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ARTICLE

Maternal *ABCA1* genotype is associated with severity of Smith–Lemli–Opitz syndrome and with viability of patients homozygous for null mutations

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The Smith–Lemli–Opitz syndrome (SLOS [MIM 270400]) is an autosomal recessive malformation syndrome that shows a great variability with regard to severity. SLOS is caused by mutations in the Δ7sterol-reductase gene (*DHCR7*), which disrupt cholesterol biosynthesis. Phenotypic variability of the disease is already known to be associated with maternal apolipoprotein E (*ApoE*) genotype. The aim of this study was to detect additional modifiers of the SLOS phenotype. We examined the association of SLOS severity with variants in the genes for ApoC-III, lecithin-cholesterol acyltransferase, cholesteryl-ester transfer protein, ATP-binding cassette transporter A1 (ABCA1), and methylene tetrahydrofolate reductase. Our study group included 59 SLOS patients, their mothers, and 49 of their fathers. In addition, we investigated whether *ApoE* and *ABCA1* genotypes are associated with the viability of severe SLOS cases ($n=21$) caused by two null mutations in the *DHCR7* gene. Maternal *ABCA1* genotypes show a highly significant correlation with clinical severity in SLOS patients ($P=0.007$). The rare maternal p.1587Lys allele in the *ABCA1* gene was associated with milder phenotypes. ANOVA analysis demonstrated an association of maternal *ABCA1* genotypes with severity scores (logarithmised) of SLOS patients of $P=0.004$. Maternal *ABCA1* explains 15.4% (R^2) of severity of SLOS patients. There was no association between maternal *ApoE* genotype and survival of the SLOS fetus carrying two null mutations. Regarding *ABCA1* p.Arg1587Lys in mothers of latter SLOS cases, a significant deviation from Hardy–Weinberg equilibrium (HWE) was observed ($P=0.005$). *ABCA1* is an additional genetic modifier in SLOS. Modifying placental cholesterol transfer pathways may be an approach for prenatal therapy of SLOS.
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INTRODUCTION

Smith–Lemli–Opitz syndrome (SLOS) was first described in 1964 as a multiple malformation syndrome with intellectual disability.[1] The phenotypic manifestations range from minimal dysmorphism and mild mental impairment to severe malformations, resulting in intrauterine death.[2] This variability is, in part, explained by different mutations in the *DHCR7* gene.[3] Most severely affected patients carry null mutations. At present, more than 100 pathogenic mutations are known in the *DHCR7* gene. The population distribution of these mutations is variable, for example, the mutation p.Trp151* has a high allele frequency in Eastern Europe, while c.964-1G>C is common in North-Western Europe.[4]

The primary defect-causing SLOS is a deficiency in the last step of cholesterol biosynthesis catalysed by the endoplasmic reticulum enzyme, Δ7-sterol reductase (DHCR7; E.C.1.3.1.21).[5–8] It is presently unclear how exactly this metabolic disturbance results in the clinical phenotype, but dysfunction of the cholesterol-dependent SHH pathway is a possible mechanism.[9] Cholesterol supply during embryogenesis is likely to be the most important factor affecting the SLOS phenotype.[10] Cholesterol provision for the growing embryo occurs mostly through endogenous synthesis, and also from exogenous sources, predominantly by transport of lipoproteins from the mother.[11] The SLOS phenotype may therefore be modified by genetic variants in the sterol transport systems of the mothers. At present, little is known about the mechanisms of cholesterol transport from the mother to the embryo in humans. Studies of knockout mice showed that apolipoprotein B (ApoB)-containing lipoproteins and their receptors may play a role.[12]

Previous studies have shown a correlation of SLOS severity with *DHCR7* genotype and maternal *ApoE* genotype, but a large part of the clinical variability remains unexplained.[3,13] We have now investigated genes involved in lipid metabolism and transport as candidate modifiers of the clinical severity of SLOS. These include ApoC-III, lecithin-cholesterol acyltransferase (LCAT), cholesteryl-ester transfer protein (CETP),[14] and ATP-binding cassette transporter A1 (ABCA1). They were selected because single-nucleotide polymorphisms (SNPs) in these genes have been previously reported to affect lipid as well as lipoprotein levels (Table 1).[18,19,23–26] Some common variants in the *ABCA1* gene, such as the amino-acid exchange p.Arg1587Lys (R1587K), are known to result in low HDL cholesterol concentrations

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Table 1 Variants used for association study in SLOS patients

