"FEDERICO II"
UNIVERSITY OF NAPLES

Faculty of Medicine and Surgery

Doctorate in
"Human Reproduction, Development and Growth"

Academic Year 2005-2006

Ph. D. Thesis

"New insights in pediatric metabolic medicine: pathogenesis of cholesterol biosynthesis defects and innovative therapies for Pompe disease"

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This study is dedicated to my wife Floriana.

CONTENTS

SUMMARY

In the last decades two issues have significantly changed the management of pediatric patients affected by inherited diseases. First, the discovery that metabolic disorders can present as multiple malformation/mental retardation syndromes has brought innovative tools for the diagnostic work-up, and has emphasized the pathogenic role of specific metabolic pathways derangement in morphogenesis defects. Second, the development of the 'genetic medicines' techniques has determined the feasibility of various innovative therapeutic approaches for a few genetic errors of metabolism, based on enzyme replacement, enzyme enhancement, substrate reduction, RNA modification and gene transfer. These two issues represent an exciting research field for pediatricians with a special interest in clinical genetics.

This study is focused on the results of a four years Doctorate Research program developed at the Department of Pediatrics of the University of Naples "Federico II", including two different projects.

The first project is focused on the characterization of specific clinical, biochemical, morphological and molecular aspects of defects of cholesterol biosynthesis, which can be considered as typical examples of multiple congenital anomalies/mental retardation syndromes with metabolic bases.

The second project is focused on the development of innovative therapeutic approaches to an inherited disorder of glycogen metabolism named Pompe disease, including the results of a clinical trial of enzyme replacement therapy and an "in vitro" study focused on an enzyme enhancement approach. The implications of the research results for the development of future studies are also discussed.

CHAPTER 1

GENERAL INTRODUCTION

In the last decades, two issues have significantly changed the management of genetic disorders. Traditionally, clinical genetics has been considered to be divided in two clearly separated fields: dysmorphology, dealing with multiple malformation syndromes, and metabolic medicine, dealing with inborn errors of metabolism. In the last few years it has become clear that these two apparently separated fields in fact significantly overlap. To date, various multiple malformation syndromes due to metabolic defects have been characterized, emphasizing that metabolic derangements can play a major role in the pathophysiology of morphogenesis defects (Jaeken and Carchon, 2004; Martin, 2006; Porter, 2003; Wanders and Waterham, 2005).

The feasibility of innovative therapeutic approaches recently experimented for a few inherited metabolic disorders, is another issue which has had a great impact on the management of genetic disorders. The treatment of the more than 1,800 known monogenic hereditary disorders depends on the ongoing development of 'genetic medicines', such as therapies that use the transfer of molecular drugs to modify gene expression to correct or compensate for an abnormal phenotype (O'Connor et al., 2006). In particular, strategies include provision of the enzyme specifically lacking in the disease, transferring the protein itself (enzyme replacement therapy), the gene encoding the lacking enzyme (gene therapy), or cells producing the lacking protein (hematopoietic stem cell transplantation), and, finally, RNA modification in order to suppress mRNA levels, or to correct or add functions to the mRNA (Brady, 2006; Hodges and Cheng, 2006; O'Connor et al., 2006). Other therapeutic strategies include the possibility of giving drugs enhancing the activity of mutated proteins (enzyme enhancement therapy), or reducing the biosynthesis of substrates whose accumulation results to be toxic (substrate reduction therapy) (Cox, 2006; Desnick, 2004; Hodges and Cheng, 2006).

The study here reported is the result of a four years Doctorate Research program developed at the Department of Pediatrics of the University of Naples "Federico II" from November 2002 to October 2006. It is made of two main projects, dealing respectively with some specific aspects related to metabolic morphogenesis and genetic medicine. These issues represent an exciting research field

for pediatricians with a special interest in clinical genetics, who can play a major role in counseling families in which there is a child affected by an inherited condition, and in treating genetic disorders (Cunniff et al., 2004). The first project is focused on the characterization of specific clinical, biochemical, molecular and histological issues of different defects of cholesterol biosynthesis, which are a typical example of metabolic syndromes. The second project is aimed at developing innovative therapeutic approaches for an inborn error of glycogen metabolism named Pompe disease (glycogenosis type II). The implications of the research results for the development of future studies are discussed.

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CHAPTER 2.

PROJECT 1: MORPHOGENESIS DEFECTS WITH METABOLIC BASES: THE MODEL OF THE DEFECTS OF CHOLESTEROL BIOSYNTHESIS

2.1 Backgorund

2.1.1 Dysmorphic syndromes with metabolic bases.

A few inborn errors of metabolism can present as multiple congenital anomalies/mental retardation syndromes (Fernandes et al., 2000; Gorlin et al., 2001; Leonard and Morris, 2006, Scriver et al., 2001). In these disorders, a wide pattern of malformations can be recognized, including craniofacial, renal, skeletal and gastrointestinal abnormalities, and central nervous system involvement. These defects include the defects of cholesterol biosynthesis, peroxisomal disorders, and congenital defects of glycosylation (Table 1; section 2.2.4). Lysosomal storage disorders are generally characterized by a progressive onset of dysmorphic features rather than multiple congenital anomalies. Amino acid disorders are generally not associated with multiple malformations, with the exception of the defect of glutamine synthase, presenting severe brain malformations, limb defects, and dysmorphic facies. Dysmorphic features associated with central nervous system involvement can be noted in various other metabolic conditions (Table 1, section 2.2.4), including amino acid disorders, such as homocystinuria, respiratory chain disorders, and molybdenum cofactor deficiency. Nevertheless, these disorders are generally not associated with the presence of major malformations of internal organs. Finally, a few skeletal dysplasias, also presenting with dysmorphic features, are caused by metabolic defects, such as the chondrodysplasia punctata group, defects of bone mineralization and some lysosomal storage disorders (Hall, 2001). The definition of the clinical and biochemical phenotypes of the metabolic syndromes, and the characterization of their molecular bases, is crucial to increase the rate of diagnoses and to correlate specific patterns of anomalies to the underlying pathogenic mechanisms.

2.1.2 Defects of cholesterol biosynthesis (DCB).

Defects of cholesterol biosynthesis (DCB) are a heterogeneous group of metabolic disorders associated in the majority of cases with multiple malformations and mental retardation.

Smith-Lemli-Opitz syndrome (SLOS) is the most frequent and better characterized DBC (Yu and Patel, 2005). This condition is characterized by developmental delay, typical facial dysmorphisms, limb anomalies, incomplete development of male genitalia and possible association with malformations of internal organs. The clinical phenotype is extremely variable, ranging from miscarried fetuses with multiple malformations including holoprosencephaly, to patients presenting with mild developmental delay and no major malformations.

SLOS is due to the deficiency of the 7-dehydrocholesterol reductase (DHCR7), the enzyme that catalyzes the last step of cholesterol biosynthesis. SLOS patients typically have low levels of cholesterol and high levels of its precursor 7-dehydrocholesterol (7-DHC) in the blood and other tissues. The gene of the SLOS has been cloned (*DHCR7*) and to date more than 120 mutations have been reported in the literature (Yu and Patel, 2005). The inheritance of the syndrome is autosomal recessive and the overall incidence is estimated to be around 1:40000 live births. To date there is no effective therapy for this condition. Patients can follow a high cholesterol diet (100-300mg/Kg/day of pure cholesterol added to a normal diet). The rationale of this approach is to provide the lacking metabolite, and to inhibit at the same time the endogenous biosynthesis of cholesterol and, therefore, the production of 7-DHC. Nevertheless, unfortunately, this therapeutic approach does not seem to have a significant effect on the neurological outcome (Yu and Patel, 2005). The other DCB known to date are reported in Table 2 (section 2.2.4).

2.1.3 Diagnosis of SLOS and other DCB at the University of Naples Federico II

Since 1994 the biochemical diagnosis of the SLOS has been performed at the Department of Pediatrics by UV Spectrophotometry (UVS) qualitative analysis of the 7-DHC (Guzzetta et al., 1996). The samples which resulted to be positive to the qualitative analysis by UVS, and negative samples collected from patients with clinical features possibly suggesting DCB other than SLOS, were analyzed also by quantitative Gas Chromatography/Mass Spectrometry (GC/MS) at the Department of Biochemistry and Medical Biotechnology of our University (Corso et al., 2002;

Guzzetta et al., 1996). Until November 2002, 132 biological samples had been analyzed, and 19 SLOS cases had been diagnosed. In 9 patients, molecular analysis of the *DHCR7* gene was carried out at the Department of Pediatrics, Federico II University (De Brasi et al., 1998).

Furthermore, in collaboration with Department of Biochemistry and Medical Biotechnology of this University, and the Department of Pediatrics of the University of Modena and Reggio Emilia, Italy, we described in 2002 the first patient affected by lathosterolosis (Brunetti-Pierri et al., 2002). This rare DCB, due to the deficiency of 3-beta-hydroxysteroid-delta-5-desaturase (SC5D), is characterized by multiple malformations, mental retardation and liver involvement, associated with high levels of the cholesterol precursor lathosterol in the biological fluids and tissues (Brunetti-Pierri et al., 2002).

Since the year 2000, our Hospital has been recognized as a national referral centre for the diagnosis of DCB.

2.2 EXPERIMENTAL WORK.

2.2.1 Outline of the Project

The overall aim of this project was the creation of a database of Italian patients affected by DCB in order to characterize specific clinical, biochemical, molecular and morphological aspects, and possible pathogenic mechanisms of DCB, and to promote international collaborative studies in this field.

2.2.2 Patients and Methods

In the years 2002-2006, we collected 176 biological samples (blood, skin fibroblasts, pathological samples) from patients presenting with clinical pictures compatible with DCB, as evaluated by geneticists and pediatricians in Italy. Plasma and serum samples were collected and kept frozen at -20°C protected from light until analysis. These samples were analyzed by UVS at the Department of Pediatrics Federico II University and/or GC/MS at the Department of Biochemistry and Medical

Biotechnology Federico II University. In three cases, skin fibroblasts were collected, cultivated in delipidated medium for 7 days and analyzed by GC/MS for sterol profiling, as previously reported (Brunetti-Pierri et al., 2002; Corso et al., 2002; Guzzetta et al., 1996). For one historical case (multiple malformed fetus previously aborted), only pathological samples were available and sterol analysis was therefore not possible. DNA was extracted from the pathological samples available, and analyzed by molecular analysis of the *SC5DL* gene (Brunetti-Pierri et al., 2002) at the Department of Pediatrics, Federico II University.

A Database for Italian DCB patients diagnosed at the Federico II University of Naples was set up. The information collected included the main clinical features, sterol profile at diagnosis, and molecular background when known. A clinical severity score was assessed according to previously proposed standards (Kelley and Hennekam, 2000; Witsch-Baumgartner et al., 2000) for all SLOS patients with clinical features available.

2.2.3 Results

During the research period, we diagnosed:

- A further patient with lathosterolosis by molecular analysis of the *SC5DL* gene, in a multiple malformed fetus, sibling of the first case described. This patient is extensively described in section 2.2.5.
- A patient with chondrodysplasia punctata X-linked dominant (CDPX2, Conradi-Hunermann-Happle), by sterol profiling of skin fibroblasts. To the best of our knowledge, this is the first patient biochemically diagnosed in Italy.
- Six further SLOS patients.

Data from the newly diagnosed cases were pooled together with those of the patients previously diagnosed at the Federico II University of Naples in the Database. The main clinical, biochemical, molecular features of the diagnosed patients with DCB are summarized in Table 3 (section 2.2.4).

A few studies were then performed on these patients, focusing on the following specific topics:

- Characterization of the phenotype of lathosterolosis, extensively reported in the "Specific aim 1", and appendix 2.5.1 of this chapter.
- Characterization of liver involvement in DCB, extensively reported in the "Specific aim 2" and appendix 2.5.2.
- Vitamin D status in patients affected by Smith-Lemli-Opitz syndrome, extensively reported in the "Specific aim 3" and appendix 2.5.3.

These studies particularly focused on possible pathogenic mechanisms responsible for clinical and biochemical abnormalities found in the DCB patients recruited.

Given the availability of the information collected in the Database of the Italian DCB patients, and of the biological material collected for biochemical and molecular diagnosis, we could join an international scientific collaborative study coordinated by Dr. M. Witsch-Baumgartner, Department of Medical Genetics, Molecular and Clinical Pharmacology, Innsbruck, Austria. This study was aimed at characterizing the genetic background of SLOS patients by molecular analysis of the *DHCR7* gene according to previous reports (Witsch-Baumgartner et al., 2000), in order to investigate possible pathogenic mechanisms of the disease. More in details, these studies focused on the following specific topics:

- Multicentric collaborative study on factors modifying the phenotypic severity in the Smith-Lemli-Opitz syndrome, extensively reported in the "Specific aim 4" and appendix 2.5.4.
- Characterization of the *DHCR7* mutational spectra in Spain and Italy, extensively reported in the "Specific aim 4" and appendix 2.5.5.

2.2.4 Tables 1-3.

Table 1
Examples of inborn errors of metabolism presenting with congenital anomalies and/or dysmorphic features.

PRESENTING PHENOTYPES	DISEASES
Multiple congenital anomalies/mental retardation syndromes	Defects of cholesterol biosynthesis Peroxisomal disorders Congenital disorders of glycosylation Glutaric aciduria type II (multiple acyl-Coa dehydrogenase deficiency) Carnitine palmitoyl-transferase-II deficiency Other very rare disorders (defect of glutamine synthase; beta-hydroxyisobutyryl-CoA deacylase deficiency)
Dysmorphic features and/or central nervous system abnormalities, generally without major congenital anomalies of internal organs	Pyruvate decarboxylase deficiency Respiratory chain disorders, congenital lactic acidosis Other organic acidurias (i.e., 3-hydroxyisobutyric aciduria) Homocystinuria Glycine encephalopathy Molybdenum cofactor deficiency Lysosomal storage disorders *
Predominant skeletal dysplasia	Chondrodysplasias Punctata (X-linked recessive; Peroxisomal disorders; Defects of cholesterol biosynthesis) Sulfate transport disorders (Diastrophic dysplasia group) Dyplasia with defective mineralization (Hypophosphatasia; Hypophosphatemic rickets) Inborn errors of collagen Lysosomal storage disorders *

* Generally characterized by a progressive development of dysmorphic features without major congenital abnormalities

Table 2.
Defects of cholesterol biosynthesis known to date.

DBC	OMIM	Enzymatic defect	Gene	Inheritance	Main phenotypic features
Mevalonic aciduria	#610377	Mevalonate Kinase	*MVK*	Autosomal recessive	Developmental delay, cerebellar ataxia, cataracts, dysmorphic facies, anemia, hepatosplenomegaly, diarrhea and malabsorption
Hyper IgD syndrome	#260920	Mevalonate Kinase	*MVK*	Autosomal recessive	Recurrent febrile crises, lymphadenopathy, splenomegaly, abdominal pain, diarrhea, vomiting, arthritis, rash, elevated polyclonal IgD
Smith-Lemli-Opitz syndrome	#270400	3-beta-hydroxysterol-delta-7-reductase	*DHCR7*	Autosomal recessive	Microcephaly, typical facies, mental retardation, limb defects including 2-3 toe syndactyly and possible polydactyly, incomplete development of male genitalia, midline defects, malformations possibly involving every organs and systems
Desmosterolosis	#602398	3-beta-hydroxysterol-delta-24-reductase	*DHCR24*	Autosomal recessive	Microcephaly or macrocephaly, facial dysmorphisms, gingival nodules, mental retardation, abnormal development of male and female genitalia, heart defects, possible osteosclerosis and rhizomelic shortening
Lathosterolosis	#607330	3-beta-hydroxysteroid -delta-5-desaturase	*SC5DL*	Autosomal recessive	Microcephaly, typical facies, mental retardation, skeletal defects including toe syndactyly and polydactyly, renal anomalies, incomplete development of male genitalia, liver involvement
CHILD (Congenital Hemidysplasia with Ichthyosiform erythroderma and Limb Defects) syndrome	#308050	NAD(P)H steroid dehydrogenase-like protein	*NSDHL*	X-linked dominant	Hemilateral limb defects and ichthyosiform erythroderma, with ipsilateral brain hypoplasia and possible malformations of internal organs, scoliosis, heart defects, epiphyseal stippling
CDPX2/Chondrodysplasia punctata X-linked dominant/ Conradi-Hunermann-Happle Syndrome	#302960	3-beta-hydroxyste-roid-delta(8),delta(7)-isomerase/emopamil-binding protein	*EBP*	X-linked dominant	Cataract, epiphyseal stippling, tracheal calcifications, scoliosis, hemivertebrae, congenital ichthyosiform erythroderma, follicular atrophoderma, coarse, sparse hair, patchy areas of alopecia, Dandy-Walker malformation, ventriculomegaly
Pelger-Huet anomaly	#169400	Lamin B receptor	LBR	Autosomal dominant	Heterozygote: hypolobulated (bilobed or rod-like) granulocyte nuclei Homozygote: ovoid granulocyte nuclei, macrocephaly, dysmorphisms, ventricular septal defect, polydactyly, short metacarpals, developmental delay, seizures.
Greenberg dysplasia/ Hydrops-Ectopic Calcification-Moth-Eaten Skeletal Dysplasia	#215140	Lamin B receptor	LBR	Autosomal recessive	Heterozygote: hypolobulated (bilobed or rod-like) granulocyte nuclei Homozygous: large head, punctate calcifications, laryngeal and tracheal calcifications, platyspondyly with multiple extra ossification centers, rhizomelia, moth-eaten (fragmented) long bones, polydactyly, extramedullary erythropoiesis, severe hydrops
Related disorder: Antley-Bixler Syndrome-Like Phenotype with disordered steroidogenesis	#201750	Cytochrome P450 oxidoreductase	*POR*	Autosomal recessive	Craniosynostosis, choanal atresia or stenosis, proptosis, narrow chest, ambiguous genitalia (males and females) , femoral bowing, fractures, camptodactyly

Table 3.
Database of the clinical, biochemical and molecular features of patients affected by defects of cholesterol biosynthesis with clinical information available, diagnosed at the Federico II University of Naples.

	DIAGNOSIS		
	SLOS	**LS**	**CDPX2**
FEATURES	**(% of cases showing the feature)**	**(number of cases)**	
Gender	15M/10F	2F	1F
Mental retardation	+ (100)	+ (1/1)	-
Postnatal growth retardation	+ (96)	+ (1/1)	+
Microcrephaly	+ (88)	+ (2/2)	-
Structural brain anomalies	+ (52)	+ (1/2)	-
Facial dysmorphisms	+ (100)	+ (2/2)	+
Cataract	+ (12)	+ (1/2)	+
Cleft palate*	+ (56)	-	-
Congenital heart defect	+ (28)	-	-
Renal anomalies	+ (8)	+ (1/2)	-
Male genital anomalies	+ (100)	NA	NA
2/3 toe syndactyly	+ (92)	+ (1/2)	-
Polydactyly	+ (28)	+ (2/2)	-
Vertebral midline defects	-	+ (2/2)	-
Pyloric stenosis	+ (36)	-	-
Colonic aganglionosis	+ (12)	-	-
Severity score (mean ± SD)	5.4 ± 2.8	NA	NA
7-DHC qualitative analysis by UV Spettrophotometry	Positive (100%)	Negative (1/1)	NA**
Sterol profile at diagnosis analyzed by GC/MS (mean ± SD)	Ch: 25,3 ±14.9 mg/dl 7DHC: 25 ± 22.8 mg/dl	Ch: 89.9 mg/dl Lth: 6.69 mg/dl	Ch: 60.5 µg/mg/pr Zym: 13.3 µg/mg/pr**
***DHCR7* alleles identified in 15/25 SLOS patients (Number of alleles identified)**	p.T93M (8) IVS8-1G>C (6) p.E448K (4) p.H119L (2) p.R352W (2) p.I58F (1) p.W151X (1) p.E288K (1) p.R404C (1) p.N407Y (1) p.F239L/N240S (cis) (1) (385-412 and IVS5+1-5)del (1)	NA	NA
***SC5DL* alleles identified in LS patients (siblings) (Number)**	NA	R29Q (2) G211D (2)	NA

SLOS: Smith-Lemli-Opitz syndrome; LS: Lathosterolosis; CDPX2: Chondrodysplasia Punctata X-Linked Dominant; GC/MS: Gas Chromatography/Mass Spectrometry; UVS: UV Spettrophotometry; Ch: cholesterol; 7DHC: 7-dehydrocholesterol; Lth: lathosterol; Zym: zymosterol; NA: not applicable; * including cleft soft palate, submucous cleft and cleft uvula; ** the patient affected by CDPX2 was diagnosed by sterol analysis of skin fibroblasts after 7 days of culture in delipidated medium by GC/MS.

2.2.5 Specific Aim 1.

CHARACTERIZATION OF THE PHENOTYPE OF LATHOSTEROLOSIS.

Lathosterolosis is a rare DCB due to the deficiency of 3-β-hydroxysteroid-Δ^5-desaturase. Only two patients have been described to date, both presenting with multiple malformations, mental retardation, and liver involvement. In addition in one of them pathological examination revealed mucolipidosis-like inclusions on optic microscopy analysis, and peculiar lysosomal lamellar bodies on electron microscopy analysis. This study is focused on a better characterization of the clinical phenotype of LS.

We describe a further case in a fetus, sibling of the first patient reported, presenting with neural tube defect, craniofacial and limb anomalies, and prenatal liver involvement. The fetal phenotype suggests the possible occurrence of significant intrafamilial variability in LS, expands the phenotypic spectrum of the disease, and suggests an important role of cholesterol biosynthesis derangement in neural tube closure.

Histological examination of autoptic samples from the fetus and skin fibroblasts from the living sibling suggested that the mucolipidosis-like picture previously reported is not a constant feature of LS, being possibly associated with the most severe phenotypes, but confirmed the ultrastructural finding of lamellar inclusions.

The LS phenotype appears to be characterized by the peculiar association of a recognizable pattern of multiple malformations, involving particularly axial and appendicular skeleton, central nervous and urogenital systems, and lyososmal storage. This condition shows a significant overlapping with other DCB such as the Smith-Lemli-Opitz syndrome, and shares also similarities with defects of cholesterol trafficking (Niemann-Pick type C disease), and defects of plant sterols metabolism (Sitosterolemia), suggesting intriguing pathogenic links among these conditions.

This study if fully reported in appendix 2.5.1.

2.2.6 Specific Aim 2.

CHARACTERIZATION OF LIVER INVOLVEMENT IN DEFECTS OF CHOLESTEROL BIOSYNTHESIS

Inborn defects of cholesterol biosynthesis are a group of metabolic disorders presenting with mental retardation and multiple congenital anomalies (MCA/MR syndromes). Functional and structural liver involvement has been reported as a rare (2.5-6%) complication of the Smith-Lemli-Opitz syndrome (SLOS) and it has not been fully characterized.

In this study we report on a long-term follow-up study of 4 patients with SLOS, and 1 case with lathosterolosis who presented with liver disease and underwent an extensive diagnostic work-up. Reports of liver involvement in cholesterol biosynthesis defects are reviewed.

Two main different patterns of liver involvement emerged: progressive cholestasis, and stable isolated hypertransaminasemia. In our series, the first pattern was found in 2 patients with SLOS and 1 with lathosterolosis, and the second in 2 SLOS cases. Cholestasis was associated with early lethality and normal serum γ-glutamyl-transferase (GGT) levels in SLOS, while possible prolonged survival and high GGT levels were seen in lathosterolosis. Hepatic fibrosis was present in both conditions. Liver biopsy performed in one of our SLOS patients with isolated hypertransaminasemia, showed only mild hydropic degeneration of the hepatocytes. The presence of liver involvement in 16% of the SLOS patients diagnosed at our Center suggests that this complication might have been underestimated in previously reported cases, possibly overshadowed by the severity of multiple malformations. Fetal hepatopathy, cholestasis, and isolated hypertransaminasemia can occur also in other disorders of cholesterol biosynthesis, such as mevalonic aciduria, desmosterolosis, Conradi-Hünermann syndrome, Greenberg dysplasia and Pelger-Huët homozygosity syndrome. This group of inherited disorders should be considered in the differential diagnosis of patients presenting with liver disease associated with developmental delay and/or multiple malformations. Periodic liver function evaluations are recommended in these patients. This study is fully reported in Appendix 2.5.2.

2.2.7 Specific Aim 3.

VITAMIN D STATUS IN PATIENTS AFFECTED BY SMITH-LEMLI-OPITZ SYNDROME.

Smith-Lemli-Opitz syndrome (SLOS) is an inborn error of cholesterol biosynthesis characterized by developmental delay and multiple malformations. Some of the patients have skin photosensitivity and therefore tend to avoid direct exposure to sunlight. SLOS patients typically have low levels of cholesterol and abnormally high levels of its precursor 7-dehydrocholesterol (7-DHC) in biological fluids and tissues. 7-DHC is also a precursor in the cutaneous synthesis of vitamin D. Sunlight exposure plays a major role in this pathway and reactions transforming 7-DHC into vitamin D and then into 25-hydroxy-vitamin D are known to be not specifically regulated. The aim of this study is to evaluate vitamin D status in SLOS patients.

We measured 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D serum levels and markers of calcium metabolism in 5 SLOS patients.

Despite abnormally high levels of 7-DHC circulating levels of vitamin D metabolites were not significantly different from appropriate controls matched for sex, age and season of blood collection. The analysis of historical serum samples stored in our laboratory from the same cases plus 10 other SLOS patients further supported these findings.

Our data suggest that SLOS patients have a peculiar vitamin D metabolism preventing them from vitamin D intoxication. This appears to be due in most cases to decreased transformation of 7-DHC into 25-hydroxy-vitamin D, perhaps depending on reduced sunlight exposure as a consequence of photosensitivity. Speculations on possible alternative mechanisms are discussed.

This study is fully reported in Appendix 2.5.3.

2.2.8 Specific aim 4

DATABASE OF ITALIAN PATIENTS WITH DCB AND INTERNATIONAL COLLABORATIVE STUDIES.

The clinical information collected in the Database, and the availability of biological material collected for biochemical or molecular diagnoses, made it possible to join the international collaborative studies coordinated by the Department of Medical Genetics, Molecular and Clinical Pharmacology Innsbruck, Austria on the characterization of specific molecular aspects of the Smith-Lemli-Opitz syndrome. DNA was collected from six further SLOS patients and their parents when available. In one case, DHCR7 molecular analysis was performed in our laboratory according to previous report (De Brasi et al., 1998). In five SLOS cases, DHCR7 molecular analysis was performed in Austria. DNA was collected from parents of SLOS patients and sent to Austria for molecular study on SLOS heterozygouses. The following collaborative studies were performed.

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

In the Smith-Lemli-Opitz syndrome, the growing affected embryos are dependent on exogenous (maternal) sources of cholesterol. This study is focused on evaluating 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.

Common apo E, DHCR7, and LDLR genotypes were determined in 137 biochemically characterized European SLOS patients and 59 of their parents.

A significant correlation was found 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 e2 genotypes were associated with a severe Smith-Lemli-Opitz syndrome phenotype, whereas apo E genotypes without the e2 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.

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. This study is extensively reported in Appendix 2.5.4.

Delineation of the *DHCR7* Mutational Spectra in Spain and Italy

The DHCR7 mutational spectra differ significantly in different areas of Europe, and several common putative founder mutations account for a substantial fraction of all mutations in some ethnic groups. This study was focused on the molecular analysis of 47 SLOS patients of Ashkenazi Jewish, Austrian, British, German, Italian, Irish, Polish, Portuguese, and Spanish origins. Fourteen newly identified mutations were found in 18 SLOS patients. Half of the new mutations were in the transmembrane domains of the protein. In addition, there were two null mutations, one mutation in the 4th cytoplasmic loop, two mutations in the first and last codons, and three mutations in other regions such as the second cytoplasmic loop and the first endoplasmic loop. The analysis included 20 Spanish and 12 Italian SLOS patients and revealed very different mutation spectra in these patients compared to previously described patients from Czechoslovakia, Germany, Poland, and the UK and implicated p.Thr93Met on the J haplotype as the most frequent Mediterranean founder mutation. This study is extensively reported in Appendix 2.5.5.

2.3 Conclusions

The setting up of a database of Italian patients affected by DCB allowed us to perform a few research studies aimed at characterizing specific clinical, biochemical, morphological and molecular aspects of SLOS and lathosterolosis, focusing particularly on the possible pathogenic mechanisms of these diseases.

Given their clinical variability, the definition of the phenotypic spectrum of DCB is crucial to increase the rate of diagnosis: our data suggest that patients presenting with a variable association of multiple malformations including neural tube defects, developmental delay, and liver disease should undergo plasma or tissue sterol analysis. The DCB clinical variability has been partially explained by one of the molecular collaborative studies performed, which demonstrated that maternal ApoE genotype can play a crucial role in modifying the SLOS phenotypic severity. Further studies are necessary to evaluate the possible presence of phenotypic modifier also for other DCB. The other collaborative molecular study confirmed our previous observation that the T93M mutation is particularly frequent in SLOS patients coming from the Mediterranean area (De Brasi et al., 1998).

The pattern of malformations observed in DCB might suggest a correlation with specific underlying pathogenic mechanisms. First, the axial and appendicular skeletal malformations observed in lathosterolosis might be partially explained by a functional disruption in the hedgehog signaling pathway, secondary to the primary defect in cholesterol biosynthesis. Furthermore, our data suggested that various different factors might contribute to the pathogenesis of the liver involvement observed in DCB, including insufficient cholesterol availability, a possible secondary disruption in bile acid metabolism in SLOS, and a secondary disruption in plant sterol metabolism in lathosterolosis. Finally, the lysosomal lamellar inclusions demonstrated in lathosterolosis and SLOS, supported the existence a secondary disruption of the intracellular cholesterol trafficking at least in these two diseases and, possibly, in other DBC.

Our data suggested that SLOS patients have a peculiar vitamin D metabolism, protecting them from vitamin D intoxication. It has been hypothesized that the relatively high incidence of SLOS in some

European ethnic groups, the relatively low percentage of patients reported to be born to consanguineous parents, and the more severe enzymatic defects caused by the most common mutations compared with the less common ones, are suggestive of a heterozygote advantage. An increased vitamin D biosynthesis rate has been proposed as a possible heterozygote advantage (Kelly and Hennekam, 2000): therefore, further studies are required to evaluate vitamin D metabolism in SLOS carriers.

2.4 References

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2.5 Appendices of project 1

APPENDIX 2.5.1

Massimiliano Rossi, Maria D'Armiento, Ida Parisi, Paola Ferrari, Christine Hall, Mariarosaria Cervasio, Francesco Rivasi, Raffaella Vecchione, Gaetano Corso, Generoso Andria, and Giancarlo Parenti.

Clinical phenotype of lathosterolosis.

Submitted for publication.

Clinical Phenotype of Lathosterolosis.

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Clinical phenotype of Lathosterolosis.

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ABSTRACT

Lathosterolosis (LS) is a defect of cholesterol biosynthesis (DCB) due to the deficiency of 3-β-hydroxysteroid-Δ^5-desaturase. Only two patients have been described to date, both presenting with multiple malformations, mental retardation, and liver involvement. In addition in one of them pathological examination revealed mucolipidosis-like inclusions on optic microscopy analysis, and peculiar lysosomal lamellar bodies on electron microscopy analysis. This study is focused on a better characterization of the clinical phenotype of LS.

We describe a further case in a fetus, sibling of the first patient reported, presenting with neural tube defect, craniofacial and limb anomalies, and prenatal liver involvement. The fetal phenotype suggests the possible occurrence of significant intrafamilial variability in LS, expands the phenotypic spectrum of the disease, and suggests an important role of cholesterol biosynthesis derangement in neural tube closure.

Histological examination of autoptic samples from the fetus and skin fibroblasts from the living sibling suggested that the mucolipidosis-like picture previously reported is not a constant feature of LS, being possibly associated with the most severe phenotypes, but confirmed the ultrastructural finding of lamellar inclusions.

The LS phenotype appears to be characterized by the peculiar association of a recognizable pattern of multiple malformations, involving particularly axial and appendicular skeleton, central nervous and urogenital systems, and lyososmal storage. This condition shows a significant overlapping with other DCB such as the Smith-Lemli-Opitz syndrome, and shares also similarities with defects of cholesterol trafficking (Niemann-Pick type C disease), and defects of plant sterols metabolism (Sitosterolemia), suggesting intriguing pathogenic links among these conditions.

INTRODUCTION

Defects of cholesterol biosynthesis (DCB) are a group of inherited metabolic diseases associated with multiple congenital anomalies and mental retardation (MCA/MR). Smith-Lemli-Opitz syndrome (SLOS, OMIM: #270400), due to the deficiency of the enzyme 7-dehydrocholesterol reductase (DHCR7), is the most frequent and better characterized example of DCB [Kelley and Hennekam, 2000; Porter et al., 2003]. SLOS patients present with typical facial dysmorphisms, limb anomalies, incomplete development of male genitalia, and malformations possibly involving all organs and systems. Lathosterolosis (LS, OMIM: #607330) is an additional example of this group of metabolic syndromes [Brunetti-Pierri et al., 2002; Krakowiak et al., 2003; Parnes et al., 1990; Rossi et al., 2005]. This disorder is due to the deficiency of 3-β-hydroxysteroid-Δ^5-desaturase (SC5D), the enzyme immediately upstream DHCR7, which catalyzes the transformation of lathosterol into 7-dehydrocholesterol. Inheritance is autosomal recessive. LS is very rare: in fact after the characterization of the first case [Brunetti-Pierri et al., 2002], only another LS patient has been diagnosed post-mortem [Krakowiak et al., 2003]. Interestingly the latter case showed massive mucolipidosis-like inclusions [Krakowiak et al., 2003; Parnes et al., 1990], a feature not evident on histological examination of a liver biopsy performed in the first case reported [Rossi et al., 2005]. In this study, we describe a new LS case further expanding the clinical spectrum of the disease, and we report updated clinical and morphological information about the first patient diagnosed [Brunetti-Pierri et al., 2002; Rossi et al, 2005]. We also compare the phenotypic features observed in LS with those reported in the SLOS, and other defects of sterol metabolism. The aim of this study is a better characterization of the clinical phenotype of LS.

CLINICAL REPORTS

PATIENT 1

Clinical features. The first sibling of the living girl with LS [Brunetti-Pierri et al., 2002, Rossi et al, 2005], was a historical fetal case, aborted at 21 weeks gestation, after a routine ultrasound scan revealed multiple malformations. Neither maternal diabetes, nor teratogenic exposure was reported. Around conceiving the mother was on adequate folate supplementation. Clinical features were reviewed after a diagnosis of LS was made in the living sister. On macroscopic examination, weight and length appeared to be appropriate for gestational age (290 g and 21 cm respectively). Post-mortem findings included multiple congenital anomalies such as Type II Arnold Chiari malformation, microcephaly, postaxial hexadactyly of upper and lower limbs, bilateral clubfeet and lumbosacral meningocele (1.5 x 1 cm). No malformations were reported in other organs. Unfortunately neither photos nor radiographs were available. Chromosome analysis showed a normal female karyotype. A diagnosis of LS was suspected on the bases of the pattern of limb and craniofacial anomalies. Molecular analysis of the *SC5DL* gene, performed as previously described [Brunetti-Pierri et al., 2002] in DNA samples obtained from stored pathological specimens, revealed the same two mutations found in the living sister (R29Q/86G→A in exon 1, and G211D/632G→A in exon 4), thus confirming the diagnosis.

Histological features. Histological examination of fetal samples from thymus, spleen, pancreas, liver, kidneys, adrenals, uterus, ovaries, heart, lungs, spinal cord and brain was carried out by optic microscopy using the following stains: hematoxylin-eosin; special histochemical stains like PAS and PAS after diastase digestion, Hale's dialyzed colloidal iron for acid MPS; immunostaining for HepPar1 in liver histology. In none of the histological specimens examined, cells with storage or foamy material were noted. In particular, liver tissue showed normal portal tracts, and marked extramedullary hematopoiesis leading to atrophy of hepatocytic laminae (Fig.1). Hemosiderinic pigment within periportal hepatocytes and Kupffer cells were also present (Fig.1 detail). Hypoxic

changes were noted in cerebral cortex and white matter, likely due to abortion procedures. No significant histological changes were noted in the other organs.

PATIENT 2

Clinical features. This girl with LS, presented with developmental delay, craniofacial anomalies, post-axial polydactyly of the left foot, syndactyly of toes, horseshoe kidneys, bilobate gallbladder and progressive intra-hepatic cholestasis [Brunetti-Pierri et al., 2002; Rossi et al., 2005]. She was reviewed at the age of 7 years. During follow-up, her gestaltic facial appearance had significantly changed. In the neonatal period the girl showed a striking microcephaly, with receding forehead, eyelid ptosis, prominent nose with bulbous nasal tip and micrognathia with protruding upper lip (Fig.2A). In the following years, physical examination revealed microcephaly with bitemporal narrowing, epicanthic folds, eyelid ptosis, a small nose with anteverted nares, a small chin, puffy cheeks, and a long philtrum (Fig.2B-C). At 6 years of age, small bilateral lens opacities became evident on ophthalmological examinations, which subsequently evolved in total cataracts of the right eye, requiring surgery. The cholestasis severity was progressive with persistently elevated serum levels of transaminases, bilirubin and ammonia as previously described [Rossi et al., 2005], and portal hypertension was noted on abdominal Doppler ultrasound at 7 years of age. In spite of multi-vitamin supplementation, low levels of vitamin A and E were repeatedly detected, as well as abnormal clotting tests. The girl experienced two pathological fractures and DEXA scan performed at 7.5 years of age, revealed severe generalized osteoporosis (Z-score: - 4.9). She also developed a peculiar feeding behavior showing frequent vomiting and a striking fishy body odor after tasting particular foods, such as fish, cow meet, milk and derivatives, which she tended to avoid spontaneously: urine trimethylamine analysis by Protonic Nuclear Magnetic Resonance resulted to be negative [Maschke et al., 1997], and no clear explanation was found for this phenomenon.

Blood films revealed the presence of abnormally shaped red cells with anisopoikilocytosis (MCV: 89.3fl; normal values: 82-98; RDW: 18.4%; normal values 11-14), acanthocytes,

schistocytes, and large platelet (mean platelet volume: 12.4 fl; normal range: 9.1-12.3); vacuolated monocytes were also noted (Fig. 3). Full blood count repeatedly showed normal hemoglobin levels (range: 12.2-13.2 g/dl) and low to normal platelet count (range: 121-219 x 10^3/μl; normal range: 150-400 x 10^3/μl).

At 7 years of age, plasma sterol profile by Gas-Chromatography-Mass Spectrometry showed normal levels of cholesterol (155 mg/dl), persistently high levels of lathosterol (17 mg/dl), and abnormally high levels of other methylated sterols, such as in the previously performed determinations [Brunetti-Pierri et al., 2002]: these metabolites were subsequently identified as the plant sterols sitosterol (0.7mg/dl), campesterol (0.5mg/dl) and stigmasterol (0.2mg/dl) (total plant sterols: 0.8% of total sterols detected; control range: 0.36 $\pm$ 0.08) (Pianese and Corso, personal communication).

Radiological features. A skeletal survey, performed at 11 months of age, showed sagittal clefting of the eight thoracic vertebra ("butterfly" shaped) (Fig. 4A), as previously mentioned [Brunetti-Pierri et al., 2002]. In addition, the left foot showed postaxial polydactyly characterized by ossification of a proximal phalanx, and an abnormal widening of the fifth metatarsal representing an attempt at duplication. Radiologically, postaxial polydactyly was evident also in the right foot, as shown by the abnormally wide fifth metatarsal, with no phalangeal ossification (Fig. 4B).

