ORIGINAL ARTICLE

Maternal apo E genotype is a modifier of the Smith-Lemli-Opitz syndrome

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Background: Smith-Lemli-Opitz syndrome (MIM 270400) is an autosomal recessive malformation and mental retardation syndrome that ranges in clinical severity from minimal dysmorphism and mild mental retardation to severe congenital anomalies and intrauterine death. Smith-Lemli-Opitz syndrome is caused by mutations in the Δ7 sterol-reductase gene (DHCR7; EC 1.3.1.21), which impair endogenous cholesterol biosynthesis and make the growing embryo dependent on exogenous (maternal) sources of cholesterol. We have investigated whether apolipoprotein E, a major component of the cholesterol transport system in human beings, is a modifier of the clinical severity of Smith-Lemli-Opitz syndrome.
Method: Common apo E, DHCR7, and LDLR genotypes were determined in 137 biochemically characterised patients with Smith-Lemli-Opitz syndrome and 59 of their parents.
Results: There was a significant correlation between patients' clinical severity scores and maternal apo E genotypes ($p = 0.028$) but not between severity scores and patients' or paternal apo E genotypes. In line with their effects on serum cholesterol levels, the maternal apo ε2 genotypes were associated with a severe Smith-Lemli-Opitz syndrome phenotype, whereas apo E genotypes without the ε2 allele were associated with a milder phenotype. The correlation of maternal apo E genotype with disease severity persisted after stratification for DHCR7 genotype. There was no association of Smith-Lemli-Opitz syndrome severity with LDLR gene variation.
Conclusions: These results suggest that the efficiency of cholesterol transport from the mother to the embryo is affected by the maternal apo E genotype and extend the role of apo E and its disease associations to modulation of embryonic development and malformations.

In 1964 Smith, Lemli, and Opitz[1] described a multiple malformation syndrome characterised by microcephaly, structural brain anomalies, cleft palate, a characteristic facial appearance, syndactyly of toes 2 and 3, polydactyly, structural anomalies of the heart and kidney, ambiguous genitalia in males, failure to thrive, and mental retardation. Subsequent studies revealed a wide range in the phenotypic appearance of patients with Smith-Lemli-Opitz syndrome (Online Mendelian Inheritance in Man, www.ncbi.nih.gov/Omim/ (MIM 270400)), from minimal dysmorphism and mild mental impairment to severe malformations resulting in intrauterine lethality.[2]

The basic defect causing Smith-Lemli-Opitz syndrome is a deficiency in the last step of the Kandutsch-Russell pathway of cholesterol biosynthesis[3 4] caused by mutations in the endoplasmic reticulum enzyme, Δ7-sterol reductase (DHCR7; EC 1.3.1.21).[5–7] As a result, the concentration of cholesterol is low, while the concentrations of precursors 7-dehydrocholesterol and 8-dehydrocholesterol (DHC) are elevated in blood and tissues of patients. It is presently unclear how this metabolic disturbance results in the clinical phenotype, but disturbance of the cholesterol dependent SHH pathway is a likely mechanism.[8] The clinical phenotype, especially mental retardation, may also result from the absence of cholesterol during synaptogenesis. It was suggested that glia-derived cholesterol is imported in apo E containing lipoproteins by the neruones to form synaptic connections.[9 10] Only a small fraction of the large phenotypic variability of the Smith-Lemli-Opitz syndrome is explained by allelic heterogeneity at the DHCR7 locus.[11]

Cholesterol supply during embryogenesis is likely to be the most important factor affecting the Smith-Lemli-Opitz syndrome phenotype.[12] Cholesterol supply to the growing embryo is through endogenous synthesis (which is defective in Smith-Lemli-Opitz syndrome) and from exogenous sources—that is, transport of lipoproteins from the mother.[13] Genetic differences in the mother's as well as the embryo's sterol transport system may therefore modify the Smith-Lemli-Opitz syndrome phenotype. Little is presently known about the mechanisms of cholesterol transport from the mother to the embryo or fetus in humans. However, lipoproteins containing apolipoprotein (apo) B and lipoprotein receptors may play a role, as is evident in studies in knockout mice.[14]

Apolipoprotein E, which is a constituent of lipoproteins in plasma and body fluids, is one possible component of the maternal-embryonal cholesterol transport system, illustrated in fig 1. A genetic polymorphism of apo E is characterised by three common alleles ε2, ε3, ε4, which differ by base substitutions in two codons of the apo E gene[15 16] resulting in amino acid replacements in positions 112 (Cys to Arg) and 158 (Arg to Cys) of the apo E protein. Apolipoprotein E is a ligand involved in the transport and receptor mediated uptake of lipoproteins by various cell types and tissues and a participant in processes as distinct as lymphocyte activation, cholesterol homeostasis in macrophages, and neuronal plasticity.[10 16 17] Apo E isoforms differ in their binding affinities to lipoprotein receptors and have profound effects on plasma cholesterol concentrations.[15] In particular apo E2 is