Gene reference sequence	*SNP ref.*	*TaqMan SNP genotyping assay*	*Presumed consequence of SNP*
MTHFR	p.Ala222Val	C_1202883	Induces increased enzyme activity, significantly increased in mothers of
NM_005957.4	rs1801133		children with cleft lip and cleft palate[15–17]
LCAT	p.His173Arg	C_8731782	Esterification of extracellular cholesterol in HDL leads to decrease of plasma
NM_001907.2	rs2301246		HDL-cholesterol[18]
ApoC-III	c.340G>C	C_8907537	Association with LDL- and HDL-cholesterol and ApoA-I concentrations in
NM_000040.1	rs5128		cord blood[19]
ABCA1	p.Lys1587Arg	C_2741104	Lys1587 is associated with decreased plasma HDL- and LDL-cholesterol
NM_005502.3	rs2230808		mainly in women[20–22]
CETP	p.Val422Ile	C_790057	Influences the CETP expression, Ile422 is associated with low CETP and
NM_000078.2	rs5882		high HDL-cholesterol[14]

in the plasma, especially in women, as well as in higher plasma cholesterol and LDL levels.[20,21] Higher risk for ischemic heart disease and coronary heart disease was postulated in persons carrying the KK genotype (homozygous for p.Lys1587).[22] As 70% of SLOS patients show anomalies of the palate, the methylene tetrahydrofolate reductase (MTHFR) was also postulated as a possible modifier candidate.[27] The known variant p.Ala222Val leads to lowered enzyme activity, which was found in a significantly higher proportion of mothers of children with a cleft lip and without cleft palate.[15,16]

ApoE is one possible component of the maternal–embryonal cholesterol transport system. ApoE is a ligand involved in the transport and receptor-mediated uptake of lipoproteins by various cell types, as well as a participant in processes as distinct as lymphocyte activation, cholesterol homeostasis in macrophages, and neuronal plasticity.[17,28] ApoE isoforms (common alleles $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$) differ in their binding affinities to lipoprotein receptors and have profound effects on plasma cholesterol concentrations.[28,29] There is a significant effect of the maternal *ApoE* on severity score and on cholesterol concentrations in SLOS patients.[13]

Thus, we have investigated if there is another modifier of the clinical severity of the SLOS by candidate gene approach. Another approach was to study whether maternal *ABCA1* and maternal *ApoE* modify the viability of the most severily affected cases of SLOS who carry two homozygous- or compound heterozygous-null mutations.

SUBJECTS AND METHODS

Patients

The first study population included 59 unrelated SLOS patients of European descent, their mothers, and 49 of their fathers, described previously.[3,13] In all patients, sterols were quantified by gas chromatography and mass spectrometry.[30] Concentrations of relevant metabolites (cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol) were available for most SLOS patients. Patients were characterised by the previously described scoring system with strictly defined criteria to ensure the comparability of scoring results.[2] Malformations were scored as '0', '1', or '2' for absent, mild or moderate to severe in a minimum of 5 out of 10 embryological distinct areas. The sum was normalised to 100. In this SLOS cohort, the average severity score was 31 for all patients. Of the patients analysed, 25 SLOS patients showed cleft palate (soft or hard) and/or midline cleft lip, and 27 SLOS patients showed no oral manifestations. In the other SLOS patients, no indication about oral malformations was given.

The second study population comprised 21 SLOS patients (inclusive fetuses) carrying two *DHCR7*-null mutations. Three of them (5, 7, and 8 in Table 5) were already included in the first study population mentioned above. They were of mixed Caucasian origin, mostly of European descent, from Germany (5), UK (3), Italy (1), Switzerland (3), Austria (5), Hungary (1), Argentina (1), and Spain (2). DNA was available from all 21 mothers and 15 fathers of SLOS patients. Patients were characterised by their ability to survive until birth.

Mutation analysis and genotyping

DNA was isolated from peripheral blood leukocytes according to a standard protocol.

In patients, mutations in exons 1–9 of the *DHCR7* gene (reference sequence NM_001360.2) were detected by a stepwise procedure of first sequencing exons 6 and 9 for most common null alleles. Sequencing was performed on the ABI Genetic Analyzer 3100.[3]

The SNPs had been chosen depending on the function of the gene and on the known effect of the different alleles with regard to lipid metabolism, maternal cholesterol transport, and embryogenesis. ApoE (NM_000041.2) genotyping was performed by sequencing a fragment of exon 4 encoding the alleles $\varepsilon 2$ (p.Cys130, p.Cys176), $\varepsilon 3$ (p.Cys130, p.Arg176), and $\varepsilon 4$ (p.Arg130, p.Arg176). These positions correspond to amino-acid residues 112 and 158 of the previous nomenclature.[31]

SNP genotyping for variants in the genes *ABCA1* (NP_005493.2: p.Lys1587Arg; minor allele count: A = 0.4113/900), LCAT (NP_001898.1: p.His173Arg), *CETP* (NP_000069.2: p.Val422Ile), *LDLR*, *ApoC-III*, and *MTHFR* (NP_005948.3: p.Ala222Val) was performed with the ABI SDS 7000 probes and primers that are part of predesigned assays from Applied Biosystems, Carlsbad, CA, USA (TaqMan SNP Genotyping Assays) (Table 1). Validation of all predesigned assays was carried out by sequencing several DNA samples and including wild-type, heterozygous, and homozygous control DNA in every genotyping assay.