A comparison was made between the limb radiological anomalies noted in patient 2 and those found in six unrelated SLOS cases diagnosed at the Department of Pediatrics, Federico II University of Naples with available skeletal surveys. These patients showed significant similarities, such as toe syndactyly (6/6), and a polydactyly pattern including either post-axial (4/6) or interdigital (1/6) extra-digits (Fig.4 C).

Histological features. Skin fibroblasts from patient 2 were propagated in DMEM supplemented with 10% fetal bovine serum. Cells were harvested at T0 and after 3 and 7 days of culture in

delipidated medium, as previously described [Brunetti-Pierri et al., 2002]. Subsequently, fibroblasts were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide and dehydrated through graded alcohols for Epon embedding; sections were stained doubly with uranyl acetate and lead citrate and examined by a Zeiss EM-109 electron microscope.

These cells were compared with skin fibroblasts from two non related SLOS patients diagnosed at the Department of Pediatrics Federico II University, and one control, cultured in the same conditions.

The LS cells showed lamellar lysosomal inclusion bodies at T0 (Fig.5A). Fibroblasts from two SLOS patients also showed almost indistinguishable lamellar inclusions (Fig.5C). In both cases the lamellar vacuoles appeared to be progressively degradated within the lysosomes after three and, more extensively, after seven days of culture in delipidated medium (Fig.5B). Control cells did not show any sign of storage (Fig.5D).

DISCUSSION

Cholesterol plays a crucial role in several biological pathways including steroid hormone biosynthesis, embryonic development, and the definition of cellular membrane functional microdomains interacting with various signaling pathways [Kelley and Herman, 2001; Gondré-Lewis et al., 2006]. As a consequence of this biological pleiotropy, a disruption in cholesterol biosynthesis can lead to a wide range of congenital anomalies with significant clinical variability [Kelley and Herman, 2001]. This is well exemplified by the desmosterolosis, a DCB due to the deficiency of 3-β-hydroxysterol-Δ^{24}-reductase (OMIM: #602398): the two patients described to date showed striking differences in their clinical pictures, including micro- and macrocephaly and very different patterns of bone mineralization [FitzPatrick et al., 1998; Andersson et al., 2002]. The definition of the clinical phenotypes of DCB is crucial to increase the rate of diagnoses and to correlate specific patterns of anomalies to the underlying pathogenic mechanisms. LS represents a further example of a rare DCB showing significant phenotypic variability even within the same

family, as demonstrated by the new case here described. Nevertheless, based on the very few patients reported to date, the phenotype of LS appears to be characterized by a recognizable pattern of multiple malformations, involving particularly axial and appendicular skeleton, central nervous and urogenital systems, partially overlapping with SLOS (Table I).

Facial dysmorphisms

The neonatal cranio-facial phenotype of our patient 2 is characterized by a marked microcephaly (Fig. 2), and shows remarkable similarity to the non related previously described case [Parnes et al., 1990]. Figure 2 clearly shows the significant change in her facial appearance occurred in the following years, with a gestalt progressively becoming more similar to the SLOS facies.

Axial skeleton

Patient 1 presented with lumbar meningocele. Neural tube defects (NTD) are a group of congenital anomalies ranging from anencephaly, resulting from failure of fusion of the cranial neural tube, to spina bifida, resulting from a failure of the vertebral laminae to fuse to complete the neural arch, with an overall incidence of approximately 1/1000 [Merbs, 2004; Mitchell, 2005]. These malformations have been reported either in MCA/MR syndromes due to chromosomal abnormalities (e.g. trisomy 13, 18, and 21), single gene defects (e.g. Meckel-Gruber syndrome, OMIM: #249000), and teratogenic exposures (e.g. maternal diabetes), or as isolated defects [Mitchell, 2005]. The vast majority of isolated cases are multifactorial and periconceptional folate supplementation to the mother has been proved to significantly reduce the incidence of these developmental defects [Mitchell, 2005]. Although theoretically there is no definite evidence that the occurrence of NTD in the case here described is surely related to the underlying metabolic disease and the disruption in cholesterol metabolism, a few clinical and experimental observations might support this hypothesis. First, although to the best of our knowledge NTD have never been reported to date in biochemically confirmed patients with DCB [Porter, 2003; Kelley and Hennekam, 2000],

lumbar meningomyelocele has been described in a case with a clinical diagnosis of CHILD syndrome (OMIM: #308050) [Hebert et al., 1987]. Secondly, mice lacking squalene synthase, an enzyme involved upstream in cholesterol biosythesis, whose deficiency has not been reported in association with human phenotypes until now, do show severe neural tube defects [Tozawa et al., 1999]. In addition, although studies evaluating apoE and apoB genotypes in patients with NTD have failed to find a significant association [Volcik et al., 2002], NTD have been unexpectedly described in mice homozygous or heterozygous for mutations of the apoB gene, which plays a key role in cholesterol transport [Huang et al., 1995; Homanics et al., 1995].

Patient 2 presented with an eight thoracic "butterfly" vertebra. Sagittal clefting is a rare anatomical defect which has been reported as a part of multiple malformation syndromes, such as Alagille syndrome (OMIM: #118450), or as an isolated anomaly [Sonel et al., 2001]. This is the result of a developmental error involving the cartilaginous precursor of the vertebral centrum, associated with a failure of the notochord to recede [Kjaer et al 1994; Merbs, 2004]. It has been demonstrated that DCB are associated with a functional disruption in different steps of the Sonic Hedgehog (Shh) pathway, which is essential for embryogenesis and, particularly, for normal midline development [Porter, 2003; Hennekam, 2005]. In particular, Shh is expressed in the floor plate and the notochord, is required for the specification of the ventral spinal cord [Gofflot et al., 1999; Lupo et al., 2006], and represents one of the major factors from the notochord and floor plate promoting and controlling the formation of the sclerotome, which is the primary origin of the axial skeleton [Kornak and Mundlos, 2003]. Therefore, it might be possible to hypothesize that an impairment of the Shh pathway, secondary to the defective cholesterol biosynthesis, is responsible of the observed vertebral midline defect.

There is some evidence suggesting that the Shh pathway closely interacts with Zic2 [Brown et al., 2003], a zinc-finger transcription factor whose deficiency has been associated in humans with either severe midline defects (holoprosencephaly) or NTD [Grinberg and Millen, 2005]. This might

suggest a possible pathogenic mechanism explaining the occurrence of either NTD or vertebral sagittal defect in LS.

Appendicular skeleton

The two LS patients here described, presented with postaxial polydactyly associated, in patient 2, with toe syndactyly. Both these features were also shown by the non related previously reported case, showing hexadactyly with complete metatarsus and phalanges in the left foot, and fused fifth and sixth metatarsal bones on the right, associated with bilateral 2-3 toe syndactyly [Parnes et al, 1990]. The LS mouse model shows postaxial polydactyly and, in addition, interdigital defects with bifurcation of the fourth medial phalanges [Krakowiak et al., 2003]. We compared the limb anomalies observed in LS with those found in the SLOS, reviewing the radiological features noted in six unrelated SLOS patients, and the findings previously reported in the literature. It is very well known that SLOS patients generally present with toe syndactyly, frequently involving the second and third toe with a distinctive "Y-shape", and can have postaxial polydactyly [Ryan et al., 1998; Kelley and Hennekam, 2000]: in addition, we observed the presence of the less common interdigital polydactyly (Fig. 4C). Postaxial polydactyly has been reported also in some of the other DCB, such as Greenberg dysplasia (OMIM #215140) and Pelger-Huët homozygosity syndrome (OMIM #169400), both of them caused by mutation in the *LBR* gene [Oosterwijk et al., 2003], and X-linked dominant chondrodysplasia punctata (CDPX2, OMIM #302960), due to the deficiency of the enzyme Δ^8-Δ^7-sterol isomerase emopamil-binding protein [Kelley and Herman, 2001]. Therefore, the spectrum of limb anomalies, including both postaxial and interdigital polydactyly possibly involving the four limbs, and toe syndactyly, appears to be a significant common pattern of abnormalities of the appendicular skeleton in DCB.

Both Sonic and Indian hedgehog are expressed in limbs during embryogenesis, playing a central role in regulating the anteroposterior patterning of the limb bud, and the chondrocyte

proliferation and hypertrophy, respectively [Kornak and Mundlos, 2003]. Therefore, a functional disruption in the hedgehog pathway might well explain the pathogenesis of the DCB common pattern of limb anomalies.

Condrodysplasia punctata was not noted on skeletal surveys performed in infancy in two LS patients [patient 2; Parnes et al., 1990]. Although this is a major feature of some of the DCB such as CDPX2, Greenberg dysplasia, and CHILD syndrome, to the best of our knowledge it has been reported in only one biochemically confirmed SLOS case, who had also a chromosomal translocation [Kelley and Hennekam, 2000], and has not been associated to other DCB, such as desmosterolosis, and mevalonic aciduria (OMIM: #610377).

Other congenital anomalies

Both our patient 2 and the non related case previously reported [Parnes et al, 1990; Krakowiak et al., 2003] developed bilateral cataracts. Cataract has been reported in some of the other DCB such as SLOS, mevalonic aciduria, and CDPX2 and in animal models of DCB such as rats treated with the DHCR7 inhibitor AY9944 [Sakuragawa, 1976]. It has been hypothesized that the accumulation of abnormal cholesterol precursors might significantly affect lens metabolism and that cataract might be considered as a storage disease-like manifestation at least in the SLOS [Cenedella, 1996; Elias et al., 1997]. On the other hand, the recent characterization of a rat model of hereditary cataract due to the deficiency of another enzyme involved in cholesterol biosynthesis named lanosterol synthase, and characterized by reduced cholesterol levels without significant precursors accumulations within the lens, has suggested that cholesterol deficiency might itself contribute to cataractogenesis too [Mori et al., 2006]. Other ocular findings associated with LS are eyelid ptosis, downslanting palpebral fissures, microcorneae and corneal clouding, the latter possibly representing another example of storage disease-like manifestations. As in the SLOS, the development of male genitalia can be affected in LS [Parnes et al., 1990], while female genitalia appeared to be normal in both cases here described. The two patients described post-natally had

very severe developmental delay [patient 2; Parnes et al., 1990], in one case associated with seizures [Parnes et al., 1990]. The neurological structural abnormalities reported include Type II Arnold Chiari malformation, brain atrophy (hydrocephalus "ex vacuo"), demyelination and dystrophic calcification: further clinical reports are required to understand whether there is a definite pattern of central nervous system malformations in this condition.

It has been recently reported that LS and SLOS mice models show a marked decrease in the number of secretory granules, and an increase of morphologically aberrant granules in exocrine and endocrine glands [Gondré-Lewis et al., 2006]. This seems to be the result of the abnormally high levels of cholesterol precursors, which significantly affect cell membrane functional microdomains playing a key role in granule budding. Further studies are required to clarify whether these functional and morphological anomalies are detectable also in LS and SLOS patients, and their clinical relevance.

Signs of storage

An intriguing finding, reported in the non related LS case previously described, was a widespread storage of mucopolysaccharides and lipids within histiocytes and the white matter, strikingly sparing the neurons, and evident as massive inclusions on optic microscopy; these findings were initially misleading, and mucolipidoses were considered in the differential diagnosis [Parnes et al., 1990]. No signs of storage were visible on histological examination by optic microscopy of either a liver biopsy from patient 2 [Rossi et al., 2005], or various tissues from our patient 1, or tissues from LS mouse model [Krakowiak et al., 2003], suggesting that the widespread mucolipidosis-like picture previously described does not appear to be a constant feature of LS. Since the patient described by Parnes et al. [1990] showed a biochemical defect more severe than our patient 2 [Krakowiak et al., 2003], it might be possible to hypothesize that the mucolipidosis-like picture is associated only with the most severe phenotypes. In this respect, the lack of overt

lysosomal storage in both our patient 1 and the LS mouse model might be explained by their pre- or peri-natal lethality [Krakowiak et al., 2003].

On the other hand, vacuolated monocytes were noted on blood film examination of patient 2, a feature not investigated in the unrelated described patient [Parnes et al., 1990]. Moreover, electron microscopy examination of fibroblasts from our patient 2 revealed the presence of lysosomal vacuoles with concentric lamellar inclusion, as observed in the non related previously reported LS case and in the LS mouse model [Parnes et al., 1990; Krakowiak et al., 2003]. We found similar lamellar inclusions also in fibroblasts from SLOS patients (Fig.5C) according to a previous observation [Wassif et al., 2002]. These findings share striking morphological similarities with the typical lysosomal vacuoles visible in fibroblasts from patients affected by Niemann-Pick type C disease (NPC, OMIM: #257220, #607625) [Patterson et al., 2001; Wassif et al., 2002; Krakowiak et al., 2003], a lysosomal storage disorder due to mutations of the *NPC1* or *NPC2* genes, and characterized by a defect in cholesterol trafficking. This morphological overlapping has been partially explained. In the SLOS, it has been shown that 7-dehydrocholesterol impairs low density lipoproteins (LDL) intracellular trafficking and degradation, probably interacting with the NPC1 protein through its sterol sensitive domain. This results in an accumulation of LDL-derived unesterified cholesterol, which can be demonstrated as an increased filipin staining [Wassif et al., 2002]. A similar effect has been postulated also for lathosterol in LS [Krakowiak et al., 2003]. Interestingly the same inclusions can be found also in either a rat model treated with the DHCR7 inhibitor AY9944 [Sakuragawa, 1976], or in a cell model treated with class 2 amphiphiles, a various group of compounds disrupting intracellular lipid trafficking possibly through an inhibition of the NPC1 protein function [Lange et al., 2000; Wassif et al., 2002]. In fact, the lamellar bodies might be considered the morphological expression of a disruption in intracellular cholesterol trafficking, which is the primary defect in NPC, and a secondary defect in LS and SLOS.

Similar inclusions have been described also in dermal cells from a case with CHILD syndrome [Hashimoto et al., 1998], supporting the hypothesis that disruptions in various steps of cholesterol biosynthesis might be associated with an abnormal intracellular lipid trafficking.

The NPC clinical phenotype shares some similarities with LS, such as developmental delay and intrahepatic cholestatic liver disease with high levels of gamma-glutamyl transferase [Yerushalmi et al., 2002; Rossi et al., 2005]. Although the presence of lysosomal lamellar inclusions on electron microscopy examination and increased filipin staining in skin fibroblasts is generally considered pathognomonic of NPC [Patterson et al., 2001], these morphological findings can be demonstrated also in LS [Krakowiak et al., 2003]. The lack of multiple malformations, and the presence of specific neurological signs such as ataxia or the typical vertical supranuclear ophthalmoplegia can clearly differentiate NPC from LS, and in selected cases, tissue sterol profiling or molecular analysis of *NPC1* and *NPC2* genes can be performed for a definite differential diagnosis.

Hematology

Patient 2 showed large platelets, inconstantly low platelet count, and abnormally shaped red cells (Fig. 3). Unfortunately no information is available on platelet size and erythrocyte shape for the unrelated reported patient [Parnes et al., 1990]. Several factors might contribute to the red cells shape abnormalities noted in our patient, such as malabsorption secondary to the severe hepatic disease, and abnormal erythrocyte membrane fluidity secondary to the abnormal sterol metabolism [Stevenson and Hardie, 2001]. In this respect, it might be interested to notice that achantocytosis is also found in disorders of cholesterol transport, such as abetalipoproteinemia (OMIM: #200100) and hypobetalipoproteinemia (OMIM: +107730).

On the other hand, large platelets possibly associated with thrombocytopenia, and abnormally shaped red cells (stomatocytes) have been described in Sitosterolemia (OMIM: #210250) as part of a complex clinical phenotype including hemolysis, xanthomas, arthritis,

accelerated atherosclerosis, and normal psychomotor development. This condition is due to mutations in the *ABCG5* or *ABCG8* genes, which cause an increased intestinal absorption and impaired bile excretion of plant sterols and cholesterol, leading to abnormally high plasma levels of these compounds. Interestingly our patient 2 repeatedly showed normal to high plasma levels of cholesterol in spite of the its biosynthetic defect [Brunetti-Pierri et al., 2002; Rossi et al., 2005], and abnormally high levels of plant sterols, even if at lower concentrations comparing with Sitosterolemia, this suggesting the presence of a secondary disruption in the metabolism of these compounds. The intestinal absorption of cholesterol and plant sterols is regulated by a protein named NPC1L1 due to its significant homology to the protein mutated in NPC disease [Davies et al., 2000]. Comparing with its homologous, NPC1L1 shows a similar putative sterol sensing domain but interacts with different intracellular targeting signals [Davies et al., 2000; Davis et al., 2004]; therefore it might be possible to speculate that abnormally high levels of lathosterol could interfere also with the NPC1L1 function, determining a secondary disruption in sterols intestinal absorption, which could explain the abnormally high plasma levels of plant sterols, the normal to high levels of cholesterol, and the platelet abnormalities found in LS patient 2.

Liver involvement

The liver involvement in patient 2 has been previously extensively described [Rossi et al., 2005]. Concerning patient 1, some of the hepatic histological abnormalities noted have been previously reported among non specific signs of prenatal liver disease in other DBC, such as desmosterolosis and Greenberg dysplasia [Clayton, 2003]. Our data suggest that liver involvement in LS may range from prenatal hepatopathy to postnatal severe progressive intrahepatic cholestasis.

A few observations might suggest a possible link between the abnormal plant sterol metabolism observed and liver involvement in LS. Mildly high levels of sitosterol have been reported in patients with advanced liver diseases such as primary biliary cirrhosis, in association with markedly increased levels of cholestanol [Nikkila et al., 2005]; the lack of the latter

biochemical abnormality in our patient 2 seems against the possibility that the high levels of plant sterols detected are the consequence of her liver disease (Pianese and Corso, personal communication). On the other hand, patients affected by Sitosterolemia can present with abnormal liver function, cholestatic liver disease and cirrhosis [Miettinen et al., 2006], and it has been demonstrated that abnormally high levels of plant sterols can specifically determine cholestasis with high levels of GGT in patients on total parenteral nutrition [Clayton et al., 1993; Clayton et al., 1998]. These observations might suggest that the high levels of plant sterols observed in the LS patients, even if at lower concentrations comparing with Sitosterolemia, might contribute to the pathogenesis of her liver damage: further studies are necessary to clarify this point and to evaluate the possible therapeutic effects of a decreased plant sterol intake on liver function in LS.

Conclusion

In conclusion, we report a further case of LS, contributing to the delineation of the clinical phenotype of this condition, which is characterized by the peculiar association of a recognizable pattern of multiple malformations and signs of lysosomal storage. Our observation supports a role of cholesterol biosynthesis derangement in neural tube closure: patients presenting with a variable associations of multiple malformations including NTD, developmental delay, and liver disease should undergo plasma or tissue sterol analysis, or molecular analysis of the *SC5DL* gene. The clinical phenotype of LS shows a significant overlapping with other DCB such as the SLOS, and shares also similarities with defects of cholesterol trafficking (NPC), and defects of plant sterol metabolism (Sitosterolemia), suggesting intriguing pathogenic links among these conditions.

ACKNOWLEDGEMENTS

We are grateful to Dr. Pierluigi Pianese, Dipartimento di Biochimica e Biotecnologie Mediche, Federico II University, Naples, Italy, for his important contribution in the biochemical analyses, and

to Dr. Antonio Risitano, Dipartimento di Ematologia, Federico II University, and Dr Roberta Migliorati, S.C. Pediatria Oncologica, Ospedale Pausilipon, Naples, Italy, for their opinion on the hematological anomalies reported in the paper.

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TABLE I. Phenotype of lathosterolosis, and comparison with the Smith-Lemli-Opitz syndrome.
IA. Congenital anomalies.

CLINICAL FEATURES	**LS**		**SLOS**
	Patients (n. of reports)	**LS mouse model**	**(frequency %, if known)**
Gender	2 female,1 male	NA	NA
Growth delay	+ (2/3)	+	+
Intrauterine growth retardation	+ (1/3)	+	+ (16)
Postnatal growth retardation	+ (2/2)	NA*	+ (82-88)
Facial dysmorphisms	+ (3/3)	+	+ (100)
Microcephaly	+ (3/3)	-	+ (80-84)
Prominent metopic suture	+ (1/3)	-	+
Eyelid ptosis	+ (2/3)	NA	+ (59-70)
Downslanting palpebral fissures	+ (1/3)	NA	+
Cataract	+ (2/2)	-	+ (12-22)
Corneal clouding/Microcorneae	+ (1/3)	-	+
Short nose	+ (2/3)	+	+
Anteverted nares	+ (1/3)	NA	+ (69-78)
Micrognathia	+ (2/3)	+	+ (67)
Downturned mouth	+ (2/3)	NA	+
High arched palate	+ (2/3)	-	+ (29)
Cleft palate	- (0/3)	+	+ (37-47)
Gingival hypertrophy	+ (1/3)	NA	+ (37)
Appendicular skeleton anomalies	+ (3/3)	+	+
Postaxial polydactyly	+ (3/3)	+	+ (48-49)
Interdigital defects (complete polydactyly, bifurcated phalanges)	- (0/3)	+	+
Toe syndactyly	+ (2/3)	-	+ (90-97)
Clubfeet	+ (1/3)	NA	+ (27)
Axial skeleton anomalies	+ (2/3)	-	+
Vertebral clefting	+ (1/3)	-	-
Neural tube defect	+ (1/3)	-	-
Brain structural anomalies	+ (2/3)	-	+ (21-37)
Type II Arnold Chiari malformation	+ (1/3)	NA	-
Hydrocephalus “ex vacuo”, demyelination, dystrophic calcification	+ (1/3)	-	+
Developmental delay	+ (2/2)	NA	+ (92-95)
Seizures	+ (1/2)	-	+ (<5)
Hearing loss/ abnormal auditory evoked potentials	+ (1/2**)	NA	+ (10)
Renal malformations	+ (1/3)	-	+ (29-43)
Male genital anomalies	+ (1/1)	-	+ (65-91)
Liver involvement* **	+ (3/3)	+	+ (2.5-16)
Platelet/red cell abnormalities	+ (1/1)	NA	-
References	Our patients; [Brunetti-Pierri et al, 2002; Krakowiak et al, 2003; Parnes et al, 1990; Rossi et al, 2005]	[Krakowiak et al, 2003]	Our patients; [Kelley and Hennekam 2000; Rossi et al, 2005; Ryan et al,1998]

IB. Signs of storage.

STORAGE	**LS**		**SLOS**
	Patients (n. of reports)	**LS mouse model**	
Lamellar inclusions (EM)	+ (2/2)	+	+
Mucolipidosis-like inclusions (OM)	+ (1/3)	-	-
Vacuolated monocytes (blood film, OM)	+ (1/1)	NA	-
Fundus oculi cherry red spot	- (0/2)	NA	-
Urine mucopolysaccharides and oligosaccharides	- (0/2)	NA	-
References	Our patients; [Krakowiak et al., 2003; Parnes et al., 1990]	[Krakowiak et al, 2003]	Our patients; [Wassif et al., 2002]

LS: Lathosterolosis; SLOS: Smith-Lemli-Opitz syndrome; n.: number.
+: reported; - not reported; OM: Optic microscopy; EM: electron microscopy; NA: not applicable.
*: early lethality; **: the patient reported by Parnes et al. [1990] had abnormal brainstem auditory evoked potentials; our patient 2 had only transient conductive hearing loss; ***ranging from mild prenatal involvement to postnatal severe progressive intra-hepatic cholestasis.

Figure 1

Patient 1: liver histology showing normal portal tracts with marked extramedullary hematopoiesis leading to atrophy of hepatocytic laminae. Haemosiderinic pigment within periportal hepatocytes and Kupffer cells was also noted (detail) (H&E, X200; detail: HepPar, X400).

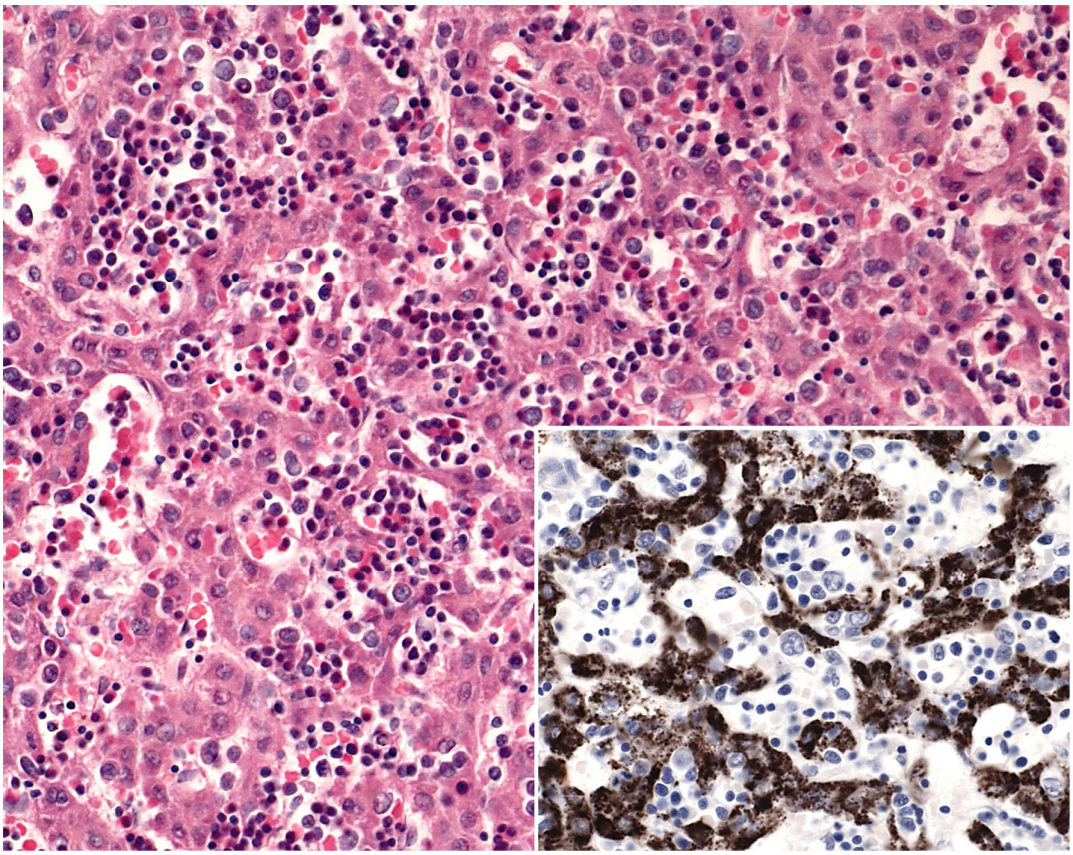
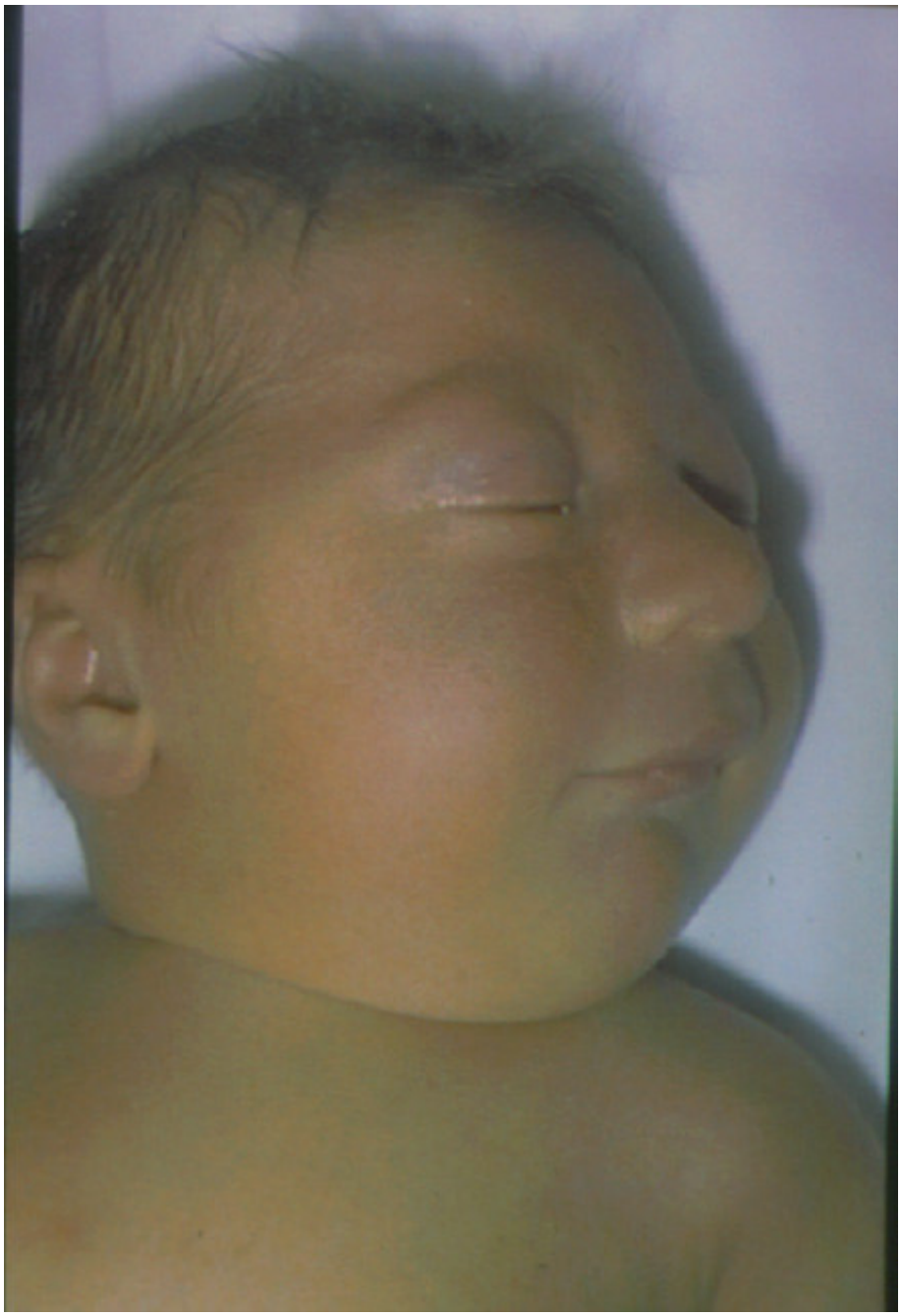


Figure 2

Patient 2: evolution of facial appearance at different ages.

2A. Neonatal period: note microcephaly, receding forehead, prominent nose with bulbous nasal tip and micrognathia with protruding upper lip.



2B. At two years of age; note a significant change in gestalt appearance comparing with the neonatal age.

2C. At 7 years of age: note microcephaly, epicanthic folds, eyelid ptosis, a small nose with anteverted nares, a small chin, and a long philtrum.

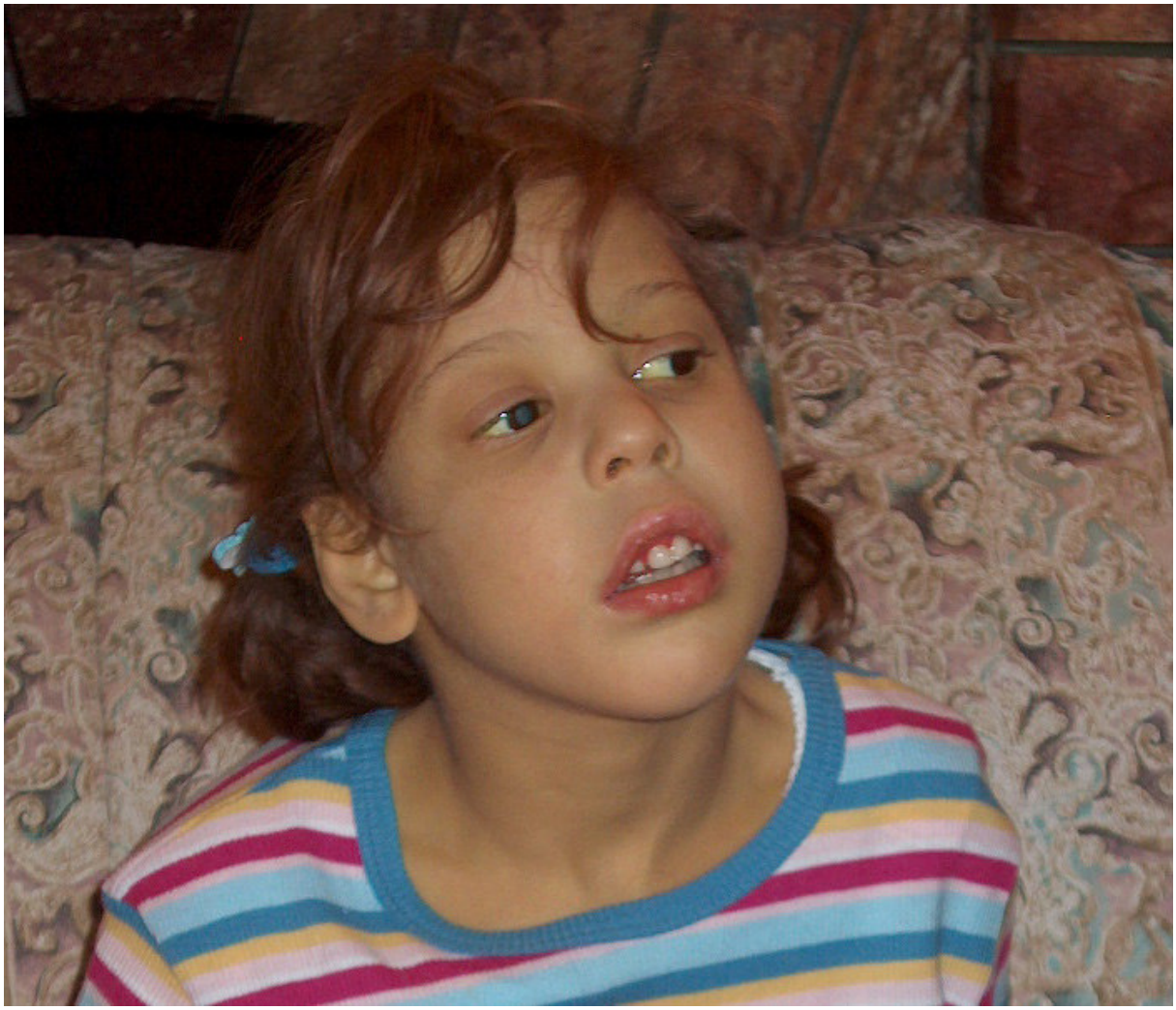


Figure 3

Patient 2: hematological features: mp: macroplatelets; aca: acanthocytes; sch: schistocytes; vm: vacuolated monocytes (detail) (May-Grunwald Giemsa).

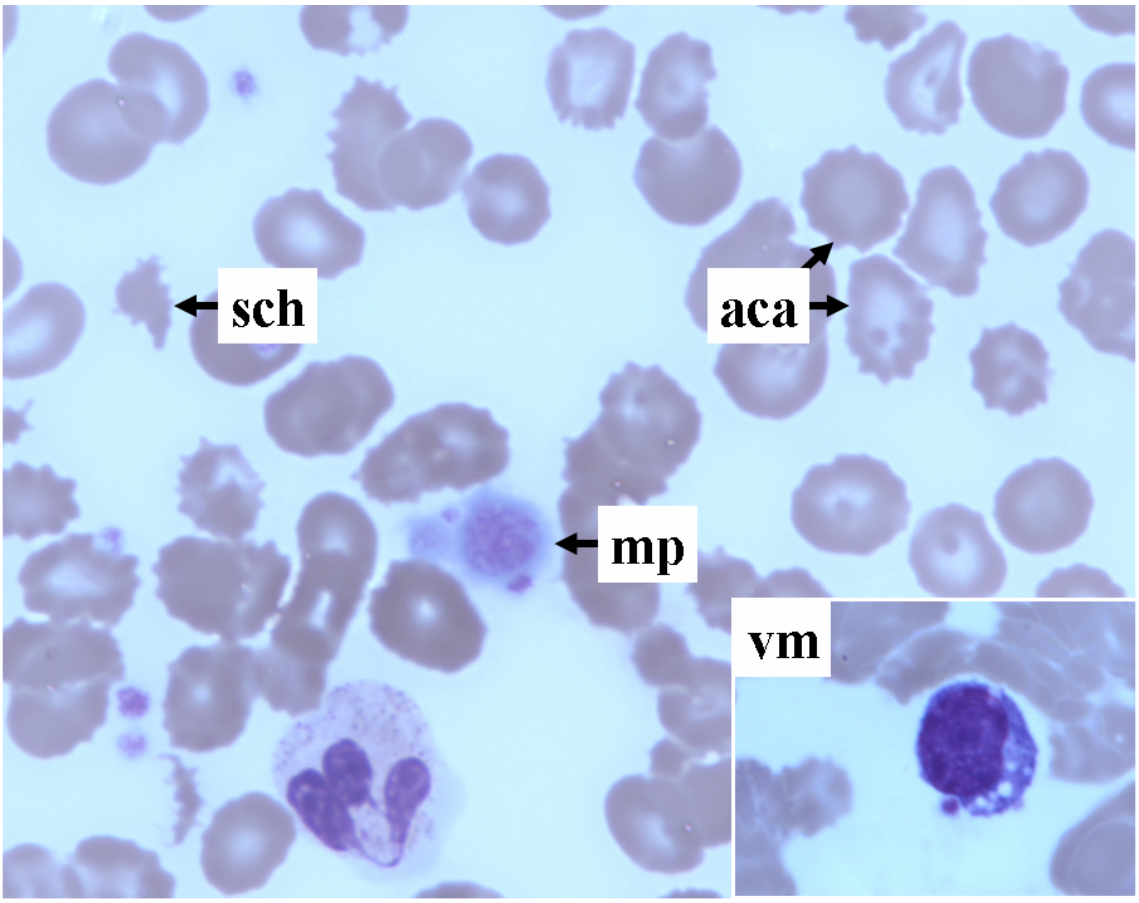
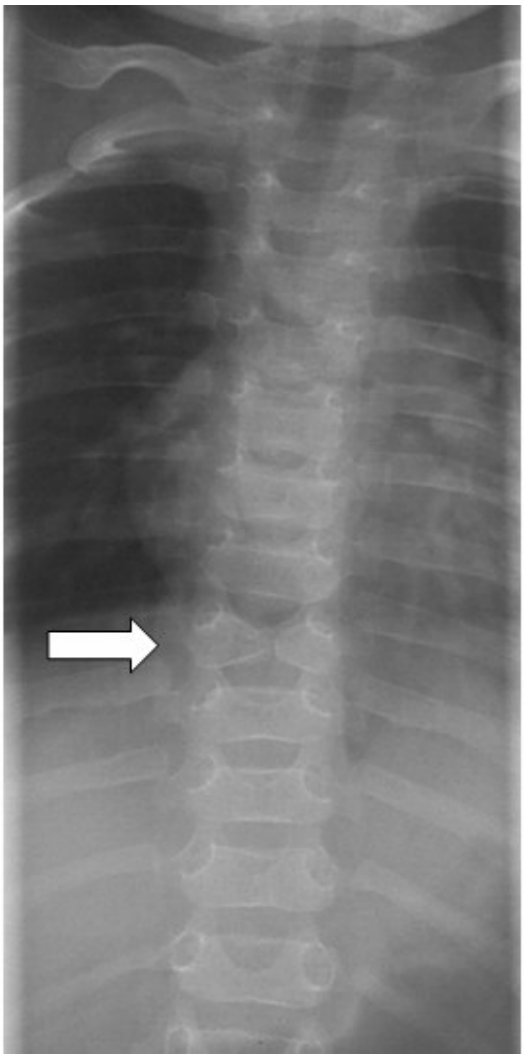


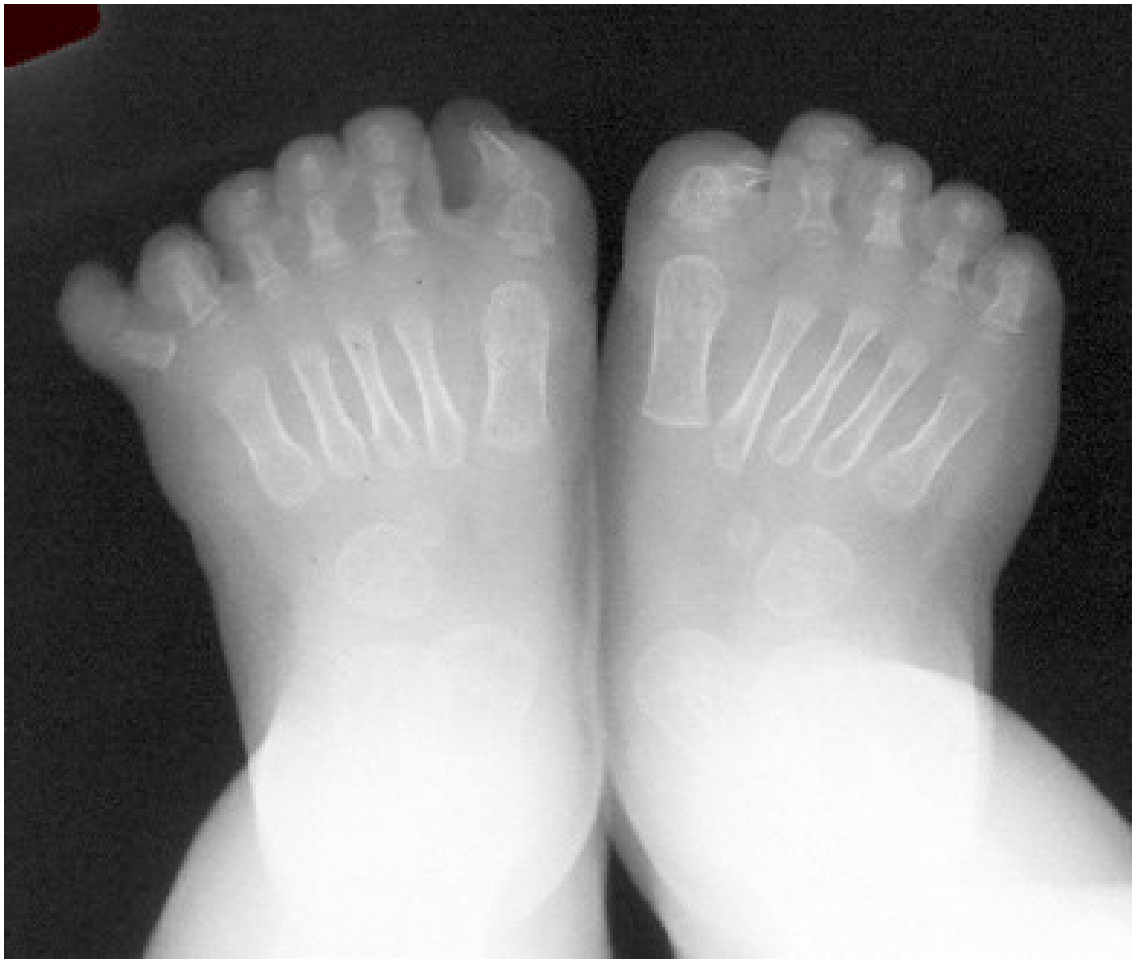
Figure 4

Details from a skeletal survey performed in patient 2 at 11 months of age, and comparison with an unrelated patient affected by Smith-Lemli-Opitz syndrome.