Abbreviations: apo, apolipoprotein; DHC, dehydrocholesterol; DHCR7, Δ7-sterol reductase; SHH, sonic hedgehog homologue; 0, "null" mutations; 4L, mutations located in the 4th cytoplasmic loop; CT, mutations located in the C terminal region of the protein; TM, mutations located in transmembrane domains

defective in binding to the LDL receptor and total plasma cholesterol is very low in most apo E2 homozygotes with some developing type III hyperlipoproteinaemia. These diverse functions may explain the association of apo E with several diseases, including dyslipidaemia,[18] atherosclerotic vascular disease,[14] and Alzheimer's disease.[17 19] We have investigated whether or not apo E gene variation also modifies the clinical severity of Smith-Lemli-Opitz syndrome. For a control, we analysed common variations in the LDLR gene, which has no known effect on plasma lipid levels and which is not expected to affect the severity of the Smith-Lemli-Opitz syndrome.

SUBJECTS AND METHODS

Patients

The study population included 137 unrelated white patients with Smith-Lemli-Opitz syndrome of almost exclusively European descent from the United States (58), Germany (25), Poland (24), the UK (13), Italy (9), and Spain (8). All DNA samples were obtained after informed consent. In all patients sterols were quantified by gas chromatography and mass spectrometry.[21] Patients were further characterised by the same scoring system with strictly defined criteria to ensure the comparability of scoring results, where malformations in a minimum of 5 out of 10 embryologically distinct areas were scored as either "0", "1", or "2" for absent, mild, or moderate to severe, respectively, and the sum was normalised to 100, which yielded a score between 5 and 100 with an average of 39 for all biochemically identified patients with Smith-Lemli-Opitz syndrome.[2] DNA was available from 59 mothers and 49 fathers of our patients. The sex of the patients was known in 52 cases (16 women, 36 men). Cholesterol, 7-dehydrocholesterol, 8-dehydrocholesterol, and dehydrocholesterol fraction were obtained in 105, 112, 98, and 93 patients respectively. Severity scores were calculated in 131 cases of Smith-Lemli-Opitz syndrome.

Mutation analysis and genotypes

DNA was isolated from peripheral blood leucocytes, according to a standard protocol. Mutations in exons 1–9 of the *DHCR7* gene were detected by a stepwise procedure of SSCP and PCR followed by sequencing on the ABI Genetic Analyzer 310.[11] Genotypes were categorised into groups as outlined previously.[11]

Apo E genotyping was performed with the APO E detection kit for the ROCHE light cycler and with TaqMan probes (E2: forward primer, 5'TCCGCGATGCCGATG3'; reverse primer, 5'CGGCCCTGTTCCACCA3'; specific wild-type probe, 5'TGCA GAAGCGCCT3'; specific mutated probe, 5' GCAGAAGt GCCTG3'; E4: forward primer, 5'GAGACGCGGGCACGG3'; reverse primer, 5'TCCTCGGTGCTCTGGCC3'; specific wild-type probe, 5'AGGACGTGTGCGGC3'; specific mutated probe, 5' GAGGACGTGcGCGG3') for the ABI 7000 SDS. Probes and primers were designed using the software Primer Express™ 1.5a from Applied Biosystems. LDLR R471 single nucleotide polymorphism genotyping was also performed with TaqMan probes (LDLR R471: forward primer, 5'GCGTCTCTTCCTA TGACACCG3'; reverse primer, 5'GGCTGGCTGTGGACTG GAT3'; specific wild-type probe, 5'CAGCAGAGACATCC3'; specific mutated probe: 5'CAGCAGgGACATC 3').

