1. Biochemical and clinical data of 13 patients with SLOS

Patients	Karyotype	Plasma sterols			Ratio *)	Ancestry	Clinical Presentation	Clinical Severtity Score (1 - 20)***)	Age at diagnosis (Age at death)
		Cholesterol (μM)	7DHC (μM)	8DHC (μM)					
P1.	46,XX	20	430	266	21.5	Dutch	Poly/syndactyly, microcephaly M. Hirschsprung, pyloric stenosis, nuclear cataract	7	day 1 (day 40)
P2.	46,XY	↓	↑	↑	-	Dutch	Complete sex reversal, horse-shoe kidney cardiac defect, Poly/syndactyly	8	day 1 (day 1)
		in post mortem tissue							
P3.	46,XY	260	300	385	1.15	Dutch	Intersex, microcephaly, poly/syndactyly failure to thrive, photo-sensitivity	4	day 5
P4.	46,XY	595	559	-	0.94	Dutch	Microcephaly, poly/syndactly ambiguous genitalia	5	2 months
P5.	46,XY	900	750	433	0.83	German	Syndactyly, intersex, M. Hirschsprung cleft palate, cataract	8	9 months (11months)
P6.	46,XY	1233	410	342	0.33	Dutch	Syndactyly, cryptorchidism failure to thrive	2	day 10
P7.	46,XX	1338	362	263	0.27	Dutch	Syndactyly failure to thrive	1	3 months
P8.	46,XY	2600	154	108	0.06	Dutch	Syndactyly, cleft palate	2	20 months
P9.	46,XY	1281	239	168	0.19	German	Syndactyly failure to thrive	1	10 months
P10.**	46,XY	1212	181	157	0.15	German	Syndactyly failure to thrive	1	5 months
P11.**	46,XX	2835	57	54	0.02	German	Syndactyly failure to thrive	1	7 years
P12.	46,XY	1456	749	543	0.51	Scottisch	Syndactyly Failure to thrive	1	2 months
P13.	46,XY	300	370	-	1.23	Dutch	Microcephaly, cleft palate, syndactyly small penis, cryptorchidism	3	1 month

*) Ratio = 7DHC/Cholesterol in plasma, **) Siblings, ***) Clinical Severity Score according to Kelley *et al.* 2000.

Table 2. DHCR7 Mutations in 13 patients with SLOS

Patient	Exon	Transmembrane Domain	Nucleotide Change	Amino acid Substitution	Effect on Coding sequence	Clinical Phenotyped
P1	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Severe
	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	
P2c	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Severe
	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	
P3	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Moderate
	6	-	529A>G	Lys198Glu	Missense	
P4c	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Moderate
	7	5	744G>T	Trp248Cys	Missense	
P5	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Severe
	5	-	326T>C	Leu109Pro	Missense	
P6	9	8	IVS8-1G>Ta	G963 134bp-insertion	Frameshift	Mild
	7	6	765C>Aa	Phe255Leu	Missense	
P7	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Mild
	6	3	461C>T	Thr154Met	Missense	
P8	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Mild
	6	3	461C>T	Thr154Met	Missense	
P9	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Mild
	6	4	545G>Ta	Trp182Leu	Missense	
P10b	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Mild
	6	4	548G>Aa	Cys183Tyr	Missense	
P11b	9	8	IVS8-1 G>C	G963 134bp-insertion	Frameshift	Mild
	6	4	548G>Aa	Cys183Tyr	Missense	
P12	4	2	278C>T	Thr93Met	Missense	Mild
	7	5	725G>A	Arg242His	Missense	
P13c	5	-	356A>T	His119Leu	Missense	Moderate
	7	5	730G>A	Gly244Arg	Missense	

a) New mutation, b) Siblings, c) Patients whose mutations have been previously reported, d) Phenotype according to the severity score of Kelley *et al*. 2000.

DISCUSSION

SLOS is an autosomal recessive disorder. The DHCR7 gene mutations identified in our SLOS-cohort and by others (Fitzky *et al*. 1998; Wassif *et al*. 1998) are likely to have deleterious effects on the function of the DHCR7 protein. Cholesterol is an important constituent of the cell membrane of most eukaryotic cells and has important interaction with proteins, which control embryonic development. In addition, cholesterol acts as the precursor for steroid hormones, bile acids and myelin formation in the brain, spinal cord and peripheral nervous system.

The IVS8-1 G>C mutation causes aberrant splicing producing an mRNA with 134 base-pairs of retained intron 8 sequence at nucleotide position 963 which leads to a frame shift and a stopcodon at nucleotide 1235 (TGA). This premature termination of translation results in a truncated and inactive DHCR7 protein as was confirmed by enzyme activity measurements in the patients homozygous for this mutation (Waterham *et al*. unpublished). Fitzky predicted that homozygotes for this mutation would be lethal if affected, which is confirmed by patients 1 and 2 from our study. These two SLOS individuals manifest a severe and lethal phenotype with microcephaly, cataract, polydactyly, multiple organ (cardiac, renal and intestinal) malformations leading to early death. In patient 2, with a 46 XY karyotype, this mutation led to apparent sex reversal (Waterham *et al*.1998). Remarkably, in a recent paper it has been reported that not all SLOS-individuals with two *null* mutations, although severely affected, are lethal (Witsch-Baumgartner *et al*. 2000).

Patients 3 - 11, who overall presented with a moderate to mild SLOS phenotype all were compound heterozygotes for a IVS8-1 splice acceptor site mutation on one allele in combination with a missense mutation on the other allele. Patient 5, with a severe clinical phenotype, is the only exception. The Leu109Pro mutation may affect the enzymatic activity more severly than the other missense mutations in this group. The clinical severity and biochemical features of our patient 5 are similar to patient D73 with the same combination of mutations described by Witsch-Baumgartner *et al*. 2000.

The five most frequent mutations described by Witsch-Baumgartner *et al*. 2000 in 168 SLOS-alleles are: IVS8-1 G>C, R404C, T93M, W151X, V326L with a frequency of: 29, 11, 8, 8 and 7 percent, respectively. Apart from the IVS8 G>C mutation, most of the mutations detected in SLOS are single amino acid substitutions. Mutations occur further throughout the whole gene without evident hotspots. In the region between codon 242 to 248, however, 5 different mutations have been detected until now (R242C, A247V: Witsch-Baumgartner *et al*. 2000; G244R, W248C: Waterham *et al*. 1999; R242H: Patient 12 from this paper). In two of these SLOS patients codon 247 and 248 are affected by a point mutation, changing an alanine into a valine and a tryptophan into a cystein, respectively. These two missense mutations are found within ten basepairs downstream of a "TGGA" motif at nucleotide 742 (according to the nucleotide sequence of Waterham *et al*. 1998). This motif is hypothesised to be, in general, a hotspot for point mutations or deletions (Cooper *et al*. 1995).

Most of the missense mutations found in patients 3, 4, 6, 9-13 are within the so called "sterol-sensing domain" containing five transmembrane segments as proposed by Fitzky *et al*. 1999 and Bae *et al*. 1999 (Figure 1).

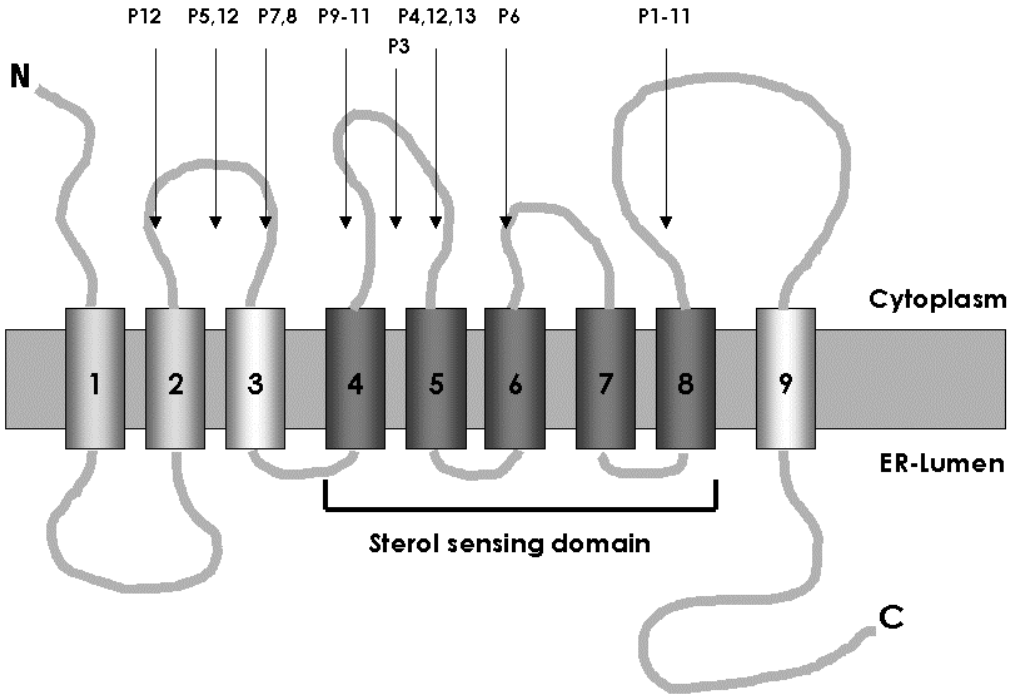

Figure 1. Predicted membrane topology of the 7-dehydrocholesterol reductase. The topology model for DHCR7 is based on the data of Fitzky et al., 1998. Transmembrane segments 4 to 8 (dark grey) represent the putative, highly conserved, sterol-sensing domain (Bae *et al.* 1999). Arrows (↓) mark the position of the various mutations found in patients 1 to 13.

Sequence comparisons of these specific DHCR7 membrane-spanning segments show strong homology to segments found in four other human proteins. These proteins in nature all have crucial interaction with sterols: 1] HMG-CoA reductase (Olender *et al*. 1992), 2] Niemann-Pick C1 gene product (Loftus *et al*. 1997), 3] Sterol regulatory element-binding protein-SCAP (Nohturfft *et al*. 1998), and 4] the morphogene receptor PATCHED (Loftus *et al*. 1997). In HMG-CoA reductase the orientation of the sterol-sensing domain (membrane-spanning segments 2 to 6) has a similar configuration as segments 4 to 8 of DHCR7. According to the hydrophobicity in the DHCR7 protein, membrane-spanning segments 4 to 8 are closely spaced and joint by relatively short hydrophilic amino acid loops. In contrast to DHCR7, SCAP and HMG-CoA reductase have a long COOH-terminal domain that projects into the cytosol (Nohturfft *et al*. 1998). Mutations that alter, interfere with or truncate the sterol-sensing domain of the DHCR7 protein are likely to cause a more severe biochemical and clinical SLOS-phenotype however making predictions of the SLOS phenotype from genotype, continues to be difficult since there is significant clinical and biochemical variability among genetically identical or comparable infants (see patients 7, 8 and 10, 11).