4A. Patient 2: detail of spine radiograph, showing the sagittal clefting of the eight thoracic vertebra, "butterfly" shaped (white arrow).



4B. Patient 2: detail of feet showing bilateral postaxial polydactyly characterized on the left side by an ossified proximal phalanx and abnormal widening of the 5th metatarsal representing an attempt at duplication, and, on the right side, abnormally wide fifth metatarsal, with no phalangeal ossification.



4C. Unrelated SLOS patient: detail of feet. The left foot shows soft tissue syndactyly and interdigital polydactyly, with a small extra proximal phalanx in the third and forth web space. In the right foot there is postaxial polydactyly with fusion at the base of the fifth and sixth metatarsals.

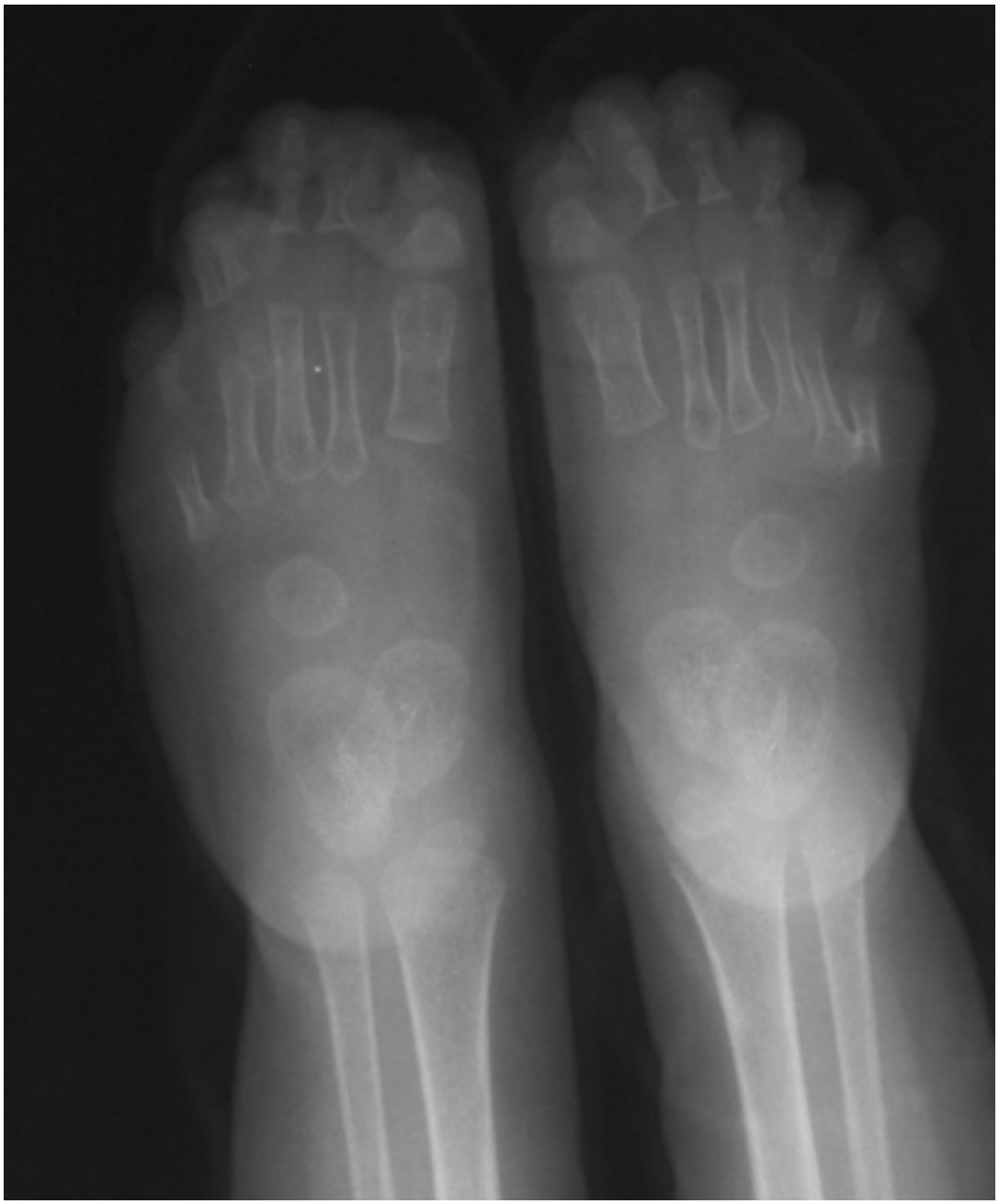
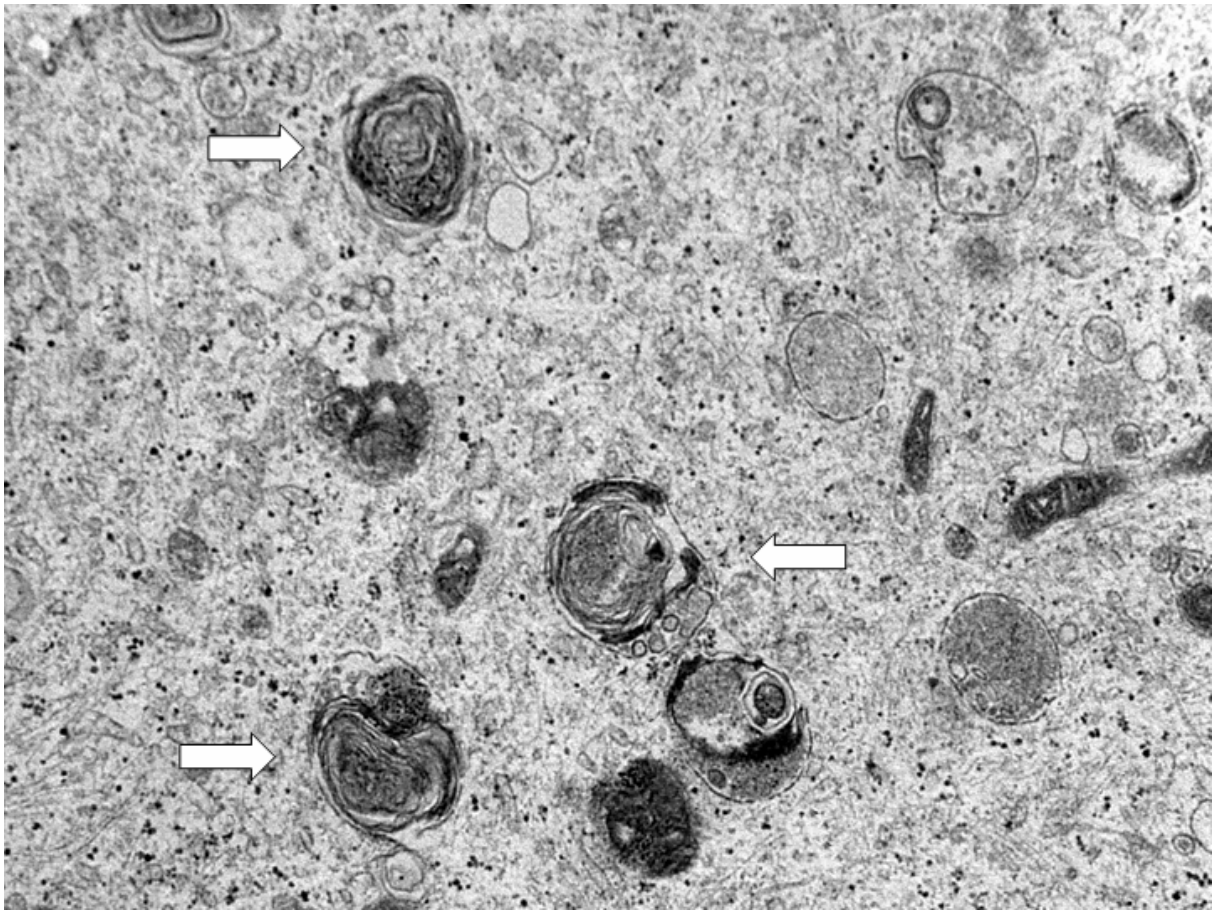


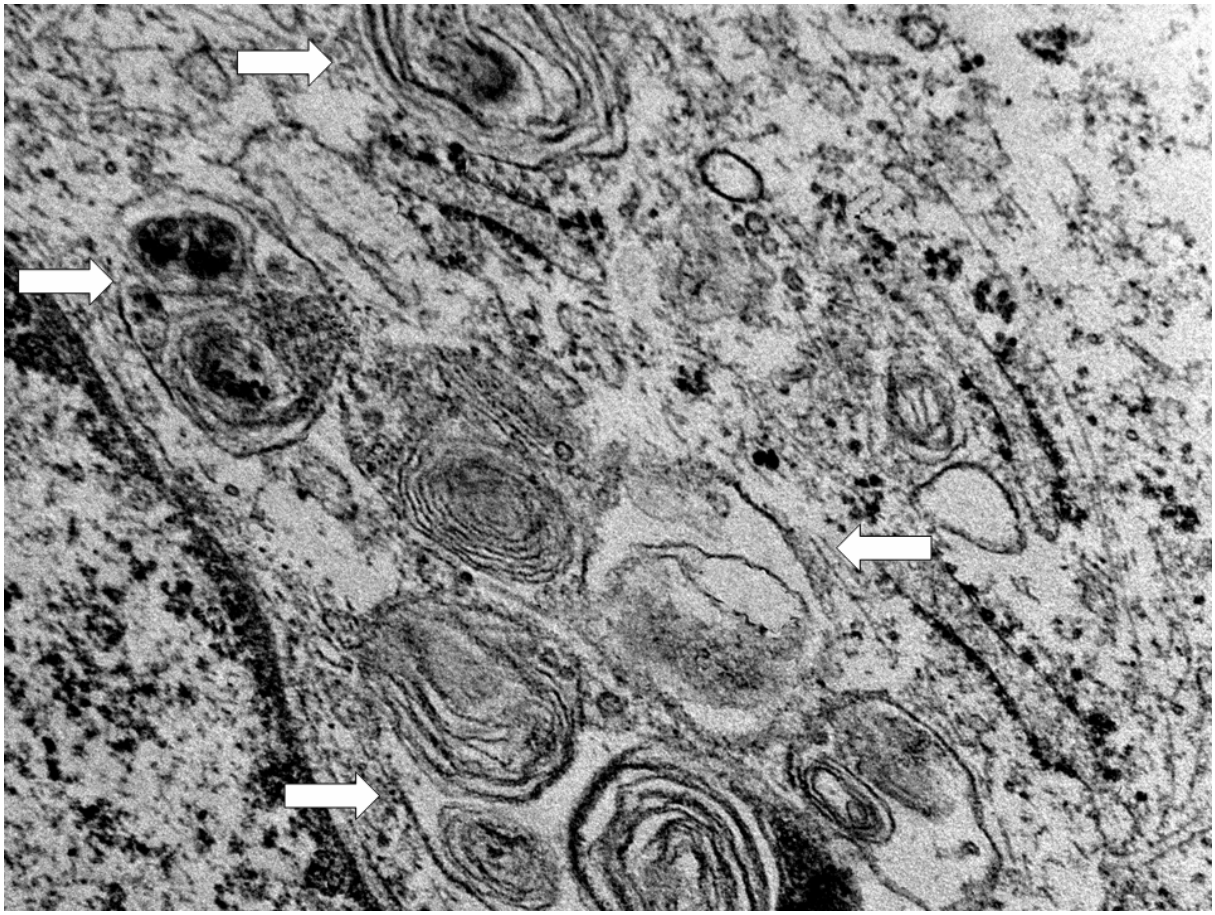
Figure 5

Electron microscopy examination.

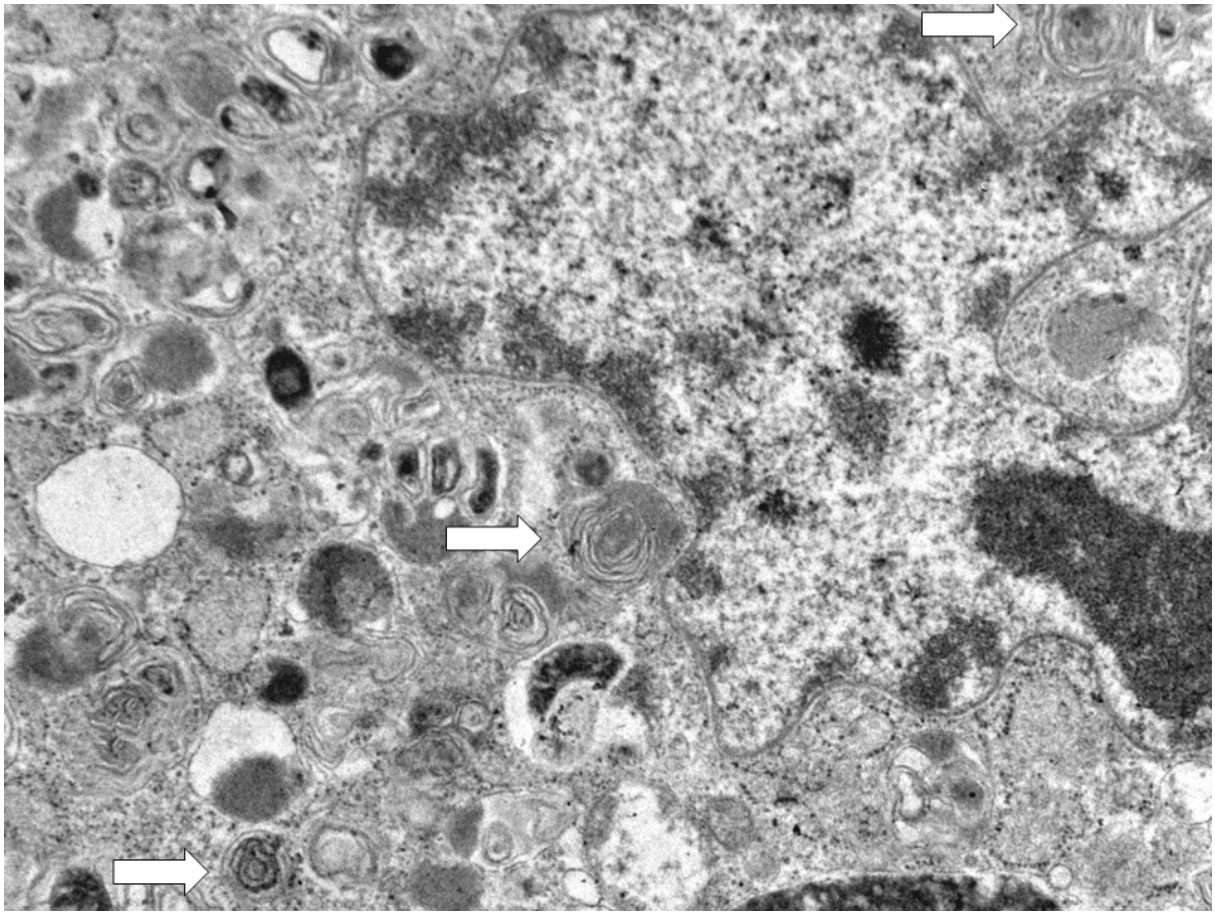
5A. Skin fibroblasts from patient 2, at baseline, 16000X. Note the lamellar inclusions (white arrows).



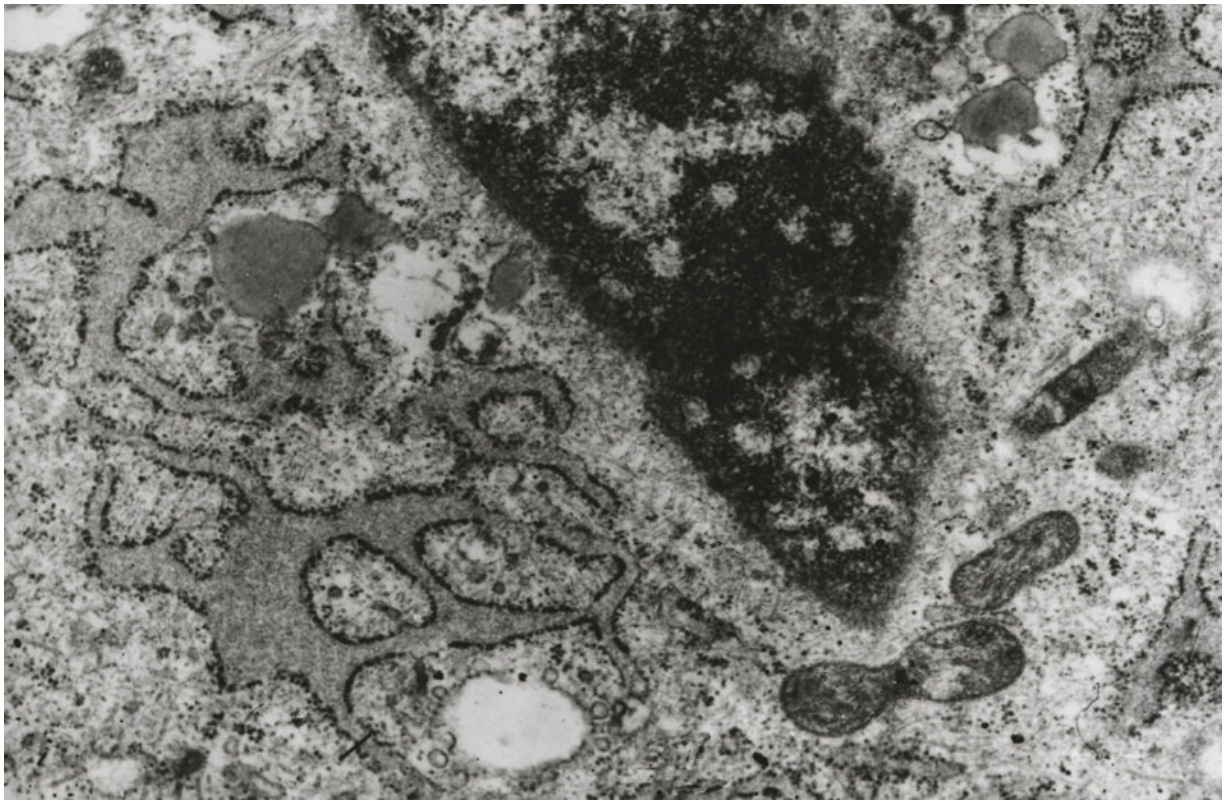
5B. Skin fibroblasts from patient 2, after 7 days of culture in delipidated medium, 40000X. The lamellar vacuoles appear to be progressively degradated within the lysosomes (white arrows).



5C. Skin fibroblasts from an unrelated Smith-Lemli-Opitz patient at baseline, 13000X, showing almost indistinguishable lamellar inclusion bodies (white arrows).



5D. Skin fibroblasts from a control, after 7 days of culture in delipidated medium, 12000X**,** showing no sign of storage.



APPENDIX 2.5.2

Rossi M, Vajro P, Iorio R, Battagliese A, Brunetti-Pierri N, Corso G, Di Rocco M, Ferrari P, Rivasi F, Vecchione R, Andria G, Parenti G.

Characterization of liver involvement in defects of cholesterol biosynthesis: long-term follow-up and review.

American Journal of Medical Genetics A

2005 15;132(2):144-51.

American Journal of Medical Genetics 132A:144–151 (2005)

Characterization of Liver Involvement in Defects of Cholesterol Biosynthesis: Long-Term Follow-Up and Review

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Inborn defects of cholesterol biosynthesis are a group of metabolic disorders presenting with mental retardation and multiple congenital anomalies (MCA/MR syndromes). Functional and structural liver involvement has been reported as a rare (2.5–6%) complication of the Smith–Lemli–Opitz syndrome (SLOS) (OMIM: #270400) and it has not been fully characterized. Here, we report on a long-term follow-up study of four patients with SLOS, and one case with lathosterolosis (OMIM: #607330) who presented with liver disease and underwent an extensive diagnostic work-up. Reports of liver involvement in cholesterol biosynthesis defects are reviewed. Two main different patterns of liver involvement emerged: progressive cholestasis, and stable isolated hypertransaminasemia. In our series, the first pattern was found in two patients with SLOS and one with lathosterolosis, and the second in two SLOS cases. Cholestasis was associated with early lethality and normal serum γ-glutamyl-transferase (GGT) levels in SLOS, while possible prolonged survival and high GGT levels were seen in lathosterolosis. Hepatic fibrosis was present in both conditions. Liver biopsy performed in one of our SLOS patients with isolated hypertransaminasemia, showed only mild hydropic degeneration of the hepatocytes. The presence of liver involvement in 16% of the SLOS patients diagnosed at our Center suggests that this complication might have been underestimated in previously reported cases, possibly overshadowed by the severity of multiple malformations. Fetal hepatopathy, cholestasis, and isolated hypertransaminasemia can occur also in other disorders of cholesterol biosynthesis, such as mevalonic aciduria (OMIM: +251170), desmosterolosis (OMIM: #602398), Conradi–Hünermann syndrome (OMIM: #302960), Greenberg dysplasia (OMIM: #215140), and Pelger–Huët homozygosity syndrome (#169400). This group of inherited disorders should be considered in the differential diagnosis of patients presenting with liver disease associated with developmental delay and/or multiple malformations. Periodic liver function evaluations are recommended in these patients.

KEY WORDS: **Smith–Lemli–Opitz syndrome; lathosterolosis; cholesterol biosynthesis defects; liver; cholestasis; gamma-glutamyl-transferase; GGT**

INTRODUCTION

Inborn defects of cholesterol biosynthesis are a group of metabolic disorders presenting variably with mental retardation, multiple congenital anomalies, and abnormal bone calcification, i.e., as MCA/MR syndromes [Kelley and Herman, 2001]. The Smith–Lemli–Opitz syndrome (SLOS) (OMIM: #270400), due to 7-dehydrocholesterol reductase (DHCR7) deficiency, is the most frequent and best characterized condition in this group of diseases, with an incidence of approximately 1 in 40,000 liveborn infants. In this condition developmental abnormalities may affect virtually all organs and systems, in most instances with a severe impact on prognosis and quality of life.

Lathosterolosis (OMIM: #607330), due to the deficiency of 3-β-hydroxysteroid-Δ^5-desaturase (SC5D), the enzyme involved in the metabolic step immediately upstream of DHCR7, was recently identified by our group [Brunetti-Pierri et al., 2002], and is also a MCA/MR syndrome. Only a single living patient affected by this disorder is known; a second male patient was diagnosed post-mortem [Parnes et al., 1990; Krakowiak et al., 2003].

Functional and structural liver involvement is a known manifestation of SLOS [Kelley and Hennekam, 2000] although it has not been fully characterized. It is possible that variable liver involvement may be overshadowed by the severity of malformations in SLOS and, possibly, other related disorders. The aim of this study is to better characterize liver involvement in defects of cholesterol biosynthesis. Here, we report on a long-term follow-up of four SLOS patients and the living patient with lathosterolosis, all presenting with signs of liver disease. We also reviewed the literature about liver involvement in defects of cholesterol biosynthesis. The presence of hepatic

Grant sponsor: Ministero dell'Istruzione, Università e Ricerca; Grant number: PRIN 2002 prot. 2002068222_003.

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Received 22 July 2004; Accepted 28 August 2004
DOI 10.1002/ajmg.a.30426

involvement in different defects of this pathway suggests a role of cholesterol metabolism in the pathogenesis of liver diseases.

PATIENTS

Five patients with defects of cholesterol biosynthesis presented with abnormal liver function. Among 24 SLOS patients diagnosed in the Department of Pediatrics, Federico II University, Naples, Italy in the last 10 years, clinically overt or sub-clinical liver disease were observed in 4. In all patients the diagnosis of SLOS was suspected on the bases of the association of typical facial features, limb anomalies including 2–3 toe syndactyly, and multiple malformations, and it was confirmed by gas chromatography–mass spectrometry plasma sterol profiling, showing increased amounts of 7-dehydrocholesterol (Table IA). Molecular analysis of the *DHCR7* gene was performed only in patient 4, who was a genetic compound for the R352W and IVS8-1G > C mutations [patient 8 in De Brasi et al., 1999]. A clinical severity score was assessed by previous standards [Kelley and Hennekam, 2000; Witsch-Baumgartner et al., 2000].

The patient with lathosterolosis (case 5) was identified by gas chromatography–mass spectrometry plasma and fibroblast sterol profiling, showing increased amounts of lathosterol (Table IA). The diagnosis of lathosterolosis was confirmed by SC5D assay in fibroblasts cultured in cholesterol-free medium, and by mutational analysis of the *SC5D* gene [Brunetti-Pierri et al., 2002].

Patients were followed either at the Department of Pediatrics, Federico II University of Naples, or in collaboration with local hospitals with periodic clinical assessment, routine blood tests including liver function tests, liver ultrasound and biopsy when appropriate, with follow-up periods ranging from 2.5 months to 6 years (Table IB).

None of the patients was obese at the time of presentation of liver involvement. In none of them drugs, possibly causing hepatotoxicity, were used. All SLOS patients had low plasma cholesterol levels. Cases 1 and 4 were supplemented with oral cholesterol (85–300 mg/kg/day of pure cholesterol added to a normal diet for age).

Liver function tests, imaging and histological findings are summarized in Table IB.

Case 1

This girl with SLOS, presented at birth with malformations (Table IA) and neonatal jaundice, treated with phototherapy. Results of maternal screening tests for infectious diseases, including hepatitis B, were negative. At 2 weeks, aspartate amino transferase (AST) and alanine amino transferase (ALT) were normal (40 and 13 U/L, respectively). Persistent jaundice and pale stools were reported during the first months and, at that time, physical examination showed a hard enlarged liver, mild splenomegaly, and undernourishment. Since the age of 2 months, blood chemistry tests showed raised levels of AST (ranging from 318 to 1,017 U/L), ALT (197–521 U/L), total and conjugated bilirubin (9.60–9.70 mg/dl; 4.70–7.57 mg/dl, respectively), alkaline phosphatase (ALP) (1,162 U/L), whereas γ-glutamyl-transferase (GGT) was normal (12 U/L) (Table IB). Alpha-fetoprotein was greatly elevated (>100,000 ng/ml; normal range: <10). Raised white cell count (21,200–32,800/μl; neutrophils: 10,320–19,980/μl), platelets (470,000–1,002,000/μl) and markers of inflammation were also detected, as well as mild transient anemia at the age of 4 months (hemoglobin lowest level 8.6 g/dl). Serial blood cultures, serology for toxoplasma, rubella, cytomegalovirus, herpes simplex, urinary cytomegalovirus, α-1-antitrypsin, immunoglobulins, lymphocyte subtyping, thyroid function tests, chromosome analysis, and screenings for common metabolic disorders were negative or normal. Creatine kinase (CK) was normal, ruling out muscle-derived hypertransaminasemia.

Ultrasonography of liver showed an enlarged bright liver with no other anomalies. Laparotomy showed a cirrhotic liver and intraoperative cholangiography demonstrated a normal biliary tract.

Histologic studies showed a distorted hepatic architecture with marked portal, periportal and pericellular fibrosis, bridging and nodular features, ductular proliferation, diffuse giant cell transformation, biliary pigment within hepatocytes, canaliculi and Kupffer cells, and hemosiderin granules in Kupffer cells (Fig. 1).

Ursodeoxycholic acid supplementation (30 mg/kg/day), high cholesterol diet (85 mg/kg/day) and wide-spectrum antibiotic therapy were started at the age of 2 months. Despite lowering of total and direct bilirubin (3.9 mg/dl; 2.6 mg/dl, respectively), and amino transferases serum levels (AST: 135 U/L; ALT 111 U/L) (Table IB), in the following weeks the patient's general conditions worsened progressively and she eventually died in another hospital at age 5 months.

Case 2

This girl with SLOS, was born at 36 weeks of gestation. Asphyxia and multiple severe malformations were noted (Table IA), as well as neonatal jaundice since the 2nd day of life (total bilirubin: 14.3 mg/dl in the 3rd day of life), treated with phototherapy. In the following weeks, results of liver function tests were found to be abnormal (AST: 135–417 U/L; ALT: 198–337 U/L; total bilirubin: 7.99–8.59 mg/dl; conjugated bilirubin: 2.6–3.5 mg/dl; ALP: 449–1200 U/L), except GGT levels (34 U/L) (Table IB). Transient thrombocytopenia (22,000/mm^3) and anemia (hemoglobin 6.8 g/dl) were noted. A sepsis was diagnosed and treated with antibiotics. An abdominal ultrasound scan was normal at 11 days of life.

During the following month, a hard and enlarged liver was noted on clinical examination and abnormalities of liver function tests persisted. Blood cultures, standard karyotype were negative or normal. Due to persistent vomiting and feeding difficulties, parenteral nutrition was needed intermittently. No further investigations on liver involvement were performed as, due to the severe clinical conditions of the patients, parents refused liver biopsy and a regular follow-up. The patient died at age 2.5 months.

Case 3

This girl with SLOS, presented with congenital anomalies including severe heart malformations (atrioventricular canal, patent ductus arteriosus, hypoplastic pulmonary truncus) (Table IA). Neonatally amino transferases levels were normal for age (AST: 39 U/L; ALT: 76 U/L). From the age of 1.5 months, a persistent increase of AST (68–193 U/L) and ALT (108–690) was noted (Table IB). Due to heart malformation and generalized seizures she was treated with dipyridamole and multidrug anticonvulsant therapy (phenobarbital, clonazepam, ethosuximide). Since the beginning of this therapeutic regimen a slight increase of GGT and ALP was noted (35–178 and 418–702 U/L, respectively) with normal total bilirubin levels (0.3–0.4 mg/dl). These abnormalities were considered the result of the anticonvulsant therapy.

Since the age of 3 years liver ultrasonography showed hepatomegaly. Screening tests for common infectious diseases, including hepatitis A, B, and C, Epstein–Barr virus, toxoplasma, rubella virus, and cytomegalovirus, were negative. Screening tests for inborn metabolic disorders were also negative. Liver histology was normal, apart from slight hydropic degeneration of some hepatocytes (Fig. 2). The patient died at age 6 years of heart failure.

TABLE I. Manifestations in Our Patients

Patients	1	2	3	4	5
Diagnosis	SLOS	SLOS	SLOS	SLOS	Lathosterolosis
A: Clinical findings and sterol pattern at diagnosis					
Clinical findings					
Gender	Female	Female	Female	Male	Female
Developmental delay	+	+	+	+	+
Microcephaly	+	+	+	+	+
Brain anomalies	+	+	−	+	−
Cataract/lens opacities	−	+	−	−	−
Cleft palate/bifid uvula	−	+	+	+	−
Congenital heart defect	+	−	+	−	−
Pyloric stenosis	−	+	−	+	−
Renal anomalies	−	−	−	−	+
Genital anomalies	−	−	−	+	−
Polydactyly	−	−	−	−	+
Toe syndactyly	+	+	+	+	+
Cholestatic liver disease	+	+	−	−	+
Mildly raised amino transferases	−	−	+	+	−
SLOS severity score[a]	8	9	6	4	
Sterol pattern at diagnosis					
Cholesterol (mg/dl)	9	24	21	33	170
7-dehydrocholesterol (mg/dl)	34	86	17	9	−
Lathosterol (mg/dl)	−	−	−	−	13
B: Liver serum function tests (range of lowest and highest values measured during follow-up), imaging, and histology					
Age of recruitment	Birth	Birth	Birth	5 years	1 year
Follow-up length	5 months	2.5 months	6 years	2 years	4 years
Liver function tests					
AST (times UNL)	<1–14	2–6	<1-4	<1–2	3–8
ALT (times UNL)	<1–6	2–4	<1–20	1–4	2–7
GGT (times UNL)	<1	<1	≤1–5[b]	<1	5–19
ALP (times UNL)	≥1–3	1–3	1–3[b]	<1	5–15
Total bilirubin (times UNL)	3–9	2–8	<1	<1	<1–11
Direct bilirubin (times UNL)	7–25	9–12	<1	<1	<1–35
Total bile acid (times UNL)	≥1–3	NP	NP	NP	3–12
Quick time %	42–66	NP	80-85	93–102	43–94
INR	1.74–1.31	1.04	NP	1.11–0.99	1.12–1.71
Liver ultrasounds	Enlarged bright liver	Enlarged liver[c]	Enlarged liver	Bright liver	Bilobed gall-bladder
Liver histology	Cirrhosis with portal periportal and pericellular fibrosis, giant cells, cholestasis	NP	Slight hydropic hepatocytes changes; absence of specific lesions	NP	Portal fibrosis, cholestasis

+, present; −, absent; NP, not performed; UNL, upper normal limit for age.
[a]Score range: 0–20.
[b]Case 3 was on multi-drug anticonvulsant therapy when GGT and ALP levels were measured.
[c]Detected on clinical examination only.

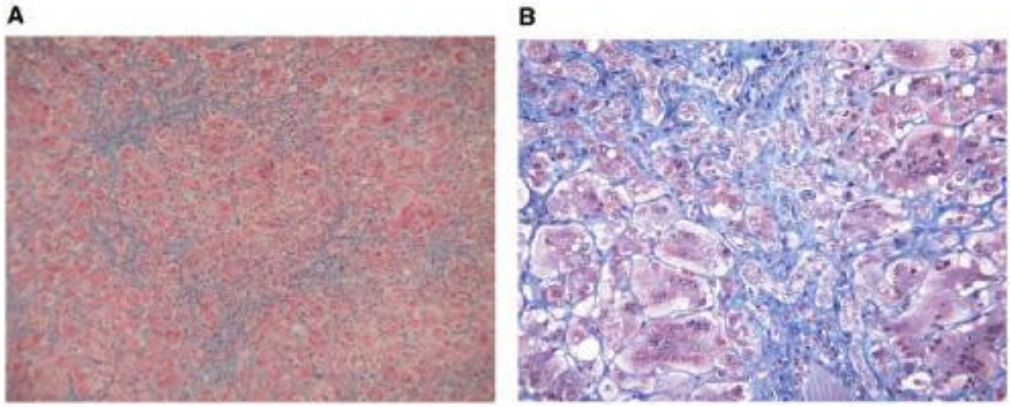

Fig. 1. Case 1. Surgical liver biopsy. **A**: Portal, periportal and pericellular fibrosis with porto-portal bridging (CAB, ×100). **B**: Giant cells, portal tract with ductular proliferation (CAB, ×200).

Case 4

This boy with SLOS, presented with multiple anomalies and developmental delay (Table IA). Mildly increased serum amino transferases levels were noted since the age of 5 years, on follow-up laboratory evaluation (AST: 35–91 U/L; ALT: 54–158 U/L). On physical examination liver size was normal. Serum markers of cholestasis were within the normal range (Table IB). Tests for common causes of hypertransaminasemia, including serologic tests for infectious diseases (toxoplasma, cytomegalovirus, Epstein–Barr virus, hepatitis A, B, and C), screening tests for celiac disease, serum copper and ceruloplasmin, α-1-antitrypsin, anti-nucleus, anti-liver-kidney microsomes, and anti-smooth muscle autoantibodies, CK, serum immunoglobulins were negative. Since the age of 7 years, abdominal ultrasound scans showed mild steatosis; AST was normal (45 U/L) and ALT was only slightly elevated (68 U/L). The patient was on a very high cholesterol supplementation (up to 300 mg/kg/day).

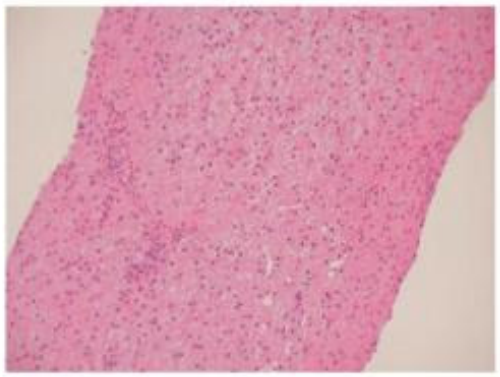

Fig. 2. Case 3. Needle liver biopsy. Hepatic tissue without any evident lesions, apart from slight hydropic degeneration of some hepatocytes (H&E, ×200).

