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RESEARCH REPORT

Favourable Outcome in Two Pregnancies in a Patient with 3-Hydroxy-3-Methylglutaryl-CoA Lyase Deficiency

David Santosa · Markus G. Donner · Stephan vom Dahl · Markus Fleisch · Thomas Hoehn · Ertan Mayatepek · Katrin Heldt · Tim Niehues · Dieter Häussinger

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Abstract In patients with 3-hydroxy-3-methylglutaryl (HMG)-CoA lyase deficiency (OMIM 246450), five pregnancies have been described worldwide, which were either terminated or resulted in severe metabolic sequelae during pregnancy or delivery. Here, we report on a patient with HMG-CoA lyase deficiency, who underwent two uncomplicated pregnancies. The 19-year-old patient was admitted because of recurrent vomiting and nausea. Diagnostics revealed pregnancy at week 8 of gestation. Metabolic analyses revealed normal lactate and blood glucose levels and normal acid-base status. Urine organic acid analysis showed an elevated excretion of 3-CH_3-glutaric acid, 2,3-CH_3-glutaconic acid, and 3-CH_3-3-OH-glutaric acid. Oral treatment with carnitine and glucose wes administered intravenously during the period of nausea and vomiting. After clinical recovery, a diet with 0.89 g/kg of protein/d and 38 kcal/kg body weight/d was given. Meals were taken every 3 h. Additionally, 70 g of starch was given at midnight to maintain normoglycemia at night time. Peripartum, a complete parenteral nutrition, was delivered through a central venous catheter. The patient delivered a healthy male infant by Caesarean section at week 38 of gestation (Apgar 9/10/10) and remained metabolically stable throughout the peripartum period. Postpartum nutrition was gradually changed from parenteral to oral diet. Two years later, the patient became pregnant again and presented with hyperemesis gravidarum. With metabolic monitoring and treatment as before no decompensation occurred. At week 38 of gestation, she delivered a healthy female infant by elective Caesarian section (Apgar 9/10/10). This case report describes the metabolic and obstetric management of two pregnancies in a patient with HMG-CoA lyase deficiency with favorable outcome without metabolic complications.

Communicated by: John H Walter, MD FRCPCH

"David Santosa" and "Markus G. Donner" contributed equally to this work.

D. Santosa (✉) · M.G. Donner · S. vom Dahl · D. Häussinger
Department of Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine University, Moorenstrasse 5, 40225 Düsseldorf, Germany
e-mail: david.santosa@med.uni-duesseldorf.de

M.G. Donner
Gastroenterologie Brugg, Brugg, Switzerland

M. Fleisch
Department of Gynecology, Heinrich-Heine University, Düsseldorf, Germany

T. Hoehn · E. Mayatepek
Department of General Pediatrics, Neonatology and Pediatric Cardiology, University Children's Hospital, Heinrich-Heine University, Düsseldorf, Germany

K. Heldt · T. Niehues
Childrens Hospital, HELIOS Klinikum Krefeld, Krefeld, Germany

Introduction

3-Hydroxy-3-methylglutaryl(HMG)-CoA lyase deficiency (OMIM 246450) is an autosomal recessive error of metabolism characterized by an impaired leucine catabolism and ketogenesis (Fig. 1). So far, 43 mutations in 124 patients have been reported in the HMGCL gene (Menao et al. 2009). Common clinical features of the disease at first presentation are hypoketotic hypoglycemia, acidosis, vomiting, and a reduced level of consciousness. Moreover, epilepsy and severe coma can develop. On MRI, white matter changes in the corticospinal tract, and the pons have been described (Yýlmaz et al. 2006). White matter changes

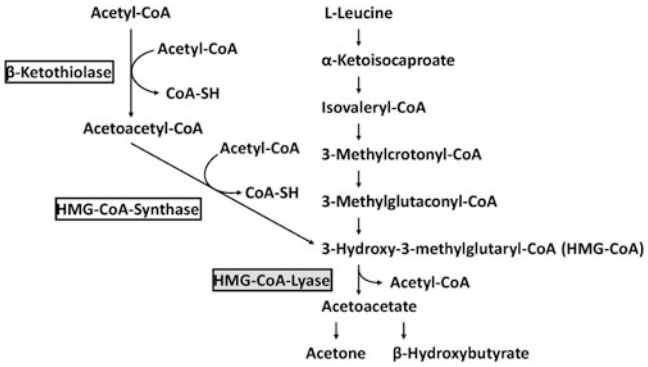

Fig. 1 Ketone body formation and leucine degradation are impaired in 3-hydroxy-3-methylglutaryl(HMG)-CoA lyase deficiency. Ketone bodies are synthesized in liver mitochondria. The enzyme β-ketothiolase catalyzes the formation of two molecules acetyl-CoA to acetoacetyl-CoA. Mitochondrial HMG-CoA synthase catalyzes the condensation of acetyl-CoA to the carbonyl group of acetoacetyl-CoA to form HMG-CoA. HMG-CoA lyase then cleaves HMG-CoA to form acetyl-CoA and acetoacetate. Acetoacetate is reduced to β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase, which occurs also in extrahepatic tissues. Acetone is produced by nonenzymatic decarboxylation of acetoacetate. In extrahepatic tissues, HMG-CoA lyase is necessary for the degradation of leucine to ketones. Deficiency of HMG-CoA lyase leads to impairment of ketogenesis and leucine degradation

have been reported to be reversible on serial brain MRI in patients on leucine-restricted diet (Zafeiriou et al. 2007). With prompt diagnosis, prognosis of HMG-CoA lyase deficiency is good (Gibson et al. 1988). Today, an increasing number of patients reach adulthood.

To our knowledge, five pregnancies in three patients with HMG-CoA lyase deficiency have been described (Langendonk et al. 2012; Pipitone et al. 2016). One patient underwent the first pregnancy with recurrent metabolic decompensation, but delivered a healthy child. During the second pregnancy, the patient developed severe lactic acidosis and hypoglycemia and died in the following course due to sepsis and pulmonary edema. The second patient with underlying polycystic ovarian syndrome (PCOS) developed lactic acidosis with hyperammonemia during her first pregnancy. Subsequently, spontaneous abortion occurred. Three years later, she presented again at week 6 of gestation and, although well, decided to terminate pregnancy (Langendonk et al. 2012). The third patient had an uneventful pregnancy and delivered a healthy child, but developed several complications during labor and delivery including metabolic acidosis (Pipitone et al. 2016).

Pregnancy in these patients presents a challenge to the mother and the caring physician due to pregnancy-associated altered energy and nutrient expenditure. Here, we describe the uncomplicated clinical course and successful metabolic management in a patient with HMG-CoA lyase deficiency during two pregnancies.

Case Report

The diagnosis of HMG-CoA lyase deficiency in our patient was made at the age of 15 months by analysis of organic acids in the urine. Already 1 h after birth, the female newborn of consanguineous Turkish parents had to be treated because of hypoglycemia (1.5 mmol/l). Repeated decompensations with hypoglycemic episodes down to blood glucose level of 0.2 mmol/l at age of 15 months and unconsciousness in the course of infections (mainly gastroenteritis and respiratory infections) resulted in emergency treatments with intravenous glucose treatment and finally led to the diagnosis. During childhood and adolescence, treatment consisted of oral carnitine with a regular high-carbohydrate, low-protein, and low-fat diet (3-hourly meals). Infections with hypoglycemia resulted in inpatient treatment every 2–3 years. At the age of 19 years, the patient was treated at her local hospital for 4 days because of nausea and vomiting. Having reached adulthood, she was referred to our department.

On admission, we found the patient to be pregnant at week 8 of gestation. Nausea and vomiting was attributed to hyperemesis gravidarum. Organic acids in urine were elevated (3-methyl-glutaric acid, 3-methyl-glutaconic acid, and 3-methyl-3-hydroxy-glutaric acid). Glucose 10% i.v. was commenced, and vomiting was successfully controlled with intravenous dimenhydrinate. After improvement of clinical symptoms, diet with 3-hourly meals (containing 50 g carbohydrates per meal) was reinitiated, and intravenous glucose was discontinued. In addition to 3-hourly meals, maltodextrin was given orally in instances of blood glucose levels <4.4 mmol/l (initially up to 215 g maltodextrin/24 h divided in 8 doses, in the following course 30 g maltodextrin/24 h with one dose at night and one in the morning). Starch (70 g) was given at midnight due to repeated low glucose levels at night (down to 3.3 mmol/l; range, 3.3–5.9 mmol/l). Detailed nutritional instruction and dietary protocol (40 g protein, 1,711 kcal/d, 0.89 g/kg) were given, and the patient was discharged from hospital.

Gynecologic ultrasound revealed an intact pregnancy without any fetal dysplasia. Metabolic monitoring showed lactate and blood glucose levels within the reference range and no episodes of hypoglycemia during the whole pregnancy. C5-OH + HMB acylcarnitines (1.26 μmol/l, reference range <1 μmol/l) and C6DC acylcarnitines (0.18 μmol/l, reference range <0.15 μmol/l) were slightly elevated and remained stable during repeated measurements. At week 28 of gestation, deficiencies of zinc and vitamin D required oral supplementation. At week 33 of gestation, the patient complained of recurrent nausea and flatulence. Lactose intolerance was suspected and after reduced lactose intake symptoms improved. Further regular medication consisted of 100 mg iron/d, multivitamin

supplement, and carnitine 3 × 1 g per day. Daily protein intake was increased to 60.5 g per day (0.96 g/kg) with a total of 2,031 kcal/d at week 35 of gestation. The patient's weight gain during the whole pregnancy was 16 kg. Three days prior to the elective Caesarean section at week 36 + 5 days, the patient was admitted to hospital for metabolic monitoring. Due to vomiting 2 days prior to delivery, a total parenteral nutrition via a central venous line was started immediately (2,131 kcal/d, 50 g of amino acids/d, 400 g of glucose/d, and 48 g of 20% MCT lipid infusion/d). Acid-base status, lactate, electrolytes, and ammonia measured every hour prior to delivery were within normal limits. Delivery was performed by elective Caesarean section under spinal anesthesia at week 37 + 1 day of gestation. The healthy male newborn had a length of 49 cm, head circumference of 34 cm, and birth weight of 3,055 g, and Apgar scores were 9/10/10. Regular screening examination at postnatal day 3–10 and metabolic screening were uneventful.

Following a Caesarean section, nutrition was gradually changed from parenteral to enteral (50.1 g protein, total of 1,929 kcal/d, starting with 50% in the first 24 h). Regular metabolic review in hospital and after discharge revealed stable parameters. Afterward, the dose of carnitine (3× 1 g/d) and starch (70 g at midnight) remained unchanged, and 2-hourly meals were taken from 6:00 a.m. until 10 p.m.

Two years later, the patient presented at the outpatient clinic with vaginal spotting and nausea. The patient was pregnant at week 6 of gestation. Dietary assistance was immediately provided, and the patient prepared a 3-day dietary record. Six weeks later, the patient continued to have nausea and also complained of intermittent vomiting attributed to early pregnancy. The patient refused hospital admission and was subsequently seen in the metabolic outpatient clinic at 2–3-week intervals. Intake of 70 g of starch was recommended at midnight because of low blood glucose levels in the morning (3.3 mmol/l). The mother of the patient was appointed as a legal custodian. Intelligence quotient was 68, as measured by the nonverbal intelligence test SON-R 2.5–7. At 34 gestational weeks, an additional intake of 20 g protein as a caloric drink was started (total protein 70 g/d). Amino acids, free carnitine, lactate, acid-base status, and blood glucose were within normal limits. Obstetric ultrasound 2 weeks prior to delivery showed good placental function and normal fetal growth.

Elective Caesarean section was planned at week 38 of gestation. Inpatient treatment was started 2 days prior to the planned procedure with metabolic monitoring (blood glucose levels every 3 h, peripartum 8-hourly monitoring of electrolytes, acid-base status, ammonia, and blood count). Peripartum nutrition was started on day of Caesarean section and consisted of 2,113 kcal per day with an intravenous 50% glucose infusion (=1,200 kcal/d), a 20% MCT lipid infusion (=687 kcal/d), and vitamin supplementation (1 amp. Soluvit®, 1 amp. Vitalipid®). Furthermore, a 10% infusion of amino acids was given (=226 kcal/d, 54 g/d). A single dose of 2 g cefazolin was given perioperatively. An uneventful Caesarean section was performed at week 37 and 6 days under epidural anesthesia delivering a healthy female newborn (length 47 cm, head circumference 33 cm, weight 2,795 g, Apgar scores 9/10/10). Clinical examination of the child at postnatal day 3–10 and metabolic screening were within normal limits.

At the follow-up presentations, the patient was free of any clinical symptoms. The abovementioned metabolic parameters remained stable.

Molecular genetics following the second pregnancy, which was performed by MVZ Dr. Eberhard & Partner Dortmund, Laboratoriumsmedizin, Dortmund, revealed a homozygous mutation at position 876 of the HMG-CoA lyase gene with transversion of G → C (c.876 + G > C). This transversion at position +1 of the intron is downstream from the exon containing the catalytic site in the encoded protein and has been reported in a Turkish patient with HMG-CoA lyase deficiency (Buesa et al. 1996).

Discussion

An increasing number of young women affected by inborn errors of metabolism reach child-bearing age. Pregnancy-related changes in energy expenditure and altered nutritional requirements represent a challenge for monitoring and treatment of metabolic diseases in pregnant patients. Here, we present a case report of a favorable outcome of two pregnancies and offspring in a patient with HMG-CoA lyase deficiency.

Possible complications in pregnant patients with HMG-CoA lyase deficiency are metabolic decompensation with metabolic acidosis and hypoglycemia, potentially leading to death of the patient and the fetus. Indeed, four pregnancies in two patients reported previously followed a complicated course with recurrent metabolic decompensations, abortions, and death of one mother (Langendonk et al. 2012), while one patient developed metabolic decompensation during labor and delivery (Pipitone et al. 2016). One reason for decompensation may be the reportedly low compliance of one patient, which was also due to learning difficulties. One woman did not attend the outpatient clinic for metabolic review for several years. She also did not attend medical follow-up after undergoing her first successful pregnancy with several episodes of metabolic acidosis. During her second pregnancy, missing emergency ID and past medical records led to underestimation of the clinical situation, and subsequent coagulopathy resulted in spontaneous abortion. Further clinical course was complicated by

hyperammonemia, sepsis, and pulmonary and cerebral edema eventually leading to cardiac arrest. The second patient with HMG-CoA lyase deficiency and polycystic ovarian syndrome (PCOS) also experienced metabolic acidosis with hyperammonemia and respiratory distress during her first pregnancy. Subsequently, she had spontaneous abortion. The same patient, although well at the 6th gestational week, decided to have termination of her second pregnancy (Langendonk et al. 2012). The third reported patient (Pipitone et al. 2016) had an uneventful pregnancy, but developed complications during labor and delivery including metabolic acidosis as well as premature rupture of membranes, intrapartum bleeding requiring transfusion, tachycardia, and hypertension.

The patient reported here had a favorable outcome of two pregnancies without any metabolic decompensation. The favorable course in our patient may on the one hand be due to the clinical assumption that she had a less severe form of the underlying disease. Genetic analysis demonstrated a donor splice site mutation, c.876 + G > C. To date, there are no data on the clinical phenotype of this mutation. As stated by Menao et al. (2009), it is generally difficult to establish a genotype-phenotype correlation in patients with HMG-CoA lyase deficiency. Further, there is no specific clinical or laboratory parameter that can predict the severity of the disease, especially in pregnancy. A tight clinical observation and metabolic monitoring therefore is key to the successful metabolic management in these patients, especially in pregnancy.

Furthermore, our patient showed a good compliance, as she attended for follow-up appointments during and between the two pregnancies. Her mother provided excellent support supervising her disease monitoring and treatment, e.g., continuous blood glucose monitoring, strict diet, etc. To ensure most optimal care within the family, her mother was appointed as the legal custodian. Good compliance was achieved by repeated tuition regarding medical and dietary instructions given during repeated visits in the outpatient clinic. In contrast, lack of compliance, which was also due to learning difficulties, promoted metabolic decompensations and eventually led to fatal decompensation in the case described above (Langendonk et al. 2012).

Third, total parenteral peripartum nutrition and delivery by elective Caesarean section in our case kept the time of delivery short. This is thought to be a crucial factor for an uneventful delivery in HMG-CoA lyase deficiency. As known from various other inborn metabolic diseases, delivery presents a high risk period for complications due to a catabolic state.

Preconceptional counseling of patients with inborn metabolic diseases should be the central part of the transition from pediatric to adult outpatient clinic. It is suggested that female patients with inborn metabolic diseases, although metabolically stable, should be seen in metabolic outpatient clinic every year and repeatedly trained in pregnancy-related risks, the special metabolic needs in pregnancy, and the crucial importance of reliable contraception. Pregnancy should be planned with the caring obstetrician and the metabolic clinic.

In summary, successful metabolic and obstetric management of a patient with HMG-CoA lyase deficiency undergoing two pregnancies is reported. This report illustrates that a favorable maternal and neonatal outcome in patients with HMG-CoA lyase deficiency without complications is possible. The course of the presented patient elicits recommendations for metabolic management of patients with HMG-CoA lyase deficiency at risk for hypoglycemia and metabolic acidosis under the circumstances of altered metabolic needs during pregnancy.

Take-Home-Message

Uneventful pregnancy and a favorable neonatal outcome are possible in patients with HMG-CoA lyase deficiency under continuous metabolic review and treatment.

Conflict of Interest

David Santosa, Markus G. Donner, Stephan vom Dahl, Markus Fleisch, Thomas Hoehn, Ertan Mayatepek, Katrin Heldt, Tim Niehues, and Dieter Häussinger declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

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RESEARCH REPORT

Lysosomal Acid Lipase Deficiency in 23 Spanish Patients: High Frequency of the Novel c.966+2T>G Mutation in Wolman Disease

Carla Ruiz-Andrés · Elena Sellés · Angela Arias · Laura Gort · The Spanish LAL Deficiency Working Group

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Abstract Lysosomal acid lipase (LAL) is a lysosomal key enzyme involved in the intracellular hydrolysis of cholesteryl esters and triglycerides. Patients with very low residual LAL activity present with the infantile severe form Wolman disease (WD), while patients with some residual activity develop the less severe disorder known as Cholesteryl ester storage disorder (CESD). We present the clinical, biochemical, and molecular findings of 23 Spanish patients (22 families) with LAL deficiency. We identified eight different mutations, four of them not previously reported. The novel c.966+2T>G mutation accounted for 75% of the Wolman disease alleles, and the frequent CESD associated c.894G>A mutation accounted for 55% of the CESD alleles in our cohort. Haplotype analysis showed that both mutations co-segregated with a unique haplotype suggesting a common ancestor. Our study contributes to the LAL deficiency acknowledgement with novel mutations and with high frequencies of some unknown mutations for WD.

Communicated by: Carla E. Hollak, MD
This chapter does not contain any studies with animal subjects performed by any of the authors.

C. Ruiz-Andrés · E. Sellés · A. Arias · L. Gort
Errors Congènits del Metabolisme-IBC, Serv. Bioquímica i Genètica Molecular, CDB, Hospital Clínic, C/Mejía Lequerica, s/n, Edifici Helios III, Barcelona 08028, Spain

A. Arias · L. Gort (✉)
CIBERER, Centro de Investigación Biomédica en Red de Enfermedades Raras and IDIBAPS, Barcelona, Spain
e-mail: lgort@clinic.ub.es

Introduction

Lysosomal acid lipase (LAL; EC 3.1.1.13) is a lysosomal key enzyme involved in the intracellular hydrolysis of cholesteryl esters (CE) and triglycerides (TG) that have been internalized via receptor-mediated endocytosis of plasma lipoprotein particles. In this process, the released free cholesterol regulates its own endogenous synthesis, the uptake of LDL, and cholesterol esterification (Assman and Seedorf 2001). LAL is a 399-amino acid protein encoded by the *LIPA* gene (MIM *613497) located in chromosome 10 (10q23.2-q23.3) (Anderson et al. 1993; Aslanidis et al. 1994). Mutations in *LIPA* gene cause LAL deficiency, which is associated with a wide spectrum of clinical manifestations. Patients with very low residual LAL activity present with Wolman disease (WD, MIM 278000), while patients with some residual activity (i.e. 3–8% of controls in blood lymphocytes or fibroblasts) develop the less severe disorder known as cholesteryl ester storage disorder (CESD, MIM 278000) (Assman and Seedorf 2001). Wolman disease is a rare recessive disorder characterized by massive storage of CE and TG in most tissues, vomiting, diarrhea, anemia, failure to thrive, hepatosplenomegaly, adrenal calcification, and early death (usually before 1 year of age) (Assman and Seedorf 2001). Patients with CESD show a broad spectrum of severity of clinical presentation, but usually they present in childhood with hepatomegaly, hypercholesterolemia, hypertriglyceridemia and most of them are diagnosed by their second decade (Assman and Seedorf 2001).

About 50 mutations have been reported in the *LIPA* gene (see HGMD® Human Gene Mutation Database), most of them always associated with Wolman disease or with CESD, and most of them located in exon 8 (Assman and Seedorf 2001; Aslanidis et al. 1994). One of the most

frequent mutation associated with CESD is c.894G>A, that affects the last nucleotide of exon 8, does not change the amino acid (p. Q298Q), but causes the skipping of exon 8 causing an in-frame deletion of 24 amino acids (p. S275_Q298del) (Klima et al. 1993).

Here we present the clinical, biochemical, and molecular findings of 23 Spanish patients with LAL deficiency corresponding to 22 different families.

Materials and Methods

We analyzed 23 patients corresponding to 22 families originated from different regions in Spain. All patients were Caucasian and ten of them were of gipsy origin. No relation was reported between these ten families, but some of the surnames are shared, so we cannot be sure they are not related. The diagnoses of the patients were made between 1992 and 2015 in our lab. Clinical information was provided by the patient's physician and in all cases the diagnosis was confirmed biochemically by determination of the acid lipase activity in fibroblasts.

Acid lipase activity was measured in fibroblasts with the fluorogenic substrates 4-methylumbelliferyl-Palmitate (MU-palmitate) and/or 4-methylumbelliferyl-Oleate (MU-Oleate) (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Kelly and Bakhru-Kishore 1979; Koster et al. 1980). Protein concentration was determined using the Lowry method.

Genomic DNA was extracted from cultured fibroblasts using standard protocols. Exons two to ten and their flanking intronic regions of the *LIPA* gene were PCR-amplified using self-designed oligonucleotides. PCR reactions were performed according to standard protocols. Fragments were directly sequenced by the dideoxy termination method. Gene nucleotides were numbered according to the RefSeq NM_000235 sequence considering nucleotide +1 the A of the ATG start codon in exon 2. The ATG codon represents +1 for the amino acid numbering according to preprotein NP_000226 sequence.

Single nucleotide polymorphisms (SNPs) were analyzed using the same oligonucleotides as for mutation analysis, to construct haplotypes co-segregating with the changes found in WD and CESD patients: (1) c.1-65G>C, rs2250781; (2) c.46A>C (p. Thr16Pro), rs1051338; (3) c.67G>A (p. Gly23Arg), rs1051339; (4) c.676-42G>A, rs1556478; (5) c.966+46C>T, rs3802656.

Results

We studied 23 patients of 22 families with LAL deficiency. Thirteen of them were diagnosed as WD, while ten patients were diagnosed as CESD. Clinical and molecular findings of all patients are shown in Table 1.

Regarding the clinical manifestations, all WD patients were diagnosed at early infancy with a mean age at diagnosis of 48 days and all deceased before the year of life. They showed the typical intestinal malabsorption, hepatic and adrenal failure pattern. The CESD patients were diagnosed at a mean age of 13 years of life and their clinical affectation was mainly hepatomegaly and hyperlipidemia.

Biochemical analyses allowed the LAL deficiency diagnosis of the patients. WD patients showed a mean activity of 11.7% (±10.2) and 4.7% (±2.6) respect to controls using MU-Oleate and MU-palmitate respectively, while CESD patients showed a mean activity of 12.4% (±7.5) and 8.2% (±3.2) respect to controls using the same substrates respectively.

Molecular analysis of 23 LAL patients let us to identify eight different mutations, four of them not reported before. The novel changes were one missense mutation (c.256C>T, p. H86Y), one amino acid deletion (c.1055_1057delACG, p. D352del), one splicing alteration (c.966+2T>G), and a change in the same base as the common c.894G>A mutation that also changes amino acid (c.894G>C, p. Q298H). Mutation p. H86Y was predicted to be probably damaging by Polyphen-2 predictor (http://genetics.bwh.harvard.edu/pph2/) and MutPred (http://mutpred.mutdb.org/). The c.966+2T>G change affecting the invariant dinucleotide "GT" of the intron 9 donor splice site was expected to alter pre-mRNA splicing. To assess its potential effect on mRNA splicing the relative strength of the 5′ splice-site signal of intron 9 was evaluated using the BDGP Splice Site prediction (http://www.fruitfly.org/seq_tools/splice.html), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), and Splice Site Score Calculation (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html). All the algorithms predicted that c.966+2T>G mutations abolished the function of the donor splice site of intron 9. Finally, the novel change c.894G>C causes a missense mutation, p. Q298H, also predicted to be probably damaging by Polyphen-2 predictor and MutPred, but also affects the same exon 8 last base that the common mutation c.894G>A. All the new changes were not found in 100 control chromosomes.

The described mutations that were also identified in our cohort were the common c.894G>A (p. S275_298del) splicing mutation, the small deletion c.398delC (p. S133X), the complex mutation c.230-33_230dup;c.232_245del (p. G77fsX82), and the missense change c.386A>G (p. H129R).

We also performed haplotype analysis to investigate the origin of the common mutations in our cohort (Table 2). We observed that both the two common mutations in our series

Table 1 Clinical and molecular findings in Spanish LAL deficiency patients

Family	DNA	Age at diagnosis	LAL form	Genotype (cDNA)	Genotype (protein)	Cons.	AD	D/MD	H	PD	HM	SM	SGS	V	D	HCH	LC	HT	HTR
F1	W1	15 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	–	Yes	Yes	Yes	Yes	–	Yes	Yes	–	–	–	–
F2	W2	1 month	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	–	–	–	–
F3	W5	ND	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F4	W6	15 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	Yes	–	–	–	Yes	Yes	Yes	–	Yes	–	–	Yes	–
F5	W13	2 months	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	Yes	Yes	Yes	–	–	–	–	–	–
F6	W15	41 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	–	–	Yes	–	–	Yes	Yes	Yes	–	–	–	–
F7	W16	2 months	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	Yes	Yes	Yes	Yes	–	Yes	–	Yes	–	–	–	–
F8	W18	15 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	Yes	–	–	Yes	Yes	Yes	Yes	Yes	–	–	–	–	–
	W23	4 months	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F9	W22	25 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F10	W7	45 days	Wolman	**[c.894G>C]; [c.894G>C]**	**[p. Q298H]; [p. Q298H]**	–	Yes	–	–	Yes	Yes	Yes	–	Yes	Yes	–	–	–	–
F11	W10	1 month	Wolman	**[c.894G>C]; [c.894G>C]**	**[p. Q298H]; [p. Q298H]**	–	Yes	–	–	Yes	Yes	Yes	Yes	–	–	–	–	–	–
F12	W14	2 months	Wolman	[c.398delC]; [c.398delC]	[p. S133X]; [p. S133X]	Yes	–	–	–	–	Yes	Yes	Yes	Yes	–	–	–	–	–
F13	W3	33 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	–	Yes	Yes	Yes
F14	W8	9 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	–	–	–	–
F15	W9	7 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	Yes	–	–	Yes
F16	W17	2.5 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	Yes	–	Yes	Yes
F17	W11	3 years	CESD	[c.894G>A]; **[c.256C>T]**	[p. S275_298del]; **[p. H86Y]**	–	–	–	–	–	Yes	–	–	–	–	Yes	–	Yes	–
F18	W12	15 years	CESD	[c.894G>A]; [c.386A>G]	[p. S275_298del]; [p. H129R]	–	–	–	–	Yes	Yes	–	–	–	–	Yes	–	Yes	Yes
F19	W21	10 years	CESD	[c.894G>A]; [c.398delC]	[p. S275_298del]; [p. S133X]	–	–	–	–	Yes	Yes	–	–	–	–	Yes	–	Yes	Yes
F20	W20	11 years	CESD	[c.386A>G]; [c.386A>G]	[p. H129R]; [p. H129R]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F21	W19	11 years	CESD	[c.386A>G]; [c.230-33_230dup; c.232_245del]	[p. H129R]; [p. G77fsX82]	–	–	–	–	Yes	Yes	Yes	–	–	–	–	Yes	Yes	Yes
F22	W4	26 years	CESD	[c.386A>G]; **[c.1055_1057del]**	[p. H129R]; **[p. D352del]**	–	–	–	–	Yes	Yes	Yes	–	–	–	–	Yes	Yes	Yes

In bold novel mutations. *Cons* consanguinity, *AD* abdominal distension, *D/MD* dystonia/muscular dystrophy, *H* hypotonia, *PD* ponderal delay, *HM* hepatomegaly, *SM* splenomegaly, *SGS* suprarenal gland stones, *V* vomiting, *D* diarrhea, *HCH* hypercholesterolemia, *LC* liver cirrhosis, *HT* hypertriglyceridemia, *HTR* high transaminases, *ND* no data, (−) no data

Table 2 Haplotypes co-segregating with LIPA mutations

Mutation (cDNA level)	Mutation (protein level)	*n*	c.1-65G>T rs2250781	c.46A>C (p. Thr16Pro) rs1051338	c.67G>A (p. Gly23Arg) rs1051339	c.676-42G>A rs1556478	c.966+46C>T rs3802656
c.966+2T>G	IVS9+2T>G	20	T	C	G	G	C
c.894G>A	p. S275_298del	11	T	A	G	A	T
c.230-33_230dup; c.232_245del	p. G77fsX82	1	T	C	G	G	T
c.256C>T	p. H86Y	1	T	A	G	A	C
c.386A>G	p. H129R	5	G/T	A	G	G	C
c.398delC	p. S133X	3	G/T	A	G	G/A	C
c.894G>C	p. Q298H	4	G	A	G	G	T/C
c.1055_1057del	p. D352del	1	G	A	G	G	T

n Number of alleles

(c.966+2T>G and c.894G>A) segregated with a unique haplotype each one in all the patients. On the contrary, the other seven mutations co-segregated with different haplotypes (Table 2).

Discussion

The thirteen patients with WD were diagnosed between 15 days and 4 months of life, with a mean age at diagnosis of 48 days. The majority of them showed a severe systemic affectation with abdominal distension, hypotonia, ponderal delay, hepatosplenomegaly, suprarenal gland stones, diarrhea, and vomiting. Only one patient showed Hypertriglyceridemia. On the contrary, the ten patients showing CESD form of the disease were diagnosed between 2,5 and 33 years of life, being 13 years the mean age at diagnosis. Nearly all of them showed hepatomegaly as the main affectation trait and some of them showed ponderal delay, hypercholesterolemia, and high transaminases (Table 1). Regarding the clinical point of view, the WD patients in our cohort showed the typical clinical WD presentation, while CESD patients also showed the typical milder form with hepatomegaly and hyperlipidemia.

Biochemical analyses showed that using MU-palmitate as substrate gave lower percentage of activity respect to controls in both WD and CESD patients (4.7 and 8.2%), than using MU-Oleate, which resulted in higher residual activity in both series of patients (11.7 and 12.4%). Both substrates allowed the diagnosis of LAL deficient patients but, to our point of view, using both of them in the diagnosis protocol gives more reliable results when analyzing patients.

After molecular analysis, we detected eight different mutations in our cohort of patients, four of them novel. The novel mutations were c.256C>T (p.H86Y), c.1055_1057delACG (p.D352del), c.966+2T>G, and c.894G>C (p.Q298H). All of them were predicted presumably damaging and were not detected in 100 control chromosomes.

The described mutations that were identified in our cohort were the common c.894G>A (p.S275_298del) splicing mutation (Klima et al. 1993), the small deletion c.398delC (p.S133X) (Sadhukhan et al. 2014), the complex mutation c.230-33_230dup;c.232_245del (p.G77fsX82) (Pisciotta et al. 2009), and the missense change c.386A>G (p.H129R) (Ries et al. 1998).

Regarding genotype-phenotype correlation, we detected a clear differentiation between Wolman and CESD patients. The novel splicing mutation c.966+2T>G in intron 9 was detected in 75% (20/26) of the Wolman patients alleles, always in homozygosity. The novel c.894G>C change was the second most frequent being detected in 17% (4/26) of the alleles also in homozygosity. In CESD patients, the most frequent mutation was the common CESD associated c.894G>A which accounts for 55% (11/20) of the alleles, followed by the already reported missense change p.R129H, which represents 25% (5/20) of the alleles. Four out of ten CESD patients were homozygous for the c.894G>A change.

All mutations detected in WD patients were not detected in CESD patients and any mutation detected in CESD patients was detected in WD patients, except for the described deletion c.398delC that was identified in homozygosity in one WD patient and in heterozygosity with the common c.894G>A in one CESD patient.

The novel highly prevalent c.966+2T>G change affects the second base of the canonic donor splicing site in intron 9. It is predicted to disrupt the correct splicing process and presumably it would cause an insertion or a

deletion of some amino acids in the protein, but studies on cDNA must be performed to know the exact effect it causes. Anyway, as when present in homozygosity it is always associated with WD, it is presumed that the protein codified by this mRNA would show very low activity. Regarding the clinical point of view, patients presenting this mutation in homozygosity did not show any differential trait compared with the other WD patients with other mutations.

The second frequent WD associated c.894G>C mutation affects the same base as the common described CESD associated c.894G>A mutation. This mutation is known to result not only in a major non-functional transcript with the skipping of exon 8, causing the deletion of 24 amino acids (p.S275_Q298del), but also in a minor normally spliced transcript producing 5–10% residual LAL activity (Klima et al. 1993). The Change c.894G>C not only would destroy the same canonic splicing donor site as c.894G>A, presumably causing the same splicing effect, but also would introduce a missense mutation (p.Q298H) in the 5–10% of protein correctly synthesized. As the mutation p.Q298H is predicted to be damaging by the mutation effect predictors, its presence would cause the loss of the activity of the protein translated from the correctly spliced mRNA. So c.894G>C would generate 90–95% of inactive protein with a 24 amino acid deletion and 5–10% of inactive protein with the missense mutation p.Q298H. The presumably lack of activity correlates with the fact that we identified the c.894G>C mutation in homozygosity only in WD patients, the severe form, unlike c.894G>A, that has only been identified in CESD patients.

The other mutation detected in a WD patient of our cohort was c.398delC. It was detected in homozygosity in one WD patient but also in compound heterozygosity with the common c.894G>A in one CESD patient. These findings agree with the literature, as this mutation was previously described in homozygosity in one WD case (Sadhukhan et al. 2014) and in heterozygosity in CESD patients (Benlian et al. 2014), pointing out that the protein carrying this deletion presents very low activity, correlating with the WD genotype in homozygosity, but may be present in milder CESD patients when it is in heterozygosity with a mild mutation as c.894G>A.

Regarding CESD patients, the majority showed the reported frequent CESD associated c.894G>A mutation in homozygosity or in heterozygosity with another mutation, confirming its association with the mild forms of the disease (Klima et al. 1993) also in our cohort.

One CESD patient showed the reported p.H129R mutation in homozygosity and three patients carried the same mutation in heterozygosity, giving the idea that this change is associated with some residual activity and being associated with a milder phenotype (Ries et al. 1998).

Regarding the other mutations detected in heterozygosity in CESD patients of our cohort (p.H86Y, p.D352del and c.230-33_230dup;c.232_245del), expression studies should be performed to elucidate to what extent they allow some residual LIPA activity to explain this phenotype or if the milder form of the patient is due to the percentage of active protein caused by the mutation in the other allele.

We also performed haplotype analysis to investigate the origin of the common mutations in our cohort (Table 2). We observed that both the two common mutations in our series (c.966+2T>G and c.894G>A) segregated with a unique haplotype each one in all the patients. On the contrary, for the other six mutations, three of them were identified in only one allele, so we cannot conclude anything consistent about their co-segregation, and the other three mutations co-segregated with different haplotypes (Table 2). c.894G>A mutation co-segregates with the same haplotype previously described by Fasano et al. (2012), except for the rs2071509 polymorphism that we didn't analyze in our cohort and we do not have data. This observation strongly supports the hypothesis of a common ancestor for this mutation suggested by Fasano et al. (2012). Regarding the novel c.966+2T>G mutation that also co-segregates with a unique haplotype, it is also presumed to be transmitted by a common ancestor. For the other mutations identified in more than one allele, as they co-segregates with different haplotypes, it is to be assumed that they are recurrent mutations affecting hot spots in *LIPA* gene.

In conclusion, we identified four novel and four previously described mutations in a cohort of 23 Spanish LAL deficiency patients. The majority of the CESD patients carried the common mutant allele c.894G>A and the great majority of the WD patients carried the novel mutation c.966+2T>G. These two mutations co-segregated with a unique haplotype suggesting a presence of a common ancestor. The other mutations in WD or CESD patients co-segregated with different haplotypes.

Our study contributes to the LAL deficiency acknowledgement with novel mutations and with high frequencies of some unknown mutations for WD.

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The Spanish LAL Deficiency Working Group Includes All Physicians Who Referred Patients' Samples for This Study

Drs: Sampedro (C.H. Cristal Piñor, Ourense), Barba, Gutiérrez, Pérez (H. Gral. U. De Albacete), Bousoño (H. Central de Asturias), Mateos, Merino (H.U. Salamanca), Rodríguez (H. Da Costa, Burela), Saitúa (H. Basurto, Bilbao), Rodríguez, Velasco (H. Bierzo, Ponferrada), Sasieta (H. Cruces), Pastor (H. Elche), Arana Amurrio (H. Gregorio Marañon, Madrid), Jara, Pérez Rodríguez (H. La Paz, Madrid), Martinez-Pardo (H. Ramon y Cajal, Madrid), Artuch (H. Sant Joan de Déu, Barcelona), Gallego, Reparaz (H. Materno Inf. Teresa Herrera, A Coruña), González (H. Vall Hebrón, Barcelona).

Take-Home Message

In Spanish acid lipase deficient patients, the novel mutation c.966+2T>G is highly frequent (75% of the alleles) in Wolman disease patients, while the common CESD associated c.894G>A is also the most frequent among these patients in our cohort.

Compliance with Ethics Guidelines

Author Contributions

Carla Ruiz-Andrés collected the clinical data and performed the molecular analyses, analyzed and interpreted the data; Elena Sellés performed the enzymatic analyses and interpreted the data; Angela Arias cultured the fibroblasts and Laura Gort contributed to conception and design, analysis and interpretation of data and writing the chapter. All the authors revised the manuscript critically.

Corresponding Author

Laura Gort.

Competing Interest Statement

All the authors declare that they have no conflict of interest.

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Informed Consent

All the procedures were approved by the ethics committee of the Hospital Clínic, Barcelona. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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RESEARCH REPORT

Guanidinoacetate Methyltransferase Activity in Lymphocytes, for a Fast Diagnosis

Lisette M. Berends · Eduard A. Struys · Birthe Roos · Ulbe Holwerda · Erwin E. W. Jansen · Gajja S. Salomons · Mirjam M. C. Wamelink

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Abstract *Introduction*. Guanidinoacetate methyltransferase (GAMT) deficiency is an inborn error of metabolism (IEM), clinically characterized by intellectual disability, developmental delay, seizures, and movement disorders. Biochemical diagnosis of GAMT deficiency is based on the measurement of creatine and guanidinoacetate in urine, plasma, or CSF and is confirmed genetically by DNA analysis or by enzyme assay in lymphoblasts or fibroblasts. To obtain enough cells, these cells need to be cultured for at least 1 month. A less time-consuming diagnostic functional test is needed, since GAMT deficiency is a candidate for newborn screening (NBS) programs, to be able to confirm or rule out this IEM after an initial positive result in the NBS.
Methods. Stable-isotope-labeled $^{13}C_2$-guanidinoacetate and $^{2}H_3$-*S*-adenosylmethionine (SAM) were used, which are converted by GAMT present in lymphocyte extracts into $^{2}H_3$-$^{13}C_2$-creatine. The formed $^{2}H_3$-$^{13}C_2$-creatine was butylated and subsequently measured by liquid chromatography tandem mass-spectrometry (LC-MS/MS).
Results. We measured GAMT enzyme activity in lymphocyte extracts of 24 controls, 3 GAMT deficient patients and of 2 parents proven to be carrier. Because GAMT activity decreases when isolation time after venipuncture increases, reference values were obtained for 2 control groups: isolation on the day of venipuncture (27–130 pmol/h/mg) and 1 day afterwards (15–146 pmol/h/mg). Deficient patients had no detectable GAMT activity. The two carriers had GAMT activity within the normal range.
Conclusion. We designed a fast, less invasive, and valid method to measure GAMT activity in lymphocytes using LC-MS/MS analysis without the need of time-consuming and laborious cell culture.

Nonstandard Abbreviations

AGAT	Arginine:glycine amidinotransferase
EDTA	Ethylenediaminetetraacetic acid
GAMT	Guanidinoacetate methyltransferase
HBSS	Hanks balanced salt solution
LC-MS/MS	Liquid-chromatography tandem mass-spectrometry
MRS	Magnetic resonance spectroscopy
NBS	Newborn screening
SAM	S-adenosylmethionine
tris	Tris(hydroxymethyl)aminomethane
WES	Whole exome sequencing

Introduction

Guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2; MIM 601240) is one of the enzymes involved in creatine biosynthesis. Creatine is synthesized in liver, pancreas, and kidney in a two-step enzymatic pathway. First, arginine and glycine are converted into ornithine and guanidinoacetate by arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1; MIM 602360), taking place mainly in the kidney. Next step is the formation of creatine out of guanidinoacetate by GAMT as enzyme, using S-adenosylmethionine as methyl-donor, which takes place mainly in the liver (Wyss and Kaddurah-Daouk 2000; Almeida et al. 2004; Mercimek-Mahmutoglu and Salomons 2009; Joncquel-Chevalier Curt et al. 2015; Hanna-El-Daher and Braissant 2016).

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L.M. Berends · E.A. Struys · B. Roos · U. Holwerda · E.E.W. Jansen · G.S. Salomons · M.M.C. Wamelink (✉)
Metabolic Laboratory, Department of Clinical Chemistry, Amsterdam Neuroscience, VU Medical Center, Amsterdam, The Netherlands
e-mail: m.wamelink@vumc.nl

Creatine is known for its use as energy buffer in muscle and brain in the form of phosphocreatine. Creatine kinase (EC 2.7.3.2) converts creatine into phosphocreatine, after creatine is transported into the cell by the creatine transporter (MIM 300036). Phosphocreatine is a phosphagen which can convert ADP to ATP with creatine kinase as enzyme when energy demand is high (Joncquel-Chevalier Curt et al. 2015; Wallimann et al. 2011; Wyss and Kaddurah-Daouk 2000).

GAMT deficiency is an autosomal recessive disorder, with an estimated incidence varying from 1:114,000 newborns in Utah (US), to 1:250,000 newborns in the Netherlands and in Australia of 1:≥770,000 (Pitt et al. 2014; Mercimek-Mahmutoglu et al. 2016). It results in the accumulation of guanidinoacetate and depletion of creatine.

Patients with GAMT deficiency can suffer from mental retardation, developmental delay, seizures, and movement disorders. After diagnosing GAMT deficiency, treatment consists of supplementation of creatine and a protein-, or arginine-restricted diet to decrease guanidinoacetate in plasma and CSF. If treated at birth, the symptoms can be prevented (El-Gharbawy et al. 2013), therefore newborn screening (NBS) for GAMT-deficiency is advised by the Ministry of Health in the Netherlands (Health Council of the Netherlands 2015) and is implemented in the Utah (US) newborn screening program since July 2015. Newborn screening will most likely be done by measuring guanidinoacetate/creatine ratio in dried blood spots with flow injection-MS/MS, with a second tier test using LC-MS/MS (Pasquali et al. 2014) followed by genetic/enzymatic confirmation (Verhoeven et al. 2004). Our current enzyme assay measures GAMT activity in lymphoblasts and fibroblasts. To obtain sufficient amount of cells, lymphoblasts and fibroblasts need to be cultured for at least 1 month, which is time consuming and delaying the enzymatic confirmation. Since GAMT deficiency is a candidate to be included in the NBS, an enzymatic confirmation method in readily available cells, i.e. lymphocytes is needed. We developed an enzyme assay in lymphocytes for the detection of GAMT activity by using tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemicals and Reagents

Tris(hydroxymethyl)aminomethane (tris), and methanol (99.8%), butanol (99.5%), acetylchloride and acetonitrile (99.5%) were purchased from Merck (Darmstadt, Germany). $^{13}C_2$-guanidinoacetate was manufactured by Dr. H.J. ten Brink (Organic Synthesis Laboratory VU Medical Center, Amsterdam). $^{2}H_3$-S-adenosylmethionine; 99 atom-% D) and $^{2}H_3$-creatine (99 atom-% D) were purchased from C/D/N isotopes (Quebec, Canada). Leucosep™ tubes with Histopaque were purchased from Greiner Bio-One. Bicinchoninic acid protein assay kit was purchased from Sigma-Aldrich (St. Louis, MO). All other solvents and chemicals were of analytical grade.