In our study, six of the eight Dutch SLOS patients were carriers for the IVS8-1 G>C mutation on one or both alleles. The high proportion of this severe frameshift mutation could explain the lower incidence of SLOS occurring in the Dutch population. One could hypothesise that in homozygous individuals this particular genetic defect may lead to intra-uterine or early neonatal death before the diagnosis SLOS is made. On the other hand, in healthy carriers, mutations in cholesterol biosynthesis could result in an evolutionary advantage either reducing the risk of cardiovascular morbidity by decreasing individual plasma cholesterol levels or by increased Vitamin D levels, preventing the occurrence of rickets (a common pediatric disease in the past) as suggested by Kelley and Hennekam (2000). We analysed plasma of some parents of our SLOS-patients (data not shown) and found plasma cholesterol levels below the 50th centile with a mean of 4.4 mmol/l. We found the IVS8-1 G>C in SLOS-patients not only from different sites in the Netherlands but also in patients from Germany. The same IVS8-1 G>C mutation was detected in one of 90 normal adult Caucasian Americans; but not among 121 Africans from Sierra Leone, 120 Caucasians from Finland, 95 Chinese or 103 Japanese adults by Yu *et al.* 2000. From the number of cases diagnosed in our laboratories we estimate a SLOS frequency of ~ 1/100,000 in the Netherlands. This frequency is lower than the incidence of 1 / 40,000 - 50,000 for the SLOS described by Opitz *et al.* 1994 and Cunniff *et al.* 1997. Currently, we are working on a more accurate estimation of the carrier frequency of the mutation in the Dutch population. The high incidence found in our European SLOS-study (50%) confirms and exceeds the findings of Yu *et al.* 2000 and Bataille *et al.* 2000 who identified the IVS8-1G>C mutation in 21 of 66 (32%) and 18 of 52 (35%) SLOS-alleles, respectively. The development of a simple PCR-RFLP can be used as a screening-method for detecting this frequent SLO-mutation (Yu *et al*. 2000). The mutation seems to be an ancient variant frequently occurring in SLOS. In order to determine whether this and other mutations have been introduced into the European, and later

American, population by a single founder, it is necessary to study the chromosome 11 haplotype of the patients carrying mutations.

In conclusion, our study showed that of the known SLOS mutations (including missense, nonsense, frameshift and deletions) the IVS8-1 G>C is the most frequent one occurring in our cohort of SLOS patients from Western-Europe and found in 13 of 26 studied alleles. This occurrence of 50% is the highest of all published studies, leading to lethal phenotypes in two homozygotes, and mild to moderate SLOS-phenotype when combined with missense mutations. We identified four novel mutations in the DHCR7 gene. Further molecular genetic studies will enable insight in genotype-phenotype correlations in SLOS and determine carrier frequency of specific DHCR7 mutations in various populations. Additional search for environmental, other genetic and compensatory determinants that can modify the phenotypical consequences of the functional DHCR7 deficiency in SLOS is needed.

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Chapter 9

Smith-Lemli-Opitz-like phenotype caused by a distal 7q36 deletion disrupting the human Sonic Hedgehog (SHH) gene

Submitted

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ABSTRACT

We report on a newborn boy with clinical manifestations of the Smith-Lemli-Opitz syndrome (SLOS, also known as RSH syndrome) including prenatal growth retardation, microcephaly, hyperplasia of the upper alveolar ridges, micropenis with glandular hypospadias and bilateral 2-3-toe syndactyly, he also had unilateral choanal atresia and severe an/microphthalmia. Gas chromatographic analysis of plasma sterols showed normal concentrations of the cholesterol precursor 7-dehydrocholesterol, thereby excluding a Δ^7-dehydrocholesterol reductase deficiency, the primary cause of SLOS. Chromosome analysis demonstrated a distal 7q36.1 deletion. Extensive fluorescence *in situ* hybridization analysis showed that the holoprosencephaly (HPE3) critical gene region on chromosome band 7q36, inclusive the *Sonic Hedgehog (SHH)* gene, was deleted. A mutation in the *SHH* gene of the non-deleted allele could be excluded. This case suggests that in addition to patients with a defect in cholesterol biosynthesis, patients with a distal 7q deletion may present with a SLOS-like phenotype. Some, if not all, of the clinical findings of SLOS may be due to a defective SHH signalling pathway caused by sterol disturbances, which is consistent with results obtained in experimental animals.

KEY WORDS: Smith-Lemli-Opitz syndrome (SLOS), Sonic Hedgehog (SHH) gene, terminal 7q deletion, holoprosencephaly (HPE3).

INTRODUCTION

Smith-Lemli-Opitz (SLOS or RSH syndrome), is a multiple congenital anomalies/mental retardation syndrome caused by a deficient Δ^7-dehydrocholesterol-reductase activity, the final enzyme of the cholesterol biosynthesis pathway (Smith *et al.* 1964; Tint *et al.* 1994; Tint *et al.* 1995; Kelley & Hennekam, 2000). The clinical diagnosis of SLOS requires experience in clinical morphology. The holoprosencephaly (HPE) sequence is a genetically heterogeneous malformation complex that affects midline development of forebrain and midface. The strongly variable clinical presentation within types of HPE ranges from severe brain malformations (lethal expression) to minimal facial anomalies (single central maxillary incisor). Distal deletions of the long arm of chromosome 7 are associated with HPE (Young *et al.* 1984; Lurie *et al.* 1990; Bogart *et al.* 1990; Frints *et al.* 1998a).

Sonic Hedgehog (SHH) is one of the human segmentation genes. Mutations in this gene have been shown to cause familial holoprosencephaly in the subset linked to chromosome band 7q36 (Roessler *et al.* 1996; Belloni *et al.* 1996). Cytogenetic deletions and/or rearrangements of this region on the distal long arm of chromosome 7 containing *SHH*, and translocations that may suppress gene expression through a position effect are common mechanisms leading to HPE (Gurrieri *et al.* 1993; Benzacken *et al.* 1997; Roessler *et al.* 1997; Frints *et al.* 1998b; Vance *et al.* 1998). Evidence was obtained from animal experiments that cholesterol biosynthesis is essential for normal SHH protein function and suggested that the phenotypic characteristics of SLO syndrome may be mediated by SHH. Here we describe a patient with apparent SLOS and HPE with a terminal deletion of the long arm of chromosome 7. This observation reinforces the notion that the pathogenesis of SLOS involves dysfunction of the SHH signalling pathway and that impairment of sterol disturbances causes a wide range of genetic defects.

CLINICAL REPORT

The index patient, a boy, the first child of nonconsanguineous caucasian parents, was born at term, after an uneventful pregnancy. His birthweight was 2,590 g (15th centile), length was 43 cm (3rd centile), and occipitofrontal circumference (OFC) was 29 cm (<< 3rd centile, -3 SD). Clinical examination showed microcephaly, inner canthal distance/outer canthal distance = 1.8 cm / 6.5 cm (10^{th} centile / 60^{th} centile), deeply set invisible eyes, bilateral epicanthus, narrow upslanting palpebral fissures, midface hypoplasia, depressed nasal bridge, small nose, microphthalmia, retro/micrognathia with upper alveolar ridge hyperplasia. His ears were small, malformed, and apparently low-set with a prominent antihelix, and large earlobes (Figure 1A and 1B). There was complete bilateral syndactyly of toe 2 and 3 (Figure 1C). External genitalia were abnormal with a chordee and micropenis of 1.5 cm (-2SD) (Figure 1D).

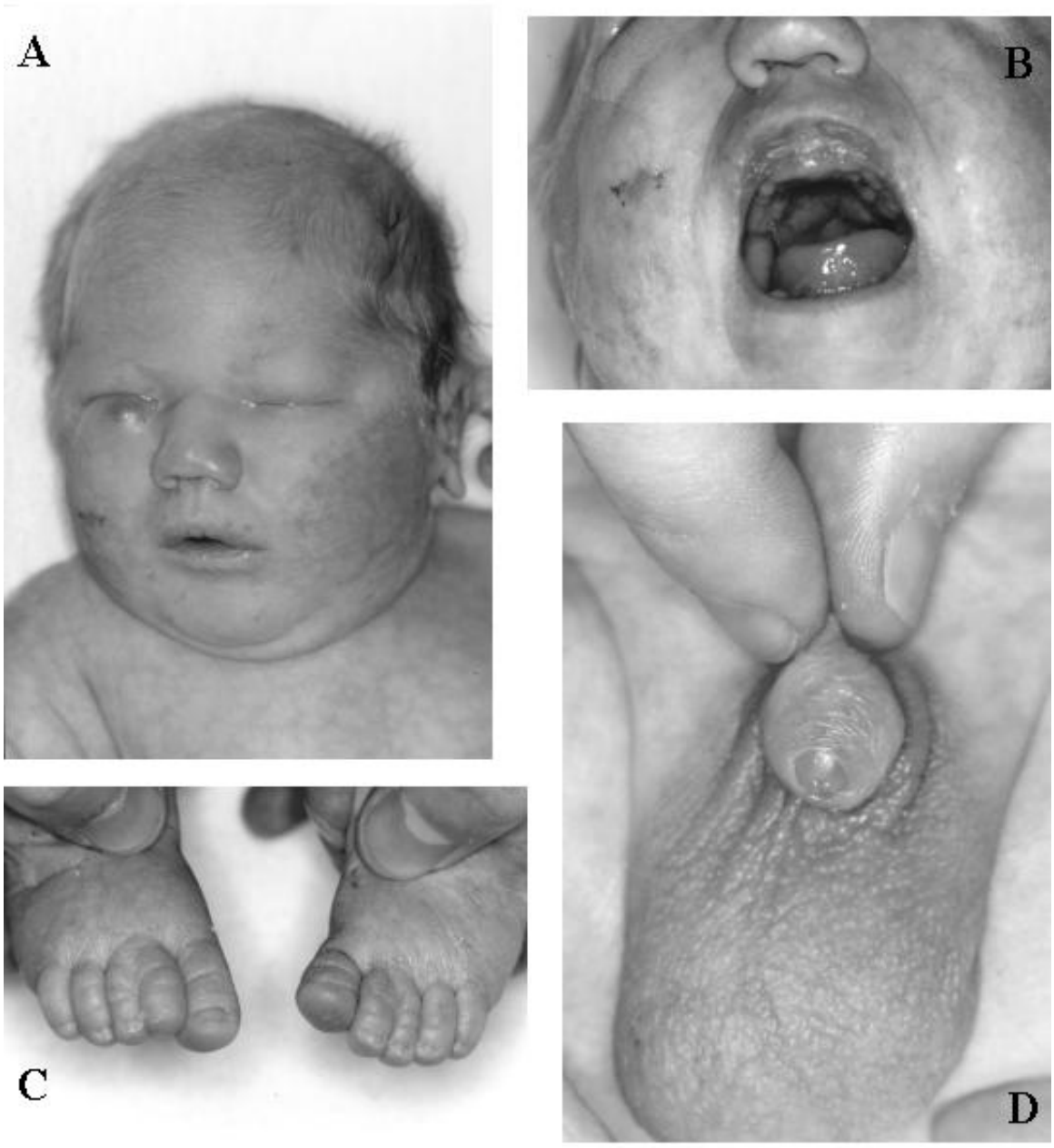

Figure 1. Patient's appearance at birth.