Statistical analysis

Spearman's correlation coefficients (r_s) and partial correlation coefficients ($r_{x,y,z}$) were calculated using Superior Performance Software System SPSS (release 11.0 for Windows). The Kruskal-Wallis test and the Mann-Whitney

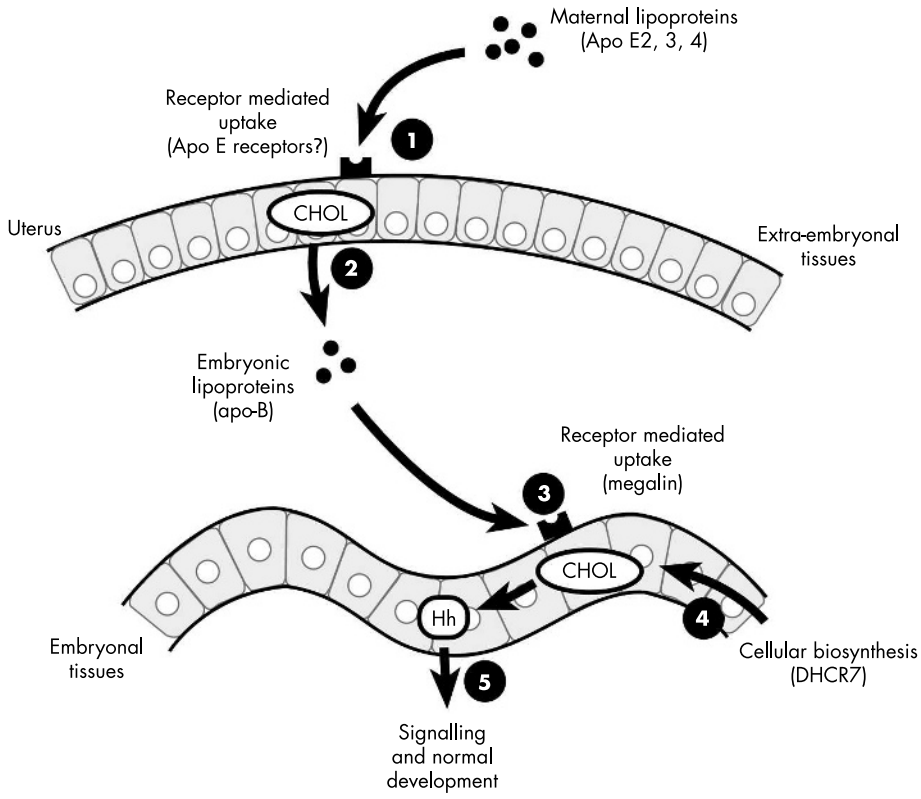


Figure 1 Simplified model of the possible role of apo E in maternal-embryonal cholesterol transport and development. In Smith-Lemli-Opitz syndrome patients with defective DHCR7 the embryo's cholesterol supply depends entirely on the transport of lipoproteins from the mother. Apo E isoforms have different affinities from lipoprotein receptors[18] which make the efficiency of transport dependent on the maternal apo E genotype. (Modified from[20]).

U test were applied as non-parametric tests, as indicated. After logarithmic transformation, the cholesterol concentrations and severity scores became normally distributed, and additional tests could then be applied (ANOVA, univariate variance analysis, multinomial logistic regression). The problem of multiple testing was accounted for by applying the Bonferroni correction.

RESULTS

Characterisation of patients with Smith-Lemli-Opitz syndrome

The diagnosis of Smith-Lemli-Opitz syndrome in the patients was established biochemically by quantification of sterols using gas chromatography and mass spectrometry.[22] Clinically, all patients were further characterised by a scoring system.[2] Not all concentrations of relevant metabolites (cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol) were available for all patients, therefore the n values varied. The severity scores of the Smith-Lemli-Opitz syndrome patients correlated significantly with their plasma cholesterol levels ($n = 100$; $r_s = -0.552$, $p<0.001$), 7-dehydrocholesterol levels ($n = 106$; $r_s = 0.440$, $p<0.001$), and the dehydrocholesterol fraction (the sum of 7-dehydrocholesterol and 8-dehydrocholesterol expressed as the fraction of total sterols ($n = 89$; $r_s = 0.610$, $p<0.001$).

DHCR7 mutations were identified in all of the patients using a standard protocol.[11] The severity scores also correlated significantly with DHCR7 genotypic class as defined in Witsch-Baumgartner et al[11] ($n = 128$; $r_s = -0.303$, $p = 0.001$) (fig 2A). Because DHCR7 genotypes, which were classified from severe genotypes including two ''null'' mutations to mild genotypes with two mutations corresponding to the C terminal region of the protein (0/0 → 4L/4L → 4L/0 → 0/TM → 0/TM → TM/TM → TM/CT → CT/CT), also correlated significantly with cholesterol levels ($n = 101$, $r_s = 0.469$, $p<0.001$) and with the dehydrocholesterol fraction ($n = 90$; $r_s = -0.488$, $p<0.001$) (fig 2B), this genotype-phenotype correlation probably reflects different residual activities of DHCR7. These results confirm and extend previously published data[11] and are summarised in table 1.