Sample Collection

Lymphocytes were isolated from venous blood samples in ethylenediaminetetraacetic acid (EDTA) containing tubes. Collection of venous blood was done at the venipuncture unit in VU medical center. Written consent was obtained from each participant. Material was then sent to the metabolic laboratory. Frozen lymphocyte cell pellets of three known patients with GAMT deficiency were available at the metabolic laboratory of VU medical center, as well as from two parents from one of the patients who were proven to be heterozygous for a pathogenic mutation. The three patients were diagnosed by measurement of metabolites and brain magnetic resonance spectroscopy (MRS) and the diagnosis was confirmed by genetic analysis. Lymphocytes of these patients and the two parents were isolated within 1 day after venous blood collection.

Sample Preparation

Isolating lymphocytes was performed using Leucosep™-tubes with Histopaque. 3–8 mL blood was gently transferred into the Leucosep™ tube which was centrifuged 20 min at 1,000 g with switched-off brakes. Then the enriched cell fraction was pipetted into another tube and washed twice with 5 mL of Hanks balanced salt solution (HBSS) and centrifuged 6 min at 340 g. An erythrocyte shock was performed by dissolving the cell pellet in 1 mL HBSS, after which 3 mL cold distilled water was added, and after 1.5 min 1 mL 3.6% NaCl was added. Then the tube was centrifuged again for 6 min at 340 g. Pellets were dissolved in 1 mL HBSS, and centrifuged 3 times at 10,000 g for 8 s. The dry pellets were stored at −80°C. Cell pellets were dissolved in 250 µL of 200 mmol/L tris-HCl buffer (pH 8.5) and sonicated 3 times 10 s on ice. Lysates were centrifuged for 5 min at 4°C and 10,000 g, then protein concentration within the supernatant was measured with bicinchoninic acid protein assay kit.

An adapted method of Verhoeven et al. (2004) was used to form labeled creatine from stable isotope labeled substrates in the lymphocyte extract, which is a direct measure for GAMT activity. In short, 25 µL 8 mmol/L $^{13}C_2$-guanidinoacetate, and 12.5 µL 25 mmol/L $^{2}H_3$-SAM were used as substrates. 0.1 mg protein of cell homogenate was used. Finally, 200 mmol/L tris-HCl buffer (pH 8.5) was added till a final volume of 0.2 mL. The reaction mixture

was incubated for 2 h at 37°C. At the start of incubation and after 2 h, an 85 μL sample was taken and stored at −20°C until preparation for LC-MS/MS analysis. This preparation method and LC-MS/MS analysis was derived from the method used by Mercimek-Mahmutoglu et al. (2016) to measure guanidinoacetate and creatine in dried bloodspots. An assay blank was prepared with 100 μL distilled water instead of cell homogenate. To quantify the formation of $^{2}H_3$-$^{13}C_2$-creatine, 6.25 pmol of $^{2}H_3$-creatine was added to the samples as internal standard. Samples were deproteinized with 330 μL methanol and were centrifuged 2 min at 5,000 g. The mixture was transferred to a vial and evaporated to dryness with nitrogen at 40°C. Then 100 μL butylating-reagens (consisting of acetylchloride and *n*-butanol in a ratio of 1:17) was added. Derivatization took place for 15 min at 60°C, after which it was evaporated with nitrogen again. After dissolving in 50 μL acetonitrile, samples were injected on the LC-MS/MS.

Liquid Chromatography

Chromatography was performed using an LC-30AD UPLC-system (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved by using a Symmetry-C_8 column: 4.6 * 100 mm, 3.5 μm (Waters, Milford, USA) with isocratic elution of 30% acetonitrile (with 0.4% formic acid) at a flow rate of 0.35 mL/min. 5 μL of sample was injected into the column and the total run time was 5 min.

Mass Spectrometry

Tandem mass spectrometry experiments were carried out using an API5000 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Toronto, Canada) equipped with a Turbo Ion Spray Source. The mass spectrometer operated in ESI-positive mode with a needle potential of 5,500 V and a source temperature of 300°C. Optimized collision energy and voltages were used and the compound of interest was analyzed using multireaction monitoring mode (*m/z* 193.0 → 95.0 for $^{2}H_3$-$^{13}C_2$-creatine and *m/z* 191.0 → 93.0 for $^{2}H_3$-creatine).

Performance Characteristics

To optimize the enzyme assay in lymphocytes, a protein curve was made (0–25–50–75–100–150–200 μg protein), as well as a sequence of incubation time (for 0–30–60–90–120–180 min). To determine the stability of GAMT enzyme in blood samples, an experiment was performed using each three blood samples of two adult controls which were isolated immediately, 24 or 48 h after venipuncture. After this, they were prepared for enzyme assay as described above.

The intra- and inter-assay variation were determined. The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the signal to noise (S/N) ratio. LOD was determined as 3 times (S/N)-ratio, LOQ was determined as 10 times (S/N)-ratio. Obtained values were the mean of three independent experiments. Since GAMT appeared to be instable, reference values were obtained for lymphocytes isolated directly after venous blood collection and 1 day afterwards. Lymphocytes from three established GAMT deficient patients and two carriers were used to determine the enzymatic activity.

Statistical Analysis

Statistical analysis, such as calculating intra- and inter-assay variation, was performed using the software of Microsoft Excel 2010. Linear regression analysis was used to calculate GAMT activity.

Results

The protein curve showed an optimum of 100 μg, which was used in further experiments. The incubation curve was performed in duplicate, which showed an optimum of 120 min (data not shown).

The stability of the GAMT enzyme in blood was tested in two different cell lines (A and B) and was as follows. When isolated immediately after venipuncture, the activities were 31 pmol/h/mg and 130 pmol/h/mg, respectively; after 24 h, the activities were 17 pmol/h/mg and 50 pmol/h/mg; after 48 h, the activities were 12 pmol/h/mg and not detectable in cell lines A and B, respectively.

Table 1 GAMT activity in lymphocytes from controls and individuals with GAMT deficiency and carriers. Controls were isolated on the day of venipuncture and 1 day afterwards

	N	Days after venipuncture	Range (pmol/h/mg)	Mean ± sd (pmol/h/mg)
Lymphocytes of adult controls	13	0	27–130	58 ± 18
Lymphocytes of adult controls	11	1	15–146	62 ± 46
GAMT deficient patients	3	0–1	Not detectable	Not detectable
GAMT carriers	2	0–1	106; 115	

The limit of detection is 3.8 pmol/h/mg.

Reference values were determined by measuring GAMT activity in lymphocytes of unaffected adult controls (Table 1). Because of the decline of GAMT activity with increased isolation time, reference values were obtained for two groups: isolation on the day of venous blood collection and 1 day afterwards. The intra-assay variation ($n = 8$) was 6.3%. The inter-assay variation ($n = 6$) was 6.7%.

The LOD and LOQ were 3.8 pmol/h/mg and 12.7 pmol/h/mg, respectively, and were sufficient to diagnose patients with GAMT deficiency. Measurement of three known GAMT deficient patients showed no detectable GAMT activity. The two parents tested who were carrier showed GAMT activity within the normal range.

Discussion

Historically, the first enzymatic assays of GAMT were performed in liver biopsies, since GAMT is mainly active in the liver (Stöckler-Ipsiroglu et al. 1996) while later, assays were developed for lymphoblasts and fibroblasts (Verhoeven et al. 2004; Ilas et al. 2000), which were less invasive, but due to the need to have enough protein, cell culturing was necessary. Since quick diagnosis and treatment is of great importance in patients with GAMT deficiency, and because of the advice of the Ministry of Health in the Netherlands to include GAMT deficiency in the NBS program (Health Council of the Netherlands 2015), we developed a method to measure GAMT activity in readily obtainable lymphocytes. Also with the increased findings of genetic variants by whole exome sequencing (WES), it is important to functionally confirm, by enzymatic (functional) tests, whether these variants are clinically relevant.

The different isolation times showed that GAMT activity decreases when the venous blood sample is older. Therefore, two groups were made for reference values: immediately after collecting the venous blood sample, and 1 day afterwards. Blood isolated after 48 h is not reliable to use for lymphocyte analysis for GAMT enzymatic analysis.

The measurement of known deficient patients showed no detectable GAMT activity, as suspected based on results in lymphoblasts and fibroblasts. Our new method for measurement of GAMT activity in lymphocytes is validated according to ISO15189 accreditation for medical laboratories and is therefore valid for diagnostics.

Future Perspectives

Because GAMT deficiency is a candidate to be included in the NBS program, an enzyme assay measuring GAMT activity in dried blood spots would be ideal. In the current enzyme assay, the sensitivity was enhanced by changing the original described workflow for GAMT activity in fibroblasts and lymphoblasts from GC-MS to LC-MS/MS. Measuring GAMT activity in dried bloodspots will require even more sensitivity compared to our here presented GAMT enzyme assay in lymphocytes, and will be our next goal to achieve.

Conclusion

We developed a fast, less invasive, reproducible, reliable, and sensitive method to measure GAMT activity in lymphocytes.

Synopsis

A new, fast, less invasive and valid method to measure GAMT activity in lymphocytes using LC-MS/MS analysis of a $^{2}H_{3}$-$^{13}C_{2}$-creatine butyl derivative was designed.

Compliance with Ethics Guidelines

Lisette M. Berends, Eduard A. Struys, Birthe Roos, Ulbe Holwerda, Erwin E. W. Jansen, Gajja S. Salomons, and Mirjam M. C. Wamelink declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Author Contribution

Lisette M. Berends: conception and design; analysis and interpretation of data; drafting the article; revising it critically.

Eduard. A. Struys: analysis and interpretation of data; revising it critically.

Birthe Roos: conception and design; analysis and interpretation of data; revising it critically.

Ulbe Holwerda: analysis and interpretation of data; revising it critically.

Erwin E. W. Jansen: analysis and interpretation of data; revising it critically.

Gajja S. Salomons: conception and design; revising it critically.

Mirjam M. C. Wamelink: conception and design; analysis and interpretation of data; revising it critically, guarantor.

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RESEARCH REPORT

Galactose Epimerase Deficiency: Expanding the Phenotype

Filipa Dias Costa • Sacha Ferdinandusse • Carla Pinto • Andrea Dias • Liesbeth Keldermans • Dulce Quelhas • Gert Matthijs • Petra A. Mooijer • Luísa Diogo • Jaak Jaeken • Paula Garcia

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Abstract Galactose epimerase deficiency is an inborn error of metabolism due to uridine diphosphate-galactose-4′-epimerase (GALE) deficiency. We report the clinical presentation, genetic and biochemical studies in two siblings with generalized GALE deficiency.

Patient 1: The first child was born with a dysmorphic syndrome. Failure to thrive was noticed during the first year. Episodes of heart failure due to dilated cardiomyopathy, followed by liver failure, occurred between 12 and 42 months. The finding of a serum transferrin isoelectrofocusing (IEF) type 1 pattern led to the suspicion of a congenital disorder of glycosylation (CDG). Follow-up disclosed psychomotor disability, deafness, and nuclear cataracts.

Patient 2: The sibling of patient 1 was born with short limbs and hip dysplasia. She is deceased in the neonatal period due to intraventricular hemorrhage in the context of liver failure. Investigation disclosed galactosuria and normal transferrin glycosylation.

Next-generation sequence panel analysis for CDG syndrome revealed the previously reported c.280G>A (p.[V94M]) homozygous mutation in the *GALE* gene. Enzymatic studies in erythrocytes (patient 1) and fibroblasts (patients 1 and 2) revealed markedly reduced GALE activity confirming generalized GALE deficiency. This report describes the fourth family with generalized GALE deficiency, expanding the clinical spectrum of this disorder, since major cardiac involvement has not been reported before.

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F. Dias Costa (✉) · L. Diogo · P. Garcia
Unidade de Doenças Metabólicas, Centro de Desenvolvimento da Criança, Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra, EPE, Avenida Afonso Romão, Coimbra 3000-206, Portugal
e-mail: filipacdcosta@gmail.com

S. Ferdinandusse · P.A. Mooijer
Laboratory Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

C. Pinto · A. Dias
Serviço de Cuidados Intensivos Pediátricos, Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra, EPE, Coimbra, Portugal

C. Pinto · A. Dias · L. Diogo
Faculdade de Medicina da Universidade de Coimbra, Coimbra, Portugal

L. Keldermans · G. Matthijs
Center for Human Genetics, University of Leuven, Leuven, Belgium

D. Quelhas
Unidade de Bioquímica Genética, Centro de Genética Médica Jacinto de Magalhães, Centro Hospitalar do Porto, Porto, Portugal

D. Quelhas
Unit for Multidisciplinary Research in Biomedicine, Abel Salazar Institute of Biomedical Sciences, University of Porto – UMIB/ICBAS/UP, Porto, Portugal

J. Jaeken
Department of Pediatrics, Centre for Metabolic Disease, University Hospital Gasthuisberg, KU Leuven, Leuven, Belgium

J. Jaeken
Metabool Centrum – Universitair Ziekenhuis Leuven, Leuven, Belgium

Introduction

Uridine diphosphate-galactose-4′-epimerase (GALE) deficiency is a very rare disease of galactose metabolism and the Leloir pathway. This enzyme catalyzes the conversion of uridine diphosphate galactose (UDP-Gal) to uridine diphosphate glucose (UDP-Glc) (Segal and Berry 1995), as well as the reverse reaction when other sources of UDP-

Gal are scarce. GALE also plays a role in the interconversion of uridine diphosphate-*N*-acetylgalactosamine and uridine diphosphate-*N*-acetylglucosamine (Wohlers et al. 1999). These uridine diphosphate sugars are essential for the glycosylation of proteins and lipids, and their restriction can lead to the production and accumulation of hypogalactosylated glycans, which are suggested to contribute to the long-term complications in galactosemia (Charlwood et al. 1998). Thus, galactosemia is a cause of secondary hypoglycosylation (Sturiale et al. 2005).

The clinical severity of GALE deficiency ranges from benign to potentially lethal, depending on the degree of reduction of GALE activity and the tissues affected. The first reported case (Gitzelmann 1972) was described as a benign condition, a "peripheral" form of the disease, in which the GALE impairment was restricted to the circulating blood cells. In 1981, (Holton et al. 1981) described the first case of "generalized" GALE deficiency, a patient with a severe clinical presentation, similar to classic galactosemia, with reduced GALE activity in both circulating red blood cells and fibroblasts. As pointed out by Openo, GALE deficiency is not a binary condition but a continuum disorder (Openo et al. 2006). There are three forms currently recognized: (1) a peripheral form, with decreased enzyme activity in red blood cells and leucocytes, with normal or near normal levels in all other tested tissues; (2) an intermediate form, with decreased enzyme activity in circulating blood cells, and less than 50% in all other tissues tested; and (3) a generalized form, with a profound generalized decrease of enzyme activity (Fridovich-Keil 2013). The peripheral form is reported to occur with a frequency of 1:6,700 to 1:60,000 depending on the ethnic background (Alano et al. 1998), while the severe generalized form is extremely rare (Holton et al. 2000) with only three families (six patients) described (Holton et al. 1981; Sardharwalla et al. 1988; Walter et al. 1999; Sarkar et al. 2010).

The present report is on two sisters with different phenotypes, expanding the clinical spectrum of generalized GALE deficiency, and reviews the literature on this GALE deficiency form.

Patient Reports

Patient 1 (Fig. 1)

This first female child of non-consanguineous Caucasian parents was born after a full-term pregnancy. The mother was treated for hypothyroidism. The biochemical and ultrasound screening showed a high risk for trisomy 21, but there was a normal karyotype on amniocentesis.

The newborn was admitted to the NICU during the first week due to a dysmorphic syndrome: relative macrocephaly, hypertelorism, micrognathia, finger contractures, short limbs, hip dislocation, positional talipes, and ligament laxity. During the first year of life, failure to thrive, hypotonia, and psychomotor disability were noticed. Cerebral ultrasound and electromyography were normal.

At 12 and 16 months old, she was hospitalized in the PICU with heart failure due to dilated cardiomyopathy, followed by hepatomegaly and acute liver failure. She recovered under supportive therapy and was discharged on diuretic medication. Infectious etiologies were excluded, and pituitary hormones and brain MRI were normal.

At the ages of 18, 41, 42, and 43 months, she was hospitalized with heart decompensation that responded well to anticongestive heart therapy.

Results of other investigations included persistently raised serum aminotransferases (3–4 times the upper limit of normal) until 4 years old, negative urinary-reducing sugars, and unspecific alterations in urinary organic acids, plasma amino acids, and acylcarnitine profile. A diagnosis of congenital disorder of glycosylation (CDG) was hypothesized based on a high serum level of carbohydrate-deficient transferrin (CDT) on several occasions (3–10.2%; reference range <2.6%). Serum transferrin isoelectrofocusing (IEF) showed a type 1 pattern, and the fibroblast LLO (lipid-linked oligosaccharides) profile was normal (small mannose 2 glycan accumulation). Phosphomannomutase and phosphomannose isomerase activities in fibroblasts were normal, and there were no mutations in *ALG2, DPM3, DK1*, and *SRD5A3* genes.

During follow-up (Fig. 1a–c), on regular diet and till the present age of 12 years, the dysmorphic features attenuated. She showed a short stature (height, −2.9 SD; weight, −0.17 SD), global developmental disability with slow acquisitions (15–19 months at 10 years old on Vineland Adaptive Behavior Scales) (Sparrow et al. 2005), a behavior disorder controlled with risperidone, sensorineural hearing loss detected at 3 years old, and bilateral nuclear cataracts diagnosed and surgically treated at 8 years old. Cardiac function normalized under furosemide and spironolactone and dilated cardiomyopathy has progressively improved. The medication is being gradually withdrawn.

Patient 2 (Fig. 2)

After the birth of a healthy male sibling, the third child of the same couple, a female, was born following an uneventful term pregnancy. She presented relative macrocephaly, short limbs, and bilateral hip dislocation and was discharged under breastfeeding. At the 8th day of life, she was admitted to the local hospital for poor feeding and lethargy. Hypotonia, hypothermia, and hypoglycemia were noticed. After initial stabilization and because of persistent depressed consciousness, she was transferred to the PICU.

Fig. 1 (**a**–**c**) Case 1, older sibling with generalized GALE deficiency

On admission, encephalopathy, jaundice, and hepatomegaly were observed. Acute liver failure was confirmed, with severe coagulopathy, thrombocytopenia, and anemia, requiring multiple transfusions. Death occurred on the 17th day, with severe intraventricular hemorrhage, despite a lactose-free regimen.

Investigations revealed positive urine-reducing sugars (20 g/L) without glycosuria; generalized hyperaminoaciduria; high plasma glycine, glutamine, alanine, tyrosine, and phenylalanine, and unspecific abnormalities in urinary organic acids. Acylcarnitine profile on Guthrie card, pituitary hormones, and serum transferrin IEF were normal. Viral infections, neonatal hemochromatosis, and hemophagocytic lymphohistiocytosis were excluded. Autopsy showed a small liver with massive hepatic necrosis, cholestasis, large kidneys with intratubular renal calcifications, hepatopancreatic siderosis, and subarachnoid and intraventricular hemorrhages. *Postmortem* mitochondrial respiratory chain functional studies showed deficits of complexes I, II, IV, and V in the liver and heart and normal activities in the muscle, with normal results for mtDNA copy number, mtDNA genome, and DGUOK gene analysis.

Gene panel using massive parallel sequencing (target capture CDGv1, NimbleGen) revealed the previously reported homozygous c.[280G>A] p.[V94M] mutation in *GALE* in both sisters, leading to the diagnosis of GALE deficiency (MIM 230350). The parents are heterozygous for the same mutation. GALE activity was markedly reduced in erythrocytes of patient 1 [0.6 μmol/(h.gHb); reference range 5.7–22.1] and also in fibroblasts of both patients 1 and 2 confirming generalized GALE deficiency. In patient 1, erythrocyte galactose 1-phosphate and urinary polyols chromatography were normal on an unrestricted diet.

Table 1 shows clinical, biochemical, and genetic characteristics of the present patients and the reported ones.

Discussion

Generalized GALE deficiency has only been reported in six patients from three families (Table 1). In one of the patients previously described (case 8), GALE was only assayed in red cells, and no genetic studies were undertaken therefore making it difficult to confirm the diagnosis of generalized GALE deficiency, besides the severe clinical presentation described (Sarkar et al. 2010). The other five patients belong to two highly consanguineous families. This constitutes a difficulty in attributing some features to generalized GALE deficiency with certainty. Although the parents of patients 1 and 2 affirmed to be non-consanguineous, a farthermost possibility of a shared ancestry couldn't be ruled out. Nevertheless, excluding cardiac involvement, all of the other features described can be attributed to GALE deficiency, according to the previous published descriptions of this disease. A majority of patients showed hepatic symptoms (7/8), hypotonia (5/8), and, during follow-up, short stature (7/7), developmental disability (7/7), and sensorineural hearing loss (4/7). Symptoms present in a minority of patients were micrognathia, flexion deformities of the fingers, dislocatable hip/hip dysplasia, and positional talipes equinovarus. Galactosemia was not considered in patient 1, since liver involvement happened outside the neonatal period, reducing sugars were negative, recovery was almost complete under a normal diet, and cataracts appeared only at 8 years. Thus, the lack of the classic newborn severe liver presentation, which emerged in the younger sister, contributed to the diagnostic delay in this family. This

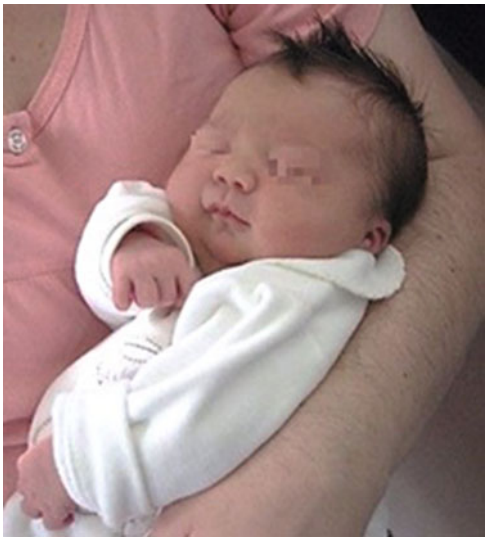

Fig. 2 Case 2, younger sibling with generalized GALE deficiency

different severity in clinical presentation overlaps the description made by Walter et al. (1999), in which the index cases for each family presented earlier with severe clinical illness similar to that seen in classic galactosemia, with poor feeding, weight loss, liver disease, and tubulopathy, while the other members of those families (cases 4, 6, and 7 of Table 1), who had the same homozygous mutation, were diagnosed before noticeable disease has developed. Another curious fact is that the older sibling (case 1) didn't show any sign of milk intolerance until date. The available knowledge about GALE deficiency is still insufficient to explain these differences in clinical presentation. In patient 1, the multisystem involvement with dysmorphism, cardiomyopathy, and developmental disability associated with high levels of serum CDT, and a type 1 pattern on IEF led to the suspicion of a CDG, which remained "type X," since no enzyme deficiency or mutation was found. Galactosemia type 1 and 3 are known causes of secondary hypoglycosylation of proteins and lipids (Charlwood et al. 1998, Sturiale et al. 2005). Hypertrophic and dilated cardiomyopathy is a known feature of some CDG, most commonly in a multisystem presentation, early in life, although late-onset and non-syndromic cases have also been reported (Lefeber et al. 2011). Dilated cardiomyopathy was the main clinical feature in patient 1 for some years, but it was absent in her younger sister. To our knowledge, this is the first generalized GALE-deficient patient with dilated cardiomyopathy.

The c.[280G>A] (p.[V94M]) mutation in the *GALE* gene has been found in a homozygous form in all of the patients tested with the severe phenotype (Table 1). Other mutations are associated with the intermediate or asymptomatic phenotype (Openo et al. 2006; Wasilenko et al. 2005).

A peculiar feature in patient 1 was the absence of accumulation of galactose metabolism products [total galactose and galactose-1-phosphate (Gal-1-P)]. A similar situation was already described in patients with intermediate and peripheral GALE deficiency (Openo et al. 2006).

Galactose is an essential constituent of the glycosphingolipids, required for brain growth and development. The galactose metabolism includes the Leloir and the pyrophosphorylase pathways, and epimerase is a key enzyme in both. In the Leloir pathway, the conversion of Gal-1-P to glucose-1-phosphate (Glc-1-P) is made by the enzyme galactose-1-phosphate uridyltransferase. In this reaction, UDP-GIc is converted to UDP-Gal. Epimerase regenerates UDP-Glc from UDP-Gal, allowing the maintenance of this cycle. The pyrophosphorylase constitutes an alternative pathway, which allows the synthesis of UDP-Gal in its direct route in patients with transferase deficiency or, in its reversal route, when galactose intake is restricted (Holton et al. 1981). In epimerase deficiency, the patients are unable to synthetize UDP-Gal by this way and depend on exogenous galactose for its synthesis (Holton et al. 1981).

Henderson et al. (1983) suggested that, in galactose epimerase deficient patients, a small amount of administered galactose will be metabolized via the Leloir pathway to produce UDP-Gal. UDP-Glc cannot be regenerated from UDP-Gal but could be formed from UTP and Glc-1-P and used as a cofactor in the maintenance of transferase activity. Thus, under these circumstances, galactose and Gal-1-P will not accumulate but UDP-Gal will. When dietary galactose is increased further, it was suggested that the synthesis of UDP-Glc might become limiting for the

Table 1 Clinical and laboratory features of the present patients and the previously reported ones

	Patient 1	Patient 2 (sibling of 1)	3 Holton et al. (1981)	4 Walter et al. (1999) (sibling of 3)[a]	5 Sardharwalla et al. (1988)	6 Walter et al. (1999) (sibling of 5)[a]	7 Walter et al. (1999) (cousin of 5,6)[a]	8 Sarkar et al. (2010)
Year of birth; gender	2004; Female	2012; Female	1980; Female	1991; Female	1984; Female	1985; Male	1994; Female	?; Male
Clinical presentation	Hypotonia Failure to thrive Dilated cardiomyopathy Cardiac failure Acute liver failure	Hypotonia Poor feeding Jaundice Hepatomegaly Acute liver failure	Hypotonia Weight loss Jaundice Vomiting	No clinical illness	Poor feeding Irritability Jaundice Hepatomegaly Cataracts	Hypotonia Poor feeding	Hypotonia	Poor feeding Jaundice Lethargy Vomiting Hepatomegaly
Dysmorphic features	Micrognathia Relative macrocephaly Hypertelorism Short limbs Flexion deformity of fingers Positional talipes Bilateral hip dysplasia Ligament laxity Short stature	Relative macrocephaly Bilateral hip dysplasia Short limbs	Hypotelorism Increased palpebral fissure length Posteriorly rotated ears Short philtrum Short stature	Short stature	Micrognathia Posteriorly rotated ears Ligament laxity Persistent femoral anteversion Internal tibial torsion Short stature	Micrognathia High palate Pigeon chest Flexion deformity of fingers Dislocatable hip Positional talipes Ligament laxity Short stature	Micrognathia Flexion deformity of fingers and toes Chest deformity Thick gums Short stature	?
Evolution	Severe SN hearing loss Global developmental disability Cataracts Cardiac function normalized under medication Normal pubertal development	Death at 17 days	Developmental disability Mod. learning difficulties Severe SN hearing loss Short stature Normal ovarian function	Mod. learning difficulties No SN deafness	Severe SN hearing loss Mod. learning difficulties Normal pubertal development	Tracheostomy (upper airway obstruction) Poor feeding Mod. developmental disability No SN deafness	Severe SN hearing loss Global developmental disability	No visual or hearing impairment Walking with support at 15 months
Laboratory investigations	↑ CDT Abn. transferrin IEF Urinary-reducing substances Ø Gal-1-P normal	Urinary-reducing substances +	Urinary-reducing substances + Galactosuria Gal-1-P and UDP-Gal increased Mod. generalized aminoaciduria		Gal-1-P increased	Gal-1-P increased Abn. transferrin IEF	Gal-1-P increased Abn. transferrin IEF	Urinary-reducing substances +
GALE activity	RBC: ↓↓↓ Fibroblasts: ↓↓↓	Fibroblasts: ↓↓↓	RBC: ↓↓↓ Fibroblasts: Ø Liver tissue:10%	Amniocytes: 5%	RBC: Ø Fibroblasts: Ø	RBC: Ø	RBC: ↓↓↓	RBC: ↓↓↓
GALE mutation	Homozygous c.280G>A (p.[V94M])	Homozygous c.280G>A (p.[V94M])	Homozygous c.280G>A (p.[V94M])	–	–	Homozygous c.280G>A (p.[V94M])	Homozygous c.280G>A (p.[V94M])	–

[a] Presymptomatic diagnosis; ↓↓↓, markedly reduced; Ø, absent; *Abn* abnormal, *CDT* carbohydrate-deficient transferrin, *Gal-1-P* galactose-1-phosphate, *Mod.* moderated, *RBC* red blood cells, *SN* sensorineural, *UDP-Gal* uridine diphosphate galactose

transferase reaction, possibly because of the limited availability of UTP, and at this point, Gal-1-P and galactose will begin to accumulate also. The responsibility of the different accumulating metabolites in the genesis of clinical manifestations and long-term complications remain unclear.

On the basis of present knowledge, the main objective in the therapeutic approach of generalized epimerase-deficient galactosemia seems to be the achievement of a balance between dietary restriction of galactose to prevent galactose toxicity and supplying enough galactose for essential requirements (Henderson et al. 1983, Walter et al. 1999, Openo et al. 2006, Sarkar et al. 2010, Fridovich-Keil 2013). However, the galactose intake for optimum outcome remains unknown, and, as in classical galactosemia, there is no guarantee that long-term complications will be completely prevented by this approach (Fridovich-Keil 2013).

In conclusion, the present report expands the clinical spectrum of GALE deficiency.

Take Home Message

Generalized GALE deficiency (galactosemia type 3) is a very rare inherited metabolic disease, associated with a secondary CDG and with a variable clinical spectrum that can include cardiomyopathy.

Compliance with Ethics Guidelines

Conflict of Interest

Filipa Dias Costa, Sacha Ferdinandusse, Carla Pinto, Andrea Dias, Liesbeth Keldermans, Dulce Quelhas, Gert Matthijs, Petra A. Mooijer, Luísa Diogo, Jaak Jaeken, and Paula Garcia declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from patients' legal guardians for being included in the study.

Additional informed consent was obtained from them for which identifying information is included in this article.

Animal Rights

This article does not contain any studies with animal subjects performed by any of the authors.

Details of the Contributions of Individual Authors

FDC gathered the data and drafted the manuscript to report the work. PG and LD were responsible for planning and conducting the diagnostic investigation and patients' follow-up and for the process of critical review of the manuscript. AD and CP assisted both patients during their hospitalizations in PICU and made significant contributions to the content of the manuscript. DQ collaborated in diagnostic investigation and reviewed the manuscript. SF and PAM performed GALE enzymatic studies and critically reviewed the data. JJ, LK, and GM were responsible for the biochemical, enzymatic, and genetic CDG studies. JJ gave significant contributions in the process of manuscript's revision. All authors gave their final approval of the version to be published.

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RESEARCH REPORT

Development and Psychometric Evaluation of the MetabQoL 1.0: A Quality of Life Questionnaire for Paediatric Patients with Intoxication-Type Inborn Errors of Metabolism

Nina A. Zeltner · Matthias R. Baumgartner · Aljona Bondarenko · Regina Ensenauer · Daniela Karall · Stefan Kölker · Chris Mühlhausen · Sabine Scholl-Bürgi · Eva Thimm · Julia Quitmann · Peter Burgard · Markus A. Landolt · Martina Huemer

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Abstract *Introduction*: This study is part of the "European network and registry for intoxication type metabolic diseases" (E-IMD) project. Intoxication-type inborn errors of metabolism (IT-IEM) such as urea cycle disorders (UCD) and organic acidurias (OA) have a major impact on patients' lives. Patients have to adhere to strict diet and medication and may suffer from metabolic crises and neurocognitive impairment. Disease-specific health-related quality of life (HrQoL) assessment questionnaires are the method of choice to estimate the subjective burden of a

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"Peter Burgard," "Markus A. Landolt," and "Martina Huemer" are contributed equally to this project.

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N.A. Zeltner · M.R. Baumgartner · M. Huemer (✉)
Division of Metabolism, Children's Research Center, University Children's Hospital, Zurich, Switzerland
e-mail: nina.zeltner@kispi.uzh.ch; matthias.baumgartner@kispi.uzh.ch; martina.huemer@kispi.uzh.ch

N.A. Zeltner · M.A. Landolt
Department of Psychosomatics and Psychiatry, Children's Research Center, University Children's Hospital, Zurich, Switzerland
e-mail: nina.zeltner@kispi.uzh.ch; markus.landolt@kispi.uzh.ch

N.A. Zeltner · M.R. Baumgartner · M. Huemer
radiz – Rare Disease Initiative Zurich, Clinical Research Priority Program for Rare Diseases, University of Zurich, Zurich, Switzerland
e-mail: nina.zeltner@kispi.uzh.ch; matthias.baumgartner@kispi.uzh.ch; martina.huemer@kispi.uzh.ch

N.A. Zeltner · M.A. Landolt
Division of Child and Adolescent Health Psychology, Department of Psychology, University of Zurich, Zurich, Switzerland
e-mail: nina.zeltner@kispi.uzh.ch; markus.landolt@kispi.uzh.ch

M.R. Baumgartner
Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland
e-mail: matthias.baumgartner@kispi.uzh.ch

A. Bondarenko · S. Kölker · P. Burgard
Center for Paediatric and Adolescent Medicine, Division for Neuropaediatrics and Metabolic Medicine, Heidelberg University Hospital, Heidelberg, Germany
e-mail: aljona.bondarenko@med.uni-heidelberg.de; stefan.koelker@med.uni-heidelberg.de; peter.burgard@med.uni-heidelberg.de

R. Ensenauer · E. Thimm
Division of Experimental Pediatrics and Metabolism, Department of General Pediatrics, Neonatology and Pediatric Cardiology, University Children's Hospital, Heinrich Heine University Düsseldorf, Düsseldorf, Germany
e-mail: regina.ensenauer@med.uni-duesseldorf.de; eva.thimm@med.uni-duesseldorf.de

D. Karall · S. Scholl-Bürgi
Clinic for Pediatrics, Inherited Metabolic Disorders, Medical University of Innsbruck, Innsbruck, Austria
e-mail: daniela.karall@i-med.ac.at; sabine.scholl-buergi@tirol-kliniken.at

C. Mühlhausen
University Children's Hospital, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
e-mail: muehlhausen@uke.de

J. Quitmann
Department of Medical Psychology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
e-mail: j.quitmann@uke.de

M. Huemer
Department of Paediatrics, LKH Bregenz, Bregenz, Austria
e-mail: martina.huemer@kispi.uzh.ch

disease. To date, no such instrument is available for IT-IEM.

Methods: Disease-specific patient- and parent-reported HrQoL questions were constructed in German based on focus group interviews with patients and parents. Questionnaires for patients from 8 to 18 years were piloted with 14 participants ($n = 9$ children and adolescents, $n = 5$ parents) by cognitive debriefing and tested psychometrically with 80 participants ($n = 38$ patients, $n = 42$ parents) for item characteristics, validity, and reliability to construct the first version of a disease-specific HrQoL questionnaire.

Results: Twenty-eight questions were selected based on item descriptives. Scales of self- and proxy questionnaires demonstrated acceptable to excellent reliability in terms of internal consistency (Cronbach's $\alpha = 0.70–0.93$). Scales and total scores correlated with those of generic HrQoL questionnaires, showing convergent validity.

Discussion: The MetabQoL 1.0 questionnaire exhibits sound psychometric properties and is a promising step towards assessing patient-reported outcomes in research and clinical practice. It provides a solid basis for translation into other languages and further elaboration and psychometric exploration in larger populations.

Introduction

This study is part of the "European network and registry for intoxication type metabolic diseases" (E-IMD) project, focusing on intoxication-type inborn errors of metabolism (IT-IEM) such as urea cycle disorders (UCD) and organic acidurias (OA). Estimated incidences are 1:35,000 for UCD (Summar et al. 2014) and 1:21,000 for OA (Dionisi-Vici et al. 2002). Recently, the natural course of the diseases has been described in two large samples (Kölker et al. 2015a; Kölker et al. 2015b; Waisbren et al. 2016). These reports highlight that IT-IEM have a major impact on patients' lives: Strict diet, daily intake of medication, the permanent risk of severe metabolic crises, and neurological sequelae are only some of the issues that the growing number of long-term surviving patients and their families face. Therefore, it is of utmost importance to consider health-related quality of life (HrQoL) as a major outcome parameter for this patient group besides medical and biochemical measures (Matza et al. 2004).

HrQoL is defined as "a patient's perception of the impact of disease and treatment on functioning in a variety of dimensions, including physical, psychological, and social domains" (Varni et al. 1999, p. 126). Due to the subjectivity of this construct, self-assessments by patients are the preferred data source (Matza et al. 2013). However, although self- and proxy assessments, e.g. by parents, often differ, parents can be very valuable as an additional source of information, especially in young or severely affected patients (Upton et al. 2008).

There are three main types of HrQoL assessment tools. Generic tools such as the PedsQL (Varni et al. 1999) target the general population and allow comparison between healthy individuals and individuals affected by any kind of disease. Chronic-generic tools such as the DISABKIDS (The DISABKIDS Group Europe 2006) allow more specific comparison between individuals affected by different diseases. Disease-specific tools such as the PKU-QOL (Regnault et al. 2015) investigate the impact of a particular disease or disease group on patients' life. They have shown high responsiveness to change of HrQoL (Wiebe et al. 2003) and are therefore the method of choice for measuring this outcome parameter in clinical trials or long-term patient management.

No such disease-specific instrument was available for IT-IEM (Zeltner et al. 2014). We therefore developed a questionnaire of this type, the MetabQoL 1.0, following the ISPOR Guidelines (Matza et al. 2013). Four versions were constructed: self- and parent reporting versions for patients from 8 to 18 years and adapted self- and parent reporting versions for patients younger than 8 years. The development process encompassed three main steps. First, focus group interviews were performed to identify core topics with high content validity; details of the procedure and results have been reported elsewhere (Zeltner et al. 2016). Items were constructed based on focus group results and the available literature (e.g. The DISABKIDS Group Europe 2006; Regnault et al. 2015).

This paper describes the second and third steps of the questionnaire's development for the patients' group aged from 8 to18 years. The second step was the exploration and adaptation of item comprehensibility and clarity ("cognitive debriefing") in children, adolescents, and adults. The third step was the psychometric evaluation of the instrument in a larger group. Item descriptives (e.g. mean, missing values, selectivity) served to select the most useful items, internal consistency of the questionnaire was calculated to assess reliability, and correlations between scores of the new instrument and those of well-established generic HrQoL questionnaires were used to test for convergent validity.

Methods

Subject Recruitment

For the cognitive debriefing, healthy children and adolescents as well as paediatric patients with UCD, OAs, or maple syrup urine disease (MSUD) from 8 to18 years were

recruited in Innsbruck and Zurich to test the comprehensibility and feasibility of the questionnaire booklet. Patients who had received a liver transplant for treatment of their metabolic disease were asked to recall the period before transplantation.

For psychometric evaluation, a sample of families with at least one child diagnosed with UCD or OA aged 8–18 years from the metabolic centres of Düsseldorf, Hamburg, Heidelberg, Innsbruck, and Zurich was invited by a member of the local medical team to participate in the study. Transplanted patients were not included.

For both study phases (cognitive debriefing and psychometric evaluation), individuals were excluded if they had insufficient command of the German language or were incapable of answering the questions due to neurocognitive constraints.

Materials

Questionnaire booklets were created for patients and parents. Patient self-report questionnaire booklets and parent proxy-report questionnaire booklets both contained basic demographic items, the newly developed questions for the MetabQoL 1.0 instrument, and well-established HrQoL questionnaires (described below). Parents worked on the booklet independently, while patients answered all questions in a one-to-one interview with a trained interviewer with medical or psychological background at their homes or at the hospital.

MetabQoL 1.0

Patient and parent questionnaires included the set of newly developed items for the MetabQoL 1.0 instrument. The items were elaborated by discussion among four of the authors (N.A.Z., M.L., M.B., M.H.); they were originally written in German and were translated into English for presentation in this report.

A set of 52 questions was developed for parallel self- and proxy assessment for patients from 8 to 18 years. Fifty questions are answered using 5-point Likert frequency scales (options: never, seldom, sometimes, often, always), with an additional answer option (e.g. “no problem with this”) for questions not applicable for all patients (e.g. tube feeding). Two questions assess disease severity during the last 12 months: (1) the disease has been “not bad at all,” “slightly bad,” “medium bad,” “bad,” or “very bad” and (2) number of hospital admissions “never,” “once,” “twice,” “three to five times,” or “six times or more.” Item scores can be aggregated to scale scores, which represent the core dimensions of physical, mental, and social HrQoL and a HrQoL total score.

PedsQL and DISABKIDS

Patients’ generic and chronic-generic HrQoL was assessed using self- and proxy assessment versions of the PedsQL (Varni et al. 1999; Felder-Puig et al. 2004) and the DISABKIDS-37 (The DISABKIDS Group Europe 2006); both instruments are reliable in terms of psychometric properties.

The PedsQL is a well-established instrument to assess the generic HrQoL of children and adolescents from 8 to18 years with a recall period of 4 weeks. Twenty-three items are answered on a 5-point Likert frequency scale. The PedsQL has scale scores for physical, social, emotional, and school-related HrQoL. Social, emotional, and school-related HrQoL can be aggregated to a psychosocial health score. Sum scores of all scales represent the HrQoL total score (Varni et al. 1999). The internal consistency of the PedsQL total scale scores in the current sample was good to excellent, with Cronbach’s $\alpha = 0.88/0.93$ (self-/proxy report).

The DISABKIDS assesses HrQoL in children with chronic disease from 8 to 16 years with a recall period of 4 weeks. The answering format comprises 5-point Likert frequency scales. Six scales represent the three main dimensions of HrQoL: limitation and medication (physical HrQoL), independence and emotion (mental HrQoL), and inclusion and exclusion (social HrQoL). Furthermore, a total HrQoL score can be computed including all scales (The DISABKIDS Group Europe 2006). The internal consistency of the DISABKIDS total scale scores in the current sample was good to excellent, with Cronbach’s $\alpha = 0.87/0.95$ (DISABKIDS self-/proxy report).

Cognitive Debriefing

The MetabQoL 1.0 questionnaire was tested for comprehensibility, relevance, and feasibility in a sample of five patients ($n = 2$ females, $n = 3$ males; age range = 8.72–16.77 years, mean = 12.42 ± 4.05 years; $n = 1$ liver transplanted) and their parents ($n = 5$ mothers). After completing the questionnaire, one-to-one interviews were conducted at the patient’s home, at the hospital, or by phone. The feasibility of the whole booklet for psychometric evaluation containing all three HrQoL questionnaires (MetabQoL 1.0, PedsQL, DISABKIDS) was assessed by interviewing four healthy partic-

ipants ($n = 2$ females, $n = 2$ males; age range = 9.83–18.09 years, mean = 12.66 ± 4.36 years) at home. All comments were discussed, and two of the authors (N.A.Z., M.H.) decided adaptations to the booklet.

Psychometric Evaluation

Cases were excluded if ≥20% of the MetabQoL 1.0 data were missing. The randomness of the remaining missing data was analysed with Little's MCAR test to ensure that the imputation method was appropriate. Missing values in the MetabQoL 1.0 were then imputed using the full information maximum likelihood (FIML) method (Arbuckle 1996).

The PedsQL and the DISABKIDS were scored according to the corresponding manuals (Varni et al. 1999; The DISABKIDS Group Europe 2006). Original scores of the MetabQoL 1.0 (never/not applicable =0, seldom =1, sometimes =2, often =3, always =4) were rescaled to values between 0 and 100 (0 = 100, 1 = 75, 2 = 50, 3 = 25, 4 = 0). Values of positively formulated items were reversed to allow comparability with PedsQL and DISABKIDS. Accordingly, the best HrQoL was indicated by values of 100, worst by 0. Scales of physical, mental, and social HrQoL were computed by the mean of the corresponding item values. A total score was computed by the mean of all item values of the physical, mental, and social scales.

Item selection was performed in two steps. The first step was based on cut-offs derived from the literature (Bühner 2011) and the distribution of item descriptives in the current sample. Items were considered for exclusion if their mean value was ≥90/85 (self-/proxy report), or if selectivity was <0.3, or if correlation with other items was ≥0.80, or missing raw data was >5% (e.g., indicating low acceptance of an item), or if lack of comprehensibility had been documented in the interview setting.

The second selection step consisted of screening these problematic items. Items remained in the instrument if their content was vital to cover main issues from the focus groups, or in the interest of parallel content and comparability of the self- and proxy-report questionnaires.

Reliability defined as internal consistency for total and scale scores was determined using Cronbach's alpha. Scores ≥0.7 were considered acceptable (Scientific Advisory Committee of the Medical Outcomes Trust 2002). Concurrent validity between MetabQoL 1.0 and PedsQL/DISABKIDS subscales and total scores was determined by Spearman correlations. Due to the small sample size, factor analysis models were not applicable (Bühner 2011).

Analyses were performed with the statistical software package SPSS, version 22.0, and Amos Version 23.0 for Windows (IBM Corp. IBM SPSS Statistics for Windows. Armonk, NY: IBM Corp). A predefined significance level of $p < 0.05$ was set for all tests.