Statistical analysis

χ^2 test and Spearman correlation coefficients were calculated using the Superior Performance Software System SPSS package (release 19.0 for windows). In addition, a univariate variance analysis on a general linear model (after logarithmising the severity score) was applied. The Mann–Whitney *U*-test was used to compare genotype groups in case of non–normally distributed variables. Power analysis for evaluating frequency differences in unrelated samples was used for validation of results in the second study group. The problem of multiple testing was accounted for by applying the Bonferroni correction.

RESULTS

Description of SLOS patients

The patients in the first study group were diagnosed by quantification of sterols using gas chromatography and mass spectrometry as mentioned.[13] Phenoytpe severity was characterised by the scoring system mentioned earlier.[2] The number of persons analysed in the association studies varied depending on availability of variables

(concentrations of relevant metabolites cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol, genotypes for patients and parents). *DHCR7* genotypes were identified in all patients as described previously.[3] *DHCR7* genotypes that were classified from severe genotypes (including two null mutations) to mild genotypes (two mutation in the C-terminal region of the protein) correlate significantly with the DHC fraction and severity scores (data not shown). These results confirm previously published data.[3]

The 21 newborn and intrauterine deaths of the second study group have been diagnosed prenatally or perinatally by clinical suspicion, followed by molecular analysis in the parental DNAs in case of nonavailibility of DNA from the proband.

Maternal MTHFR, ApoC-III, CETP, *and* LCAT *genotypes do not show any association with SLOS severity*

The unrelated Caucasian SLOS patients ($n=59$), their fathers ($n=49$), and their mothers ($n=59$) were genotyped for known SNPs in the *ApoC-III*, *CETP*, *LCAT*, and *MTHFR* genes, characterised in NCBI (http://www.ncbi.nlm.nih.gov/) as rs5128, rs5882, rs2301246, and rs1801133, respectively. The frequency distribution of the alleles in SLOS patients, their mothers, and their fathers were not statistically different from already described frequencies (see NCBI) (Table 2). The genotype frequencies in SLOS patients and their parents (Table 3) showed no significant deviation from HWE. Association was calculated between gene dose of the rare maternal alleles with patients' cholesterol concentrations and clinical severity scores (Table 4a), and also between gene dose of the rare patients' and paternal alleles with patients' clinical severity score (data not shown). The maternal rare alleles of *ApoC-III*, *MTHFR*, *CETP*, and *LCAT* did not show any significant correlation with regard to severity score or cholesterol levels in SLOS patients.

Maternal ABCA1 *genotype correlates with severity score of SLOS patients*

Genotype analysis of maternal *ABCA1* genotypes with regard to the polymorphism p.Arg1587Lys showed a significant correlation (Tables 4a and b and Figure 1), with severity score in SLOS patients of $r=-0.350$ and $P=0.007$. The correlation persisted after Bonferroni correction for multiple testing (significance is given at $P\leq 0.0083$, $\alpha=0.05/6$). No other investigated SNPs in mothers showed correlation with disease severity of SLOS patients. No correlation with severity scores was detected for patients' or paternal *ABCA1* genotypes (Table 4b).

The rare maternal Lys1587 allele in the *ABCA1* gene was associated with milder SLOS phenotypes (Figure 1). Univariate variance analysis

Table 2 Allele frequencies of SNPs in SLOS patients and their parents

	ApoC-III *rs5128*	*MTHFR* *rs1801133*	*ABCA1* *R1587K* *rs2230808*	*LCAT* *rs2301246*	*CETP* *rs5882*
NCBI frequency	0.09	0.26	0.34	0.19	0.35
Patients	0.05	0.29	0.20	0.19	0.27
$P=$	0.912	0.956	0.824	1	0.903
Mothers	0.09	0.33	0.27	0.19	0.31
$P=$	1	0.914	0.914	1	0.952
Fathers	0.11	0.33	0.19	0.23	0.34
$P=$	0.960	0.914	0.819	0.945	0.988