Case 5

This patient with lathosterolosis presented with multiple congenital anomalies including minor facial anomalies, limb abnormalities, horseshoe kidneys, a "butterfly" vertebra, and developmental delay (Table IA) [Brunetti-Pierri et al., 2002]. Maternal serology, performed during pregnancy, ruled out congenital cytomegalovirus, toxoplasma, rubella, hepatitis B and C, HIV, and syphilis. Neonatal jaundice was treated with phototherapy. Abnormal liver function was first documented at 12 months on a follow-up laboratory evaluation, showing raised levels of amino transferases (AST: 262 U/L; ALT: 188 U/L), ALP (1,731 U/L), GGT (539 U/L), and total bile acids (52 μmol/L; normal range <16 μmol/L); at that time clotting test (international normalized ratio, INR) was normal (1.13; normal range: 0.84–1.25) (Table IB). Total and direct bilirubin levels were normal (0.97 and <0.3 mg/dl, respectively) (Table IB) as well as cholesterol levels (Table IA).

Results of screening tests for hepatitis B and C, Epstein–Barr virus, and Adenovirus were negative. Cyomegalovirus infection, initially suspected based on a positive urinary cytomegalovirus antigen at the age of 9 months, was not confirmed by PCR analysis (performed at the age of 13 months and retrospectively on a neonatal Guthrie card), negative plasma cytomegalovirus antigen and specific IgM. Total immunoglobulins and lymphocyte subtyping were normal, as well as screening for other possible causes of chronic liver disease including cystic fibrosis, celiac disease, and common metabolic disorders. An abdominal ultrasound scan showed normal liver size and structure and a bilobed gallbladder with regular walls and no gallstones.

A liver needle biopsy, performed at the age of 14 months, showed bile stasis, severe portal fibrosis, proliferation of bile ducts, which were irregular and abnormally displaced around blood vessels resembling a ductal plate malformation; granulocyte and monocyte cholangiolitis was evident; no inflammation was noted in the parenchyma (Fig. 3). Periportal hepatocytes had diffuse expression of biliary cytokeratin CK7. No viral inclusions or findings suggestive intracellular lipid storage were noted.

Therapy with ursodeoxycholic acid (20 mg/kg/day) was started at the age of 15 months. Nevertheless, in the following years, cholestasis and liver function worsened progressively; at the age of 5 years AST levels were 400 U/L, ALT 157 U/L, ALP

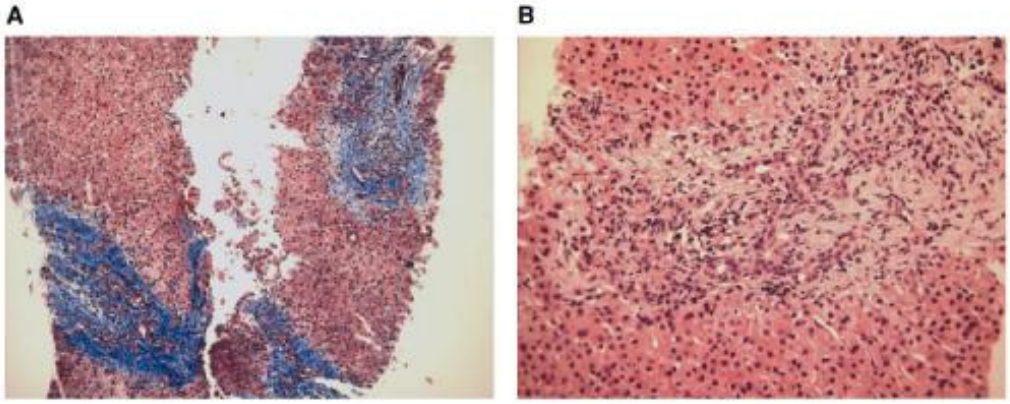

Fig. 3. Case 5. Needle liver biopsy. **A**: Three portal tracts with severe fibrosis (CAB, ×100). **B**: Portal fibrosis with ductular proliferation and mixed inflammatory infiltrate (H&E, ×200).

2704 U/L, GGT 317 U/L; total and direct bilirubin were 11.60 and 10.40 mg/dl, respectively; total bile acids were >200 μmol/L; INR was 1.71; at that time plasma cholesterol and lathosterol levels (analyzed by gas chromatography–mass spectrometry) were 250 and 25 mg/dl, respectively (Table IB).

DISCUSSION

We report a long-term follow-up of five unrelated patients with SLOS and lathosterolosis, two different defects of cholesterol biosynthesis. These metabolic disorders are characterized by disturbed morphogenesis affecting central nervous system, limbs, genitalia, and virtually all internal organs. Liver disease has been reported infrequently. Nevertheless, the severity of malformations may overshadow a liver involvement. A review showed that in a few studies of SLOS patients [Bialer et al., 1987; Curry et al., 1987; Pierquin et al., 1995; Cunniff et al., 1997; Ness et al., 1997; Nwokoro and Mulvihill, 1997; Ryan et al., 1998; Herman, 2003] (Table II) in which liver disease was reported, the frequency of this manifestation appeared low, with estimates of 2.5% [Cunniff et al., 1997], 5% [Herman, 2003], and 6% [Ryan et al., 1998]. Nevertheless, Nwokoro and Mulvihill [1997] reported a higher frequency, with three patients out of six examined showing mild and transient hypertransaminasemia. Liver histological abnormalities were documented in a single patient with normal liver function [Pierquin et al., 1995] (Table IIA). In our study we found 4 SLOS patients with abnormal liver function tests among 24 SLOS cases diagnosed at our Center; the frequency of liver involvement in our SLOS population appeared therefore to be approximately 16%.

We also found liver disease in a patient with lathosterolosis. Lathosterolosis is a rare disorder that was identified recently [Brunetti-Pierri et al., 2002]. To date, only a second male patient has been reported [Parnes et al., 1990; Krakowiak et al., 2003] and, therefore, it is not possible to estimate the frequency of liver involvement in this condition. Anyway, this patient, presenting with a more severe phenotype as compared to case 5, did show signs suggesting cholestatic liver disease, in addition to multiple facial, genital, and limb malformations, cataract, severe neurological signs and early death. Liver histology showed fibrosis in portal and periportal areas, and large inclusions of lipids and mucopolysaccharides [Parnes et al., 1990; Krakowiak et al., 2003]. Interestingly, lathosterolosis mice have enlarged liver and multiple malformations [Krakowiak et al., 2003].

The degree of hepatic involvement in our patients was variable, ranging from severe cholestasis (cases 1 and 2, accounting for 8% of the SLOS patients, and case 5 with lathosterolosis), to a mild/moderate increase of serum amino transferases (cases 3 and 4; 8% of the SLOS patients). Severe progressive cholestasis was striking and common in both SLOS and lathosterolosis and, among SLOS patients, seemed more frequently associated with severe phenotypes, as defined by a validated severity score [Kelley and Hennekam, 2000; Witsch-Baumgartner et al., 2000], while isolated hypertransaminasemia was associated with milder phenotypes.

GGT serum levels were normal in our two SLOS cases with severe cholestatic liver disease and increased in the lathosterolosis patient; these findings might be an interesting clue to differential diagnosis between these disorders, which show a significant clinical overlap.

Although progressive cholestasis has been occasionally reported as a possible severe, life-threatening complication of SLOS, to the best of our knowledge, a full hepatic histological description has been published to date in only one biochemically confirmed SLOS case [Ness et al., 1997]. In our patients, histologic analysis showed fibrosis in both SLOS and lathosterolosis with cholestasis, fitting with previous observations [Parnes et al., 1990; Ness et al., 1997]; it involved both portal spaces and the parenchyma in the former condition, and particularly portal tracts in the latter. On the other hand, this is the first description of a liver biopsy in a patient with SLOS and isolated hypertransaminasemia, showing mild hydropic degeneration of the hepatocytes without fibrosis.

Common causes of liver disease or isolated hypertransaminasemia were ruled out in our patients, based on their biochemical and immunological profile. Particularly, in patient 2 cholestasis associated with asphyxia and parenteral nutrition was excluded as these forms are usually characterized by raised GGT serum levels [Vajro et al., 1997; Forchielli and Walker, 2003], and we found normal GGT in case 2.

Two patients with SLOS were on oral cholesterol supplementation; although we noticed a decrease of liver enzymes serum levels after the start of therapy especially in case 1, overall this therapeutic regimen did not improve significantly the patient's prognosis.

TABLE II. Types of Occurrence of Liver Involvement in Defects of Cholesterol Biosynthesis

A: Post-natal liver disease

Liver disease	SLOS	Lathosterolosis	Mevalonic aciduria
Progressive intra-hepatic cholestasis	+	+	+
Hepatomegaly	+	+	+
Splenomegaly	+	+	+
Reported histological findings			
Pericellular fibrosis/early septal fibrosis	+	−	−
Portal/periportal fibrosis	+	+	+
Giant cells	+	−	−
Increased extra-medullary hematopoiesis	+	−	+
Iron in hepatocytes/Kupffer cells/portal tracts	+	+	−
Bile duct proliferation	+	+	−
Fibrotic extra-hepatic bile ducts	+[a]	−	−
Cholangiolitis	−	+	−
Mucopolysaccharide and lipid inclusions	−	+	−
Hypertransaminasemia without cholestasis	+	−	+
Hepatomegaly	+	−	+
Splenomegaly	−	−	+
Reported histological findings:			
Slight hydropic hepatocytes changes, absence of evident specific lesions	+	−	−
Asymptomatic liver involvement	+	−	−
Reported histological findings			
Slight fibrosis	+	−	−
Parenchymal inclusions (lipofuscins)	+	−	−
Increased number of peroxysomes	+	−	−
Liver and/or biliary tract malformations	+	+	−
Absent liver lobulation	+[a]	−	−
Extra-hepatic biliary atresia	+[a]	−	−
Atretic gall-bladder	+	−	−
Bilobed gall-bladder	−	+	−
Gallstones	+	−	−
References	Case 1–4: Bialer et al. [1987], Curry et al. [1987], Pierquin et al. [1995], Cunniff et al. [1997], Ness et al. [1997], Nwokoro and Mulvihill [1997], Ryan et al. [1998]	Case 5: Parnes et al. [1990], Brunetti-Pierri et al. [2002], Krakowiak et al. [2003]	Hoffmann et al. [1993], Hinson et al. [1998], Clayton [2003]

B: Pre-natal liver disease

	SLOS	Desmosterolosis	Conradi–Hunermann	Greenberg dysplasia[b]
Hydrops	+	−	−	+
Hepatomegaly	−	+	+	+
Splenomegaly	−	+	−	−
Reported histological findings:				
Portal fibrosis	−	+	−	−
Excessive extra-medullary hematopoiesis	−	+	−	+
References	Clayton [2003]	Fitzpatrick et al. [1998], Clayton [2003]	Clayton [2003]	Clayton [2003], Oosterwijk et al. [2003]

+, reported; −, not reported.
[a]Feature reported only in SLOS case not biochemically confirmed.
[b]Isolated hepatomegaly has been reported, post-natally, also in one case of Pelger–Huët homozygosity, allelic to Greenberg dysplasia [Oosterwijk et al., 2003].

A variable functional involvement of the liver was found in other disorders of cholesterol biosynthesis. It is very well known that isolated hypertransaminasemia and hepatomegaly are part of the phenotype of mevalonic aciduria (OMIM: +251170), due to mevalonate kinase deficiency, together with recurrent fever, splenomegaly, and gastrointestinal symptoms [Hoffmann et al., 1993]. Severe progressive cholestasis has been reported as well [Hinson et al., 1998] (Table IIA). Hepatomegaly or liver dysfunction with post-natal or even pre-natal onset was reported in desmosterolosis (OMIM: #602398) due to 24-dehydrocholesterol reductase deficiency, Conradi–Hünermann syndrome (OMIM: #302960) due to 3-β-hydroxysteroid-Δ8,Δ7-isomerase deficiency, Greenberg dysplasia (OMIM: #215140) and the Pelger–Huët homozygosity syndrome (#169400) both of them due to mutations in the lamin B receptor gene (Table IIA,B) [Fitzpatrick et al., 1998; Clayton, 2003; Oosterwijk et al., 2003].

The pathogenesis of liver damage in SLOS and lathosterolosis is unclear. In SLOS, insufficient cholesterol availability for cell membranes may cause cellular dysfunction and multi-organ failure including liver disease. Alternatively, very young severely affected SLOS children may produce and secrete inadequate quantities of normal bile acids, resulting in severe cholestasis and reduced intestinal cholesterol absorption.

The differential diagnosis of cholestasis with normal GGT and giant cells on histological liver examination, includes the ARC syndrome (OMIM: #208085) due to mutations of the *VPS33B* gene, defects of bile acids transport, such as progressive familial intra-hepatic cholestasis (PFIC) 1 and 2 due to mutations of the *ATP8B1* and *ABCB11* genes, respectively (OMIM: #211600 and #601847, respectively), and a few conditions characterized by abnormal bile acids metabolism such as primary defects of bile acids biosynthesis (PFIC 4, due to 3-β-hydroxy-Δ-5-C27-steroid oxidoreductase deficiency, OMIM: #607765) and generalized defects of peroxisomal biogenesis [Bove et al., 2000; Elferink, 2003; Gissen et al., 2004]. Our cases 1 and 5 during follow-up developed high levels of total serum bile acids, likely secondary to bile stasis (Table IB). It might be interesting to emphasize that an abnormal bile acid biosynthesis and metabolism has been demonstrated in a rat model of severe SLOS [Honda et al., 1999], and abnormal bile acid species have been demonstrated in SLOS patients [Natowicz and Evans, 1994; Steiner et al., 2000]. It has been hypothesized in the past that these metabolites, even in small concentrations, may contribute to liver dysfunction in SLOS patients [Honda et al., 1999].

Over time, the patient with lathosterolosis developed high levels of serum cholesterol, as reported in other congenital cholestasis conditions with high GGT levels, such as PFIC3 (OMIM: #602347), due to ABCB4 mutations, and Alagille syndrome (OMIM: #118450), due to Jagged 1 mutations [Elferink, 2003]. It might be interesting to emphasize some clinical similarities between our patient with lathosterolosis and Alagille syndrome, i.e., cholestatic liver disease and a "butterfly" vertebra. Moreover, although bile duct paucity is the typical histological picture found in Alagille syndrome, duct proliferation has been reported as well [Deutsch et al., 2001]. Finally, the Sonic Hedgehog (SHH) protein signal transduction pathway, which is impaired in cholesterol biosynthesis defects [Kelley and Herman, 2001], is linked to the NOTCH/Jagged signaling pathway [Frézal, 1998; Lopez et al., 2003]. These observations suggest a possible pathogenic link for liver involvement in these conditions. Hence, further studies are necessary to clarify the pathogenic mechanisms of liver diseases in cholesterol biosynthesis defects.

ACKNOWLEDGMENTS

We are thankful to Dr. M. Witsch-Baumgartner, Institute of Medical Biology and Human Genetics, Innsbruck, Austria, for identifying the mutation IVS8-1G > C in case 4, to Dr. L. Zelante, CSS-IRCCS, S. Giovanni Rotondo (FG), Italy, for providing some clinical information about case 3.

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APPENDIX 2.5.3

Rossi M, Federico G, Corso G, Parenti G, Battagliese A, Frascogna AR, Della Casa R, Dello Russo A, Strisciuglio P, Saggese G, Andria G.

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Journal of Inherited Metabolic Disease

2005;28(1):69-80.

J. Inherit. Metab. Dis. 28 (2005) 69–80

Vitamin D status in patients affected by Smith–Lemli–Opitz syndrome

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MS received 17.11.03 Accepted 19.07.04

Summary: Smith–Lemli–Opitz syndrome (SLOS) is an inborn error of cholesterol biosynthesis characterized by developmental delay and multiple malformations. Some of the patients have skin photosensitivity and therefore tend to avoid direct exposure to sunlight. SLOS patients typically have low concentrations of cholesterol and abnormally high concentrations of its precursor 7-dehydrocholesterol (7-DHC) in biological fluids and tissues. 7-DHC is also a precursor in the cutaneous synthesis of vitamin D. Sunlight exposure plays a major role in this pathway and reactions transforming 7-DHC into vitamin D and then into 25-hydroxyvitamin D are known not to be specifically regulated. The aim of this study was to evaluate vitamin D status in SLOS patients. We measured 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D serum concentrations and markers of calcium metabolism in five SLOS patients. Despite abnormally high concentrations of 7-DHC, circulating concentrations of vitamin D metabolites were not significantly different from appropriate controls matched for sex, age and season of blood collection. The analysis of historical serum samples stored in our laboratory from the same cases plus 10 other SLOS patients further supported these findings. Our data suggest that SLOS patients have a peculiar vitamin D metabolism that protects them from vitamin D intoxication. This appears to be due in most cases to decreased transformation of 7-DHC into 25-hydroxyvitamin D, perhaps depending on reduced sunlight exposure as a consequence of photosensitivity. Possible alternative mechanisms are discussed.

Smith–Lemli–Opitz syndrome (SLOS) (McKusick 270400) was first described as a syndrome of multiple congenital anomalies/mental retardation (MCA/MR) characterized by developmental delay, craniofacial and limb anomalies, incomplete development of male genitalia and possible association with internal malformations (Smith et al 1964). Some of the patients have skin photosensitivity (Anstey 2001) and therefore tend to avoid direct exposure to sunlight. In 1993, Irons and colleagues demonstrated that SLOS is due to the deficiency of 7-dehydrocholesterol reductase (7-DHCR) (McKusick 602858), the enzyme that catalyses the last steps of cholesterol biosynthesis. In 1998, the 7-DHCR gene was cloned (Moebius et al 1998; Wassif et al 1998; Waterham et al 1998) and to date more than 90 mutations have been described in SLOS patients (Jira et al 2003). The frequency of the syndrome has been estimated as ranging between 1/10 000 and 1/60 000 (Nwokoro et al 2001). Owing to the biochemical defect, SLOS patients have abnormally high concentrations of 7-dehydrocholesterol (7-DHC) in the biological fluids and tissues, usually associated with low levels of cholesterol (Nwokoro et al 2001).

7-DHC is also a precursor of vitamin D. Although normal values of nonhydroxylated vitamin D have occasionally been reported in SLOS patients (Nwokoro and Mulvihill 1997), to the best of our knowledge no studies have been performed to date evaluating vitamin D status in this condition.

Note: Some preliminary results of the present study were presented in 1997 at the Annual Symposium of the Society for the Study of Inborn Errors of Metabolism (patients 1, 2, 3, 13 and 14 are respectively patients 5, 1, 2, 4 and 3 of Guzzetta et al 1997).

SUBJECTS AND METHODS

Patients: We studied 5 SLOS patients (patients 1 to 5; age 0.7–18 years, median 6.7 years) to assess circulating serum concentrations of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and markers of calcium metabolism including serum calcium, phosphate, creatinine, alkaline phosphatase, albumin and parathyroid hormone (PTH) together with urinary excretion of calcium and phosphate. Patients 1 to 5 were on a high-cholesterol diet (50–100 mg/kg per day of pure cholesterol added to a normal diet for age) and have been followed by our department in collaboration with local hospitals for 1–10 years. Periodic clinical assessment and routine blood tests have never shown significant anomalies in calcium metabolism in any of the patients but one. In the first year of life, patient 1 was reported to have reduced bone density on skeletal radiography, associated with high serum levels of alkaline phosphatase (702–830 mU/ml), serum calcium at the lower limit (8.3–8.7 mg/dl), and normal serum phosphate (4.9–5.9 mg/dl), urinary calcium (0.44 mg/kg per day) and tubular phosphate reabsorption (TPR) (88%). At that time she was treated with a single dose of 300 000 IU of vitamin D, followed by intermittent supplementation with 400 IU daily. DEXA scan of the lumbar spine performed at 18 years of age showed a normal bone density (BMD 1.033 g/cm; *z*-score +0.17; *T*-score −0.12).

In the 6 weeks preceding the present study, all patients but one were not on vitamin D or other medications that might have affected circulating concentrations of vitamin D metabolites (as steroids, anticonvulsants, bile acids or statins); only patient 4 was on vitamin D (400 IU/day) and ursodeoxycholic acid (6 mg/kg per day) when his blood sample was collected.

Patient 1 had marked photosensitivity so that she needed to limit sunlight exposure; patients 2 and 3 showed a mild photosensitivity; patients 4 and 5 did not have skin problems. Mean exposure to sunlight was <1, 1, 3, <1 and 2 hours per day for patients 1, 2, 3, 4 and 5, respectively. All patients 1 to 5 came from southern Italy and blood samples were collected in the period from May to July from all patients except case 4, whose sample was collected in November; no patients were reported to use sunscreens. No patients had either liver or kidney problems that could possibly affect vitamin D hydroxylation or clinical or biochemical findings suggestive of generalized intestinal malabsorption.

We also measured vitamin D metabolite concentrations in historical samples previously collected for 7-DHC analysis and stored in our laboratory from patients 1, 2, 3 and 5 and from 10 additional SLOS patients (patients 6 to 15, ages ranging from 0 to 14 years, median 2 years). Although detailed clinical data were not available for them, no anomalies of calcium metabolism had ever been reported in their medical history.

Controls: Since 25-hydroxyvitamin D circulating concentrations vary depending on the season, and 1,25-dihydroxyvitamin D concentrations vary at different ages (Bertelloni et al 1993; Saggese et al 1986), we compared patients' circulating concentrations of vitamin D metabolites with those of 14 normal controls matched for sex, age and season of blood collection.

Methods: Immediately after blood collection, serum was separated, protected from light and stored at −20°C until assayed as previously described (Lissner et al 1981; Saggese et al 1986). SLOS was confirmed in all cases by demonstration of abnormally high serum levels of 7-DHC by gas chromatography–mass spectrometry analysis performed as previously reported (Corso et al 2002; Guzzetta et al 1996). 7-DHC analysis was performed in all specimens, either new or historical, within one week from blood collection.

Serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations were detected by radioreceptor assay, as previously described (Bertelloni et al 1993; Saggese et al 1985). Our 25-hydroxyvitamin D normal reference values were 45.7 ± 12.6 ng/ml (winter, 41.6 ± 5.3 ng/ml; summer, 58.4 ± 6.8 ng/ml); normal reference values for 1,25-dixydroxyvitamin D were: <20 days of life, 19.8 ± 3 pg/ml; 20 days to 23 months, 86.5 ± 8.4 pg/ml; 2 years to 10.7 years, 38.3 ± 7.2 pg/ml; >11 years, 74.6 ± 7.1 pg/ml (Bertelloni et al 1993 Saggese et al 1985, 1986). For all measurements, inter-assay variability was less than 9% and intra-assay variability was less than 7%. Historical serum samples, initially collected for 7-DHC analysis, were stored protected from light, frozen at −20°C, until vitamin D metabolites were assayed (storage time 3.2 ± 0.75 years; range 1–7 years). Concerning vitamin D metabolite stability, in our laboratory we observed a loss of ≤5%/year of

25-hydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations in serum samples stored as described, when assayed yearly over 4 years (data not shown).

Serum intact PTH levels were determined immediately after blood collection by immunochemiluminescent assay for the biologically intact sequence of the hormone (PTH Intact, Diagnostic Products Corporation, Los Angeles, CA, USA). Serum levels of calcium, phosphate, creatinine, albumin, and alkaline phosphatase, as well as urinary excretion of calcium, phosphate and creatinine were measured by standard laboratory methods on samples collected at the same time as those for vitamin D metabolites. TPR was calculated using the formula {[1 − (urine phosphorus × serum creatinine)/(serum phosphorus × urine creatinine)] × 100}. A renal ultrasound was performed in all cases 1 to 5. We used the Student *t*-test and linear regression analysis for statistical analysis. Results are expressed as mean ± standard error of mean (statistical significance: $p < 0.05$).

RESULTS

Our results are summarized in Figure 1 and Table 1. In cases 1 to 5, vitamin D metabolite concentrations were not significantly different from those in appropriately matched controls, in spite of abnormally high concentrations of 7-DHC (41.2 ± 9.2 mg/dl; normal values; ≤0.27) (Figure 1A,B). Serum concentrations of 25-hydroxyvitamin D were 45.8 ± 9.6 ng/ml in cases and 54.4 ± 3.7 ng/ml in controls (not significant, NS). Circulating concentrations of 1,25-dihydroxyvitamin D were 55.8 ± 11.9 pg/ml in cases and 57.4 ± 7.8 pg/ml in matched controls (NS). Ratios between 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations (expressed as pg/ml) were 1019.4 ± 272.2 in cases and 1035.6 ± 182.2 in matched controls (NS). In particular, serum 25-hydroxyvitamin D concentrations were reduced in patient 1 and within the normal range in patients 2 to 4; case 5 had slightly increased concentrations of 25-hydroxyvitamin D on only one occasion (Figure 1A). Blood 1,25-dihydroxyvitamin D was reduced in patient 1, normal in patients 4 and 5 and increased in patients 2 and 3, compared with normal values for age (Figure 1B).

Markers of calcium metabolism were not diagnostic of a specific disturbance (Table 1). A slight increase in serum alkaline phosphatase (ALP) was noted. Circulating PTH was assayed in 4 out of 5 cases: concentrations were in the high normal range in patient 3, in the normal range in patients 1 and 5, and reduced in patient 4 (Table 1). Patient 3 had normocalcaemic hypercalciuria with no other anomalies (Table 1). Renal ultrasonography was normal in all cases, without evidence of renal stones or nephrocalcinosis. In patients 1 to 5 we found a mild linear correlation between 7-DHC and 25-hydroxyvitamin D concentrations ($R^2 = 0.44$) and no correlation between 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations ($R^2 = 0.09$).

Concerning the historical samples, again in spite of abnormally high concentrations of 7-DHC (20.5 ± 5.7 mg/dl; normal values ≤0.27), circulating concentrations of vitamin D metabolites were not significantly different from those in the control group (Figure 1C, D). Circulating concentrations of 25-hydroxyvitamin D were 48.6 ± 9.9 ng/ml in historical samples and 46.7 ± 4.5 ng/ml in controls

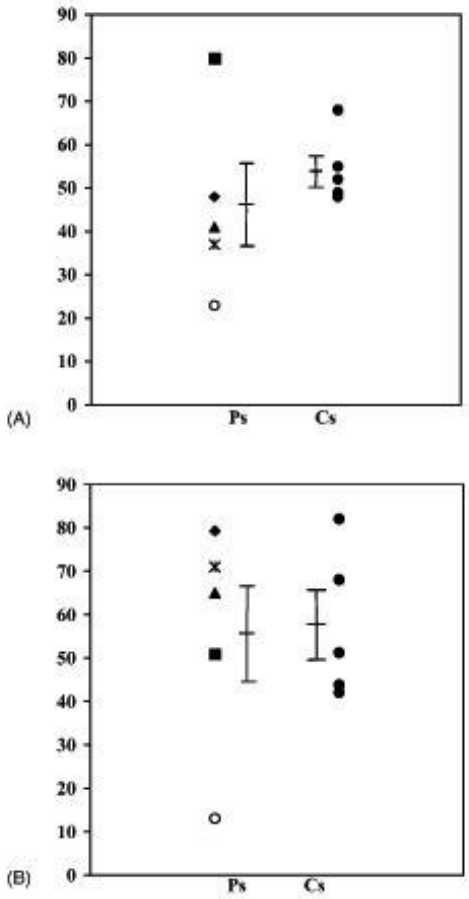

Figure 1 Circulating levels of vitamin D metabolites in our patients compared with normal controls, expressed as single values and mean ± standard error of mean (bars). (A) 25-Hydroxyvitamin D serum concentrations (ng/ml) in cases 1 to 5 and matched controls. (B) 1,25-Dihydroxyvitamin D serum concentrations (pg/ml) in cases 1 to 5 and matched controls. (C) 25-Hydroxyvitamin D serum concentrations (ng/ml) in historical samples compared with matched controls. (D) 1,25-Dihydroxyvitamin D serum concentrations (pg/ml) in historical samples compared with matched controls. Ps, patients; Cs, controls; h, historical: O, P1(h); ⋇, P2(h); ▲, P3(h); ◆, P4; ■, P5(h); □, P6h; •, P7h; § , P8h; -, P9h; ×, P10h; ◇, P11h;#, P12h; −, P13h; △, P14h; +, P15h; •Cs

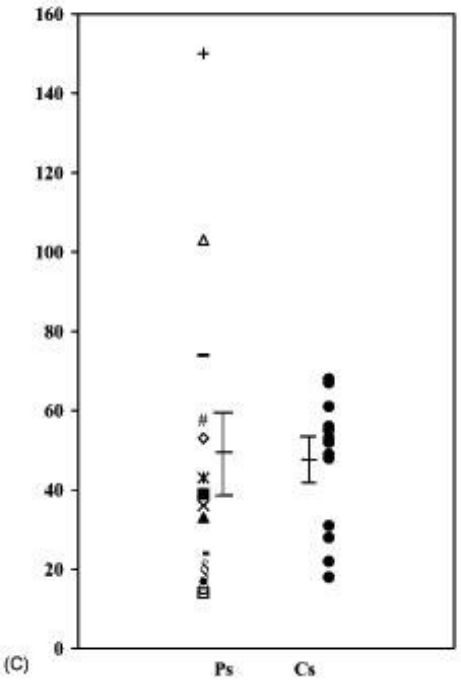

Figure 1 Continued

(NS); 1,25-dihydroxyvitamin D serum concentrations were 58.8 ± 11.5 pg/ml in cases and 55.1 ± 5.9 pg/ml in controls (NS). Ratios between 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations (pg/ml) were not significantly different (cases 1557.2 ± 672.1; controls 975.2 ± 138.2; NS). In particular, concerning patients 6 to 15, serum 25-hydroxyvitamin D concentrations were reduced or at the lower normal limit in 4/10 cases (6 to 9), in the normal range in 4/10 cases (10 to 13), and higher than normal in 2/10 patients (14, 15) (Figure 1C). Circulating concentrations of 1,25-dihydroxyvitamin D were lower than normal or at the low-normal limit in 3/9 cases (8, 11 and 15), normal in 3/9 case (7, 10, 12) and higher than normal in 3/9 patients (6, 9 and 14) (Figure 1D). Although we could not correlate 7-DHC and vitamin D metabolite concentrations because we did not have enough clinical information to rule out with certainty vitamin D supplementation in all cases, historical samples clearly confirmed that the majority of patients did not have

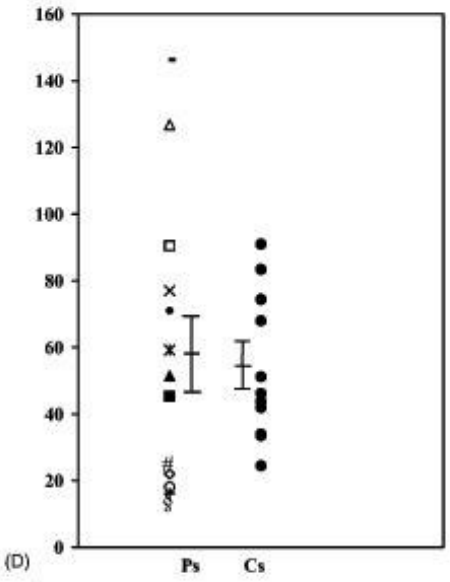

Figure 1 Continued

high concentrations of 25-hydroxyvitamin D despite abnormally high concentrations of 7-DHC (Figure 1C).

DISCUSSION

The aim of this study was to evaluate vitamin D status in SLOS patients. Humans can obtain vitamin D either from endogenous synthesis (vitamin D_3 or cholecalciferol) or from diet (cholecalciferol and ergocalciferol or vitamin D_2, derived from plants and fungi). Both vitamin D_2 and vitamin D_3 have the same metabolism and actions in human beings (Liberman and Marx 2001).

In humans cholecalciferol is mainly synthesized in the skin: during exposure to sunlight, UVB photons penetrate the epidermis and photolyse 7-DHC (provitamin D_3) to previtamin D_3; once formed, previtamin D_3 undergoes a thermally induced isomerization to vitamin D_3 (Holick, 2003). Alternatively, this thermally labile product can isomerize to biologically inert compounds such as lumisterol and tachysterol (Bringhurst et al 2003; Liberman and Marx 2001). Cutaneous production of vitamin D_3 from 7-DHC is known not to be specifically regulated, depending mainly on sunlight exposure (Holick 2003). Circulating vitamin D is hydroxylated at position

Table 1 Markers of calcium metabolism of SLOS patients 1 to 5

	Patient no., sex and age					
	1 F 18 y	*2 M 6 y 8 m*	*3 F 10 y 5 m*	*4 M 9 m*	*5 F 3 y 2 m*	*Normal range for age*
Serum calcium (mg/dl)	8.8	9.8	9	9.5	10.1	Child: 8.8–10.8 Thereafter: 8.4–10.2[a]
Serum albumin (g/dl)	ND	4.4	3.7	3.7	4.0	<5 y: 3.9–5 5–19 y: 4–5.3[a]
Serum phosphate (mg/dl)	4.2	5.8	4.8	5.7	6.2	1–3 y: 3.8–6.5 4–11 y: 3.7–5.6 16–19 y: 2.7–4.7[a]
Serum ALP (U/L)	159	456	637	636	564	1–9 y: 145–420 10–11 y: 130–560 16–19 y: 50–130[a]
Serum creatinine (mg/dl)	0.8	0.8	0.6	0.5	0.5	Infant: 0.2–0.4 Child 0.3–0.7 Adolescent 0.5–1 Adult F: 0.5–1.1[a]
Serum PTH (pg/ml)	42	ND	67	6.8	35	9–65[a]
TPR (%)	85	ND	89	89	91	≥85%[b]
Urinary calcium (mg/kg per 24 h)	0.78	ND	5.3	3.4	0.51	<4[c]

y, years; m, months; ND, not determined
[a] Nicholson and Pesce (2003)
[b] Gertner (2003)
[c] Elder (2003)

25 and 1α in liver and kidney, respectively. 25-Hydroxylation is known not to be specifically regulated (Bringhurst et al 2003; Liberman and Marx 2001); accordingly an excessive intake of nonhydroxylated vitamin D can lead to abnormally high levels of 25-hydroxyvitamin D and vitamin D intoxication (Bringhurst et al 2003; Heird 2003). 25-Hydroxyvitamin D is the major circulating metabolite and is considered the most sensitive index of vitamin D status (Bringhurst et al 2003). 1-Hydroxylation is known to be strictly regulated, 1,25-dihydroxyvitamin D being the main active metabolite (Bringhurst et al 2003; Holick 2003).

In 1987 Bonjour and colleagues studied the effects of an inhibitor of 7-DHCR in a rat model and demonstrated that raised concentrations of skin 7-DHC induced a significant increase in plasma concentrations of 25-hydroxyvitamin D. Furthermore, it has been demonstrated that the administration of an inhibitor of 7-DHCR is able to reverse ineffective vitamin D biosynthesis in cats, increasing 7-DHC and 25-hydroxyvitamin D concentrations (Morris, 1998). It was therefore reasonable to hypothesize abnormally high concentrations of 25-hydroxyvitamin D also in SLOS patients, as a consequence of the abnormally high concentrations of 7-DHC in biological fluids and tissues. Our data demonstrate that this is not a frequent finding in SLOS patients, even if on vitamin D supplementation (case 4).

A minority of cases (patients 5, 14 and 15) did show increased 25-hydroxyvitamin D concentrations; it is worth emphasizing that vitamin D supplementation could be surely ruled out in case 5 and 14, although we did not have detailed clinical information about patients 15. To explain these findings, it might be hypothesized that an increased amount of 7-DHC forces the cutaneous synthesis of vitamin D, as observed in the experimental rat and cat models (Bonjour et al 1987; Morris 1998). None the less, if this assumption is correct, the regulation of vitamin D metabolism should be quite heterogeneous in SLOS patients.

More than 30% of our patients showed reduced concentrations of 25-hydroxyvitamin D, which was within the normal range in more than 50% of cases. These findings might be explained by a reduced exposure to sunlight. It has been reported that about 57% of patients with SLOS have severe photosensitivity, so that they tend to avoid sunlight exposure (Anstey 2001). It has been demonstrated that insufficient sunlight exposure is associated with reduced vitamin D biosynthesis (Holick 2003). Our findings appear to fit with these observations: 3 out of 5 patients (cases 1 to 3) had photosensitivity, which was marked in patient 1 who had low vitamin D metabolites concentrations. Other speculations are possible. Interestingly, it has been reported that the conversion of previtamin D into vitamin D is enhanced in the skin of poikilothermic animals and possibly of warm-blooded vertebrates too, including humans (Holick et al 1995). This seems to be because the membrane phospholipid bilayer stabilizes previtamin D into a *cis* conformation required for its transformation into vitamin D (Holick et al 1995). It has been demonstrated that increased concentrations of 7-DHC are likely to determine an abnormal formation of membrane domains (rafts) rich in sterols and sphingolipids, closely interacting with phospholipid domains (Xu et al 2001). One might therefore speculate that, owing to the abnormal cell membrane lipid composition, SLOS

patients might have a limited number of vitamin D biosynthesis-enhancing sites, with a reduced transformation rate of 7-DHC into cholecalciferol. Other factors affecting vitamin D metabolism might also be hypothesized, such as unknown secondary metabolic pathways that play a major role in removing excessive amounts of previtamin D or vitamin D metabolites in SLOS patients. Finally, the possibility of a feedback effect of the vitamin D metabolites on sterol biosynthesis cannot be ruled out, although it has been excluded in a mouse model (Feingold et al 1987).

Patients showing high concentrations of 25-hydroxyvitamin D had low (case 15), normal (case 5) or high (case 14) 1,25-dihydroxyvitamin D concentrations; patients showing normal concentrations of 25-hydroxyvitamin D had normal (cases 4, 10 to 12) or elevated 1,25-dihydroxyvitamin D (cases 2 and 3). This lack of correlation between 25-hydroxy- and 1,25-dihydroxyvitamin D concentrations has also been reported in other conditions. It is interesting to note that in vitamin D intoxication generally associated with abnormally high concentrations of 25-hydroxyvitamin D, mildly elevated, normal or even low concentrations of 1,25-dihydroxyvitamin D have been described (Bringhurst et al 2003). Although markers of calcium metabolism in patients 1 to 5 showed mild anomalies such as slight increase of ALP (5/5), isolated normocalcaemic hypercalciuria (1/5) and different levels of PTH (Table 1), it is worth emphasizing that none of the patients with increased serum concentrations of 1,25-dihydroxyvitamin D had hypercalcaemia, not even those with abnormally high 25-hydroxyvitamin D blood concentrations. This fits with the observation that vitamin D intoxication has never been reported in SLOS (Jira et al 2003; Kelley and Hennekam 2000; Nwokoro et al 2001; Waterham, 2002).

Despite abnormally high concentrations of 7-DHC, four patients showed 25-hydroxyvitamin D concentrations below or at the lower limit of normal range (cases 1, 6, 7 and 9). We did not have clinical information, including possible vitamin D supplementation, about patients 6, 7 and 9, that would explain the high concentrations of 1,25-dihydroxyvitamin D found. It is in any case interesting to note that either low, normal or increased serum concentrations of 1,25-dihydroxyvitamin D have been reported in vitamin D deficiency rickets, depending on the particular phase of the disease (Liberman and Marx, 2001).

It has been hypothesized that the relatively high incidence of SLOS in some European ethnic groups, the relatively low percentage of patients reported to be born to consanguineous parents, and the more severe enzymatic defects caused by the most common mutations compared with the less common ones, are suggestive of a heterozygote advantage (Kelley and Hennekam 2000). An increased vitamin D biosynthesis rate has been proposed as a possible heterozygote advantage, since there is evidence suggesting that rickets was a common paediatric disease in ancient times (Kelley and Hennekam 2000). We found increased vitamin D metabolites only in a few patients, but normal or even low concentrations of vitamin D metabolites in the majority of cases: further studies are required to evaluate vitamin D metabolism in SLOS carriers.

In conclusion, this is the first study evaluating vitamin D status in SLOS patients. Our data suggest that despite abnormally high concentrations of 7-DHC, SLOS

patients have a peculiar vitamin D metabolism protecting them from vitamin D intoxication. A reduced transformation of 7-DHC into 25-hydroxyvitamin D is likely, perhaps depending on reduced sunlight exposure or, possibly, other unknown mechanisms. A minority of patients did have abnormally high concentrations of vitamin D metabolites without a clinical picture of vitamin D intoxication. Further studies are required to explain these findings.