Results

Sample Characteristics

Of 87 families approached, 46 (53%) participated in the psychometric evaluation. This resulted in a sample of 80 participants: 38 patients ($n = 17$ females, $n = 21$ males; age range = 7.86–17.77 years, mean age = 12.56 ± 3.03 years; $n = 25$ OA, $n = 13$ UCD) with IT-IEM and 42 parents ($n = 35$ mothers, $n = 7$ fathers) of children with IT-IEM ($n = 19$ females, $n = 23$ males; age range = 8.49–18.34 years, mean age = 13.42 ± 3.04 years; $n = 27$ OA, $n = 15$ UCD; 32 parent-child pairs).

Cognitive Debriefing Results

Overall, patients and parents reported good feasibility of the MetabQoL 1.0 and the validation booklet. The majority of the questions were considered comprehensive and relevant to patients and parents. The guidance of the interviewer was important to improve patients' concentration and ensure that they understood the questions. Some questions (e.g., addressing motoric function, tube feeding) were not applicable for all patients, but their parents were aware of their relevance for other patients. Five questions had to be rephrased to increase comprehensibility and one to increase relevance.

Psychometric Evaluation Results

The self- and proxy questionnaires showed 6.01%/1.31% of randomly missing data in the MetabQoL 1.0 (Little's MCAR test, $x^2 = 10.02/x^2 = 464.94$, DF = 292/DF = 492, $p = 1.00/p = 0.80$) and therefore are qualified for the application of data imputation.

Item Selection

The item selection process resulted in a final sample of 28 items. Selected items and their scale affiliation, representing the first version of the MetabQoL 1.0, are listed in Table 1. Detailed item descriptives and the selection process of all items are shown in Supplementary Table 1.

Reliability

Psychometric properties of the MetabQoL 1.0 scales and their correlations are summarised in Tables 2 and 3. Means and skewness were higher for self-reported HrQoL than for

Table 1 Items included in the first version of the MetabQoL 1.0 for self-assessment[a]

	Items included based on item analysis[b]	Scale
1	Does it bother you that you are not allowed to eat anything you want?	Physical
2	Does it bother you that you have to eat even when you are not hungry?	Physical
3	Does it bother you that you have to take medications?	Physical
4	Does the taste of your medications bother you?	Physical
5	Does it bother you that you have regular check-ups?	Physical
6	Are you afraid of having blood taken?	Physical
7	Do you worry that you may have to go to the hospital due to an emergency?	Physical
8	Do you worry about the results of your blood test?	Physical
9	Does your metabolic disorder bother you when you are playing or during other activities?	Physical
10	Does it bother you that you cannot move as well as others?	Physical
11	Does it bother you that you get tired quickly?	Physical
12	Does it bother you that you often feel sick to your stomach?	Physical
13	Does it bother you that you have a feeding tube?	Physical
14	Do you have trouble keeping up in school/in your apprenticeship because of your metabolic disorder?	Mental
15	Are you happy?	Mental
16	Are you worried about your metabolic disorder?	Mental
17	Are you sad because you have a metabolic disorder?	Mental
18	Are you angry at having a metabolic disorder?	Mental
19	Are you afraid of the future because of your metabolic disorder?	Mental
20	Are you having problems doing things with friends because of your metabolic disorder?	Social
21	Are others less willing to be friends with you because of your metabolic disorder?	Social
22	Does it bother you that your parents or others in your family are particularly worried about you because of the metabolic disorder?	Social
23	Does it bother you that people treat you differently because of your metabolic disorder?	Social
24	Does it bother you that many people do not understand your metabolic disorder?	Social
25	Do you get left out because of your metabolic disorder?	Social
26	Does it bother you when other people feel sorry for you?	Social
27	How bad were your problems with your metabolic disorder over the last 12 months?	Severity
28	In the past 12 months, how often did you have to be admitted to the hospital in an emergency?	Severity

[a] The proxy assessment questionnaire consists of parallel rephrased items (e.g. item 1: Does it bother your child that he/she is not allowed to eat anything he/she wants?)

[b] Answering options: never, seldom, sometimes, often, always (items 1–26); not bad at all, slightly bad, medium bad, bad, very bad (item 27); never, once, twice, three to five times, six times or more (item 28)

proxy-reported HrQoL. Floor effects were not present, in contrast to ceiling effects, which were more dominant in self-reports than in proxy reports. Overall, reliability in terms of internal consistency was acceptable to excellent throughout all scales and total scores with a range of Cronbach's $\alpha = 0.70–0.93$, which was generally higher in proxy reports than in self-reports. All scale intercorrelations of the MetabQoL 1.0 were significant, ranging $r = 0.60–0.96$.

Validity

Convergent validity between the MetabQoL 1.0 and PedsQL/DISABKIDS was present and generally higher for the DISABKIDS than for the PedsQL (Table 4). Correlations were not limited to corresponding scales but also present between noncorresponding scales (e.g. MetabQoL 1.0 physical scale with PedsQL scales).

Discussion

This study presents the development process of the first disease-specific HrQoL questionnaire for paediatric patients with IT-IEM, the MetabQoL 1.0. The content validity of the questionnaire in general was ensured by involving patients and their parents in focus group interviews at the very beginning of the questionnaire development process (Zeltner et al. 2016). Questions for self- and proxy assessment were constructed based on statements from the focus groups. Cognitive debriefing was performed to further refine and focus the items of the questionnaire and to gain a first impression concerning the practical applicability of the instrument. Testing of the questionnaire in a larger sample of IT-IEM patients was conducted to analyse its psychometric properties.

Reliability in terms of internal consistency was acceptable to excellent for all scales and total scores. A general tendency towards high HrQoL was observed. This is consistent with data from other disease-specific questionnaires (Regnault et al. 2015; Bullinger et al. 2015). Correlation with the PedsQL and DISABKIDS scales was investigated to examine concurrent validity. Correlations in a medium range indicated that beyond measuring the construct of HrQoL in general, the MetabQoL 1.0 – as intended – adds specific content and information. This result underscores the benefit of this disease-specific questionnaire. As expected, correlations with the chronic-generic instrument, DISABKIDS, were higher than with the generic instrument, PedsQL. Since the DISABKIDS specifically addresses a population with health conditions, conceptualization of HrQoL was closer to the MetabQoL 1.0. Nevertheless, due to its more specific content, we

Table 2 Psychometric properties of the MetabQoL 1.0 questionnaire

Scale	Descriptive statistics							Reliability
	N items	Mean	SD	Median	Skewness	% Floor	% Ceiling	Cronbach's α
		Self/proxy	Self/proxy	Self/proxy	Self/proxy	Self/proxy	Self/proxy	Self/proxy
Physical	13	81.68/69.92	14.70/20.75	84.16/71.15	−0.92/−0.78	0/0	13.2/2.4	0.77/0.90
Mental	6	85.42/71.63	15.43/20.58	89.58/77.08	−1.36/−0.27	0/0	23.7/9.5	0.70/0.81
Social	7	86.47/72.87	15.60/20.22	92.86/73.21	−1.19/−0.36	0/0	26.3/14.3	0.70/0.81
Total score	**26**	**83.83/71.11**	**13.39/18.68**	**87.98/71.15**	**−0.95/−0.32**	**0/0**	**5.3/2.4**	**0.88/0.93**
Disease severity	2	81.25/75.30	21.89/26.69	87.50/87.50	−1.34/−1.12	0/0	39.5/31	

Table 3 Scale intercorrelations of the MetabQoL 1.0

Scales	Correlation coefficient
	Self/proxy
Physical – mental	0.67*/0.75*
Mental – social	0.60*/0.77*
Social – physical	0.66*/0.77*
Total score – physical	0.96*/0.95*
Total score – mental	0.78*/0.86*
Total score – social	0.79*/0.90*
Severity – physical	0.53*/0.57*
Severity – mental	0.48 */0.41*
Severity – social	0.48*/0.55*
Severity – total score	0.55*/0.57*

*$p < 0.05$

hypothesise that the MetabQoL 1.0 will be more responsive to disease-related changes than the chronic-generic DISABKIDS (Wiebe et al. 2003) and thus most valuable for clinical practice and research settings. This hypothesis will be followed up in long-term studies.

Generally, the results of proxy assessment were more favourable in terms of psychometric validity than the results of self-assessment. Self-assessment revealed higher means, increased skewness, lower reliability scores, and considerable ceiling effects for the mental and social scales. The modality of data collection may have influenced the answers; parents completed the questionnaires independently, while children were interviewed. The focus groups performed at the beginning of the questionnaire's development allowed space to freely express and discuss opinions and feelings, encouraged by exchange with other affected patients. The standardised questionnaire interview is a less open communication situation and may have favoured socially desirable answers. This idea is supported by the observation that in contrast to focus group interviews, where stigmatisation was a central topic (Zeltner et al. 2016), patients neglected this issue in the individual interview situation.

Higher self-ratings than proxy ratings of children's HrQoL in healthcare are well known from the literature (Eiser and Jenney 2007; Jamiolkowski et al. 2016). Children have a more intuitive, spontaneous view of a situation than adults and a tendency towards extreme answers (Chambers 2002). Furthermore, the effects of fatigue may be more prominent in children and adolescents than in adults and may have led to a lack of concentration during the interviews. This assumption is supported by the results of the cognitive debriefing, which emphasised the necessity of an interviewer guiding the patients through the questionnaire booklet to maintain concentration. Some IT-IEM patients have neurocognitive deficits, and their chronological age may not fully reflect their development age and concentration abilities. To reduce this bias, the questionnaire was kept as short as possible, with 28 items after psychometric evaluation. Furthermore, the more comprehensible 10-item smiley version of the MetabQoL 1.0 instrument for patients younger than 8 years, which is currently under development, may also prove useful in older patients with cognitive impairment.

Notably, 83% of the participating parents were female. In research about paediatric patients, higher representation of mothers compared to fathers is a well-known phenomenon (Goldstein et al. 2013) and may limit the generalisability of parent reports.

The pattern of correlation between the MetabQoL 1.0 scales and the PedsQL and DISABKIDS scales showed not only correlations between corresponding scales of the three instruments but also correlations between noncorresponding scales. Furthermore, correlations were observed between total score and physical, mental, and social scales of the MetabQoL 1.0. Highest correlations were found between physical HrQoL and the total score, which is, however, partly due to the large number of items these scores share. Overall, these findings lead to the hypothesis that there may be only a single dimension behind the items of the MetabQoL 1.0. The concept of the classical three

Table 4 Correlation coefficients between the MetabQoL 1.0 and the PedsQL (generic)/DISABKIDS (chronic generic)

		PedsQL						DISABKIDS						
								Physical		Mental		Social		
		Physical	Emotional	Social	School	Psychosocial	Total	Limitation	Medication	Independence	Emotion	Inclusion	Exclusion	Total
MetabQoL 1.0 self-report[a]	Physical	0.36*	0.43*	0.44*	0.41*	0.57*	0.52*	0.59*	0.57*	0.57*	0.55*	0.41*	0.47*	0.70*
	Mental	0.20	0.34*	0.23	0.43*	0.47*	0.36*	0.50*	0.57*	0.57*	0.69*	0.41*	0.53*	0.69*
	Social	0.33*	*0.47	0.28	0.26	0.42*	0.40*	0.53*	0.47*	0.53*	0.56*	0.27	0.43*	0.57*
	Total	0.37*	0.46*	0.45*	0.41*	0.57*	0.52*	0.63*	0.64*	0.63*	0.61*	0.43*	0.52*	0.75*
MetabQoL 1.0 proxy report	Physical	0.62*	0.51*	0.63*	0.52*	0.69*	0.72*	0.84*	0.62*	0.65*	0.67*	0.57*	0.74*	0.79*
	Mental	0.45*	0.60*	0.59*	0.47*	0.67*	0.62*	0.68*	0.79*	0.58*	0.86*	0.52*	0.86*	0.84*
	Social	0.67*	0.61*	0.72*	0.46*	0.73*	0.75*	0.82*	0.57*	0.73*	0.71*	0.70*	0.76*	0.85*
	Total	0.63*	0.59*	0.71*	0.53*	0.75*	0.75*	0.86*	0.72*	0.71*	0.78*	0.65*	0.83*	0.89*

* $p < 0.05$

[a] Sample size for convergent validity analysis was $n = 37$ in self-report ($n = 1$ excluded due to complete missing of DISABKDIS and PedsQL scores) and thereby different from all other psychometric analyses

dimensions of HrQoL is under discussion, and others have proposed the sole use of a single total HrQoL score (Solans et al. 2008). Particularly, in IT-IEM, the influence of the disease on patients' lives may be global. Physical aspects such as diet have a strong influence on social and mental aspects (feeling different or socially excluded). IT-IEM predominantly affect the brain. Therefore, cognitive functioning is strongly associated with the physical dimension. This association seems specific and is not present in the majority of generic HrQoL questionnaires for children (Rajmil et al. 2004) in which cognitive and emotional functioning constitutes an independent mental dimension.

Factor analysis, which might have elucidated the structure of the MetabQoL 1.0 in more detail, could not be performed due to the sample size (MacCallum et al. 1999). Although the international character of this study increased sample size, the diseases are rare, and large patient samples can only be gathered over time. Therefore, the questionnaire will be translated to be applied in larger samples in the near future. Further psychometric exploration will also include analyses of criterion validity, which was not addressed in this study. Criterion validity refers to the ability of a questionnaire to distinguish between different groups of patients. Furthermore, a larger sample may allow providing normative data. Normative data form the basis to compare between patients and to describe an individual's position within the reference group, which clearly is of long-term scientific interest. For now, the MetabQoL 1.0 is a tool to identify profiles of concerns and strains an individual patient experiences and opens the field for targeted clinical counselling. Furthermore, changes of a patient's HrQoL over time or under different treatment conditions can be monitored. These uses are not bound to normative data.

The MetabQoL 1.0 may be applied in clinical practice as well as in research, especially to detect changes in HrQoL over time. In clinical practice, monitoring HrQoL over time facilitates the identification of patients' needs and emotional and social aspects of the disease, which may not easily be detected in clinical routine. Notably, impaired social HrQoL has been shown for IT-IEM before (Fabre et al. 2013). Interestingly, the use of HrQoL instruments generally improves communication between patients and the medical team, which results in enhanced patients' well-being (Velikova et al. 2004). This is of particular importance during transitional phases such as transition to kindergarten, to school, and to adolescence (Packman et al. 2012; Khangura et al. 2015), when the impact of the disease and specific needs may change.

Considering research, disease-specific HrQoL measures are a most interesting additional approach for measuring outcome in clinical trials (Wiebe et al. 2003). The MetabQoL 1.0 is a promising tool for assessing disease-related HrQoL changes in IT-IEM. Furthermore, the questionnaire facilitates the exploration of predictors of HrQoL in IT-IEM patients and the development of interventions targeting patients' needs.

Conclusion

The MetabQoL 1.0 is the first psychometrically evaluated HrQoL questionnaire addressing the specific impact of IT-IEM on patients. Its targeted approach – in contrast to

generic measures – renders the MetabQoL 1.0 a valuable measure in clinical and research settings. Translation into other languages and further evaluation will allow broader application of the instrument.

Intellectual Property and Conditions of Use

Researchers or clinicians interested in using the MetabQoL 1.0© may contact the corresponding author (martina.huemer@kispi.uzh.ch).

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One Sentence Take-Home Message

A newly developed disease-specific health-related quality of life questionnaire for intoxication-type inborn errors of metabolism (MetabQoL 1.0) allows insight into the subjective burden of disease among children and adolescents.

Details of the Contributions of Individual Authors

N.A.Z. was involved in designing the study, collected and analysed the data, and drafted the manuscript. M.R.B. was involved in designing the study, contributed patient data, and critically reviewed the manuscript. A.B., R.E., D.K., S.K, C.M., S.S.B., and E.T. contributed patient data. J.Q. was involved in study design and gave advice on data analysis. P.B. was involved in coordination of the study and contributed patient data. M.A.L. was involved in designing the study, gave advice on data collection and analysis, and critically reviewed the manuscript. M.H. provided the original concept of the study, coordinated the study, and revised the manuscript. All authors read and approved the final version of the manuscript.

Conflict of Interest

Nina A. Zeltner and Martina Huemer have received research grants from Milupa Metabolics. Matthias R. Baumgartner, Aljona Bondarenko, Regina Ensenauer, Daniela Karall, Stefan Kölker, Chris Mühlhausen, Sabine Scholl-Bürgi, Eva Thimm, Julia Quitmann, Peter Burgard, and Markus A. Landolt declare no conflict of interest.

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RESEARCH REPORT

Widening the Heterogeneity of Leigh Syndrome: Clinical, Biochemical, and Neuroradiologic Features in a Patient Harboring a NDUFA10 Mutation

Francesca Minoia • Marta Bertamino • Paolo Picco • Mariasavina Severino • Andrea Rossi • Chiara Fiorillo • Carlo Minetti • Claudia Nesti • Filippo Maria Santorelli • Maja Di Rocco

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Abstract Leigh syndrome (LS) is an early-onset progressive neurodegenerative disorder, characterized by a wide clinical and genetic heterogeneity, and is the most frequent disorder of mitochondrial energy production in children. Beside its great variability in clinical, biochemical, and genetic features, LS is pathologically uniformly characterized by multifocal bilateral and symmetric spongiform degeneration of the basal ganglia, brainstem, thalamus, cerebellum, spinal cord, and optic nerves. Isolated complex I deficiency is the most common defect identified in Leigh syndrome. In 2011, the first child with a mutation of *NDUFA10* gene, coding for an accessory subunits of complex I, was described. Here, we present an additional description of a child with Leigh syndrome harboring a homozygous mutation in *NDUFA10*, providing insights in clinical, biochemical, and neuroradiologic features for future earlier recognition.

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F. Minoia (✉) · P. Picco
Second Division of Pediatrics, Istituto Giannina Gaslini, Genoa, Italy
e-mail: francescaminoia@gaslini.org

M. Bertamino · M. Di Rocco
Rare Diseases Unit, Istituto Giannina Gaslini, Genoa, Italy

M. Severino · A. Rossi
Neuroradiology Unit, Istituto Giannina Gaslini, Genoa, Italy

C. Fiorillo · C. Minetti
Neuromuscular Diseases Unit, Istituto Giannina Gaslini, Genoa, Italy

C. Nesti · F.M. Santorelli
Molecular Medicine and Neuromuscular Disorders, IRCCS Stella Maris, Calambrone, Pisa, Italy

Introduction

Defects in mitochondrial energy production are the most frequent group of inherited metabolic disorders, with an incidence of at least 1 in 5,000 live births (Skladal et al. 2003). The most common clinical manifestation of mitochondrial disease in children is Leigh syndrome (Leigh 1951) (LS), an early-onset progressive neurodegenerative disorder, characterized by a wide clinical and genetic heterogeneity. Clinical presentation frequently includes psychomotor delay or regression, acute neurological or acidotic episodes, hypotonia, ataxia, spasticity, movement disorders, and corresponding anomalies of the basal ganglia and brainstem on MRI (Tetreault et al. 2015). Up to now, more than 75 disease genes have been linked to LS, involving the oxidative phosphorylation (OXPHOS) system, the pyruvate dehydrogenase complex (PDHc), and multiple other enzymes mostly linked to OXPHOS system or to a broader pathway of energy generation (Lake et al. 2016).

Isolated complex I deficiency is the most common biochemical defect identified in LS, accounting for approximately 25% of all children with OXPHOS deficiencies (Smeitink et al. 2001). Complex I (NADH: ubiquinone oxidoreductase) is the first and the largest multiheteromeric respiratory chain (RC) enzyme complex being composed of 45 protein subunits (7 of which encoded by mtDNA), with a pivotal role in ATP synthesis, transferring electrons from reduced NADH to coenzyme Q10, and pumping protons across the inner mitochondrial membrane. Moreover, its maturation, assembly, and stability rely on a number of ancillary proteins. Up to now, pathogenic mutations have been reported in all the seven mtDNA-encoded subunits

and in several nuclear DNA-encoded subunits or assembly factors, including NADH dehydrogenase 1α subcomplex 10 (NDUFA10) (Lake et al. 2016). NDUFA10 is located in the hydrophobic protein fraction and is homologous to deoxyribonucleoside kinases, but it has no enzymatic activity (Elurbe and Huynen 2016). The function of this accessory subunit is still not clear, but it has been suggested to have a role in the transfer of protons across the inner membrane (Hoefs et al. 2011). Until now, only two cases of pathogenic *NDUFA10* mutations have been reported (Hoefs et al. 2011; Haack et al. 2012), both presenting with LS and complex I deficiency. In the present paper, we further characterize the clinical, biochemical, and neuroradiologic features of a child with LS due to *NDUFA10* mutation.

Case Report: Biochemical and Molecular Analyses

Case Report

We present the case of an Italian boy, the third child of consanguineous parents (third degree cousins), born at 41 weeks of gestation after a normal pregnancy. Family history is remarkable for a paternal cousin who died at the age of 4 months for an unspecified cardiomyopathy, two maternal cousins manifesting an unspecified myopathy, and another cousin affected by an early-onset epileptic encephalopathy. In the proband, birth weight was 3,460 g and length 52 cm. The neonatal period was uneventful, but nystagmus was reported since the first month of life. He reached all his milestones but with a slight delay. At the age of 2 years and 8 months, after a febrile illness, he presented acute-onset ataxia with severe hyposthenia and inability to stand and walk. This condition lasted for approximately a week and was followed by a slow and partial neurological improvement but without complete recovery. A first brain MRI was performed at that age and showed rather symmetrical lesions of the putamina and globi pallidi, characterized by swelling, necrotic changes, and mixed cytotoxic and vasogenic edema. Asymmetric focal lesions of the caudate nuclei were also noted, as well as focal involvement of the left cerebral peduncle (Fig. 1). MR spectroscopy detected a lactate doublet peak.

The child was then referred to our attention for further evaluation. On clinical examination, we observed a generalized hypotonia with muscular hypotrophy especially in the lower limbs; tendon reflexes were slightly increased, and oscillatory nystagmus was present. Gait ataxia and clumsiness were also observed. The psychomotor evaluation showed a moderate delay in his development. Blood and urine tests were unremarkable with the exception of levels of plasma lactate slightly above normal range (39.2 mg/dl, normal range 8–22 mg/dl). The clinical, neuroimaging, and biochemical features strongly suggested a LS, and a defect of PDHc or OXPHOS pathway was considered. At first, on the basis of his clinical presentation and mostly of intermittent ataxia, molecular analysis on PDHc genes was performed and thiamine-biotin therapy was started.

In the next year, the child presented a slight improvement in neurological development with a partial recovery of gait and language. However, severe difficulties in oculomotor abilities persisted with extrapyramidal signs such as dystonic features, nystagmus, and ataxia. Parents reported intermittent relapses, usually associated with febrile illness, with only partial recovery after each episode, resulting in a progressive neurologic deterioration. Cardiologic evaluations were always unremarkable. A second brain MRI, performed at the age of 3 years and 6 months, showed the chronic evolution of the putaminal lesions and the presence of new symmetric lesions of the caudate heads and Meynert basal nuclei and asymmetric lesions at the level of the right globus pallidus and cerebral peduncle. MR spectroscopy, performed on the right caudate nucleus head, revealed a lactate doublet peak (Fig. 2).

Biochemical and Molecular Analyses

Routine morphology, histochemical stains for oxidative metabolism, and spectrophotometric determination of mitochondrial RC complexes in muscle homogenate were assayed according to standard methods.

Deltoid muscle biopsy was performed under the hypothesis of a mitochondrial disease: histological examinations showed only a slight reduction of cytochrome oxidase activity. Biochemical analyses of the respiratory chain revealed a reduction of complexes I (1.12 mmol/min/g tissue; normal range 1.56–2.60), I + II (0.08 mmol/min/g tissue, normal range 0.11–0.25), and II + III (0.02 mmol/min/g tissue, normal range 0.05–0.08) (details available on request).

Genomic blood and muscle DNA from the patient were purified by standard methods and analyzed by Sanger sequencing to exclude pathogenic variants in mitochondrial DNA (mtDNA) and *PDHA1* gene. Then, we used a customized targeted resequencing panel able to investigate the coding regions of 168 genes linked to mitochondrial disorders applying standard methodologies as outlined elsewhere (Mignarri et al. 2016). The identified pathogenic variants in *NDUFA10* (NM_004544.3) were confirmed by Sanger sequencing and were tested for segregation in the family.

Molecular analysis identified the homozygous mutation c.296G>A in exon 3 of the *NDUFA10* gene (Fig. 3a), causing a substitution of the extremely conserved throughout evolution glycine 99 for glutamate (p.G99E) (Fig. 3b). Both parents carried the heterozygous c.296G>A variant

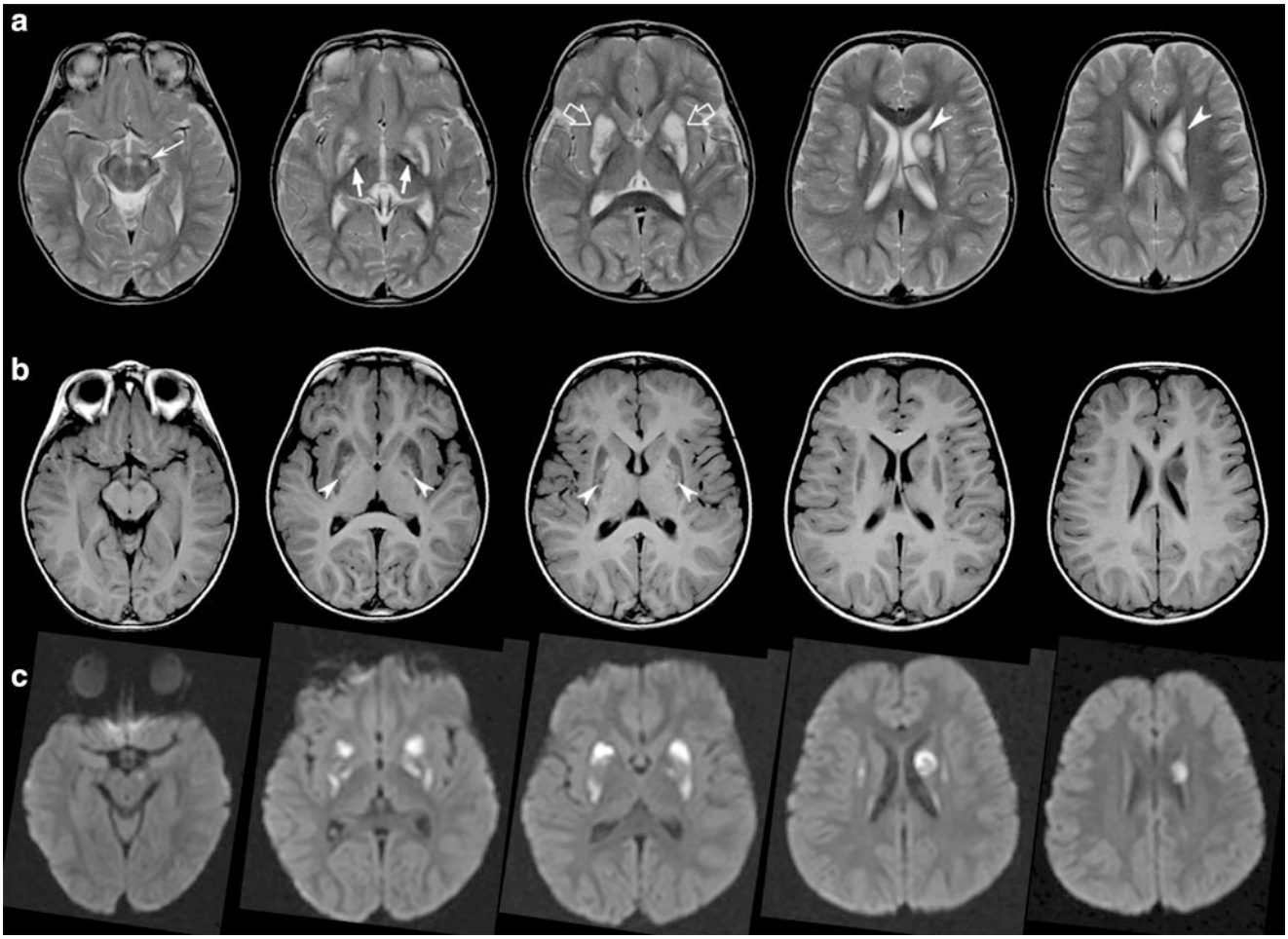

Fig. 1 Brain MRI performed at 2 years and 8 months. (**a**) Axial T2-weighted images demonstrate symmetric swelling and T2 hyperintensity of the putamina (*empty arrows*) and asymmetric involvement of globi pallidi (*arrows*), left cerebral peduncle (*thin arrows*), and left caudate nucleus (*arrowheads*). Note that the lesion of the left caudate head is round and well demarcated, with moderate mass effect. (**b**) Hyperintense necrotic foci are present at the level of the medial part of the putamina and globi pallidi on T1-weighted images (*arrowheads*). On diffusion-weighted images (**c**), most of the lesions are characterized by restricted diffusion in keeping with cytotoxic edema

which was predictably damaging when examined in silico using Polyphen2. The mutation has been already reported in a child presenting with severe complex I deficiency (Haack et al. 2012).

Discussion

The case described here further supports the role of *NDUFA10* mutations as a rare cause of LS with reduced complex I activity, providing new data on the related clinical, biochemical, and neuroradiologic features and contributing to the genotype-phenotype correlation in LS.

LS is the most frequent disorder of mitochondrial energy production in children, as the result of a heterogeneous group of defects in the OXPHOS or in the PDHc systems. Therefore, it is unsurprising that a wide variety of multisystemic symptoms may be reported, including cardiac, hepatic, gastrointestinal, and renal dysfunction (Lopez et al. 2006; Monlleo-Neila et al. 2013). The age of presentation is typically set within 2 years of age (Lake et al. 2016), but there is a wide range of disease onset. As in the case here presented, affected children generally have an acute presentation of symptoms, often during an infection, after an initial period of normal development. Progression is typically episodic, with relapses commonly related to febrile illnesses; often, there may be some initial recovery, but never back to the baseline (Lake et al. 2016; Rahman et al. 1996; Sofou et al. 2014). The overall survival is variable, but most patients die after few years from diagnosis. Poor prognosis predictors include age at disease onset less than 6 months, failure to thrive, seizures resistant to pharmacologic treatments, brainstem lesions on neuroimaging, and intensive care unit admission (Lopez et al.

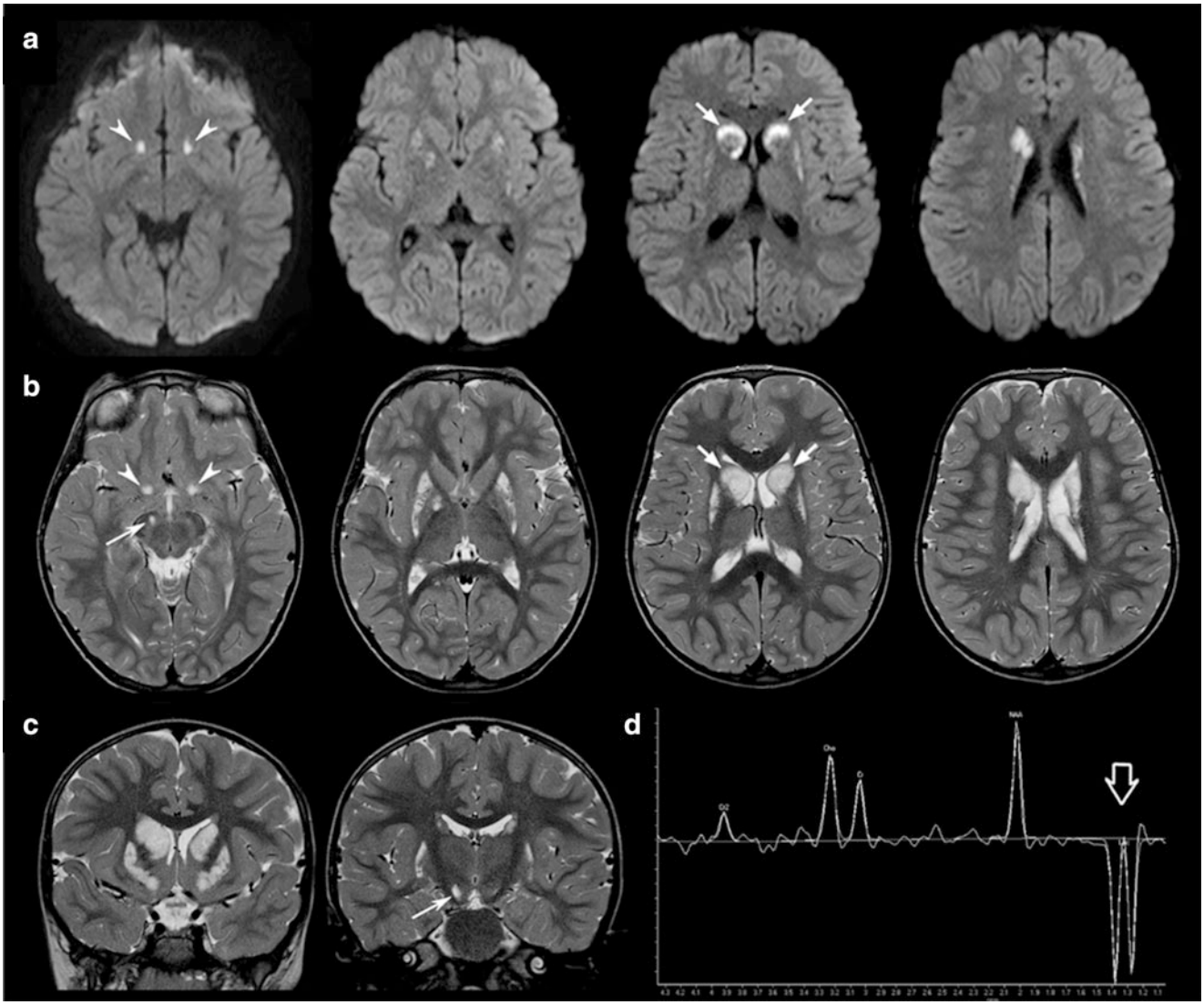

Fig. 2 Follow-up brain MRI and MR spectroscopy performed at 3 years and 6 months. Axial diffusion-weighted images (**a**) and T2-weighted images (axial, **b** and coronal, **c**) reveal new acute lesions characterized by T2 hyperintensity and restricted diffusion due to cytotoxic edema at the level of the basal nuclei of Meynert (*arrow-heads*), right cerebral peduncle (*thin arrow*), and caudate nuclei (*arrows*). Note the peculiar involvement of the heads of caudate nuclei, with prevalent restricted diffusion in the anterior peripheral portions and increased diffusion in the central portions. The putamina are markedly reduced in size with prevalent increased diffusion, in keeping with chronic evolution of the previous lesions. (**d**) The MR spectroscopy performed at the level of the right basal ganglia demonstrates slightly reduced NAA peak and markedly elevated lactate peak (*empty arrow*)

2006; Monlleo-Neila et al. 2013; Rahman et al. 1996; Sofou et al. 2014; Fassone et al. 2011; Benit et al. 2003; Ruhoy and Saneto 2014).

Hoefs et al. firstly described a patient harboring two heterozygous *NDUFA10* mutations, one disrupting the start codon in exon 1 (c. c.1A>G) and the other resulting in an amino acid substitution in exon 3 (p.Gln142Arg). The disease was characterized by an early onset, with hypotonia and developmental delay, and by an aggressive course with severe lactic acidosis, progressive respiratory failure, convulsions, hypertrophic cardiomyopathy, and exitus at 23 months of age (Hoefs et al. 2011). Conversely, in the present patient harboring a homozygous mutation c.296G>A in exon 3 of the *NDUFA10* gene, the clinical course was less severe with later age of onset, longer survival, and absence of hypertrophic cardiomyopathy. In particular, the developmental delay was very mild, and the prevailing neurological features were the episodes of ataxia with initial intercritical recovery. Interestingly, hypertrophic cardiomyopathy, a potential life-threating finding in many patients with complex I deficiency (Fassone et al. 2011; Benit et al. 2003; Fassone and Rhaman 2012), was absent also in the patient reported by Haack et al., who harbored the same homozygous *NDUFA10* mutations in exon 3,

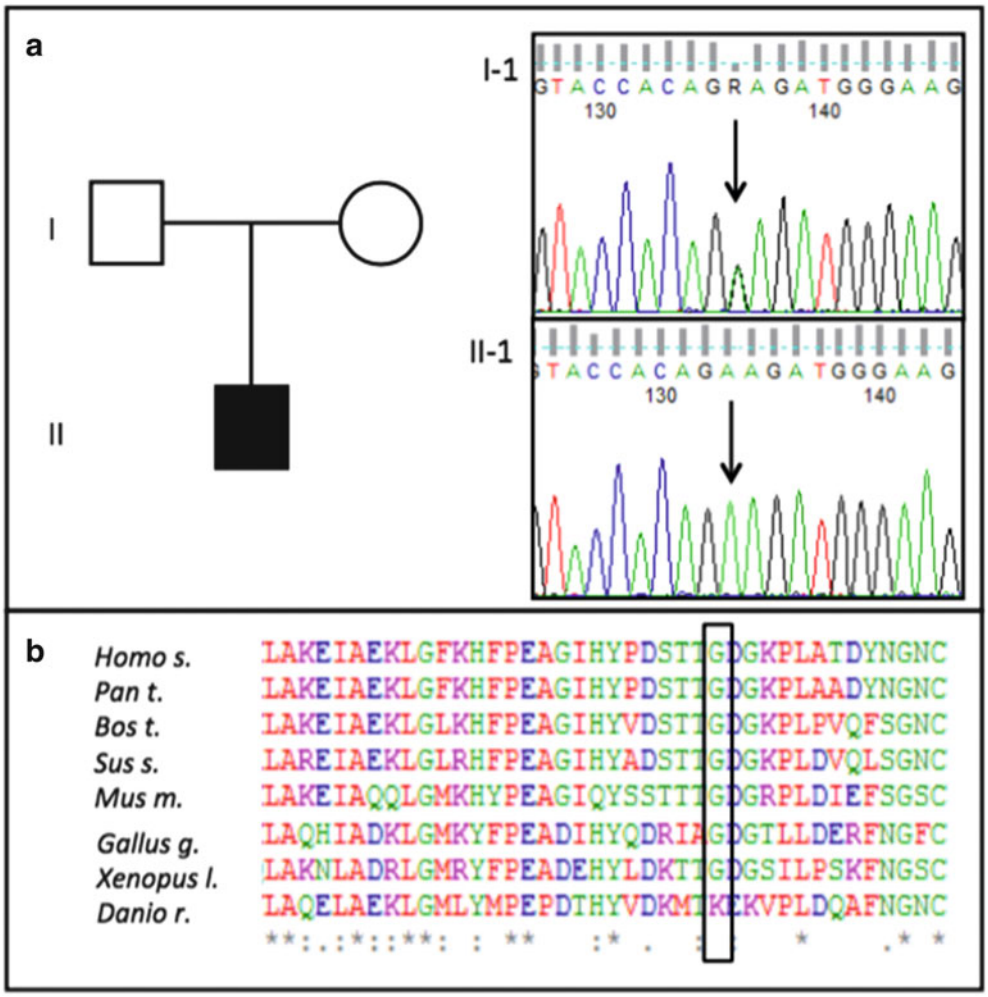

Fig. 3 Molecular analysis of *NDUFA10* gene. Electropherograms present the nucleotide change in fibroblasts of the patient (II-1); *arrows* point out the nucleotide substitution c.296G>A in exon 3 (**a**). *Bottom figure* show the high conservation of the altered amino acid (p. G99E) throughout evolution (**b**)

suggesting the hypothesis of different effects of specific *NDUFA10* mutations in brain and cardiac muscle (Haack et al. 2012).

Of note, the relapsing-remitting course of the disease, and the first positive response to thiamine, initially led us to consider a PDH complex defect as the most likely diagnostic hypothesis (Giribaldi et al. 2012). However, this diagnosis was dismissed by the results of the analysis of RC enzyme activity revealing a defect in the OXPHOS system, subsequently confirmed by molecular assays. In particular, we noticed a clear reduction in complex I activity, associated to a milder decrease in complex I + III and II + III activities. These results are similar to those described by Hoefs et al., underlying the importance of screening for the presence of disease-causing NDUFA10 mutations not only in patients with isolated complex I deficiencies but also in patients with a reduced complex I deficiency combined with a reduction in complex II or III activity. From a histopathologic point of view, similarly to Hoefs et al., we did not found major abnormalities in the patient's muscle biopsy. Indeed, ragged red fibers, a typical sign of many mitochondrial disorders, are rare in LS, and muscle biopsy histology may be completely normal (Sofou et al. 2014; Gerards et al. 2016; Finsterer 2008).

Besides its wide clinical, biochemical, and genetic heterogeneity, LS is uniformly characterized by the presence of multifocal bilateral and symmetric spongiform degeneration of the basal ganglia, thalamus, cerebellum, brainstem, spinal cord, and optic nerves, variably associated to demyelination and gliosis (Rahman et al. 1996; Ruhoy and Saneto 2014). These pathological changes, also described in the autopsy of the child with NDUFA10 mutation reported by Hoefs et al., are well related to the abnormalities commonly identified on brain MRI.

MR imaging in LS usually reveals symmetrical hyperintense lesions of the basal ganglia and brainstem on T2-

weighted images. Cerebellar dentate nuclei are typically abnormal, while spinal cord, hemispheric white matter, and cerebral cortex changes are less frequently found (Lake et al. 2016; Gerards et al. 2016; Lee et al. 2009; Arii and Tanabe 2000). Acutely affected areas may show restricted diffusion, and MR spectroscopy typically reveals elevated lactate, most prominent within the lesions, due to the impairment of the mitochondrial function with secondary energetic failure and cytotoxic edema (Ruhoy and Saneto 2014; Saneto et al. 2008). Interestingly, beside the classical putaminal lesions, the present patient showed a rather unusual asymmetric involvement of the caudate nuclei, globi pallidi, and cerebral peduncles. In particular, peculiar asynchronous round lesions were noted at the level of the caudate heads, characterized by mass effect and mixed restricted and increased diffusion, consistent with the presence of both cytotoxic and vasogenic edema (Fig. 1). Of note, asymmetric involvement of the basal ganglia and brainstem may be found in other mitochondrial diseases. For instance, mutations in *MTFMT*, encoding a mitochondrial methionyl-tRNA formyl-transferase, may cause variably asymmetric Leigh encephalopathy and cystic or multifocal leukoencephalopathy (Haack et al. 2014; Baertling et al. 2016). Brain MRI images are not provided in the two reported patients with *NDUFA10* mutations, but the basal ganglia involvement is described as symmetric in both cases. Further studies are therefore needed to establish if *NDUFA10* mutations may cause a specific and recognizable neuroradiologic pattern. In literature, several attempts have been made to correlate the brain MR findings to a specific mutation in LS. For instance, the subthalamic nuclei and brainstem are consistently abnormal in patients with SURF1 mutation (Rossi et al. 2003). On the other hand, abnormalities in the subthalamic nuclei are identified also in patients with other genetic defects in LS, demonstrating the complexity and wide variability of neuroimaging features in these conditions. Indeed, despite its typical features, MRI imaging alone may lack specificity in LS detection, as other mitochondrial conditions and non-mitochondrial diseases can show similar basal ganglia alterations on MRI. Therefore, LS diagnosis is currently based on a combined set of clinical, biochemical, and neuroimaging findings (Rahman et al. 1996; Baertling et al. 2014).

In conclusion, our description adds further evidence on the pathogenic role of NDUFA10 in LS, expanding the spectrum of clinical and brain MRI manifestations. Our data suggest that even in patients apparently sharing the same genotype, the variability of single mutations may lead to different clinical, biochemical, and neuroradiologic phenotype.

Short Running Title (Synopsis)

New Clinical and Neuroradiologic Features of NDUFA10 Mutation.

Authors Contribution

We confirm that all listed authors have provided a significant contribution in the manuscript preparation, in intellectual revision, and in patient's care.

Conflict of Interest

Francesca Minoia, Marta Bertamino, Paolo Picco, Maria-savina Severino, Andrea Rossi, Chiara Fiorillo, Carlo Minetti, Claudia Nesti, Filippo Maria Santorelli, and Maja Di Rocco declare that they have no conflict of interest related to the present manuscript.

Details of Funding

This study received no specific funding.

Details of Ethical Approval

Specific ethics committee was not requested for anonymous data report.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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RESEARCH REPORT

Normal Neurological Development During Infancy Despite Massive Hyperammonemia in Early Treated NAGS Deficiency

Hallvard Reigstad • Berit Woldseth • Johannes Häberle

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Abstract A girl born at term was admitted to the neonatal intensive care unit because of mild respiratory distress after a complicated delivery. She recovered, but was readmitted at 58 h of life with mild respiratory distress and increased muscle tone. Neonatal abstinence syndrome because of maternal use of lithium, clomipramine, and quetiapine during pregnancy was suspected, but at 115 h of life she became unresponsive, and an immediate work-up for coma was initiated. An ammonia of 2,235 μmol/l was found, and treatment with sodium benzoate, sodium phenylacetate, arginine, glucose, and *N*-carbamylglutamate (NCG, Carbaglu®) was started. This treatment normalized plasma ammonia levels within 16 h.