Table 3 Genotypes of analysed SNPs in SLOS patients and their parents

		Frequencies		
Genotypes		*Absolute*	*Percent*	*HWE*
ApoC-III (rs5128)	1/1	49	89	0.89
	1/2	6	11	0.09
Patients	2/2	0	0	0.02
	Sum	55	100	$P=0.77$
	1/1	40	78.4	0.78
	1/2	11	21.6	0.19
Fathers	2/2	0	0	0.02
	Sum	51	100	$P=0.59$
	1/1	48	81.3	0.83
	1/2	11	18.6	0.17
Mothers	2/2	0	0	0.00
	Sum	59	100	$P=0.81$
MTHFR (rs1801133)	1/1	28	50	0.48
	1/2	23	41	0.41
Patients	2/2	5	09	0.09
	Sum	56	100	$P=0.99$
	1/1	23	46	0.44
	1/2	21	42	0.44
Fathers	2/2	6	12	0.1
	Sum	50	100	$P=0.94$
	1/1	29	50	0.45
	1/2	20	34	0.45
Mothers	2/2	9	16	0.11
	Sum	58	100	$P=0.49$
ABCA1 (rs2230808)	1/1	36	65	0.64
	1/2	16	29	0.32
Patients	2/2	3	6	0.04
	Sum	55	100	$P=0.86$
	1/1	31	62	0.66
	1/2	19	38	0.30
Fathers	2/2	—		0.04
	Sum	50	100	$P=0.28$
	1/1	32	55	0.53
	1/2	21	36	0.40
Mothers	2/2	5	9	0.07
	Sum	58	100	$P=0.90$
LCAT (rs2301246)	1/1	37	66	0.63
	1/2	17	30	0.38
Patients	2/2	2	4	0
	Sum	56	100	$P=0.29$
	1/1	28	58	0.60
	1/2	17	36	0.35
Fathers	2/2	3	6	0.06
	Sum	48	100	$P=0.90$
	1/1	37	66	0.66
	1/2	17	30	0.30
Mothers	2/2	2	4	0.04
	Sum	56	100	$P=1$
CETP (rs5882)	1/1	31	57	0.54
	1/2	17	32	0.39
Patients	2/2	6	11	0.07
	Sum	54	100	$P=0.64$
	1/1	22	44	0.44
	1/2	22	44	0.44
Fathers	2/2	6	12	0.12
	Sum	50	100	$P=1$
	1/1	29	52	0.48
	1/2	19	34	0.43
Mothers	2/2	8	14	0.09
	Sum	56	100	$P=0.51$

Abbreviation: HWE, Hardy–Weinberg equilibrium. *n* fathers >49, because some of them had SLOS children without available maternal DNA.

Table 4a Correlation between patients' severity score, —plasma cholesterol, and gene dose of rare maternal alleles

	Severity score	*ApoE maternal*	*ApoC-III maternal*	*MTHFR maternal*	*ABCA1 maternal*	*LCAT maternal*	*CETP maternal*
Severity score							
Correlation coefficient		−0.307[a]	−0.027	0.087	−0.335[a]	−0.089	0.015
Significance (two-sided)		0.020	0.839	0.514	0.010	0.514	0.910
N= number of DNAs		57	59	58	58	56	56
Plasma cholesterol							
Correlation coefficient	−0.387[b]	0.394[b]	0.149	−0.145	0.035	−0.089	0.149
Significance (two-sided)	0.005	0.007	0.324	0.336	0.819	0.562	0.323
N= number of DNAs	50	46	46	46	46	45	46

[a]Correlation is significant at 0.05 level.
[b]Correlation is significant at 0.01 level.
Correlation coefficient: Spearman rank.

Table 4b Correlation between patients'- severity score,—plasma cholesterol, and gene dose of *ABCA1* in maternal, patients, and paternal alleles

	ABCA1[a] *maternal*	*ABCA1 patient*	*ABCA1 paternal*
Severity score			
Correlation coefficient	−0.350**	−0.217	−0.090
Significance (two-sided)	0.007	0.108	0.532
N= number of DNAs	58	56	50
Plasma cholesterol			
Correlation coefficient	0.149	0.160	0.167
Significance (two-sided)	0.324	0.305	0.310
N= number of DNAs	46	43	39

[a]The genotypes p.Lys1578Arg heterozygous and p.Lys1578 homozygous are grouped together (see Figure 1). **Correlation is significant at 0.01 level

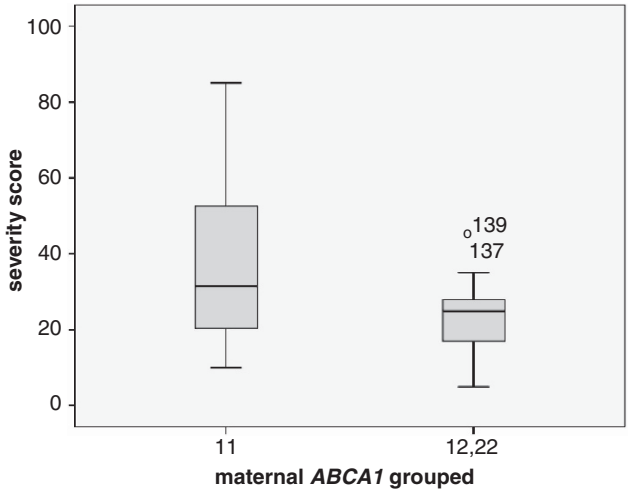

Figure 1 Box-plot diagram demonstrating the difference between maternal *ABCA1* genotypes (Arg1587 = 11; Arg1587Lys = 12; Lys1587 = 22) with regard to the severity scores of SLOS patients; $P = 0.008$ (Mann–Whitney *U* test).