Apo E allele and genotype frequencies

The unrelated white patients with Smith-Lemli-Opitz syndrome ($n = 137$) and their fathers ($n = 49$) and mothers ($n = 59$) were genotyped for the common apo E alleles ε2, ε3, and ε4. The frequency distribution of apo E alleles from patients ($\epsilon2 = 0.06$, $\epsilon3 = 0.80$, and $\epsilon4 = 0.14$), mothers ($\epsilon2 = 0.09$, $\epsilon3 = 0.78$, and $\epsilon4 = 0.13$) and fathers ($\epsilon2 = 0.051$, $\epsilon3 = 0.86$, and $\epsilon4 = 0.09$) were not statistically different from white population samples (Germans $\epsilon2 = 0.077$; $\epsilon3 = 0.773$; $\epsilon4 = 0.15$)[15] ($p_{patients} = 0.998$, $p_{fathers} = 0.999$, $p_{mothers} = 0.987$). The genotype frequencies of apo E in patients with Smith-Lemli-Opitz syndrome and their parents (table 2) show no significant deviation from Hardy-Weinberg equilibrium ($p_{patients} = 0.869$, $p_{fathers} = 0.976$, $p_{mothers} = 0.993$).

Correlation of apo E genotypes with Smith-Lemli-Opitz syndrome severity and Smith-Lemli-Opitz syndrome patients' cholesterol levels

Because the severity scores and cholesterol concentrations are not distributed normally, the data were first analysed by non-parametric tests. No overall genotype effect was observed when severity scores were correlated with apo E genotypes of patients with Smith-Lemli-Opitz syndrome and their mothers and fathers by Spearman rank correlation coefficients ($p_{patients} = 0.252$, $p_{mothers} = 0.914$, $p_{fathers} = 0.787$). However significant differences regarding severity scores and sterol parameters were observed between maternal apo E genotypes when the Kruskal-Wallis test was applied (for severity scores, $p = 0.007$; for cholesterol, $p = 0.034$, see table 3). An intriguing difference of the severity scores between the apo E genotypes containing the ε2 allele and those that did not became obvious. The difference between maternal apo E genotypes *E*2/*E*3 and *E*3/*E*3 was highly significant ($p = 0.002$, Mann-Whitney *U* test). Therefore the data were re-analysed for an effect of the ε2 allele. Comparing two groups E2(+) and E2(−) of apo E genotypes, the first including genotypes with the ε2 allele (E2(+):ε2/2, ε3/2, ε4/2), and the second without ε2 alleles (*E*2(−): ε3/3, ε4/3, ε4/4), there was a highly significant difference in the severity scores ($p = 0.029$, Mann-Whitney *U* test, fig 3B, table 4). There was also a highly significant difference between maternal apo E genotype groups with regard to cholesterol ($p = 0.006$, Mann-Whitney *U* test, figure 3A, table 4). When compared both within and between maternal apo E genotype groups E2(+) and E2(−), the cholesterol levels are significantly more similar within apo E groups than between them. Therefore the variance of cholesterol is also dependant on apo E alleles (ANOVA $p = 0.015$).

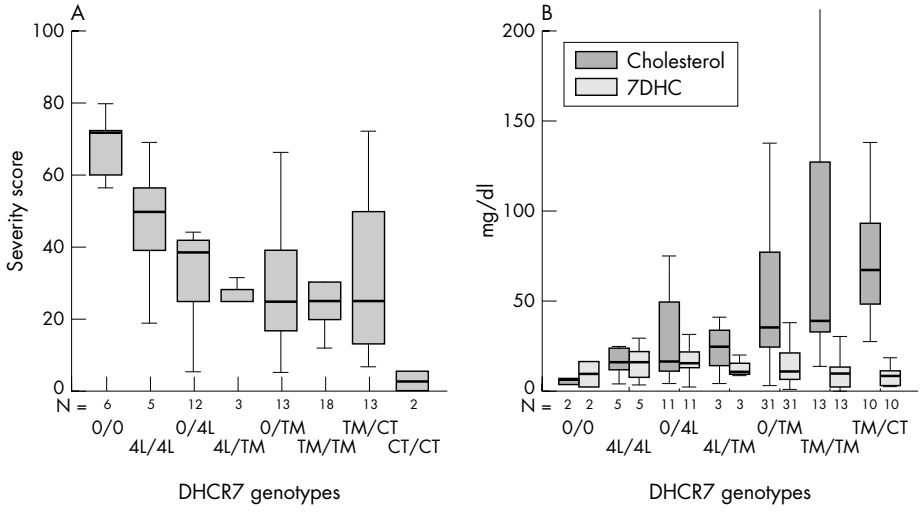


Figure 2 Box plot demonstrating the correlation of DHCR7 genotypes with the Smith-Lemli-Opitz syndrome phenotype (with (A) severity score (B) cholesterol and 7-dehydrocholesterol (7DHC)). DHCR7 genotypes classified as previously described,[11] ordered to get a reasonable sequence.