Unilateral choanal atresia was suspected and confirmed on CT-scanning. Deep tendon reflexes were diminished. MRI-scanning, ophthalmic examination and orbital ultrasonography showed bilateral anophthalmia with a dysgenetic cyst on the right with normal corpus callosum and (midline) brain structures (Figure 2).

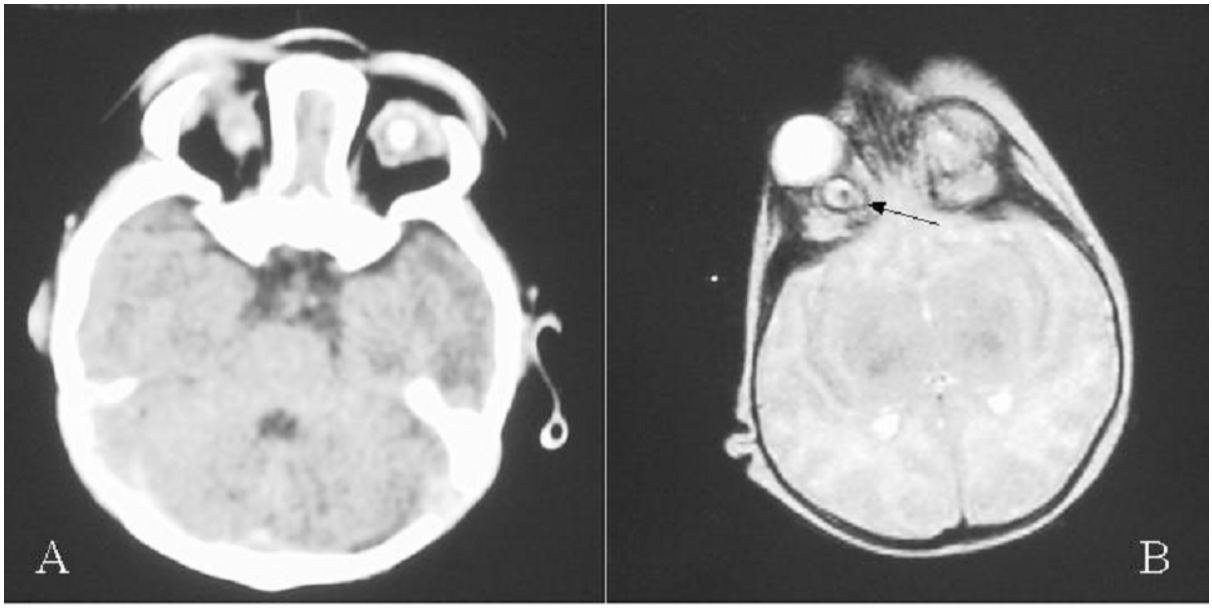

Figure 2. CT- (A) and MRI-scan (B) of the face and brain disclosing anophthalmia with cyst (arrow).

Radiological examination of the spine and hips showed a normal vertebral spine and sacrum but left hip dysplasia with an acetabular angle of 36 degrees. Echocardiography excluded cardiac malformations. No renal abnormalities were detected by ultrasound. The electro-encephalography was normal. Since early detection of deafness in this blind patient is important, a brain stem auditory evoked potential investigation was performed. No respons could be obtained with this test, suggesting the patient might be deaf. Endocrine status was normal; gonadotrophins, LH: 2.0 U/l, FSH: <0.6 U/l, testosterone: 6.0 nmol/l, ACTH: 4.1 pmol/l, cortisol: 0.20 mmol/l, TSH: 2.47 mU/l, tyroxine; 191 nmol/l, free T4: 16.6 pmol/l. TORCHES screening negative.

Feeding problems were treated by initial nasogastric tube feeding. At the age of 4 months he developed severe vomiting, pyloric stenosis was surgically corrected and during this operation a percutaneous gastric tube was implanted. At this age his weight was 5220 g (3rd centile) and the OFC 36.2 cm (still below 3rd centile). The inner canthal distance measured 22 mm (50th centile). He was delayed in psychomotor development, however, due to his severe sensory impairment a judgement about the severity of the development retardation could not be made at that age. At the age of 14 months a central maxillary incisor became visible. At the age of 16 months he started to roll over and at 24 months he was able to sit without support.

At the age of 5 years and 9 months the boy still has a percutaneous gastric tube and is not interested in oral feeding. His height is 104 cm (-3.3 SD), weight is 16.5 kg (-2.5 SD) and OFC 45.5

cm (-4.9). Although he can stand and walk independently from the age of four his gait is limited. Hearing has improved gradually and a slight hearing impairment of 25 dB is detectable after one adenoidectomy and three times placement of bilateral myringotomy tubes between the age of 2 ½ and 5 ½ years. Nevertheless there is no passive or active language development. At the age of 5 9/12 years his developmental status was examined in the context of degree of visual the Reynell-Zinkin scales (Reynell *1978*). His scores on the five developmental areas of social adaptation, sensori-motor understanding, exploration of environment, verbal comprehension and expressive language were 9-11 months, 1.1-1.4 years, 9-11 months, 1.2-1.7 years and 5-7 months, respectively.

MATERIALS AND METHODS

Defects of cholesterol biosynthesis investigated at birth by gas chromatographic analysis of plasma sterols and chromosome analysis of two peripheral blood lymphocyte cultures were performed. Parents had normal chromosomes, establising a *de novo* origin of the 7q deletion. In order to define more precisely the deletion-breakpoint and the presence/absence of genes located at this chromosomal region, which are important for normal extremity, facial and brain development, we performed fluorescence *in situ* hybridization (FISH) analysis on metaphase chromosomes. A panel of informative C.E.P.H. yeast artificial chromosomes (YACs) was selected all containing well-defined marker loci and one cosmid clone; 738D7, 761H5, 744A8, 850G5, Y965C12, COS2000 (Morton *et al.* 1987). The deletion-breakpoint was concluded to reside between YACS 761H5 and 744A8, as illustrated in Fig. 3.

To exclude the possibility of a coincidental mutation on the other allele we performed mutational analysis of the complete coding region and intron-exon junctions of the non-deleted *SHH* gene by direct sequencing using primers as described before (Roessler *et al.* 1996, 1997a; Nanni *et al.* 1999). No mutation in the other SHH allele could be detected.

RESULTS AND DISCUSSION

Sterol analysis revealed normal concentrations of cholesterol (2235 µmol/l), 7-dehydrocholesterol (<15 µmol/l) and 8-dehydrocholesterol (<15 µmol/l) (Jira *et al.* 1997). Chromosome analysis of two peripheral blood lymphocyte cultures demonstrated a terminal 7q deletion: 46,XY,del(7)(q36.1→qter). The patient reported here illustrates the clinical presentation of SLOS and minimal expression of HPE. After ruling out a Δ^7-sterol reductase deficiency, the hallmark of SLOS, chromosomal investigation