(ANOVA) demonstrated an association of maternal *ABCA1* genotypes (11 *vs* 12 + 22) with severity scores (logarithmised) in SLOS patients of $P = 0.004$ and β-coefficient of −0.439, and accordingly for non-logarithmised severity scores β-coefficient of −14.053 (taken from non-logarithmised model for better interpretability). Maternal *ABCA1* genotype explains 15.4% (adjusted $R^2 =$ 13.7%) of severity of SLOS patients. Association of maternal *ABCA1* with the severity score is reduced ($P = 0.03$) if the model was adjusted for cholesterol concentration. The β-coefficient of −0.348 and R^2 of 22.5% (adjusted $R^2 = 18.6\%$) did change marginally compared with the unadjusted model.

Analysis of ApoE *and* ABCA1 *genotypes in SLOS patients carrying two null mutations*

Patients were diagnosed clinically as well as by molecular analysis of *DHCR7* gene. Cholesterol levels were obtained only in two living patients. In addition to the frequent null mutations, c.964-1G>C and p.Trp151*, rare ones such as p.Gln98* and c.964-1G>T were detected. In this sample of 21 patients and fetuses, the c.964-1G>C represented the most common mutation (26 out of 42 alleles). P.Trp151* was the second most common mutation with 13 alleles; c.964-1G>T and p.Gln98* were detected twice and once, respectively. The effect of these mutations on sterol reductase activity was already shown for the splice site mutations c.964-1G>C and c.964-1G>T.[6,32] The stop mutations p.Gln98* and p.Trp151* show nonsense-mediated decay without any enzyme activity and total loss of function.[33] The mutations c.964-1G>C and p.Trp151*, described as non-functional, and their corresponding phenotypes are associated with the highest severity scores and patients usually die perinatally.[33] In all, 12 patients with these mutations survived birth, and one lived for 7 weeks. Nine pregnancies ended in intrauterine death. *ApoE* and *ABCA1* alleles were determined in mothers and fathers. *ApoE* was also determined in SLOS patients (Table 5). Fathers and mothers of unrelated Caucasian SLOS patients ($n = 21$) carrying two null mutations were genotyped for the common *ApoE* alleles (Table 5). The frequency distribution of *ApoE* alleles from SLOS patients ($\varepsilon2 = 0.08$, $\varepsilon3 = 0.75$, and $\varepsilon4 = 0.17$), their mothers ($\varepsilon2 = 0.05$, $\varepsilon3 = 0.83$, and $\varepsilon4 = 0.12$), and their fathers ($\varepsilon2 = 0.1$, $\varepsilon3 = 0.77$, and $\varepsilon4 = 0.13$) were not statistically different from Caucasian population samples for patients, fathers, and mothers.[29] The genotype frequencies of *ApoE* in mothers from SLOS patients did not show significant deviation from the fathers ($P = 0.67$). Mothers of patients who died neonatally carried genotype E2E3 once, E3E3 seven times, and E3E4 four times. Mothers of fetuses also carried E2E3 once, E3E3 7 times, and E3E4 once. These two groups did not differ significantly with regard to their genotype frequencies ($P = 0.496$).

With regard to the frequency distribution of the *ABCA1* alleles in fathers of SLOS patients, they expressed no statistically different

Table 5 SLOS patients, their *DHCR7* mutations, and *ABCA1* and *ApoE* genotypes of mothers, fathers, and patients (only ApoE), and IUFD (intrauterine fetal death)

	Sex	*Age at death*	*Maternal DHCR7*	*Paternal DHCR7*	*Maternal ApoE*	*Patients ApoE*	*Paternal ApoE*	*Maternal ABCA1*	*Paternal ABCA1*
1	F	3 weeks	W151X	W151X	E3E3	ND	E2E3	ND	RK
2		1 day	W151X	W151X	E3E3	E3E3	E3E3	RR	RK
3	F	4 weeks	IVS8-1G>C	W151X	E3E3	ND	E3E3	RK	RK
4	F	Neonatal	IVS8-1G>C	W151X	E3E3	ND	E3E3	RR	RR
5	M	Some weeks	IVS8-1G>C	W151X	E3E4	ND	ND	RR	ND
6		5 weeks	W151X	IVS8-1G>C	E3E3	ND	ND	RR	ND
7	M	7 weeks	IVS8-1G>C	IVS8-1G>C	E3E4	ND	E3E4	RR	ND
8	M	4 weeks	IVS8-1G>C	IVS8-1G>C	E3E4	E3E4	E3E4	RR	RR
9		1 day	IVS8-1G>C	IVS8-1G>C	E3E4	E3E3	E3E3	RR	RR
10	M	Neonatal	IVS8-1G>C	Q98X	E3E3	E3E3	ND	RR	ND
11	F	2 weeks	IVS8-1G>C	W151X	E2E3	E2E3	E3E4	RR	ND
12	M	Neonatal	IVS8-1G>C	IVS8-1G>C	E3E3	ND	E3E3	RR	KK
13		IUFD	IVS8-1G>T	IVS8-1G>T	E3E3	ND	E3E3	RR	RK
14	F	IUFD	IVS8-1G>C	W151X	E3E3	ND	E2E3	RR	RR
15		IUFD	W151X	IVS8-1G>C	E2E3	ND	ND	RR	ND
16		IUFD	IVS8-1G>C	IVS8-1G>C	E3E4	ND	E3E4	RR	ND
17		2 IUFD	IVS8-1G>C	IVS8-1G>C	E3E3	ND	E3E3	RR	RK
18		IUFD	IVS8-1G>C	IVS8-1G>C	E3E3	ND	ND	RR	ND
19		IUFD	IVS8-1G>C	IVS8-1G>C	E3E3	ND	E3E3	RR	RK
20		IUFD	IVS8-1G>C	IVS8-1G>C	E3E3	E3E4	ND	RR	ND
21	F	IUFD	W151X	W151X	E3E3	ND	E2E3	ND	RK