ACKNOWLEDGEMENTS

We are grateful to Dr V. Guzzetta for his collaboration in the early steps of this study; to Dr L. Zelante, CSS-IRCCS, S Giovanni Rotondo (FG), Dr A. Donadio, Arcispedale S Maria Nuova, Reggio Emilia, Dr M. A. Ambruzzi, Ospedale Pediatrico Bambino Gesù, Rome, Italy, for providing blood samples of patients 11, 12 and 15 respectively, and to Dr V. Bzduch and Dr D. Behulova, University Children's Hospital, Bratislava, Slovakia, for providing samples of cases 8 and 10. This work was supported partially by Ministero dell'Istruzione, Università e Ricerca, PRIN 2002 prot. 2002068222.003.

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APPENDIX 2.5.4

Witsch-Baumgartner M, Gruber M, Kraft HG, Rossi M, Clayton P,

Giros M, Haas D, Kelley RI, Krajewska-Walasek M, Utermann G.

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

Journal of Medical Genetics

2004 Aug;41(8):577-84.

ORIGINAL ARTICLE

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

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J Med Genet 2004;**41**:577–584. doi: 10.1136/jmg.2004.018085

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Revised version received 12 March 2004
Accepted for publication 12 March 2004

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.nlm.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 neurones 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.[18] 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,[10] atherosclerotic vascular disease,[11] and Alzheimer's disease.[12 13] 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.[12]

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

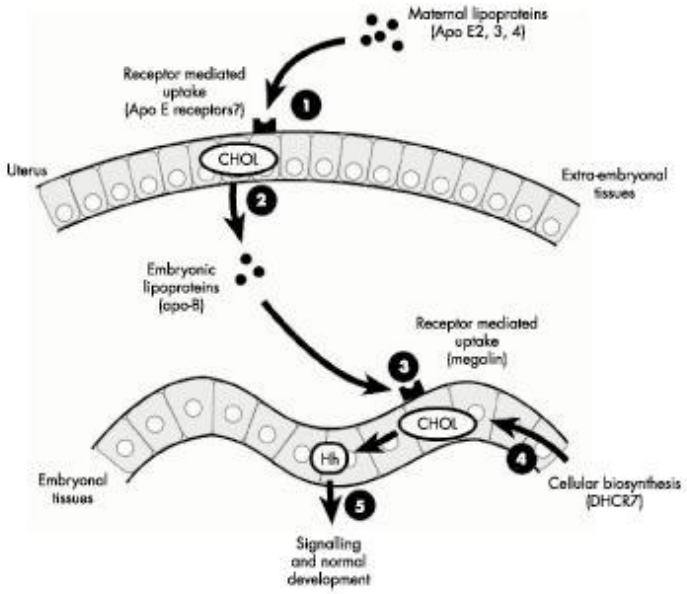

Figure 1 Simplified model of the possible role of apo E in maternal-embryonal cholesterol transport and development. In Smith-Lemli-Opitz syndrome patients with defective DHCR7 the embryo's cholesterol supply depends entirely on the transport of lipoproteins from the mother. Apo E isoforms have different affinities from lipoprotein receptors[18] which make the efficiency of transport dependent on the maternal apo E genotype. (Modified from[22]).

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.[3] 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 ($\varepsilon2 = 0.06$, $\varepsilon3 = 0.80$, and $\varepsilon4 = 0.14$), mothers ($\varepsilon2 = 0.09$, $\varepsilon3 = 0.78$, and $\varepsilon4 = 0.13$) and fathers ($\varepsilon2 = 0.051$, $\varepsilon3 = 0.86$, and $\varepsilon4 = 0.09$) were not statistically different from white population samples (Germans $\varepsilon2 = 0.077$; $\varepsilon3 = 0.773$; $\varepsilon4 = 0.15$)[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 *E2*/*E3* and *E3*/*E3* 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 (*E2*(−): 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 $p = 0.015$).

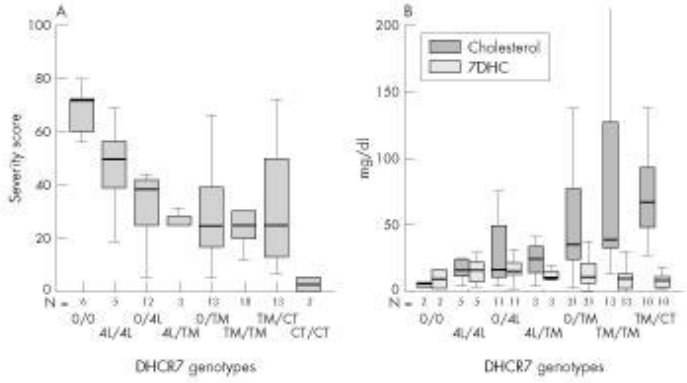

Figure 2 Box plot demonstrating the correlation of DHCR7 genotypes with the Smith-Lemli-Opitz syndrome phenotype (with (A) severity score (B) cholesterol and 7-dehydrocholesterol (7DHC)). DHCR7 genotypes classified as previously described,[11] ordered to get a reasonable sequence.

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: Absolute	Frequencies: Percentage	Expected number of patients according to Hardy-Weinberg equilibrium
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 SNP1D: rs5930 R471 mothers	G/A	15	27.8	
	G/G	32	59.3	
	A/A	7	13	
	sum	54		
LDL receptor SNP1D: 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 O/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).[11] 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 = 0·015). 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

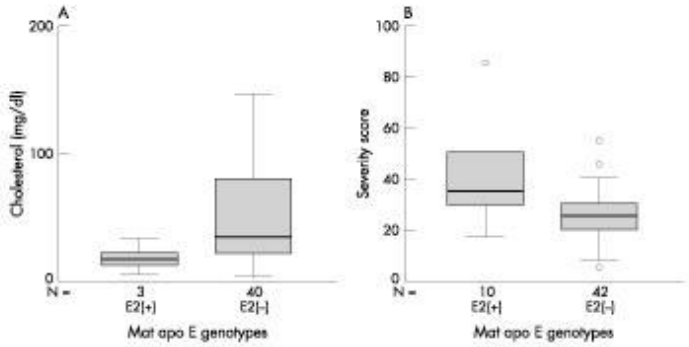

Figure 3 Box plot demonstration of correlation of maternal apo E genotype (E2 present versus absent) with cholesterol levels (A) and disease severity (B) in patients with Smith-Lemli-Opitz syndrome.

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,[18] 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.[25] 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.[26] 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,[11 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.[29 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,[32] 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,[33] 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.[35] The clearance of maternal lipoproteins by the placenta, yolk sac, and decidua is mediated by receptor mediated processes,[36] which may involve apo E. The strongest association of the severity of the Smith-Lemli-Opitz syndrome is with postpartal cholesterol levels.[34] 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.

ACKNOWLEDGEMENTS

We thank Rüdiger Emshoff for his help with the statistical analysis.

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This work was supported by grant no T161 from the Austrian Science Fund (FWF) to MW-B, P-15480 GEN from the Austrian Science Fund to GU, grant no 4P05E09118 from the State Committee for Scientific Research of the Republic of Poland to MK-W and grant from "MIUR Rome, Italy, PRIN 2002 prot. 2002068222_003" to MR.

Conflicts of interest: none declared.

M Witsch-Baumgartner and M Gruber contributed equally to this work.

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APPENDIX 2.5.5

Witsch-Baumgartner M, Clayton P, Clusellas N, Haas D, Kelley RI,

Krajewska-Walasek M, Lechner S, Rossi M, Zschocke J, Utermann G.

Identification of 14 novel mutations in DHCR7 causing the Smith-Lemli-Opitz syndrome and delineation of the DHCR7 mutational spectra in Spain and Italy.

Human Mutation

2005 Apr;25(4):412.

MUTATION IN BRIEF

Identification of 14 Novel Mutations in *DHCR7* Causing the Smith-Lemli-Opitz Syndrome and Delineation of the *DHCR7* Mutational Spectra in Spain and Italy

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Grant sponsor: Austrian Science Fund (FWF); Grant number: T161 to M.W.-B., P-12819 GEN to G.U.

Communicated by Mark H. Paalman

The Smith-Lemli-Opitz syndrome (SLOS) is a phenotypically variable metabolic malformation and mental retardation syndrome for which more than 80 mutations in the DHCR7 disease-causing gene have been described. The DHCR7 mutational spectra differ significantly in different areas of Europe, and several common putative founder mutations account for a substantial fraction of all mutations in some ethnic groups. Here we have analysed 47 SLOS patients and describe 14 newly identified mutations in 18 SLOS patients of Ashkenazi Jewish, Austrian, British, German, Italian, Irish, Polish, Portuguese, and Spanish origins. Half of the new mutations are in the transmembrane domains of the protein. In addition, there were two null mutations, one mutation in the 4th cytoplasmic loop, two mutations in the first and last codons, and three mutations in other regions such as the second cytoplasmic loop and the first endoplasmic loop. The analysis included 20 Spanish and 12 Italian SLOS patients and revealed very different mutation spectra in these patients compared to previously described patients from Czechoslovakia, Germany, Poland, and the UK and implicated p.Thr93Met on the J haplotype as the most frequent Mediterranean founder mutation. © 2005 Wiley-Liss, Inc.

KEY WORDS: Smith-Lemli-Opitz syndrome; DHCR7; sterol reductase, Δ7; cholesterol; Spain; Italy

INTRODUCTION

The Smith-Lemli-Opitz syndrome (SLOS [MIM# 270400]) is an autosomal recessive metabolic disorder characterized by variable congenital defects, characteristic facial dysmorphism, failure to thrive, and mental

Received 14 October 2004; accepted revised manuscript 13 January 2005.

DOI: 10.1002/humu.9328

retardation. The most characteristic malformations include microcephaly, cleft palate, syndactyly of toes 2/3, polydactyly, visceral malformations, variable anomalies of the heart and kidneys, and ambigous genitalia in males. The clinical presentation ranges from mild dysmorphism with moderate or even no mental impairment to multiple malformations and intrauterine death (Opitz, 1994, Cunniff et al., 1997, Kelley, 1997, Kelley and Hennekam, 2000, Langius et al., 2003). The basic defect of the disorder is the last step of the Kandutsch-Russell pathway of cholesterol biosynthesis, which is catalysed by the Δ7-sterol reductase (DHCR7, E.C.1.3.1.21) (Irons et al., 1993, Kelley, 1995). This deficiency results in the biochemical hallmarks of the disorder, namely, low plasma and tissue levels of cholesterol and elevated levels of 7-(7DHC) and 8-dehydrocholesterol (8DHC).

The cDNA for the human DHCR7 was first cloned by Moebius et al. (1998), and mutations in the *DHCR7* gene (MIM# 602858) were soon identified as the cause of the SLOS independently by three groups (Fitzky et al 1998, Wassif et al., 1998, Waterham et al., 1998). To date, more than 80 mutations have been described in SLOS patients (Fitzky et al., 1998, Wassif et al., 1998, Waterham et al., 1998, Witsch-Baumgartner et al., 2001a, Yu et al., 2000, DeBrasi et al., 2000, Neklason et al., 1999). Although there exists a clear correlation of *DHCR7* genotype with the severity of the SLOS phenotype (Witsch-Baumgartner et al., 2000), severity is also affected by the apo E genotype of the mothers of the SLOS patients (Witsch-Baumgartner et al., 2004). The mutational spectra of *DHCR7* have been described in several populations of European origin. The frequently occurring mutations c.964-1G>C (former nomenclature: IVS8-1G>C) and p.Trp151X, show decreasing frequency gradients in SLOS patients from Western to Eastern Europe and vice versa, respectively (Witsch-Baumgartner et al 2001b).

Here we report on 14 previously undescribed SLOS-causing *DHCR7* mutations together with their phenotypes and associated haplotypes. The analysis of 20 Spanish SLOS patients (40 alleles) and 12 Italian SLOS patients (20 alleles, because four patients have parents of non-Italian origin) also allowed us to describe the Spanish and the Italian *DHCR7* mutational spectra.

MATERIALS AND METHODS

Patients

A total of 47 SLOS patients of various European origins were examined in the present study, including 20 patients from Spain and 12 patients from Italy. In 15 out of 18 patients the diagnosis of SLOS had been previously confirmed by increased concentrations of 7-DHC and 8-DHC by gas-chromatography/mass spectrometry (GC-MS) (Kelley 1995), which was performed before any treatment. Clinical symptoms of patients were scored by a system (Kelley & Hennekam, 2000), that classifies SLOS patients into mild (5 - 19), moderate (20 - 50) and severe (> 50) phenotypes. After obtaining informed consent, DNA was extracted according to standard procedures. Mutations and their Mendelian inheritance were confirmed by analysis of the parents when DNA samples were available for molecular analysis. For the 18 SLOS patients with new mutations, the diagnosis of SLOS was made between age two days and 35 years (Table 1). The sex proportion was 2:1 males:females. The severity scores ranged from 2 to 66. Cholesterol concentrations were close to the normal range in all patients, but 7- and 8-DHC were markedly elevated.

Mutation Analysis by Direct Sequencing

High-molecular-weight DNA of the probands was isolated from peripheral blood leukocytes or fibroblasts with established procedures. Exons 3 – 9 and their flanking sequences were amplified using the polymerase chain reaction (Saiki et al., 1985). The amplification primers and the PCR conditions were described previously (Fitzky B. et al., 1998). The amplified DNA was subjected to agarose gel electrophoresis, then the excised fragments were purified with Quiagen KIT and sequenced with Perkin Elmer Big Dye sequencing reaction on the ABI Genetic Analyzers 310 and 3100. Both strands (forward and reverse) were sequenced for each fragment. The *DHCR7* cDNA sequence (GenBank acc.no. AF034544.1) was used as a reference sequence where the A of ATG translation initiation starts represents nucleotide +1 (c.1).

RESULTS AND DISCUSSION

We here have analyzed the *DHCR7* gene from 47 previously undescribed SLOS patients and present fourteen not yet described mutations in 18 of these SLOS patients which were from 17 unrelated families of various European origins together with phenotypic descriptions, ethnic origins, and SNP haplotypes. We compared the

novel mutations, the resulting genotypes, and the patients' phenotypes with previous findings concerning the phenotypic spectrum of SLOS. In addition, we present here the mutational spectra of Spanish and Italian SLOS patients. Alltogether we have now analyzed 218 SLOS DNA samples from different origin and identified a total of 82 different *DHCR7* mutations, 68 of which had been described previously (Yu et al., 2000; Waterham et al., 2000; Jira et al., 2001; Witsch-Baumgartner et al., 2001a).

Table 1. Clinical Features of SLOS Patients with Newly Identified Mutations

Patient ID	Ethnic origin	Age at dx	Sex	Severity score	Severity class	Cholesterol mg/dl	7 DHC mg/dl	8 DHC mg/dl	DHC fraction	Genotype
P1	Ashk.J.	2 d		9	mild	115	0,28	0,94	0.010	p.Met1Val/c.964-1G>C
P2	AT	5 mo	M	25	moderate	40	0,3	-		p.Ala50Asp/c.964-1G>C
P3	PL	22 y	M	11	mild	149,6	19,6	18,9	0.204	p.Leu68Pro/p.Val326Arg
P4*	PL	25 y	M	10	mild	123,2	10,2	12,2	0.153	p.Leu68Pro/p.Val326Arg
P5	UK/PT	2 y	F	no data	no data	no data	no data	no data		p.Gln98X/Tp.Thr93Met
P6	DE	15.5 y	M	no data	mild	151	15,4	20,1	0,19	p.Gln98X/n.d.
P7	DE	fetal			severe	no data	no data	no data		p.His119fsX8/c.964-1G>C
P8	DE	1 y	M	30	moderate	262	0,37	-		p.Ile178Phe/p.Arg242His
P9	DE	5 mo	F	4	mild	96	0,2	0,3	0.005	p.Ile178Phe/p.Trp151X
P10	ES	2 y	F	22	moderate	29,2	11,7	11,3	0.440	p.Trp182Leu/p.Glu224Lys
P11	ES	2 mo	M	20	moderate	27,1	12,2	9,7	0,45	p.Arg228Trp/p.Thr93Met
P12	IE	35 y	F	15	mild	no data	very high	no data		p.Ser192Phe/p.Thr93Met
P13	TR	3 y	F	1	mild	139	27	20,1	0,25	p.Val273Gly/p.Tyr432Cys
P14	UK	7 mo	M	33	moderate	no data	0,26	no data.		p.Gly347Ser/p.Trp151X
P15	PL	1 mo	F	66	severe	34	27,3	18,7	0.575	p.Leu360Pro/p.Trp151X
P16	PL	1 mo	M	no data		no data	no data	no data		p.Leu360Pro/p.Val326Arg
P17	DE	6 mo	F	2	mild	34	13,1	15,9	0,46	p.Tyr432Cys/c.964-1G>C
P18	IT	no data		10	mild	114,9	14	23	0.243	p.Phe475Ser/c.964-1G>C

* brother of P3
7 DHC – 7-dehydrocholesterol
8 DHC – 8-dehydrocholesterol

Novel mutations

Clinical details of the 18 SLOS patients are given in Table 1. As noted before (Witsch-Baumgartner et al., 2000), the sum of 7- and 8-DHC expressed as a fraction of total sterols in plasma (DHC fraction) appears to have the best predictive value for clinical severity of patients. Mildly affected patients exhibit DHC fractions < 0.4 while moderately and severely affected patients have DHC fractions ranging from 0.44 to 0.575, with the exception of patient P17 who is mildly affected with a DHC fraction of 0.46 (Table1).

Table 2 provides molecular data for all novel mutations, including nucleotide change, affected exon, effect on the coding sequence, occurence at conserved amino acid or not, protein domain involved, and the mutation-bearing haplotype. These new mutations were not detected in 100 wild type alleles (data not shown). In all but two patients where no parental DNA was available, inheritence in trans was revealed for all mutations.

Mutation p.Met1Val changes the first codon in exon 1 from ATG to GTG. P.Met1Val might cause reduced or absent translation of the protein, but the actual consequences are not completely clear. Although patient P1 with p.Met1Val carried a "null" (0) mutation on his other allele, he was only mildly affected. This suggests that p.M1V despite changing the ATG start codon is not a severe mutation. Indeed the first patient described with a mutation in codon 1 p.Met1Ile (Waterham & Wanders, 2000) was also very mildly affected. The reason may be that GTG in this position also may function as a start codon, as described by Kozak (1997). Further, a second ATG at codon 59 might be used as a translation start codon. The second ATG is a closer match than the first to the Kozak sequence (Kozak M., 1991) that facilitates translation initiation. Hence, it seems possible that both ATGs are used as

initiation sites. Indeed, Wassif et al., (1998) demonstrated that a cDNA clone encoding a 417-amino-acid open reading frame with a deletion of 58 amino acids at the 5´end resulted in fully active enzyme when expressed in vitro. It has however never been ascertained whether or not both forms of the protein exist in vivo.

In the cohort of 18 SLOS patients with new mutations, mild phenotypes (corresponding to severity scores in between 2 and 15) were found associated with genotypes p.Met1Val/0, p.Leu68Pro/TM, p.Ile178Phe/0, p.Ser192Phe/TM, pTyr432Cys/0, and p.Phe475Ser/0. The 2nd mutation in the mildly affected SLOS patient (P18) carrying the p.Phe475Ser mutation was a 0 mutation. The mutation in the last codon affects the last amino acid believed to locate in the lumen of the endoplasmic reticulum (ER). Another mutation also located in the terminal part of the enzyme and associated with mild phenotype is the p.Tyr432Cys, also found in combination with a 0 mutation (P17). Multiple other mutations in the ER domain of the protein had already been identified, and all of them were found associated with mild phenotypes (Nezarati et al., 2002, Witsch-Baumgartner et al., 2000). Therefore, the described mutations in the terminal part of the enzyme situated in the lumen of the ER all seem to retain some DHCR7 enzymatic activity.

Table 2. Novel Mutations in SLOS Patients

nt substitution[•]	exon	aa exchange	Conserved aa	Mutation type	Protein domain[a]	Haplotype ID[b]	Remarks
c.1A>G	3	p.Met1Val	no	missense	NT	B	no parents available, if A for IVS8-1G>C
c.149C>A	4	p.Ala50Asp	no	missense	TM 1	B	
c.203T>C	4	p.Leu68Pro	yes	missense	1.EL	A	2 related patients
c.292C>T	4	p.Gln98X	no	stop	TM 2	K	no parents available, if J for T93M
c.355delC	5	p.H119fsX8[c]	no	frame shift	TM 2	F	
c.532A>T	6	p.Ile178Phe	no	missense	TM 3/4	K	2 unrelated patients
c.670G>A	7	p.Glu224Lys	no	missense	2. CL	A	
c.682C>T	7	p.Arg228Trp	yes	missense	2. CL	J	
c.575C>T	6	p.Ser192Phe	no	missense	TM 4	n.i.	family haplotype data not informative
c.818T>G	7	p.Val273Gly	yes	missense	TM 6	A	
c.1039G>A	9	p.Gly347Ser	no	missense	TM 8	A	
c.1079C>T	9	p.Leu360Pro	no	missense	4. CL	A	2 unrelated patients
c.1289A>G	9	p.Tyr432Cys	yes	missense	CT	F	2 unrelated patients
c.1423T>C	9	p.Phe475Ser	no	missense	CT	A	

[a] NT – N terminus, TM – transmembrane, 1.EL – first endoplasmic loop, 2./4. CL – second/fourth cytoplasmic loop, CT – C terminus

[b] see EJHG 9:45-50, haplotype J in JMGe31, K: c.189G, c.207T, c.231C, c.438T, c.969G, c.1158T, c.1272C

[c] fs - frameshift

• c.1 is +1 from the ATG (according to GenBank acc.no. AF034544.1)

The phenotypic evaluation of p.Leu68Pro and p.Ser192Phe is more difficult because these mutations were found in mildly affected SLOS patients (P3, P4, P12) in association with the transmembrane (TM) mutations p.Val326Leu and p.Thr93Met respectively. Homozygous or compound heterozygous presence of these latter mutations generally leads to moderate phenotypes as shown previously (Witsch-Baumgartner et al., 2000). Therefore p.Leu68Pro and p.Ser192Phe presumably are less severe than p.Thr93Met and p.Val326Leu. p.Leu68Pro is located in the 1st endoplasmic loop, not hitherto shown to carry SLOS causing mutations. Although the paucity of mutations in this domain may be a chance finding, an alternative explanation is that mutations in this loop are not particularly deleterious. p.Ser192Phe is in a predicted transmembrane region where *DHCR7* mutations are associated with mild to moderate severity.

The TM mutation p.Ile178Phe in combination with a 0 mutation was found in a mildly affected SLOS patient (P9) with only slightly increased 7- and 8- dehydrocholesterol levels in plasma. Biochemical analysis of cholesterols in fibroblast culture showed the typical increase in 7-DHC as a fraction of total sterols. In combination with p.Arg242His, however, p.Ile178Phe caused a moderate phenotype in patient P8. This discrepancy between the clinical and mutation severity probably reflects the presence of unknown modifying factors.

The mutations p.Ala50Asp and p.Gly347Ser in combination with 0 mutations were found to be associated with moderate severity (scores from 20 – 33) in two patients (P2, P14). Both mutations are in TM domains of the protein. Mutations in these regions combined with 0 mutations usually lead to moderate phenotypes.

The new mutations p.Glu224Lys, and p.Arg228Trp, in combination with transmembrane mutation p.Trp182Leu and p.Thr93Met, respectively, also cause moderate disease severity (P10, P11). Both are located in the 2nd cytoplasmic loop (Table 2), which has not been found to be affected by mutations in previous studies.

Severe phenotypes resulted from a 0 mutation in combination with the newly identified mutation p.Leu360Pro (P15) and with the frame shift mutation p.His119fsX8 in patient P7. P.Leu360Pro is located in the 4th cytoplasmic loop. Previously described mutations in this domain, such as p.Arg404Cys and p.Gly410Ser, lead also to severe phenotypes. Therefore the phenotype found in patient P15 with the p. Leu360Pro mutation is consistent with the severe effect expected of mutations in the 4th cytoplasmic loop. The p.His119fsX8 change leads to a truncated enzyme that is predicted to be non-functional. This is comparable to the effect of the stop mutation, p.Trp151X, and explains the severe phenotype. An analysis of genotype - phenotype correlations in a much larger number of SLOS patients might answer questions about the importance and functions of individual protein domains in the enzyme.

Mutation associated haplotypes

Many of the novel mutations (p.Leu68Pro, p. Glu224Lys, p.Val273Gly, p. Gly347Ser, p. Leu360Pro, p. Phe475Ser) are associated with the common haplotype A. These mutations originated on A alleles from Poland, Spain, United Kingdom, Italy, and Turkey. The mutations, p.Met1Val and p.Ala50Asp, were found associated with haplotype B, which differs from A only at one postion (c.189A>G). Two mutations p.His119fsX8 and p.Tyr432Cys were found on haplotype F in two German SLOS patients (P7, P17), F is the third among the common haplotypes in Europeans. The missense mutation p.Arg228Trp was found associated with the J haplotype in a Spanish patient (P11). The K haplotype was found associated with the newly described mutations p.Ile178Phe in a German patient (P8), and p.Gln98X in a German patient (P6) and a patient of British and Portuguese origins (P5). K is a rare haplotype not detected in 30 other European alleles (M. Witsch-Baumgartner, unpublished). The emerging of p.Gln98X and p.Ile178Phe with this rare haplotype K might be by chance and likewise the association of the B haplotype with p.Met1Val and p.Ala50Asp. In patients P9 and P12 the SNP data were not informative, and therefore no haplotypes could be constructed.

Table 3. Relative Frequencies of Common *DHCR7* Mutations in Caucasian Populations

	Great Britain	Germany	Poland	Italy	Spain	Czech
No. of chromosomes	**44**	**44**	**30**	**20**	**40**	**20**
c.964-1G>C	**34%**	**20%**	3%	**20%**	**30%**	5%
p.Trp151X	<5%	**18%**	**33%**	<5%	<5%	**50%**
p.Thr93Met	7%	<5%	no	**45%**	**23%**	no
p.Val326Leu	<5%	**18%**	**23%**	no	no	**25%**
p.Arg404Cys	9%	<5%	no	<5%	no	no
p.Arg352Trp	<5%	7%	**13%**	<5%	<5%	no
p.Phe302Leu	<5%	no	no	no	**10%**	no
p.Leu157Pro	no	<5%	7%	no	no	5%
p.Gly410Ser	<5%	<5%	no	no	7.5%	**10%**
p.Arg446Gln	no	no	no	no	7.5%	5%
ref.	a	a	a	this article	this article	b

a Witsch-Baumgartner M. et al., 2001
b Kozak L. et al., 2000

SLOS mutational spectra in Spain and Italy

In 20 SLOS patients of Spanish origin, we analysed the *DHCR7* mutations to determine the spectrum of SLOS-causing mutations (Table 3) in that population. 30% of these Spanish alleles carry the most common SLOS-causing mutation, c.964-1G>C, always associated with the A haplotype. The p.Thr93Met mutation accounts for

nearly 23% of alleles and is associated most often with the J haplotype. Only one p.Thr93Met allele was found associated with the F haplotype. The p.Phe302Leu mutation is the third common *DHCR7* mutation in Spanish SLOS alleles. This mutation was always detected on the F haplotype. p.Gly410Ser and p.Arg446Gln, both located on haplotype A alleles, were each detected in 7.5% of Spanish SLOS-causing alleles. Ten mutations appeared only once, and several of these were detected only in the Spanish SLOS patients: p.Ile145Thr, p.Trp182Leu, p.Glu224Lys, p.Arg228Trp and p.Asp300Asn.

Twelve Italian patients carried 20 different SLOS alleles (four alleles were not of Italian origin). The p.Thr93Met mutation was found to be the most common (45%) (Table 3). Apart from one case with the F haplotype, this mutation was always found associated with the J haplotype (Table 4). 20% of alleles carry the c.964-1G>C mutation, always associated with the A haplotype. The remaining alleles carry each different mutations from which some were only detected among the Italian alleles: p.Cys380Arg, p.Asn407Tyr and p.Phe475Ser.

The Spanish and Italian mutational spectra obtained here are different from those seen in patients from Germany, Poland, Czechoslovakia and the UK (Kozak et al., 2000; Witsch-Baumgartner et al., 2001b) (Table 3). Although in Spanish SLOS patients, as in those from Germany and the UK, the most common mutation is the c.964-1G>C splice site mutation, the second and third most common mutations are p.Thr93Met and p.Phe302Leu. In contrast to Spanish patients, the most common mutation found in Italian patients was p.Thr93Met (45% of alleles), while c.964-1G>C was only the second most frequent. Other mutations were only found once. Taking into account previous frequency data for the splice site mutation c.964-1G>C it appears that there is a decreasing frequency gradient for this mutation from the British Isles to the Southern and Eastern parts of Europe (Witsch-Baumgartner et al., 2001b; Kozak et al., 2000). The p.Thr93Met, which is an important mutation in Spain and Italy, is less common in the UK and rare in German, Czech and Polish SLOS patients. Hence, this mutation might be a mediterranean founder mutation. In nearly all patients from Spain and Italy p.Thr93Met is found associated with the J haplotype, supporting the notion of a founder effect, which was already proposed by Novaczyk et al., (2004) based on the analysis of SLOS patients of Spanish origin from Canada and Cuba. In two cases, one from Spain and one from Italy, the mutation appears on the F haplotype. F and J haplotypes differs only at position c.231. Therefore either the p.Thr93Met occurred at least twice and hence is a recurrent mutation, or the mutation is old and occurred originally on a J haplotype that subsequently changed to a F haplotype by mutation or gene conversion. The British p.Thr93Met alleles are associated with haplotype A (Witsch-Baumgartner, unpublished), and a SLOS patient of Ukrainian/Irish ancestry carried the p.Thr93Met mutation on a K haplotype (Novaczyk et al., 2004). These observations and the fact that the mutation occurs at a CpG might be an evidence for a recurrent origin of this mutation.

Among the Spanish SLOS alleles, the p.Phe302Leu mutation is relatively frequent and is always associated with haplotype F. In contrast, p.Phe302Leu is a very rare mutation in other populations, we observed it only in one British SLOS patient so far. Therefore also this mutation might be a Spanish founder mutation.

Further investigations of mutational spectra and haplotype associations in different ethnic populations may give insights into the origins and propagation of *DHCR7* mutations and perhaps answer the question why the SLOS appears to be much less frequent in non-Caucasian than in Caucasian populations and why some *DHCR7* mutations have reached relatively high frequencies.

ACKNOWLEDGMENTS

This work was supported by grant no. T161 from the Austrian Science Fund (FWF) to M.W.-B., P-12819 GEN from the Austrian Science Fund to G.U. Acknowledgment to Gabriela Huber for technical assistence and to medical doctors contributing one or two SLOS patients: E. Steichen-Gersdorf (Innsbruck, Austria), P. Müller (Leipzig, Germany), T.A. Miettinen (Helsinki, Finland), G. Gillessen-Kaesbach (Essen, Germany), A. Green (Dublin, Ireland), Fusch (Greifswald, Germany).

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CHAPTER 3.

PROJECT 2: INNOVATIVE THERAPEUTIC APPROACHES FOR INHERITED DISORDERS: THE MODEL OF POMPE DISEASE

3.1 Background

3.1.1 Pompe disease (Glycogenosis type II).

Pompe disease, also known as glycogenosis type II, is an autosomal recessive progressive metabolic myopathy with an estimated incidence of 1:40,000 live births (Hirschhorn and Reuser, 2001; Kishnani and Howell, 2004). This condition is due to mutations of the acid α-glucosidase gene, (*GAA*) encoding the lysosomal hydrolase α-glucosidase (acid maltase, GAA), which is synthesized as a 110 kDa precursor and is processed into the active polypeptides of 76 and 70 kDa, through an intermediate molecular form of 95 kDa (Hirschhorn and Reuser, 2001; Kishnani and Howell, 2004). The biochemical defect determines tissue glycogen accumulation and secondary muscle destruction. Different mutations of the *GAA* gene result in a wide phenotypic variability, spanning a continuum range. The severe classic infantile form is characterized by early onset, severe hypertrophic cardiomyopathy, marked hypotonia, macroglossia, hepatomegaly, and invariably fatal outcome by one year of age. The late onset (childhood, juvenile or adult) form is characterized by skeletal myopathy, possible respiratory failure and absence of significant cardiac disease. Current classifications identify intermediate phenotypes, such as the non-classic infantile form, presenting within the first two years of life, with less severe heart involvement and absence of left ventricular outflow obstruction (Hirschhorn and Reuser, Kishnani and Howell, 2004; 2001; Slonim et al., 2000). It has been shown that an inverse correlation generally exists between degrees of clinical severity and different ranges of residual enzymatic activity (Hirschhorn and Reuser, 2001; Kishnani and Howell, 2004).

Enzyme replacement therapy (ERT) with recombinant human alpha-glucosidase derived from either rabbit milk or Chinese Hamster Ovary cells, has been recently introduced and is currently under evaluation (Amalfitano et al., 2001; Van den Hout et al., 2000; Winkel et al., 2004). Although some promising experimental data on gene therapy have been reported in animal models (Sun et al., 2005), to date no therapeutic approaches are available for Pompe patients, other than ERT.

3.2 Experimental work

3.2.1 Outline of the project

The overall aim of this project was to develop innovative therapeutic approaches for Pompe disease. The different steps of the project specific aims are summarized hereafter, and the clinical, biochemical and molecular results of this studies are reported "in extenso" in the appendices 3.5.1, 3.5.2, and 3.5.3 of this chapter.

3.2.2 Patients

We collected eight Pompe patients followed up by our Department or other local hospitals: their clinical features are summarized in Table 1 (section 3.2.3). In all cases the diagnosis was confirmed by GAA enzymatic assay in cultured skin fibroblasts or muscle biopsy, carried out as previously described (Hirschhorn and Reuser, 2001). The patients were characterized also by molecular analysis of the GAA gene performed in collaboration with Dr. Pittis and Dr. Bembi, Unità di Malattie Metaboliche, I.R.C.C.S. Burlo Garofolo, Trieste, Italy, and Dr. Filocamo, Laboratorio di Diagnosi Pre e Postnatale Malattie Metaboliche, I.R.C.C.S. G. Gaslini, Genoa, Italy: some of the molecular results were included in an Italian multicenter collaborative study, enclosed in appendix 3.5.3 (patients BAP, VPO, DPF are respectively patients 6, 40 and 1 in the paper by Montalvo et al., 2006).

3.2.3. Table 1.

Pompe patients recruited for our research project 2.

Patients		Age at diagnosis	Main clinical features at diagnosis
DCME	Classic infantile	5m	Muscle hypotonia, severe cardiomyopathy, pneumonias, raised serum muscle enzymes
RGG	Classic infantile	6y	Muscle hypotonia, severe cardiomyopathy, pneumonias, raised serum muscle enzymes
RP	Infantile onset non-classic	1y	Hypotonia, motor delay, pneumonias, raised serum muscle enzymes
GM	Infantile onset non-classic	11m	Motor delay, frequent vomiting and failure to thrive, raised serum muscle enzymes
BAP	Childhood-onset	4y	Muscle weakness, raised serum muscle enzymes
PP	Juvenile	11y	Muscle weakness, scoliosis, low respiratory tract infections, raised serum muscle enzymes
VPO	Juvenile	2y2m	Motor delay, hepatomegaly, raised serum muscle enzymes
DPF	Juvenile	3y	Hypotonia, raised serum muscle enzymes

Note: m: months; y: years.

3.2.4 Specific aim 1.

EVALUATION OF THE LONG TERM EFFECTS OF ENZYME REPLACEMENT THERAPY OF POMPE DISEASE WITH RECOMBINANT HUMAN ALPHA-GLUCOSIDASE (DERIVED FROM CHINESE HAMSTER OVARY CELLS).

We studied the long term safety and efficacy of ERT with recombinant human alpha-glucosidase derived from Chinese Hamster Ovary cells on three of the Pompe cases recruited (GM, RP and VPO), all of whom presenting without cardiomyopathy. The patients were treated at dose regimens of 20-40mg/Kg/every other week. Follow-up length ranged from 20 weeks to 140 weeks of therapy. The first patient (GM), who underwent enzyme replacement therapy earlier and in better baseline conditions, showed motor improvement and no respiratory failure. The second case (RP) who started the therapy early, in severe general conditions, showed improvement in motor function and degree of disability, but not in muscle strength. Significant respiratory improvement was also noted: following enzyme replacement therapy, the frequency of low airway infections markedly decreased and the patient, who was ventilator-dependent at baseline, became steadily ventilator-free for short periods. The third case (VPO), who started the therapy at a terminal stage of a long lasting disease, died shortly after. Our study suggests that enzyme replacement therapy can lead to significant motor and respiratory improvement in the subgroup of patients who start the therapy before extensive muscle damage has occurred. The recombinant enzyme derived from Chinese Hamster Ovary cells, administered at doses significantly higher than previously reported, appears to have the same safety as the rabbit milk derived drug. This study is fully reported in appendix 3.5.1.

3.2.5 Specific aim 2.

EVALUATION OF THE EFFECTS OF IMINO SUGARS ON MUTATED ALPHA-GLUCOSIDASE ACTIVITY IN FIBROBLASTS FROM PATIENTS WITH POMPE DISEASE

In this study, we tried to evaluate the feasibility of an alternative therapeutic approach in an "in vitro" model of Pompe disease. Our aim was to provide the proof of principle for developing an enzyme enhancement therapy using pharmacological chaperones. We studied the effects of two imino sugars, deoxinojirimycin (DNJ) and N-butyldeoxynojirimycin (NB-DNJ), on residual GAA activity in fibroblasts from the 8 patients recruited, presenting with different forms of Pompe disease (2 classic infantile, 2 non-classic infantile onset, 4 late onset forms), and with different mutations of the *GAA* gene. We demonstrated a significant increase of GAA activity (1.3 to 7.5-fold) after imino sugar treatment in fibroblasts from patients carrying the mutations L552P (3 patients) and G549R (1 patient). GAA enhancement was confirmed in HEK293T cells where the same mutations were overexpressed. No increase of GAA activity was observed for the other mutations. Western blot analysis showed that imino sugars increase the amount of mature GAA molecular forms. Immunofluorescence studies in HEK293T cells overexpressing the L552P mutation showed an improved trafficking of the mutatnt enzyme to lysosomes after imino sugar treatment. These results provide a rationale for an alternative treatment, other than enzyme replacement, to Pompe disease. This study is fully reported in appendix 3.5.2.

3.3 Conclusions

In this study we evaluated the effects of two experimental innovative therapeutic strategies for Pompe disease. Our data suggested that ERT has controversial results on Pompe patients: in fact this treatment appears to prevent motor regression and lead to motor and respiratory improvement in Pompe patients who start the therapy before a significant muscle damage has occurred. Nevertheless, the lack of significant effects of the treatment on severely compromised skeletal muscle, supports the need of alternative therapeutic approaches. The results of the "in vitro" study evaluating the use of small molecules with "chaperone" effect on Pompe cells can provide the rationale for an alternative therapy. Future studies might be focused on evaluating the "in vitro" effects of the combination of enzyme enhancement therapy and ERT and, ultimately, on the feasibility of clinical trials based on this innovative therapeutic approach.