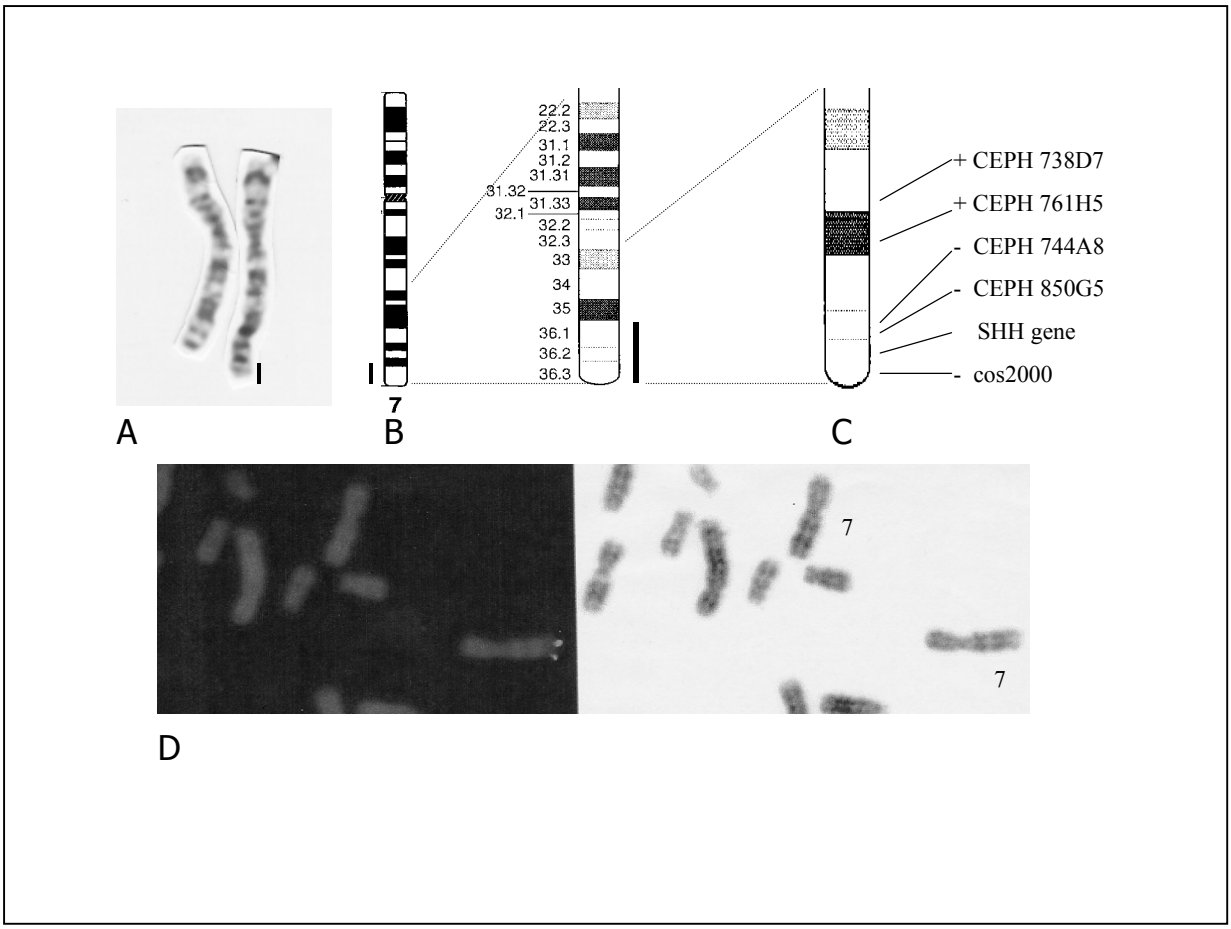

Figure 3. A. Chromosomes 7 of the patient. De left chromosome has a terminal deletion. The size of the deletion is indicated by a bar along the normal chromosome 7. B. Ideogram of the entire (left) and terminal part of the long arm (right) of chromosome 7. The deletion extends from band q35 to qter. C. Location of the yacs (CEPH-probes) and cosmid used for in-situ-hybridisation. The probes located in band q36are deleted on the derivative chromosome 7. The SHH-gene is located between CEPH850G5 and cos2000 and thus deleted in the patient. D. FISH results with the subterminal probe cos2000. A fluorescence signal is visible on the normal, but not on the derivative chromosome 7.

applying FISH showed a deletion of the terminal long arm of chromosome 7. The deletion-breakpoint resides between YAC clone markers 761H5 and 744A8, preserving the *Eng2 (Hox1)* gene but eliminating the *SHH* gene. The deleted distal arm contains about 12 cM and at least 30 known human genes.

SLOS is an autosomal recessive disorder showing considerable phenotypic variability including craniofacial abnormalities (microcephaly, ptosis, cataract, cleft palate, upper alveolar ridge hyperplasia), limb anomalies (postaxial syndactyly and/or polydactyly), incomplete development of male genitalia, failure to thrive, gastrointestinal abnormalities such as pyloric stenosis and Hirschprung disease, mental retardation and various internal anomalies including holoprosencephaly in some cases (Smith *et al.* 1964; Tint *et al.* 1994; Tint *et al.* 1995; Nowaczyk *et al.* 2001). Defective conversion of 7-dehydrocholesterol (7-DHC) to cholesterol in SLOS patients is due to mutations in the

Δ^7-sterol-reductase gene that was mapped to chromosome band 11q13 (Fitzky *et al.* 1998; Waterham *et al.* 1998; Wassif *et al.* 1998). Less severe symptoms of HPE can occur in SLOS (Kelley *et al.* 1996). However, not all patients clinically suspected of SLOS can be confirmed biochemically (Guzetta *et al*. 1996; Krajewska-Walasek *et al.* 1999).

Some of the malformations seen in SLOS can be mimicked in experimental animal models utilizing specific cholesterol lowering drugs: BM 15.766, AY-9944 and YM 9429 inhibiting the Δ^7-reductase enzyme in cholesterol biosynthesis (Honda *et al.* 1996; Kolf-Clauw *et al.* 1996; Dehart *et al.* 1997; Roux *et al.* 2000). The most characteristic malformations of this rat/mouse "holoprosencephalic syndrome" include cyclocephaly, cyclopia, monorhinia, palatoschisis and agenesis of the pituitary gland in up to 80 % of subjects (Honda *et al.* 1996; Kolf-Clauw *et al.* 1996).

Although some of these defects, especially holoprosencephaly, were described previously in cholesterol-deficient rats (Dehart *et al.* 1997), limb and external genital defects as seen in SLOS have not been seen in animal experiments. Some similarities between the SLO syndrome in humans and the AY-9944/BM 15.766 animal experimental teratogenic effects suggest, but do not explain, that the Δ^7-reductase enzyme deficiency is directly responsible for the malformations.

SHH (MIM 600725) is one of the genes identified to cause HPE in humans. It maps to 7q36 that is near the HPE3 locus (MIM 142945) (Gurrieri *et al.* 1993; Belloni *et al.* 1993; Muenke *et al.* 1994; Roessler *et al.* 1997). *SHH* mutations have been identified in HPE patients and include missense, nonsense, deletion, insertion, and frameshift mutations located throughout the gene (Roessler *et al.* 1996; Roessler *et al.* 1997; Nanni *et al.* 1999; Odent *et al.* 1999; Wallis *et al.* 2000). Proper function of SHH depends on cholesterol modification. Specifically, cholesterol is important for the way Hedgehog proteins spread through tissues where they split in two. The hydrophobic modification is apparently necessary to restrict hedgehog signaling to the proper spatial pattern in the developing embryo (Porter *et al.* 1996a; Porter *et al.* 1996b; Cooper *et al.* 1998). The cholesterol-Hedgehog complex attaches to its 12-span transmembrane receptor proteins Patched-1 and Patched-2 in target cells (Villavicencio *et al.* 2000; Ingham and McMahon, 2001). Although the mechanism of Shh-induced signaling pathway in vertebrates is not completely defined, it is known to regulate dorso-ventral patterning within the neural tube, limb, lung, genital, ocular, and retinal development as well as craniofacial morphogenesis (Marigo *et al.* 1995; Hall *et al.* 1995; Kumar *et al.* 1996; Krishnan *et al.* 1997; Imokawa *et al.* 1997; Levine *et al.* 1997, Helms *et al.* 1997; Hayes *et al.* 1998; Ingham and McMahon 2001). Dysregulation of the sonic Hedgehog-Pathed-Gli pathway leads to several human diseases, including birth defects and cancers (Villavicencio *et al.* 2000).

Through recent animal studies there is growing evidence linking cholesterol deficiency-based birth defect and the *SHH* gene. In SLOS the effects of perturbed cholesterol biosynthesis are shown confirming the important role of cholesterol in transport, activation and receptor-binding of embryonic signaling *SHH* proteins (Porter *et al.* 1996a; Porter *et al.* 1996b; Cooper *et al.* 1998; Ingham and McMahon 2001). *SHH* gene studies in the mouse showed that this gene plays a critical role in

patterning of vertebrate embryonic tissues, including brain, spinal cord, axial skeleton and limbs. Shh mutated mice show a SLOS-like phenotype with craniofacial and limb abnormalities (Chiang *et al.* 1996).

Cholesterol deficiency *in utero*, whether genetic, environmental or multifactorial, can result in malformations representing a wide spectrum of severity. In patients presenting with malformation of brain, face, eye, limbs, or genitalia defects in cholesterol biosynthesis and SHH-functioning should be considered and ruled out (Lanoue *et al.* 1997; Kelley *et al.* 1996).

Our patient presented with a SLOS more than an HPE phenotype. MRI scanning of the brain revealed no gross abnormalities. Initially there were no signs of holoprosencephaly at all. Later development of a single central maxillary incisor disclosed a minor HPE-variant. Microphthalmia has been reported once previously in a fetus with holoprosencephaly and sacral agenesis due to a deletion 7q36→qter (Morichon-Delvallez *et al.* 1993). Thus, we would like to reinforce two other observations made by Berry (*et al.* 1998) and Warburg (*et al.* 1995). The first described a family of apparent SLOS and Miller-Dieker syndrome (MDS) with segregating translocation t(7;17)(q34;p13.1) presenting with a phenotype similar to that of our patient. The patient with MDS had inherited the derivative chromosome 17, thus lacking 17p13.1, resulting in MDS. The patient with SLOS phenotype had inherited the abnormal chromosome 7 with a deletion of 7q34→qter. The latter authors documented the clinical findings of a boy with blepharophimosis, epicanthus inversus, right atresia and left choanal stenosis and developmental delay with genital malformations. This patient had a single mesial maxillary incisor suggesting the HPE anomaly but a normal CT scan of the brain. Chromosome analysis showed a *de novo* terminal deletion of the long arm with a breakpoint at band 7q36.

In the perspective of the facial anomalies (a long philtrum, upper alveolar ridge hyperplasia, and microcephaly) documented above we conclude that the patient had the SLOS. A single central maxillary incisor, as apparent in our patient, can also occur in biochemically and genetically proven SLO patients (own observation). In SLOS genital anomalies (65%) 2-3 toe syndactyly (97%) and pyloric stenosis (14%) as disclosed by our patient are common (Kelley, Hennekam, 2000). These phenomena have to be mediated by impaired SHH function rather than a primary disruption of cholesterol biosynthesis. Mental retardation and failure to thrive are non-specific manifestations in many syndromes.

The existence of phenocopies of SLOS after exclusion of a Δ7-reductase deficiency may point to a defect in important morphogenetic signaling pathways, such as the Sonic Hedgehog-Patched-Gli pathway, causing similar malformations. High-resolution cytogenetic studies and mutation analyses focusing on chromosome band 7q36, and other pathway genes in these patients therefore should always be considered.

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Chapter 10

Summary and General Discussion

SUMMARY AND GENERAL DISCUSSION

The Smith-Lemli-Opitz syndrome (SLOS) is a developmental disorder associated with a broad spectrum of congenital anomalies and is one of the recently discovered defects of cholesterol biosynthesis in humans (Smith *et al.* 1964; Irons *et al.* 1993; Tint *et al.* 1995; Fitzky *et al.* 1999; Battaile & Steiner 2000). In **chapter 1** the Smith-Lemli-Opitz syndrome and its enzymatic defect, the 7-dehydrocholesterol reductase (DHCR7) deficiency, is introduced. The aim of our study is described in **chapter 2** and current knowledge about diagnosis, clinical presentation, therapy, molecular genetics and pathophysiology in SLOS are reviewed in **chapter 3** (Jira *et al.* 2003).