Biochemical results suggested a mitochondrial urea cycle defect, either of *N*-acetyl glutamate synthase (NAGS) or carbamoyl phosphate synthetase 1. DNA analysis later confirmed a diagnosis of NAGS deficiency. Under long-term treatment with NCG, the patient developed normally at last follow-up at 7 months of age.

In conclusion, the standard neonatal situation of a neurologically compromised newborn turned out as a treatable rare inborn error of metabolism. In all neonates with somnolence and coma and hence the suspicion of a bacterial sepsis, plasma ammonia should be included in the work-up. NCG was immediately beneficial for the patient described and should be considered for the emergency treatment of neonatal hyperammonemia. Even a very high ammonia may allow for a normal neurological development in infancy (and possibly beyond).

Communicated by: Bridget Wilcken

H. Reigstad (✉)
Neonatal Intensive Care Unit, Children's Department, Haukeland University Hospital, 5021 Bergen, Norway
e-mail: hallvard.reigstad@helse-bergen.no

B. Woldseth
Department of Medical Biochemistry, Oslo University Hospital, 0424 Oslo, Norway

J. Häberle
Division of Metabolism and Children's Research Center, University Children's Hospital Zurich, Eleonore Foundation, Steinwiesstrasse 75, 8032 Zürich, Switzerland

Case Report

Our patient, a baby girl, is the first child of unrelated, healthy parents. In pregnancy the mother was treated with clomipramine, lithium, and quetiapine due to bipolar disease. She was born 2 days before estimated day of delivery, 64 h after rupture of membranes. A vacuum extraction was performed because of suspected intrauterine hypoxia. She had Apgar scores of 6, 7, and 8 after 1, 5, and 10 min, respectively, and a birth weight of 3,380 g.

Shortly after birth she was admitted to the neonatal intensive care unit because of mild respiratory distress and was treated with nasal continuous positive airway pressure (CPAP) and antibiotics, and recovered in a few hours. She was fed formula by bottle.

Apart from a blood glucose of 1.1 mmol/l (normal $\geq$2.2 mmol/l) 6 h after birth, blood count, electrolytes, ionized calcium, and capillary acid base were normal. CRP was a maximum of 20 mg/l (normal <5 mg/l). Blood cultures remained negative.

She was discharged to the maternity unit at 48 h postnatal age but readmitted 10 h later with mild respiratory distress and general increased muscular tone. Blood pressure was 112/78 mmHg, capillary pCO_2 5.0 kPa with

a pH of 7.44. Neonatal abstinence syndrome due to maternal quetiapine use was suspected, and treatment was started with phenobarbital. Formula was given by nasogastric tube.

From 88 h of age she had apneic episodes, and after failure of nasal CPAP she was intubated at 100 h of postnatal age because of respiratory failure, after analgesia with fentanyl 3 μg/kg. At 115 h of age she did not respond to pain or suction, and had no spontaneous ventilation. Therefore, a work-up for neonatal coma was immediately started. Ultrasound of brain was normal, CRP was negative, but arterial ammonia was highly elevated (2,235 μmol/l, normal <100 μmol/l). Buffer base was 2.1 mmol/l (normal −3.0 to +3.0), alanine aminotransferase 21 U/l (normal 7–40), quetiapine was undetectable, lithium 0.3 mmol/l (therapeutic range for adults 0.5–1 mmol/l).

With ammonia levels above 400–500 μmol/l, invasive extracorporeal detoxification is recommended (Häberle et al. 2012). Since the prognosis in the presence of severe hyperammonemia was considered grim, it was decided, after discussion with the parents, to limit treatment to a trial of sodium benzoate, sodium phenylacetate, L-arginine, and NCG.

On start of this emergency treatment for hyperammonemia (at 120 h of age), ammonia was further increased to 2,455 μmol/l, but 3 h later already declined to 1,746 μmol/l, and 16 h after treatment was started ammonia was in the normal range (36 μmol/l).

With normalized ammonia the patient became awake again but was extremely irritable. She also had abdominal distension and bloody diarrhea, and on day 7 required laparotomy for a partial gangrenous large bowel resection and enterostomy.

Plasma for analysis of amino acids was taken approximately 30 min after start of loading dose of sodium benzoate, sodium phenylacetate, and L-arginine, which was likely the cause for the elevation of arginine (938 μmol/l, normal 17–120), while glutamine was only slightly elevated to 872 μmol/l (normal 400–850), and citrulline was unmeasurably low. In urine, excretion of orotic acid was normal. Analysis of urine and plasma did not show evidence of an organic aciduria or a fatty acid oxidation defect. Biochemical results therefore suggested a urea cycle defect, either of *N*-acetyl glutamate synthase (NAGS) or of carbamoyl phosphate synthetase 1 (CPS1).

Given the fast response to the above treatment NAGS deficiency was suspected. This was later confirmed by DNA-analysis, which identified a known homozygous nonsense mutation in exon 4 of the *NAGS* gene, c.971G>A (p.Trp324*) (University Children's Hospital, Zürich, Switzerland) (Sancho-Vaello et al. 2016). The parents were confirmed to be heterozygous for the same mutation.

After gradual decrease of the above medical treatment, the patient was discharged at 5 weeks of age. By that time she was feeding well, on treatment with NCG 40 mg/kg/day, without any dietary restriction or other medication. She smiled at 7 weeks. At 7 months she develops normally. Her enterostomy has been closed without any complications. Plasma ammonia levels on follow-up remained normal.

Discussion

Hyperammonemia

Elevated ammonia is a major problem and the main cause of disease in urea cycle defects (Gropman et al. 2007; Msall et al. 1984). Ammonia may also be elevated in organic acidemias, fatty acid oxidation disorders, and in other inherited and a few acquired conditions (Häberle 2013). Ammonia is extremely toxic for the central nervous system (Braissant et al. 2013), and levels above 360 μmol/l are associated with a poor prognosis, either death or severe developmental delay (Kido et al. 2012). Prognosis is considered very poor with ammonia levels above 1,000 μmol/l, although the impact of the absolute value also depends on the duration of hyperammonemia (Häberle et al. 2012; Picca et al. 2001). With timely treatment several patients with NAGS deficiency have had a normal outcome (Sancho-Vaello et al. 2016).

Standard treatment for moderate hyperammonemia without known diagnosis has been sodium benzoate, sodium phenylbutyrate or sodium phenylacetate, and L-arginine, and recent guidelines suggest NCG to be added to the initial treatment (Häberle et al. 2012). Hemofiltration or hemodialysis is recommended with ammonia levels above 400–500 μmol/l, but this threshold is rather based on empirical and circumstantial evidence than on proper studies. In many patients it will take several hours to establish the vascular access to start dialysis, and there is a risk of complications with this technique.

N-Acetyl Glutamate Synthase (NAGS) Deficiency

NAGS deficiency is probably the rarest urea cycle disorder, with just over 50 published cases worldwide (Sancho-Vaello et al. 2016). Diagnosis is best achieved by genetic

analysis (Häberle et al. 2012). As this diagnosis is not obvious from ordinary metabolic screening, cases may remain undiagnosed. It is the only disorder within the urea cycle which can be specifically and effectively treated by a drug, with no dietary restriction outside catabolic circumstances (Gessler et al. 2010; van Leynseele et al. 2013).

Clinical Significance

- Any somnolence or coma in the neonate, especially following a short symptom-free interval after birth, should be investigated for hyperammonemia, even without hyperventilation with corresponding low pCO_2 and elevated pH.
- *N*-carbamylglutamate should be added to the standard protocol for treatment of hyperammonemia, together with sodium benzoate, sodium phenylbutyrate, and L-arginine.
- Response to *N*-carbamylglutamate in NAGS deficiency may be so rapid that dialysis may be omitted.
- Even with very high ammonia levels later development may be good in cases in which hyperammonemia lasts for only a few hours.

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Compliance with Ethics Guidelines

Conflict of Interest

Hallvard Reigstad and Berit Woldseth declare no conflict of interest. Johannes Häberle has received travel support and honoraria as an invited speaker from Orphan Europe Recordati.

Informed Consent

Informed consent was obtained from the parents of the patient.

This chapter does not contain any studies with human or animal subjects performed by any of the authors.

Hallvard Reigstad has been responsible for the treatment and follow-up of the patient, and is the main author of this chapter.

Berit Woldseth has been responsible for the biochemical analyses and has revised the chapter.

Johannes Häberle has been responsible for the genetic analyses and has revised the chapter.

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RESEARCH REPORT

Dihydropyrimidine Dehydrogenase Deficiency: Metabolic Disease or Biochemical Phenotype?

M. Fleger · J. Willomitzer · R. Meinsma · M. Alders · J. Meijer · R. C. M. Hennekam · M. Huemer · A. B. P. van Kuilenburg

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Abstract Dihydropyrimidine dehydrogenase (DPD) deficiency is an autosomal recessive disorder of pyrimidine metabolism that impairs the first step of uracil und thymine degradation. The spectrum of clinical presentations in subjects with the full biochemical phenotype of DPD deficiency ranges from asymptomatic individuals to severely affected patients suffering from seizures, microcephaly, muscular hypotonia, developmental delay and eye abnormalities.

We report on a boy with intellectual disability, significant impairment of speech development, highly active epileptiform discharges on EEG, microcephaly and impaired gross-motor development. This clinical presentation triggered metabolic workup that demonstrated the biochemical phenotype of DPD deficiency, which was confirmed by enzymatic and molecular genetic studies. The patient proved to be homozygous for a novel c.2059-22T>G mutation which resulted in an in-frame insertion of 21 base pairs (c.2059-21_c.2059-1) of intron 16 of DPYD. Family investigation showed that the asymptomatic father was also homozygous for the same mutation and enzymatic and biochemical findings were similar to his severely affected son. When the child deteriorated clinically, exome sequencing was initiated under the hypothesis that DPD deficiency did not explain the phenotype completely. A deletion of the maternal allele on chromosome 15q11.2-13-1 was identified allowing the diagnosis of Angelman syndrome (AS). This diagnosis explains the patient's clinical presentation sufficiently; the influence of DPD deficiency on the phenotype, however, remains uncertain.

Communicated by: Verena Peters

M. Fleger · M. Huemer (✉)
Department of Paediatrics, Landeskrankenhaus Bregenz, Carl-Pedenz-Straße 2, 6900 Bregenz, Austria
e-mail: martina.huemer@lkhb.at

J. Willomitzer
Department of Paediatrics, Krankenhaus Dornbirn, Dornbirn, Austria

R. Meinsma · M. Alders · J. Meijer · R.C.M. Hennekam · A.B.P. van Kuilenburg
Departments of Clinical Chemistry, Laboratory Genetic Metabolic Diseases, Department of Pediatrics, and Department of Clinical Genetics, Academic Medical Center, University of Amsterdam, Emma Children's Hospital, Amsterdam, The Netherlands

M. Huemer
Division of Metabolism and Children's Research Center, University Children's Hospital Zürich, Zürich, Switzerland

Introduction

Dihydropyrimidine dehydrogenase (DPD) deficiency is a rare autosomal recessive disorder that was first described in the 1980s (van Gennip et al. 1981; Bakkeren et al. 1984). Dihydropyrimidine dehydrogenase catalyses the first and rate-limiting step of uracil and thymine degradation. Biochemical hallmark of enzyme deficiency is the excretion of uracil and thymine in urine, which is easily detected by basic metabolic work-up. DPD deficiency has been associated with severe, early-onset neurological symptoms such as seizures, microcephaly, muscular hypotonia, global developmental delay and eye abnormalities (e.g. microcornea, nystagmus and hypoplastic macula) (Yau et al. 2004; Van Kuilenburg et al. 1999; Braakhekke et al. 1987; Bakker et al. 1994). EEG-abnormalities encompass various types of epileptic discharges (Braakhekke et al. 1987). However, the biochemical phenotype has also been observed in asymptomatic individuals (Van Kuilenburg et al. 1999; Braakhekke et al. 1987; Van Gennip et al. 1994).

Brain magnetic resonance imaging (MRI) in individuals with DPD deficiency showed normal brain morphology in some individuals; non-specific findings (e.g. cerebral atrophy or white matter T2-hyperintensity) have also been described (Enns et al. 2004).

Angelman syndrome (AS) has initially been described in 1965 in three unrelated children with "puppet-like" gait, microcephaly, dysmorphic facial features, paroxysms of laughter and severe mental disability (Angelman 1965). AS is caused by loss of function of the ubiquitin protein ligase E3A (UBE3A) gene on the maternally derived chromosome 15. In 75% of cases, an interstitial deletion of the maternal part of the chromosome is causative. Genomic imprinting defects, paternal uniparenteral disomy of chromosome 15 and intragenic mutations or deletions of UBE3A gene have also been reported as causes for AS (Buiting et al. 2016).

As in DPD deficiency, the MRI-features in AS are rather unspecific. Thinning of the corpus callosum, enlargement of the lateral ventricles and cerebral atrophy with frontal and temporal predominance have been described. The EEG changes in AS encompass three quite distinctive main patterns: rhythmic high amplitude 2–3 Hz activity predominantly over the frontal regions; rhythmic high amplitude 4–6 Hz activity or 4–5 Hz spike waves over the parieto-occipital regions after eye closure. These patterns may appear isolated or in various combinations and often evolve over time in affected individuals (Buiting et al. 2016; Boyd et al. 1988). The major characteristic in AS is, however, neither the MRI nor the EEG but development and behaviour, which can be characterised as severe intellectual disability, absence of speech, paroxysmal periods of laughter, sleeping disturbances including reversal of the day-night rhythm, and extreme hyperactivity which can result in uncontrolled movements due to ataxia.

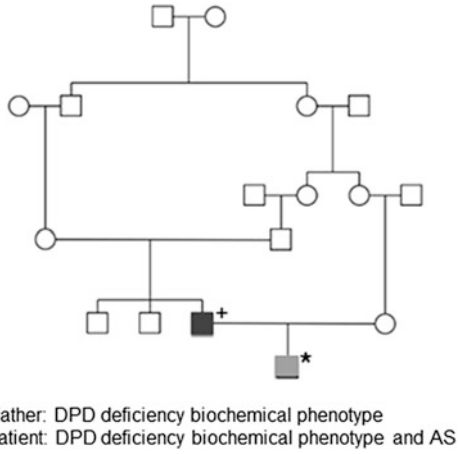

Fig. 1 Family tree over four generations

Table 1 DPD activity and pyrimidine bases in affected family members

	Tissue	DPD activity (nmol/mg/h)	Uracil (μmol/mmol creatinine)	Thymine (μmol/mmol creatinine)
Son	PBM cells	0.2		
	Fibroblasts	< 0.02		
	Urine		562	288
Father	PBM cells	0.1		
	Fibroblasts	<0.02		
	Urine		141	42
Controls	PBM cells[a]	9.9 ± 2.8		
	Fibroblasts[b]	1.1 ± 1.2		
	Urine[c]		11.8 ± 9.1	0.5 ± 0.6

[a] Data from van Kuilenburg et al. (2002)
[b] Data from van Kuilenburg et al. (2000b)
[c] Data from van Kuilenburg et al. (2004b)

Case Report

Our patient was the first male child born to healthy, consanguineous (second cousins) Turkish parents (Fig. 1). He was born at term by secondary caesarean section; auxologic parameters were normal and neonatal adaptation uneventful.

At 4 weeks, the child had been hospitalised with an acute infection, feeding difficulties and failure to thrive, and metabolic workup was initiated. Determination of uracil and thymine concentrations in urine using reversed-phase HPLC hyphenated with electrospray tandem mass spectrometry (van Lenthe et al. 2000) demonstrated excessive excretion of uracil and thymine, indicating DPD deficiency (Table 1).

For enzymatic testing, peripheral blood mononuclear (PBM) cells were isolated and the activity of DPD was determined in a reaction mixture containing 35 mM potassium phosphate (pH 7.4), 2.5 mM MgCl2, 1 mM dithiothreitol, 250 μM NADPH and 25 μM [4-14C]-thymine. Separation of radiolabeled thymine from radiolabeled dihydrothymine was performed by reversed-phase HPLC with online detection of the radioactivity, as described before (van Kuilenburg et al. 2000b). The patient showed an almost complete deficiency of DPD activity (Table 1).

DNA was isolated from blood using standard techniques. PCR amplification of all 23 coding exons and flanking intronic regions of DPYD was carried out using intronic primer sets, essentially as described before (van Kuilenburg et al. 2000a). Sequence analysis of genomic fragments

amplified by PCR was carried out on an Applied Biosystems model 3,730 automated DNA sequencer using the dye-terminator method (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The DPYD sequence of DPD deficient patients were compared to those observed in controls and the reference sequence of DPYD (Ref Seq NM_000110.3; Ensembl ENST00000370192). Total RNA was isolated from cultured fibroblasts using Trizol extraction (Invitrogen, Carlsbad, CA, USA). Subsequently, cDNA was prepared using a first strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). PCR amplification of a part of the cDNA sequence containing exon 13–19 was performed using the forward primer (5′-TTGATCTGGTGGACATTAGTG-3′) and reverse primer (5′-GAAACTGAAGACCACTTTCAG-3′), corresponding to the nucleotides c.1583_1603 (exon 13) and c.2396_2416 (exon 19), respectively. Genetic testing in the patient revealed a novel homozygous mutation in intron 16 (c.2059-22T>G) of the *DPYD* gene, leading to homozygosity for an in-frame insertion of 21 bp (2058_2059ins21) corresponding to the nucleotides c.2059-21_c.2059-1 of intron 16 of *DPYD* (Fig. 2). Consequently, additional seven amino acids (p. Gln686_Asp687ins7) are included into the mature DPD protein, which most likely has a deleterious effect on protein function.

Work-up of the family showed that the mother was a heterozygous carrier. Surprisingly the completely asymptomatic father was also homozygous for the c.2059-22T>G mutation and had comparable enzymatic and biochemical findings as observed in his affected son (Table 1). The father had never encountered any neurological problems, had completed junior high school and was presently employed as a semiskilled worker.

From the age of 12 months, the paediatrician noticed developmental impairment and generalised muscular hypotonia. At 19 months, the child presented with failure to thrive and microcephaly (body weight and head circumference <3rd centile). He was able to sit unsupported but had not yet started to walk. His movements were poorly coordinated. Speech development, play behaviour and social skills corresponded to a developmental age of approximately 10 months. MRI of the brain revealed unspecific symmetric T2-hyperintense lesions in the occipital white matter region. EEG registered 4–5 Hz

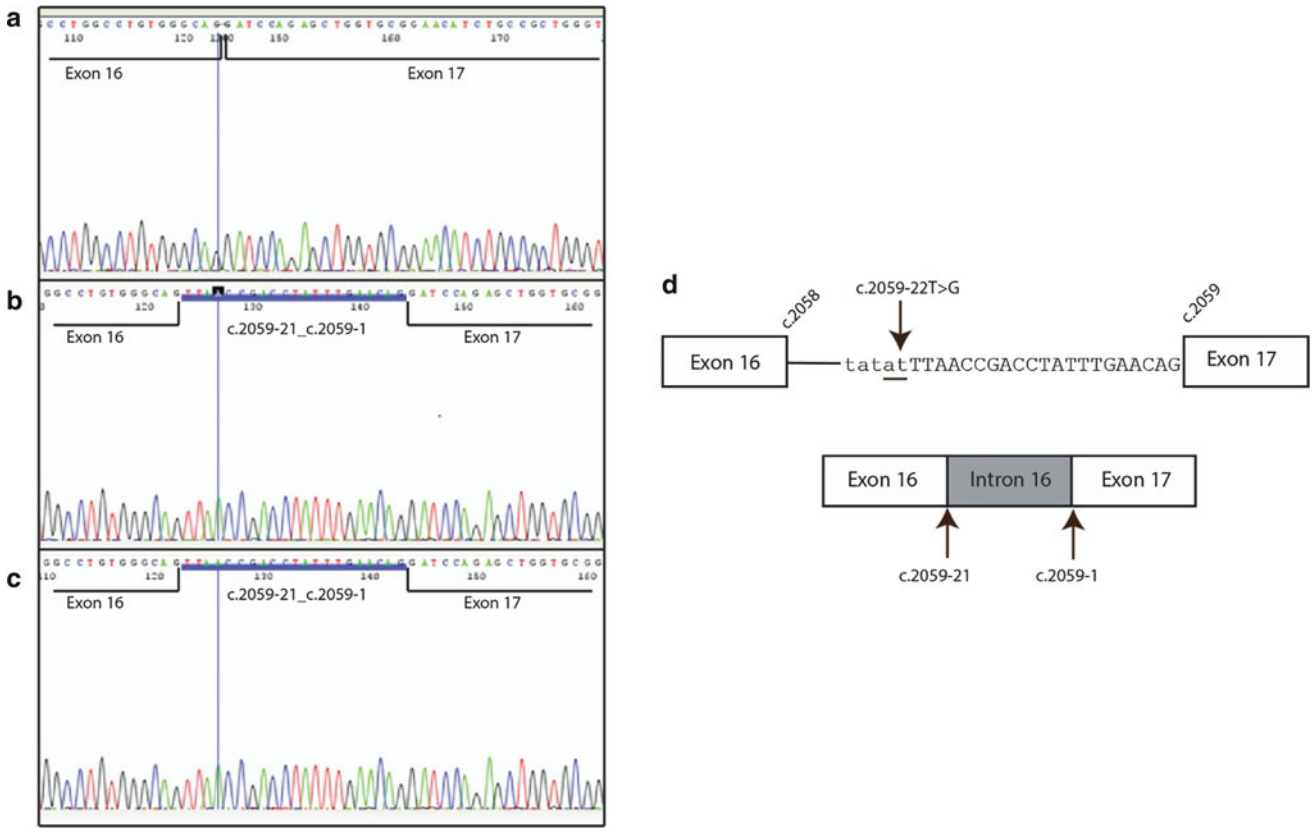

Fig. 2 Alternative DPD pre-MRA splicing due to the c.2059-22T>G mutation in DPYD. The cDNA sequence of part of exon 16 and 17 is shown for a control (panel **a**), the father (panel **b**) and the affected son (panel **c**). The in-frame insertion of 21 base pairs (c.2059-21_c.2059-1) of intron 16 is underlined in *blue*. Panel (**d**) shows a schematic representation of the region of intron 16 upstream of exon 17 and the effect of the c.2059-22T>G mutation on splicing of the DPD pre-mRNA. The intronic sequence inserted into the DPD mRNA is indicated in *capital letters*. The cryptic splice acceptor site is *underlined*

parieto-occipital spike waves; the parents had never perceived seizures. Treatment with valproic acid improved the patient's overall condition and development and alleviated the EEG. He learned to ambulate one hand-guided and made remarkable progress in speech comprehension, play behaviour and social skills. Until age 3.5 years, he has not developed active speech but learned to use basal augmentative communication; his movements remain uncoordinated.

Against the background of the genetic, enzymatic and biochemical findings in the asymptomatic father and the severe, but at this time rather nonspecific phenotype in the child we decided to search for further causes for developmental delay and initiated exome sequencing. A deletion of the maternal allele of chromosome 15q11.2-13-1 causative for AS was identified (Fig. 3) (Buiting et al. 2016).

During the further course, the clinical phenotype of AS evolved more and more. At 3 years, the parents reported paroxysm of laughter, he showed hyperactivity and ataxia, he was microcephalic and the characteristic EEG pattern with rhythmic frontal high amplitude 2–3 Hz activity and rhythmic high amplitude 4–6 Hz activity in addition to the previously described 4–5 Hz spike waves over the parieto-occipital regions was recorded.

Discussion

In the patient presented here, overall developmental delay triggered basic metabolic work-up, which resulted in the identification of DPD deficiency. Enzymatic and molecular genetic studies were consistent with the biochemical phenotype. However, as the healthy father had the identical genetic and biochemical phenotype and further studies in the index patient showed signs unusual for DPD deficiency, additional studies were performed that finally demonstrated AS. Characteristic signs of AS such as microcephaly, delayed development and characteristic EEG features were encountered in the patient (Buiting et al. 2016).

Until now, firm genotype–phenotype correlations in DPD deficiency have not been established and -like in the reported family- asymptomatic individuals have repeatedly been identified (Van Kuilenburg et al. 1999; Braakhekke et al. 1987; Van Gennip et al. 1994).

This broad phenotypic spectrum indicates a variable penetrance of DPD deficiency. Additional factors such as other metabolites in pyrimidine catabolism, and oxidative stress or febrile infections as triggers may be involved in the development of the clinical phenotype and may even lead to sudden, eventually devastating neurologic disease in

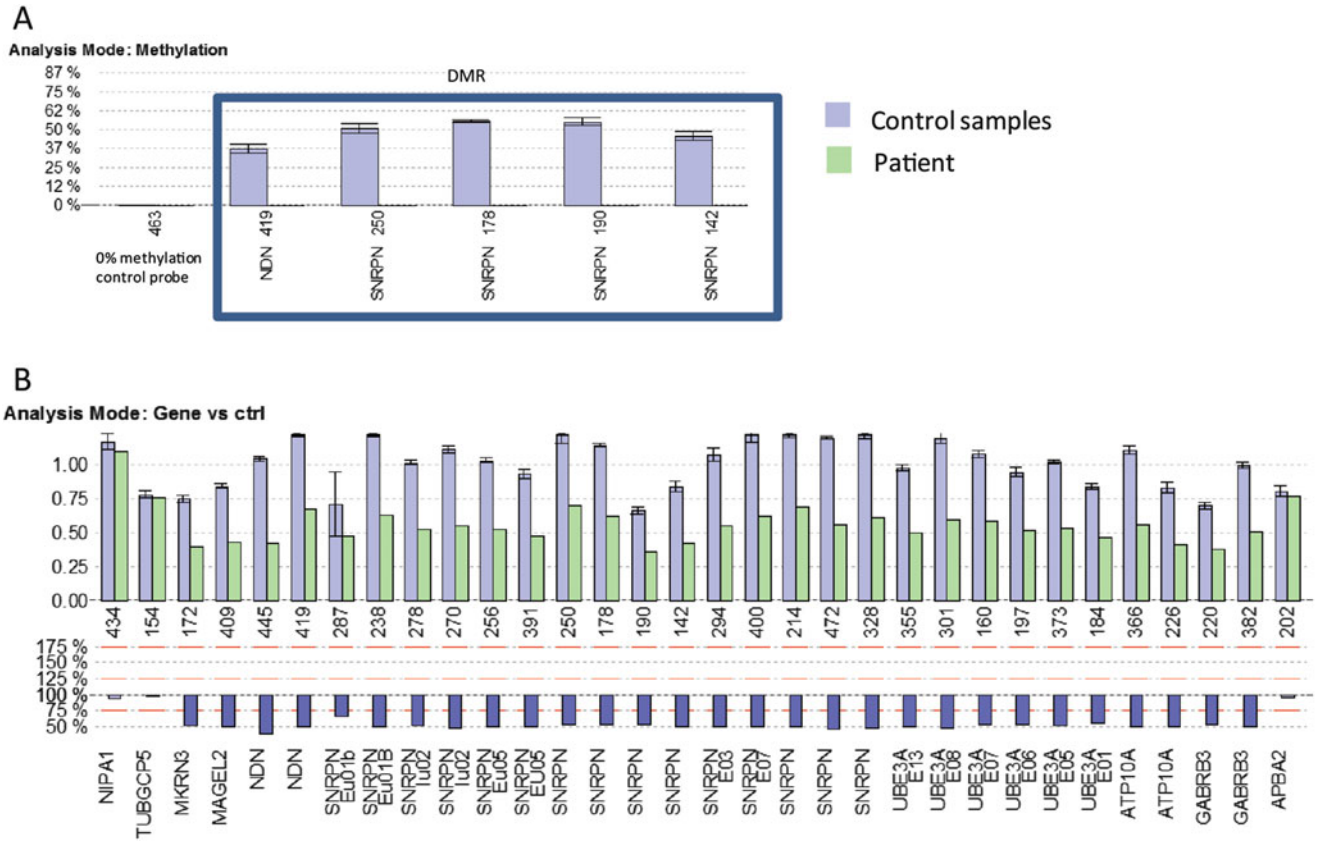

Fig. 3 Methylation specific MLPA of the PWS/AS region on chromosome 15q11–q13. (**a**) Absence of methylation at the differential methylated sites (DMRs) at the SNRPN locus. (**b**) Heterozygous deletion between genes *TUBGCP5* and *APBA2*

formerly asymptomatic individuals (Fiumara et al. 2003; Van Kuilenburg et al. 2006). The mechanism behind this, however, has not been elucidated yet. Furthermore, it has been suggested that mutations in other, yet unknown genes and epigenetic influences may be linked to mutations in *DPYD* and thus involved in the development of the clinical phenotype (Van Kuilenburg et al. 1999).

Epilepsy is a feature often encountered in DPD deficiency. Therefore, it is an interesting observation that in DPD patients, plasma beta-aminoisobutyric acid (ß-AIB), a downstream metabolite in thymine catabolism, is significantly decreased (van Kuilenburg et al. 2004a). The molecular structure of ß-AIB is very similar to gamma-aminobutyric acid and glycine, two of the most important inhibitors of neuronal synaptic transmission. It has therefore been hypothesised that ß-AIB may also be involved in inhibitory synaptic regulation. Following this line of thought, low ß-AIB may contribute to the imbalance between excitatory and inhibitory agents and thus play a role in seizure aetiology in DPD. Since plasma concentrations of ß-AIB levels are very low in DPD patients this parameter may unfortunately not be useful to differentiate between mild and severely affected individuals (Van Kuilenburg et al. 2004a).

We conclude that more investigations are necessary to elucidate possible pathogenic mechanisms behind the variable phenotype of DPD. We recommend thorough diagnostic workup of patients with the DPD biochemical phenotype beyond DPD in case the clinical course is suggestive of another disease or not consistent with clinical observations in DPD deficiency.

Compliance with Ethical Guidelines

All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Conflict of Interest

M. Fleger, J. Willomitzer, R. Meinsma, M. Alders, J. Meijer, R. C. M. Hennekam, A. B. P. van Kuilenburg and M. Huemer declare that they have no relevant conflict of interest.

Details of the Contributions of Individual Authors

M. Fleger, M. Huemer and A. B. P. van Kuilenburg designed the research and drafted the manuscript. R. Meinsma, M. Alders, J. Meijer and R. C. M. Hennekam performed the experiments and critically revised the manuscript. J. Willomitzer provided clinical data and revised the manuscript.

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RESEARCH REPORT

Potential Misdiagnosis of Hyperhomocysteinemia due to Cystathionine Beta-Synthase Deficiency During Pregnancy

Sally P. Stabler · Cynthia Freehauf · Robert H. Allen · Janet Thomas · Renata Gallagher

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Abstract Extreme hyperhomocysteinemia with low cystathionine and cysteine is virtually diagnostic of cystathionine beta-synthase (CBS) deficiency since remethylation defects and hypermethioninemia due to other inborn errors cause elevated serum cystathionine. However, a pregnant CBS deficient patient was found to have elevated cystathionine in addition to elevated total homocysteine and methionine at 23 weeks of gestation and post-delivery cystathionine decreased to the lower level of normal. A second patient with cystathionine values during gestation also showed a rise from the low pre-pregnant value to massive elevation by delivery. Her infant had severe hyperhomocysteinemia in cord blood with a massive elevation of cystathionine, S-adenosylmethionine, and S-adenosylhomocysteine. The infant corrected her homocysteine value by 2 months and is not affected. This data demonstrates that the fetus when exposed to high homocysteine and methionine has increased synthesis of cystathionine which cannot be cleared because the fetus lacks cystathionine gamma-lyase, and thus cystathionine is returned to the mother's circulation. This situation could lead to a misdiagnosis of the cause of hyperhomocysteinemia in a previously undiagnosed pregnant CBS deficient patient. Assays combining homocysteine with cystathionine measurements are commonly available from commercial laboratories in the USA. The recognition of CBS deficiency vs. remethylation disorders is important in order to maximize treatment. The cord blood values revealed a major disturbance in methionine metabolism including a potential for impaired transmethylation reactions in the fetus due to the buildup of S-adenosylhomocysteine. It is possible that monitoring maternal cystathionine during gestation could provide another measure of fetal exposure to homocysteine.

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S.P. Stabler (✉) · R.H. Allen
Division of Hematology, Department of Medicine, University of Colorado School of Medicine, 12700 E. 19th Avenue, Room 9122, Bldg. RC2, Campus Box B170, Aurora, CO, USA
e-mail: Sally.Stabler@ucdenver.edu

C. Freehauf · J. Thomas
Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO, USA

R. Gallagher
Department of Pediatrics, University of California at San Francisco, 330 Post Street, 6th Floor, San Francisco, CA, USA

Introduction

Severely elevated homocysteine (tHcy) with low cystathionine and low cysteine is virtually diagnostic of cystathionine beta-synthase (CBS) deficiency (classical homocystinuria) since remethylation defects and hypermethioninemia due to other inborn errors cause elevated serum cystathionine and normal cysteine concentrations (Orendac et al. 2003; Stabler et al. 2013; Bártl et al. 2014) (Fig. 1a–c). We report the presence of elevated serum cystathionine during pregnancy in two patients with CBS deficiency. This demonstrates that elevated cystathionine with hyperhomocysteinemia might lead to misdiagnosis of a remethylation disorder rather than classical homocystinuria. Severe hyperhomocysteinemia is a well-documented cause of thrombosis, and pregnancy is a high-risk condition for thrombotic disease. Measuring homocysteine is often part of routine testing for causes of

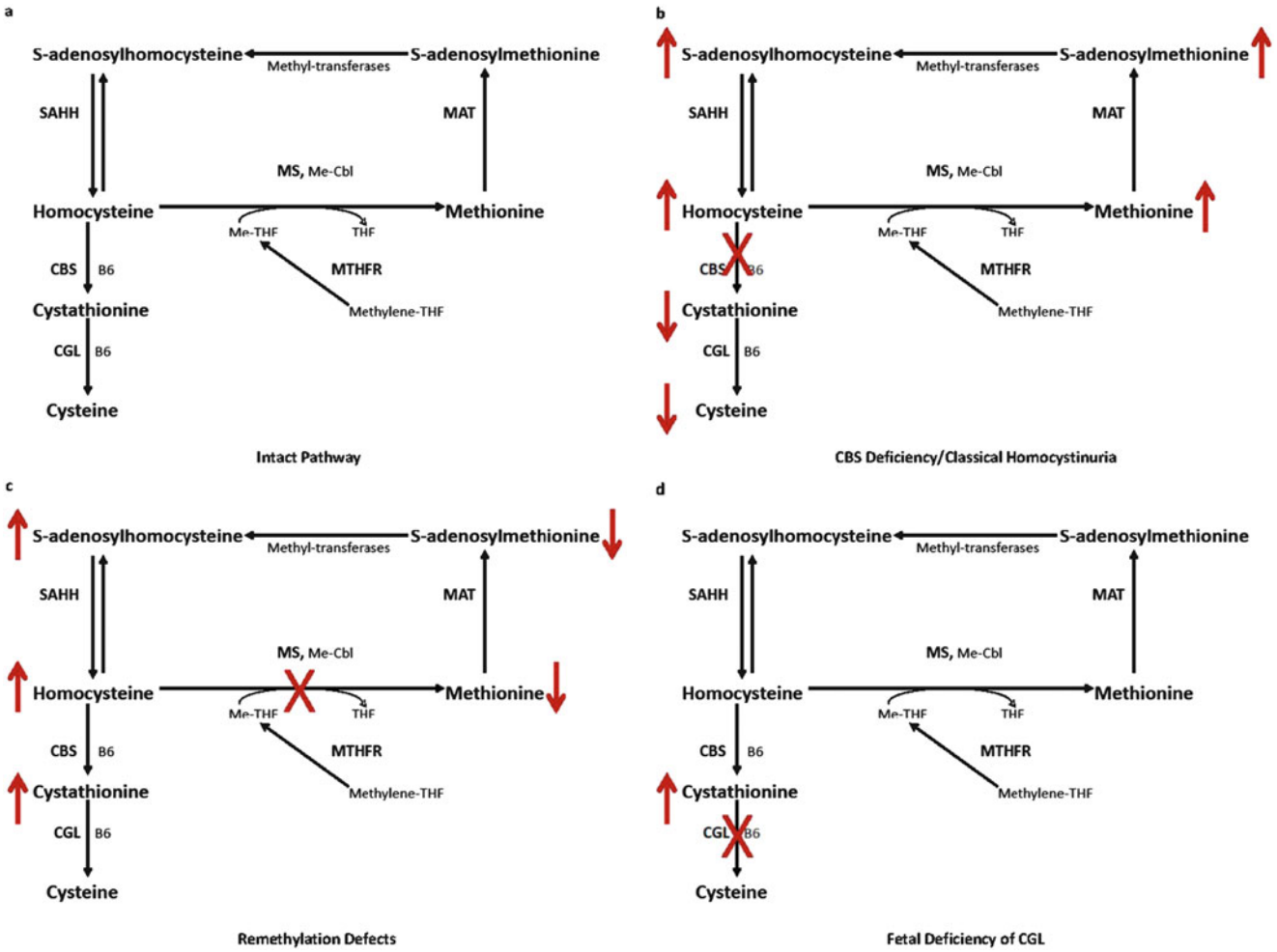

Fig. 1 Pathways of homocysteine and methionine metabolism. (**a**) Homocysteine is either remethylated to methionine by methionine synthase (MS) utilizing methyl-cobalamin (Me-Cbl) and N5-methyl-tetrahydrofolate (Me-THF) as co-factors, or it is condensed with serine (not shown) to form cystathionine by cystathionine beta-synthase (CBS) which uses pyridoxal phosphate (B6) as a cofactor. Cystathionine is converted to cysteine by cystathionine gamma-lyase (CGL) in another pyridoxal phosphate dependent reaction. Me-THF is demethylated to THF in the methionine synthase reaction. Methionine can be activated to S-adenosylmethionine by methionine adenosyltransferase (MAT I/III or MAT II). Many methyltransferase reactions utilize S-adenosylmethionine, generating S-adenosylhomocysteine, which is hydrolyzed to homocysteine by S-adenosylhomocysteine hydrolase (SAHH). The S-adenosylmethionine dependent methylations are important in the production of phospholipids, myelin, creatine, and many other reactions. Methylene tetrahydrofolate (Methylene-THF) is reduced to Me-THF by methylenetetrahydrofolate reductase (MTHFR). (**b**) In classic homocystinuria cystathionine beta-synthase activity is severely deficient or absent, leading to elevated levels of homocysteine, methionine, S-adenosylhomocysteine, and S-adenosylmethionine and low levels of cystathionine and cysteine. (**c**) In disorders of remethylation, including severe deficiencies, or absence, of methionine synthase (MS) or methylenetetrahydrofolate reductase (MTHFR) activities, there are elevated levels of homocysteine and S-adenosylhomocysteine, low levels of methionine and S-adenosylmethionine, and high levels of cystathionine. (**d**) In the fetus there is a severe deficiency of cystathionine gamma-lyase activity; data in this report and others support that this results in high levels of cystathionine in the presence of elevated maternal homocysteine

thrombophilia and some commercially available metabolite panels include cystathionine in combination with homocysteine. The reported case shows how a previously undiagnosed patient with CBS deficiency might be thought to have a remethylation defect and counseled and treated inappropriately.

Methods

Patients

Both patients were seen at the Inherited Metabolic Diseases Clinic at the Children's Hospital Colorado on a sporadic basis but were also followed by obstetricians and other physicians in their communities. The two subjects provided written informed consent for themselves and their children to participate in this retrospective chart review through a University of Colorado (COMIRB) IRB approved protocol.

Metabolite Studies

tHcy, cystathionine, methionine, total cysteine, *N*,*N*-dimethylglycine, and *N*-methylglycine were assayed in serum by capillary stable isotope dilution gas chromatography/mass spectrometry as previously described (Stabler et al. 1988, 1993; Allen et al. 1993). S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were assayed in serum by stable isotope dilution liquid chromatography/mass spectrometry (Stabler and Allen 2004). The homocysteine value obtained on patient 2's baby at a later date was done in a commercial laboratory.

Results

Case Reports

Patient 1

Patient 1 was 32 years old and pregnant with a gestational age of 23 weeks when referred to the metabolic clinic for evaluation of hyperhomocysteinemia. She had been referred to Genetics during pregnancy due to a clinical diagnosis of Marfan Syndrome. A homocysteine level was ordered as classical homocystinuria is a phenocopy of Marfan Syndrome. She had been given the diagnosis of Marfan syndrome as she had been found to have upward dislocating ectopia lentis when she was 18 with high myopia. At age 25 she developed a seizure disorder controlled by medication and at age 26 she developed phlebitis following a transatlantic flight and was placed on warfarin anticoagulation. She had a history of mild developmental delay but had been in a normal classroom setting and graduated from high school. Physical exam revealed scoliosis. The biochemical data shown in Table 1 revealed severely elevated tHcy and methionine and low total cysteine. The markedly elevated cystathionine initially suggested a remethylation disorder until it was revealed by the clinic that the patient was pregnant, and had a history consistent with CBS deficiency. She was treated with B_6, methionine restriction, B12, increased folate, betaine and cysteine supplements. Her tHcy and methionine decreased, total cysteine increased, and *N*,*N*-dimethylglycine and *N*-methylglycine increased documenting her compliance with betaine treatment (Stabler et al. 2013). Cystathionine fluctuated but was essentially the same at 24 and 32 weeks of pregnancy. Post delivery the cystathionine decreased to the lower end of the normal range and methionine increased markedly. CBS deficiency was confirmed by enzyme analysis.

Patient 2

The laboratory data of another patient with CBS deficiency was reviewed as she had cystathionine values measured throughout pregnancy. Case 2 was 23 years of age at the time of her pregnancy and had been diagnosed with classical homocystinuria the year prior when she presented with cavernous sinus thrombosis, which led to visual loss and right-sided hemiparesis. She also had a history of myopia and bilateral lens dislocation. She had been treated with warfarin anticoagulation since her thrombosis and was treated with vitamin B_6, folic acid, B12 injections, betaine and methionine restriction, although compliance was poor. Additional data was also available 8 years later during a fourth pregnancy, when she was treated with low molecular weight heparin anticoagulation alone. The biochemical data for the two pregnancies are shown in Table 1. In the first pregnancy, the first trimester serum values showed a low-normal cystathionine, mildly elevated methionine, and low *N*,*N*-dimethylglycine despite presumed betaine treatment. The cystathionine rose markedly in the second trimester and remained high at the time of delivery but fell to a value at the lower level of normal after delivery. In her fourth pregnancy at age 30 values were available at 31 weeks and at delivery, and these again demonstrated a marked elevation of cystathionine. The baby's cord blood sample revealed severely elevated homocysteine, a massive elevation of cystathionine (tenfold greater than maternal serum), elevated methionine, and low total cysteine. In addition, serum was assayed for S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) values, both of which were elevated in mother and baby. A repeat homocysteine in the child, age 2 months, was normal at 8.4 uM.

Metabolite Ratios

We have previously reported that the ratio of tHcy to cystathionine had a median of 2.8 in 118 patients with CBS deficiency as compared to 0.1 for 132 patients with remethylation defects (Stabler et al. 2013). The values in the two case patients while not pregnant were 2.46, 3.4, 3.6, and 5.5 but fell to between 0.1 and 0.19 in patient 1 and

Table 1 Clinical and biochemical data on two patients with cystathionine β-synthase deficiency

	Gestation[a] (week)	tHcys (μM)	Cystat (nM)	Meth (μM)	tCyst (μM)	DMG (μM)	MG (μM)	SAM (nM)	SAH (nM)	Treatment
Patient 1	23	181	983	315	83	2.1	5.3			None except AC[b]
	24	124	691	64	96	1.9	4.3			
	25	83	423	165	128	34.1	14.5			B6, methionine restriction, betaine and cysteine
	26	71	479	152	136	76.8	20.1			" "
	32	67	639	158	207	225.1	28.6			" "
	Post	50	81	1,283	222	234	86.5			" "
	Post	123	50	1,560	178	109.2	29.5			" "
Patient 2	Pre	310	85							AC[b]
First pregnancy	First Tri	136	85	55	108	3	6.1			B6, folic acid, B12, betaine and methionine restriction (unknown compliance)
	Second Tri	182	1,432							
	Delivery	134	2,942	279	99					
	Post	232	42	118	89	5.3	15.7			
Fourth pregnancy	31	232	4,354							Only LMWH[c]
Mother	Delivery	166	3,115	73	98	2.6	7	266	382	
Baby	Cord blood	169	30,443	119	111	4.1	8.6	840	366	
	2 months	8.4								
Normal ranges		(5.1–13.9)	(44–342)	(13–45)	(200–361)	(1.4–5.3)	(0.6–2.7)	(71–168)	(8–26)	

[a] Gestation is noted in weeks or as to trimester, at time of delivery or pre- or post-pregnancy state
[b] AC is anticoagulant therapy
[c] LMWH is low molecular weight heparin injections

0.045 and 1.6 in patient 2, completely overlapping the values previously seen for remethylation disorders. The ratio of methionine to cystathionine is also useful in distinguishing CBS deficiency from remethylation defects since it is quite elevated in the former (Stabler et al. 2013; Bártl et al. 2014). The methionine to cystathionine ratios were much lower in the 2 patients while pregnant ranging from 23 to 647 as compared to the non-pregnant values of 2,809 to 31,200 but were always higher than that reported for remethylation defects (Bártl et al. 2014).