Table 1 Spearman rank correlation coefficients between severity scores, DHCR7 genotypes and sterol concentrations

		cholesterol	7DHC	DHC fraction	DHCR7 genotype class
severity score	r_s	0.552**	0.440**	0.610**	0.303**
	p	0.000	0.000	0.000	0.001
	n	100	106	89	128
DHCR7 genotype class	r_s	0.469**	0.186	0.488**	
	p	0.000	0.054	0.000	
	n	101	108	90	

**correlation is significant at the 0.01 level (2 sided)
*correlation is significant at the 0.05 level (2 sided)
r_s = Spearman's correlation coefficient, p = significance, n = number of persons analysed. DHC, dehydrocholesterol
DHC fraction = (7DHC+8DHC)/(7DHC+8DHC+cholesterol).

The apo E genotypes of patients with Smith-Lemli-Opitz syndrome and their fathers were similarly divided into groups E2(+) and E2(−) and statistically compared, as described for the mothers. However, for both patients and fathers, the differences between the two groups concerning severity scores and cholesterol were not significant (table 4). On average, patients with Smith-Lemli-Opitz syndrome whose mothers had ϵ2 alleles had the highest severity scores and the lowest cholesterol concentrations and those with mothers having ϵ3 or ϵ4 alleles had the lowest severity scores and highest cholesterol concentrations.

Partial correlation analysis was performed to discriminate the effects of cholesterol, DHCR7 genotype, and maternal apo E genotype on Smith-Lemli-Opitz syndrome severity. Spearman's rank correlations revealed an effect of the DHCR7 genotype on the 7DHC level ($p = 0.054$), on the dehydrocholesterol fraction ($p<0.001$), on cholesterol ($p<0.001$), and on the clinical severity score ($p = 0.001$). In addition, Spearman's rank correlation applied to maternal apo E genotypic groups instead of to several apo E genotypes showed an effect of the maternal apo E genotypic groups E2(+) against E2(−) on patients' cholesterol ($p = 0.006$) and on the severity scores ($p = 0.029$). There was no significant effect of the maternal apo E genotypic groups on the level of 7-dehydrocholesterol and the dehydrocholesterol fraction.

The effect of DHCR7 genotype on Smith-Lemli-Opitz syndrome severity did not persist after stratification for cholesterol ($p = 0.292$), and also the significant effect of the maternal apo E groups disappeared with stratification for cholesterol ($p = 0.78$). Interestingly, when the DHCR7 genotype was treated as a possible factor determining the

Table 2 Genotypes of patients with Smith-Lemli-Opitz syndrome and their parents

Genotypes		Frequencies		Expected number of patients according to Hardy-Weinberg equilibrium
		Absolute	Percentage	
DHCR7	0/0	6	4.3	–
	4L/4L	5	3.5	–
	0/4L	14	9.9	–
	4L/TM	3	2.1	–
	0/TM	77	54.6	–
	TM/TM	17	12.1	–
	TM/CT	13	9.9	–
	CT/CT	2	1.4	–
	sum	134	100	–
Apo E patients	E2/E2	0	0	0.543
	E2/E3	17	12.1	13.5
	E3/E3	83	58.9	84.5
	E3/E4	29	20.6	29.5
	E4/E4	4	2.8	2.57
	sum	133		
Apo E mothers	E2/E2	1	1.7	0.51
	E2/E3	9	15.3	8.57
	E3/E3	35	59.3	35.8
	E3/E4	13	22	11.6
	E4/E4	1	1.7	0.953
	sum	59		
Apo E fathers	E2/E2	0	0	0.127
	E2/E3	5	10.2	4.29
	E3/E3	36	73.5	36.24
	E3/E4	7	14.3	7.58
	E4/E4	1	2	0.39
	sum	49		
LDL receptor SNPID: rs5930 R471 mothers	G/A	15	27.8	
	G/G	32	59.3	
	A/A	7	13	
	sum	54		
LDL receptor SNPID: rs5930 R471 patients	G/A	45	35.7	
	G/G	60	47.6	
	A/A	21	16.7	
	sum	126		

Table 3 Association of maternal apo E genotypes with severity scores and sterol levels (medians) of patients with Smith-Lemli-Opitz syndrome (Kruskal-Wallis test)