Biochemical Diagnosis

The biochemical hallmark of SLOS was disclosed in 1993 (Irons *et al.* 1993). In **chapter 4** we studied cholesterol levels in plasma of 8 SLOS patients with gas-chromatography (GC) and with the common oxidase method used routinely in clinical chemistry laboratories to measure cholesterol in body fluids. We described pitfalls in measuring plasma cholesterol in SLOS with this common cholesterol-oxidase method.

First of all, healthy newborns normally may have low plasma cholesterol levels around or even below 2000 μmol/L. All SLOS patients studied had increased 7DHC (143 - 848 μmol/L) and 8DHC (107 - 555 μmol/L) plasma levels, diagnostic for SLOS. In healthy control groups, cholesterol-precursor concentrations are below 10 μmol/L. Secondly, we could demonstrate that the oxidase method for measuring cholesterol in plasma cannot discriminate between cholesterol, 7DHC or 8DHC. When measured with GC, all patients had a plasma cholesterol concentration (20 - 2030 μmol/L) below the reference range for age. In contrast, only 6 of 8 patients had decreased cholesterol plasma levels when measured with the cholesterol-oxidase method. Since this method in fact measures the sum of cholesterol and its precursors 7DHC and 8DHC, it gives falsely high cholesterol levels. This could potentially lead to erroneous conclusions. The GC technique detects separate precursor and cholesterol peaks. This enables a proper biochemical diagnosis and can be of use in scoring severity and in monitoring therapy in SLOS (Jira *et al.* 1997a).

Therapy

Chapter 5 and 6 describe new treatment strategies in SLOS. Conventionally, patients were treated with cholesterol and/or bile acid suppletion without impressive biochemical or clinical response.

In **chapter 5** erythrocytes of SLOS patients were incubated in normal donor plasma and we observed a rapid exchange between precursors (7DHC and 8DHC) from the erythrocyte membrane and cholesterol from the plasma. When normal erythrocytes were incubated in SLOS plasma, a similar uptake velocity of precursors into normal erythrocyte-membranes could be detected. This motivated our group to evaluate the effect of blood exchange transfusions in two young SLOS patients. Repeated

blood exchange transfusions showed a significant reduction in circulating cholesterol-precursor levels and increases of cholesterol to normal levels in plasma. The procedure, however, is invasive for the patient, time-consuming and the biochemical effect of blood exchange transfusions in plasma was limited to three days (Jira *et al.* 1997b). Therefore, this cannot be considered as a long-term therapeutical approach.

We incubated cultured SLOS fibroblasts with a HMG-CoA reductase inhibitor, simvastatin, to study its effect on sterol synthesis. As expected, we observed a marked reduction of accumulated 7DHC and 8DHC precursors. However, the unexpected observation that cholesterol concentration increased was surprising. This improvement in precursor/cholesterol ratio in cultured SLOS fibroblasts motivated us in treating SLOS patients with simvastatin. **Chapter 6** describes the long-term effect (23 and 14 months) of oral HMG-CoA reductase inhibition with simvastatin in two SLOS patients. A significant reduction of total precursors (7DHC+8DHC), to 28% and 33% of the initial plasma values, could be documented. Cholesterol concentration normalised unexpectedly by a more than two-fold increase in that period for both patients. The 7DHC+8DHC/Cholesterol ratio improved from 0.47 to 0.06 and 0.32 to 0.04 in plasma and from 1.22 to 0.12 and from 0.62 to 0.07 in erythrocyte membranes, respectively. During the follow-up period, morphometric parameters (length, weight, head circumference) and neuromotor development improved in both patients. The therapy was well tolerated and no unwanted clinical or biochemical side effects occurred. This is the first study in which the blood cholesterol level in SLOS patients normalised with a simultaneous significant decrease in precursor levels. There was a lasting biochemical improvement with an encouraging clinical effect (Jira *et al.* 2000).

The decreased accumulation of 7DHC and 8DHC in SLOS patients treated with an oral HMG-CoA reductase inhibitor could be expected. The beneficial effect in increasing cholesterol concentration in SLOS patients treated with statin, however, was unexpected. Further studies were designated to explain this observation in both fibroblasts and patients' plasma. **Chapter 7** describes three relatively mild SLOS patients treated with simvastatin for 80, 49 and 31 months, respectively. We determined the efficacy of the treatment on development and general well being of the patients. In addition, we studied *in vitro* the effect of simvastatin on DHCR7 expression and activity in primary skin fibroblast cells of the patients. All patients tolerated the simvastatin therapy well without apparent clinical or biochemical side effects. During treatment, a marked decrease of plasma 7DHC levels in all three and a concomitant increase of the plasma cholesterol concentration was observed in two patients. Moreover, two patients showed good and one patient moderate clinical improvement of growth and development. The *in vitro* studies with cultured primary skin fibroblast cells of the patients showed that simvastatin induces an increase in gene transcription of the *DHCR7* gene, which results in an increase of DHCR7 protein and enzyme activity. An increase in (residual) DHCR7 activity by a factor 6.1, 3.6 and 4.2, respectively could be documented in the three SLOS cell lines. DHCR7 catalyses the rate-limiting step in SLOS, and an increase in residual DHCR7 activity leads to an increased flux

through the cholesterol biosynthetic pathway and explains lower 7DHC and (almost normalizing) cholesterol levels, as observed.

Biochemical and clinical improvement was strongly correlated with the residual DHCR7 activity. Up-regulation of DHCR7 activity in cultured fibroblasts by simvastatin was also correlated with the patients' clinical severity and genotype. Our study is the first to demonstrate that simvastatin therapy in SLOS patients with a relatively mild phenotype and residual activity of 7DHCR in cells results in increased DHCR7 expression. This increased DHCR7 activity level leads directly to a lowering of 7DHC and an increase of cholesterol levels. Since the efficacy and outcome of the simvastatin treatment appears to correlate well with the residual 7DHCR activity and clinical severity in patients, the treatment may provide a good therapeutic option in SLOS patients with a mild to moderate presentation (Jira *et al.* 2005a, *submitted*). Statin therapy is a promising, novel approach in SLOS that deserves further studies in larger series of patients. Currently, the European NISLOS (Nijmegen International Simvastatin SLOS) Study is being performed on the long-term biochemical and clinical effects of simvastatin with and without cholesterol suppletion. Estimation of residual DHCR7 enzyme activity, without any doubt, will be useful in predicting statin effect in SLOS patients before therapy is initiated (Ginat *et al.* 2004). From the initial precursor/cholesterol ratio or genotype, an attempt will be made to predict the SLOS patients that will benefit most from long term treatment with simvastatin.

Molecular Genetics

In 1998, the human DHCR7 gene was identified and assigned to chromosome region 11q12-q13. More than one hundred different mutations in the DHCR7 gene have been described to date. In **chapter 8** we describe new and known mutations found in the DHCR7 gene in 13 European patients with SLOS. We, and other groups, observed that most (~80%) of the patients are compound heterozygous for a severe and a mild mutation in the DHCR7 gene. The most frequent mutation in Western Europe is the severe IVS8-1G>C mutation, causing aberrant splicing. This mutation results in a mRNA with 134 base-pairs of retained intron 8 sequence at nucleotide position 963. Upon translation, this leads to a frame shift and a stop codon at nucleotide 1235 (TGA), producing an inactive, truncated protein lacking 154 amino acids of its original C-terminal sequence. The patient in our study, who was homozygous for the IVS8-1G>C null mutation, indeed showed a very severe lethal phenotype with the lowest plasma cholesterol concentration (20 μmol/L) ever reported (Jira *et al.* 2001).

A severe clinical phenotype in SLOS patients is due to *null* allele and fourth loop mutations, whereas C-terminal and transmembrane mutations cause a mild to moderate clinical phenotype. Further molecular genetic studies will enable insight in carrier frequency of specific DHCR7 mutations in various populations. Making predictions of the SLOS phenotype from genotype, however, continues to be difficult since there is significant clinical and biochemical variability among

genetically identical SLOS patients (such as siblings). Additional clues for prenatal, maternal, environmental, other genetic and compensatory biochemical determinants that can modify the phenotypical consequences of the functional DHCR7 deficiency in SLOS is needed.

Pathogenesis

Chapter 9 describes a patient with a "Smith-Lemli-Opitz-like" phenotype caused by distal chromosome 7q36 deletion disrupting the human Sonic Hedgehog (SHH) gene. This remarkable finding is in line with SLOS pathogenesis and the role of cholesterol in the important, recently revealed morphogenetic Cholesterol-SHH-Patched-Smoothened-Gli cascade. The newborn boy presented clinical manifestations of SLOS, including prenatal growth retardation, microcephaly, hyperplasia of the upper alveolar ridges, micropenis with glandular hypospadias and bilateral 2-3-toe syndactyly. He also revealed an unilateral choanal atresia and severe an/microphthalmia. Gas chromatographic analysis of plasma sterols showed normal concentrations of the cholesterol precursors 7DHC, 8DHC and also of cholesterol, thereby excluding a DHCR7 deficiency, the primary cause of SLOS. Chromosome analysis demonstrated a distal 7q36.1 deletion. Extensive fluorescence *in situ* hybridization analysis showed that the holoprosencephaly (HPE3) critical gene region on chromosome band 7q36, including the SHH gene, was deleted. Mutations in or microdeletion of the SHH gene of the non-deleted allele could be excluded. This case suggests that, in addition to patients with a defect in cholesterol biosynthesis, patients with a distal 7q deletion may present a SLOS-like phenotype (Jira *et al.* 2005b, *submitted).*

Morphogenesis

SHH genes are highly conserved and have been identified within a variety of species, including humans, mice, frogs and chickens. Mouse and human Shh protein are 92% identical at the amino acid level. In the human embryo, Shh is expressed in the notochord, the floor plate of the neural tube, the gut, and in the developing limbs. The Shh protein is a secreted intercellular signaling molecule , which is synthesized as a precursor, that undergoes autocatalytic cleavage into a highly conserved N-terminal domain (Shh-N) and a more divergent C-terminal domain (Shh-C), as shown in the Figure. During the auto-processing reaction, a cholesterol moiety is covalently attached to the C-terminus of Shh-N (Porter *et al.* 1996). Shh-N contains all the known signaling activities. In contrast, Shh-C mediates both the enzymatic cleavage and cholesterol modification of the protein. This modification is crucial for proper patterning activity. The covalently attached moiety tethers the N-terminal signaling fragment to the cell membrane proteins Patched and Smoothened. Cholesterol-modified Shh-N binds to Patched. Binding results in the dissociation and activation of Smoothened. Active Smoothened triggers the activation of transcription factor Gli-1 and subsequent factors involved in a variety of developmental processes, including neurogenesis, myogenesis, skeletal patterning, and left-right axis establishment (Lum & Beachy 2004; Ogden *et al.* 2004). Some, if not all, of the clinical findings of

SLOS may be due to a defective SHH signaling pathway caused by sterol disturbances, which is consistent with results obtained in experimental animals. Much has been learned about the mevalonate-cholesterol biosynthetic pathway in the last decade. Eight clinical entities are caused by defects in the cholesterol biosynthesis (Table in Chapter 1).