Discussion

Both patients with CBS deficiency delivered healthy babies, although the tHcy was not controlled for most of patient 1's gestation nor for both gestations of patient 2. Patient 1 demonstrated the massive elevations in *N*,*N*-dimethylglycine seen with adequate betaine treatment, but patient 2 had normal range *N*,*N*-dimethylglycine suggesting low or no intake of betaine (Allen et al. 1993). The high cystathionine values with a low tHcy to cystathionine ratio were initially unexpected, as the ratio of tHcy to cystathionine is considered to be a robust way to diagnose probable CBS deficiency since remethylation disorders, both due to inborn errors and to severe vitamin B12 or folate deficiency, show a much lower ratio of these compounds (Stabler et al. 2013). The cord blood for patient 2's baby was very abnormal with severe hyperhomocysteinemia, massive cystathioninemia with a tenfold elevation compared to maternal serum, and a 100-fold elevation compared with normal values. There were also large elevations in SAM and in particular, SAH, which was 10 times normal. The normal tHcy later in infancy showed that the baby did not have homocystinuria. Thus, the values in the cord blood must reflect in utero hypermethioninemia and hyperhomocysteinemia due to the elevated values of these in the mother. Cystathionine is converted to cysteine by

cystathionine gamma-lyase, an enzyme that is deficient in fetal life, which increases post-natally in the first weeks of life (Gaull et al. 1972). A likely explanation for the hypercystathioninemia in the two women reported here and in the infant, are that the fetus with presumed 50% of normal CBS activity, converted elevated homocysteine derived from the mother to cystathionine and returned it to the mother's circulation since the fetus could not metabolize cystathionine (Fig. 1d). This is the first report of a massive elevation of cord blood cystathionine in maternal CBS deficiency.

The fact that cystathionine can be markedly elevated during pregnancy in women with CBS deficiency is important to know in order to prevent errors in diagnosis and might also be monitored in pregnancy in order to assess efficacy of therapy. Women with CBS deficiency are at increased risk for thrombosis during pregnancy and the literature documents CBS patients diagnosed, as was our patient, in the peripartum period (Kurczynski et al. 1980; Constantine and Green 1987; Yap et al. 2001; Levy et al. 2002; Pierre et al. 2006). Patients without severe connective tissue complications may present in adulthood (Maclean et al. 2002; Magner et al. 2011). In addition some patients are confused with Marfan Syndrome as was patient 1 (Berry and Levy 2010). THcy values are frequently determined as part of a hypercoagulable screen in adult medicine. A panel measuring serum/plasma tHcy, cystathionine, and methylmalonic acid is widely available from commercial laboratories in the USA and the results could lead to the erroneous conclusion that a patient had a remethylation defect such as severe methylenetetrahydrofolate reductase deficiency as the cause of elevated tHcy and cystathionine. The necessity to also measure serum methionine which may be low in remethylation defects and elevated in CBS deficiency may not be recognized by hematologists or obstetricians who are not familiar with inborn errors of metabolism.

While there are many reports of successful pregnancy with delivery of healthy infants in patients affected by CBS deficiency (Levy et al. 2002; Pierre et al. 2006), increased fetal loss and malformations have also been reported (Constantine and Green 1987). The severe hyperhomocysteinemia in the baby's cord blood and marked abnormalities of SAM and SAH could contribute to fetal anomalies. SAH is an inhibitor of most transmethylation reactions, and it is not known whether this massive increase in SAH throughout gestation would be detrimental. Elevated homocysteine could also impair uptake of other amino acids by the placenta (Regnault et al. 2007). The time course of the accumulation of these metabolites can be estimated by viewing the results of patient 2 in the first pregnancy, the first and second trimester, and time of delivery. Cystathionine and methionine were normal in the first trimester but by the second trimester cystathionine had increased more than tenfold suggesting that the fetus had already developed hyperhomocysteinemia and hypermethioninemia. The lower cystathionine and homocysteine values in patient 1 as compared to patient 2 likely reflect the effect of betaine treatment as suggested by elevated *N*,*N*-dimethylglycine in patient 1 and not patient 2 as well as better compliance with dietary restriction of methionine. This would lower the homocysteine exposure of the fetus.

There are only a few previous reports of total homocysteine or the disulfide, homocystine, and methionine values in amniotic fluid, fetal tissues, or cord blood in the fetuses of mothers with CBS deficiency. In one case report, amniotic fluid at about 16 weeks gestation showed severely elevated methionine, 590 μmol/L, (control fluid: 20 μmol/L) elevated homocystine, 20 μmol/L (control fluid: not detected), and no detectable cystathionine. The affected mother had an elevated plasma methionine of 400 μmol/L and a homocystine of 40 μmol/L; total homocysteine was not reported. The fetus was aborted at 20 weeks and had massively increased liver and brain cystathionine as well as elevated methionine (Rassin et al. 1979). Another case report identified a similar elevation of methionine in amniotic fluid at 18 weeks gestation of 590 μmol/L (Kurczynski et al. 1980). It appears from this limited data that the fetus of an affected mother is exposed to high concentrations of methionine and homocysteine, starting from the second trimester at least. Levy et al. (2002) reported that total homocysteine, 155 μmol/L, and methionine 117 μmol/L were elevated in umbilical cord plasma when a mother with homocystinuria had elevated homocysteine at delivery. Cystathionine was not measured.

We have previously reported that serum cystathionine rose massively in a patient with moderately severe methionine adenosyl-transferase (MAT I/III) deficiency during several pregnancies (Mudd et al. 2003). We proposed that the elevated methionine in the mother was delivered to the fetus who produced cystathionine but since the fetus lacked cystathionine gamma-lyase, the cystathionine was returned to the mother's circulation. The present cases further support this mechanism of maternal cystathionine elevation in pregnancy in both disorders.

It is unclear if elevated cystathionine is harmful to the fetus; however, in post-natal life cystathionine gamma-lyase deficiency (cystathioninuria) is a benign disorder (Kraus et al. 2009). In addition, a report of maternal cystathionine gamma-lyase deficiency showed massive increases of cystathionine in amniotic fluid in 2 pregnancies of 45 and 54 μmol/L as compared to normal (expected <1 μmol/L) as reported for their laboratory. The infants had cord blood

values that were also massively elevated at 12 and 16 μmol/L (Vargas et al. 1999), which are lower than that of the infant of patient 2, at 30 μmol/L (30,443 nmol/L).

Conclusions

We have assayed methionine-related metabolites in two patients with CBS deficiency during pregnancy and demonstrate that cystathionine values are increased above the pre- and post-pregnancy values by 10- to 20-fold during gestation. The cystathionine value in the cord blood of the one infant evaluated was tenfold elevated above the concurrent maternal serum value. Recognition that cystathionine is elevated during pregnancy will prevent diagnostic errors in pregnant CBS patients who may have not been previously diagnosed with classical homocystinuria prior to their pregnancy. Maximum biochemical control should be encouraged in order to limit exposure of the fetus to the lowest concentrations of homocysteine and methionine possible.

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Take Home Message

Cystathionine is low in patients with cystathionine beta-synthase deficiency except during pregnancy when fetal exposure to high homocysteine results in fetal synthesis of cystathionine and transfer to the mother's circulation, which may confound the specific diagnosis of hyperhomocysteinemia.

Contributions of Authors

Sally Stabler, Janet Thomas, and Renata Gallagher were responsible for planning the details in the manuscript. Sally Stabler and Robert Allen were involved in the conduct of the laboratory measures in the manuscript. Janet Thomas, Cynthia Freehauf, and Renata Gallagher were involved in the conduct of obtaining the clinical measures. Sally Stabler and Renata Gallagher wrote the bulk of the manuscript with contributions from the other authors.

Guarantor for Chapter

Sally P Stabler serves as the guarantor for this chapter, accepting full responsibility for the work. She had access to all the data and controlled the decision to publish.

Conflict of Interest

Sally Stabler and Robert Allen and the University of Colorado have competing interests because a company has been formed at the University of Colorado to assay homocysteine, methylmalonic acid, and cystathionine. Cynthia Freehauf, Janet Thomas, and Renata Gallagher declare that they have no conflict of interest.

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Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Written informed consent was obtained from all patients and their children for being included in the study.

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RESEARCH REPORT

Clinical and Molecular Variability in Patients with *PHKA2* Variants and Liver Phosphorylase b Kinase Deficiency

Deeksha S. Bali • Jennifer L. Goldstein • Keri Fredrickson • Stephanie Austin • Surekha Pendyal • Catherine Rehder • Priya S. Kishnani

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Abstract Glycogen storage disease (GSD) type IX is a rare disease of variable clinical severity affecting primarily the liver tissue. Individuals with liver phosphorylase b kinase (PhK) deficiency (GSD IX) can present with hepatomegaly with elevated serum transaminases, ketotic hypoglycemia, hyperlipidemia, and poor growth with considerable variation in clinical severity. PhK is a cAMP-dependent protein kinase that phosphorylates the inactive form of glycogen phosphorylase, phosphorylase b, to produce the active form, phosphorylase a. PhK is a heterotetramer; the alpha 2 subunit in the liver is encoded by the X-linked *PHKA2* gene. About 75% of individuals with liver PhK deficiency have mutations in the *PHKA2* gene; this condition is also known as X-linked glycogenosis (XLG). Here we report the variability in clinical severity and laboratory findings in 12 male patients from 10 different families with X-linked liver PhK deficiency caused by mutations in *PHKA2*. We found that there is variability in the severity of clinical features, including hypoglycemia and growth. We also report additional *PHKA2* variants that were identified in 24 patients suspected to have liver PhK deficiency. The basis of the clinical variation in GSDIX due to X-linked PHKA2 gene mutations is currently not well understood. Creating systematic registries, and collecting longitudinal data may help in better understanding of this rare, but common, glycogen storage disorder.
Synopsis: Liver phosphorylase b kinase (PhK) deficiency caused due to mutations in X-linked *PHKA2* is highly variable.

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"Deeksha S. Bali" and "Jennifer L. Goldstein" contributed equally.

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D.S. Bali · J.L. Goldstein · K. Fredrickson · S. Austin · S. Pendyal · P.S. Kishnani
Department of Pediatrics, Duke Medicine, Durham, NC, USA

C. Rehder
Department of Pathology, Duke Medicine, Durham, NC, USA

D.S. Bali (✉)
Biochemical Genetics Laboratory, Duke Medicine, 801-6 Capitola Drive, Durham, NC 27713, USA
e-mail: deeksha.bali@duke.edu

Introduction

Glycogen storage diseases (GSDs) are a heterogeneous group of inherited disorders characterized by the storage of glycogen in various tissues, particularly the liver and/or muscle. GSDs are caused by deficiencies of enzymes and transport proteins that are important in the metabolism of glycogen. Deficiency of liver phosphorylase b kinase (PhK), also known as glycogen storage disease type IX (GSD IX), accounts for about 25% of individuals with GSD. PhK is one of the key regulatory enzymes in glycogen metabolism. In response to physiological conditions, PhK activates phosphorylase enzyme in the liver and muscle, which in turn catalyzes glycogen breakdown in these organs (Newgard et al. 1989; Kishnani et al. 2009). It is estimated to affect about 1 in 100,000 people (Maichele et al. 1996) but may be underdiagnosed due to the variable presentation in some individuals. Children with liver PhK deficiency typically present in the first 2 years of life with hepatomegaly,

growth delay, elevated liver transaminases, and ketotic hypoglycemia of variable severity. Mild elevations in serum cholesterol and triglyceride are common, and postprandial lactic acid elevations may also be present (Wolfsdorf et al. 1999; Davit-Spraul et al. 2011). Based on available reports in the literature, symptoms generally improve with age, and most adults are virtually asymptomatic (Willems et al. 1990).

PhK is a complex enzyme composed of four copies of four different subunits (alpha, beta, gamma, and delta/calmodulin). The activity of the catalytic gamma subunit is regulated by the phosphorylation state of the alpha and beta subunits and by calmodulin via calcium levels (Brushia and Walsh 1999). Tissue-specific expression and alternative splicing of different genes for the various PhK subunits gives rise to differential expression of different isoforms of PhK in different tissues. In the liver, *PHKA2*, *PHKB*, and *PHKG2* encode the alpha, beta, and gamma subunits of PhK, respectively. While mutations in each of these genes can cause liver PhK deficiency, mutations in the X-linked *PHKA2* gene (Xp22.13; OMIM# 300798) are by far the most common cause of liver PhK deficiency, accounting for about 75% of cases (Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Roscher et al. 2014). Mutations in *PHKB* (16q12.1; OMIM# 172490) and *PHKG2* (16p11.2; OMIM# 172471) genes cause autosomal recessive forms of liver PhK deficiency and are responsible for most, if not all, of the remainder of PhK deficiency cases.

Traditionally, analysis of glycogen content and PhK enzyme activity in affected tissue samples, such as liver biopsy specimens and erythrocytes, has been important in the diagnosis of liver PhK deficiency. In liver biopsy specimens, glycogen content is highly elevated, with normal structure. PhK activity is usually reduced or absent. However, in a subgroup of patients (those with X-linked glycogenosis type 2, XLG 2), PhK activity can be normal or equivocal in blood cells and variable in liver specimens (Hendrickx et al. 1996; Burwinkel et al. 1997a, b, 1998a, b; Hendrickx et al. 1999). Therefore, a normal in vitro PhK activity does not rule out the diagnosis of liver PhK deficiency. DNA analysis, by sequencing individual genes, gene panels, or whole exome sequencing (WES), can confirm the diagnosis and circumvent the need for liver biopsy if causative pathogenic mutations are detected.

Although there is no clear gene/phenotype correlation seen in PhK deficiency, patients with mutations in *PHKG2* have been reported to have more severe symptoms and increased risk of developing liver cirrhosis in childhood (Maichele et al. 1996; van Beurden et al. 1997; Burwinkel et al. 1998a, b, 2000, 2003; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Fahiminiya et al. 2013; Albash et al. 2014; Bali et al. 2014; Roscher et al. 2014), while those with mutations in *PHKB* tend to have milder involvement (Burwinkel et al. 1997a, b; van den Berg et al. 1997; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Roscher et al. 2014). A wide variability in clinical presentation and severity has been recognized among patients with the most common subtype, X-linked PhK deficiency, ranging from mild involvement to severe, recurrent hypoglycemia and liver cirrhosis in some reported cases (Burwinkel et al. 1998a, b; Morava et al. 2005; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Tsilianidis et al. 2013; Roscher et al. 2014). In this manuscript we further report the variability in clinical severity and laboratory findings in 12 male patients from 10 different families with X-linked liver PhK deficiency caused by mutations in *PHKA2*. We also describe *PHKA2* variants identified in another 24 patients who were suspected to have liver PhK deficiency but for whom limited clinical information was available and/or the variants were not clearly pathogenic.

Materials and Methods

Patients

This study includes 12 male patients from 10 unrelated families. All subjects have a "pathogenic" or "likely pathogenic" variant in *PHKA2*, based on the American College of Medical Genetics and Genomics guidelines for the interpretation of sequence variants (Richards et al. 2015). Patients 1 and 2 are maternal first cousins, and Patients 6 and 7 are maternal second cousins. All of the patients were followed at medical centers in the United States.

Medical records were reviewed to obtain relevant clinical and laboratory data. Hypoglycemia was defined as blood glucose level <70 mg/dL. Height and weight percentiles were calculated according to the CDC stature-for-age and weight-for-age data (http://www.cdc.gov/growthcharts/cdc_charts.htm). Short stature was defined as height that is two standard deviations or more below the mean for children of that gender and chronologic age. This study was performed in accordance with the Institutional Review Board requirements of Duke University Health System.

PHKA2 Gene Sequencing

PHKA2 gene variants were detected through full gene Sanger sequencing of all 33 coding exons of the gene (NM_000292.2). Sequencing was performed either in a clinical diagnostic laboratory or by a research laboratory at Duke followed by confirmation in the Duke Molecular Diagnostics Laboratory. Sequence variants were named according to the recommendations of the Human Genome Variation Society (www.hgvs.org). The pathogenicity of

PHKA2 variants was investigated using the following in silico tools: the Berkeley Drosophila Genome Project (BDGP) Splice Site Prediction Tool (http://www.fruitfly.org/seq_tools/splice.html) for alterations to the splice site consensus sequences, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (Ramensky et al. 2002), Sorting Intolerant from Tolerant (http://sift.jcvi.org/) (Kumar et al. 2009) for amino acid substitutions, and Mutation Taster (http://www.mutationtaster.org/) for splice site consensus sequence changes, amino acid substitutions, and small in-frame deletions. To determine if any novel variants identified in this study are common variants, we searched the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Snp) and ExAC databases (http://exac.broadinstitute.org/). Variants were classified as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign based on the American College of Medical Genetics and Genomics guidelines for the interpretation of sequence variants (Richards et al. 2015).

Results

Subjects were aged <1 year–4 years at the time of diagnosis (median 2 years 6 months) and 5–17 years at the last evaluation (median 8 years 6 months). Subjects were followed for 2–14 years (median 5 years). A detailed summary of the clinical features and other findings in the 12 patients included in this study is given in Table 1.

Diagnosis

Six patients were initially diagnosed by PhK enzyme analysis in red blood cells ($n = 5$) or liver ($n = 1$) with enzyme activity levels of <10% of normal means. The diagnosis was further confirmed for these patients by the finding of a pathogenic/likely pathogenic mutation in *PHKA2*. One subject (Patient 8) had normal PhK activity in the liver but was later found to have a likely pathogenic variant in *PHKA2* that was previously reported to be associated with X-linked glycogenosis type 2 (XLG 2). Five patients were diagnosed by DNA testing alone.

Initial Clinical Presentation

For all of the patients, symptoms were first noticed in early childhood (3 years and under). Abdominal distension/hepatomegaly was the first symptom to be noticed for the majority of cases ($n = 8$). For another two patients (Patients 9 and 11), the initial concern was growth failure. Upon further evaluation, both of these patients had hepatomegaly and elevated serum transaminases. Another patient (Patient 7) first came to medical attention due to hypoglycemic seizures, but had additional features of GSD on further evaluation including hepatomegaly, elevated serum transaminases, elevated serum triglycerides and cholesterol, and a "doll-like face." Although Patient 2 had a known family history of X-linked PhK deficiency (affected first cousin; Patient 1), his family sought an evaluation after noticing that he fed frequently and had a cherubic face.

Hepatomegaly and Abdominal Imaging

Hepatomegaly, based on palpation, varied from 3 to 10 cm below the right costal margin, at the time of first evaluation. Abdominal imaging results (ultrasound $n = 10$; ultrasound and CT scan $n = 1$) were available for 11 patients. Typical findings on abdominal ultrasound were heterogeneously/diffusely echogenic and enlarged liver. One (Patient 1) had hepatosplenomegaly identified on ultrasound. Results of serial ultrasounds, performed approximately every 2 years, were available for eight patients. No signs of liver cirrhosis, adenoma, or focal abnormality were found on abdominal imaging for any of the patients at any time in the study.

Liver Histology

Liver histology reports were available for eight patients (Table 1), all of whom were less than 5 years old at the time of the biopsy. Liver histology universally showed distended hepatocytes with cytoplasmic glycogen accumulation shown by PAS staining. Mild to moderate steatosis was described for two patients (Patients 7 and 9). On electron microscopy, glycogen accumulation in hepatocytes appeared granular or particulate. Four patients had evidence of fibrosis (two with portal fibrosis, two with bridging fibrosis), but none had any signs of liver cirrhosis (Table 1). Muscle histology was performed for one patient (Patient 6) and was normal.

Glucose and Ketone Levels

Glucose levels were not measured routinely when patients were asymptomatic; therefore, the degree and frequency of hypoglycemia is not known. Blood glucose levels were available for seven patients who were treated with cornstarch. Pretreatment glucose levels for these patients varied from fasting lows of 60's mg/dL ($n = 1$) to 50's mg/dL ($n = 3$) to 40's mg/dL ($n = 3$). One of these patients (Patient 7) had hypoglycemic seizures prior to diagnosis, with glucose levels in the 30's mg/dL. After initiation of cornstarch treatment, occasional low blood glucose levels were still seen during times of illness or poor food intake. Four patients were not on regular cornstarch treatment; of these two patients had no documented low blood glucose levels, one had very occasional levels in the 60's, and one had some documented

Table 1 Key clinical features in the male patient cohort with *PHKA2* variants

Patient	Age at diagnosis (years)	Age at last evaluation (years)	Family history[a]	Hepatomegaly (liver cm below RCM[b] by palpation at time of diagnosis)	Liver pathology[c] (age at evaluation)	Fasting hypoglycemia[d]	Growth[e]	Musculoskeletal	Others
1	2	8	Yes	Hepatomegaly (10 cm); splenomegaly	ND	Hypoglycemia	Normal	Mild hypotonia in early childhood	Cherubic face, loose stools
2	0	5	Yes	Hepatomegaly (2 cm)	ND	Hypoglycemia	Normal	Normal	Cherubic face, occasional constipation
3	2	9	No	Hepatomegaly (10 cm)	No fibrosis, glycogen accumulation, no fatty change (2 years)	Hypoglycemia	Normal	Bilateral external rotation of the feet, mild spinal asymmetry[f]	Asthma, constipation, speech delay, learning difficulties
4	1	10	Yes	Hepatomegaly (7 cm)	No fibrosis, glycogen accumulation (1 year)	Mild hypoglycemia	Normal	Bilateral pronation of the feet, scoliosis, pectus excavatum	ECG change of incomplete right bundle branch block
5	1	9	Yes	Hepatomegaly (4 cm)	ND	Hypoglycemia	Normal	Bilateral pronation of the feet and mild scoliosis, resolved	
6	3	5	Yes	Hepatomegaly (10 cm)	Portal fibrosis, glycogen accumulation (3 years)	Hypoglycemia	Short stature	Medial pronation of feet and broad gait[f]	Premature birth with bowel obstruction caused by necrotizing enterocolitis
7	3	7	Yes	Hepatomegaly (9 cm)	Minimal portal fibrosis with areas of bridging fibrosis, moderate steatosis, glycogen accumulation (3 years)	Hypoglycemia	Short stature	Mild postural deviations, genu valgus[f]	Cherubic face, loose stools, exercise intolerance
8	2	17	No	Hepatomegaly (ND)	Mild portal fibrosis with areas of bridging fibrosis, glycogen accumulation (1 year)	Mild hypoglycemia	Normal	Mild genu recurvatum and pronation and eversion at feet and ankles[f]	Hyperflexibility
9	4	8	No	Hepatomegaly (mild)	No fibrosis, glycogen accumulation, mild steatosis (4 years)	No documented hypoglycemia	Short stature	Normal	Mild right hydronephrosis, meatal stenosis, flat top of the head, learning difficulties, ADD

(continued)

Table 1 (continued)

Patient	Age at diagnosis (years)	Age at last evaluation (years)	Family history[a]	Hepatomegaly (liver cm below RCM[b] by palpation at time of diagnosis)	Liver pathology[c] (age at evaluation)	Fasting hypoglycemia[d]	Growth[e]	Musculoskeletal	Others
10	4	11	No	Hepatomegaly (10 cm)	Bridging fibrosis, glycogen storage (4 years)	Hypoglycemia	Normal	Normal	
11	3	8	No	Hepatomegaly (ND)	No fibrosis, glycogen storage, no steatosis (3 years)	No documented hypoglycemia	Short stature	Medial pronation of the feet	Cherubic face
12	1	3	No	Hepatomegaly (ND)	ND	Mild hypoglycemia	Normal	Lumbar lordosis, bilateral pronation of the feet, mild muscle weakness[f]	Food allergies, hypothyroidism, global developmental delay

[a] Patients 1 and 2 are maternal first cousins; Patients 6 and 7 are maternal second cousins. Patients 4 and 5 each have a maternal male relative with a reported diagnosis of liver PhK deficiency
[b] *RCM* right costal margin, *ND* not done or not defined
[c] Based on liver histology
[d] Severity of fasting hypoglycemia varied. Hypoglycemia = episodes of fasting hypoglycemia <60 mg/dL; mild hypoglycemia = episodes of fasting hypoglycemia <70 mg/dL
[e] Growth delay is defined as <2 standard deviations below the mean
[f] Identified on physical therapy evaluation

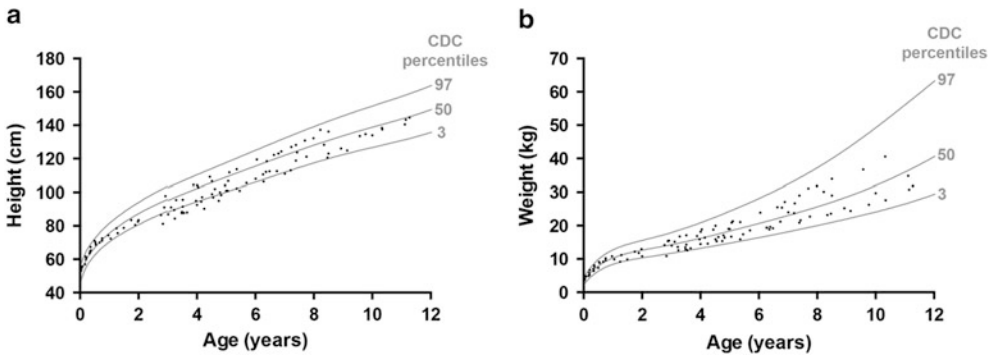

Fig. 1 Height (**a**) and weight (**b**) at different ages for our patient cohort. CDC percentiles were plotted using height and weight data for the 3rd, 50th, and 97th percentiles for 1–36 months and 3–20 years using data tables from the CDC (http://www.cdc.gov/growthcharts/percentile_data_files.htm)

lows in the 40–50's after overnight fast. Of note, two patients had blood glucose levels in the 50's mg/dL range without overt clinical symptoms.

Less data is available on ketone levels ($n = 4$). However, where data is available, blood ketone levels began to rise as glucose levels fell, and ketones were elevated in the presence of hypoglycemia, as expected (Brown et al. 2015).

Growth

Both growth and weight were variable in this group of patients (Fig. 1). Four of the 12 patients had short stature at some point during childhood (height <2 standard deviations below the mean). For all subjects, including those with normal stature, our data show a general trend of

reduction in growth velocity in early childhood and later improvement in growth over time. Due to variations in treatment strategy, it was not possible to separate the effects of age and treatment on growth. One patient was treated with growth hormone from age 3 years; his height increased from the 1st to the 16th percentile over the next 14 months, and by age 8 years his height was at the 90th percentile.

Cardiology Evaluations

Seven patients had an echocardiogram (ages 2–4 years for six patients and age 17 years for one patient); three of them also had an EKG. Cardiac findings were present in two patients; one had incomplete right bundle branch block that did not affect cardiac function, and the other had a very small patent foramen ovale that did not require intervention. Otherwise, no cardiac problems were identified.

General Laboratory Testing and Evaluations

A summary of serum transaminase results is given in Table 2. At the time of diagnosis, serum transaminases (AST, ALT) were elevated by 2–36 times the upper limit of normal in all patients. Serum transaminase levels decreased with increasing age in all patients, regardless of treatment. Elevations of serum cholesterol and triglyceride were common at diagnosis and tended to decrease with increasing age. Plasma lactate levels were available for seven patients and were usually normal with occasional mild elevations. However, one patient (Patient 8) had several unexplained elevated lactate levels (around 2× upper limit), with increased anion gap, which later resolved. Plasma uric acid level, where measured ($n = 6$), was normal. Creatine kinase level was normal in all patients in which it was analyzed ($n = 7$). Urine organic acid analysis was performed for four patients. Results were normal for two patients (Patients 7 and 10); one patient had marked elevation of lactate and prominent ketosis in one sample (Patient 6); another patient (Patient 9) had slight elevation of 3-methylglutaconic acid which subsequently normalized. Serum total protein and albumin levels at diagnosis and prior to initiating dietary treatment were normal in patients in whom this data was available ($n = 6$).

Treatment

Treatment strategies varied considerably between patients; most (8 out of 12 patients) took oral uncooked cornstarch and some of those patients ($n = 4$) also had protein supplementation; the amount and frequency of cornstarch and protein supplementation varied between patients according to age and current needs. General treatment guideline is to recommend small frequent meals to avoid hypoglycemia.

Cornstarch dose was individualized based on the risk of hypoglycemia and varied between 0.4 g and 2 g/kg throughout the day or at bedtime, protein to provide ~20% calories (3–4 g protein/kg/day) and distributed throughout the day, and fat to meet the general criteria of a heart healthy diet.

Current need for CS and protein was assessed by measuring blood glucose and ketone levels over a 2–3-day period, upon waking in the morning, before meals, and after activity. Due to the clinical variability and differences in individualized treatment regimens, it was not possible to compare the effects of treatment on parameters such as growth and laboratory values (AST, ALT).

Table 2 Key laboratory findings in the male patient cohort with *PHKA2* variants

Patient	AST – multiples of ULN at the time of initial diagnosis	AST – multiples of ULN at the time of last evaluation	ALT – multiples of ULN at the time of initial diagnosis	ALT – multiples of ULN at the time of last evaluation
1	16.2	0.7	36.4	1.0
2	7.5	3.8	8.9	4.4
3	4.7	1.8	3.1	1.6
4	2.7	0.6	3.8	0.4
5	23.9	0.8	16.8	0.9
6	13.5	1.4	6.9	1.6
7	30.7	3.0	20.8	3.6
8	23.4	0.6	16	1.2
9	2.0	1.7	1.8	1.5
10	2.7	1.3	2.9	0.8
11	3.6	0.7	2.9	0.3
12	11.1	0.9	11.6	1.1

ULN upper limit of normal, *AST* aspartate transaminase, *ALT* alanine transaminase

PHKA2 Gene Sequencing

The *PHKA2* mutations in these patients are listed in Table 3 and in the Supplementary Tables 1, 2, and 3. The Supplementary Tables 1, 2, and 3 also includes *PHKA2* variants that were identified in another 24 families for whom limited clinical information was available. Overall, we identified 17 different pathogenic/likely pathogenic mutations (7 missense, 5 frameshift, 2 splice site, 2 nonsense mutations, and 1 multi-exon deletion), 9 variants of unknown significance, and 6 benign/likely benign variants. Ten of the pathogenic/likely pathogenic variants are novel (Supplementary Tables 1, 2, and 3).

Discussion

Here, we present the clinical and laboratory features of 12 patients with PhK deficiency caused by mutations in the *PHKA2* gene. Similar to previous case series, symptoms of hepatomegaly, elevated serum transaminases, ketotic hypoglycemia, and growth delay were common among our cohort, and there was variability in severity (Beauchamp et al. 2007a, b; Achouitar et al. 2011; Davit-Spraul et al. 2011; Kido et al. 2013; Roscher et al. 2014). Historically, liver PhK deficiency was reported to be "benign," but more recently, patients have been described with symptoms ranging from growth retardation alone (Hirono et al. 1998) to asymptomatic hepatomegaly (Willems et al. 1990; Kim et al. 2015), to severe recurrent hypoglycemia and growth delay (Hidaka et al. 2005; Achouitar et al. 2011) and progression to liver cirrhosis (Johnson et al. 2012; Tsilianidis et al. 2013). There are considerable differences in severity among patients with the same *PHKA2* mutation (Hirono et al. 1998; Achouitar et al. 2011) suggesting that other genetic and environmental factors may influence disease severity. In this regard, it is interesting that two of the more severely affected patients in our cohort (Patients 6 and 7) are also heterozygotes for a pathogenic mutation, c.79delC, in GSDI gene, *G6PC*. This gene encodes glycogen-6-phosphatase, which catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis. It is possible that there is a synergistic effect between carrier status for GSD Ia and liver PhK deficiency in Patients 6 and 7 which results in a more severe phenotype for these patients.

Interestingly for two patients in our cohort, low blood glucose levels (in the 50's mg/dL) were not associated with any obvious clinical symptoms but were found by chance during a clinic visit. Another patient was ketotic in the absence of symptoms and with normal glucose levels. The presence of ketosis with minimal hypoglycemia has been reported in patients with liver PhK deficiency (Tsilianidis et al. 2013). These findings, both of asymptomatic hypoglycemia and ketosis, indicate the importance of monitoring glucose and ketone levels routinely, even in the absence of symptoms, because undetected hypoglycemia and ketosis can affect growth and development, and dietary intervention may be needed.

Table 3 *PHKA2* variants, predicted to be pathogenic or likely pathogenic, identified in our patient cohort

Patient	Location	cDNA change	Amino acid change	Reference
1	Exon 2	c.133C>T	p.Arg45Trp	Davit-Spraul et al. (2011), Tsilianidis et al. (2013), Wang et al. (2013), and Brown et al. (2015)
2	Exon 2	c.133C>T	p.Arg45Trp	Davit-Spraul et al. (2011), Tsilianidis et al. (2013), Wang et al. (2013), and Brown et al. (2015)
3	Exon 2	c.134G>A	p.Arg45Gln	This study
4	Intron 5	c.537+2T>C		This study (c.537+5G>A) reported (Davit-Spraul et al. 2011; Choi et al. 2016)
5	Exon 8	c.811delG	p.Glu271Lysfs*3	This study
6	Exon 9	c.883C>T	p.Arg295Cys	Ban et al. (2003)
7	Exon 9	c.883C>T	p.Arg295Cys	Ban et al. (2003)
8	Exon 9	c.884G>A	p.Arg295His	Hendrickx et al. (1999) and Choi et al. (2016)
9	Intron 16	c.1715-2A>G		This study
10	Exon 21	c.2238-2239delTG insGAACAGGCC	p.Ser746Argfs*11	This study
11	Exon 31	c.3334G>T	p.Glu1112*	This study
12	Exon 33	c.3614C>T	p.Pro1205Leu	van den Berg et al. (1995), Hirono et al. (1998), Achouitar et al. (2011), Davit-Spraul et al. (2011), and Roscher et al. (2014)

Patient 3 also has a variant of unknown significance, p.Phe71Val (c.211T>G), in *PHKB*

Of the four patients for whom urine organic acid analysis was performed, one (Patient 9) had mild elevations of 3-methylglutaconic acid. Elevation of urine 3-methylglutaconic acid has previously been reported in some patients with liver PhK deficiency caused by *PHKA2* (Achouitar et al. 2011) and *PHKG2* mutations (Bali et al. 2014) and also in patients with GSD I (Law et al. 2003). The cause of urinary 3-methyglutaconic acid elevations in some patients with glycogen storage disease is unknown but may reflect abnormal cholesterol metabolism or general mitochondrial dysfunction (Law et al. 2003).

Three patients in our cohort had developmental delays including speech delay with learning difficulties (Patient 3); motor delays, dyslexia, ADD, language-based learning disability, and behavior problems (Patient 9); and global developmental delay (Patient 12). While most individuals with liver PhK deficiency do not have these problems, cognitive impairment or speech delay has been reported (Burwinkel et al. 1998a, b; Beauchamp et al. 2007a, b; Roscher et al. 2014). It is not clear if PhK deficiency contributes to neurocognitive problems in any way. It is possible that undetected hypoglycemia, particularly early in life, could be a factor. It is known that neonatal hypoglycemia can cause brain injury leading to developmental delay/cognitive impairment, particularly if it is severe and prolonged (Montassir et al. 2009). We do not have evidence for severe, prolonged hypoglycemia in any of our subjects with developmental delays/learning difficulties, but this cannot be ruled out. It is also possible that PhK deficiency may have a more direct effect on brain function. ESTs from PhK subunit genes, including *PHKA2*, are found in the brain (Winchester et al. 2007) suggesting that PhK activity may have a role in brain function. Further studies are needed to determine whether developmental delays are any more common in children with liver PhK deficiency when compared to the general population.

In this study, we identified ten novel pathogenic/likely pathogenic variants in *PHKA2*, further showing the genetic heterogeneity involved in X-linked liver PhK deficiency as indicated in previous studies (Hendrickx et al. 1999; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Roscher et al. 2014). One of these novel variants, p. Arg45Gln, was found in three families; it is not known if these families are related or whether this variant is recurrent.

Molecular testing for patients suspected to have GSD type IX (PhK deficiency) is very helpful in confirmation of the diagnosis and identifies which PhK subunit gene is altered and provides information on the inheritance pattern (Davit-Spraul et al. 2011). Finding of rare pathogenic mutations in families with this rare but variable GSD disorder could provide important education about genotype and phenotype correlations. Gene panels or whole exome sequencing (WES) can be helpful because similar symptoms can present in individuals with various types of glycogen storage disease including GSD III, VI, and IX. However, due to the limitations of WES, including the possibility of missing a mutation if coverage is poor or if a large deletion or duplication is present, single gene or panel sequencing should be considered if the clinical suspicion for liver PhK deficiency is high.

In conclusion, liver PhK deficiency caused by mutations in the *PhKA2* gene is a highly variable condition in terms of clinical presentation and severity. Although our study highlights the variability among our patient cohort, it has the inherent limitations of a retrospective review of medical records. Since some subjects had limited data, while certain trends were noted, we do not know if they apply to the population as a whole. Secondly, early data availability was very limited; therefore, analysis of trends early in life was not possible except for growth parameters. In addition, there may be bias toward more severely affected patients because very mildly affected patients may not come to medical attention (Willems et al. 1990). It would be ideal to follow the early development data and parameters in a population of children with *PHKA2* mutations, from birth, and look for environmental and genetic factors that alter the presentation of the condition. In order to achieve this, creating a detailed registry for individuals diagnosed with liver PhK deficiency would be very helpful and educational for GSD treating community.

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RESEARCH REPORT

Hyperphenylalaninemia Correlated with Global Decrease of Antioxidant Genes Expression in White Blood Cells of Adult Patients with Phenylketonuria

Charlotte Veyrat-Durebex • Christelle Debeissat • Hélène Blasco • Franck Patin • Hélène Henique • Patrick Emond • Catherine Antar • Valérie Gissot • Olivier Herault • François Maillot

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Abstract *Background*: Several studies have highlighted disturbance of redox homeostasis in patients with phenylketonuria (PKU) which may be associated with neurological disorders observed in patients, especially during adulthood when phenylalanine restrictive diets are not maintained. The aim of this study was to assess the antioxidant profile in a cohort of PKU patients in comparison to the controls and to evaluate its relation to biochemical parameters especially phenylalaninemia.

Methods: We measured RNA expression of 22 antioxidant genes and reactive oxygen species (ROS) levels in white blood cells of 10 PKU patients and 10 age- and gender-matched controls. We also assessed plasma amino acids, vitamins, oligo-elements, and urinary organic acids concentrations. Then we evaluated the relationship between redox status and biochemical parameters.

Results: In addition to expected biochemical disturbances, we highlighted a significant global decrease of antioxidant genes expression in PKU patients in comparison to the controls. This global decrease of antioxidant genes expression, including various isoforms of peroxiredoxins, glutaredoxins, glutathione peroxidases, and superoxide dismutases, was significantly correlated to hyperphenylalaninemia.

Conclusion: This study is the first to evaluate the expression of 22 antioxidant genes in white blood cells regarding biochemical parameters in PKU. These findings highlight the association of hyperphenylalaninemia with antioxidant genes expression. New experiments to specify the role of oxidative stress in PKU pathogenesis may be useful in suggesting new recommendations in PKU management and new therapeutic trials based on antioxidant defenses.

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The authors "Olivier Herault" and "François Maillot" contributed equally.

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C. Veyrat-Durebex (✉) · H. Blasco · F. Patin · C. Antar
Laboratoire de biochimie et biologie moléculaire, hôpital Bretonneau CHRU de Tours, Tours, France
e-mail: charlotte.veyrat@live.fr; charlotte.veyratdurebex@chu-angers.fr

C. Veyrat-Durebex · H. Blasco · F. Patin · P. Emond · C. Antar
INSERM U930, Université François Rabelais Tours, Tours, France

C. Debeissat · O. Herault
CNRS UMR 7292 - équipe LNOx, Université François Rabelais Tours, Tours, France

C. Debeissat · O. Herault
Service d'hématologie biologique, CHRU de Tours, Tours, France

V. Gissot
INSERM CIC 1415, CHRU de Tours, Tours, France

H. Henique · F. Maillot
Service de médecine interne, CHRU de Tours, Tours, France

H. Henique · F. Maillot
INSERM U1069, Université François Rabelais Tours, Tours, France

Abbreviations

BMI	Body mass index
CAT	Catalase
Ct	Cycle threshold
GLRX	Glutaredoxin
GPX	Glutathione peroxidase
GSR	Glutathione-disulfide reductase
Phe	Phenylalanine

PKU	Phenylketonuria
PRDX	Peroxiredoxin
ROS	Reactive oxygen species
RQ	Relative quantification
SOD	Superoxide dismutase
TXN	Thioredoxin

Introduction

Phenylketonuria (PKU, OMIM #26160) is an autosomal recessive disease caused by the deficiency of phenylalanine hydroxylase activity leading to an accumulation of phenylalanine (Phe) and its metabolites in blood and tissues of affected patients (Krause et al. 1985; Mazzola et al. 2013). Neonatal newborn mass-screening of this disorder allows early diagnosis and consequently a better and earlier dietary management leading to a normal development of PKU children (Groselj et al. 2014). In some countries, Phe-restricted treatment was generally not maintained over the second decade of life, after the development period (de Baulny et al. 2007). However, evidences suggest that high plasma Phe concentrations can cause neurological and psychological disorders in adulthood (Waisbren et al. 2007; ten Hoedt et al. 2011; Sharman et al. 2012; Bilder et al. 2013; Okano and Nagasaka 2013; Weglage et al. 2013). Thus, maintaining a Phe-restricted diet in adult PKU patients is still debated (Demirkol et al. 2011; ten Hoedt et al. 2011; Trefz et al. 2011; Hanley 2013; Mutze et al. 2016). Several hypotheses about the pathophysiology of brain injury in PKU have been discussed but the mechanisms remain unclear and seem to be multifactorial (Bone et al. 2012).

Oxidative stress was observed in several diseases such as neurological diseases (Halliwell 2006) and some inborn errors of metabolism including PKU (Sirtori et al. 2005; Sitta et al. 2006; Deon et al. 2015), maple syrup urine disease (Barschak et al. 2006), tyrosinemia (Bird et al. 1995), urea cycle defects, homocystinuria (Streck et al. 2003; Vanzin et al. 2011), and organic acidurias (Wajner et al. 2004). In PKU, accumulation of Phe and/or its metabolites may lead to a disturbance of redox homeostasis (Artuch et al. 2004; Mazzola et al. 2013). Some studies showed an increase of lipoperoxidation markers (Sanayama et al. 2011) and DNA damages (Sitta et al. 2009), or a reduction of various antioxidant compounds, especially selenium, coenzyme Q10, or antioxidant system of glutathione (Menzel et al. 1983; Sitta et al. 2006; Sanayama et al. 2011). Decrease of antioxidant compounds was also observed in animal and in vitro models of PKU (Ercal et al. 2002; Fernandes et al. 2010; Mazzola et al. 2011; Moraes et al. 2014). Imbalance in redox homeostasis in PKU is now admitted, but its link with metabolic disturbances observed in the disease remains unclear. Understanding and characterization of oxidative mechanisms in PKU are essential for management and treatment of the disease. It remains crucial to assess whether oxidative unbalance may be associated with plasma Phe concentration, and potentially controlled by a diet limiting the plasma Phe concentration and/or antioxidants supplementation strategy, which may be useful for PKU management and patients' outcome (Mazzola et al. 2013).

A better understanding of the relations between PKU biochemical markers and oxidative stress may provide a new light on pathophysiological mechanisms and open new therapeutic perspectives. Thus, the aim of the study was to characterize the antioxidant status in PKU by quantifying the expression of major antioxidant genes in white blood cells and by assessing the link of antioxidant status with biochemical markers.

Methods

Patients and Controls

Blood and urinary samples were obtained from ten untreated PKU adult patients and ten sex- and age-matched healthy controls. Patients and controls were recruited from September 2014 to February 2015 in the University Hospital of Tours. All PKU patients were diagnosed at birth. For each patient, clinical data including information on diagnosis of PKU, gender, age, age of dietary management cessation, treatment, smoking status, pollutant exposure, height, and weight were collected. Body mass index (BMI) was calculated for each included subject. PKU patients did not undergo Phe-restricted diet at time of sample collection or study. Controls were selected in accordance to age and sex following the inclusion of each PKU patient. This study was approved by the ethics committee of University Hospital of Tours (CPPRB, for Comité de Protection des Personnes) and registered on ClinicalTrials.gov (study ID number PHAO14/FM-StressOX-PCU, "Antioxidant Signature in Adults Patients with Phenlyketonuria"). Informed consent was signed by all patients and controls prior to inclusion.

Blood and urinary samples were all collected in the morning fasting. Urines were directly stored at −80°C until organic acids analysis. Blood biochemical parameters were measured upon arrival at the laboratory following routine recommendations. For the measurement of oxidative stress parameters, a gentle lysis of red blood cells was performed after samples' collection. Reactive oxygen species level was directly assessed on leukocytes and leukocytes samples

used for antioxidant genes expression were stored in Trizol® at −80°C until RNA extraction.

Biochemical Tests

Biochemical tests were performed in routine laboratory using nationally validated reference methods (with validated or ongoing accreditation).

Plasma concentrations of amino acids were determined using ion exchange chromatography with post-column derivation by ninhydrin. Relative urinary concentrations of organic acids were assessed using gas chromatography coupled with mass spectrometry (GC-MS) (Supplementary Methods).

Blood vitamins A, C, and E concentrations were determined by High Performance Liquid Chromatography (HPLC) with UV detection at 245 nm using assay kits provided by Chromsystems (Chromsystems Instruments & Chemicals GmbH).