3.4 References

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3.5 Appendices of project 2

APPENDIX 3.5.1

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Long term enzyme replacement therapy of Pompe disease with recombinant human alpha-glucosidase (derived from Chinese Hamster Ovary cells)

Journal of Child Neurology. In press.
2006

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ABSTRACT

Pompe disease is a rare autosomal recessive myopathy due to the deficiency of lysosomal acid alpha-glucosidase. Clinical phenotypes range from the severe classic infantile form, characterized by hypotonia and hypertrophic cardiomyopathy, to the childhood, juvenile and adult forms characterized by skeletal myopathy and absence of significant heart involvement. Enzyme replacement therapy with recombinant human alpha-glucosidase derived from either rabbit milk or Chinese Hamster Ovary cells, has been recently introduced and employed in still ongoing clinical trials. We report on a long term follow-up of three Pompe cases presenting without cardiomyopathy, treated with recombinant human alpha-glucosidase derived from Chinese Hamster Ovary cells at dose regimens of 20-40mg/Kg/every other week. The first patient, who underwent enzyme replacement therapy earlier and in better baseline conditions, showed motor improvement and no respiratory failure. The second case who started the therapy early, in severe general conditions, showed improvement in motor function and degree of disability, but not in muscle strength. Significant respiratory improvement was also noted: following enzyme replacement therapy, the frequency of low airway infections markedly decreased and the patient, who was ventilator-dependent at baseline, became steadily ventilator-free for short periods. The third case, who started the therapy at a terminal stage of a long lasting disease, died shortly after. Our study suggests that enzyme replacement therapy can lead to significant motor and respiratory improvement in the subgroup of patients who start the therapy before extensive muscle damage has occurred. The recombinant enzyme derived from Chinese Hamster Ovary cells, administered at doses significantly higher than previously reported, appears to have the same safety as the rabbit milk derived drug.

INTRODUCTION

Pompe disease, also known as glycogenosis type II, is an autosomal recessive progressive metabolic myopathy with an overall incidence of 1:40000 live births. [1,2] This condition is due to the deficiency of the lysosomal enzyme alpha-glucosidase, leading to tissue glycogen accumulation and secondary muscle destruction. [2] The phenotype of the disease is characterized by clinical variability, spanning a continuum range. The severe classic infantile form is characterized by early onset, severe hypertrophic cardiomyopathy, marked hypotonia, macroglossia, hepatomegaly, and invariably fatal outcome by one year of age. [3] The late onset (childhood, juvenile or adult) form is characterized by skeletal myopathy, possible respiratory failure and absence of cardiac disease. [4,5] Current classifications identify intermediate phenotypes, such as the non-classic infantile form, presenting within the first two years of life, with less severe heart involvement and absence of left ventricular outflow obstruction. [1,2,4,6] It has been shown that an inverse correlation generally exists between degrees of clinical severity and different ranges of residual enzymatic activity. [2]

Enzyme replacement therapy with recombinant human alpha-glucosidase derived from either rabbit milk or Chinese Hamster Ovary cells, has been recently introduced and is currently under evaluation. To date the results of only four trials have been published, enrolling a total of nine cases with classic infantile form and three cases with late onset Pompe disease; [7,8,9,10,11,12,13] in particular, information about treatment with the recombinant alpha-glucosidase derived from Chinese Hamster Ovary cells is to date available only for three patients presenting with significant cardiomyopathy (classic infantile Pompe disease), and followed up for one year. [9]

Given the phenotypic variability of this condition, the limited number of patients on treatment, the different sources of recombinant human alpha-glucosidase and the various dosages used for enzyme replacement therapy in the few published studies, even single observations may provide useful information on either the clinical course or the therapeutic outcome. We report on three Pompe patients presenting without significant cardiac involvement, treated with recombinant human alpha-

glucosidase derived from Chinese Hamster Ovary cells, who provide additional information on the effects of this experimental therapeutic approach.

PATIENTS AND METHODS

Three patients with Pompe disease were recruited in an observational study; their clinical and biochemical features are summarized in Table 1. Written informed consent was obtained from either patients and/or parents, and the therapy was approved by the local ethical committee. The drug was provided by Genzyme Corporation (Myozyme ®). Case 1 was enrolled in the expanded access protocol for infantile onset Pompe disease (AGLU 02203); case 2 received the drug for compassionate use; case 3 was enrolled in the protocol for severely affected cases with late onset Pompe disease (AGLU 02503).

Cases 1, 2 and 3 started the enzyme replacement therapy at 3 years and 8 months, 2 years and 8 months, and 19 years and 9 months of age respectively. Case 1 and 3 were on 20mg/Kg/every other week (qow); case 2 started the therapy at the dosage of 10mg/Kg/week, which was gradually increased to 40mg/Kg/qow (Table 1). Periodic clinical assessments included physical examinations, and neuro-developmental assessments performed by a pediatric neurologist and psychologist. Muscle function was evaluated with the Gross Motor Function Measure. This scoring system evaluates the patients' skills on the bases of the following five different dimensions: "lying and rolling"; "sitting"; "crawling and kneeling"; "standing"; "walking, running and jumping". The calculated scores for each dimension are the result of the percentage of performance items reached by the patient. After the age of 4 years, muscle functional impairment was also measured according to the Walton and Gardner-Medwin scale, an 11-points ordinal scale ranging from grade 0 (patient performing all activities) to grade 10 (patient confined to bed and requiring help for all activities). Manual Muscle Testing according to the Medical Research Council score [14] was used to assess specifically muscle strength after the age of 4 years. The following muscles were evaluated, separately for each side of the body when appropriate: neck flexors and extensors, deltoids, biceps,

triceps, wrists flexors and extensors, hip flexors and abductors, knee flexors and extensors, foot dorsal and plantar flexors. Scores were given to each muscle (scores ranging from 0: no movement, to 5: normal strength) and were added to obtain a total muscle score for upper body (maximum score: 50), lower body (maximum: 60) and total body (maximum: 110). Mental and cognitive status was evaluated by Bayley Scales of Infant Development II and WPPSI tests, according to the age and general conditions of the patients. In all cases, the degree of disability was evaluated using the Pediatric Evaluation of Disability Inventory: [15] the Normative Standard Score, expressed as a percentile (mean $\pm$ 2 standard deviations corresponding to a score of 50 $\pm$ 20), was evaluated to measure the child overall functional performance relative to peers; the Scaled Score provided an estimate, regardless of age, of the child's functional performance along a continuum of items scored from 0 to 100.

Audiometry was performed every 6 months. Biochemical analyses included full blood count, serum levels of alanine transaminase, aspartate transaminase, lactate dehydrogenase, creatine kinase, bilirubin, alkaline phosphatase, electrolytes, glucose, creatinine, urea, uric acid, bicarbonate, protein, albumin, and urine analysis. Anti-recombinant human alpha-glucosidase antibodies were analyzed by enzyme-linked immunosorbent and radioimmunoprecipitation assays by Genzyme Corporation. Muscle biopsy was repeated during enzyme replacement therapy at week 12 and week 77 only in case 2.

RESULTS

Growth parameters and general conditions

A total of 153 infusions were performed during the observational period. Our three cases underwent enzyme replacement therapy with variable degrees of muscular damage and general conditions severity (Table 1).

Case 1, who started the therapy before a significant muscular damage occurred, remained in good conditions during the whole observational period. Comparing baseline with week 70, weight

improved from the 5th to the 10-25th centile, while stature and head circumference remained between the 10th and the 25th centile, and between mean and -2 standard deviations, respectively.

In case 2 baseline weight, length and head circumference were all below the 5th centile (Kg 10.220, cm 85 and cm 47 respectively, at 32 months of age). During enzyme replacement therapy, a gradual improvement in growth parameters was noted and, at week 135, her weight was Kg 20 (75th), length cm 108 (25th) and head circumference cm 51 (between mean and +2 standard deviations). Her general conditions, which were critical at baseline due to significant motor impairment and respiratory failure, slowly improved. After a 57 weeks admission in a Pediatric Intensive Care Unit (37 weeks of enzyme replacement therapy) she was allowed to go home for a few hours-days/week and, at week 124, she eventually became an outpatient.

At baseline, case 3 was already at a terminal stage of the disease and had a very wasted build (weight: Kg 26). It was not possible to measure stature, because of the severe kypho-scoliosis and multiple joint contractures.

Motor assessment

Case 1, who had only a mild motor impairment at baseline, showed a significant improvement of muscular function during follow-up, and no regression. The "walking, running and jumping" dimension of the Gross Motor Function Measure score was 86% at baseline, 93% at week 12, and steadily 100% since week 26; the other dimensions were steadily 100% (Figure 1A). Walton and Gardner-Medwin scale grade and Manual Muscle Testing scores were always normal.

Case 2, who had been able to stand at 2 years of age, at baseline could not hold her head, but was only able to lift her arms for a short time, with her lower limbs laying down in a frog-like position. After enzyme replacement therapy was started, she stopped losing motor skills, and re-achieved some major motor milestones: at week 70, the patient was able again to sit unsupported and to manipulate objects, but she could stand only with multiple orthopedic devices. Although this girl was not able to speak properly, partly due to the prolonged use of invasive respiratory support, she could efficiently communicate with gestures. The dose of the recombinant enzyme was gradually

increased up to a maximum of two times the initial dosage, and motor function evaluated by Gross Motor Function Measure showed a significant improvement. In the first year of therapy, the scores for the "lying and rolling" and "sitting" dimensions gradually improved from 9.8% to 37.3%, and from 0% to 43.3% respectively (Figure 1B); nevertheless, after two years of therapy, the girl reached a plateau score (52.9% and 55% respectively) and subsequently did not further improve, despite continuous physiotherapy (Figure 1B). The other Gross Motor Function Measure dimensions were steadily 0% during follow-up. The Walton and Gardner-Medwin scale grade, evaluated from week 70, confirmed the impression of a steady state, being constantly 8. Muscle strength, evaluated from week 70 with Manual Muscle Testing, initially improved but subsequently declined in spite of the drug dose increase (Figure 2).

Case 3 underwent enzyme replacement therapy with a baseline skeletal muscle function very severely compromised by the long lasting disease (Table 1) (Manual Muscle Testing total scores for upper body: 23, lower body 11, total body: 34)**.**

In all three cases cognitive and intellectual skills were not impaired (data not shown).

Orthopedic complications

Case 1, who underwent enzyme replacement therapy with a very mild impairment of muscle function, never experienced orthopedic complications.

Case 2, who underwent enzyme replacement therapy with a significant degree of muscular damage, experienced a few orthopedic complications. Despite continuous physiotherapy, she developed joint contractures at lower limbs from week 27: at week 77 she underwent surgical operation for clubfeet. At week 84, a pathologic fracture of the left femoral neck occurred, requiring surgical reduction, immobilization for 19 weeks and vitamin D supplementation for a complete recovery. Total body DEXA scan performed at week 124 did not show generalized osteoporosis (Z score: -0.1, normal values for age >-2.5), although it was not possible to rule out the presence of localized areas of osteopenia secondary to immobility. Bilateral hip dislocation and scoliosis of the thoracic and lumbar tract were noted at week 88 and 130 respectively.

Case 3 had severe contractures of knees and ankles and a very marked kypho-scoliosis from baseline.

Disability evaluation

Case 1 had a Pediatric Evaluation of Disability Inventory score within normal range through the whole follow-up period, without any significant decrease. Case 2 showed a significant increase particularly in the social function and self care domains (Figure 3), although the mobility domain scores remained steadily below the 10^{th} centile, as compared with peers. Case 3 showed a significant degree of disability (baseline Functional Skills Scaled Score: Self care: 30.7; Mobility: 11.4; Social function: 52).

Respiratory function

During the follow-up period, case 1 had recurrent upper airway infections and otitis, but he neither experienced pneumonia nor developed respiratory failure.

At baseline case 2 was on intermittent mandatory ventilation with 20-25 breaths/min, and oxygen supplementation (FiO2: 0.6-0.7) (Figure 4). In the following weeks, she had several lung infections (Figure 4) causing left lung atelectasia. A tracheostomy was performed at week 12. During treatment, the frequency of respiratory infections progressively decreased and a gradual improvement in respiratory function was observed. She was gradually weaned from intermittent mandatory ventilation and, since week 25, she was on continuous positive airway pressure only (Figure 4). From week 37, the patient could stay without any respiratory support, initially for a few hours during the day and then, gradually, for a few days (up to 8 days between weeks 56 and 57). At week 58, continuous permanent continuous positive airway pressure was required after another episode of low respiratory tract infection and, since week 61, she was on a pressure support ventilation, with no need of active ventilation (positive end-expiratory pressure: 0-4 cmH2O; pressure support: 10 cmH2O); since week 68, she was again steadily able to stay without respiratory support for 1-2 hours/day (Figure 4)

During the whole follow-up period, case 3 was on continuous intermittent mandatory ventilation through tracheostomy, with oxygen supplementation during most of the nights for about 8 hours/night, and daytime only during upper respiratory tract infections.

Cardiac status

Electrocardiogram and echocardiography, scheduled every 3 months for infantile Pompe patients and every 6 months for the late onset case, did not show signs of hypertrophic cardiomyopathy.

Hearing assessment

Case 1 had recurrent otitis but no significant hearing problems. Conductive hearing loss due to recurrent otitis was demonstrated in case 2 since week 36, and at week 134 grommets were inserted. Mild mixed hearing loss was detected in case 3 by audiometry at week 9.

Global outcome

While a significant improvement was noted in case 1 and 2, case 3 unfortunately suddenly died after only 20 weeks of enzyme replacement therapy; it was not possible to perform autopsy, and the death was considered to be likely related to the severe pre-therapy general conditions.

Laboratory tests

In all cases, creatine kinase serum levels tended to increase, transaminases levels tended to decrease and lactate dehydrogenase levels were variable over time with no definite trend: these variations were statistically significant for alanine transaminase serum levels in case 1 (R2: 0,7802) and creatine kinase serum levels in case 2 (R2: 0.3054) (Figure 5).

For case 3, creatine kinase and aspartate transaminase levels were respectively 302 and 92 IU/L at baseline and 395 and 89 IU/L at week 20.

Repeated muscle biopsies, performed only in case 2 at weeks 12 and 77 during surgical procedures, did not show major histological changes as compared to the sample obtained at diagnosis.

Safety evaluation

In all cases, blood pressure and heart rate showed only minor changes during the infusions.

Case 2 experienced a few adverse events from week 2, consisting of transient abdominal pain, accumulation of secretions in the upper airways, irritability, fatigue, transient hypoxia and moderate fever (up to 38.5°C), easily managed with administration of paracetamol, reduction of the infusion rate and occasional pre-medication with prometazine and paracetamol. Anti-recombinant human alpha-glucosidase IgG were positive since week 6 with increasing titers (at week 6: 6400; at week 7: 3200; at weeks 10 and 13: 25600; at weeks 17, 70, 76, 114, 124 and 140: steadily 12800).

Case 1 and 3 never experienced infusion related adverse events. In case 1, the anti-drug IgG were negative until week 20, and then became positive at variable titers (at weeks 20, 26, 38, 52, and 64: 200, 400, 800, 800, 400 respectively). Concerning case 3, anti-drug IgG were negative until week 12 and then became positive at increasing titers (at weeks 12, 16 and 20: 400, 400, 800 respectively).

Repeated urine analyses were normal and, in particular, did not show proteinuria.

DISCUSSION

This observational study reports on the use of recombinant human alpha-glucosidase derived from Chinese Hamster Ovary cells in Pompe patients presenting with no cardiac involvement, focusing particularly on long term safety and efficacy. Little information about the effects of this drug is currently available in the literature: the only published study described the drug effects observed in classic infantile Pompe patients during a yearly follow-up at a dose regimen of 5mg/Kg/twice weekly. [9] Subsequently it has been suggested that the administration of a higher quantity of recombinant enzyme, less frequently might be more effective. [11] In the present study, the drug was administered at a dose of 20mg/Kg/qow in 2 cases. Moreover, case 2 was treated with increasing doses of recombinant enzyme, up to a maximum of 40mg/Kg/qow, and was followed for up to three years. Our data confirm that enzyme replacement therapy with recombinant human alpha-glucosidase derived from Chinese Hamster Ovary cells, performed at doses significantly higher (two times) than previously reported, has the same safety as the rabbit milk derived drug.

Only a single trial has been reported to date, enrolling Pompe patients presenting with no heart involvement: [11] in this study, three juvenile Pompe cases were treated with the rabbit milk derived recombinant enzyme and showed motor improvement and stabilization of pulmonary function. [11] Our observation suggests that the recombinant enzyme derived from Chinese Hamster Ovary cells has a similar efficacy in childhood Pompe disease. In particular, concerning respiratory status, our case 2 showed a remarkable decrease of the frequency of lung infections and a significant reduction in the need of respiratory support after the therapy was started (Figure 4). Ventilator dependency has been considered an exclusion criterion in studies on classic infantile Pompe patients. [7,10] Two previously described late onset patients, who were ventilated and showed severe scoliosis at the start of therapy, did not show significant improvement in pulmonary function. [11] This is the first description of a Pompe patient who was ventilator-dependent at baseline and, following enzyme replacement therapy, eventually became steadily ventilator-free for short periods. Our observation suggests that improvement in respiratory function is possible in baseline ventilated patients, if the therapy is started before the occurrence of significant muscle destruction and/or significant scoliosis.

Concerning motor status, case 1 showed no regression and a complete normalization of his motor skills. On increasing drug doses, case 2 showed controversial results. She had a gradual improvement in muscle function, stopping losing motor skills and progressing significantly in motor development. Nevertheless, she reached a plateau at around week 114 and neither major improvement nor decline was subsequently noted (Figure 1). The degree of motor disability improved significantly (Figure 3). On the other hand, after week 70, muscle strength initially slightly improved and subsequently showed a mild decline (Figure 2).

A few different factors might explain the different outcome of the observed cases. The phenotypic clinical variability of the disease is likely to affect the global outcome of the patients. Before starting enzyme replacement therapy, case 2 had a significantly worse clinical course than case 1, in spite of an early presentation in both cases; this might fit with the previous observation that, among

patients presenting before the age of 15 years, there is a subgroup with a more severe and rapid course of the disease. [16] It has been suggested that glycogen clearance by enzyme replacement therapy is likely to lead to a more significant clinical improvement, the earlier the therapy is started, and the less skeletal muscle fibers have been damaged to an irreversible degree. [13] It is possible to hypothesize that the lack of significant effects of enzyme replacement therapy on muscle strength and the occurrence of orthopedic complications in case 2, might be due to the severe muscle destruction already established at baseline, and not further amenable to treatment. In this respect, the higher baseline degree of clinical severity associated with the longer disease duration [16] may well explain the very poor global outcome of case 3.

Although to the best of our knowledge pathological fractures and hip dislocations have not been reported in the previously published trials, we believe that Pompe patients should be strictly monitored for these events, which are very likely to be secondary to reduced mobility.

We did not observe significant changes in muscular histology after one year and a half of therapy in case 2; this is in agreement with previous observations suggesting a possible little of even absent histological change in Pompe patients on enzyme replacement therapy, [7,10] and emphasizing that muscle pathology can vary significantly between different muscle bundles examined. [11]

During enzyme replacement therapy, we observed an increasing trend for creatine kinase levels and a decreasing trend for transaminases. While muscle enzymes have been reported to be useful biochemical diagnostic tools for Pompe disease, [5] their significance in the follow-up of Pompe patients is currently a matter of debate. Lactate dehydrogenase and transaminases have been proposed to be better markers of disease progression in the natural history of classic infantile Pompe disease, as compared with creatine kinase; [3] on the other hand creatine kinase levels have been considered to be the best serologic marker of enzyme replacement therapy efficacy in late onset Pompe disease. [11] It might be reasonable to hypothesize that both muscle and liver contribute to the abnormally high levels of transaminases found in Pompe patients. The discrepancy between decreasing transaminases serum levels and increasing values of creatine kinase might suggest that

enzyme replacement therapy is more effective on liver than muscle, probably because the drug is up-taken more efficiently by the hepatocytes compared with skeletal muscle fibers. [17] Comparing the increasing creatine kinase levels found in our patients treated with the Chinese Hamster Ovary cells derived drug, with the decreasing creatine kinase levels previously reported in three cases treated with the rabbit-milk derived recombinant drug, [11] one might hypothesize a more significant effect on skeletal muscle of the latter drug. No studies have been reported to date systematically comparing the effects of the two drugs available. Nevertheless, in the very few observational studies published to date, the efficacy of both alternative drugs on motor status seems to be variable and overall comparable. [18]

The presence of anti-drug antibodies in our cases was difficult to interpret. Case 2 developed anti-drug antibodies at increasing titers in the first months of treatment, when she showed significant clinical improvement. On the other hand, when she subsequently stopped improving in muscular function and showed a slight decline in muscular strength, she had stable anti-drug serum levels. Case 1 and 3, who developed anti-drug antibodies at lower and comparable levels, had a very different outcome. On the basis of our findings and the previously reported observations, the clinical relevance of the anti-drug antibodies appears unclear and requires further investigation. [11]

Our case 3 started enzyme replacement therapy in very poor general conditions and died after only 20 weeks of treatment. We think that positive results are more likely to be submitted for publication than the negative ones. This might explain the fact that, among a total of twelve Pompe patients reported in the literature on enzyme replacement therapy, there is only one death report. [10] Further descriptions of cases with poor outcome might contribute to the identification of the subgroup of patients who might be more likely to improve on enzyme replacement therapy.

The lack of significant effects of the treatment on severely compromised skeletal muscle, suggests the need of alternative therapeutic approaches: experimental data about gene therapy in animal models, [19] and preliminary data on the effects of small molecules with chaperone effect in “in-vitro” models of Pompe disease, [20] seem to be promising.

In conclusion, our observations provide additional information on the efficacy and safety of enzyme replacement therapy with recombinant human alpha-glucosidase derived from Chinese Hamster Ovary cells, suggesting that this experimental therapeutic approach can prevent motor regression and lead to motor and respiratory improvement in Pompe patients who start the therapy before significant muscle damage has occurred.

Acknowledgements

We are grateful to: Dr. D. Melis and Dr. M. Sibilio, Department of Pediatrics, Federico II University, Naples, Italy for their important contribution to the clinical management of the patients. We are also grateful to Prof. N. Rizzuto, Neurology Department, University of Verona; Dr. C. Dionisi Vici, Metabolic Medicine Department, and Dr. E. Bertini, Molecular Medicine Unit, Bambino Gesù Pediatric Hospital, Rome; Prof. R. Gatti and Dr. M. Filocamo, Laboratory for Pre- and Post-natal Diagnosis of Metabolic Diseases, Istituto Gaslini, Genova, Italy, for diagnosis confirmation of case 1, 2 and 3 respectively.

This work was supported partially by Ministero dell'Istruzione, Università e Ricerca, PRIN 2004068595_005 (to GP), and by Istituto Superiore di Sanità, Progetto Malattie Rare, Conv. N°526/A20 (to GA)**.**

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FIGURE LEGENDS

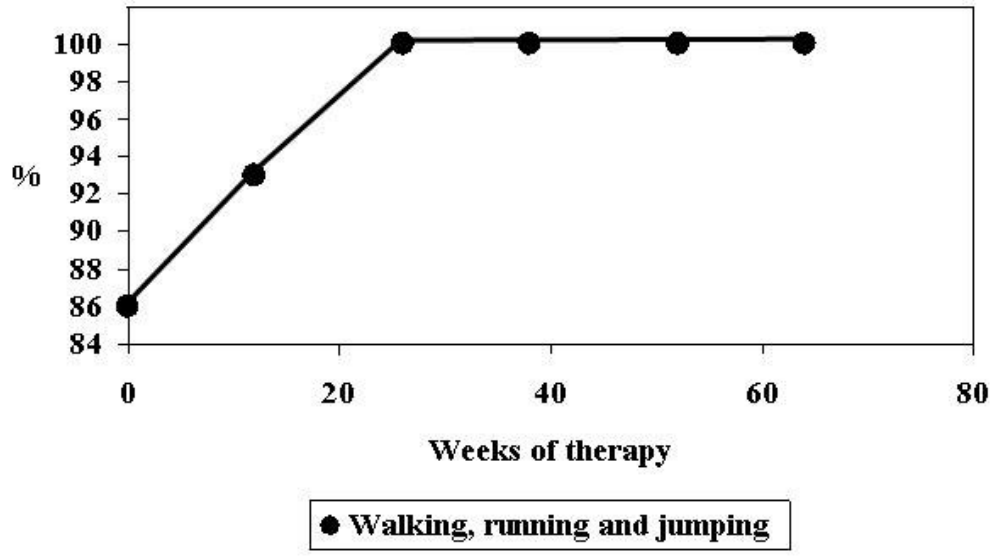
Figure 1

Cases 1 and 2: effects of the enzyme replacement therapy on muscle function measured by the Gross Motor Function Measure. For details of the scoring system: see text.

1A: Case 1. The scores for the "lying and rolling", "sitting", "crawling and kneeling" and "standing" dimensions were steadily 100% (not shown).

1B: Case 2. The scores for the "standing" and "walking, running and jumping" dimensions were steadily 0 (not shown).

Figure 1
1A



1B

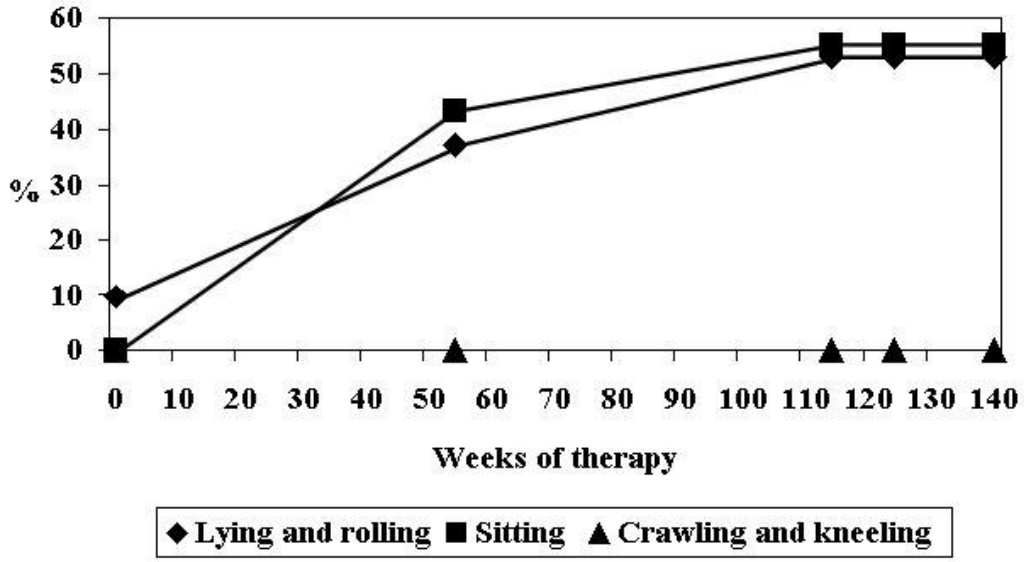


Figure 2

Case 2: Manual Muscle Test total scores, evaluated during treatment from week 70 according to the Medical Research Council, for upper body (circles), lower body (squares), and total body (triangles).

For details about the test: see the text.

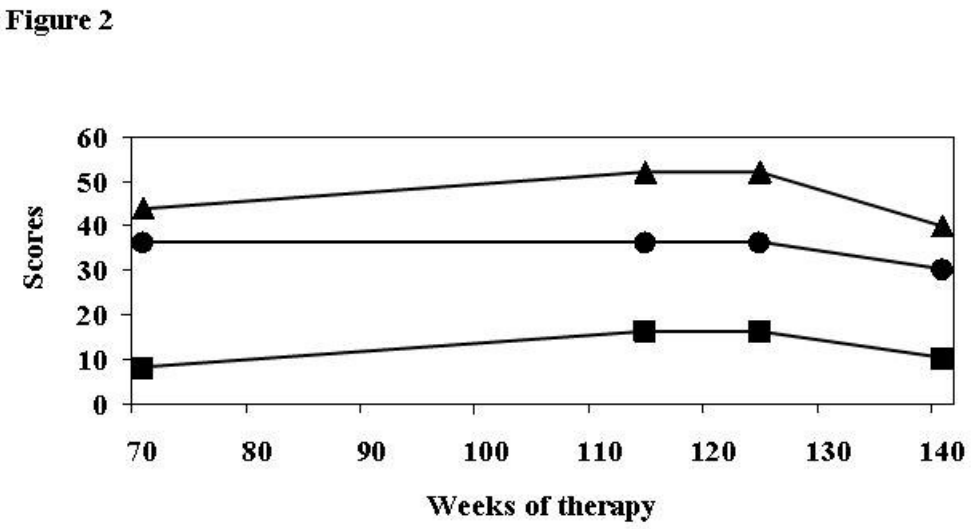
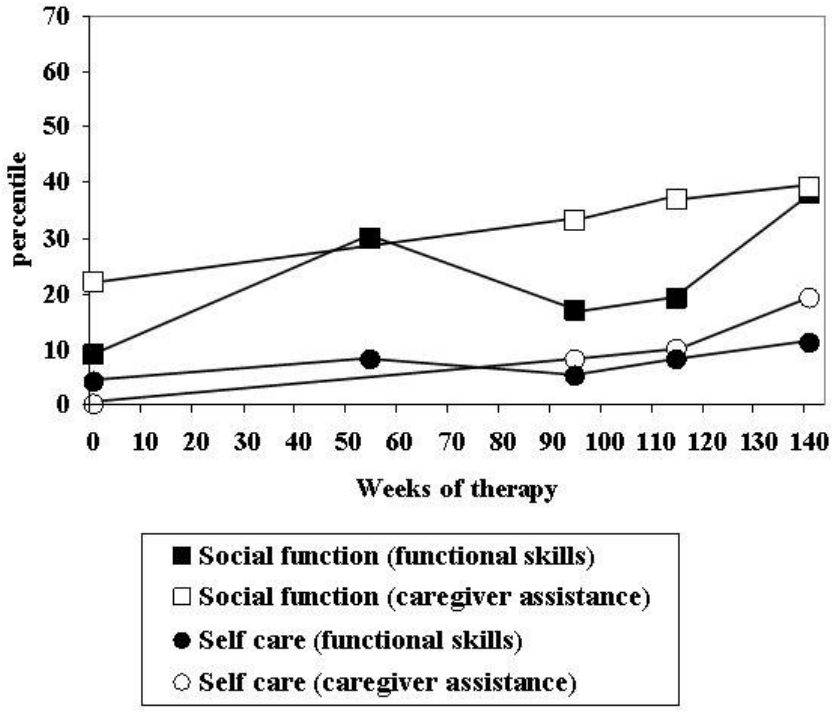


Figure 3

Case 2: degree of disability assessed during enzyme replacement therapy using the Pediatric Evaluation of Disability Inventory. For details about the test: see text.

3A: Normative Standard Scores. Normal range: 30-70. Mobility domain (both functional skills and caregiver assistance): always <10th centile (data not shown).

Figure 3
3A



3B: Scaled Score.

3B

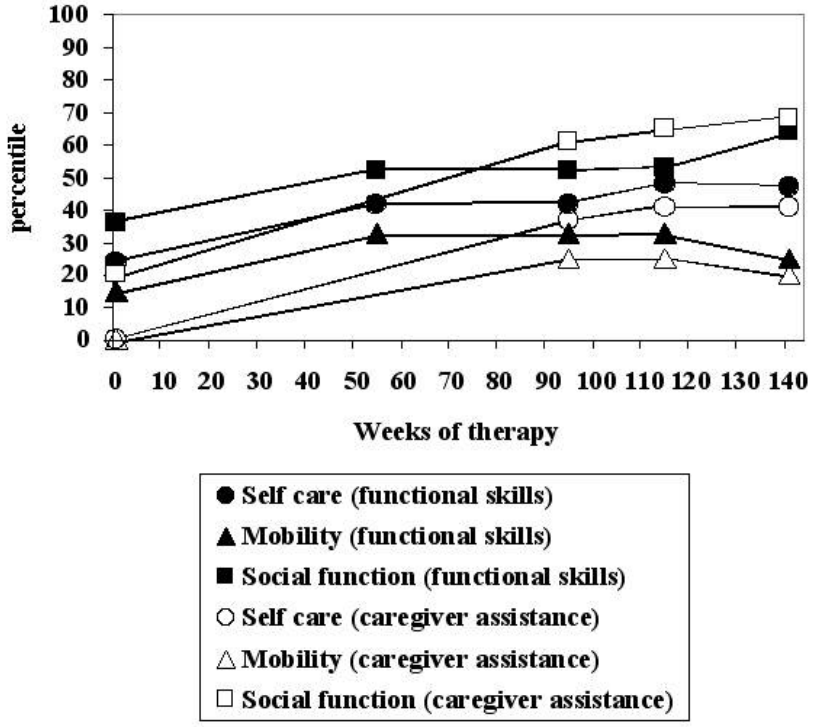


Figure 4

Case 2: infectious episodes and respiratory support. After enzyme replacement therapy was started, the frequency of lung infections (black arrows) decreased over time and respiratory function gradually improved.

Note: white arrows: start of enzyme replacement therapy, and subsequent changes in the drug dose; qow: every other week.

Respiratory support:

A: Continuous intermittent mandatory ventilation (at baseline: 20-25 breaths/min; in the following weeks: 5-25 breath/min depending on patient's conditions) and continuous oxygen supplementation (FiO2 0.5-0.7).

B: Intermittent mandatory ventilation during nighttime or infectious episodes (5-15 breaths/min depending on patient's conditions); continuous positive airway pressure during daytime in infection-free periods. Intermittent oxygen supplementation (FiO2 0.21-0.7).

C: Continuous positive airway pressure; no oxygen.

D: Continuous positive airway pressure/no oxygen/no respiratory support for short periods (few hours/few days)

E: Continuous pressure support ventilation; no oxygen. F: Pressure support ventilation/no oxygen/no respiratory support for short periods (few hours).

Figure 4

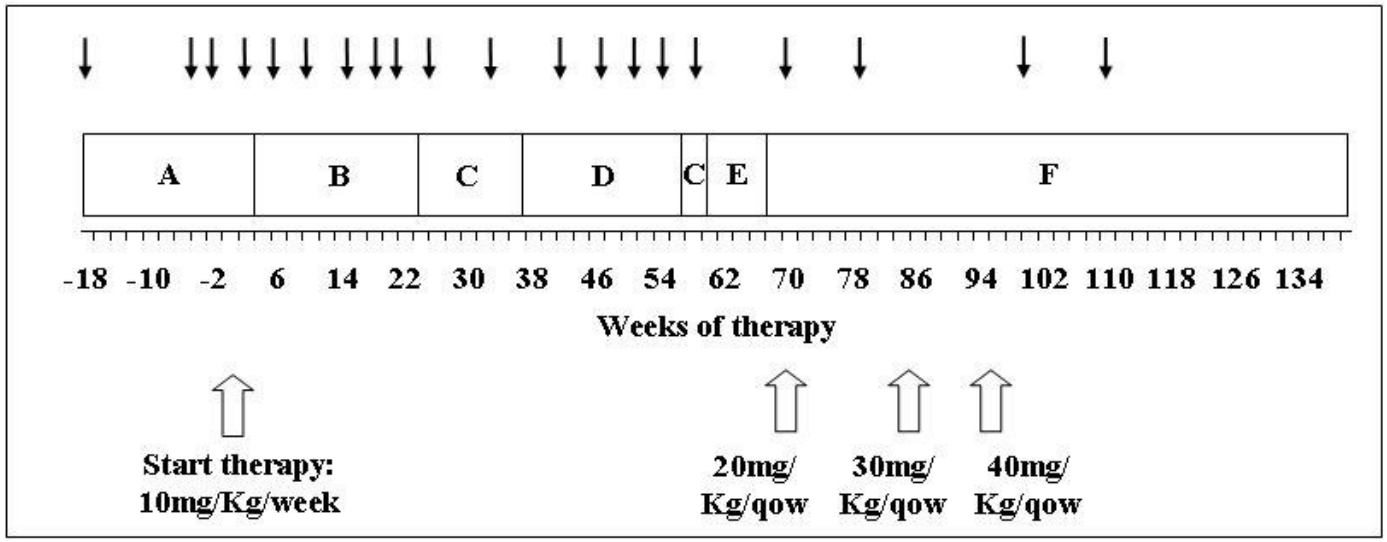

A
B
C
D
C
E
F
-18 -10 -2 6 14 22 30 38 46 54 62 70 78 86 94 102 110 118 126 134
Weeks of therapy
Start therapy:
10mg/Kg/week
20mg/
Kg/qow
30mg/
Kg/qow
40mg/
Kg/qow

Figure 5

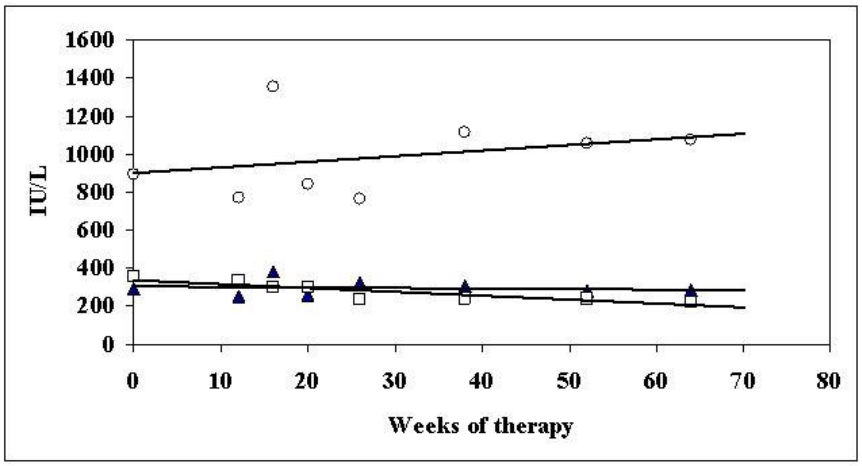
Cases 1 and 2: effects of the enzyme replacement therapy on serum levels of creatine kinase (circles), aspartate transaminase (triangles), and alanine transaminase (squares).

Note: IU: International Units; L: Liter

5A: Case 1.

5B: Case 2.

Figure 5
5A



5B

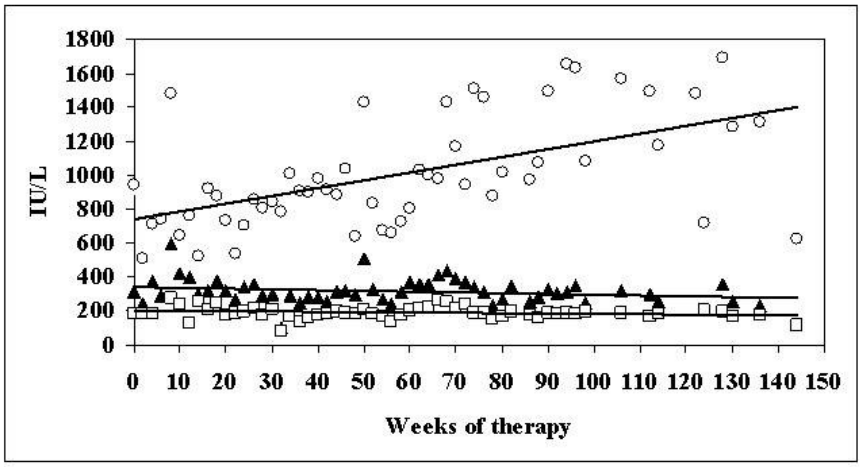


Table 1.

Features of the three Pompe patients. m: months; y: years; qow: every other week.