In addition, several malformation syndromes, diseases and malignancies are associated with genes involved down-stream in the Cholesterol-SHH-Patched-Smoothened-Gli signaling pathway, as illustrated below;

Sonic Hedgehog	Holoprosencephaly (MIM 236100)
Patched 1 and 2	Gorlin syndrome (MIM 109400)
	basal cell carcinoma, medulloblastoma, trichoepithelioma
	oesophageal squamous cell carcinoma ,
	bladder transitional carcinoma
Smoothened	basal cell carcinoma, medulloblastoma
Gli-1	basal cell carcinoma, glioblastoma, rhabdomyosarcoma,
	osteosarcoma, predicts sarcoma grade
Gli-3	Greig syndrome (MIM 175700)
	Pallister-Hall (MIM 146510)
	Postaxial polydactyly A/IV and pre-axial polydactyly A/B
CBP	Rubinstein-Taybi (MIM 180849)
Twist	Saethre-Chotzen (MIM 1014000)

Recently, disruption of the SHH pathway genes has been associated with small-cell lung cancer (Watkins *et al.* 2003) and colon cancer (Oniscu *et al.* 2004). Finally, the Hedgehog-Gli1 signaling pathway is a new candidate, therapeutic target for breast cancer (Kubo *et al.* 2004) and prostate cancer (Sanchez *et al.* 2004).

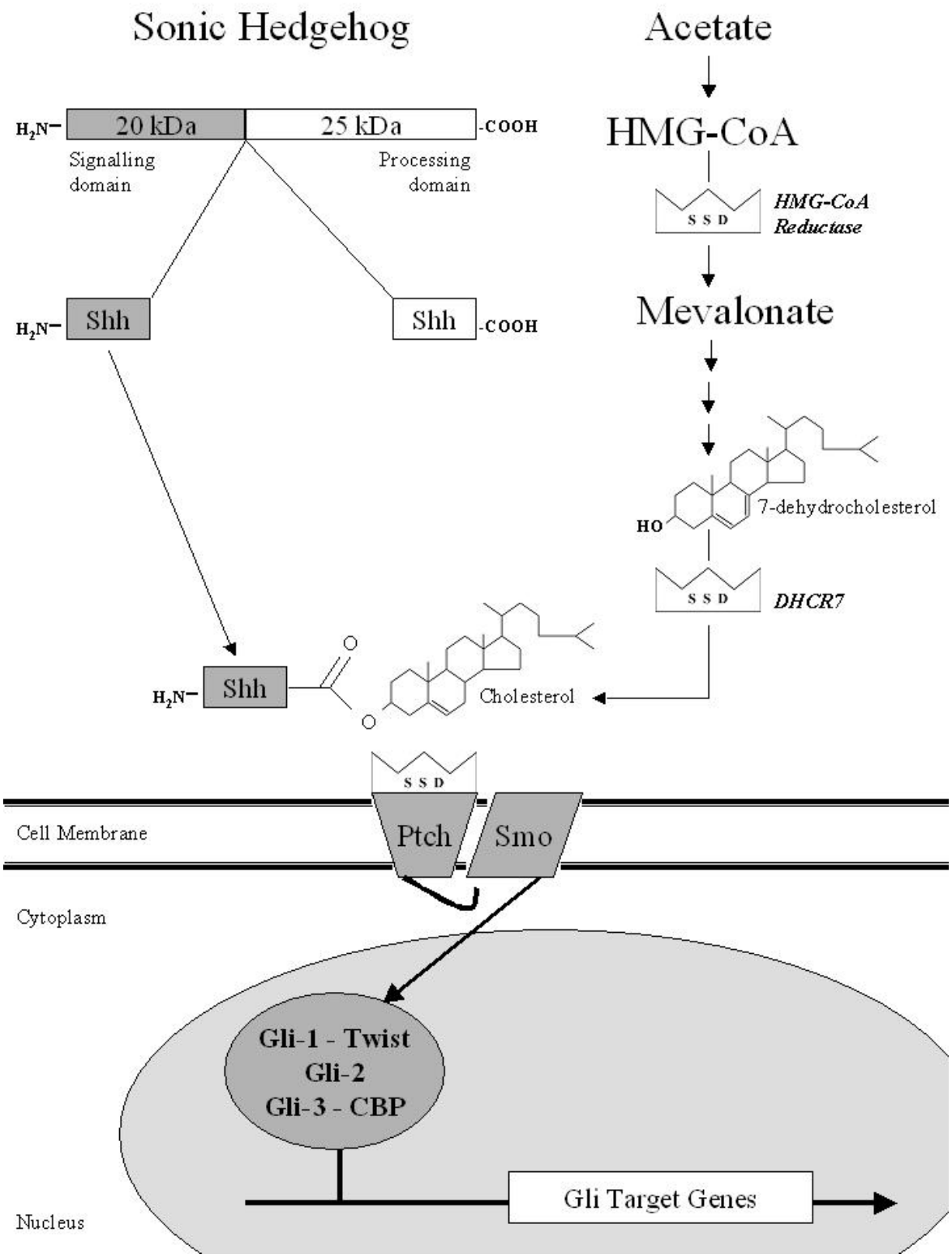

Figure. Removal of the N-terminal signal peptide from the initial Shh-translation product yields the 20-kDa precursor, which is covalently attached to cholesterol. This "Cholesterol-Shh-N" molecule attaches to Patched (Ptch) and this activates the protein Smoothened (Smo) by uncoupling. This in turn activates nuclear Gli and subsequently Gli-target genes. SSD marks the homologous "Sterol Sensing Domain" present in two key pathway-enzymes (HMG-CoA reductase and DHCR7) and the membrane-bound receptor Patched.

Precursor toxicity

The main question in the pathogenesis of SLOS remains: Is it the lack of cholesterol in plasma and tissue or the accumulation of harmful sterol precursors that cause disease? There are firm clues that the primary precursor accumulation in SLOS may lead to secondary negative effects on cholesterol metabolism and disturb intracellular trafficking.

7DHC binds to the "sterol sensing domain" site of the DHCR7 enzyme that encompasses five central transmembrane segments 4 – 8 (Fitzky *et al*. 1999; Bae *et al*. 1999). This specific DHCR7 membrane-spanning segment shows strong homology to the SSD found in five other human proteins. These proteins in nature all have crucial interaction with sterol-synthesis, metabolism, trafficking or signaling: 1] HMG-CoA reductase (Olender *et al*. 1992); 2] Niemann-Pick C1 gene product (Loftus *et al*. 1997); 3] Sterol regulatory element-binding protein-SCAP (Nohturfft *et al*. 1998); 4] the morphogene receptor PATCHED (Loftus *et al*. 1997); 5] the DISPATCHED protein (Burke *et al*. 1999). 7DHC may interfere with proper function of the five proteins that contain a similar SSD. When the SDD of DHCR7 normally recognises 7DHC, other homologous enzymes should in theory be influenced when 7DHC accumulates in hundred-to-thousand-fold increased concentrations, as in SLOS.

There are three possible sites where accumulation of 7DHC precursors can interfere with cholesterol synthesis and signaling: HMG-CoA reductase, DHCR7, and Patched (Figure). Low plasma and tissue cholesterol do not lead to poor growth, organ malformations or individual lethality, per se, as illustrated by familial abetalipoproteinaemia and many living species such as rodents. Increased 7DHC concentration in cultured skin fibroblasts from SLOS patients has been shown to lead directly to a reduced HMG-CoA reductase activity, suppressing cholesterol synthesis in the cell. HMG-CoA synthase and LDL binding were inhibited equally by 7DHC and cholesterol. Not only cholesterol but also 7DHC is a very effective feedback inhibitor of HMG-CoA reductase (Honda *et al.* 1998).

Using filipin staining, it has been shown that SLOS fibroblasts accumulate unesterified sterols (Wassif *et al.* 2002). Further studies showed that this increased filipin staining was due to an abnormal accumulation of LDL-derived cholesterol rather than to storage of endogenously synthesized 7DHC. SLOS fibroblasts failed to degrade LDL at a normal rate. Examination of SLOS fibroblasts by electron microscopy demonstrated the formation of lysosomal inclusions similar to those seen in Niemann-Pick type C (NPC) cells. 7DHC may directly or indirectly inhibit the function of the NPC protein through its sterol-sensing domain (SSD). 7DHC accumulation may have a negative effect on the function of other SSD containing proteins (Wassif *et al.* 2002). These observations and insights stress that in the future not only the important role of cholesterol but the possible negative toxic role of cholesterol precursors, intermediates and sterol-like compounds such as dietary plant sterols (phytosterols) should be intensively studied in humans and other species.

Medical management

SLOS demonstrates the fundamental importance of understanding the pathogenesis of dysmorphology in multiple malformation syndromes. The key role of sufficient and *structurally normal* (chole)sterol in humans, animals and plants is illustrated by several organ malformations and developmental delay in SLOS.

Important clinical progress however, has to be made in the near future on syndrome-specific behaviour in SLOS. Patients have severe sleep disorders, feeding problems and auto-mutilating behaviour. Autism or autistic symptoms have been observed in most SLOS patients and have great impact on patients and their families. Other medical complications may include cholestatic liver disease, subclinical or apparent adrenal insufficiency, recurrent infections and a multifactorial, severe feeding intolerance. Anaesthetic management, when needed, should use propofol and fentanyl, to prevent malignant hyperthermia, as reported after using of halothane or suxamethonium (Petersen & Crouch 1995). Further intubations could be difficult because of micrognatia, cleft palate or tongue abnormalities. The variability and complexity of features and symptoms in SLOS requires a multidisciplinary approach providing optimal care for children and their families.