Maternal apo E genotype	Severity score	Cholesterol (mg/dl)	DHC fraction	7-DHC (mg/dl)	8-DHC (mg/dl)
	n = 59	n = 48	n = 42	n = 52	n = 45
E2/E2	17 (n = 1)	34.6	0.3	9.88	5
E2/E3	35	15.8	0.526	16.9	8.5
E3/E3	25	36.1	0.279	8.9	6.15
E3/E4	25	31.4	0.538	16.2	10.1
E4/E4	10 (n = 1)	65.3	0.06	3.2	0.9
Overall	25	34	0.37	9.94	7.2
P (Kruskal-Wallis)	0.007	0.034	0.079	0.267	0.325

DHC, dehydrocholesterol.

significant correlation between maternal apo E genotype group and Smith-Lemli-Opitz syndrome severity, this effect stayed significant ($p = 0.038$), indicating that DHCR7 and apo E effects are independent.

Maternal apo E effect on the Smith-Lemli-Opitz syndrome severity score in the subgroup of DHCR7 genotypes 0/TM

Since DHCR7 genotype correlates with Smith-Lemli-Opitz syndrome severity scores, this may confound the association between maternal apo E genotype and Smith-Lemli-Opitz syndrome severity. Therefore we next analysed the severity scores between different maternal apo E genotypes in the largest DHCR7 genotype class (O/TM; n = 37), which includes compound heterozygotes with one functional null allele and a second hypomorphic allele with a mutation in the transmembrane domain (TM).[13] In this subgroup of functionally similar DHCR7 genotypes,[11] the severity score also depended on the presence of an apo ϵ2 allele in the mother. The difference between maternal apo E genotype groups with and without ϵ2 was of borderline significance (n = 37, $p = 0.066$, Mann-Whitney *U* test), which probably reflects the smaller sample size. Again however the difference between the maternal genotypes *E2E3* and *E3E3* stayed significant (n = 29, $p = 0.007$, Mann-Whitney *U* test).

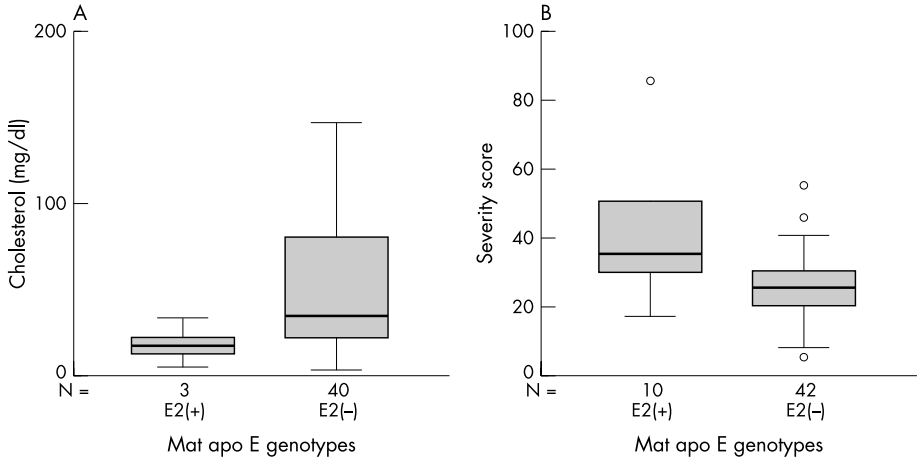
Variance and regression analysis

The variance of ln cholesterol concentrations was analysed by univariate variance analysis (ANOVA) regarding the DHCR7 genotype of the patients and the maternal apo E genotype groups E2(+) and E2(−). For the DHCR7 genotype a significant difference was found ($p<0.001$). Regression analysis revealed that 29% of the variance in ln cholesterol is explained by the DHCR7 genotype. Concerning the maternal apo E genotypes the ln cholesterol concentrations were also significantly different ($p = 0{\cdot}015$). In this case 12% of the ln cholesterol variance in the patients was explained by the maternal apo E genotype. By combining DHCR7 and apo E genotypes, the p values became 0·016 and 0·005, respectively, and the r^2 was 0.383 (corrected $r^2 = 0.275$). The significance for both DHCR7 and apo E genotypes together in the combined model was not significant because their effect is independent of each other.

Further, the effect of the DHCR7 genotypes and the maternal apo E genotypic groups on the disease severity were analysed after logarithmic transformation of the patient severity scores. For the DHCR7 genotype, a significant difference was found ($p<0.001$); about 20% of the variance of Smith-Lemli-Opitz syndrome severity was explained by the DHCR7 genotype. No significant effect of the maternal apo E genotypic groups on the severity scores could be demonstrated, which is probably because of the limits of the applicability of variance analysis in this scenario.