Plasma concentrations of selenium were measured by inductively coupled plasma–mass spectrometry, and plasma concentrations of zinc and copper by inductively coupled plasma–atomic emission spectrometry.

Oxidative Stress Parameters Measurement

Reactive Oxygen Species (ROS) Measurement

The level of reactive oxygen species (ROS) in leukocytes was evaluated by flow cytometry using the cell permeant reagent CM-H_2DCFDA (chloromethyl derivate of 2′,7′-dichlorodihydrofluorescein diacetate) (Life Technologies). This reagent is oxidized in 2′,7′-dichlorofluorescein (DCF), a highly fluorescent component, by intracellular ROS, especially H_2O_2.

Heparinized whole peripheral blood samples were incubated for 10 min at 37°C in lysis buffer (125 µM EDTA, 10 mM $KHCO_3$, 155 mM NH_4Cl) for gentle lysis of red blood cells and washed twice with X-Vivo10™ (Lonza). Cells were then incubated for 10 min at 37°C with 9.6 µmol/L of CM-H_2DCFDA, washed twice with cold phosphate buffered saline, and DCF level was analyzed by flow cytometry (Gallios™, Beckman Coulter). Data analysis was performed with FlowJo software. Results are expressed as geometric mean of fluorescence intensity.

Antioxidant Genes Expression Profile

We assessed the expression of 22 antioxidant genes (26 transcripts) by real-time PCR in white blood cells of PKU patients and controls.

Total cellular RNA was isolated from leukocytes with Trizol® (Invitrogen) according to the manufacturer's instructions and after gentle lysis of red blood cells (protocol above). RNA quality were checked on Agilent 2100 bioanalyzer (Applied Biosystems) using Agilent RNA 6000 Nano kit (Agilent Technologies) according to manufacturer's instructions.

RNA reverse-transcription was performed with SuperScript® VILO coding DNA (cDNA) Synthesis Kit (Invitrogen) according to manufacturer's instruction. cDNA samples obtained were diluted in H_2O and stored at −20°C. Real-time Polymerase Chain Reaction (PCR) was performed according to Universal Probe Library Technology with Light Cycler 480 Probe Master (Roche Diagnostics) and Real-Time Ready Custom Panel pre-coated plates (Roche Diagnostics). We used previously described antioxidant profile (Herault et al. 2012). cDNA specificity is brought by Universal Probe Library technology that uses short hydrolysis probes recognizing amplicon of a specific primer pair. Primer sequences and specifically associated probes are available on request. Three microliters of cDNA samples were mixed with 5 µL master mix in a final volume of 10 µL, distributed in pre-coated plate and run 10 min at 95°C, 10 s at 95°C, and 30 s at 60°C for 45 cycles, and finally 10 s at 40°C on Light Cycler 480 (Roche). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *B2M* (β-2-microglobulin), and *ACTB* (β-actin) were used as reference genes. The geometric mean of GAPDH, B2M, and ACTB cycle threshold (Ct) was used to normalize samples according to ΔCt method (Ct target − mean Ct reference). Relative quantification (RQ) was calculated as $RQ = 2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct = (\Delta Ct_{PKU\ patients} - \Delta Ct_{healthy\ controls})$. The numerical value of the Ct is inversely proportional to the amount of amplicon in the reaction (i.e., the lower the Ct, the greater the amount of amplicon). Results of real-time PCR assays are presented as fold change of genes expression in white blood cells of PKU patients in comparison to controls and were considered relevant for a fold change >2 (Schmittgen and Livak 2008).

Statistical Analysis

The Wilcoxon-Mann-Whitney non-parametric test was used to compare mean age, body mass index (BMI), biochemical measure values, and redox parameters between PKU patients and controls. We evaluated the relation between biological parameters and molecular antioxidant status as well as clinical data using Spearman's rank correlation coefficient (Spearman's rho). Differences were considered significant by comparing *p*-value to a threshold value which was calculated according to Benjamini and Hochberg procedure for multiple comparisons (Hochberg and Benjamini 1990; Benjamini et al. 2001).

Statistical analysis was performed with JMP statistical software version 7.0.2 (SAS Institute).

Results

Subjects

Statistical analyses were performed on data from nine PKU patients and their nine matched controls. Data from one patient and his matched control were excluded from the analysis because the patient was receiving treatment by tetrahydrobiopterin (BH4).

The mean age for the PKU patients was 31 years (±6) and was not significantly different from that of the controls ($p = 0.7186$). BMI of PKU patients was significantly higher than BMI of controls (26 ± 4 vs 22 ± 4 kg/m^2 with $p = 0.0283$). All patients followed a Phe-restrictive diet in childhood and stopped it at the average age of 6 years (age from 5 to 13 years). There is no significant differences in smoking status or pollutant exposure between controls and patients (p-values of chi^2-test respectively 0.53 and 0.28). Patients' characteristics were given in supplementary results (Table S1). Among the ten patients initially included in the study, three patients were the subject of a more detailed analysis because of their disease particularity: (1) Patient-2 received BH4 treatment (this patient was excluded from global statistical analysis); (2) Patient-9 presented a permanent moderate hyperphenylalaninemia; (3) Patient-10 was classified as an atypical PKU.

Biochemical Parameters

As expected we observed higher plasma Phe concentrations and lower plasma tyrosine concentrations in PKU patients compared to controls ($p = 0.0003$ and $p = 0.0026$, respectively) (Table S2). PKU patients showed significantly lower plasma concentrations of several amino acids: threonine, glutamine, proline, alanine, α-amino-butyric acid, valine, methionine, isoleucine, leucine, and arginine (Table S2).

Using the routine diagnostic procedure, we measured urinary relative concentrations of 30 organic acids by GC-MS (Table S3). We observed a negative correlation between plasma concentrations of Phe and urinary concentrations of glycerate (ρ Spearman $= -0.75$ and $p = 0.0002$), suberate (ρ Spearman $= -0.64$ and $p = 0.0024$), and 3-hydroxy-glutarate (ρ Spearman $= -0.63$ and $p = 0.0028$). We also showed a positive correlation of plasma Phe and urinary 2-hydroxy-phenylacetate (ρ Spearman $= 0.83$ and $p < 0.0001$), 2-keto-glutarate (ρ Spearman $= 0.69$ and $p = 0.0007$), 3-hydroxy-3-methyl-glutarate (ρ Spearman $= 0.68$ and $p = 0.001$), 4-hydroxy-phenylpyruvate (ρ Spearman $= 0.61$ and $p = 0.0046$), isovaleryl-glycine (ρ Spearman $= 0.59$ and $p = 0.0058$), 3-hydroxy-butyrate (ρ Spearman $= 0.54$ and $p = 0.0145$), and 3-hydroxy-isovalerate (ρ Spearman $= 0.54$ and $p = 0.0141$) (p-values were compared to threshold p-values calculated according to Benjamini-Hochberg formula).

Analysis of plasma concentrations of vitamins A, E, and C and trace-elements (selenium, copper, zinc) revealed no significant differences between PKU patients and controls (Table S2).

Redox Parameters

We observed a trend to higher ROS levels in monocytes of PKU patients when compared to controls without any statistical significant differences in all white blood cells subpopulations (monocytes, lymphocytes, and granulocytes) (Fig. 1).

Fold change of antioxidant genes expression in PKU patients compared to controls is illustrated in Fig. 2. PKU patients presented a global decrease of main antioxidant genes (Fig. 2). We observed a two- to threefold decreased expression of the following antioxidant genes in PKU patients when compared to healthy controls: superoxide dismutase 1 (*SOD1*), glutaredoxin 2 and 3 (*GLRX2* transcript 2 and *GLRX3*), glutathione peroxidase 1 and 4 (*GPX1* and *GPX4*), and peroxiredoxins 1, 2, 4, and 5 (*PRDX1*, *PRDX2* transcript 3, *PRDX4*, and *PRDX5*) (Fig. 2 and Table 1). We did not observe difference in the expression of the reference genes, relative to the amount of RNA, between patients and controls (*GAPDH* $p = 0.2568$, *B2M* $p = 0.2265$, *ACTB* $p = 0.2265$). Detailed analysis of antioxidant profile in PKU patients highlighted that the decrease of antioxidant genes expression of patients 2, 9, and 10 (respectively treated by BH4, permanent moderate hyperphenylalaninemia, and atypical PKU) was less pronounced as compared to the average typical PKU patients (Fig. S1).

Relationship Between Metabolic Factors and Antioxidant Status

We observed that high plasma concentrations of Phe were associated with low expression of various antioxidant genes (*PRDX1*, *GPX4*, *GLRX3*, *SOD1*, *PRDX2 transcripts 1 and 3*, *TXN2*, *PRDX4*, *GLRX5*, *GPX7*, and *SOD3*) (Table 1). We observed significant positive correlation between plasma concentrations of amino-butyric acid and expression of various antioxidant genes (correlation of low plasma amino-butyric acid with low expression of *SOD1*, *GPX4*, *GPX1(2)*, *PRDX5*, *SOD3*, *PRDX1*, *PRDX4*, *GLRX5*, *TXN2*, *GLRX2(2)*, and *GLRX3* with p-values <0.005). We also observed significant correlation between various antioxidant genes expression and plasma tyrosine and branched-chain amino acids (data not shown). Observed variations in urinary concentrations of organic acids also seemed to be correlated

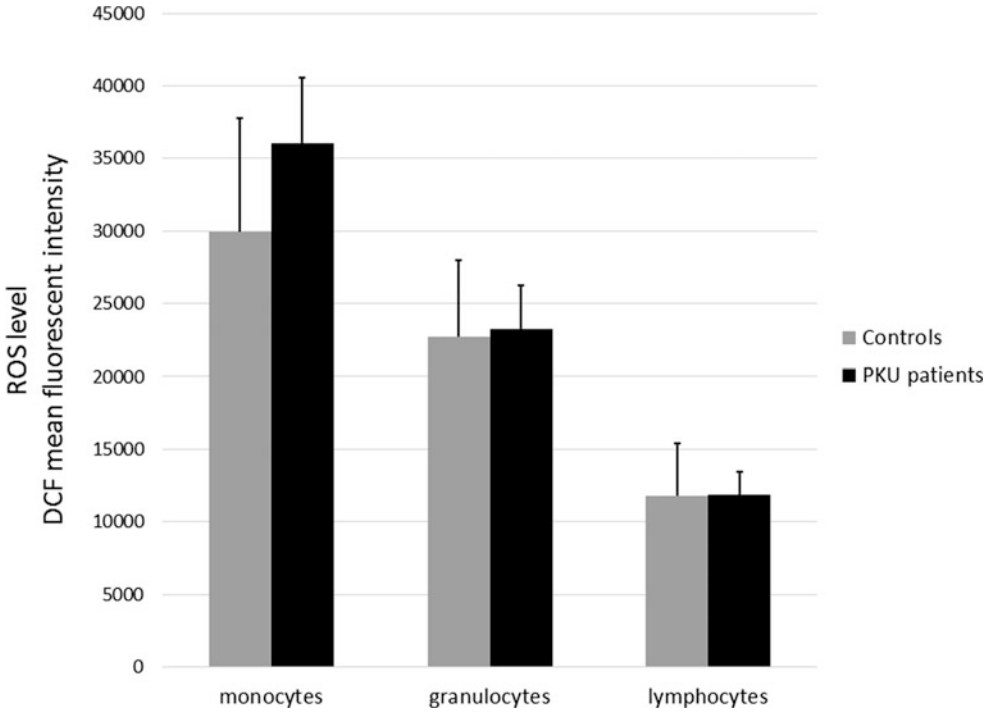

Fig. 1 ROS production in subpopulations of white blood cells. Relative ROS production in white blood cells of PKU patients and controls was determined using the CM-H_2DCFDA dye in flow cytometry. ROS levels in three subpopulations of leukocytes (monocytes, granulocytes, and lymphocytes) are shown as mean fluorescence intensity in cells (error scales are standard error of the mean)

with the decrease of expression of some antioxidant genes (p-values <0.01).

No significant correlation was observed between age or BMI and biochemical parameters or antioxidant profile (data not shown).

Discussion

This preliminary study is the first to assess the gene expression profile of 22 major antioxidant enzymes in white blood cells of adult PKU patients and to analyze their relation with the metabolic status. These data highlight a lower antioxidant arsenal in leukocytes associated with hyperphenylalaninemia.

PKU patients of this study were representative of the adult PKU population with high phenylalaninemia and low tyrosinemia (Krause et al. 1985). As expected we observed a positive correlation between plasma Phe concentrations and urinary concentrations of Phe-derived organic acids (2-hydroxy-phenylacetate, 4-hydroxy-phenylpyruvate). As previously observed, PKU patients of this study presented a higher BMI compared to controls (Belanger-Quintana and Martinez-Pardo 2011; Burrage et al. 2012; Robertson et al. 2013). This high BMI could be due to an unbalanced diet of PKU patients due to habits acquired during restrictive diet in childhood (Robertson et al. 2013), but this hypothesis is controversial (Rocha et al. 2012). We did not highlight correlation suggesting that the unbalanced oxidative status that we observed in PKU patients was related to their higher BMI.

We observed a decrease of several amino acids correlated to hyperphenylalaninemia, especially branched-chain amino acids. These lower plasma concentrations of branched-chain amino acids were already described in children with PKU (Antonozzi et al. 1987), and a deficiency of branched-chain amino acids, tyrosine, and tryptophan transport across the brain–blood barrier was described in PKU mice due to a competition with Phe transport (Matalon et al. 2003). A defect in central nervous system protein synthesis has been highlighted in PKU and correlated with plasma Phe concentrations in PKU patients, and experiments in animal or in vitro models (de Groot et al. 2013; Schuck et al. 2015). We showed a significant decrease of plasma amino-butyric acid in PKU patients, which is correlated to plasma Phe and to the decreased expression of some antioxidant genes. Amino-butyric acid is an amino acid not taking part in protein synthesis but is considered as a nonspecific biomarker of liver, malnutrition, or protein catabolism (Chiarla et al. 2011). We highlighted an increase of urinary 3-hydroxy-3-methylglutarate in hyperphenylalaninemia ($p = 0.0018$). This result is

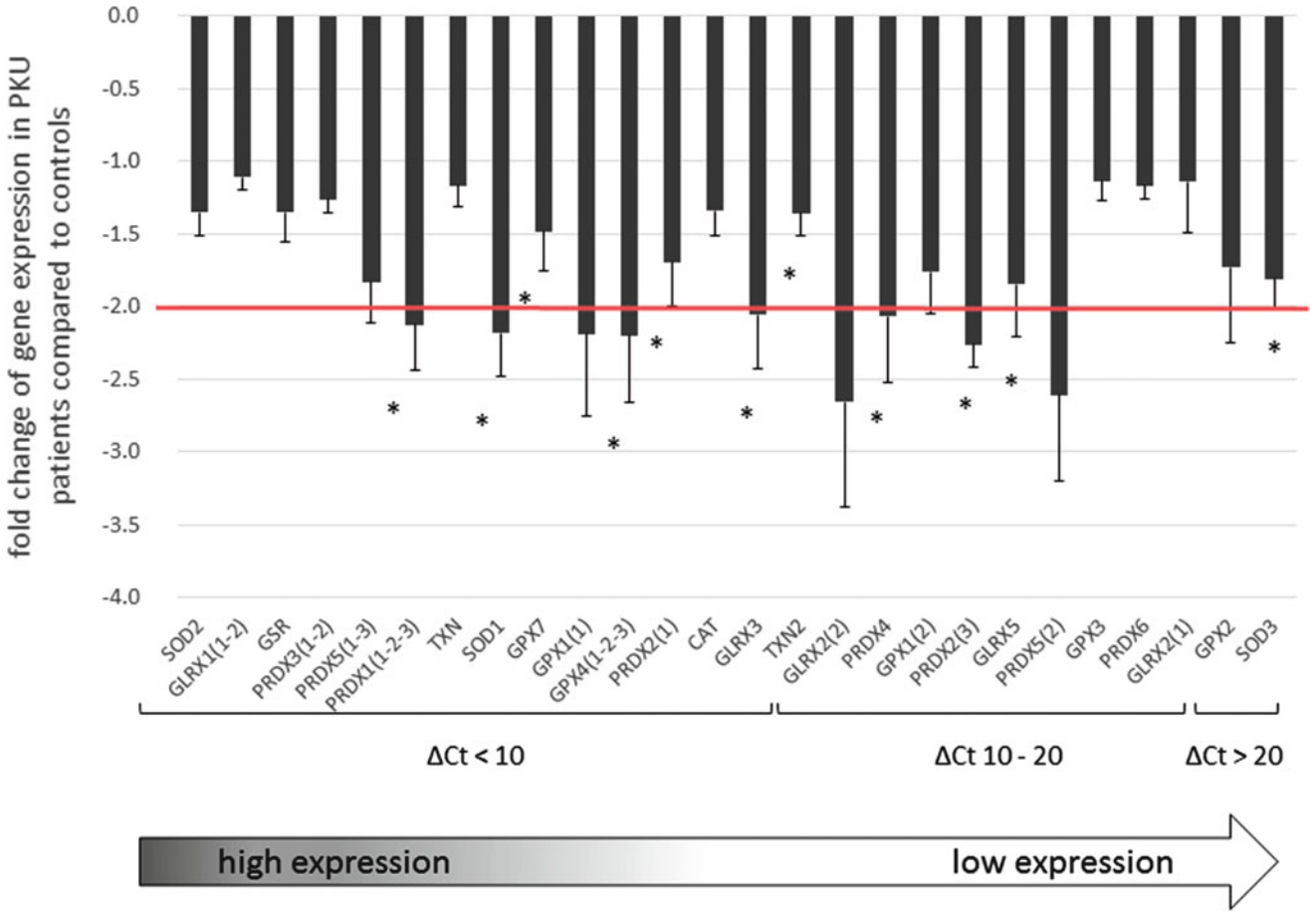

Fig. 2 Molecular antioxidant profile of PKU patients compared to healthy controls. RNA isolated from white blood cells of PKU patients and controls was reverse transcribed, and a real-time PCR using a custom panel was performed on cDNA in order to assess the expression of main antioxidant genes. Results of real-time PCR assays are presented as fold change ratio of genes expression in white blood cells of PKU patients compared to controls. Genes of the antioxidant profile are presented in order from most to least expressed in X-axis, according to their ΔCt values (versus mean Ct references). Data are presented as mean fold change ratio of genes expression in PKU patients compared to controls (error scales are standard error of the mean). *Red scale* represented the twofold change threshold. *Significant correlations between high concentrations of Phe and low expression of antioxidant gene (considered significant with a *p*-value inferior to threshold value calculated using Benjamini-Hochberg formula for multiple comparison correction)

consistent with previous experiment showing that high concentration of Phe inhibits the activity of 3-hydroxy-3-methylglutaryl-CoA reductase linked with a deficit of antioxidant coenzyme Q_{10} involved in mitochondrial respiratory chain (Hargreaves 2007). Moreover, an increase of oxidative damages was described in patients with 3-hydroxy-3-methylglutaric aciduria (Dos Santos Mello et al. 2015). We also observed significant correlations between plasma Phe concentrations and various urinary organic acids. A recent study reported 19 differentially expressed compounds in urine of PKU patients (Xiong et al. 2015). These results bring up an underlying global metabolic disturbance in PKU which needs more exploration.

The focus of this clinical study was to evaluate antioxidant status of PKU patients in relation to their metabolic condition. Our preliminary results showed a molecular antioxidant signature of PKU white blood cells characterized by a global decrease in expression of antioxidant genes (Fig. 2). Nine of these antioxidant enzymes had more than a twofold decreased gene expression in white blood cells of PKU patients as compared to controls. We noted that 11 genes had a decreased expression significantly correlated with high plasma concentrations of Phe after multiple testing correction. These data support results of previous studies highlighting disturbance in redox system with reduction of total antioxidant status or unbalance of prooxidant-antioxidant system in blood samples of PKU patients (Sitta et al. 2009; Sanayama et al. 2011; Preissler et al. 2016; Tavana et al. 2016). Several plasma amino acids and urinary organic acids seem to be correlated with antioxidant expression profile. Despite the use of restrictive multiple testing

Table 1 Comparison of antioxidant genes expression profile in PKU patients and controls and correlation with Phe concentrations

ΔCt (mean ± SD)	PKU patients	Controls	Expression fold change compared to controls	Correlation with high plasma Phe	
				ρ spearman's	*p*-value
SOD2	0.43 ± 0.26	0.53 ± 0.20	−1.36	0.20	0.4504
GLRX1	0.016 ± 0.004	0.016 ± 0.005	−1.11	0.13	0.6124
GSR	6.18 ± 0.50	5.85 ± 0.31	−1.35	0.35	0.1660
PRDX3	6.23 ± 0.31	5.94 ± 0.28	−1.26	0.44	0.0798
PRDX5	6.49 ± 0.63	5.76 ± 0.43	−1.83	0.54	0.0242
PRDX1	7.10 ± 0.65	6.18 ± 0.68	**−2.13**	**0.66**	**0.0038**[a]
TXN	7.21 ± 0.67	7.14 ± 0.62	−1.17	−0.04	0.8848
SOD1	7.44 ± 0.61	6.41 ± 0.92	**−2.18**	**0.66**	**0.0039**[a]
GPX7	8.23 ± 0.62	7.83 ± 0.48	−1.48	**0.60**	**0.0107**[a]
GPX1 (transc. 1)	8.80 ± 1.08	8.15 ± 0.57	**−2.19**	0.32	0.2123
GPX4	8.80 ± 0.78	7.88 ± 0.44	**−2.20**	**0.65**	**0.0048**[a]
PRDX2 (transc. 1)	8.86 ± 0.68	8.30 ± 0.48	−1.69	**0.73**	**0.0008**[a]
CAT	9.41 ± 0.56	9.17 ± 0.70	−1.34	0.05	0.8371
GLRX3	10.43 ± 0.76	9.66 ± 0.81	**−2.06**	**0.58**	**0.0137**[a]
TXN2	9.64 ± 0.48	9.30 ± 0.36	−1.36	**0.58**	**0.0137**[a]
GLRX2 (transc. 2)	10.90 ± 1.07	9.93 ± 0.37	**−2.66**	0.51	0.0359
PRDX4	10.44 ± 0.92	9.70 ± 0.36	**−2.07**	**0.57**	**0.0166**[a]
GPX1 (transc. 2)	10.87 ± 0.66	10.23 ± 0.73	−1.76	0.41	0.1048
PRDX2 (transc. 3)	12.58 ± 0.27	11.34 ± 1.05	**−2.26**	**0.75**	**0.0005**[a]
GLRX5	12.32 ± 0.75	11.68 ± 0.76	−1.85	**0.64**	**0.0060**[a]
PRDX5 (transc. 2)	12.38 ± 1.04	11.42 ± 0.76	**−2.61**	0.44	0.0788
GPX3	11.27 ± 0.49	11.02 ± 0.67	−1.14	0.26	0.3208
PRDX6	16.46 ± 0.38	16.32 ± 0.53	−1.17	0.41	0.1060
GLRX2 (transc. 1)	17.06 ± 0.93	17.37 ± 2.88	−1.14	0.32	0.5068
GPX2	19.30 ± 1.20	19.00 ± 2.36	−1.73	0.54	0.0242
SOD3	25.81 ± 0.56	25.05 ± 0.44	−1.81	**0.65**	**0.0050**[a]

Genes are ranked in order from most to least expressed, according to their ΔCt values
Ct cycle threshold, *ΔCt* difference between mean reference gene Ct and gene of interest Ct, *PKU* phenylketonuria, *SD* standard deviation, *transc* transcript. Differences in comparison of gene expression between PKU patients and controls were considered relevant with fold change >±2
[a] Correlation between high concentrations of Phe and low expression of antioxidant gene using Spearman's correlation coefficient (ρ Spearman) was considered significant with a *p*-value inferior to threshold value calculated using Benjamini-Hochberg formula for multiple comparison correction

corrections, this result strengthens the idea of a link between metabolic disturbances and redox status in PKU. The low-noise disturbance of global metabolism linked with antioxidant status in PKU patients observed in this study highlighted the potential benefit of the maintenance of plasma Phe concentrations in adult PKU patients. These results need more detailed exploration of both metabolic and oxidative disturbance observed here in order to improve care of adult PKU patients.

Detailed analysis of data of three patients with disease particularity highlighted a less obvious decrease of antioxidant genes expression. These data strengthen the main observation of this study that high plasma Phe concentrations are linked to a decrease of antioxidant profile. Only patient-2 receiving BH4 treatment was excluded from statistical analysis. Patients 9 and 10 were the subject of a special analysis in a second time. The inclusion of the data from these two patients in the analysis may introduce a bias. These results, although interesting, need to be confirmed in a larger cohort in order to compare various subpopulations of PKU and hyperphenylalaninemia.

Figure 3 illustrates the results of our study compared with results of several studies on PKU patients, in vitro and in vivo models. As in the present study, several studies on PKU patients, adults and children with or without restrictive diet, highlighted a decrease of GPx enzyme in various

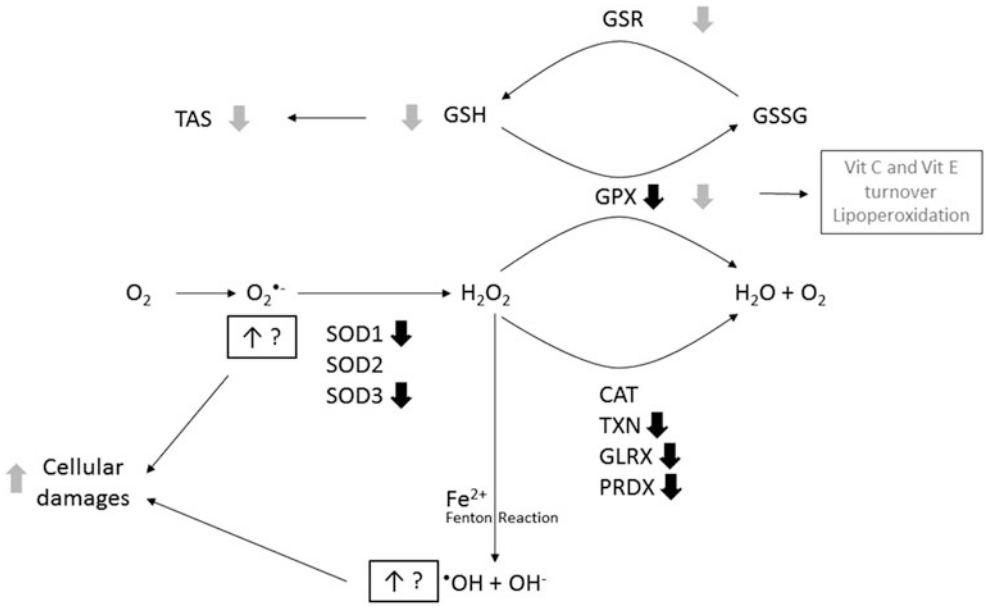

Fig. 3 Schematic representation of main results of the study (*black arrows*) compared with results of several studies on PKU patients, in vitro and in vivo models (*grey arrows*). As in this study, several studies highlighted a decrease of GPx enzyme in various tissues in PKU (Menzel et al. 1983; Reilly et al. 1990; Sierra et al. 1998; Sitta et al. 2006; Sanayama et al. 2011). The combination of a decrease in expression of GPX and GSR may suggest a reduced activity of the GSH antioxidant system. The observation of a global decrease of expression of antioxidant enzymes could be related with previous findings showing a lower total antioxidant status (TAS) in PKU patients compared to controls (Sanayama et al. 2011). The reduced expression of antioxidant systems that we observed could lead to a defect in maintenance of the redox homeostasis in response to elevation of ROS levels (superoxide and hydroxyl anions especially) responsible of cellular damages previously observed in PKU (Ercal et al. 2002; Sitta et al. 2009; Sanayama et al. 2011). *GPX* glutathione peroxidase, *GSR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *SOD* superoxide dismutase, *CAT* catalase, *TXN* thioredoxins, *GLRX* glutaredoxins, *PRDX* peroxiredoxins

tissues (Menzel et al. 1983; Reilly et al. 1990; Sierra et al. 1998; Sitta et al. 2006; Sanayama et al. 2011). The reduced expression of antioxidant systems that we observed could lead to an inefficient response to oxidative stress and by consequences to elevation of ROS levels (superoxide and hydroxyl anions especially) responsible for cellular damages previously highlighted in PKU (Ercal et al. 2002; Sitta et al. 2009; Sanayama et al. 2011) and which may lead to brain injury observed in the disease. In the present study, ROS production in PKU patients trends to increase in monocytes but interindividual variability and lack of statistical power prevent us from concluding. Evaluation of intracellular ROS in erythrocytes, which have a lower turnover than monocytes, might be a better model for highlighting increased ROS generation. Moreover, expression of some antioxidant genes of this study is under the control of the Nrf2 transcription factor (nuclear factor erythroid 2-related factor 2) (Ma 2013). It would be interesting to study the Nrf2 pathway in PKU pathophysiology.

Despite the reduced size of our cohort, an interesting link was highlighted between biochemical parameters and a large molecular antioxidant profile. It might be interesting to extend this study by performing a wide range of oxidative stress markers, especially markers of cellular damages such as 8-hydroxy-2′-deoxyguanosine (DNA damages), malondialdehyde (lipid peroxidation), and carbonylated proteins (Sitta et al. 2009; Sanayama et al. 2011), in order to validate assumptions from our data and previous studies, and to evaluate their relation with biological markers and clinical data in a longitudinal larger cohort of PKU patients. Analysis of disease phenotype, age (adults vs children), gender, metabolic status or diets, and environmental oxidative stress factors which may introduce a bias (i.e., self-medication treatments, life-style, or activities) could also bring new perspectives in PKU pathophysiology understanding. Furthermore, the assessment of a characteristic molecular antioxidant signature of different subcategories of PKU patients, using the antioxidant profile of the present study, could open perspectives on the use of this signature in disease monitoring or in the prognosis of disease complications, such as neurological deteriorations. Thus, it might be interesting to compare the blood antioxidant profile with cognitive functions in order to characterize the potential role of oxidative stress in neurological troubles observed in PKU. Studies on large neural amino acids supplementation including tyrosine in PKU mice model showed reduced brain Phe concentrations

(van Vliet et al. 2016). Large neural amino acids supplementation could be an interesting alternative dietary treatment in patients with PKU (Andersen and Avins 1976). Physical exercise has been shown to improve brain function by preventing oxidative stress in PKU (Mazzola et al. 2016). Several antioxidant supplementations have been evaluated and may be a complement or an alternative to a restrictive diet for adolescent and adult PKU patients with poor compliance (Mazzola et al. 2013). In this way, supplementation with L-carnitine and selenium seems to prevent oxidative stress observed in hyperphenylalaninemia (Sitta et al. 2011). Antioxidant treatments have been widely considered in several pathologies where oxidative stress is involved, and the necessity of more studies on this topic in PKU seems essential.

These findings highlight the association of hyperphenylalaninemia with antioxidant genes expression and emphasize the importance of the restricted protein diet in PKU, which is fundamental for the metabolic control of phenylalanine and, consequently, the redox status in these patients.

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Take-Home Message (Synopsis)

This preliminary study highlights a lower antioxidant arsenal in leukocytes associated with hyperphenylalaninemia using assessment of gene expression of 22 major antioxidant enzymes in adult PKU patients.

Conflict of Interest

All authors declare that they have no conflicts of interest with the contents of this chapter.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study. Proof that informed consent was obtained are available upon request.

Author Contributions

C.V-D designed and performed experiments, analyzed data, and wrote the manuscript; C.D performed and supervised redox status experiments and gave technical and intellectual support on redox study; H.B designed experiments, supervised the writing of the manuscript, and gave intellectual support and conceptual advice; F.P performed experiments; H.H collected clinical data; P.E supervised biochemical parameters assays; C.A performed biochemical experiments; V.G recruited healthy controls and participated in the design of study; O.H gave technical and intellectual support on redox status interpretation and supervised the writing of the manuscript; F.M designed and supervised the study, recruited PKU patients, and supervised the writing of the manuscript. All authors provided conceptual advices and comments on the manuscript.

Author who serves as guarantor: François Maillot.

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Details of Ethics Approval

This study was approved by the ethics committee of University Hospital of Tours (CPPRB, for Comité de Protection des Personnes) and registered on ClinicalTrials.gov (study ID number PHAO14/FM-StressOX-PCU, "Antioxidant Signature in Adults Patients with Phenlyketonuria"). Informed consent was signed by all patients and controls prior to inclusion.

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RESEARCH REPORT

The Impact of Fabry Disease on Reproductive Fitness

Dawn A. Laney • Virginia Clarke • Allison Foley • Eric W. Hall • Scott E. Gillespie • Myrl Holida • Morgan Simmons • Alexandrea Wadley

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Abstract Fabry disease (FD) is a pan-ethnic, X-linked, progressive lysosomal storage disorder caused by pathogenic mutations in the GLA gene. Published case reports and abstracts suggest that decreased reproductive fitness may occur in males with FD. In order to understand the impact of FD on reproductive fitness and increase the accuracy of reproductive genetic counseling, this study examines a large, multi-centered population of individuals with FD to determine if males have reduced reproductive fitness. Study data were collected on 376 patients through two, gender-specific surveys distributed across the United States and Canada. The number of biological live-born children among individuals with FD was compared to statistics from the general population. Information was also collected on reduced sperm count, depression, pain, use of assisted reproductive technology, and reproductive choice. On average, females affected by FD had more biological live-born children (1.8) than males affected by FD (1.1). However, males affected by FD had an increased mean number of biological children (1.1) compared to the mean number of biological children fathered by men in the United States (0.9). Sixteen of the 134 males with FD reported oligospermia, which suggests that an infertility work up may be indicated for males having difficulty impregnating their partners. In our large multicenter sample, males and females with FD do not exhibit reduced reproductive fitness; on average they have more biological children than the general population in the United States. This information should assist clinicians in providing accurate reproductive genetic counseling and treatment for individuals with FD.

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D.A. Laney (✉) · A. Foley · M. Simmons
Division of Medical Genetics, Department of Human Genetics, Emory University School of Medicine, 2165 North Decatur Road, Decatur, GA 30033, USA
e-mail: dawn.laney@emory.edu

V. Clarke
Neurology Department, Massachusetts General Hospital, Boston, MA, USA

E.W. Hall
Department of Epidemiology, Emory University Rollins School of Public Health, Atlanta, GA, USA

S.E. Gillespie
Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA

M. Holida
Division of Medical Genetics, Stead Family Department of Pediatrics, University of Iowa, Iowa City, IA, USA

A. Wadley
University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Introduction

Fabry disease (FD) (OMIM 301500) is a pan-ethnic, X-linked, multi-systemic, progressive lysosomal storage disorder caused by mutations in the GLA gene. These mutations result in decreased or deficient levels of the enzyme α-galactosidase A (α-gal A) (EC 3.2.1.22). This deficiency of α-gal A causes the accumulation of globotriaosylceramide (GL3 or GB3) and related glycosphingolipids in the vascular endothelium (Desnick et al. 2001; Eng et al. 2006). The glycosphingolipid storage initiates a cascade of events beginning with the dysfunction of basic metabolic processes on the cellular level and moving into cell death, inflammatory events, and progressive major organ dysfunction (Eng et al. 2006).

The incidence of FD ranges from 1 in 1,250 to 1 in 117,000 live male births worldwide, with an increased

incidence of mutations expected to result in non-classical or later onset FD (Hopkins et al. 2015; Inoue et al. 2013; Meikle et al. 1999; Mechtler et al. 2012; Lin et al. 2009; Zarate and Hopkin 2008; Hwu et al. 2009; Spada et al. 2006). The symptoms and progression of FD are highly variable, from classical FD with early and severe onset of symptoms to non-classical or later onset FD with symptoms that may have a more severe impact on a particular organ system (cardiac, neurological, or renal) or may present in adolescence or later rather than childhood (Eng et al. 2006; Smid et al. 2015; Biegstraaten et al. 2010; Desnick et al. 2001; Laney et al. 2015). These symptoms affect both males and females and vary among individuals (Eng et al. 2006; Wang et al. 2007; Desnick et al. 2001; Hopkin et al. 2008).

Unlike the characteristic features and symptoms of FD, reproductive health issues have only been reported in individual case reports and not examined systematically in a large FD population. In particular, there have been several pathology-focused case reports of males with azoospermia and FD, males with storage of GL3 in testicular biopsies, and diminished fertility and FD (de Groot 1964; Guin et al. 1976; Lacombe et al. 2010; Papaxanthos-Roche et al. 2007; Török et al. 1980). The most informative case report focused on biopsies from a deceased 32-year-old male with FD. As reported, biopsies revealed multiple areas of significant GL3 storage (Leydig cells, epithelial of ductuli efferentes, ductus of the epididymis, testicular interstitium, and seminiferous tubules) but undisturbed fertility in the patient despite the reduced diameter of the seminiferous tubules (Nistal et al. 1983). In a separate report on two testicular biopsies which found storage of GL3 in the Leydig cells by optic and electronic microscopic analysis in two individuals with azoospermia and FD, authors suggest male patients desiring children may need to use assisted reproductive technologies such as testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) (Papaxanthos-Roche et al. 2007; Lacombe et al. 2010; Török et al. 1980).

Another reproductive health issue that could have a negative impact on reproductive fitness in FD, priapism, has also been reported via case reports in males with FD (Labarthe et al. 2010; Foda et al. 1996). However, in a study of 48 male patients with FD, Biegstraaten et al. found nearly normal male sexual function and autonomic control of the cardiovascular system using the Autonomic Symptom Profile in their sampling of patient with FD as compared to a control population (Biegstraaten et al. 2010).

Similarly, a small scale study from Hauser et al. assessed ovarian, testicular, and adrenal function in a cohort of 13 patients with FD (6 females and 7 males, including 3 males on hemodialysis) and found undisturbed hormonal function and a normal fertility rate in both male and female patients when compared with the corresponding Austrian populations. The three males on maintenance hemodialysis had abnormal plasma levels of LH, prolactin, and testosterone consistent with their uremic state. All 13 patients had α-gal A enzyme levels below normal at a mean of 17.70 nMol/mg protein/h ± 19.51 (1–48) (Hauser et al. 2005).

A single-site pilot study in 2007 investigated the differing rate of live-born children between males and females with FD and found that males affected by FD who have completed their families have a reduced rate of live-born children (1.7 children per male on average) as compared to females affected by FD (3 children per female on average) (Laney et al. 2008). This data was counter to the existence of several very large families with males with many offspring that had been published in the clinical setting (Veronik et al. 2004; Spence et al. 1978).

Finally, males with FD may choose to reduce the number of or not have biological children, as previous studies have shown that individuals with genetic conditions make different reproductive decisions than individuals without known genetic risks (Beeson and Golbus 1985; Hershberger et al. 2012; Read 2002). If males with FD are choosing to have fewer children, this raises the possibility that they have reduced reproductive fitness when compared to females with FD and/or the national rates of reproduction.

The scientific literature offers several theoretical factors that could contribute to decreased fitness in males with FD including: mechanical blockage of the vas deferens due to glycolipid storage, autonomic dysfunction, reduced life expectancy, depression and decreased social adaptive functioning, or personal choice (Laney et al. 2010; Wagner et al. 2014; Arends et al. 2015; Germain et al. 2015; Waldek et al. 2009). In order to provide accurate genetic counseling about their reproductive fitness to individuals affected by FD, we conducted a multi-center, large scale study to determine if reproductive fitness is decreased in males with FD and, if so, delineate specific risk factors or issues related to decreased fitness. We anticipate this information will increase the accuracy of comprehensive monitoring and treatment plans, and help determine if there is skewing in the incidence calculation of FD.

Methods

Institutional Review Board Approval

This study has been reviewed and approved by the Emory University Institutional Review Board. All participants completed a consent process prior to participating in the

study. Documents approved for this study included recruitment flyers, recruitment emails, consent information forms, and surveys.

Participants and Recruitment

Recruitment for the study was a multifaceted effort. Recruitment flyers were mailed to Emory Lysosomal Storage Disease Center patients who met inclusion criteria. In addition, notice of this study was included on the Fabry Support and Information Group (FSIG) and the National Fabry Disease foundation (NFDF) website and Facebook pages. Study investigators attended and recruited at local and regional FD meetings sponsored by NFDF and FSIG. Recruitment flyers were also distributed to genetic counselors around the United States within Lysosomal Storage Disease Centers of Excellence to provide to qualified patients who may have an interest in participating. Participating institutions included the University of Iowa, Children's Hospital of Wisconsin, the University of Washington, and Massachusetts General Hospital.

Survey Population

Participants who completed the informed consent process were screened to determine if they met study entry criteria. Inclusion criteria for participating in the study required all participants to be affected by FD, be over age 18 years, and be able to provide informed consent. Subjects not meeting these criteria were excluded. This includes five subjects who completed the surveys, but did not record their sex on the questionnaires. Demographic and reproductive data was used to ensure that subjects' survey data was not counted more than once. The target participant population was 450 participants from all regions of the United States and Canada.

Survey Design

Following completion of the consent process, patients were asked to complete either a paper or online self-response survey entitled "Reproductive Fitness in Individuals Affected by Fabry Disease (FIT)." Separate questionnaires were completed if the subject was male or female. The survey for males consisted of 30 questions and was divided into 4 parts: demographics, reproductive history, medical history, and questions about their family and children. The survey for females consisted of 36 questions and was divided into 5 parts: demographics, reproductive history, medical history, gynecological history, and questions about their family and children. Key questions from the surveys are provided in Appendix 1.

Statistical Analysis

Raw data was obtained from self-response queries and analyzed using SAS 9.4 (SAS Institute, Cary, North Carolina, USA). Demographic variables were summarized using frequencies and proportions. Demographic variables of interest were compared by sex, using chi-square tests and Fisher's exact tests, where appropriate. The number of biological live-born children was summarized with medians and interquartile ranges. Any children born to a parent affected by FD who used a donor egg or sperm in place of their biological contribution were not reported as biological children. The proportion of male participants who reported various characteristics related to infertility was compared to reported statistics for the general population. Cohen's h was calculated to assess the standardized difference between the general population proportions and those in our sample (Cohen 1988). As Cohen suggests, h values of 0.2, 0.5, and 0.8 were interpreted as "small," "medium," and "large" effect sizes, respectively. Nonparametric Kruskal-Wallis tests were used to compare the number of biological live-born children by sex and all other demographic and survey variables of interest. These included key health and behavior issues such as azoospermia, oligospermia, depression, erectile dysfunction, environmental exposures, having burning pain in hand/feet at less than 10 years of age, renal failure, and knowing FD status prior to having children. The mean number of biological live-born children was compared with national population estimates from the United States National Health statistics report, the World Health Organization fertility data, and World Bank fertility data by using paired comparison tests (Martinez et al. 2012; The World Bank 2015; Cooper et al. 2010; Sharlip et al. 2002). Chi-square tests were used to compare the distribution of the number of live-born children among participants with FD to the distribution of live-born children among the United States. All statistical tests were assessed using an alpha $= 0.05$.

Although we did not collect data on genotypes, studies have shown that the majority of men with classical FD experience neuropathic extremity pain (acroparesthesias) in childhood (Hopkin et al. 2015; Laney et al. 2015; Smid et al. 2015). To focus on the males most likely to have classical FD, we separately analyzed the number of live-born children, infertility issues (including azoospermia and oligospermia), and use of assisted reproductive technology (ART) in males with FD who reported onset of neuropathic pain at age 10 or under.

Results

A total of 376 subjects (134 males, 242 females) consented to participate in the study and completed the survey. The mean age of participants who completed the survey was

46.5 years with a range of 19–78 years and a median of 46 years. The majority of individual respondents were Caucasian (332/376, 90%) with additional representation from the Hispanic (18/376, 4.8%), Arab American (1/376, 0.27%), Native American (2/376, 0.50%), African-American (4/376, 1.1%), and Asian (5/376, 1.3%) populations (Table 1).