Future Perspectives

In the future, against the background of the broad spectrum of the SLOS, phenotypes with only mild/moderate retardation and/or minor dysmorphias should be screened biochemically and genetically. These individuals may have two mild missense mutations in the DHCR7 gene, since the carrier frequency for some DHCR7 mutations is quite high, with 1 in 30 to 1 in 100 individuals, suggesting an incidence of 1 in 1,700 to 1 in 13,400. This high number is supported by observation of newborn and prenatal incidence of 1 in 22,000 in the Caucasian population (Witsch-Baumgartner *et al.* 2000; Waterham *et al.* 2000). In the future, patients with pylorus stenosis, intersex, hypospadias, polydactyly, single central incisors, Hirschprungs' disease, bilateral cataract, II-III toe syndactyly, microcephaly or unexplained mental retardation without other signs of SLOS may be screened biochemically for sterol abnormalities and at the molecular genetic level for mutations in the DHCR7 gene or in other genes involved in the post-squalene biosynthesis. Sterols prove to be crucial and essential for cell survival in micro-organisms, yeast, plants, animals and humans.

Millions of people being treated for hypercholesterolemia with statins showed not only a decrease in circulating lipids but statins also revealed cardio protection, even within weeks after initiation. One should explore the anti-bacterial potency of statins in inhibiting the essential cholesterol biosynthesis in the bacteria. Cholesterol biosynthesis is not only extensively studied in designing lipid-lowering agents but is also a target for antifungals. Sterol 14α-demethylase is inhibited by azole antifungals as fluconazole and ketoconazole, which are widely used for the treatment of fungal infections (Nakayama *et al.* 2001). The wide range of species that need (chole)sterol synthesis for survival and the multiple enzymes involved in this process affords researchers many opportunities.

Study designs can focus on growth and signaling cascades, hormone-production, myelin formation and cell survival. Future studies should investigate the potential benefit of statins and lipid-lowering agents in cancer research by inhibiting malignant cell division through its vital cholesterol biosynthesis.

At the end of the past millennium new insights were gained on the impressive role of cholesterol on prenatal and postnatal growth, neuromotor development, behaviour, morphogenic and signaling pathways. The Smith-Lemli-Opitz malformation syndrome is the most frequent and most widely studied inborn error of cholesterol biosynthesis. It confronted us with lack of basic knowledge about cholesterol biosynthesis. It motivated scientists, biologists, molecular engineers and physicians to enlarge their knowledge about healthy humans, patients and design new treatment strategies. These insights have opened new opportunities in understanding and improving growth, cell signaling, development, cancer treatment, and their possible use in autoimmune and infectious diseases in the twenty-first century.

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Samenvatting

SAMENVATTING

Het Smith-Lemli-Opitz Syndroom (SLOS) is een zeldzame, autosomaal recessief erfelijke stofwisselingsziekte. De belangrijkste symptomen bij patienten zijn een ontwikkelingsachterstand en overeenkomstige kenmerken zoals een karakteristiek gelaat, kleine schedelomtrek, syndactylie (versmelting) van de tweede en derde teen en een onduidelijk geslacht bij jongens. Afwijkingen kunnen verder voorkomen in alle inwendige organen inclusief cataract (staar) en hersenafwijkingen. De eerste klinische beschrijving van drie jongens die op elkaar leken dateert van 1964 en verbond het syndroom aan de namen van de drie artsen; William Smith, John Opitz en Luc Lemli.

De ontdekking in 1993 dat een stoornis in de aanmaak van cholesterol leidt tot het SLOS was een doorbraak. Er is een deficiëntie (onvoldoende werkzaamheid) van het enzym 7-dehydrocholesterol reductase (DHCR7), onmisbaar voor de cholesterol biosynthese. Dit defect geeft aanleiding tot lage concentraties van cholesterol en een ophoping van twee voorlopers van cholesterol; 7-dehydro-cholesterol (7DHC) en 8-dehydrocholesterol (8DHC) in plasma en weefsels. Deze observatie heeft talloze opwindende vragen opgeworpen zoals beschreven in **hoofdstuk twee**. De kennis en inzichten betreffende SLOS worden uiteengezet in **hoofdstuk drie.**

Dit proefschrift beschrijft de resultaten van klinisch-, biochemisch-, moleculair genetisch-, therapeutisch- en pathofysiologisch onderzoek. Hoewel het bij SLOS gaat om een zeldzaam ziektebeeld waarvan in Nederland slechts enkele patienten per jaar geboren worden is dankzij de bereidheid en enthousiasme van enkele ouders en hun kinderen dit onderzoek mogelijk gemaakt.

Biochemische diagnostiek

In **hoofdstuk vier** wordt middels gas-chromatografie (GC) de analyse van cholesterol waarden in plasma van acht SLOS patienten vergeleken met de gangbare oxidase-methode die routinematig in klinisch chemische laboratoria gebruikt wordt om cholesterol in lichaamsvloeistoffen te meten. Wij beschrijven valkuilen in de bepaling van plasma cholesterol indien de gangbare cholesterol-oxidase methode in SLOS wordt toegepast.

Allereerst hebben gezonde pasgeborenen uitgesproken lage plasma cholesterol concentraties rond of zelfs onder de 2.0 mmol/l. Alle SLOS patienten die wij bestudeerden hadden verhoogde 7DHC (143-848 μmol/l) en 8DHC (107-555 μmol/l) plasma concentraties, diagnostisch voor SLOS. In een gezonde controle-groep, zijn cholesterol-precursor concentraties onder de 10 μmol/l. Als tweede, konden wij aantonen dat de oxidase methode om cholesterol in plasma te meten geen onderscheid kan maken tussen respectivelijk cholesterol, 7DHC of 8DHC. Indien de analyse middels GC wordt toegepast, bleken alle SLOS patienten een plasma cholesterol concentratie (20 - 2030 μmol/l) te tonen onder de referentie waarden voor de leeftijd. Na toepassing van de cholesterol-oxidase methode, hadden daarentegen slechts 6 van de 8 patienten, verlaagde plasma cholesterol waarden. Doordat deze methode in feite de som van cholesterol en haar precursors 7DHC en 8DHC samen meet, komt het tot

vals hoge cholesterol-waarden. Dit kan in potentie tot foutieve conclusies leiden. De GC methode detecteert separate precursor- en cholesterol-pieken. Deze methode maakt het derhalve mogelijk bij SLOS de juiste diagnose te stellen en is bruikbaar om de biochemische ernst en de effectiviteit van behandeling te evalueren.

Behandeling

Hoofdstukken vijf en zes beschrijven nieuwe behandelstrategieën in SLOS. Conventioneel werden patienten behandeld met cholesterol en/of galzuur suppletie zonder indrukwekkende biochemische dan wel klinische verbetering. In **hoofdstuk vijf** worden allereerst erythrocyten van SLOS patienten geincubeerd in normaal donor plasma en konden wij een snelle uitwisseling tussen precursors (7DHC and 8DHC) van het eryhrocyten membraan en cholesterol uit het plasma observeren. Indien normale erythrocyten werden geincubeerd in SLOS plasma, konden wij een vergelijkbare opname-snelheid van precursors in de normale erythrocyten membranen vaststellen. Dit motiveerde onze onderzoeksgroep om het effect van bloed-wisseltransfusie bij twee jonge SLOS patienten te evalueren. Herhaalde bloed-wisseltransfusies toonden een significante daling van circulerende cholesterol-precursor spiegels en verhoogden het cholesterol in plasma tot normale concentraties. Deze procedure is echter invasief voor patienten, tijdrovend en het biochemische effect van bloed-wisseltransfusie was slechts beperkt tot drie dagen. In dit kader kan deze behandelstrategie niet beschouwd worden als een blijvende curatieve lange termijn optie. Vervolgens incubeerden wij gekweekte SLOS huidfibroblasten met een HMG-CoA reductase remmer (simvastatine) om het effect op de sterol synthese te bestuderen. Zoals verwacht, konden wij een significante daling in accumulatie van 7DHC en 8DHC aantonen. De observatie dat de cholesterol concentratie steeg kwam echter als een positieve verrassing. De indrukwekkende verbetering van de precursor/cholesterol ratio in gekweekte SLOS huidfibroblasten motiveerde ons uiteindelijk om SLOS patienten met simvastatine te gaan behandelen.

In **hoofdstuk zes** worden de lange termijn effecten (23 en 14 maanden) van de orale HMG-CoA reductase remming door simvastatine bij twee SLOS patienten beschreven. Wij konden een significante reductie van totale precursors (7DHC+8DHC) tot respectivelijk 28% en 33% van de initiële plasma waarden waarnemen. De cholesterol concentratie normaliseerde onverwacht door een meer dan verdubbeling in de behandelperiode bij beide patienten. De 7DHC+8DHC/Cholesterol ratio verbeterde in plasma van 0.47 naar 0.06 en 0.32 naar 0.04, respectievelijk, en van 1.22 naar 0.12 en van 0.62 naar 0.07 in erythrocyten membranen. Gedurende de follow-up periode verbeterden zowel de morphometrische parameters (lengte, gewicht, schedelomtrek), als de neuromotore ontwikkeling bij beide patienten. De therapie werd goed verdragen zonder dat er klinische- dan wel biochemische bijwerkingen optraden. Dit was de eerste studie waarin een simultane stijging en normalisering in bloed cholesterol naast een significante daling van precursor spiegels in SLOS patienten kon worden aangetoond. De simvastatine behandeling resulteert in een langdurige en bijvend biochemisch effect met een hoopgevende klinsche verbetering. De daling in accumulatie van 7DHC en 8DHC in SLOS

patienten behandeld met een orale HMG-CoA reductase-remmer kon worden verwacht. Het gunstige effect met stijging van de plasma cholesterol concentratie in SLOS patienten behandeld met een statine was echter onverwacht. Volgende studies werden ontwikkeld om deze observatie in gekweekte huidcellen en in plasma van patienten te verklaren.

Hoofdstuk zeven beschrijft drie klinisch relatief milde SLOS patienten die gedurende respectievelijk 80, 49 en 31 maanden, met simvastatine werden behandeld. Wij vervolgden de effectiviteit van de behandeling op hun ontwikkeling en algemeen welbevinden. In aanvulling bestudeerden wij *in vitro* het effect van simvastatine op de DHCR7-expressie en DHCR7-activiteit in primaire gekweekte huidfbroblasten van deze patienten. De simvastatine therapie werd door de patienten goed verdragen zonder evidente klinische en biochemische bijwerkingen. Gedurende de behandeling kon een duidelijke daling van de plasma 7DHC concentratie bij allen en een simultane stijging van de cholesterol concentratie in plasma bij twee van de drie patienten worden waargenomen. Belangrijker nog, twee patienten toonden een goede en één patient een matige klinische respons in groei en ontwikkeling.