ND: Not determined.

Table 6 *ABCA1* genotypes from parents of SLOS patients

		Frequencies		
Genotypes		*Absolut*	*Percent*	*HWE*
Fathers	1/1	5	42	0.44
	1/2	6	50	0.45
	2/2	1	8	0.12
	Sum	12	100	$P=0.98$
Mothers	1/1	18	94	0.44
	1/2	1	6	0.45
	2/2	—	0	0.12
	Sum	19	100	$P=0.005$

ABCA1 genotypes *(Arg1587=1/1; Arg1587Lys=1/2; Lys1587=2/2).*

deviation from already described frequencies. However, the allele and the genotype frequencies in mothers of SLOS patients showed a significant discrepancy with regard to allele frequency and HWE. Only one mother out of 19 carried a Lys allele (Tables 5 and 6), although fathers of SLOS patients ($n=12$) carried RR five times (homozygosity for p.Arg1587), RK six times (heterozygosity for p.Arg1587Lys), and KK genotype (homozygosity for p.Lys1587) once, which did not deviate from HWE ($P=0.98$). The allele frequency of one Lys allele out of 38 maternal alleles is significantly different from NCBI frequency ($P=0.0011$) and from fathers' Lys allele frequency ($P=0.0004$). Regarding the genotypes (Table 6), the deviation from HWE is significant at $P=0.005$, with a power of 1.0 at $\alpha=0.05$.

DISCUSSION

Previous studies have shown that the variability of the clinical severity of SLOS can be explained partially by the patients' *DHCR7* genotype and the maternal *Apo*E genotype.[13,31,33,34] Variations in *DHCR7* and maternal *ApoE* explain 29 and 12%, respectively, of the variance of cholesterol (logarithmised) in SLOS patients. Hence, it is probable that additional factors contribute to the variability of SLOS phenotype. As cholesterol and 7-DHC levels are the strongest predictors of disease severity, it has been speculated that these additional factors act through their effect on cholesterol levels.

In this study, we identified *ABCA1* as an additional modifier of SLOS severity. *ABCA1* is known to be involved in the transport of cellular cholesterol across membranes to acceptor molecules as ApoA-I (Figure 2) and to ApoE in extracellular fluids.[35,36] A particularly strong support for our conclusion with regard to *ABCA1* as a modifier of SLOS severity is that the protein was found to be highly expressed in the placenta.[37] It was shown that *ABCA1* is localised in the maternal tissue, namely in the syncytiotrophoblast, especially at the apical maternal facing side as well as at the fetal side (Figure 2).[37–39] Here it acts as a regulator of reverse cholesterol transport by interacting with ApoA-I, which transfers cholesterol to HDL as was shown in macrophages.[24] In our study, no association was demonstrated between maternal *ABCA1* genotype with regard to the SNP p.Arg1587Lys and the plasma cholesterol measured in the SLOS patients. However, a highly significant correlation was found between maternal *ABCA1* genotype and the severity score of the SLOS patients, which is mainly calculated by taking into account external malformations. The rare allele of the common *ABCA1* SNP p.Arg1587Lys in the mother was found to be associated with decreased plasma HDL cholesterol and higher plasma cholesterol in young women.[21] In our investigation, it was associated with a milder SLOS phenotype without an effect on cholesterol concentration in the SLOS patients, which indicates a mode of action other than direct influence on cholesterol concentration.[26] Interestingly, p.Arg1587 is located in the second extracellular domain, which interacts directly with ApoA-I.[40] This may be an explanation for the observed effect of