Fabry Related Syndrome and Health History

The majority of male respondents with FD reported experiencing the characteristic burning pain in the hands/feet seen frequently in FD (115/134, 85.8%). A subset of those males (75/134, 56%) reported onset of burning pain at age 10 or younger. Additional health issues experienced

Table 1 Demographic characteristics of study subjects with Fabry disease, United States, 2013–2015

Characteristic	Total $N = 376$		Females $n = 242$	Males $n = 134$	P value[a]
	N	%	n (%)	n (%)	
Recruitment source					0.330
Paper survey	131	(34.8)	80 (33.1)	51 (38.1)	
Survey monkey	245	(65.2)	162 (66.9)	83 (615)	
Age in 2014 (in years)					0.920
18–21	10	(2.7)	6 (2.5)	4 (3.0)	
22–29	30	(8.1)	21 (8.8)	9 (6.7)	
30–35	55	(14.8)	34 (14.3)	21 (15.7)	
36–40	43	(11.6)	29 (12.2)	14 (10.5)	
41+	234	(62.9)	148 (62.2)	86 (642)	
Race/ethnicity					0.318[b]
Caucasian, single race	332	(90.0)	211 (88.7)	121 (92.4)	
Hispanic	18	(4.9)	11 (4.6)	7 (53)	
African-American, single race	4	(1.1)	4 (17)	0 (0.0)	
Other or mixed race	15	(4.1)	12 (5.0)	3 (23)	
Highest level of education					0.327
No school	2	(0.5)	1 (0.4)	1 (0.8)	
High school or less	75	(20.1)	51 (21.3)	24 (17.9)	
Some college or technical degree	139	(37.2)	96 (40.0)	43 (32.1)	
Four-year degree	60	(16.0)	34 (14.2)	26 (19.4)	
Graduate education	98	(26.2)	58 (24.2)	40 (29.9)	
Average annual household income					0.267
$0–$24,999	58	(17.8)	42 (20.4)	16 (13.3)	
$25,000–$39,999	38	(11.7)	20 (9.7}	18 (15.0)	
$40,000–$49,999	32	(9.8)	21 (10.2)	11 (9.2)	
$50,000–$74,999	76	(23.3)	53 (25.7)	23 (19.2)	
$75,000–$99,999	47	(14.4)	27 (13.1)	20 (16.7)	
$100,000–$124,999	36	(11.0)	23 (11.2)	13 (103)	
$125,000–$149,999	14	(4.3)	8 (3.9)	6 (5.0)	
Over $150,000	25	(7.7)	12 (5.8)	13 (10.8)	
Any biological children	253	(69.7)	178 (77.4)	75 (56.4)	<0.001
	med	**(IQR)**	**med (IQR)**	**med (IQR)**	
Number of liveborn children	2.0	(0–2)	2.0 (1–3)	1.0 (0–2)	<0.001[c]

[a] Calculated by chi-square test with an alpha = 0.05, unless otherwise noted
[b] Calculated by Fisher's exact text with an alpha = 0.05
[c] Calculated by Kruskal Wallis test with an alpha = 0.05

in more than 25% of the male respondents included depression (55/134, 41.0%), anxiety (50/134, 37.3%), and renal failure (45/134, 33.6%) (Table 2). In the majority of cases, the onset of renal failure in the males occurred after the reported age range at which they had their first biological child. In males, erectile dysfunction (ED) was reported in 19 males (19/134, 14.2%), with the majority reporting onset after the birth of their first biological child (onset at an average age of 50 with a range of 30–64 years of age). Priapism was also reported in 2 out of the 134 males with FD (1.5%) and did occur during the subjects' main time of reproduction. Data on Fabry related symptoms and health history in females can be found in Table 3.

Table 2 Reproductive history of 134 males with Fabry disease, United States, 2013–2015

			Number of live-born biological children		
Characteristic	Level	*N*	Mean	Median	Kruskal-Wallis *P*-value
Age at birth of first child, in years	12–20	1	2.0	2	0.76
	21–29	32	2.0	2	
	30–35	23	1.7	2	
	36–40	7	1.7	2	
	41–45	3	1.7	2	
Age at birth of last child, in years	21–29	24	1.8	2	0.51
	30–35	28	1.9	2	
	36–40	14	2.0	2	
	41–45	4	2.5	2	
Ever had depression	No	78	1.1	1	0.53
	Yes	55	1.0	1	
Ever had renal failure	No	88	1.0	1	0.40
	Yes	45	1.2	1	
Ever smoked	No	52	1.0	1	0.06
	Yes	23	0.5	0	
Reported any pain	No	18	0.8	0	0.16
	Yes	115	1.1	1	
Burning pain before age 10	No	40	1.3	1	0.28
	Yes	75	1.0	1	
Anxiety	No	83	1.1	1	0.73
	Yes	50	1.0	1	
Erectile dysfunction	No	114	1.0	1	0.51
	Yes	19	1.3	1	
Reported any infertility problems	No	57	1.6	2	0.01
	Yes	32	0.9	0	
Ever had an infertility evaluation	No	99	1.1	1	0.96
	Yes	29	1.0	1	
Low sperm count (oligospermia)	No	117	1.1	1	0.39
	Yes	16	0.8	1	
Low sperm motility	No	127	1.1	1	0.70
	Yes	6	1.2	2	
Used assisted reproductive technology	No	121	1.1	1	0.46
	Yes	12	0.8	0	

Rate of Biological Children

The majority (253/376, 69.7%) of individuals with FD in the study reported having at least one biological child. Stratified by gender, 56.4% of males (75/134) and 77.4% of females (178/242) reported having at least one biological child (Table 4), with males having an average of 1.1 biological children and females having an average of 1.8 biological children (Table 4). Females affected by FD have significantly more live-born children than males affected by FD ($p < 0.001$, Figs. 1 and 2). However, the mean number of biological children among females with FD in our study (1.8 children) is higher than the general United States population average of 1.3 children (Table 4). This is also true when compared to the Caucasian, non-Hispanic United States population average of 1.1 children. Males affected by FD also had an increased mean number of children (1.1 in all males with FD) as compared to the mean number of biological children fathered by all men in the United States

Table 3 Reproductive history of 242 females with Fabry disease, United States, 2013–2015

		Number of live-born biological children			
Characteristic	Level	*N*	Mean	Median	Kruskal–Wallis *P*-value
Ever had depression	No	133	1.9	2	0.98
	Yes	97	1.7	2	
Ever had renal failure	No	140	1.8	2	0.16
	Yes	10	1.1	1	
Reported any pain	No	33	1.5	1	0.20
	Yes	197	1.9	2	
Burning pain before age 10	No	112	2.0	2	0.20
	Yes	85	1.8	2	
Anxiety	No	131	1.9	2	0.77
	Yes	99	1.8	2	
Panic	No	196	1.8	2	0.90
	Yes	34	1.8	2	
Reported any infertility problems	No	157	2.2	2	0.04
	Yes	40	1.6	1	
Ever had an infertility evaluation	No	194	1.8	2	0.63
	Yes	31	1.8	2	
Used assisted reproductive technology	No	200	1.9	2	0.91
	Yes	30	1.7	2	

Table 4 Mean number of children by sex and data source

Data source	*N*	Mean (S.E.)	Percent distribution (S.E.) for number of live-born children[b]				
			None	1	2	3	4+
Males (all)							
National data[a], 2006–2010	–	0.9 (0.0)	55.2 (1.1)	15.8 (0.6)	17.0 (0.7)	7.9 (0.5)	4.1 (0.3)
Fabry data, 2015	133	1.1 (0.1)	43.6 (4.3)	17.3 (3.3)	30.8 (4.0)	6.0 (2.1)	2.3 (1.3)
Females (all)							
National data[a], 2006–2010	–	1.3 (0.0)	44.4 (1.1)	16.2 (0.5)	21.0 (0.8)	11.5 (0.5)	6.9 (0.5)
Fabry data, 2015	230	1.8 (0.1)	22.6 (2.8)	20.4 (2.7)	30.9 (3.1)	13.9 (2.3)	12.2 (2.2)
White, non-Hispanic (single race)							
Women							
National data[a], 2006–2010	–	1.1 (0.0)	47.7 (1.4)	15.7 (0.7)	21.8 (1.0)	10.6 (0.6)	4.2 (0.4)
Fabry data, 2015	201	1.8 (0.1)	23.4 (3.0)	19.4 (2.8)	31.8 (3.3)	13.4 (2.4)	11.9 (2.3)
Men							
National data[a], 2006–2010	–	0.8 (0.0)	58.9 (1.5)	14.9 (0.9)	17.0 (1.0)	6.6 (0.6)	2.6 (0.3)
Fabry data, 2015	120	1.1 (0.1)	44.2 (4.6)	18.3 (3.6)	28.3 (4.1)	6.7 (2.3)	2.5 (1.0)

Abbreviations: *SE* standard error

[a] National data is based on a survey of 10,403 men and 12,279 women which were weighted to reflect approximately 62 million men and 62 million women aged 15–44

[b] Chi-square tests were used to compare the percent distribution of number of live-born children among patients with Fabry's disease to corresponding national data. All statistical comparisons were significant as a result of the large sample size in the national data

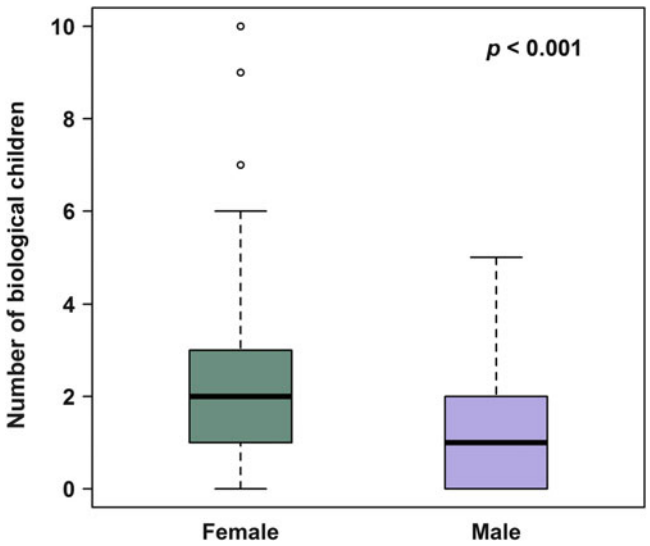

Fig. 1 Distribution of biological children counts by gender

(0.9) and among Caucasian, non-Hispanic men in the United States (0.8) (Table 4).

Infertility in Males

Approximately 32 males with FD reported issues with infertility (32/89, 36.0%). This is higher than the reported general population rate of 7% of males of reproductive age who reported having infertility issues (Krausz 2011). Similarly, 29 males (29/128, 22.7%) reported seeking an infertility evaluation to address specific male infertility issues (Table 5). Although our data did not identify the age at which the males with FD sought evaluation, the percentage of males with FD seeking an infertility evaluation is twice the general population rate in which 9.4% of all sexually experienced men younger than age 45 report undergoing an infertility evaluation (Chandra et al. 2014). There were 16 males (15/132, 11.4%) who reported being diagnosed with low sperm count (oligospermia), 1 with no sperm detected (azoospermia) (1/133, 0.8%), 4 with low testosterone (4/134, 2.99%), 1 with a varicocele (1/134, 0.75%), and 6 with low sperm motility (6/134, 4.49%). Looking at a selected population of males with FD who reported onset of neuropathic pain at age 10 or younger, 9 out of 75 (11.4%) males reported having either azoospermia or oligospermia. The rate of oligospermia in our population of males with FD (11.4%) was comparable to general population data which found that 15% of males in the general population are defined to have oligospermia (sperm concentration was below 15 million/ml) (Jørgensen et al. 2012). (Table 5) This is slightly lower than the rate of fertile men (male partners of pregnant women) in a previous Jørgensen study in which 8% had oligospermia (Jørgensen et al. 2001). The percent of males with FD and azoospermia in our study (0.75%) was lower than the general population rate in which 2% of all men are reported to have azoospermia (Willott 1982).

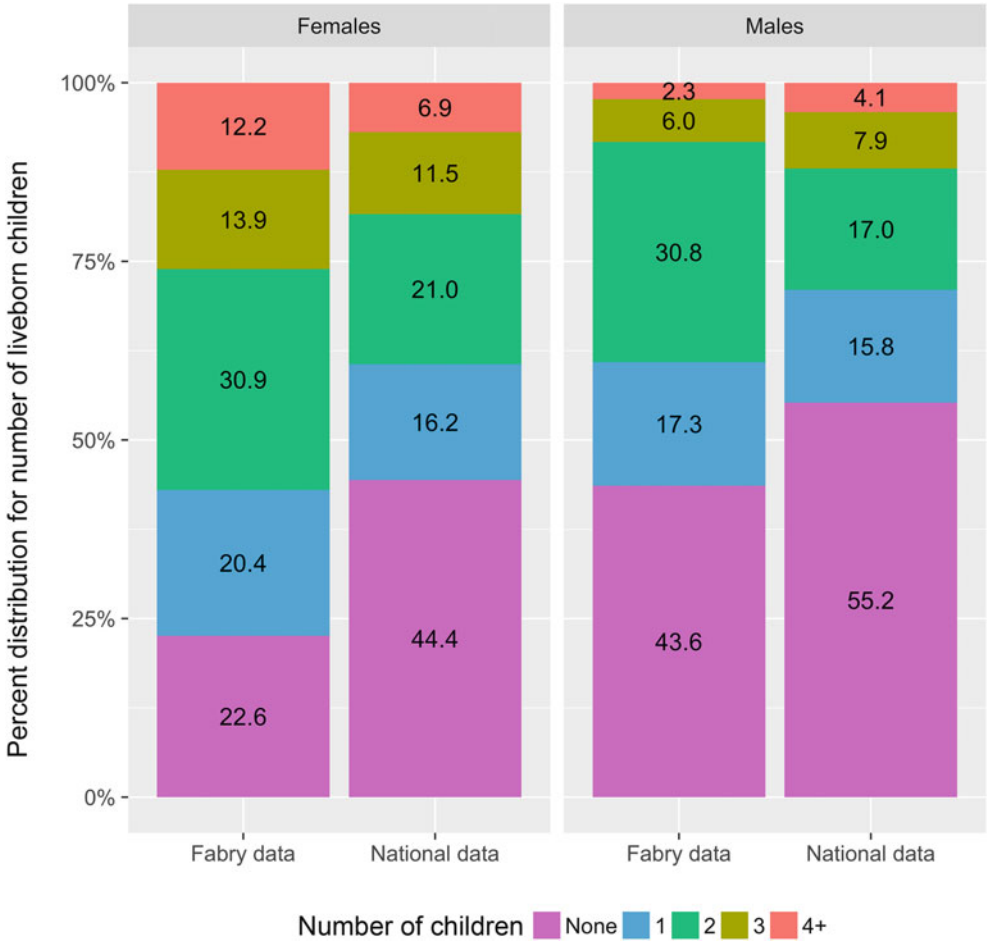

Fig. 2 Percent distribution of the number of liveborn children born to individuals in the general population as compared to individuals with Fabry disease

Table 5 Reproductive characteristics of participants with Fabry disease compared to the general population, by sex

Characteristic	Total respondents	*n*	(%)	*P* in general population (%)	Cohen's *h*[a]
Males					
Reported having infertility issues	89	32	(36.0)	7% (Krausz 2011)	0.75
Reported undergoing an infertility evaluation	128	29	(22.7)	9.4% (Chandra et al. 2014)	0.37
Low sperm count (oligospermia)	132	15	(11.4)	15% (Jørgensen et al. 2012)	0.11
Azoospermia	133	1	(0.8)	2% (Willott 1982)	0.11
Used ART	133	12	(9.0)	0.7% (Chandra et al. 2014)	0.44
Females					
Reported having infertility issues	197	40	(20.3)	12% (Chandra and Copen 2013)	0.23
Reported undergoing an infertility evaluation	225	31	(13.8)	13.7% (Krausz 2011)	0.00
Used ART	230	30	(13.0)	0.7% (Chandra et al. 2014)	0.57

[a] Cohen suggests that *h* values 0.20, 0.50, and 0.80 can be interpreted as "small," "medium," and "large" effect sizes, respectively (Cohen 1988)

Out of the 134 males with FD, 12 (9.0%) used infertility services to attempt to impregnate their partners including: intrauterine insemination, use of donor sperm, in vitro fertilization, in vitro fertilization and use of donor sperm, and partner taking hormones to increase ovulation. Of the males who used donor sperm, three reported having oligo

or azoospermia and one chose to use a donor sperm in order to avoid the risk of transmitting FD to his children.

Infertility in Females

Approximately 40 females with FD reported issues with infertility (40/197, 20.3%) with 31 females (31/225, 13.8%) seeking an infertility evaluation to address specific female infertility issues. The number of females with FD reporting issues with infertility is higher than the general population rate of 12% of women aged 25–44 who report ever having infertility issues, but equivalent to the number of females in the general population seeking an infertility evaluation (Chandra and Copen 2013; Chandra et al. 2014; Kessler et al. 2013). Out of the 242 females with FD, 30 (12.4%) used infertility services to attempt a pregnancy including: use of donor egg, in vitro fertilization, and taking hormones to increase ovulation (Tables 3 and 5). It is important to note that at least three females used Pre-implantation Genetic Screening (PGS) for FD and one used a donor egg to avoid passing on FD rather than for reasons of infertility.

Adoption

In our survey, 13 males and 15 females with FD reported adopting a least one child (28/376; 7.4%). Eleven of the 13 males with FD had adopted children and did not have any biological children. Two of the 13 males with FD had at least one biological child in addition to their adopted children. Several males reported that their adopted children were their stepchildren, their wife's biological children. Three of the females with FD reported adoptive children and no biological children, while 12 had both adopted and at least one biological study. Approximately 4% of US families on average have at least one adopted child. Individuals with Fabry disease in this study report a higher than average incidence of having an adopted child in their family at 7.4% (Kreider and Lofquist 2014).

Impact of Known Diagnosis of Fabry Disease

Elective Terminations

The psychosocial impact of having FD and making the decision to have children could impact the rate of reproductive fitness via personal choice. There were 21 out of the 273 females affected by FD that reported having an elective pregnancy termination. Of the 21 reported terminations, 3 were of male fetuses prenatally diagnosed with FD, 3 were over the fear that the baby could be affected by FD, and one was due to fetal exposure to the teratogenic medication Dilantin for treatment of FD related symptoms. In males who completed the question, 18 reported having a partner who had electively terminated at least one pregnancy. No further information about the reasons for the elective terminations was given in males. These rates are comparable to the general population data which found that 21% of all pregnancies (excluding miscarriages) end in elective terminations with non-Hispanic white women accounting for 36% of abortions (Jones and Jerman 2014; Jones et al. 2010).

Use of Preimplantation Genetic Diagnosis or Other Assisted Reproductive Technology

Preimplantation genetic diagnosis (PGD) is available in the United States for individuals with a known family *GLA* mutation or for those who wish to implant only male or female embryos. PGD is expensive and often not covered by insurance; however, it offers an alternative to prenatal testing. Using a donor sperm or egg to replace the biological contribution of an affected individual is also an option for parent who chooses not to pass on FD. Of these males with FD, only four used donor sperm and one used preimplantation genetic diagnosis for FD. Of female respondents, three used PGD for FD in the past, one used a donor egg, and four plan to use PGD to avoid passing on FD in future pregnancies (one of these already has frozen eggs to this end). Although this option may cause a larger impact on reproductive fitness in the future, at this time the option appears to be used minimally.

Reproductive Decision-Making and Fabry Disease

Survey questions specifically focused on personal reproductive choice in individuals with FD and the possibility that study subjects chose to have fewer children based on their risk of passing on FD. These data were limited in that individuals needed to know that they were affected by FD prior to attempting pregnancies in order to have a choice. In summary, 41/134 males and 71/242 females knew that they were affected by FD prior to having children and completed the survey questions about the impact of FD risk and number of children. In females, 39 (39/242) respondents said their knowledge impacted their decision-making process and 19 (19/242) said it did not. In males, 14 respondents (14/134) said having FD did affect their reproductive decision-making process and 12 respondents (12/134) said it did not. The impact that knowledge of FD had on reproductive decision making was very diverse with an interesting range of free response and comment sections about how it affected their personal choices. Some felt it made little difference in their choices and they proceeded to have the number of children they wished. Examples of free response answers in this category are available in Appendix

2. The knowledge that FD could be passed on to their children did negatively impact the choice to have children or have more children in approximately 50% of males and 67% of female respondents who knew they had FD, but many of them still had at least one live-born biological child and the rate of live-born children does not seem to be greatly reduced based on the numbers reported.

Discussion

Analysis of self-reported reproductive data in a large cross section of males and females with FD did not find reduced reproductive fitness as compared to general population data; instead, it indicated increased reproductive fitness in males and females with FD. On average, participants with FD had more biological, live-born children than the general population in the United States. There was an increased number of biological children seen in females with FD compared with males with FD, which mirrors the general population rates, but may also be affected by the expected incidence of affected individuals with FD. However, there is a subset of males with FD who did self-report infertility, oligospermia, and the existence of at least one related sperm or semen problems. Given the number of males with oligospermia, the standard genetic counseling for FD should include discussion of the option of semen analysis for sperm count and motility where appropriate as well as referral to reproductive centers and in vitro fertilization with testicular sperm extraction and intracytoplasmic sperm injection as appropriate and desired by the patient. Furthermore, the data on the impact of a FD diagnosis on reproductive decision making emphasizes the need to explore the topic with patients. These discussions would be added to the current genetic counseling recommendations including review of the inheritance pattern of FD, identification of at-risk family members, addressing the psychological impact of FD, and options for preimplantation and prenatal genetic testing (Laney et al. 2013).

The specific rationale for increased reproductive fitness in males and females with FD as compared to the general population rate was not uncovered in this study, but the authors have a few hypotheses to explore in future studies. The first is related to the impact of FD on daily life, career, and motivation. Other studies have found that individuals with FD have reduced social adaptive function, increased fatigue, and more school absences than their peers due to medical issues and appointments (Hopkin et al. 2008; Laney et al. 2010). These factors could reduce the number of individuals who continue their education after high school. When women pursue higher education, they have a delayed fertility pattern (Dye 2010). The opposite may be true for women who do not pursue high education. Another hypothesis, albeit with more anecdotal than firm evidence, could be that given relationship stress caused by having a chronic disease that includes fatigue, depression, anxiety, and decreased social adaptive functioning may lead individuals with FD to more divorces or dissolutions of long-term relationships than the general population, leading to an increased rate of serial long-term partners at younger ages increasing the chances for additional children.

The incidence of oligospermia in males diagnosed with FD still seems more frequent than in the general population and may be related to the significant glycolipid storage in the male reproductive tract seen on pathology; however, it doesn't appear to significantly affect fertility in the majority of males. This is consistent with a study that found that, despite significant GL3 storage and narrowed vas deferens, one subject still produced three children (Nistal et al. 1983). It is also consistent with other studies that found largely normal endocrine hormone profiles and normal male sexual function, and with the observed after that the fertility rate in both female and male patients was equal to or higher than the local fertility rate (Nistal et al. 1983; Hauser et al. 2005; Biegstraaten et al. 2010). However, as the National Survey of Family Growth found "sperm or semen problems" in 14% of general population couples seeking infertility evaluation, attention to the matter is still indicated (Martinez et al. 2006).

There were no significant associations between the number of biological children and other factors that could theoretically impact reproduction in males with FD including erectile dysfunction, pain, or depression. It appears that though these factors may decrease quality of life, they do not have a direct impact on reproductive fitness.

Limitations of the study include: the majority of respondents were Caucasian, there may be a skewing toward individuals with internet access and those who are active in the FD community through attending in person meetings, being on support group mailing lists, or participating in online support groups. In addition, data on reproductive history, Fabry disease related symptoms, infertility, and health history symptom data was self-reported and not verified by medical record review. The study design also did not provide specific definitions for "infertility" which was interpreted differently by different subjects based on free response answers used for clarification. The study design introduces another bias in that only those interested in this area may have participated, and we did not directly collect genotypes to distinguish between classical and nonclassical/later onset FD. In order to mimic classical FD in analysis, we attempted to focus on "classically affected males" by analyzing separately those who reported pain onset in childhood at 10 years of age or younger; however, we did not find a statistically significant difference between males with early onset of pain and those without. Assumptions about classical vs. nonclassical genotype based

on symptoms reported was not possible, given the variable presentation of symptoms in females with FD.

Future studies may choose to prospectively examine changes in sperm count over time as compared to serum glycolipid storage levels such as lysoGL3 in classically affected males, as it remains possible that fertility decreases due to glycolipid storage over time leading to lower fertility in males with classical FD later in life. Another important direction to explore would be the impact of treatment with enzyme replacement therapy (ERT) on sperm count and fertility in men with classical FD. Knowledge about the reproductive impact of early treatment with enzyme replacement therapy following newborn or family screening will help patients understand the broader effects of therapy and impact on their reproductive choices.

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Summary

Males and females with Fabry disease do not exhibit reduced reproductive fitness; on average they have more biological children than the general population in the United States.

Compliance with Ethics Guidelines

Allison Foley, Myrl Holida, Scott Gillespie, Eric Hall, Morgan Simmons, Alexandrea Wadley, and Virginia Clark declare they have no conflicts of interest.

Dawn Laney consults for Genzyme and Shire, and is a study coordinator in clinical trials sponsored by Genzyme, Amicus, and Protalix. She has also received research funding from Alexion, Amicus, Genzyme, Pfizer, Retrophin, Shire, and Synageva. Grant funding to conduct this investigator initiated studies was provided through an educational grant from Genzyme, A Sanofi company. These activities are monitored and are in compliance with the conflict of interest policies at Emory University School of Medicine.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentations (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study. This chapter does not contain any studies with animal subjects performed by any of the authors.

Contributions

Planning of this study, oversight, data collection, analysis, and reporting of work were coordinated by Dawn Laney. Statistical analysis and manuscript authorship by Scott Gillespie and Eric Hall. Subject recruitment, data collection, and manuscript creation review by Alexandrea Wadley. Data analysis and manuscript review and editing by Morgan Simmons and Allison Foley. Subject recruitment and manuscript authorship/review by Myrl Holida and Virginia Clarke.

Appendix 1: FIT Survey Sample Questions. Questions Were Presented to Be Answered as Self-Response Multiple Choice, Yes or No, and Free Response Answers

FIT survey	Sample question asked
Males	
Part 2. General Questions	"Do you have any biological children?" Yes: _____ Number of boys ____ Number of girls _____ No, but I have adopted children No, I do not have any children biological or adopted
	"Have you ever gotten one of your partners pregnant?"
	"Have you ever had difficulties getting your partner pregnant?" Yes (please describe) _____ No I have never tried to make my partner pregnant
	"Have you ever experienced issues with infertility?"
	"Have you ever been used assisted reproductive technology?" Yes, my partner took hormones to increase ovulation Yes, we used a donor egg Yes, we used a donor sperm Yes we used in vitro fertilization Yes, we used in vitro fertilization and preimplantation genetic diagnosis No, we have never used assisted reproductive technology
Part 3. Medical History	"Have you ever experienced any of the following health issues?" Hypertension/High Blood pressure, beginning at age ___

(continued)

FIT survey	Sample question asked
	Burning pain in your hands/feet, beginning at age ___ Fabry pain crises, beginning at age ___ Depression, beginning at age ___ Anxiety, beginning at age ___ Panic Attacks, beginning at age ___ Kidney failure, beginning at age ___ Liver disease, beginning at age ___ Erectile dysfunction, beginning at age ___ Low sperm count, beginning at age ___ No, none of the above.
	"Do you take any medications for high blood pressure?" Yes, _____ (medication) beginning at age _____
	"Have you ever been treated for cancer or a tumor?"
	"Have you ever worked in a job that included frequent exposure to industrial chemical, pesticides, lead, heavy metals, or radiation/ Xrays?"
Part 4. Family History	"Did you know that you had Fabry disease before you considered having children?"
	"If you did know that you had Fabry disease prior to having children, did having Fabry disease affect your decision to have children?"
	"Anything else about your reproductive history or having babies while living with Fabry disease that you think we should know?"
Females	
Part 2. Medical History	"Have you ever experienced any of the following health issues?" Burning pain in your hands/feet, beginning at age ___ Depression, beginning at age ___ Kidney failure, beginning at age ___ Polycystic ovaries, beginning at age ___ Abnormal uterine shape Endometriosis, beginning at age ___ An STD such as herpes, which one? ___ Other chronic health issue(s) (other than Fabry disease) not mentioned above
Part 3. Reproductive History	"Have you ever been pregnant" If yes, number of times and how old were you during the first trimester of each? If no, have you ever tried to become pregnant?
	"At what age did you begin having your periods?"
	"Have you ever had difficulties getting pregnant?" Yes (please describe _____) No I have never tried to become pregnant.
	"Have you ever had any concerns about your ability to have children?"
	"Have you ever experienced any issues with infertility?"
	"Have you ever been evaluated for infertility concerns?"
	"Have you ever been used assisted reproductive technology?" Yes, I took hormones to increase ovulation Yes, I used a donor egg Yes, I used a donor sperm Yes, I used in vitro fertilization Yes, I used in vitro fertilization and preimplantation genetic diagnosis No, I have never used assisted reproductive technology
	"Have you ever experienced menopause?"
Part 4. Pregnancy History	"How many times have you been pregnant?"
	"Have you ever had a miscarriage?"
	"Have you ever had an elective termination of a pregnancy?"
	"Have you ever had a termination of a pregnancy for medication reasons?"
	"How many children have you given birth to ___boys ___girls?"
	"Were any of your children stillborn or died soon after birth?"
	"Do you have any adopted children?"
Part 5. Family history	"Did you know that you had Fabry disease before having children?"
	If you did know that you had Fabry disease prior to having children, did having Fabry disease affect your decision to have children?

(continued)

Appendix 2: Reproductive Decision-Making and Fabry Disease (Selection of Free Response Answers)

Impact reported on the reproductive decision-making process of the knowledge that they had Fabry disease	Patient quotes
No negative impact	"No, it <having Fabry disease> didn't matter . . . My wife and I discussed Fabrys, and decided we wanted at least 2 children."
	"No, not at all. My dad always told me he had enjoyed his life and was glad he was born and hated when his dad told him if he knew his boys would have Fabrys he wouldn't have had them."
	"No, but it has changed that decision for some of my family members. I figure my life, even though not easy, is worth it. So

(continued)

Impact reported on the reproductive decision-making process of the knowledge that they had Fabry disease	Patient quotes
	my kids if they got it would feel the same way. Some of my family have a harder time with that feeling."
Mixed impact	"Yes, from age 18 I was certain I did not want children because I did not want the disease to continue. I was the youngest person in my family that had Fabry and the disease would end with me. My brother had two kids, but male, so I would be the last of it. But then I got married and I changed my mind and wanted children."
Negative impact	"The treat<ment> is great for many reasons, but the pain from neuropathy is not controlled enough for me to be comfortable in having a child who could possibly go through the type of pain I did growing up."
	"YES . . . I had a tubal ligation at age 24"
	"Once I knew I had Fabry it was sort of the final straw in deciding if we should have kids or not. I felt selfish possibly bringing a child into the world with Fabry. I know this doesn't take into consideration all of the other facets of a child, and that if my mom had chosen that, then I wouldn't be here. But for me I would feel so guilty watching a child suffer the symptoms of Fabry, knowing I chose that for them, in sense."

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RESEARCH REPORT

Neonatal-Onset Hereditary Coproporphyria: A New Variant of Hereditary Coproporphyria

Kosei Hasegawa • Hiroyuki Tanaka • Miho Yamashita • Yousuke Higuchi • Takayuki Miyai • Junko Yoshimoto • Ayumi Okada • Norihiro Suzuki • Keiji Iwatsuki • Hirokazu Tsukahara

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Abstract Genetic mutation of the coproporphyrinogen oxidase (*CPOX*) gene causes either hereditary coproporphyria (HCP) or harderoporphyria. HCP, a rare hepatic porphyria, causes acute attacks after puberty and rarely accompanies cutaneous symptoms. In contrast, harderoporphyria is an erythropoietic porphyria that represents photosensitivity and hemolytic anemia from the neonatal period. In patients with harderoporphyria, the p.Lys404Glu mutation is found in the homozygous or compound heterozygous state with another mutation, and a marked increase in harderoporphyrin is observed. This report describes a neonate with symptoms of erythropoietic harderoporphyria (photosensitivity of the skin, hemolytic anemia, and jaundice). However, the pattern of porphyrin metabolites of feces was consistent with that of typical HCP, not of harderoporphyria. We found a heterozygous, novel, four-base pair deletion in exon 7 of the *CPOX* gene, although other mutations including the p.Lys404Glu mutation in *CPOX* were not found. By unknown etiology, our patient had accompanying adrenocortical insufficiency and 46, XY disorders of sex development. Based on genetic mutation of the *CPOX* gene and information from a previous similar case report, we consider that neonatal-onset HCP is a variant of HCP.

Communicated by: John Christodoulou, MBBS PhD FRACP FRCPA

K. Hasegawa (✉) · H. Tanaka · M. Yamashita · Y. Higuchi · T. Miyai · J. Yoshimoto · A. Okada · H. Tsukahara
Department of Pediatrics, Okayama University Hospital, Shikatacho 2-5-1, Kita-ku, Okayama 700-8558, Japan
e-mail: haseyan@md.okayama-u.ac.jp

H. Tanaka
Department of Pediatrics, Okayama Saiseikai General Hospital, Okayama, Japan

N. Suzuki · K. Iwatsuki
Department of Dermatology, Okayama University Hospital, Okayama, Japan

Introduction

Hereditary porphyrias are disorders that are caused by abnormalities in eight enzymes of the heme biosynthesis pathway. Coproporphyrinogen oxidase (CPOX; EC 1.3.3.3) is the sixth enzyme of the heme biosynthesis pathway. CPOX catalyzes the two steps of oxidative decarboxylation from coproporphyrinogen III to protoporphyrinogen IX via an intermediate metabolite, harderoporphyrinogen (Elder et al. 1978). Genetic mutation of the *CPOX* gene causes either hereditary coproporphyria (HCP: MIM 121300) or harderoporphyria (Nordmann et al. 1983; Lamoril et al. 1995). HCP is a rare hepatic porphyria that causes acute attacks and rarely (5%) accompanies skin photosensitivity. The symptoms of HCP usually begin after puberty. Acute attacks are notable for various symptoms, and often begin with mental disturbance such as anxiety, restlessness, and so on (Kauppinen 2005; Puy et al. 2010). Gastrointestinal symptoms (abdominal pain, nausea, vomiting, and constipation) usually follow the mental disturbance. Tachycardia, excess sweating, and hypertension, which are signs of increased sympathetic activity, are frequently observed. Myopathy is a very common symptom and muscle pain in the arms and legs is also observed in early stages. Diffuse muscle weakness begins in the proximal muscles and can lead to respiratory and bulbar paralysis which can be life threatening. Sensory neuropathy such as sensory loss can develop. Hyponatremia due to inappropriate anti-diuretic hormone secretion may lead to convulsions. Convulsions

are also observed as a central nervous feature of acute porphyric attacks. Daily hem infusions for 3–4 consecutive days are enough to abolish the porphyric symptoms associated with an acute attack. To prevent an acute attack, avoidance of precipitating factors such as certain drugs, alcohol intake, infection and so on, is needed.

Harderoporphyria is an erythropoietic porphyria that is characterized by hemolytic anemia, jaundice, and photosensitivity, which begin from the neonatal period (Nordmann et al. 1983). In feces of patients with harderoporphyria, a marked increase in harderoporphyrin is observed in contrast to patients with HCP in whom a marked increase in fecal coproporphyrin III is observed.

In patients with harderoporphyria, the p.Lys404Glu mutation in *CPOX* has been found in the homozygous or compound heterozygous state with another *CPOX* mutation, which results in exon 6 skipping at the mRNA level (Lamoril et al. 1995, 1998; Schmitt et al. 2005). Amino acids around p.Asp400-Lys404 in CPOX have been reported to be a putative hot spot for harderoporphyria (Schmitt et al. 2005). Kim et al. reported that p.Asp 400 is required for dimerization of CPOX and p.Arg401-Lys404 is necessary for catalysis of the second step in oxidative decarboxylation from harderoporphyrinogen to protoporphyrinogen (Kim et al. 2013).

We describe a neonate representing with clinical symptoms of erythropoietic porphyria (photosensitivity of the skin, hemolytic anemia, and jaundice) with congenital adrenocortical insufficiency and 46, XY disorders of sex development.

Case Report

At our affiliated hospital, a neonate was delivered by cesarean section because of reduced fetal movement at 40 weeks and 4 days of gestational age. The father and mother of the neonate were non-consanguineous. His birth weight was 3,318 g (0.75 SD) with Apgar scores of 9 at 1 and 5 min. He had hypospadias, a bifid scrotum, and bilateral cryptorchidism (Fig. 1a). The testes were palpable bilaterally in the inguinal regions. Chromosomal analysis showed that he had a 46, XY male karyotype. The neonate also showed marked jaundice and hepatosplenomegaly. Biochemical studies that were conducted 1 h after birth showed leukocytosis, hypoglycemia, and an elevated total bilirubin level (183.0 μmol/L). A continuous glucose infusion and phototherapy were begun. Hypoglycemia recovered spontaneously. The firsturine, which was reddish brown, was excreted at 16 h after birth. Serum total and direct bilirubin levels increased to 218.9 and 100.9 μmol/L, respectively, in spite of continuous phototherapy. Therefore, exchange transfusion was performed twice on days 1 and 2. The serum total bilirubin level reached its highest level on day 2 (270.2 μmol/L) and decreased daily thereafter. Phototherapy was discontinued on day 4. On day 5, blisters appeared on the front side of the patient's trunk, limbs, and face. On the other hand, no blisters were observed on his back, which had not been exposed to green light (Fig. 1b). On day 9, he became anemic but recovered spontaneously by day 12. An auto-immune basis for the anemia and congenital infections were excluded. He was referred to our hospital on day 17 to confirm the cause of blistering and ambiguous genitalia.

After admission to our hospital, the blisters spontaneously healed with no specific therapy. Skin biopsy of a blister was conducted on day 19, which showed a subepidermal blister without overt inflammation, and a church spire-like protrusion of the dermal papillae (so-called festooning phenomenon) (Fig. 2a–c). Clumpy deposits of IgG were observed along the basement membrane zones and vessels (Fig. 2d). Electron microscopy showed blister formation above the basal lamina, and cytolysis of some basal cells (Fig. 2e, f). These findings were consistent with those of porphyria, excluding autoimmune blistering disease or epidermolysis bullosa hereditaria.

We speculated that photosensitivity due to porphyria might have caused the patient's skin lesions. Therefore, porphyrin levels were analyzed in erythrocytes and urine. We observed elevated concentrations of urinary uroporphyrin and coproporphyrin, and elevated concentrations of protoporphyrin and coproporphyrin in erythrocytes (Table 1). These results suggested that a disordered porphyrin metabolism caused his skin lesions. After admission, skin pigmentation developed around his perineal and axillary regions. His serum potassium level rose slightly. Because adrenal insufficiency was suspected, we analyzed plasma adrenocorticotropic hormone levels, plasma renin activity, and serum steroid metabolites to identify the cause of ambiguous genitalia (Table 2) Although a urinary steroid profile analysis conducted on day 21 showed ACTH resistance, no known steroidogenic enzyme deficiency was not suggested. Abdominal computed tomography on day 5 and ultrasonography on day 19 showed that his adrenal glands were of normal size. Oral hydrocortisone was started and serum adrenocorticotropic hormone and potassium levels were reduced to the normal range. Skin hyperpigmentation improved.

At 1 year and 9 months old, we examined the urinary steroid profile again, which was again nondiagnostic. Fecal porphyrin metabolites were also analyzed. The coproporphyrin III level was extremely elevated (Table 1). He has had no acute attack as at 1 year 9 months of age.

Based on the clinical manifestations and laboratory testing, we considered that our patient might have HCP.

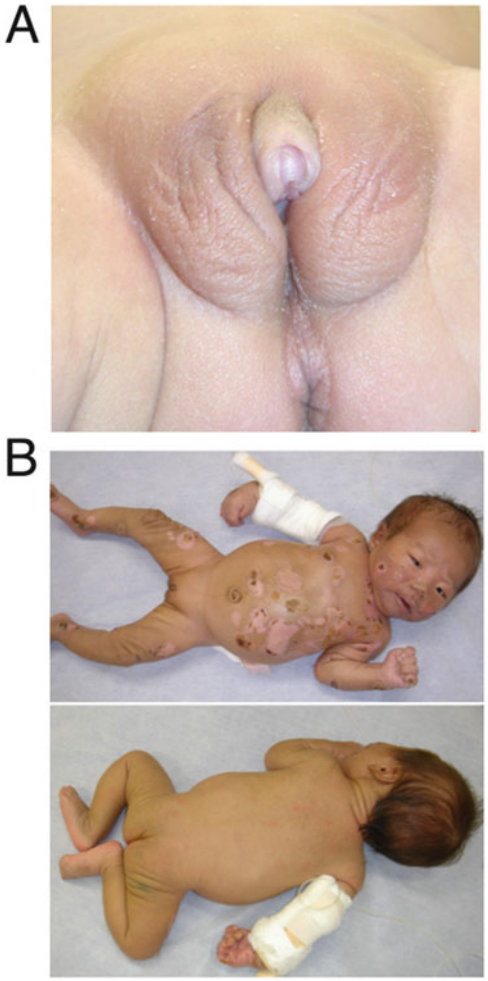

Fig. 1 Distinctive features of our patient. (**a**) External genitalia of the patient on day 17. Hypospadias, a bifid scrotum, and bilateral cryptorchidism are apparent. (**b**) Skin lesions of the patient. Blisters are apparent, restricted to his front on which green light was illuminated for phototherapy

Therefore, we conducted mutational analysis related to the *CPOX* gene.

Methods

Porphyrin metabolites in urine and erythrocytes were measured in a commercial laboratory, SRL (Tokyo, Japan). Fecal porphyrin metabolites were measured at Mayo Medical Laboratories (Rochester, MN) via LSI Medience Corporation (Tokyo, Japan).

To analyze the *CPOX* gene (reference sequence: Genomic, NG_0125944.2; mRNA, NM_000097.5), genomic DNA from our patient and the mother was extracted using the QIAamp DNA Mini Kit (Qiagen Inc., Tokyo, Japan) after informed consent was obtained from his mother. Total RNA was also extracted from peripheral blood of the patient using the RNeasy Mini Kit (Qiagen Inc.). A total of 1.0 μg of RNA was reverse-transcribed using the Omniscript RT Kit (Qiagen Inc.) to obtain cDNA. A PCR was conducted using standard PCR methods (primers are available on request). The PCR products were sequenced using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and Genetic Analyzer (ABI Prism 310; Applied Biosystems, Foster City, CA). The PCR products were then cloned into the pCR®4-TOPO vector using the TOPO TA Cloning® kit for sequencing (Thermo Fisher Scientific, Inc., Waltham, MA). The obtained clones were sequenced. The father was not analyzed because the mother had divorced the father soon after the birth of our patient.

Results

A heterozygous deletion of four base pairs was found in exon 7 of the *CPOX* gene (c.1286_1289 del ACAT; Fig. 3a, b). Other mutations, including the p.Lys404Glu mutation, were not identified in the *CPOX* gene. This four-base pair deletion was not found in single nucleotide polymorphism databases, the 1,000 Genomes and the ExAC databases. This mutation was not found in his mother and is presumed to be novel. The mutant allele with the four-base pair deletion was expressed as mRNA (Fig. 3c). This mutation is predicted to add an abnormal 64 amino acids instead of the normal 27 amino acids (p. Tyr429Cys fs64*). Enzymatic activity of CPOX was not analyzed in this patient. No other mutation was found in the respective coding regions of protoporphyrinogen oxidase, uroporphyrinogen decarboxylase, or ferrochelatase genes (data not shown).

Discussion

This report describes a case of a neonatal-onset HCP with adrenocortical insufficiency and a disorder of sex development associated with a normal male karyotype. Clinical features of our patient, such as neonatal-onset jaundice, hemolytic anemia, and photosensitivity, were not consistent with typical HCP, which causes acute attacks after puberty. However, these features are consistent with harderoporphyria. In spite of the similar clinical features to harderoporphyria, our patient showed a marked increase in the level of fecal coproporphyrin III, which is observed in patients with HCP (Table 3). Furthermore, our patient did not have previously reported harderoporphyria mutations, but had a novel, heterozygous, four-base pair deletion. These results are consistent with HCP, but not harderoporphyria.

Similar to our patient, another neonatal patient with HCP showed neonatal jaundice and hemolytic anemia with a

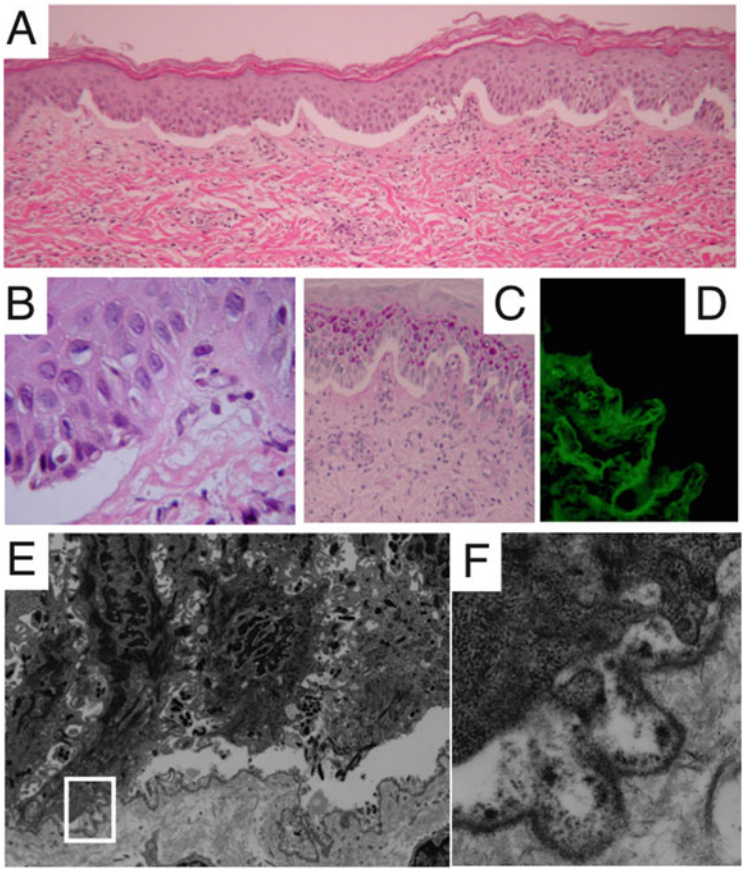

Fig. 2 Results of skin biopsy. (**a**) Subepidermal blister with church spire-like dermal papillae (festooning phenomenon). (**b**) Expanded image of (**a**). Note basal cell damage. (**c**) The blister can be seen above the periodic acid-Schiff-positive basement membrane. (**d**) Clumpy deposits of IgG can be seen along the basement membrane zone and blood vessels. (**e**)Electron microscopic findings show blister formation above the basal lamina. (**f**) Expanded image of the *white box* in (**e**)

heterozygous *CPOX* gene mutation, which caused exon 6 skipping (Table 3) (Takeuchi et al. 2001). This case also did not have a mutation that is found in patients with harderoporphyria and the coproporphyrin III level in the feces was greatly increased.