De *in vitro* studies in gekweekte huidfibroblasten van de patienten toonden aan dat simvastatine een toegenomen gen transcriptie van het *DHCR7*-gen induceert, hetgeen resulteert in een toegenomen DHCR7 eiwit- en enzym-activiteit. In de drie SLOS cellijnen kon een toename in residuele activiteit worden aangetoond met respectivelijk een factor 6.1, 3.6 en 4.2. DHCR7 katalyseert de snelheidsbepalende stap in SLOS, en een toename in residuele DHCR7 activiteit leidt tot een toegenomen flux in de cholesterol biosynthese en verklaart de verlaging van 7DHC en (vrijwel normalisering) van cholesterol concentraties zoals aangetoond. De biochemische en klinische verbetering was bovendien sterk gecorreleerd met de residuele DHCR7 activiteit. De opregulatie van de DHCR7 activiteit in gekweekte huidfibroblasten door simvastatine in onze studie was eveneens sterk gerelateerd aan de klinische score en het genotype (mutaties) van de betreffende SLOS patient.

Onze studie is het eerste onderzoek dat aantoont dat simvastatine behandeling van SLOS patienten met een relatief mild phenotype en residuele DHCR7-activiteit in gekweekte huidcellen resulteert in een toegenomen DHCR7-expressie. Deze toegenomen DHCR7-activiteit leidt rechtstreeks tot een verlaging van 7DHC en een toename in cholesterol concentratie. Doordat de effectiviteit en verbetering van de simvastatine therapie goed correleert met de residuale 7DHCR-activiteit en de klinische ernst van de patienten, kan deze behandel-strategie beschouwd worden als een goede behandel optie bij SLOS patienten met een licht tot matig ernstige klinische presentatie. Statine therapie is een veelbelovende, nieuwe benadering in SLOS dat verder onderzoek rechtvaardigt in een grotere serie patienten. Thans vindt een Europese NISLOS studie (Nijmegen International Simvastatin SLOS Study) plaats naar de lange termijn biochemische- en klinische effecten van simvastatine in combinatie met of zonder cholesterol suppletie. Bepaling van de residuele DHCR7 enzym activiteit kan inzicht geven of statine-behandeling effectief zal zijn in SLOS patienten voor start van de behandeling. Gepoogd zal worden met behulp van de initiele precursor/cholesterol ratio of

van het genotype, te voorspellen welke SLOS patienten het meest kunnen/zullen profiteren van de lange termijn behandeling met simvastatine.

Moleculaire Genetica

In 1998, kon het humane DHCR7 gen worden geidentificeerd en gelocaliseerd op chromossom 11q12-13. Inmiddels zijn er ruim honderd verschillende mutaties in het DHCR7 gen beschreven. In **hoofdstuk acht** beschrijven wij nieuwe en reeds bekende mutaties in het DHCR7 gen bij 13 Europese SLOS patienten. Zoals andere onderzoeksgroepen konden ook wij aantonen dat de meeste (~80%) patienten compound heterozygoot zijn voor combinaties van zowel een ernstige als een milde mutatie in het DHCR7-gen. De meest frequente mutatie in West-Europa is de ernstige IVS8-1G>C mutatie, die aanleiding geeft tot abberante splicing. Deze mutatie resulteert in een mRNA met een insertie van 134 baseparen vanaf intron 8 op nucleotide-postie 963. Na translatie leidt dit tot een frame shift en een stop codon ter hoogte van nucleotide 1253 (TGA), waardoor een getrunceerd eiwit wordt geproduceerd dat 154 van zijn oorspronkelijke C-terminale aminozuren mist. Bij de patient in onze studie die homozygoot was voor de IVS8-1G>C *nul* mutatie was inderdaad sprake van een ernstig en lethaal phenotype. De patient presenteerde zich met de laagste plasma cholesterol concentratie (20 μmol/l) ooit beschreven.

Het ernstige phenotype bij SLOS is vaak het gevolg van *nul* mutaties en mutaties in de vierde eiwit-loop, terwijl C-terminale- en mutaties in het transmembraan gedeelte aanleiding geven tot milde- en matig ernstige klinische phenotypes. In de toekomst zal verder moleculair genetisch onderzoek de dragerschap-frequentie van specifieke DHCR7 mutaties mogelijk maken. Het voorspellen van klinische ernst van het SLOS op basis van het genotype zal moeilijk blijven daar er een aanzienlijke klinische en biochemische variabliteit aanwezig is tussen genetisch identieke SLOS patienten (zoals bij verwanten). Gezocht zal moeten worden naar additionele mechanismen als verklaring voor het uiteindelijke phenotype bij de functionele DHCR7 deficiëntie in deze cholesterol aanmaakstoornis.

Pathogenese

Hoofdstuk negen beschrijft een patient met een op "Smith-Lemli-Opitz-gelijkend" phenotype dat wordt veroorzaakt door een terminale 7q36 deletie leidend tot afwezigheid van het Sonic Hedgehog (SHH) gen. Deze opmerkelijke bevinding bekrachtigt de pathogenese van het SLOS en de rol van cholesterol in de belangrijke, recent ontdekte, cholesterol-SHH-Patched-Smoothened-Gli cascade. Deze morphogenetische pathway is belangrijk bij de aanleg van vele organen en weefsels.

De pasgeboren jongen, toonde een aantal kenmerken zoals gezien worden bij het SLOS: een prenatale groeiachterstand, microcephalie, hyperplasie van de bovenkaak, een micropenis met een glandulaire hypospadie, en een bilaterale syndactylie van de tweede en derde teen. Verder was er sprake van een unilaterale choane (neusholte) atresie en een ernstige an/microphtalmie. Gas chromatografische analyse van de plasma sterolen toonde normale concentraties van cholesterol

precursors 7DHC, 8DHC en tevens van cholesterol waardoor een DHCR7 deficiëntie (zoals bij SLOS) kon worden uitgesloten. Chromosoom analyse toonde een distale 7q36.1 deletie aan. Uitgebreide fluorescentie in situ hybridisatie analyse bevestigde de afwezigheid van de holoprosencephaly (HPE3) critical gene region waaronder het SHH gen. Mutaties in of microdeletie van het nog aanwezig SHH gen op het nog aanwezige allel konden worden uitgesloten. Deze casus suggereert dat naast patienten met een stoornis in de cholesterol biosynthese tevens patienten met een distale 7q deletie zich kunnen presenteren met een op SLOS-gelijkend phenotype.

In **hoofdstuk tien** worden onze onderzoeksbevindingen samengevat en bediscussieerd. Het Smith-Lemli-Opitz syndroom is één van de acht recent ontdekte defecten in de cholesterol biosynthese. Dat verschillende specifieke defecten allen aanleiding geven tot ernstige ziektebeelden en syndromen, vaak met malformaties, geeft aan hoe belangrijke cholesterol is voor iedere cel. Tot slot belichten wij de rol van cholesterol en SHH voor toekomstig wetenschappelijk onderzoek naar celgroei, kanker- en infectiebehandeling.

Dankwoord

DANKWOORD

Lieve patienten en ouders, zonder jullie bereidheid, enthousiasme en nieuwsgierigheid was ons onderzoek de laatste jaren onmogelijk geweest. Jullie hebben de wetenschap en medische kennis een stapje verder gebracht. Ik bewonder en respecteer jullie om het vertrouwen en de durf nieuwe behandelingen aan te gaan. Door nieuwe paden te bewandelen helpen jullie kinderen met een zeldzame stoornis in de cholesterol-aanmaak in de toekomst beter te kunnen behandelen.
Ik dank jullie zeer.

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Prof. U. Wendel, *beste Udo*. Ik ben blij dat jij als “Europees” kinderarts nuances kon brengen in de benadering en behandeling van metabole patienten. Je kunstzinnige en relativerende filosofie leerde mij dat een arts meer is dan een lopend leerboek. Ik lach nog vaak over je “freud-hapje” dat ouders zo graag al bij drie maanden aan hun zuigeling willen geven. De totempaal in je tuin en rondslingerende saxefoon in het gastvrije Hilden vormen waardevolle herinneringen. Je exposties kom ik graag een keer bezoeken omdat jouw wetenschap en kunst minimaal één ding gemeen hebben: intuïtie.

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Sandra Hoenderop, *lieve Sandra*. Je advies over Powerpoint, Word en het submitten van manuscripten hebben mijn de afgelopen jaren enorm geholpen. Nooit was iets lastig, te veel of kwam ongelegen. Je leermomenten neem ik zeker mee.

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Beste Aad, jouw onderzoek naar CTX had vele paralellen met SLOS. Ik heb dan ook zeer goede herinneringen aan de fietstochten met een ondergaand zomerzonnetje op weg naar huis. We deelden als onderdeel van " onze Trias Lipidica " samen met Michèl de verwondering over vers verworven inzichten. Als voortrekkers van de Jonge Garde in het UMCN hebben we de strijd om de arbeidvoorwaarden van jonge specialisten in de academie te verbeteren op moeten geven.
We zijn beiden zonder rancune of spijt naar de periferie gegaan onder het motto: "a man's gotto do what a man's gotto do" (Clint Eastwood).

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Curriculum vitae

CURRICULUM VITAE

Petr Edvard Jira, werd op 17 maart 1963 geboren achter het " ijzeren gordijn" te Hradec Králové in Tsjecho-slowakije. In 1969 emigreerde hij met zijn ouders naar het beloofde land, Nederland. Hij groeide op in Nijmegen alwaar hij in 1982 het diploma Atheneum B aan het St. Dominicus college behaalde. Zijn studie geneeskunde aan de Katholieke Universiteit Nijmegen resulteerde in het artsexamen, dat hij in 1991 cum laude in ontvangst mocht nemen.
De specialisatie kindergeneeskunde vond plaats tussen 1992 en 1997 in het St. Radboud ziekenhuis (Opleider: Prof. Rob Sengers) en zijn B-opleiding in het Canisius Wilhelmina Ziekenhuis te Nijmegen (Opleider: Dr. Paul van Wieringen). Na de registratie tot kinderarts volgden twee deel-specialisties Neonatologie (Opleider: Prof. Margot van de Bor) en Kinder-nefrologie (Opleider: Prof. Leo Monnens).
Sederts 1 mei 2001 is hij full-time werkzaam als kinderarts in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch, alwaar hij deel uitmaakt van de maatschap kindergeneeskunde.
De auteur leeft gelukkig met zijn Lia, Bibi, Rens, Milou en Puck.

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