Patients (Gender, patient's initials)	*1 (Male, GM)*	*2 (Female, RP)*	*3 (Male, VPO)*
Onset	4m	6m	2y
Age of diagnosis	11m	1y	2y2m
Presenting symptoms/signs			
Hypotonia	-	+	-
Motor delay	+	+	+
Frequent vomiting, failure to thrive	+	-	-
Hepatomegaly	-	-	+
Hypertrophic cardiomyopathy	-	-	-
Raised serum muscle enzymes	+	+	+
Enzymatic activity at diagnosis (% of lower limit of normal range)			
Muscle	0.46	3	-
Fibroblasts	1.8	4	2.4
Lymphocytes	-	8	3.3
Baseline conditions			
Skeletal muscle involvement	+	+	+
Motor regression	-	+	+
Severe hypotonia	-	+	+
Wheelchair bound	-	+	+
Severe scoliosis	-	-	+
Joint contractures	-	-	+
Pathologic fractures	-	-	+
History of pneumonias	-	+	+
Respiratory failure	-	+	+
Invasive ventilation	-	+	+
ERT			
Age at the start	3y8m	2y8m	19y9m
Follow-up duration (weeks)	70	140	20
Dose (weeks)	20mg/Kg/qow (0-70)	10mg/Kg/week (0-69); 20mg/Kg/qow (70-85); 30mg/Kg/qow (86-95); 40mg/Kg/qow (96-140)	20mg/Kg/qow (0-20)
Global outcome	Improvement	Improvement	Death

APPENDIX 3.5.2

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Molecular Therapy. In press.

2006

PHARMACOLOGICAL ENHANCEMENT OF MUTATED α-GLUCOSIDASE ACTIVITY IN FIBROBLASTS FROM PATIENTS WITH POMPE DISEASE

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ABSTRACT

We investigated the use of pharmacological chaperones for the therapy of Pompe disease, a metabolic myopathy due to mutations of the gene encoding the lysosomal hydrolase α-glucosidase (GAA) and characterized by generalized glycogen storage in cardiac and skeletal muscle. We studied the effects of two imino sugars, deoxinojirimycin (DNJ) and N-butyldeoxynojirimycin (NB-DNJ), on residual GAA activity in fibroblasts from 8 patients with different forms of Pompe disease (2 classic infantile, 2 non-classic infantile onset, 4 late onset forms), and with different mutations of the *GAA* gene. We demonstrated a significant increase of GAA activity (1.3 to 7.5-fold) after imino sugar treatment in fibroblasts from patients carrying the mutations L552P (3 patients) and G549R (1 patient). GAA enhancement was confirmed in HEK293T cells where the same mutations were overexpressed. No increase of GAA activity was observed for the other mutations. Western blot analysis showed that imino sugars increase the amount of mature GAA molecular forms. Immunofluorescence studies in HEK293T cells overexpressing the L552P mutation showed an improved trafficking of the mutatnt enzyme to lysosomes after imino sugar treatment. These results provide a rationale for an alternative treatment, other than enzyme replacement, to Pompe disease.

Key words

Pompe disease, glycogen storage disease, α-glucosidase, imino sugar, deoxynojirimycin, N-butyldeoxynojirimycin, enzyme enhancement, pharmacological chaperone

INTRODUCTION

Pompe disease (PD, glycogenosis type II, OMIM 232300) is an autosomal recessive disease with an estimated incidence of 1:40,000 live births [1]. PD is due to mutations of the acid α-glucosidase (*GAA*) gene, encoding the lysosomal hydrolase α-glucosidase (acid maltase, GAA, E.C.3.2.1.20), which is synthesized as a 110 kDa precursor and is processed into the active polypeptides of 76 and 70 kDa, through an intermediate molecular form of 95 kDa [1, 2].

α-Glucosidase deficiency results in generalized tissue glycogen accumulation and secondary cardiac and skeletal muscle destruction [3]. Different mutations of the *GAA* gene result in a wide phenotypic variability, ranging from the devastating classic infantile form, characterized by early onset, severe hypertrophic cardiomyopathy, marked hypotonia, hepatomegaly, and invariably fatal outcome by one year of age, to the late onset childhood, juvenile or adult forms, characterized by skeletal myopathy, in the absence of cardiac disease [3]. Infantile onset non-classic phenotypes have been described, presenting within the first two years of life, with milder or absent heart involvement [3, 4].

Enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) derived from either rabbit milk or Chinese Hamster Ovary (CHO) cells has been recently introduced in the treatment of PD patients [5,6]. The results of the published trials, enrolling more than thirty cases with variable phenotypes, support the effectiveness of ERT on cardiomyopathy and motor function in the classic infantile PD patients, whereas the effects of ERT on skeletal myopathy in the late-onset patients were variable [7]. Concern about the efficacy of ERT on skeletal muscle manifestations also derives from a number of studies performed in animal models, showing preferential targeting of rhGAA to liver, rather than to muscle [8], little or no clearing of glycogen stores from muscle cells in advanced stage skeletal muscle involvement [8, 9], selective resistance of type II muscle fibers to ERT with rhGAA [10]. These observations suggest that a search for alternative therapeutic approaches to PD, other than ERT, is warranted, particularly for patients with late-onset forms and prominent skeletal muscle disease.

Recently, the use of pharmacological chaperones to enhance the activity of mutated lysosomal enzymes has attracted considerable interest. It has been shown that specific drugs, mostly imino sugars and their alkylated derivatives, are able to rescue the deleterious effects of missense mutations on the folding and activity of several lysosomal hydrolases, including β-glucocerebrosidase [11], α-galactosidase [12,13], β-galactosidase [14] and β-hexosaminidase [15]. It has been proposed that the enhancing effect of these compounds may have applications in the treatment of lysosomal storage disorders due to the deficiency of these enzymes.

In this study we have explored the possibility of using this approach in PD. We have investigated the effects of two imino sugars, deoxynojirimycin (DNJ) and N-butyldeoxynojirimycin (NB-DNJ), on GAA activity in fibroblasts from PD patients carrying different mutations of the *GAA* gene.

RESULTS

Molecular characterization of patients

Seven out of 8 patients included in the study were characterized on the molecular ground. The results of the molecular analysis of patients are reported in Table 1. The mutation L552P was found in three unrelated patients (28% of the alleles studied) coming from different Regions of Italy. Patients 1 was homozygous for this mutation, whereas patients 2 and 3 were heterozygotes, both carrying the L552P mutation together with a splicing mutation c.-35C>A and the A445P mutation, respectively. Patient 4 was a compound for the mutation G549R and a splicing mutation. Patients 5 and 7 were homozygous for two missense mutations (L355P and R375L, respectively). In patient 6 only a single allele, carrying a c.-45T>G mutation, commonly associated with juvenile phenotypes, was identified. Patient 8 died before the molecular analysis was performed and her parents could not be traced back to provide a parental consent to mutational analysis.

Effects of imino sugars on GAA activity in PD fibroblasts

We studied the effects on GAA activity of the imino sugar DNJ and its alkylated derivative NB-DNJ in fibroblasts from the PD patients included in the study. Treatment for 9 days with both compounds was effective in enhancing GAA activity in fibroblasts from patients 1-4 (Fig. 1). A 1.8 to 5.6-fold increase of residual GAA activity after treatment with NB-DNJ was observed in fibroblasts from patients 1, 2 and 3, carrying at least one allele with the L552P mutation and from patient 4, carrying the missense mutation G549R and a splicing mutation on the second allele.

Similar results (1.3 to 7.5-fold increase in GAA residual activity) were obtained after culturing the same fibroblast cell lines in the presence of DNJ. In all cases GAA increases from baseline were statistically significant with values of p ranging from <0.001 to 0.019.

The maximum increase of GAA activity reached approximately 10% of the low normal range in control fibroblasts. No increase was found in cells from patients 5-8. No effect on GAA activity was observed in control fibroblasts after imino sugar treatment.

NB-DNJ concentrations as low as 10 μM were sufficient to enhance GAA activity, reaching a plateau between 10 and 80 μM (Fig. 2A). The enhancing effect was already observed after 3 days of incubation and continued up to15 days (Fig. 2B).

No increase of GAA activity was observed when PD fibroblasts were cultured in the presence of 20 μM concentrations of different imino sugars (deoxygalactonojirimycin and mannojirimycin) suggesting a specific effect of DNJ and NB-DNJ on GAA (not shown).

Effects of imino sugar treatment on single mutations expressed in HEK293T cells

To study the effects of imino sugar treatment on single alleles, each of the missense mutations found in responsive patients was transiently expressed in HEK293T cells. According to the studies on the time course of imino sugar enhancement in fibroblasts a treatment period of 3 days is sufficient to obtain a detectable effect on GAA catalytic activity.

GAA activity was measured in homogenates from NB-DNJ treated and untreated transfected cells and the results were compared (Fig. 3). Imino sugar treatment of HEK293T cells transfected with constructs carrying the mutations L552P and G549R, resulted in an increased GAA activity. These results are consistent with those obtained in fibroblasts. No increase was found when the mutation A445P was expressed. This suggests that in patient 3, who is a compound for the mutation L552P and the A445P, the contribution of the latter mutation to GAA enhancement is negligible. In the HEK293T cells expressing the mutation R375L no enhancement by imino sugar treatment was observed, confirming the results obtained in fibroblasts from patient 7 (homozygous for this mutation).

Expression of GAA mRNA and protein

To investigate the mechanism involved in the enhancement of GAA activity, mRNA transcription and protein expression were studied in PD fibroblasts and in transfected HEK293T cells.

Real time PCR was performed on the cDNA prepared from imino sugar-treated and untreated fibroblasts from patient 1 (homozygous for the responsive mutation, L552P), patient 7 (homozygous for the unresponsive mutation R375L) and a normal control. Imino sugar treatment did not influence the expression of GAA mRNA in all cell lines studied (not shown).

Western blot analysis of GAA peptides was performed in imino sugars responsive and in non responsive fibroblasts. In the fibroblasts showing enhancement of GAA activity, imino sugar treatment resulted in an appreciable increase of mature GAA polypeptides. Figure 4 shows the results obtained in fibroblasts from patient 1, homozygous for the L552P mutation, and showing the highest GAA increase, and from patient 4, carrying the other imino sugar responsive mutation G549R on one allele.

The 110 kDa precursor is detectable in all untreated cells. In fibroblasts from patient 1 after NB-DNJ treatment the amount of precursor is unchanged and the 70-76 kDa peptides are faint but clearly appreciable. In fibroblasts from patient 4, a compound for the responsive G549R mutation and a splicing mutation, a reduced amount of the mature 70-76 kDa peptides is already detectable under basal conditions and remains unchanged after imino sugar treatment. In both cases the amount of mature GAA polypeptide is low, as compared to that observed in controls, consistent with increases of enzyme activity of no more than 10% of normal values. Imino sugar treatment resulted in an increased amount of mature GAA peptides also in fibroblasts from patient 7, in which no enzyme activity enhancement had been observed. It is likely that the R375L mutation, detected in the unresponsive patient, has a severe impact on GAA catalytic activity, thus explaining the lack of enzyme enhancement by imino sugars.

Western blot analysis was also performed in NB-DNJ treated and untreated transfected HEK293T cells (Fig. 5). In all cells, expressing each of the missense mutations studied, imino sugar treatment

resulted in increased amounts of both the 95 kDa intermediate and the mature GAA 70-76 kDa polypeptides, confirming the results obtained in fibroblasts. The observation of an increased amount of mature GAA in cells expressing the non-responsive mutations A445P and R375L, further supports the idea that specific mutations impact severely on the functional domains of the enzyme and that in these mutants GAA activity cannot be rescued by pharmacological chaperones, even if the maturation of the protein is improved.

Immunolocalization of mutant GAA in HEK293T cells

To investigate whether imino sugar improve the trafficking of GAA to the lysosomal compartment immunofluorescence studies were performed in HEK293T cells overexpressing the mutation L552P. In the cells expressing mutated GAA imino sugar treatment resulted in a punctate staining typical of lysosomal localization and comparable to that obtained in the same cells expressing the wild type enzyme (Fig. 6). The GAA fluorescence partly co-localized with a lysosomal protein (LAMP2), thus confirming an improved delivery of GAA to lysosomes.

DISCUSSION

We have shown that treatment with the imino sugars DNJ and NB-DNJ enhances the activity of GAA in fibroblasts from patients with specific mutations of the *GAA* gene.

DNJ and NB-DNJ are known inhibitors of glycosidases, including GAA, and ceramide-specific glucosyltransferases [16] and were initially proposed for clinical use as anti-HIV drugs [17]. NB-DNJ is presently approved for the treatment of Gaucher disease in humans, as a substrate-reducing agent, and is commercially available. In principle, the same inhibitory effect on the synthesis of glucosphingolipids may be useful for the treatment of other disorders, including G_{M1} and G_{M2} gangliosidoses and Niemann-Pick disease type C.

It has recently been shown that, in addition to the effect on substrate synthesis, NB-DNJ is able to perform as a pharmacological chaperone on β-glucocerebrosidase, and that it can rescue the effects of specific mutations of this enzyme (including the common N370S associated with Gaucher disease type 1) [11]. The concept of pharmacological chaperones that can rescue misfolded, unstable and non-functional proteins is a growing field of investigation and the possible applications in human therapy are currently under investigation [18-20]. The use of specific chemical chaperones has been proposed also in other lysosomal storage diseases, such as Fabry disease [12, 13], G_{M1} [14] and G_{M2} gangliosidoses [15]. In Fabry disease this approach had a clinical application [21] and proved to be effective in reducing cardiac hypertrophy in a patient with the cardiac variant of the disease.

We demonstrated a similar effect of imino sugars DNJ and NB-DNJ on residual GAA activity in fibroblasts from PD patients with different genetic backgrounds and different phenotypic presentations.

Since some patients were genetic compounds for different *GAA* gene mutations, the enhancing effects of imino sugars were studied in human kidney cells expressing single mutant alleles. This allowed to clarify that two of the mutations studied were responsive to imino sugar treatment. The L552P mutation results in the change of a lysine into a proline residue, which may induce a turn in the protein tertiary structure and can be implicated in protein misfolding. Although most missense mutations of the *GAA* gene are rare or private mutations, the L552P mutation appeared to be relatively frequent in our series of patients (28% of the alleles). The other responsive mutation changes a glycine into an arginine (G549R). Both the L552P and the G549R mutations are localized close to the GAA catalytic domain, where it is likely that interactions with DNJ and NB-DNJ occur, while the unresponsive mutations (A445P, L355P and R375L) are localized in different domains of the protein. Studies on the tertiary structure of GAA will probably provide an important tool to better understand the mechanisms leading to GAA enzyme enhancement by pharmacological chaperones and to identify other responsive mutations.

To study the mechanisms implicated in enzyme enhancement the effects of imino sugars on GAA mRNA transcription and protein maturation were analyzed in cultured fibroblasts from responsive patients, and compared to the results obtained in a non-responsive cell line and in controls.

Imino sugars had no significant effect on mRNA levels, that remained unchanged in all cell lines studied. Western blot analysis of fibroblasts and HEK293T cells expressing single *GAA* gene mutations showed the presence of substantial amounts of the mature and active 76-70 kDa GAA molecular forms after treatment with imino sugars. This indicates that enzyme enhancement is mediated through an improved maturation and/or stability of the enzyme. Increased amounts of GAA polypeptides was observed in all cell lines studied, also in the absence of enzyme activity enhancement. This indicates that, although imino sugars are generally effective in increasing the amount of mature GAA, in the presence of specific *GAA* gene mutations GAA catalytic activity cannot be rescued.

Immunofluorescence studies in cells overexpressing the L552P mutation showed that imino sugars enhance the trafficking and delivery of mutated GAA to lysosomes.

An important issue to be addressed is whether the enhancing effect of DNJ and NB-DNJ *in vitro* has potential relevance for therapeutic applications. In this respect, although it appears paradoxical that the same molecule that inhibits a lysosomal enzyme is able to enhance its activity by improving protein folding and stability, a number of observations suggest that the use of pharmacological chaperones may not affect the enzyme catalytic activity within the lysosomal compartment, and therefore might be beneficial for patients.

First, it has been shown that NB-DNJ does not increase muscle glycogen content in mice at plasma concentrations comparable to those reached in human therapy (between 2 and 30 μM), and that glycogen storage in muscle is observed only when high doses of drug (2400 mg/kg/day) are administered to the animals [22]. In our study we found that an enhancing effect of imino sugars on GAA is already detectable at concentrations of 10-20 μM, therefore lower than those potentially

causing glycogen accumulation.

Second, it has been suggested that imino sugars concentrations attained in lysosomes are lower that those in the endoplasmic reticulum [22] and that the interactions between imino sugars and the catalytic site of the enzymes are pH dependent and do not occur at acidic pH [12].

Also, it can be speculated that imino sugars are displaced from the catalytic site of the enzyme by the excess of natural substrate, such as glycogen, accumulated in the lysosomes.

During the preparation of our manuscript, an *in vitro* study was published showing a stabilizing effect on GAA of high concentrations of glucose in the culture medium of CHO-K1 cells expressing wild-type GAA [23]. In the same study an enhancing effect on GAA activity by glucose was described in adult onset PD fibroblasts, although no information on the molecular background of the patients was provided. Although these data are consistent with our findings on GAA enzyme enhancement in PD, high levels of glucose would not be appropriate for human therapy, due to possible deleterious metabolic effects.

Our results appear promising in terms of possible clinical applications of imino sugars for the treatment of PD. One of the drugs tested in our study, NB-DNJ, has been used for treatment of Gaucher disease for more than 5 years [16] and wide clinical experience on the use of this drug is already available. In our study we obtained a significant increase of GAA activity in fibroblasts from patients with responsive mutations, up to approximately 10% of the low normal GAA activity in fibroblasts. The GAA specific activity obtained in fibroblasts treated with imino sugars is comparable to the activity detected in muscle from *GAA* knock-out mice treated with high doses (100 mg/kg/week) enzyme replacement therapy [8]. Such an increase may impact on patients' phenotypes and clinical course. For several lysosomal diseases a threshold effect has been demonstrated, and it has been shown that even low residual enzyme activities are sufficient to prevent substrate accumulation. In PD a clear correlation between enzyme activity and the severity of the phenotype has been observed, with undetectable GAA in the severe forms of the disease and substantial residual activity in milder and late onset forms [1].

In conclusion, our results indicate an alternative therapeutic strategy based on the stimulation of endogenous residual GAA, at least in some patients with PD. An *in vitro* evaluation of imino sugars effect may be a preliminary step in recognizing individual patients in whom an enzyme enhancement-based approach could be beneficial.

PATIENTS AND METHODS

Patients

Patients 1 and 2 (Table 1) with the non-classic infantile onset form of the disease presented with generalized hypotonia and myopathy within the first year of life. They did not show cardiac involvement and are presently alive at ages of 6 and 5 years, respectively. Patient 1 is presently wheel chair-bound and needs respiratory support. Patients 3 presented at 3 years of age with respiratory infections, hypertransaminasemia and progressive motor regression. She is presently wheel chair-bound at the age of 20 years. Mild cardiac involvement is also present. Patient 4 presented at the age of 4 years with mild generalized weakness and hypertransaminasemia, without signs of cardiac disease, and is presently alive at the age of 11 years. Patient 5 presented in the third year of life with hepatomegaly and muscular weakness, progressively leading to respiratory insufficiency and death at the age of 20 years. Patient 6 presented in the third year of age with lower limb hypotonia and hypertransaminasemia. He is presently 13 years old and shows generalized weakness, without cardiac involvement. Patients 7 and 8 presented with severe cardiomyopathy and generalized hypotonia. In both cases their phenotype was typical of the classic infantile PD. They died at ages of 13 and 14 months, respectively. Patients 1-6 were on enzyme replacement therapy for variable periods.

Reagents and chemicals

Unless otherwise stated all reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). A polyclonal antiserum against human GAA developed in rabbit was available in our laboratory. DNJ

and NB-DNJ were dissolved in DMSO at a concentration of 4.5 mM; appropriate amounts of this stock solution were added to cell culture media to obtain the required final concentrations in the different experiments. Glucose concentration in the medium was 4500 mg/L.

Cell cultures

Fibroblasts cell lines were cultured from skin biopsies, after obtaining an informed consent by the patients' parents. Both fibroblasts and human kidney embryonic cells HEK293T were grown in DMEM, supplemented with antibiotics and glutamine. Glucose concentration in the medium was 4500 mg/L.

Molecular characterization of patients

The mutational analysis of the *GAA* gene was performed according to reference [24] after obtaining an informed consent from the patients' parents. Genomic DNA from the patients was obtained from peripheral blood, using commercial kits, according to standard procedures. Oligonucleotides corresponding to intronic sequences flanking all GAA exons were used as primers to amplify single exons by PCR. Cycle sequencing was performed in the forward and reverse directions with the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystem, Warrington, UK). Sequence analysis was performed on an automated ABI Prism 3700 sequencing apparatus.

Treatment of fibroblasts with imino sugars

Fibroblasts from each patient were incubated for 9 days with 20 μM DNJ and 20 μM NB-DNJ, respectively. The same cell lines were cultured in parallel in the absence of imino sugars for comparison. The optimal concentrations of imino sugars to be used and the time course of GAA enzyme enhancement were determined in fibroblasts from patient 1 that were cultured for variable times (0, 3, 6, 9, 15 days) in the presence of different concentrations (0, 10, 20, 50, 80 μM) of NB-DNJ. After each experiment the cells were harvested and cell extracts were used for the assay of

GAA activity and for Western blot analysis. To test the specificity of different imino sugars, fibroblasts were also cultured for 9 days in the presence of 20 μM concentrations of deoxy-galactonojirimycin and mannojirimycin.

Expression of GAA mRNA in PD fibroblasts

Total RNA was extracted from treated and untreated cell cultures using RNeasy mini kit protocols (Qiagen). RNA concentrations were determined using Cary 50 Bio Spectrophotometer (Varian). The SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) was used to generate cDNA for PCR from 1 μg of total RNA. Real time RT-PCR was performed using I cycler IQ Detector System (BioRad). GAA cDNA was amplified using a forward primer in GAA exon 1 (5' TCTGAAATGGGCTACACGGC 3') and a reverse primer in GAA exon 2 (5' CGCTGTTAGCTGGATTG 3'). The relative amount of transcripts was calculated by using the threshold cycle (Ct) methods, according to the manufacturer's instructions, comparing GAA *versus* Beta-Actin transcripts. Real time PCR reactions of each sample were performed in triplicate and experiments were repeated at least three times.

Site directed mutagenesis and expression of mutated GAA in mammalian cells

GAA cDNA was obtained as described [24]. The mutations identified in patients were introduced in the wild type GAA gene by site-directed mutagenesis, using commercially available kits (Quick-Change, Stratagene), according to the manufacturer's instructions and using primers carrying the mutations identified in patients. The cDNAs were subcloned in pCDNA3 and human embryonic kidney cells HEK293T were transiently transfected with 2 μg wild type and mutant cDNAs by commercially available kits (PolyFect, Quiagen), according to the manufacturer's instructions. Immediately after transfection the cells were incubated in the presence of 20 μM NB-DNJ. Seventy-two hours after transfection the cells were harvested and cell homogenates were used for the assay

of GAA activity and for Western blot analysis.

GAA enzyme assay

Fibroblasts and HEK293T cells were harvested by trypsinization and disrupted by freezing and thawing (3x). Cell homogenates (30-40 μg of protein) were incubated at 37° for 60 minutes with 2 mM 4-methylumbelliferyl α-D-glucopyranoside as substrate in a 0.2 acetate buffer buffer pH 4.0 in an incubation mixture of 100 μl. Reactions were stopped with 1.9 ml glycine carbonate buffer pH 10.7 and fluorescence was read on a Turner biosystems fluorometer Modulus 9200 (360 nm excitation, 450 nm emission). Protein concentration in cell homogenates was measured according to reference [25].

Western blot analysis

Equal amounts (20 μg protein) of fibroblast or HEK293T cell homogenates were subjected to SDS-PAGE electrophoresis and Western blotting. To detect the GAA polypeptides a polyclonal anti-GAA antiserum raised in rabbit was used as primary antiserum. The different GAA polypeptides were visualized using an alkaline phosphatase-conjugated goat anti-rabbit secondary antiserum. To detect β-actin polypeptides a monoclonal anti β-actin antibody developed in mouse was used as primary antibody and the filters were developed by ECL, according to standard procedures. Extracts from untreated cells and control fibroblasts were run in parallel for comparison.

Immunolocalization of mutant GAA in HEK293T cells

Immunofluorescence studies were performed according to ref [24], using a rabbit polyclonal anti-GAA antiserum as a primary antibody and a FITC-conjugated anti-rabbit IgG (DAKO, Glostrup, Denmark). For co-localization studies a monoclonal anti-LAMP2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody and a TRITC-conjugated anti-mouse antiserum as a secondary antibody (DAKO, Glostrup, Denmark).

Statistical analysis

The statistical significance of the effects of imino sugar on GAA activity in fibroblasts was evaluated by Mann-Whitney test. The variation of RNA expression in treated and untreated fibroblasts levels was analyzed statistically by a T-test.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Ministero dell'Istruzione dell'Università e della Ricerca (PRIN 2004068595_005 to Giancarlo Parenti) and a by grant from Istituto Superiore di Sanità (Progetto Malattie Rare) Conv. N526/A20 (to Generoso Andria). The financial support of Telethon - Italy (grant GGP05045 to Giancarlo Parenti) is gratefully acknowledged.

Fibroblasts from patient 5 were obtained from the "Cell Line and DNA Bank from Patients affected by Genetic Diseases" collection supported by Italian Telethon grants (project no GTF04002 to Mirella Filocamo).

We are grateful to Marinella Pirozzi (Microscopy and Imaging Core, TIGEM) for technical assistance.

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Figure 1

Effects of imino sugars treatment in Pompe fibroblasts.

Treatment with NB-DNJ and DNJ resulted in increased GAA activity in patients 1-4. Mean, standard deviations and statistical significance, derived from 4-10 independent experiments for each patient are indicated.

No increase was found in fibroblasts from patients 5-8.

NCI = non classic infantile. J = juvenile. CI = classic infantile.

GAA activity in control fibroblasts was 58.5 +/- 28.1 nmoles 4-methylumbelliferone liberated/mg protein/hour.

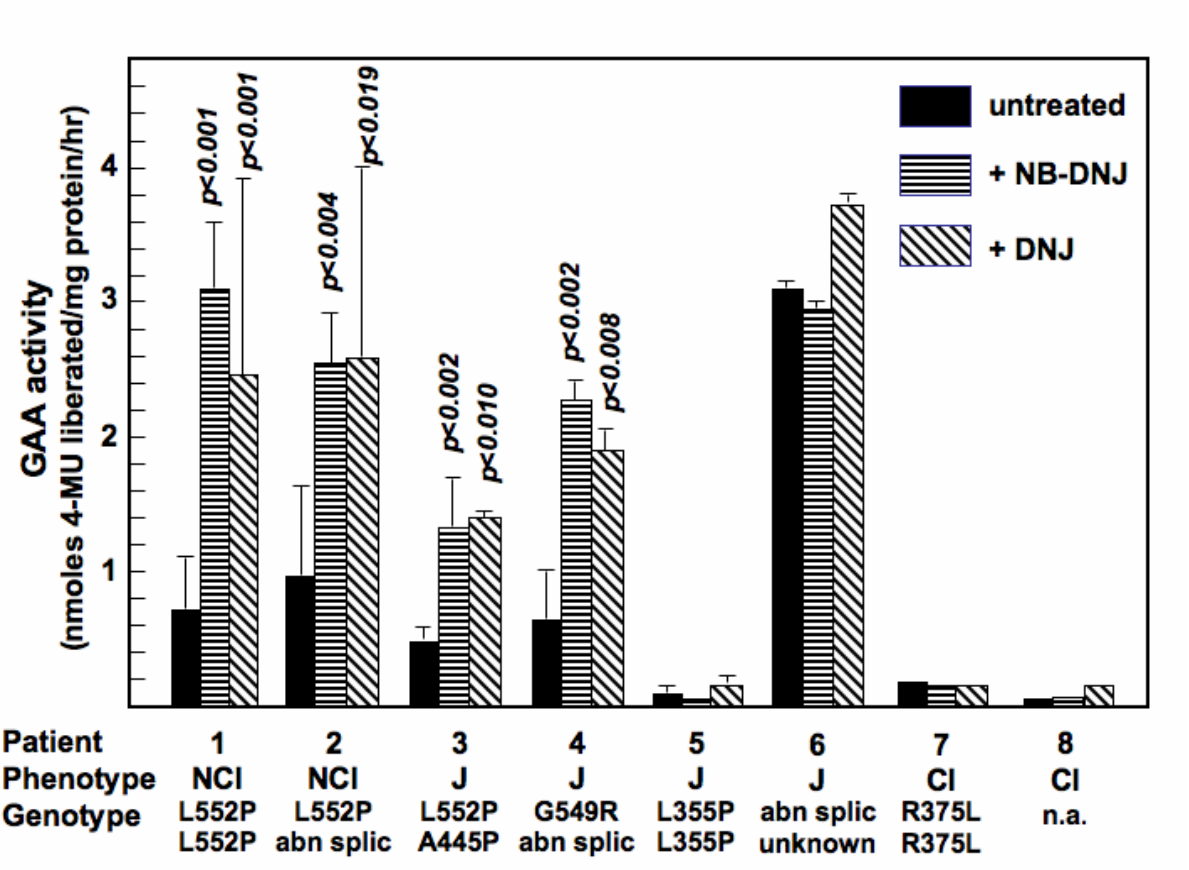

Figure 2

Effects of different imino sugar concentrations (A) and time course of GAA enhancement (B) in fibroblasts from patient 1.

Enhancement of GAA activity was observed at NB-DNJ concentrations between 10 and 80 μM. The enhancing effect of 20 μM NB-DNJ was already observed after 3 days of incubation and continued up to15 days.

Each point is mean of 2 different experiments.

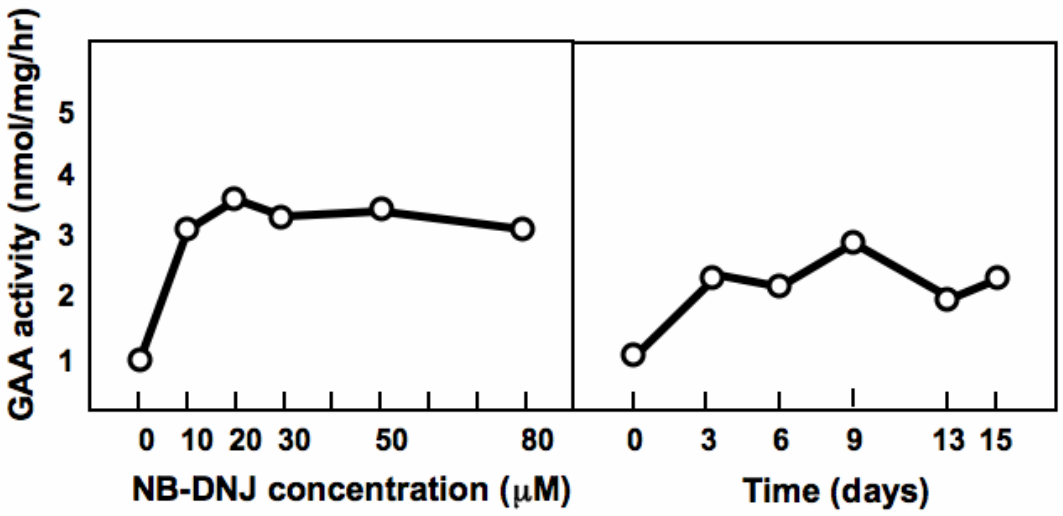

Figure 3

Effects of NB-DNJ in HEK293T cells expressing wild-type and mutated GAA.

Increase of GAA activity after NB-DNJ treatment was observed in HEK293T cells expressing the mutations L552P and G549R. No increase was observed in the cells expressing the mutations A445P and R375L. The specific activities indicated at the top of each bar are the mean of three independent experiments. The activities are expressed as nmoles 4-MU liberated/mg protein/hour.

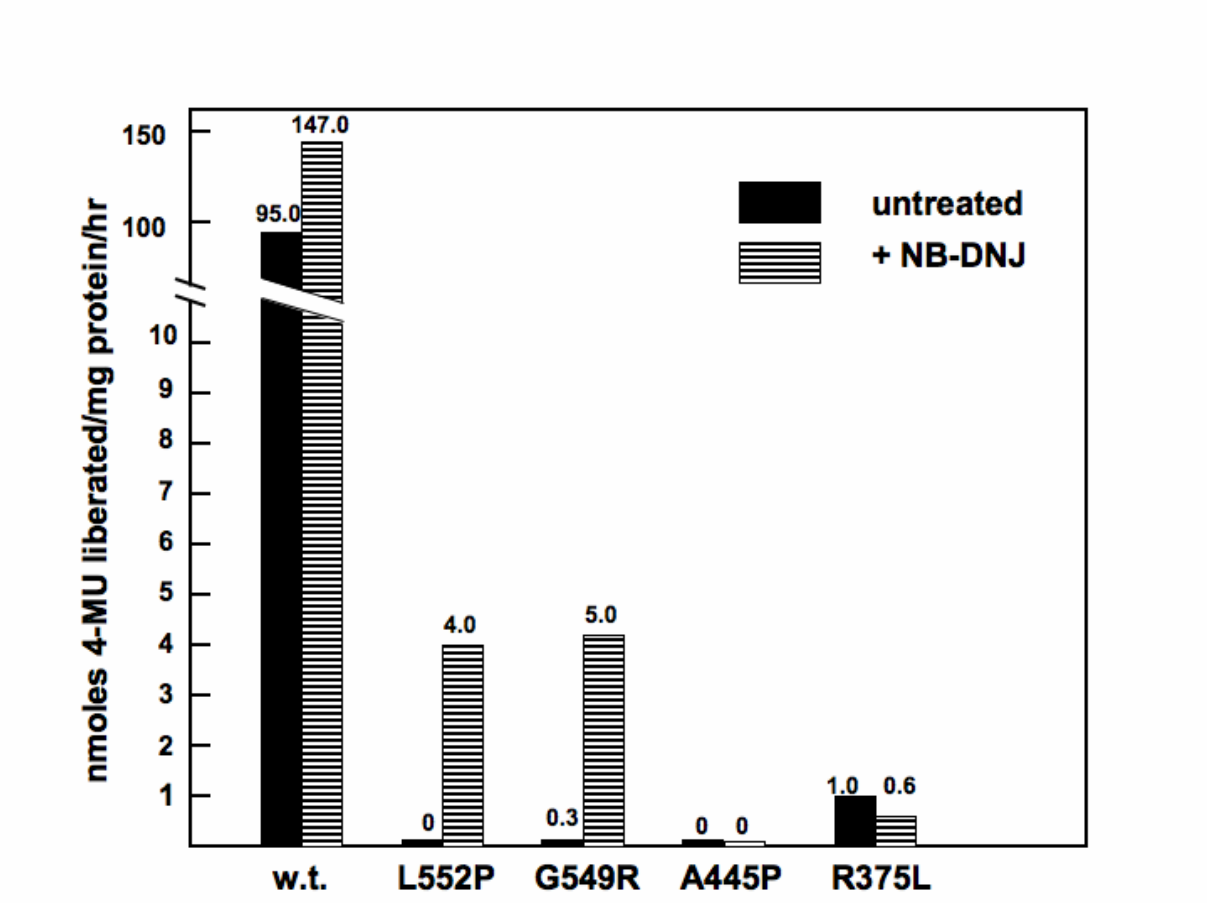

Figure 4

Western blot analysis of fibroblast.

The figure shows the results obtained in fibroblasts from patients 1, 4 (imino sugar responsive), 7 (non responsive) and from an untreated control cell line. The cells were cultured for 9 days with (+) and without (-) 20 μM NB-DNJ. The arrows indicate the bands corresponding to a precursor peptide of approximately 110 kDa and the mature active molecular forms (76-70 kDa).

In all cell lines an increase of the mature 70-76 kDa GAA molecular forms was observed. The results of Western blot analysis were consistent and reproducible in four independent experiments.

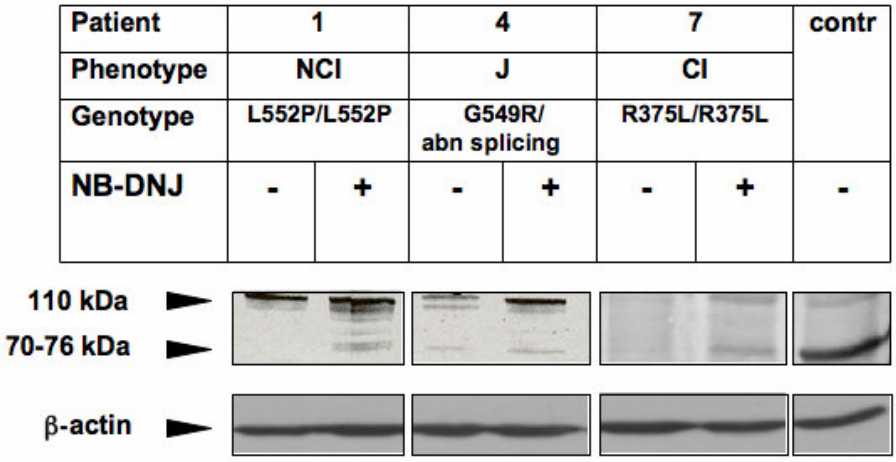

Figure 5

Western blot analysis of HEK293T cell extracts expressing single mutations of the *GAA* gene.

After transfection the cells were cultured for 72 hours with (+) and without (-) 20 μM NB-DNJ. The arrows indicate the bands corresponding to a precursor peptide of approximately 110 kDa, the intermediate 95 kDa and the mature active molecular forms (76-70 kDa). In treated cells an increase of the mature 70-76 kDa GAA molecular forms was observed.

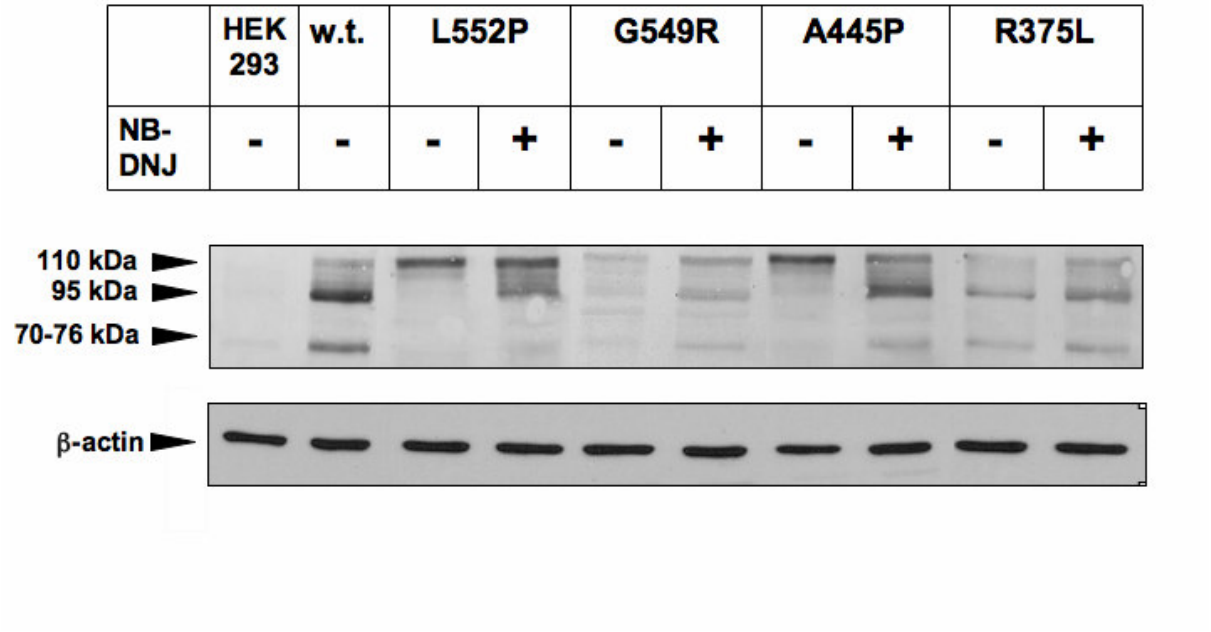

Figure 6

Confocal analysis of immunolocalization of GAA in HEK293T expressing L552P mutated GAA in the absence and in the presence of NB-DNJ and in cells expressing wild type GAA.