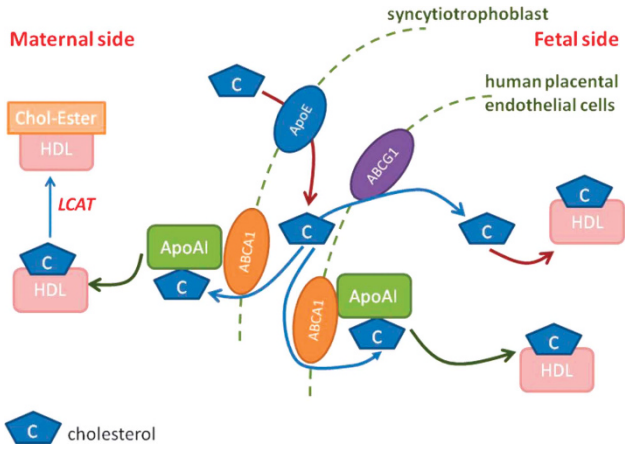

Figure 2 Schematic representation of the conclusion about transfer of maternal cholesterol to the fetus through placentally expressed ApoE, ABCA1, and ABCG1.

this variant. The main cholesterol efflux takes place from placenta, especially from syncytiotrophoblast to the acceptor ApoA-I (pre-β-HDL) (Figure 2).[39] At the fetal side, ABCG1 is localised, which also requires mature HDL as an acceptor for cholesterol efflux. In Tangier disease, a decrease of ABCA1 leads to an increase of ABCG1 activity and to an increase of HDL cholesterol in macrophages.[41] In *DHCR7* $-/-$ mice, it was shown that disruption of *ABCA1* is associated with a 30% decrease of cholesterol transfer to the fetus, suggesting that *ABCA1* plays a crucial role in placental cholesterol transfer.[42] In this setting, it is important that *ABCA1* has no effective ATPase activity and therefore does not directly facilitate the transport of cholesterol from mother to child. Rather, it has a regulatory role in this process.[35] If the consequence of the polymorphism p.Arg1587Lys would be a simple decrease of cholesterol by lowered transfer of maternal cholesterol to fetus, clinical presentation should be more severe. In contrast, we showed an association of maternal p.Arg1587Lys polymorphism with a milder clinical SLOS phenotype. Hence, we postulate that decreased HDL cholesterol on maternal side of the placenta due to *ABCA1* SNP p.Arg1587Lys results in overload of cholesterol in the syncytiotrophoblast, which is delivered to the fetus by an increase of ABCG1-mediated cholesterol efflux (Figure 2), as was shown in macrophages in Tangier disease.[41] At the moment, there is no explanation why there is no association of p.Arg1587Lys with cholesterol level in the SLOS patients. Maybe, the cholesterol levels in SLOS patients at the age of diagnosis are no more in any association with the fetal situation. Another assumption is that the amount of supplementary HDL cholesterol suffices to prevent major malformations, but may just not be adequate to elevate the patients' cholesterol level in total, which was measured disregardful of LDL- and HDL cholesterol. In conclusion, we suppose a regulatory role of *ABCA1* in the cholesterol transport as was postulated previously.[40] In this context, it would be a challenging trial to increase the activity of ABCG1, which should result in an increase of fetal HDL cholesterol and lowered severity score.

In 21 SLOS cases carrying *DHCR7*-null mutations, simultaneously maternal *ApoE* and *ABCA1* genotypes were analysed. The aim was to demonstrate a difference in viability analysing maternal *ApoE* and *ABCA1* genotypes in SLOS-affected fetuses and analysing an association with intrauterine death or neonatal survival. We expected less carriers of Apo-E2 and more *ABCA1* p.Lys1587 in the mothers of survivors. However, in this proband sample, no significant correlation between maternal *ApoE* genotypes and viability was identified. An explanation could be that in patients with two null mutations, which are the most severily affected, external cholesterol supply is inadequate to influence the severity of the phenotype.

Surprisingly, a significant deviation of maternal *ABCA1* genotypes from HWE was detected. Only one mother of 18 carried the Lys allele. All others were homozygous for the Arg allele, whereas in fathers the frequencies of *ABCA1* genotypes were in accordance with HWE. In the collective of mothers, the expected numbers of genotypes after HWE were eight for RR (p.Arg1587 homozygous), eight for RK, and two for KK genotype (p.Lys1587 homozygous). What happened with SLOS-affected fetuses whose mothers carry the Lys allele? This question and associated questions have to be answered: (1) whether the p.Lys1587 allele may be associated with embryonic loss at the beginning of pregnancy; and (2) whether there is another mode of action of *ABCA1* than to be simply a cholesterol transporter during embryogenesis. In different settings of *ABCA1* knockout mice, fetal loss could be shown.[43,44] However, also in *ABCA1* $+/-$ mice, heterozygous for an *ABCA1* knockout allele associated with decreased serum cholesterol, it was shown that placenta was malformed, the embryos showed severe growth retardation, there was fetal loss and neonatal death, and half of the remaining embryos ended as intrauterine deaths and the other half was born normally.[43] In another study, *ABCA1* $-/-$ mice demonstrated reduced to absent fertility with a supposed HDL cholesterol axis of fertility.[44] Furthermore, it seems that *ABCA1* has multitopic localisation, which changes during pregnancy. *ABCA1* is located in the cell membrane, in the membrane of the endoplasmatic reticulum, and also in intracellular compartments. During the first and third trimesters, much *ABCA1* is located in the cytotrophoblast; during first trimester, it is also located in increased concentrations in the syncytiotrophoblast; and during third trimester, *ABCA1* is much decreased in syncytiotrophoblast. It was concluded that a regulatory function of *ABCA1* in intracellular signalling with regard to cell differentiation and hormone metabolism seems to be operating during pregnancy.[37] In our study, the p.Lys1587 is not a deleterious mutation comparable to *ABCA1* gene knockout, but because of its effect on maternal cholesterol levels, we postulate a similar effect in human embryogenesis for p.Lys1587 at least in severe SLOS cases as for *ABCA1* knockout in mice.