Multinomial logistic regression

This analysis was applied to test the likelihood of a clinical outcome knowing the maternal apo E genotype and DHCR7 genotype of the child with Smith-Lemli-Opitz syndrome. The



The E2(+) group includes individuals with genotype apo E2/E3 and with genotype E2/E4
The E2(–) group includes individuals with genotype apo E3/E4 and with genotype E4/E4

Figure 3 Box plot demonstration of correlation of maternal apo E genotype (E2 present versus absent) with cholesterol levels (A) and disease severity (B) in patients with Smith-Lemli-Opitz syndrome.

Table 4 Association of plasma cholesterol concentrations (mg/dl) and severity scores of patients with apo E genotypes in patients with Smith-Lemli-Opitz syndrome, and their mothers and fathers (all values are medians)

		E2(+)	E2(−)	n	p (Mann-Whitney)
Cholesterol	patients' apo E genotype	49.3 (36.4)	49.8 (46.3)	101	0.569
	maternal apo E genotype	18.6 (8.6)	52.9 (47.84)	48	**0.006**
	paternal apo E genotype	105.9 (100.7)	44.6 (35.7)	39	0.207
Severity scores	patients' apo E genotype	37.7 (21.6)	33.3 (20.6)	123	0.418
	maternal apo E genotype	40.2 (19)	29.4 (16)	59	**0.029**
	paternal apo E genotype	26.4 (6.7)	31.5 (17)	49	0.936

model testing the effect of DHCR7 genotype and maternal apo E genotype group on Smith-Lemli-Opitz syndrome severity was significant ($p = 0.013$). When the patients' severity scores were grouped as mildly (5−25), moderately (26−50), and severely (51−100) affected, the analysis showed that, for example, the probability for a child with DHCR7 genotype TM/TM to be mildly affected is 15% if the mother has an ϵ2 allele and 74% if the mother has no ϵ2 allele. The child's chance of being moderately affected is 72% when the mother has an ϵ2 allele and 17% when she has no ϵ2 allele. The probability of being severely affected is 12% when the mother has an ϵ2 allele, and 8% when she has no ϵ2 allele.

Association of oral malformations and cholesterol uptake

The finding that maternal apo E genotype was correlated with the patients' cholesterol concentrations was puzzling. One possible explanation is that patients with more severe phenotypes also have more feeding problems, perhaps because of oral malformations. Therefore the presence or absence of oral malformations in patients with Smith-Lemli-Opitz syndrome was analysed with regard to the maternal apo E genotypes. There were significantly more Smith-Lemli-Opitz syndrome patients with oral malformations (for example, cleft lip or palate) in the group with maternal ϵ2 alleles ($p = 0.044$, χ^2). The patients with oral malformations also had lower cholesterol levels than patients without oral malformations ($n = 44$, $p = 0.047$, Mann-Whitney U test). The median cholesterol concentrations in the group with oral malformations and in the group without oral malformations were 24.0 mg/dl and 34.6 mg/dl, respectively. For a control, the presence versus absence of genital malformations was analysed. No difference was found between different maternal apo E genotype groups ($p = 0.643$, Mann-Whitney U test).

Effect of LDLR SNP on Smith-Lemli-Opitz syndrome severity

For a control we performed genotyping of the LDLR SNP R471 in patients with Smith-Lemli-Opitz syndrome ($n = 117$) and their mothers ($n = 54$). No association or correlation of LDLR genotype with the severity of the Smith-Lemli-Opitz syndrome or cholesterol concentrations was noted (data not shown).