In the cells expressing the L552P mutation in the absence of imino sugar treatment negligible immunofluorescent labelling for GAA was observed (a). NB-DNJ treatment of the cells resulted in an enhancement of immunofluorescent labelling for GAA with a punctate staining typical of lysosomal localization (b). This pattern is comparable to that observed in the same cells expressing the wild type enzyme (c).

The same cells were stained for an endogenous lysosomal marker (LAMP2) (d, e, f). In cells overexpressing the L552P mutated GAA in the presence of NB-DNJ, and in cells overexpressing the wild type enzyme, the fluorescent labelling for GAA partly co-localized (h, i), thus confirming an improved delivery of GAA to lysosomes.

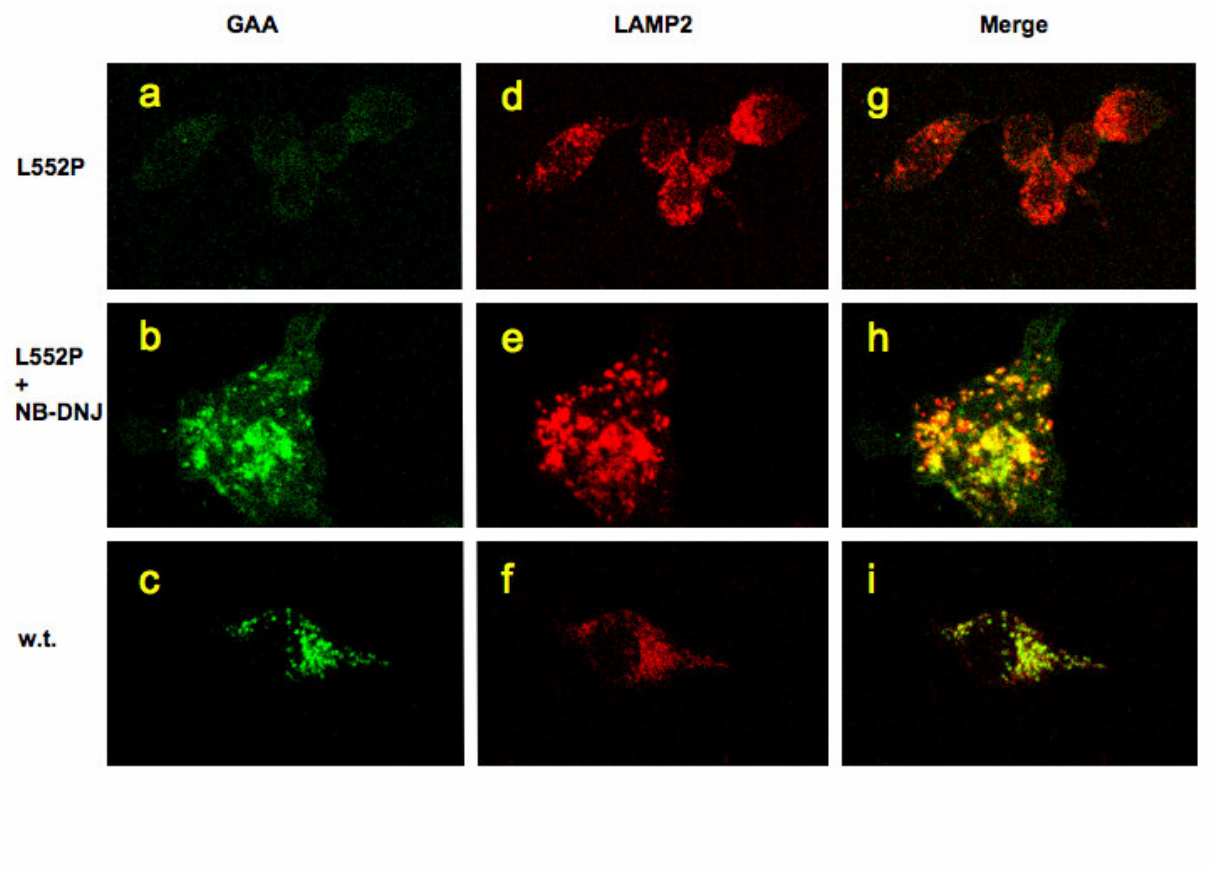

Table 1.

Phenotype and genotype of PD patients

Patient (initials)		**Genotype**	
		cDNA mutation	**Protein mutation**
1 (RP)	Infantile onset non-classic	1655T>C	L552P
		1655T>C	L552P
2 (GM)	Infantile onset non-classic	1655T>C	L552P
		c.-35C>A	abnormal splicing
3 (PP)	Juvenile	1655T>C	L552P
		1333G>C	A445P
4 (BAP)	Childhood-onset	1645G>C	G549R
		c.692+1G>C	abnormal splicing
5 (VPO)	Juvenile	1064T>C	L355P
		1064T>C	L355P
6 (DPF)	Juvenile	c.-45T>G	abnormal splicing
		unknown	
7 (DCME)	Classic infantile	1124G>T	R375L
		1124G>T	R375L
8 (RGG)	Classic infantile	n.a.	n.a.

APPENDIX 3.5.3

Montalvo AL, Bembi B, Donnarumma M, Filocamo M, Parenti G, Rossi M, Merlini L, Buratti E, De Filippi P, Dardis A, Stroppiano M, Ciana G, Pittis MG.

Mutation profile of the GAA gene in 40 Italian patients with late onset glycogen storage disease type II.

Human Mutation

2006; 27(10):999-1006.

HUMAN MUTATION 27(10), 999–1006, 2006

RESEARCH ARTICLE

Mutation Profile of the *GAA* Gene in 40 Italian Patients With Late Onset Glycogen Storage Disease Type II

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Communicated by Elizabeth Neufeld

Glycogen storage disease type II (GSDII) is a recessively inherited disorder due to the deficiency of acid α-glucosidase (GAA) that results in impaired glycogen degradation and its accumulation in the lysosomes. We report here the complete molecular analysis of the GAA gene performed on 40 Italian patients with late onset GSDII. Twelve novel alleles have been identified: missense mutations were functionally characterized by enzyme activity and protein processing in a human GAA-deficient cell line while splicing mutations were studied by RT-PCR and in silico analysis. A complex allele was also identified carrying three different alterations in *cis*. The c.-32-13T>G was the most frequent mutation, present as compound heterozygote in 85% of the patients (allele frequency 42.3%), as described in other late onset GSDII Caucasian populations. Interestingly, the c.-32-13T>G was associated with the c.2237G>A (p.W746X) in nine of the 40 patients. Genotype–phenotype correlations are discussed with particular emphasis on the subgroup carrying the c.-32-13T>G/c.2237G>A genotype. Hum Mutat 27(10), 999–1006, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: glycogen storage disease type II; Pompe disease; late onset; GAA; acid α-glucosidase; acid maltase

INTRODUCTION

Glycogen storage disease type II (GSDII, Pompe disease, acid maltase deficiency; MIM# 232300) is an autosomal recessive inherited disorder due to the deficiency of acid α-glucosidase (GAA; E.C.3.2.1.20) that results in impaired glycogen degradation that accumulates within the lysosomes. The GAA gene (MIM# 606800) has been localized to human chromosome 17q25.2–q25.3; the enzyme is synthesized as an inactive precursor of 110 kD which is transported to the pre-lysosomal and lysosomal compartment via the mannose-6-phosphate receptor where it is processed into the 95-kD intermediate and the fully active 70-kD and 76-kD forms [Hoefsloot et al., 1990a,b; Martiniuk et al., 1991; Hirschhorn and Reuser, 2001; Moreland et al., 2005].

Clinically, GSDII encompasses a continuous spectrum of phenotypes, from a rapidly progressive infantile form leading to death within the first year of life, to a slowly progressive late onset form of the disease that affects mobility and respiratory function. Classic infantile GSDII manifests soon after birth and is characterized by absent or nearly absent enzyme activity, severe muscle weakness, cardiomegaly/cardiomyopathy, and respiratory insufficiency, which typically leads to death within the first year of life [Hirschhorn and Reuser, 2001; Raben et al., 2002; van den Hout et al., 2003; Kishnani and Howell, 2004]. Some infantile patients have less severe cardiac involvement without left cardiac output obstruction, survive longer, and die because of pulmonary infections with secondary ventilatory insufficiency [Slonim et al., 2000; Winkel et al., 2005].

Late onset GSDII comprises all milder subtypes: partial enzyme deficiency manifests in children and adults as slowly progressive skeletal muscle weakness without cardiac involvement. Respiratory muscle weakness, particularly of the diaphragm, is the leading cause of death in the late onset cases [Hirschhorn and Reuser, 2001; Raben et al., 2002; Kishnani and Howell, 2004; Hagemans et al., 2004, 2005a,b].

More than 200 mutations in the GAA gene have been described up to date (www2.eur.nl/fgg/ch1/pompe) and reviewed by Raben et al. [2002], Huie et al. [2002], Lam et al. [2003], Pipo et al. [2003], Pittis et al. [2003], Hermans et al. [2004], Montalvo et al. [2004], and Anneser et al. [2005]. The leaky c.-32-13T>G (traditionally IVS1-13T>G) is the most frequent mutation among the Caucasian late onset GSDII patients [Hirschhorn and Reuser,

Received 25 November 2005; accepted revised manuscript 25 April 2006.

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Grant sponsor: Fondazione CRTrieste; Grant sponsor: IRCCS Burlo Garofolo; Grant number: RC34/02; Grant sponsor: Italian Telethon; Grant number: GTF04002.

DOI 10.1002/humu.20374

Published online 17 August 2006 in Wiley InterScience (www.interscience.wiley.com).

2001]. The c.-32-13T>G mutation gives rise to alternatively spliced transcripts with deletion of the first coding exon but still produces a low amount of normally processed mRNA. In this work, we report the molecular analysis of 40 Italian patients affected by late onset GSDII. Twelve novel GAA alleles have been identified: missense mutations were functionally characterized by enzyme activity and protein processing in a human GAA-deficient cell line, while splicing mutations were studied by RT-PCR and in silico analysis.

MATERIALS AND METHODS

Patients

We studied 40 patients with late onset GSDII coming from different parts of Italy (16 females and 24 males), one of whom is of Romanian origin (Patient 39). Among this group, 38 are unrelated while Patients 18 and 19 are siblings. The diagnosis was based on clinical data and confirmed by reduced GAA activity in lymphocytes or muscle. The mean age at diagnosis was 23.2 ± 18, ranging from 2 to 68 years. Almost all the patients underwent a program of physiotherapy, high protein diet, and respiratory management in their reference centers. Most of the patients have had mild muscular symptoms since childhood. First complaints were mostly related to mobility problems, weakness, and fatigue. Patients 3, 6, and 35 were diagnosed at a presymptomatic stage due to elevated levels of creatine kinase (CK) and transaminases.

GAA Mutation Analysis

Genomic DNA was extracted from peripheral blood leukocytes of the affected individuals with QIAamp DNA blood Mini Kit (Qiagen GmbH, Hilden, Germany). All 19 coding GAA exons and their flanking regions were PCR amplified as described elsewhere [Ko et al., 1999]. Cycle sequencing was performed in the forward and reverse direction with the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) following the manufacturer's instructions and sequences were analyzed on the ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Mutations were confirmed by sequencing duplicate PCR products and confirmed by the analysis of DNA sequence in the parents and other family members whenever possible.

RT-PCR Analysis

Total RNA was isolated from cultured fibroblasts using Trizol Reagent (Gibco, Paisley, UK) according to the manufacturer's instructions. For RT-PCR analysis the first strand cDNA was synthesized using random hexamer primers, and subsequent amplification was done in six overlapping fragments as described by Hermans et al. [1997].

Site-Directed Mutagenesis

Site-directed mutagenesis was carried out using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) according to the manufacturer's instructions. Each clone was entirely sequenced to confirm that no other mutations were introduced by the PCR-based mutagenesis procedure.

Cell Culture and Transient Transfection

Patient fibroblasts obtained from skin biopsies were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 mg/mL penicillin/streptomycin (Gibco).

TABLE 1. Mutation Profile of the GAA Gene in the Italian Late Onset GSDII Population

Location	cDNA mutation[a]	Mutation effect	References	Allele frequency
Intron 1	c.-32-13T>G	Leaky splice	Huie et al. [1994b]	42.3%
Exon 2	c.258dupC	p.N87QfsX9	Beesley et al. [1998]	1.3%
Exon 2	c.525delT	p.E176RfsX45	Hermans et al. [1994]	3.8%
Intron 3	c.692+1G>C	r.0	Present study	2.6%
Exon 5	c.925G>A	p.G309R	Kroos et al. [1998]	1.3%
Exon 6	c.1064 T>C	p.L355P	Montalvo et al. [2004]	2.6%
Intron 6	**c.1076-1G>C**	**r.1076-79_1195+89ins**	Present study	1.3%
Exon 7	c.1082C>T	p.P361L	Lam et al. [2003]	1.3%
Intron 7	c.1194+2T>A	r.spl?	Present study	1.3%
Exon 9	c.1333G>C	p.A445P	Present study	1.3%
Exon 10	c.1465G>A	p.D489N	Present study	2.6%
Intron 10	c.1551+1G>C	p.V480_I517del	Huie et al. [1994b]	1.3%
Exon 11	c.1626C>G	r.spl?	Present study	2.6%
Exon 12	c.1645G>C	p.G549R	Present study	1.3%
Exon 12	c.1655T>C	p.L552P	Bodamer et al. [2002]	2.6%
Exon 13	c. 1776del G	p.T593HfsX5	Present study	1.3%
Exon 13	**[c.1833_1839del; c.1846G>T; 1847_1848insT]**	**p.H612_D616delinsRGI**	Present study	1.3%
Exon 13	c.1836C>G	p.H612Q	Present study	1.3%
Exon 14	c.1927G>A	p.G643R	Hermans et al. [1993]	1.3%
Exon 14	c.2014C>T	p.R672W	Huie et al. [1998]	2.6%
Exon 15	c.2104C>T	p.R702C	Montalvo et al. [2004]	1.3%
Exon 16	**c.2219_2220delTG**	**p.V740GfsX55**	Present study	1.3%
Exon 16	c.2237G>A	p.W746X	Beesley et al. [1998]	10.3%
Exon 16	c.2242dupG	p.E748GfsX48	Beesley et al. [1998]; Huie et al. [1998]	1.3%
Intron 17/18	c.2481+102-2646+31del	p.G828_N882del	Huie et al. [1994a]	2.6%
Exon 18/ intron 18	**c.2646_2646+1delTG**	r.spl?	Present study	1.3%

[a]New mutations are indicated in bold; cDNA reference sequence Y00839.1. For cDNA numbering +1 corresponds to the A of the first ATG translation initiation codon.

TABLE 2. Genotype Encountered in the 40 Italian Patients Studied

Patient # (sex)	Age at diagnosis (years)	Genotype[a]	
		Allele 1	Allele 2
1 (male)[b]	2	c.-32-13T>G (leaky splice)	Unknown (r.0)
2 (female)[b]	14	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
3 (female)[b]	2	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
4 (female)	30	c.-32-13T>G (leaky splice)	**c.1836C>G (p.H612Q)**
5 (male)[b]	26	**c.1465G>A (p.D489N)**	c.2014C>T (p.R672W)
6 (female)[b]	4	**c.1645G>C (p.G549R)**	**c.692+1G>C (r.0)**
7 (female)[b]	17	c.-32-13T>G (leaky splice)	**[c.1833_1839del; c.1846G>T; 1847_1848insT] (p.H612_D616delinsRGI)**
8 (female)	30	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
9 (male)[b]	38	c.-32-13T>G (leaky splice)	**c.1465G>A (p.D489N)**
10 (female)	26	c.-32-13T>G (leaky splice)	**c.692+1G>C (r.0)**
11 (male)	27	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
12 (male)[c]	25	**c.1626C>G (r.spl?)**	**c.1626C>G (r.spl?)**
13 (female)[b]	52	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
14 (female)	52	c.-32-13T>G (leaky splice)	Unknown
15 (female)	43	c.-32-13T>G (leaky splice)	c.925G>A (p.G309R)
16 (male)[b]	13	c.-32-13T>G (leaky splice)	c.2014C>T (p.R672W)
17 (male)	30	c.-32-13T>G (leaky splice)	**c.1465G>A (p.D489N)**
18 (male)[b]	46	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
19 (male)[b]	38	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
20 (male)	6	c.-32-13T>G (leaky splice)	**c.2219_2220delTG (p.V740GfsX55)**
21 (female)	29	c.-32-13T>G (leaky splice)	c.1655T>C (p.L552P)
22 (male)	30	c.-32-13T>G (leaky splice)	c.2481+102_2646+31del (p.G828_N882del)
23 (female)[b]	15	c.-32-13T>G (leaky splice)	c.525delT (p.E176RfsX45)
24 (female)[b]	20	c.-32-13T>G (leaky splice)	c.525delT (p.E176RfsX45)
25 (male)	40	c.-32-13T>G (leaky splice)	**c. 1776del G (p.T593HfsX5)**
26 (male)[b]	3	c.-32-13T>G (leaky splice)	c.2481+102_2646+31del (p.G828_N882del)
27 (male)	23	c.-32-13T>G (leaky splice)	c.2242dupG (p.E748GfsX48)
28 (male)[b]	68	c.-32-13T>G (leaky splice)	**c.1076-1G>C (r.1076-79_1195+89ins)**
29 (male)[b]	2	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
30 (male)[b]	4	c.-32-13T>G (leaky splice)	**c.1194+2T>A (r.spl?)**
31 (male)[b]	49	c.-32-13T>G (leaky splice)	c.258dupC (p.N87QfsX9)
32 (female)	3	c.1551+1G>C (p.V480_I517del)	c.1082C>T (p.P361L)
33 (female)	10	c.-32-13T>G (leaky splice)	c.1927G>A (p.G643R)
34 (female)	10	c.1655T>C (p.L552P)	**c.1333G>C (p.A445P)**
35 (male)[b]	10	c.-32-13T>G (leaky splice)	c.2237G>A (p.W746X)
36 (male)[b]	45	c.-32-13T>G (leaky splice)	**c.2646_2646+1delTG (r.spl?)**
37 (male)[b]	28	c.-32-13T>G (leaky splice)	c.525delT (p.E176RfsX45)
38 (male)[b]	4	c.-32-13T>G (leaky splice)	Unknown
39 (male)	6	c.-32-13T>G (leaky splice)	c.2104C>T (p.R702C)
40 (male)[b]	2	c.1064 T>C (p.L355P)	c.1064 T>C (p.L355P)

[a]New mutations are indicated in bold; cDNA reference sequence Y00839.1. For cDNA numbering +1 corresponds to the A of the first ATG translation initiation codon.
[b]Genotype was confirmed by the DNA analysis in parents and/or other relatives.
[c]Consanguineous parents.

For in vitro expression assays we used the Ad5-SV40 immortalized human GAA-deficient fibroblast cell line (obtained from Dr. F. Martiniuk). GAA-deficient fibroblasts were grown on monolayers in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 mg/mL penicillin/streptomycin (Gibco). GAA-deficient cells were transfected with wild-type and mutant constructs with a standard calcium/phosphate using 4 μg of total plasmid DNA Endofree purified (Sigma, St. Louis, MO) following the manufacturer's instructions. Cells were harvested after 48 hr and assayed for GAA activity and Western blot.

Enzyme Activity Assay

Alpha-glucosidase activity was measured using the fluorogenic substrate 4-methylumbelliferyl-D glucopyranoside (Sigma) [Hermans et al., 1991]. Protein concentration of the samples was determined by the Lowry method. Enzymatic activity was expressed as nanomoles of substrate hydrolyzed per milligram of total protein per hour. All assays were done in triplicate from at least three separate transfections.

Western Immunoblot Analysis

Cell lysates (15 μg of protein/lane) were resolved on 10% SDS-PAGE gels, transferred onto a nitrocellulose membrane (Biorad, Hercules, CA). Blotted membranes were probed with an antiserum against α-glucosidase as described elsewhere [Montalvo et al., 2004]. An anti-rabbit HRP conjugated antibody (DAKO, Glostrup, Denmark) was used as a second antibody and developing was performed by enhanced chemiluminescence (ECL Amersham Biosciences, Buckinghamshire, UK). The antibody recognized all the forms of α-glucosidase: the 110-kD precursor, a 95-kD processing intermediate, and the 76-kD and 70-kD mature forms.

Mutation Nomenclature

All mutations are described according to mutation nomenclature, considering nucleotide +1 the A of the first ATG translation

initiation codon [den Dunnen and Antonarakis, 2000; den Dunnen and Paalman, 2003] (www.hgvs.org/mutnomen). Nucleotide numbers are derived from cDNA GAA sequence (RefSeq cDNA Y00839.1).

RESULTS AND DISCUSSION

Mutation Analysis and Functional Characterization of Novel Alleles

In this work, we analyzed the complete mutation profile of the GAA gene in 40 patients presenting the late onset form of the disease. As shown in Table 1, we identified 26 different alleles: 12 of them are novel including a complex allele that carried three different alterations in cis. Table 2 reports the genotype encountered in the 40 late onset GSDII patients. The GAA profile was characterized by mutations spread all over the sequence, including point mutations, both small and large deletions, small insertions, and splicing aberrations. The frequent c.-32-13T>G mutation, correlated to late onset GSDII, was present in heterozygosity in 34 out of 40 of the patients studied. Three alleles remained unknown (Cases 1, 14, and 38). However, based on genomic DNA sequencing, Patient 1 was compound heterozygote but cDNA sequencing demonstrated that only the allele carrying the c.-32-13T>G mutation, inherited from his father, was expressed. Then we assumed that the unknown allele may harbor an unidentified mutation in the non-coding regions of the GAA gene that prevent the formation of a stable mRNA.

Four novel single-base substitutions were identified: c.1333G>C, c.1465G>A, c.1645G>C, and c.1836C>G (p.A445P, p.D489N, p.G549R, and p.H612Q). Interestingly, a G>A substitution at nucleotide position 1645 has been previously reported in a Dutch juvenile onset GSD II patient; this substitution also resulted in a p.G549R mutation [Hermans et al., 2004]. To characterize the deleterious effect of the c.1333G>C, c.1465G>A, c.1645G>C, and c.1836C>G mutations, the wild-type GAA cDNA and mutant constructs were transiently transfected in the Ad5-SV40 immortalized human GAA-deficient fibroblast cell line. The mean wild-type GAA activity was 221 ± 18 nmol/mg/hr; the lack of endogenous activity in the GAA-deficient fibroblasts was confirmed in mock-transfected cells with pCDNA3-ALDP (adrenoleukodystrophy protein), a peroxisomal transmembrane protein not correlated with GSDII.

As shown in Figure 1A, c.1465G>A and c.1836C>G mutants remained as the GAA inactive precursor of 110 kD, and consequently they did not express residual enzyme activity. The combination of these severe mutations with milder mutations may explain the late onset disease in Patients 4, 5, 9, and 17 [Huie et al., 1998; Hirschhorn and Reuser, 2001]. No immunoreactive protein, and consequently no activity, was detected in the c.1333G>C construct. However, a faint 110-kD band was detected when a higher amount of protein was loaded onto the gel, suggesting that the protein is highly unstable or it is poorly expressed. The c.1645G>C and c.1645G>A, included for comparative analysis, did not express enzyme activity. Previously, a partial loss of activity was reported for the c.1645G>A mutation expressed in COS-1 cells [Hermans et al., 2004]. Interestingly, c.1645G>A was correctly processed while c.1645G>C accumulates predominantly as the 110-kD and 76-kD forms. Western blot analysis of Patient 6 fibroblasts (c.1645G>C /r.0) demonstrated a low enzyme expression, but still 110-, 95-, and 76-kD forms can be recognized (Fig. 1B). Fibroblast residual activity was 2.3% of normal values.

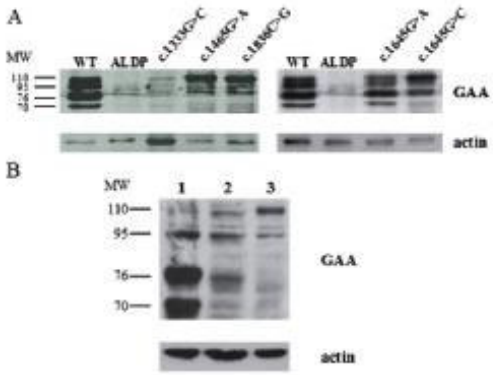

FIGURE 1. **Western blot analysis. A: GAA-deficient fibroblasts transiently transfected with wild-type pCDNA3-GAA and mutant constructs. All the forms of GAA can be detected in the wild-type transfected cells (WT), while no GAA is detected in the mock transfected cells (ALDP). Almost all the protein obtained from the c.1465G>A and c.1836C>G constructs remained as the GAA inactive precursor of 110 kD. The c.1645G>A mutant was correctly processed while c.1645G>C accumulates predominantly as the 110-kD and 76-kD forms. For the c.1333G>C construct, a faint band of 110 kD was detected when a higher amount of protein was loaded onto the gel, suggesting that the protein is highly unstable and it is rapidly degraded. B: Fibroblast lysates from a healthy subject (lane 1), Patient 6 carrying the c.1645G>C/r.0 genotype (lane 2), and an infantile patient homozygous for the c.1665T>C that results in accumulation of the inactive 110-kD precursor (lane 3).**

Patient 12 carried a novel single-base substitution in homozygosity, c.1626C>G, which did not disrupt the reading frame and codon usage and which may be considered a neutral polymorphism. However, none of the 100 control alleles analyzed carried this alteration, suggesting it could be a disease-causing mutation. No RNA was available from Patient 12. Therefore, to clarify the potential significance of this change, we used several splice-prediction programs based on the search for potential SR binding sequences in the case of ESEfinder [Cartegni et al., 2003], general enhancer sequences in the case of RESCUE-ESE [Fairbrother et al., 2004], or programs such as NNSPLICE [Reese et al., 1997], MaxEntscan [Yeo and Burge, 2004], and Spliceview [Rogozin and Milanesi, 1997] that evaluate splice site signal strengths. As shown in Table 3, all splice site consensus programs clearly indicated the creation of a novel donor site for the c.1626C>G. The preferential usage of this new splice site would cause the exclusion of 11 bp of exon 11.

The two small deletions detected (c.1776delG and c.2219_2220delTG) cause a shift in the reading frame introducing premature stop codons that lead to truncated proteins (p.T593HfsX5 and p.V740GfsX55).

As shown in Table 2, a complex allele was identified in Patient 7 due to the concomitant presence in cis of three novel mutations: (c.1833_1839del; c.1846G>T; and c.1847_1848insT) (Fig. 2A). As a result, residues from 612 to 616 (HWTGD) are replaced by RGI—p.H612_D616delinsRGI, indicating that this allele should be considered a severe mutation. The sequence analysis revealed the presence of a 3-nt repeat, TGG, which is included in the 7-bp deletion, and a second repeat at the deletion junction (Fig. 2B). This second repeat shows up in the patient sequence due to the presence of the c.1830C>T polymorphism on the same allele.

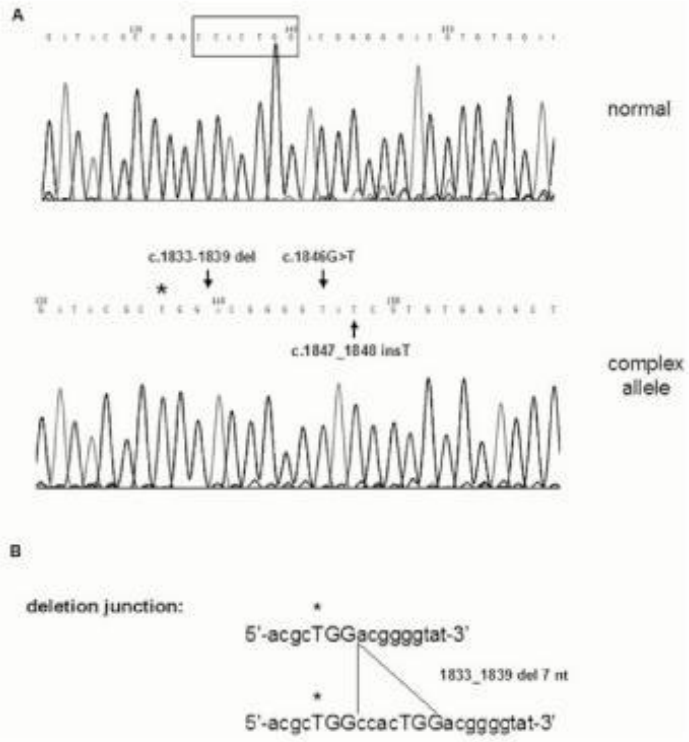

FIGURE 2. **Detection of a novel complex allele due to the concomitant presence in *cis* of three novel mutations (c.1833_1839del; c.1846G>T; and c.1847_1848insT). A:** Sequence analysis of the pGEM-cloned PCR products obtained from a normal donor and Patient 7. The c.1833_1839del is boxed in the normal sequence and the deletion junction is indicated by an arrow in the patient sequence. The asterisk indicates the presence of the c.1830C>T polymorphism on the same allele. This complex allele leads to the replacement of residues from 612 to 616 (HWTGD) by RGI: p.H612_D616delinsRGI. **B:** Deletion generation between 3 nt repeatsTGG in the patient DNA sequence. Note that the second 3-nt repeat turns up due to the presence of the c.1830 C>T polymorphism (*).

The two tandem repeats are characteristic elements that are frequently associated with deletion generation and have been previously reported in other deletions affecting the GAA gene [Pittis et al., 2003].

Finally, four novel mRNA splicing alterations were detected: c.692+1G>C, c.1194+2T>A, c.2646_2646+1delTG, and c.1076–1G>C. The first three mutations affected the consensus GT sequence in the donor splice site. The deleterious effect was confirmed for c.692+1G>C: RT-PCR analysis performed in cultured fibroblasts from Patient 6 showed the lack of the corresponding mutant transcript. Instead, the c.1076–1G>C mutation affected the acceptor splice site sequence. RT-PCR demonstrated the inclusion of intron 6 (79 bp) and intron 7 (89 bp) into the transcribed gene cDNA. Unfortunately, no RNA and/or cells were available to study aberrant RNA species in the other two cases.

Genotype–Phenotype Correlation

The mutation profile of the GAA gene in Italian late onset GSDII patients was quite heterogeneous, similar to what has

TABLE 3. **Predicted Effects of the c.1626C>G Mutation in GAA Exon 11**

Prediction program	c.1626C>G mutation (Patient 12)
ESEfinder	No effect
RESCUE-ESE	No effect
NNsplice	New 5'ss (0.77)*
	Wild-type 5'ss not recognized
MaxEntscan	
MAXENT	New 5'ss (8.98)*
MDD	Wild-type 5'ss (3.85)*
	New 5'ss (10.48)*
	Wild-type 5'ss (9.28)*
MM	New 5'ss (7.74)*
	Wild-type 5'ss (4.82)*
WMM	New 5'ss (4.52)*
	Wild-type 5'ss (5.29)*
Spliceview	New 5'ss (77)*
	Wild-type 5'ss (80)*

*Individual scores obtained from each prediction program are within parentheses.

TABLE 4. Clinical Data of Late Onset GSDII Patients Carrying the c.-32–13T>G/c.2237G>A Genotype

Patient # (sex)	Age (years)		First symptoms noted	% Residual enzyme activity	Follow-up period (years)	Mobility status		Respiratory follow-up
	Onset	Diagnosis				At diagnosis	Follow-up[a]	
2 (female)	13	14	Elevated CK, AST, ALT; lower limb weakness, fatigue	18 (M)	22	Unrestricted	With help	Reduction of pulmonary function
3 (female)	2	2	Elevated CK, AST, ALT	NA	4	Unrestricted	Unrestricted	Normal
8 (female)	30	31	Elevated CK, fatigue; generalized weakness	4.3 (L)	25	Unrestricted	With walker or wheelchair	Respiratory distress
11 (male)	18	27	Elevated CK, AST, ALT; generalized weakness	10 (M)	10[b]	Unrestricted	With help	Respiratory support by noninvasive ventilation
13 (female)	30	52	Difficulty in walking up steps; fatigue	absent	8	Unrestricted	With wheelchair	Supplemental oxygen
18 (male)	22	46	Generalized weakness; fatigue	10 (L)	8	Unrestricted	With help	Reduction of pulmonary function
19 (male)	38	38	Generalized weakness; fatigue	8 (L)	7	Unrestricted	With difficulty	Reduction of pulmonary function
29 (male)	1	2	Elevated CK, AST, ALT; muscular weakness	3.7 (M)	10	Impaired ambulation	With wheelchair	Respiratory support by noninvasive ventilation
35 (male)	1	10	Elevated CK, AST, ALT	0.02 (M)	4	Unrestricted	Unrestricted	Normal

[a]Follow-up period indicates years after confirmed diagnosis.
[b]Patient died at the age of 40 years.
L, lymphocytes; M, muscle; NA, not available.

been previously described in the French late onset GSDII population [Laforet et al., 2000]. The c.-32-13T>G, which is the most frequent mutation among the Caucasian late onset GSDII population, was also the most common mutation in our group (allele frequency 42.3%) (reviewed by Raben et al. [2002]).

Interestingly, the c.-32-13T>G was associated with the c.2237G>A (p.W746X) in nine of the 40 patients studied.

Table 4 summarizes clinical data in this subgroup. Despite the common genotype, patients presented with a wide variability in residual enzyme activity, age of appearance of clinical signs, and rate of disease progression. The first complaints started at a mean age of 17±14 years (range 1–38 years). Four patients waited 9 to 24 years for a final diagnosis, despite the presence of clinical and laboratory signs (elevated transaminases and CK). At diagnosis, muscular weakness was present in all the patients except two (Patients 3 and 35) in whom the disease had been suspected due to the fortuitous detection of high transaminases during a clinical control in the first year of life. They are also the only two patients who did not show movement and/or respiratory impairment during the follow-up.

Patient 34 carried the new c.1333G>C in association with c.1655 T>C. At the age of 3 years, she presented with frequent pulmonary infections and hypertransaminemia; GSDII diagnosis was confirmed at the age of 10 years. Muscle weakness progressed severely; at the age of 16 years, she was using a wheelchair, tracheostomy was performed, and since then she has received ventilatory assistance. The c.1655 T>C mutation was previously reported in a non-classic infantile GSDII male [Bodamer et al., 2002]. Muscle GAA activity was mildly reduced, while in vitro expression of the c.1655 T>C resulted in little residual enzyme activity. Moreover, the c.1655 T>C mutation was found in homozygosity in another late infantile GSDII patient (unpublished data) in whom significant residual GAA activity (3–8% of normal values) was detected in all the tissues tested (muscle, fibroblasts, and lymphocytes). Then, the in vivo residual c.1655 T>C activity is likely to explain the late onset GSDII in Patient 34 (lymphocyte GAA activity = 8.1% of normal values).

Patient 6 carried the c.1645G>C/c0 genotype, previously reported in juvenile onset GSDII [Hermans et al., 2004]. She was diagnosed at the age of 4 years after detection of high transaminases and CK levels and after 8 years she is still asymptomatic. In vivo enzyme activity was consistent with clinical phenotype despite functional in vitro analysis.

Patient 40, who presented with the most severe course of the disease among the group of patients studied, carried the c.1064T>C mutation in homozygosity. Interestingly, the same genotype was previously found in a 2-year-old infantile non-classic GSDII patient, with marked generalized hypotonia, respiratory insufficiency, and moderate left ventricular hypertrophy [Bembi et al., 2003; Montalvo et al., 2004]. Diagnosis in Patient 40 was also done at the age of 2 years due to muscular weakness and mild organomegaly. Subsequently, he presented with severe and progressive respiratory history including macroglossia, weakness of respiratory muscles, respiratory failure, chronic hypoxia, and hypercapnia leading to pulmonary hypertension. Tracheostomy was performed at the age of 5 years and since then he has received invasive ventilatory assistance <24 hr/day. Generalized hypotonia progressed and his growth was poor and presented with very severe progressive kyphoscoliosis. He suffered three pathologic fractures at the lower limbs, and bilateral-Achilles-tendon intervention for joints contractures was performed when he was 8 years old. Ambulation was progressively lost and at the age of 15 years he

was using a wheelchair. He received enzyme replacement therapy when he was 19 years old and died at the age of 20 years.

In this work we analyzed the mutation GAA profile in 40 patients with late onset GSDII, which represents the largest study of GSDII conducted in Italy to date. In vitro expression analysis resulted as a useful tool in discriminating disease-causing mutations and evaluating their effect on the normal enzyme function. It was less powerful in the genotype–phenotype discussion, as previously reported [Huie et al., 1998; Montalvo et al., 2004]. Our data confirmed the wide spectrum of clinical manifestations observed in GSDII and the phenotypic variability among patients, even those carrying the same genotype. Moreover, continued mutational analysis will contribute to the understanding of genotype–phenotype correlations and this may be useful in the evaluation of emerging ERT efficacy.

ACKNOWLEDGMENTS

We thank Sarah Tripepi for her assistance with the editing of the manuscript, S. Dominissini and R. Cariati for their technical contribution, and V. Guerci for clinical data collection and management of the patients. A.L.E. Montalvo is a research fellow of the Associazione Italiana Glicogenosi (AIG). Part of the samples were obtained from the "Cell Line and DNA Bank From Patients Affected by Genetic Disease" collection (www.gaslini.org/labdppm.htm).

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ACKNOWLEDGEMENTS

I would like to thank all the people who helped or supported me during the study whose results are here summarized.

In particular I would like to thank:

my supervisor Prof. Giancarlo Parenti for his tutorship and dear friendship. He significantly encouraged and supported my research, and shared with me his wide clinical expertise in metabolic medicine.

Prof. Generoso Andria for his always stimulating mentorship, for having taught me a method of work and study, and for having offered me the possibility of learning from his experience, particularly concerning morphogenesis defects with metabolic bases and innovative therapies for inherited disorders.

My professional training has significantly benefited also from:

the experienced and knowledgeable comments and suggestions of Prof. Gianfranco Sebastio

and from the opportunity to meet and work with the staff of the Clinical and Molecular Genetics Unit at Great Ormond Street Hospital and Institute of Child Health, London, UK, an experience which has given a crucial contribution to my background.

I would like to thank my family and, in particular, my father Aldo, my mother Donatella, my sister Corinna, my great uncle Memore, and my French relatives, for their important support and advice.
Finally I say a special thanks to my beloved wife Floriana, whom I have met and married in these four years, and to whom this study is dedicated.

BRIEF CURRICULUM VITAE

Massimiliano Rossi was born on May the 14th, 1972, in Naples, Italy. In 1990 he got the secondary education degree at the Liceo Umberto I of Naples. In 1997 he graduated in Medicine, at the Federico II University of Naples, with top mark cum laude. His final graduation thesis was entitled: "La sindrome di Smith-Lemli-Opitz (Smith-Lemli-Opitz syndrome)". Subsequently, he had a five years specialization training in Pediatrics, with a special training in Clinical Genetics and involvement in research projects. On October the 31st 2002 he passed the final exam of the Specialization in Pediatrics with top mark cum laude. The final thesis, entitled "I difetti della biosintesi del colesterolo (Cholesterol biosynthesis defects), has been awarded by the Italian Society of Pediatrics with the "Luigi Capotorti" prize as the best thesis on pediatric metabolic medicine. During the whole 2002, he worked as a Honorary Specialist Registrar and overseas visitor at the Clinical and Molecular Genetics Unit, Great Ormond Street Hospital for Children (GOSH) and Institute of Child Health, London. UK (Tutor: Prof. Robin Winter). In this period he also had a special training with Christine Hall, Professor of Radiology at GOSH with a special interest in skeletal dysplasias.

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