We may also be dealing with a possible patient sample bias. In our study, there are 12 out of 21 surviving SLOS cases. Regarding carrier calculations, more SLOS cases than these 12 should carry two null mutations.[45] Hence, it seems possible that a great number of pregnancies with affected fetuses are lost as miscarriages before clinical diagnosis. We do not know which genotypes the mothers of these cases carry and may only postulate that they carry the missing RK and KK genotypes, which would support our hypothesis.

An interesting exceptional case is patient 11 (Table 5 and Figure 3), who is less severily affected than expected while carrying the *DHCR7* mutations c.964-G>C and p.Trp151*. Both null mutations are known to cause the most severe phenotype of SLOS, namely intrauterine death, meaning that the embryo and the fetus are completely dependent on maternal cholesterol. The mother of this patient carries the *ApoE* genotype E2E3 and the *ABCA1* genotype p.Arg1587 homozygously. Hence, this case clearly indicates that there are supplementary modifiers that mask the effect of E2 and is independent of *ABCA1*. This is an indicator that additional work has to be carried out onto understand the process of cholesterol provisioning to the fetus. This subject requires further investigation

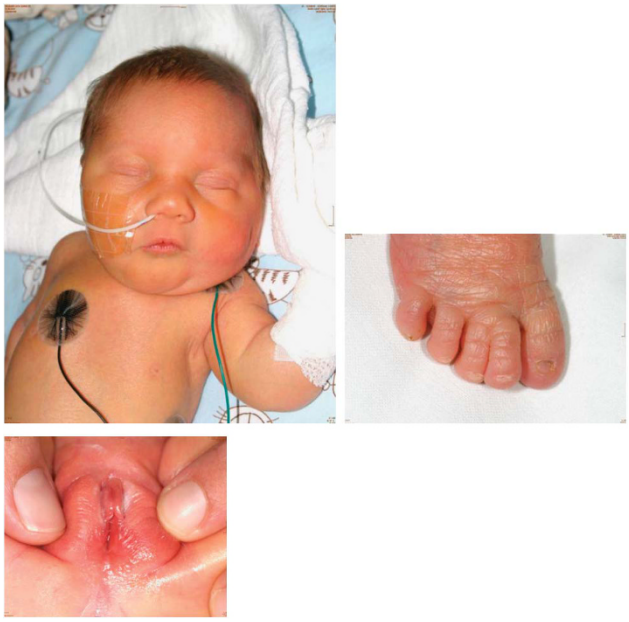

Figure 3 SLOS patient (patient 11 in Table 5).

into cholesterol efflux from syncytiotrophoblast to the fetal side during gestation.

CONCLUSION

In conclusion, through this study, it becomes obvious that there are factors influencing the phenotype of SLOS patients other than their *DHCR7* genotype and the maternal *ApoE* genotype. *ABCA1* is involved in the development of phenotypic severity of SLOS patients and it seems that it also plays a crucial role in the viability of SLOS fetuses, although the mode of action is not yet clear and cannot be completely elucidated by known data about *ABCA1* at the moment.

We described the association of maternal *ABCA1* gene variation with SLOS severity, which suggests that the growing SLOS embryo is critically dependent on *ABCA1* as a mediator of cholesterol transport from the mother and as a probable regulator of intracellular signalling. As a consequence of our findings, it will be interesting to demonstrate precisely the influence of ABCA1 and ABCG1 during embryogenesis, which could be targets of prenatal therapy in SLOS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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PATIENT CONSENT

Clinical data and DNA samples were obtained after written informed consent, including consent to use photographs in this report.

ETHICS APPROVAL

Clinical data and DNA samples were obtained after informed consent, including consent to use photographs in this report.

ELECTRONIC-DATABASE INFORMATION

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nih.gov/omim/ for SLOS [MIM 270400]:

DHCR7 Mutation Database: https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=DHCR7

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