DISCUSSION

Smith-Lemli-Opitz syndrome is caused by mutations in DHCR7, which catalyses the last step in cholesterol biosynthesis. The severity of the Smith-Lemli-Opitz syndrome varies extremely between affected individuals, ranging from severe malformations and intrauterine death to very mild forms which may easily escape correct diagnosis.[23] Only part of this variation is explained by variation of the DHCR7 locus,[11] suggesting that modifier genes may operate. Recently, some modifier genes for human monogenic diseases have been identified or suggested.[24] We demonstrate here that variation at the genetic locus for apolipoprotein E is a modifier of the phenotypic severity of Smith-Lemli-Opitz syndrome, a condition in which endogenous synthesis of cholesterol is defective but exogenous supply from the mother should be unimpaired. It should be mentioned that the statistical association we found could be obtained by chance (for example, type I error), but the results were always significant, whatever test we used. Moreover the association described here is biologically plausible. Previous studies have demonstrated the importance of components of the systems that transport cholesterol in plasma and deliver it to cells during embryonic development.[14] Targeted disruption of the megalin/gp 330 gene in mice results in holoprosencephaly,[25] which is also a frequent brain anomaly in Smith-Lemli-Opitz syndrome, and deletion of *apo B* results in lethality in mice having some features of holoprosencephaly.[14] Holoprosencephaly is also caused by mutations in *sonic hedgehog* in mice and humans beings, linking cholesterol metabolism to *sonic hedgehog* mediated signalling in embryogenesis.[26–28] The potential role of apo E for the transport of cholesterol from the mother to the embryo and the link of receptor mediated cholesterol uptake to sonic hedgehog dependent development are demonstrated in the diagram in fig 1. Notably *apo E* genetic variation, including apo E deficiency alone, is not associated with malformation in rodents or in human beings. However, in the context of limited endogenous cholesterol biosynthesis, apo E may become a critical component for embryonal cholesterol supply and homeostasis. Given that steady state cholesterol levels are on average 15 mg/dl below the population mean in ϵ2 heterozygotes and 5−10 mg/dl above the average in ϵ4 heterozygotes,[15 29] it is conceivable that apo E type may have a major effect on cholesterol delivery to the embryo and fetus. A role for apo E in the transport of exogenous cholesterol from the mother to the embryo and, in particular, to the developing brain, is suggested by the increase in apo E mRNA during pregnancy, the presence of apo E mRNA in chorionic villi and placenta, and the presence of receptors known to bind apo E in placenta and neuroepithelial cells,[28 30 31] In the light of these observations, it is intriguing that the apo E genotypes of the patients with Smith-Lemli-Opitz syndrome had no effect on the phenotype. However, this is consistent with the finding that there is no difference in cholesterol concentrations of cord blood from newborns with different apo E genotypes,[31] suggesting that the embryo's apo E genotype does not significantly modulate lipoprotein concentrations prenatally.

However maternal cholesterol turnover or concentration may be an important factor that determines cholesterol supply to the embryo. This view is supported by animal studies. For example, differences in the maternal high density lipoprotein cholesterol concentration or composition can affect the size of the fetus in the mouse,[32] and in the Golden Syrian hamster, sterol homeostasis in the fetus is affected by maternal plasma cholesterol concentration in a

gradient fashion, indicating that sterol metabolism in the fetus is dependent on sterol homeostasis in the yolk sac or placenta.[33] The clearance of maternal lipoproteins by the placenta, yolk sac, and decidua is mediated by receptor mediated processes,[34] which may involve apo E. The strongest association of the severity of the Smith-Lemli-Opitz syndrome is with postpartal cholesterol levels.[14] Since postpartal cholesterol levels do not affect prenatal development and the occurrence of malformation, they probably reflect prenatal cholesterol metabolism in the embryo-maternal system. In this context one puzzling finding of our study is that maternal apo E genotype is not only associated with disease severity but also with the Smith-Lemli-Opitz syndrome patients cholesterol level. It is difficult to see how maternal apo E genotypes could affect postnatal cholesterol levels in patients with Smith-Lemli-Opitz syndrome. One possible scenario that could explain the findings is that the presence of maternal apo E genotypes which result in a low cholesterol supply to the embryo will also result in more severe malformation. Patients with Smith-Lemli-Opitz syndrome and additional oral malformations will have more feeding problems and cholesterol intake, which is the major determinant of cholesterol concentration in blood in human beings, will be lower. Embryos with Smith-Lemli-Opitz syndrome who receive less cholesterol from the mother will be more severely affected, which in turn results in lower postnatal cholesterol levels. This is a vicious circle, which may finally result in the strong association of postnatal cholesterol concentrations with Smith-Lemli-Opitz syndrome severity. Our study was undertaken under the hypothesis that the mothers' or the patients' apo E genotypes influence disease severity but that neither the paternal apo E genotype nor the LDLR genotype of either parent or affected child have an effect on disease severity. It was reassuring that, indeed, neither correlation of paternal apo E genotype nor LDLR variation in any of the studied groups had a measurable effect on disease severity.

Notably, the effect of apo E variation on the phenotype observed here could not have been detected by a linkage approach (including TDT tests or sibling pair linkage approaches). No difference in transmitted apo E alleles or allele frequencies in siblings is expected in a scenario where exclusively the maternal genotype is a determinant of disease variability in the offspring. In conclusion, our results expand the role of apo E and its disease associations to include embryonic development and malformation and have implications for disease gene identification strategies in situations where parental genotypes determine the phenotype of offspring.

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