Based on these clinical and biological results, we concluded that our patient was an atypical case of HCP, who showed symptoms of erythropoietic porphyria from the neonatal period, as in a previous case (Takeuchi et al. 2001), thus our case is only the second reported case of neonatal-onset HCP twice. We consider that neonatal-onset HCP is a variant of HCP.

Hypospadias and adrenocortical insufficiency were observed in our patient and in the previously reported neonatal patient with HCP (Table 3) (Takeuchi et al. 2001). In patients with harderoporphyria and compound heterozygous mutations of p.Lys404Glu and IVS6 + 3A>G (Lamoril et al. 1998), which result in exon 6 skipping at the mRNA level, only hypospadias was observed. Hypospadias is the result of a lack of androgenic steroid during the embryonic period. Synthesis of steroid hormones, such as cortisol and androgen, depends on the heme-thiolate enzyme, cytochrome P450. Therefore, heme synthesis disorders might be linked to impairment of steroid hormone synthesis, although the mechanisms remain unknown. Further investigations, including the relationship between the genotype of *CPOX* and hypospadias and adrenocortical insufficiency, are needed because exon 6 skipping of *CPOX* also causes typical HCP (Delfau-Larue et al. 1994).

We conclude that the neonatal-onset HCP is a recognized variant of HCP caused by genetic mutations of *CPOX*. However, questions remain as to why some *CPOX* gene mutations cause photosensitivity, jaundice from the neonatal period, hypospadias, and adrenocortical insufficiency. Findings from our case have further expanded the clinical spectrum of diseases caused by *CPOX* gene mutations.

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Table 1 Porphyrin metabolite concentrations

Age	3 Months	5 Months	1 Year and 9 months	Reference range
Urine				
Uroporphyrin (μg/g Cre)	**336**	**89**	**117**	≤36
Coproporphyrin (μg/g Cre)	**15,235**	**14,762**	**16,284**	≤170
δ-Aminolevulinic acid (mg/L)		0.4	1.4	≤5
Erythrocytes				
Uroporphyrin (μg/dL RBC)		≤1		≤1
Coproporphyrin (μg/dL RBC)		**3**	≤1	≤1
Protoporphyrin (μg/dL RBC)		**323.0**	**122.1**	30–86
Feces				
Coproporphyrin I (μg/24 h)			452 (3.5%)	<500
Coproporphyrin III (μg/24 h)			**10,541 (82%)**	<400
Isocoproporphyrin (μg/24 h)			**622 (4.8%)**	<200
Protoporphyrin (μg/24 h)			54 (0.4%)	<1,500
Harderoporphyrin (μg/24 h)			**1,112 (8.7%)**	<200
Coproporphyrin III/coproporphyrin I			**23.3**	<1.20

Cre creatinine, *RBC* erythrocyte
Abnormal findings are shown in bold font

Table 2 Analysis of steroid metabolites and stimulating hormones

	Day 19	Day 28	Day 36	Reference range[a]
Pregnenolone(nmol/L)		0.02		0.01–0.04
Progesterone (nmol/L)		**14.9**		0.22–1.7
17α-Hydroxypregnenolone (nmol/L)		**0.34**		0.01–0.23
17α-Hydroxyprogesterone (nmol/L)		**0.08**		0.00–0.03
Cortisol (nmol/L)		**66.2**		77.3–634.8
Androstenedione (nmol/L)		**0.13**		0.01–0.03
DHEA-S (μmol/L)		0.73		0.14–3.0
Testosterone (nmol/L)	12.0	4.9	2.2	2.1–13.9
Estradiol (pmol/L)		48.4		36.7–117.4
Aldosterone (nmol/L)		1.4		0.14–2.5
ACTH (pmol/L)	**296.7**	**189.2**	**47.3**	1.63–12.3
Plasma renin activity (μg/L/h)		17		3.6–24
Luteinizing hormone (IU/L)	**4.8**			4.85–10.02
Follicle-stimulating hormone (IU/L)	**1.2**			1.22–5.19

Abnormal findings are shown in bold font
DHEA-S dehydroepiandrosterone sulfate
[a] Diamond and Bercu (2004)

Compliance with Ethics Guidelines

Conflict of Interest

Kosei Hasegawa, Hiroyuki Tanaka, Miho Yamashita, Yousuke Higuchi, Takayuki Miyai, Junko Yoshimoto, Ayumi Okada, Norihiro Suzuki, Keiji Iwatsuki, and Hirokazu Tsukahara declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. This study was approved by the Ethical Committee of Okayama University Hospital. Informed consent was obtained from patient's mother. Additional informed consent was also obtained from patient's mother for which identifying information is included in this article. Proof that informed consent was obtained is available upon request.

Details of the Contributions of Individual Authors

Study design: Kosei Hasegawa and Hiroyuki Tanaka.

Clinical data collection: Kosei Hasegawa, Miho Yamashita, Yousuke Higuchi, Takayuki Miyai, Junko Yoshimoto, Ayumi Okada, and Norihiro Suzuki.

Drafting the manuscript: Kosei Hasegawa.

Revising manuscript content: Hiroyuki Tanaka, Keiji Iwatsuki, and Hirokazu Tsukahara.

Take-Home Message

Neonatal-onset hereditary coproporphyria is a variant of HCP.

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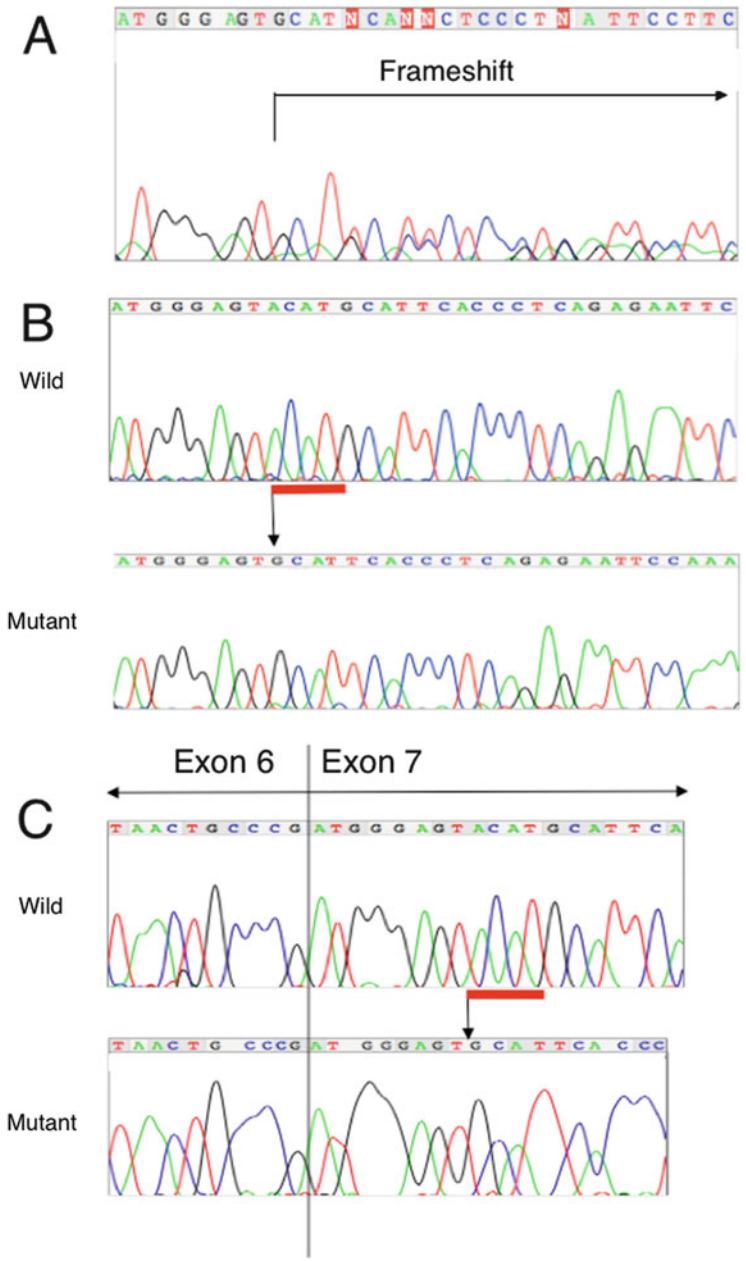

Fig. 3 Results of sequential analyses of the *CPOX* gene of our patient. (**a**) Sequence of PCR products of exon 7. A frameshift was found. (**b**) Sequence of TA cloning sample of PCR products of exon 7. A four-base pair (indicated by a *red bold line*) deletion (c.1286_1289 del ACAT) was found in the mutant allele. (**c**) Sequence of *CPOX* cDNA. The mutant allele including the four-base pair deletion (*red bold line*) was demonstrable in cDNA cloned into the sequencing vector

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Kosei Hasegawa

Table 3 Comparison of genotype–phenotype correlation and porphyrin metabolites in diseases caused by *CPOX* gene mutation

Diseases (onset)	*CPOX* mutation	Enzymatic activity	Increased metabolite in feces (ratio to total fecal porphyrin)	Neurological attack	Cutaneous lesiondue to sensitivity	Hemolytic anemia and jaundice	Adrenocortical insufficiency	Hypospadias
HCP (after puberty to adult)	Heterozygous or homozygous mutation of *CPOX*	~50% (hetero)[a,b,c] <10% (homo)[d,e,f]	COPRO III dominant	+	Rarely +	–	–	–
Harderoporphyria (neonate)	p.Lys404Gluhomo[g,h]	18–25%	COPRO III (19–29%) HARDERO (65–72%)	–	+	+	–	–
	p.Lys404Gluand IVS6 + 3A>G (exon 6 skipping at mRNA level)[i]	18%	COPRO III (6.2%) HARDERO (90%)	–	+	+	–	+
Takeuchi et al. (neonate)[j]	c.1277G>A hetero (exon 6 skipping at mRNA level)	ND	COPRO III (63%) HARDERO (7%)	–	+	+	+	+
Present case (neonate)	c.1286_1289del ACAThetero	ND	COPRO III (82%) HARDERO (8.7%)	–	+	+	+	+

HCP hereditary coproporphyria, *CPOX* coproporphyrinogen oxidase, *COPRO* coproporphyrin, *HARDERO* harderoporphyrin, *homo* homozygous mutation, *hetero* heterozygous mutation, *ND* not determined

[a] Elder et al. (1976)
[b] Nordmann et al. (1977)
[c] Grandchamp and Nordmann (1977)
[d] Grandchamp et al. (1977)
[e] Martasek et al. (1994)
[f] Kuhnel et al. (2000)
[g] Nordmann et al. (1983)
[h] Schmitt et al. (2005)
[i] Lamoril et al. (1998)
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RESEARCH REPORT

Treatment Adherence and Psychological Wellbeing in Maternal Carers of Children with Phenylketonuria (PKU)

Emma Medford · Dougal Julian Hare · Katie Carpenter · Stewart Rust · Simon Jones · Anja Wittkowski

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Abstract Phenylketonuria (PKU), a rare metabolic disorder, causes cognitive impairment unless treated with a strict, protein-restricted diet, but few studies have examined the relationship between treatment compliance and parental wellbeing. In the present study, 46 primary caregivers of children with PKU completed measures of psychological distress, parenting stress (related to caring for a child with an illness), resilience, perceived social support and child dependency. Treatment adherence was assessed using the proportion of blood phenylalanine concentrations within target range in the preceding year. Results indicated that 59% of caregivers showed clinical levels of psychological distress, which was predicted by their parenting stress and resilience. Whilst the proportion of blood phenylalanine concentrations in range was not associated with parental distress, it was predicted by child age and caregiver's perceived support from family. Despite experiencing high levels of distress, the results indicated that caregivers' ability to adhere to treatment was not affected. Interventions to reduce parenting stress and boost caregiver resilience may have a positive effect on parental wellbeing. Additionally, interventions to promote treatment adherence benefit parents of older children, with a focus on promoting support from family members. Further research with larger sample sizes and longitudinal designs is needed to further establish causal mechanisms.

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E. Medford · K. Carpenter · A. Wittkowski
Division of Psychology and Mental Health, School of Health Sciences, University of Manchester, Manchester, UK

E. Medford · A. Wittkowski (✉)
Greater Manchester Mental Health NHS Foundation Trust, Prestwich, UK
e-mail: Anja.Wittkowski@manchester.ac.uk

D.J. Hare
School of Psychology, Cardiff University, Cardiff, UK

S. Rust
Paediatric Psychosocial Service, Royal Manchester Children's Hospital, Manchester, UK

S. Jones
Central Manchester University Hospitals NHS Foundation Trust St Mary's Hospital, Manchester Centre for Genomic Medicine, Manchester, UK

Introduction

Phenylketonuria (PKU, OMIM 261600) is a rare genetic disorder in which the amino acid phenylalanine (phe) is insufficiently metabolised. Although phe accumulation causes severe and irreversible cognitive impairment, this can be prevented by a lifelong protein-restricted diet. Parents have to supervise their child's nutritional intake and facilitate regular sampling of blood phe concentrations (Smith et al. 1993). Given the effort required for treatment adherence and the consequences of poor compliance, caring for a child with PKU brings additional challenges (Awiszus and Unger 1990) that potentially affect parental wellbeing, with increased depression and anxiety reported in some parents of children with PKU (Gundaz et al. 2015; Mahmoudi-Gharaei et al. 2011), but not others (Fidika et al. 2013; Kazak et al. 1988; Ten Hoedt et al. 2011).

To support parents of children with PKU, it is important to understand the determinants of their psychological wellbeing. Fidika et al. (2013) found parental quality of life (QoL) was predicted by family stress and perceived social support, whilst Ten Hoedt et al. (2011) noted that health-related QoL was affected by emotional support and

loss of friendship. Whilst QoL was lower for parents with younger children due to greater parental responsibility for dietary adherence, Gundaz et al. (2015) found that parental anxiety and depression were not associated with child age.

There is little data on parental psychological wellbeing and treatment adherence. Reber et al. (1987) found no significant difference in external life stress between parents of children with good and poor metabolic control; Fehrenback and Peterson (1989) observed that parental anxiety and depression were not significantly associated with metabolic control. However, associations between parental wellbeing and treatment adherence have been identified in other lifelong metabolic conditions. In childhood diabetes, increased maternal depressive symptoms were associated with less parental monitoring, itself associated with poorer adherence and metabolic control (MacKey et al. 2014).

The aims of the current study were to (1) examine the psychological impact of parenting a child with PKU, (2) examine influences on parental psychological wellbeing and (3) examine the relationship between parental wellbeing and treatment adherence. It was hypothesised that parents would have high levels of psychological distress, which would be predicted by their parenting stress (related to caring for a child with an illness), resilience, perceived social support and level of child dependency. It was also hypothesised that treatment adherence would be associated with parental psychological distress and other parent factors (resilience, perceived social support and child dependency).

Method

Participants

Primary caregivers of children with PKU attending three metabolic clinics in the North West UK (Manchester, Liverpool and Bradford) were invited to participate. Caregivers were eligible to participate if their child was between 0 and 16 years old and diagnosed with PKU at birth. Caregivers were excluded if they could not comprehend English or if there were any other significant health problems or caring responsibilities that could affect their psychological wellbeing.

Measures

Psychological Distress

The 12-item *General Health Questionnaire-12* (GHQ-12; Goldberg and Williams 1988) was used to assess caregiver psychological distress. Higher scores suggest higher levels of distress (anxiety and depression), and scores over 12 indicate distress within the clinical range (Goldberg et al. 1997). The GHQ-12 has good psychometric properties (Goldberg and Williams 1988) and good internal consistency in the current study ($\alpha = 0.87$).

Parenting Stress

The *Pediatric Inventory for Parents* (PIP; Streisand et al. 2001) was used to measure caregiver stress related to caring for a child with an illness. The PIP is a self-report measure with four subscales (communication, medical care, emotional distress and role function) and overall total difficulty and total frequency scores. The total scores were used in the present study, and both had good internal consistency ($\alpha = 0.94$ and 0.95).

Resilience

Caregiver resilience was assessed using the *Resilience Scale for Adults* (RSA; Friborg et al. 2006), a self-report measure with six subscales (perception of self, planned future, social competency, structured style, family cohesion and social resources) and a total score. Higher scores indicate increased protective resilience. The RSA has good psychometric properties (Windle et al. 2011), and internal consistency in the present study was high ($\alpha = 0.88$).

Social Support

Perceived social support was assessed using the *Multidimensional Scale of Perceived Social Support* (MSPSS; Zimet et al. 1988), a self-report measure with three subscales (family, friends and significant other) and a total score. The MSPSS has strong psychometric properties (Zimet et al. 1990) and good internal consistency in the current study (family $\alpha = 0.92$, friends $\alpha = 0.93$, significant other $\alpha = 0.97$ and total scale $\alpha = 0.90$).

Child Dependency

A seven-point Likert-style question was developed to assess how much the child depended on their caregiver to adhere to the recommended dietary treatment using two opposing statements ('My child has managed their diet on their own' and 'My child has relied on me to help them stick to a protein-restricted diet'). Participants were required to select an option box closest to the end statement that best described their child best over the previous weeks.

Treatment Adherence

Two measures of treatment adherence were used: (1) the proportion of blood phe concentrations within target range

in the preceding year and (2) the proportion of required blood samples submitted in the preceding year, with information taken from existing medical records. Blood samples had been routinely collected using Guthrie card blood spots or venous samples and analysed using tandem mass spectrometry or Thermo high-performance liquid chromatography.

All three PKU clinics used the National Society for PKU recommendations (NSPKU 2014) for target blood phe concentrations and frequency of blood sampling, i.e. acceptable blood phe ranges of 120–360 μmol/L for 0–5-year-olds, 120–480 μmol/L for over 5-year-olds and 120–700 μmol/L for adults. However, one of the clinics accepted 100–400 μmol/L for 0–5-year-olds, 100–500 μmol/L for over 5-year-olds and up to 700 μmol/L for adolescents (from around 14 years old). Two clinics requested weekly blood samples for 0–5-year-olds, fortnightly samples for over 5-year-olds and monthly samples for adolescents and adults. The third clinic requested weekly samples for 0–2-year-olds, fortnightly samples for 2–5-year-olds and monthly samples for over 5-year-olds. In addition to these minimum requirements, patients were asked to provide additional blood samples on a case-by-case basis, for example, during periods of illness or when blood phe levels were high, and these were necessarily included in the data used for the present study as it was not feasible to disaggregate the data to remove these additional samples.

Demographic information (caregiver date of birth, gender, language, relationship to child, highest qualification and average family income) was also collected.

Procedure

Clinical teams at the PKU clinics identified eligible caregivers by reviewing patient notes and databases. Invitation packs (including participant information sheets, consent forms and the study questionnaires) were posted to eligible caregivers alongside a freepost envelope. If caregivers returned incomplete questionnaires, they were followed up with a phone call. An opt-out form was available for caregivers to complete if they did not want to participate. If caregivers did not respond to their initial invitation pack, a reminder pack was sent. In addition to postal invitation, researchers [EM and KC] attended PKU clinics, and the study was advertised on posters displayed in PKU clinics and social media (Facebook) and via NSPKU and clinic newsletters. On return of the questionnaires, participants received a £5 shopping voucher. Ethical approval was granted by the NHS Greater Manchester Central Research Ethics Committee (REC reference number: 15/NW/0454). The study, which was also approved by the University of Manchester Research Subcommittee and the Manchester, Liverpool and Bradford NHS Research and Development departments, was conducted in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration (1975, 2000 revision). Informed written consent was obtained from all participants.

Statistical Analysis

Average score substitution was used for minor missing data on GHQ-12 and/or PIP. As one participant had an incomplete PIP, she/he was excluded from analyses involving this measure. Assumptions of normality were met for all variables, except MSPSS significant other subscale and the child dependency scale, which were negatively skewed. Pearson's and Spearman's correlations were therefore used, depending on data distribution, to examine associations between demographic variables, caregiver measures and treatment adherence. Although multiple correlational analyses were performed, Bonferroni corrections were not utilised (Nakagawa 2004) with attention paid to the post hoc effect size of significant results. Multiple regression analysis was used to examine the predictors of parental psychological distress (GHQ-12 score) and exploratory regression analyses to explore predictors of parenting stress (frequency and difficulty) and treatment adherence. With minimum $N = 10$ participants per predictor variable to prevent overfitting, four predictor variables were included in the regression models using $p = 0.05$ for all analyses. All analyses were performed using IBM SPSS 20.

Results

Forty-six (24%) of 192 invited caregivers participated, mean age 36 years and 11 months (SD = 8 years and 4 months, range = 22 years to 66 years and 2 months), with 28/94 from Manchester, 6/44 from Liverpool and 12/54 from Bradford, comprising of 44 mothers (96%), one father (2%) and one grandmother (2%). Two caregivers completed questionnaires for two siblings with PKU, and for the purposes of analysis, one child from each sibling pair was selected at random. Twenty-nine (63%) of the children were male and 17 (37%) were female, with a mean age of 6 years and 11 months (SD = 4 years and 10 months, range = 4 months to 15 years and 10 months). English was the caregivers' first language, with the exception of one mother (Punjabi). Educational attainment included General Certificate of Secondary Education (GCSE) ($N = 10/22\%$), A Levels ($N = 8/17\%$), diploma ($N = 4/9\%$), degrees (N = 13/28%), masters ($n = 5/11\%$) and

doctorate ($N = 1/2\%$) [no information $N = 5/11\%$]. Thirty-six (78%) caregivers reported a mean income of £37,157 (SD = £24,598; range = £6,500–100,000).

The mean GHQ-12 score was 13.09 (see Table 1) with 27 caregivers (59%) scoring above the clinical cut-off score of 12, indicative of clinically significant anxiety and depression. The mean care dependency score was 5.78, indicating that as might be expected given their age, the children were highly dependent on their carers for dietary treatment adherence.

Caregiver psychological distress and stress related to caring for a child with an illness were strongly and negatively associated with caregiver level of resilience (see Table 2). Levels of psychological distress and parenting stress were positively correlated. Conversely, level of psychological distress was not significantly associated with perceived social support, child dependency or demographic factors (caregiver and child age, caregiver qualifications and household income).

Whilst there was a moderate negative correlation between caregivers' stress related to caring for a child with an illness (both frequency and difficulty) and perceived support from friends, it was not significantly correlated with their perceived support from family or significant other. Although level of child dependency was moderately and positively associated with stress difficulty score, it was not associated with stress frequency score. Finally, stress was not significantly associated with demographic factors.

Regarding treatment adherence, the percentage of blood phe concentrations in target range was moderately negatively correlated with parent and child age and moderately positively correlated with caregivers' perceived support from family, but was not associated with caregivers' perceived support from friends or significant other, child dependency, resilience, psychological distress, stress related to caring for a child with an illness or other demographic factors. Percentage of blood samples submitted was moderately negatively associated with the proportion of blood phe concentrations in target range but was not correlated with any other variables.

Table 1 Descriptive statistics for caregiver measures and treatment adherence

Measure	*N*	Mean (SD)	Range of data (scale range)
GHQ-12	46	13.09 (5.09)	5–27 (0–36)[a]
PIP total frequency	45	102.04 (25.92)	50–158 (42–210)
PIP total difficulty	45	99.98 (30.79)	45–169 (42–210)
RSA	46	167.46 (24.12)	114–227 (33–231)
MSPSS friend	46	19.61 (6.62)	4–28 (4–28)
MSPSS family	46	20.93 (5.78)	7–28 (4–28)
MSPSS sig. other	46	21.57 (7.51)	4–28 (4–28)
MSPSS total	46	62.11 (14.785)	27–84 (12–84)
Care dependency	46	5.78 (2.086)	1–7 (1–7)
% phe in target	46	69.09 (23.992)	0–100
% blood samples	46	107.17 (60.484)	8–333

[a] Scores over 12 indicate distress within the clinical range

Multiple regression analysis was used to identify predictors of parental psychological wellbeing using the variables that were hypothesised to influence GHQ-12 score (Table 3). As parenting stress frequency and difficulty scores were highly intercorrelated ($r = 0.93$), only the latter was used.

When parenting stress, resilience, perceived social support and child dependency were entered simultaneously, the regression model explained 36.7% of the variance in GHQ-12 score. Caregivers' parenting stress and resilience independently accounted for significant variance ($\beta = 0.061$ and $-0.0.08$, $p = 0.032$ and 0.049, respectively), but perceived social support and child dependency did not ($p = 0.497$ and 0.673, respectively). Exploratory regression analyses were used to examine predictors of parenting stress (frequency and difficulty, Table 4). Given the close relationship between child dependency and age (Spearman's $\rho = -0.63$), age was entered first to determine whether child dependency could explain additional variance. Perceived support from friends was entered rather than total social support, given the higher correlation with parenting stress.

Child age did not significantly predict parenting stress related to caring for a child with an illness (frequency or difficulty). Whilst caregivers' level of resilience explained significant variance in PIP difficulty and frequency after accounting for child age ($\beta = -0.677$ and -0.538, $p = <0.001$ and 0.001, respectively), perceived support from friends and level of child dependency did not.

When examining predictors of the proportion of blood phe concentrations within target range (Table 4), child age was entered first, given previously reported associations with metabolic control (MacDonald et al. 2010). Parental age was not selected as a predictor variable given the high correlation with child age ($r = 0.73$), and perceived support from family was used rather than total social support score given the stronger correlation with percentage of blood phe concentrations in target range. Child age accounted for significant variance in the proportion of blood phe concentrations in target range ($\beta = -1.879$, $p = 0.009$, $r^2 = 0.147$), followed by perceived social support ($\beta = 1.844$, $p = 0.002$). Inclusion of perceived support from family, parental wellbeing and level of child depen-

Table 2 Correlations between demographic variables, caregiver measures and treatment adherence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Parent age														
2. Child age	0.73**													
3. Parent qualification	0.20	0.01												
4. Parent income	0.29	0.15	0.65**											
5. MSPSS total	−0.02	0.00	−0.07	0.27										
6. MSPSS friend	0.15	0.27	−0.11	0.12	0.72**									
7. MSPSS family	−0.08	−0.04	−0.18	−0.04	0.71**	0.30*								
8. MSPSS sig. other	−0.18[a]	−0.26[a]	0.14[a]	0.57**[a]	0.76**[a]	0.34*[a]	0.42**[a]							
9. Child dependency	−0.34*[a]	−0.63**[a]	0.06[a]	−0.09[a]	−0.28[a]	−0.45**[a]	−0.19[a]	−0.02[a]						
10. RSA total	0.09	0.11	0.17	0.21	0.52**	0.41**	0.30*	0.40**[a]	−0.24[a]					
11. GHQ-12	−0.06	−0.05	0.18	0.01	−0.19	−0.16	−0.09	−0.16[a]	0.16[a]	−0.54**				
12. PIP frequency	0.08	−0.08	0.03	−0.16	−0.30*	−0.37*	−0.18	−0.21[a]	0.27[a]	−0.62**	0.51**			
13. PIP difficulty	−0.04	−0.14	0.04	−0.24	−0.29	−0.39**	−0.2	−0.16[a]	0.30*[a]	−0.63**	0.55**	0.93**		
14. % phe in target	−0.36*	−0.38**	−0.13	−0.03	0.11	−0.16	0.43**	0.19[a]	0.19[a]	−0.04	−0.09	−0.10	−0.08	
15. % blood samples	0.14	0.13	0.15	−0.02	0.13	0.26	−0.04	−0.02[a]	−0.20[a]	0.17	−0.10	0.04	0.06	−0.43**

Note: *$p < 0.05$, **$p < 0.01$

[a] Spearman's rho correlation, all other correlations are Pearson's

Table 3 Multiple regression analysis using parenting stress, resilience, social support and child dependency to predict caregiver psychological distress

Enter	*B*	SE B	*p*	R^2	*F* change
Parenting stress: difficulty	0.061	0.027	0.032		
Resilience	−0.08	0.039	0.049		
Social support: total	0.035	0.051	0.497		
Child dependency	0.145	0.341	0.673	0.367	5.791**

Note: $^{**}p < 0.01$
Criterion variable: GHQ total score

Table 4 Hierarchical regression analyses predicting parenting stress (difficulty and frequency) and the proportion of blood phe concentrations within target range

Criterion variable: PIP difficulty					
Enter	*B*	SE B	*p*	R^2	*F* change
1. Age	−0.875	0.946	0.360	0.019	0.855
2. Age	0.536	0.988	0.590		
Social support: friends	−0.649	0.638	0.316		
Resilience	−0.677	0.177	<0.001		
Child dependency	2.495	2.528	0.330	0.435	7.713**
Criterion variable: PIP frequency					
Enter	*B*	SE B	*p*	R^2	*F* change
1. Age	−0.413	0.802	0.609	0.006	0.265
2. Age	1.014	0.833	0.231		
Social support: friends	−0.537	0.539	0.324		
Resilience	−0.538	0.149	0.001		
Child dependency	3.062	2.133	0.159	0.433	7.642**
Enter	*B*	*SE B*	*p*	R^2	*F change*
1. Age	−1.879	0.683	0.009	0.147	7.571**
2. Age	−1.249	0.835	0.143		
Social support: family	1.844	0.547	0.002		
Parental wellbeing	−0.432	0.612	0.484		
Child dependency	2.02	2.01	0.321	0.341	5.309**
Enter	*B*	*SE B*	*p*	R^2	*F change*
1. Age	−1.807	0.623	0.006		
Social support: family	1.706	0.529	0.002		
Parental wellbeing	−0.320	0.601	0.597	0.325	6.741**

Note: $^{**}p < 0.01$

dency in the regression model increased the percentage of variance explained from 14.7 to 34.1%, but age was no longer significant after adjusting for the other variables ($p = 0.143$). In another model that excluded child dependency and included child age, social support from family and parental distress (Table 4), child age and social support explained significant independent variance, whilst the inclusion of child dependency only explained an additional 1.6% of the variance in the proportion of blood phe levels in target range (r^2 [including child dependency] = 0.341; r^2 [excluding child dependency] = 0.325).

Discussion

As predicted, caregivers had high levels of clinically significant psychological distress, reflecting the additional challenges of caring for a child with PKU. This is consistent with previous research in both PKU and other disorders (Gundaz et al. 2015; Mahmoudi-Gharaei et al. 2011; Cousino and Hazen 2013). Increased resilience was also associated with reduced psychological distress in the present study. In contrast to earlier studies (Fidika et al. 2013; Ten Hoedt et al. 2011), caregivers' psychological

distress was not associated with their perceived social support in the present study.

In the present study, level of dependency on the caregiver for dietary adherence was not associated with level of psychological distress. Although it correlated with parenting stress difficulty, it was not a significant predictor when entered with other variables. In addition, child age and caregiver wellbeing were not significantly associated, which is congruent with Gundaz et al. (2015), but not with other studies reporting an association between child age and parental quality of life (Fidika et al. 2013; Ten Hoedt et al. 2011).

Contrary to prediction, caregiver levels of psychological distress, parenting stress and resilience were not associated with treatment adherence. Although at variance with other metabolic conditions such as type 1 diabetes (Driscoll et al. 2010; MacKey et al. 2014; Skocic et al. 2012; Whittemore et al. 2012), this is consistent with previous research in PKU (Fehrenback and Peterson 1989; Reber et al. 1987) and implies that experiencing increased levels of psychological distress does not affect adherence to treatment.

As per previous research (MacDonald et al. 2010), child age accounted for nearly 15% of the variance in blood phe concentration data, but child dependency was not significantly correlated with treatment adherence, suggesting that other factors around food and lifestyle may be involved (Levy and Waisbren 1994). After accounting for age, higher levels of caregiver's perceived support from family predicted better metabolic control, consistent with other metabolic conditions (Miller and DiMatteo 2013). Such increased family support may negotiate the challenges of dietary adherence, for example, through greater assistance with meeting the child's dietary needs and making necessary adjustments. Finally, socioeconomic factors were not associated with parental wellbeing or treatment adherence.

Limitations

In the present study, it was identified that using proportion of required blood samples submitted had limitations as an estimate of treatment adherence, because it was not possible to obtain the number of additional samples requested. The correlation between the proportion of submitted samples and the proportion of phe concentrations in target range ($r = -0.43$) indicated that more samples were requested when metabolic control was poor, suggesting that using the proportion of blood samples as a measure of treatment adherence should be treated with caution. However, exploratory t-tests were carried out to examine differences between children with 100% blood samples submitted and children with less than 100%, with no significant differences for any caregiver or demographic variable. Other limitations include the cross-sectional design of the study and the lack of an appropriate control group, whilst acknowledging the difficulties involved in identifying the latter.

Implications for Clinical Practice

The current findings highlight consideration of the psychological impact of parenting a child with PKU and the need for appropriate support to caregivers, such as referral to appropriate psychological services or support groups (Awiszus and Unger 1990). With regards to treatment adherence, interventions to promote dietary compliance may be particularly important for older children, as metabolic control decreases with age. In addition, interventions such as family therapy, involving communication training and cognitive restructuring to promote metabolic control (Wysocki et al. 2006), may be required.

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Synopsis

This study identified that for parents of children with phenylketonuria, parental psychological wellbeing was predicted by parenting stress and resilience, whereas treatment adherence was predicted by child age and perceived support from family.

Compliance with Ethics Guidelines

Conflict of Interest

Emma Medford, Dougal Hare, Katie Carpenter, Stewart Rust, Simon Jones, and Anja Wittkowski declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all participants for being included in the study.

Details of the Contributions of Individual Authors

Emma Medford contributed to identifying the research question, designing and planning the research, all aspects of the method section, and the majority of the writing of the article. She was the main researchers on this project.

Dougal Hare contributed to identifying the research question, designing and planning the research, and writing the article.

Katie Carpenter contributed to identifying the research question, deciding on the scope of the research and data collection.

Stewart Rust contributed to identifying the research question and designing and planning the research.

Simon Jones contributed to identifying the research question and designing and planning the research.

Anja Wittkowski contributed to the statistical analysis and writing the article. She oversaw the research process and the writing of the final manuscript.

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RESEARCH REPORT

Systematic Review and Meta-analysis of Intelligence Quotient in Early-Treated Individuals with Classical Galactosemia

Lindsey Welling • Susan E. Waisbren • Kevin M. Antshel • Hugh-Owen Colhoun • Matthias Gautschi • Stephanie Grünewald • Rebecca Holman • Johanna H. van der Lee • Eileen P. Treacy • Annet M. Bosch

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Abstract *Introduction*: Cognitive impairment is a well-known complication of classical galactosemia (CG). Differences in patient characteristics and test methods have hampered final conclusions regarding the extent of intellectual disabilities in CG. The primary aim of this systematic review was to assess intellectual performance in early-treated (≤4 weeks of life) individuals with confirmed CG (defined by absent or barely detectable GALT enzyme activity and/or the presence of two null or severe missense variations), assessed with comparable test instruments. The full-scale IQ (FSIQ) was the variable of interest.

Methods: A clinical librarian developed search strategies, and two independent investigators performed the study selection, risk of bias assessment and data extraction. Individual patient data were pooled for meta-analysis using linear mixed-effect models with a random intercept per study and including covariates (age or gender) as fixed effects where appropriate.

Results: Four articles were included in this meta-analysis. Data of 87 individuals (median age 13 years, range 3–38 years) were used to assess mean FSIQ in CG. The FSIQ ranged from 47 to 122, and the mean score was 87 (95% CI, 81–94). Forty-five percent of individuals attained scores <85, almost 40% attained scores of 85–100, and a minority (15%) attained scores above 100. There was no significant correlation between FSIQ and age.

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L. Welling · A.M. Bosch (✉)
Department of Pediatrics, Division of Metabolic Disorders, Academic Medical Center, University Hospital of Amsterdam, Amsterdam, The Netherlands
e-mail: lindsey.welling@amc.uva.nl; a.m.bosch@amc.uva.nl

S.E. Waisbren
Division of Genetics and Genomics, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA
e-mail: susan.waisbren@childrens.harvard.edu

K.M. Antshel
Department of Psychology, Syracuse University, Syracuse, USA
e-mail: kmantshe@syr.edu

H.-O. Colhoun
Department of Paediatrics, Trinity College, Dublin, Ireland
e-mail: colhounh@tcd.ie

M. Gautschi
University Children's Hospital, Pediatric Endocrinology, Diabetes and Metabolism, and Institute of Clinical Chemistry, Inselspital, University of Bern, Bern, Switzerland
e-mail: matthias.gautschi@insel.ch

S. Grünewald
Metabolic Unit, Great Ormond Street Hospital and Institute of Child Health, University College London, NHS Trust, London, UK
e-mail: Stephanie.grunewald@gosh.nhs.uk

R. Holman
Clinical Research Unit, Academic Medical Center, Amsterdam, The Netherlands
e-mail: r.holman@amc.uva.nl

J.H. van der Lee
Pediatric Clinical Research Office, Emma Children's Hospital, Academic Medical Center, Amsterdam, The Netherlands
e-mail: j.h.vanderlee@amc.uva.nl

E.P. Treacy
National Centre for Inherited Metabolic Disorders, Temple St. Children's University Hospital and Mater Misericordiae University Hospital, Dublin, Ireland
e-mail: etreacy@mater.ie

Conclusions: Results from this meta-analysis fortify conclusions from previous studies that early-treated individuals with CG are at risk for having impaired cognitive abilities. However, IQ varies considerably between affected individuals.

Introduction

Classical galactosemia (CG, MIM 230400) is an inborn error of galactose metabolism, caused by a severe deficiency of the enzyme galactose-1-phosphate uridyltransferase (GALT; EC 2.7.7.12). The incidence in Europe and the United States has been estimated for several countries and varies from 1 case per 19,000 to 1 case per 44,000 individuals (Bosch 2006; Ounap et al. 2010; Waisbren et al. 2012; Coss et al. 2013). When exposed to galactose from breast milk or infant formula, affected newborn infants develop life-threatening symptoms that quickly resolve once a galactose-restricted diet is initiated. A very strict life-long galactose-restricted diet is the only available treatment since CG was first reported in more detail (Jones and Leak 1959). Despite good dietary adherence, individuals are at risk for developing long-term complications including speech and language impairment and impaired cognitive abilities (Bosch 2006). Evaluation of cognitive abilities, including measurement of intelligence, is therefore an important aspect of the follow-up of children and adults. Studies show mean IQ scores below average, however, with large interindividual variability (Donnell et al. 1961; Komrower and Lee 1970; Waggoner et al. 1990; Schweitzer et al. 1993; Kaufman et al. 1994, 1995; Hansen et al. 1996; Rasmussen et al. 1996; Badawi et al. 1996; Shield et al. 2000; Widhalm et al. 2002; Antshel et al. 2004; Doyle et al. 2010; Schadewaldt et al. 2010; Coss et al. 2013; Rubio-Agusti et al. 2013), with many patients (45–72%) scoring in the low to very low range (IQ <85) (Schweitzer et al. 1993; Rasmussen et al. 1996; Shield et al. 2000; Hoffmann et al. 2011). Start of treatment after the 8th week of life seems to increase the risk for decreased cognitive abilities (Waggoner et al. 1990; Schweitzer et al. 1993). A negative correlation between age and IQ in individuals is reported in cross-sectional studies (Komrower and Lee 1970; Waggoner et al. 1990; Doyle et al. 2010), but was not confirmed by longitudinal studies (Fishler et al. 1966; Waggoner et al. 1990; Manis et al. 1997; Schadewaldt et al. 2010). Drawing conclusions from the individual studies about the extent of cognitive impairment in CG remains controversial, because many studies did not (1) define the diagnosis of CG, possibly including individuals with a variant form of galactosemia who have a better outcome; (2) define inclusion criteria (patients may have been tested on indication [cognitive impairment]), resulting in sampling bias; and (3) report age at start of dietary treatment. Patient samples were frequently small, results of different instruments could not be compared, and study results were presented in variable manners. Because results may vary from one study to another, validity of a hypothesis cannot be based on the results of single studies. An individual patient data (IPD) meta-analysis systematically assesses the results of previous studies to derive conclusions about that body of research (Haidich 2010). It pools patient-level data from multiple studies in a statistically responsible manner, including individual patients according to predefined inclusion and exclusion criteria. A meta-analysis can increase power of the results and may include a more precise estimate of the outcome than any individual study contributing to the pooled analysis (The Cochrane Collaboration 2011).

The primary aim of this systematic review with meta-analysis was to assess cognitive abilities in a large group of individuals with a confirmed diagnosis of CG, who commenced dietary treatment before the age of 4 weeks and who were not tested on because of (suspected) cognitive impairment. The primary variable of interest was full-scale intelligence quotient (FSIQ), determined by comparable test instruments. Secondary aims were to assess a possible correlation between FSIQ and age and to evaluate performance IQ (PIQ) and verbal IQ (VIQ) scores in individuals with this disorder.

Methods

Eligibility Criteria

Inclusion and Exclusion Criteria

In the meta-analysis, these criteria apply to individual patients.

Patients Individuals of all ages with a confirmed diagnosis of CG, defined by a profound impairment of GALT enzyme activity (absent or barely detectable) and/or the presence of two null or severe missense variations. Individuals with reported residual GALT enzyme activity >1%, as well as individuals from the Traveller community, were excluded. To avoid any influence of later treatment on cognition, we decided to include only patients with age at diagnosis/start of dietary treatment ≤4 weeks of life.

Outcome Cognitive testing with any of the following test instruments yielding full/total IQ score: any version of the Wechsler Preschool and Primary Scale of Intelligence

(WPSSI), Wechsler Intelligence Scale for Children (WISC), Wechsler Adult Intelligence Scale (WAIS), the McCarthy Scales of Children's Abilities or Stanford–Binet (and any translated, validated versions of these test instruments).

Study Design Randomized controlled trials (RCT), non-RCT, cohort studies, case-control studies, cross-sectional studies and case series were included; case reports and (systematic) reviews were excluded. Only studies in which the majority of patients treated in a specific hospital participated were included, aiming to avoid bias caused by only testing patients because of (suspected) cognitive impairment. Studies published ≥1980 were included, as it was not considered feasible to contact authors of the articles published before 1980.

Characteristics Human studies, studies conducted in any year, and articles (including conference abstracts) written in English were included.

It is well known that the Irish Traveller community may have decreased access to full educational opportunities. Traveller pupils are reported to have lower levels of achievement and are more likely to be identified as having special educational needs than pupils from other ethnic groups in Ireland (Wilkin et al. 2010). It was not possible to correct for parental educational or socioeconomic status as they were not reported in any of the original studies. Therefore, to prevent bias caused by reduced access to schooling by members of the Traveller community, we excluded the FSIQ data reported in Coss et al. (2013) from this group.

Identifying Studies

A computerized search was conducted by a clinical librarian and one of the investigators (LW) in EMBASE, MEDLINE, PsycInfo and the Cochrane Library up to June 2015, who also hand-searched reference lists of included articles, conference abstracts and narrative and systematic reviews for additional relevant articles. Search strategies for each database are presented in Supplementary Data 1.

Study Selection Process

Two independent investigators (AMB, LW) performed the initial selection of potentially eligible articles, by screening titles of identified records. Thereafter, these investigators read abstracts (or the whole article in case an abstract was lacking), to make a more precise selection of potentially eligible articles. Of these studies, the full-text articles were read and selected based on predefined eligibility criteria. Study selection process for the meta-analysis of FSIQ (Fig. 1) and the study selection process of the analysis of PIQ and VIQ were performed separately. Based on the IPD, patients not meeting all inclusion criteria were excluded from the meta-analysis.

Data Collection Process: Individual Patient Data

Authors of all relevant articles were contacted via e-mail with the request to share IPD. Multiple attempts, contacting different authors, were undertaken to obtain IPD.

Data Items: Individual Patient Data

Variables collected at a participant level were sex, age at diagnosis and/or initiation of dietary treatment, age at testing, test instrument used and individual FSIQ. If applicable: if patients were from a Traveller community. If individuals had been tested multiple times, the most recent score was used in the analysis.

Individual Patient Data Integrity

All IPD received from the authors were checked for completeness. If data were incomplete or if data included a sample that differed from the original article, authors were asked to explain these differences. Additional data were incorporated in the dataset only if they met inclusion criteria used for the systematic review, thus preventing selection bias.

Risk of Bias Assessment in Individual Studies

The Scottish Intercollegiate Guidelines Network (SIGN) quality appraisal checklists to assess bias were used (for RCT, non-RCT, cohort study and case-control study designs). For cross-sectional studies and case series, no standardized critical appraisal checklists were available, and risk of bias assessment was performed by systematically judging the study population in terms of reliability of the diagnosis of CG. Two independent investigators performed risk of bias assessment (AMB, LW).

Specification of Outcomes and Effect Measures

The primary outcome investigated in this systematic review is cognitive ability, expressed as a FSIQ. Secondary outcomes are VIQ and PIQ.

Statistical Analysis

We present age in terms of median and range, with no correction for clustering within studies. We tested for homogeneity of variances of FSIQ across the studies using

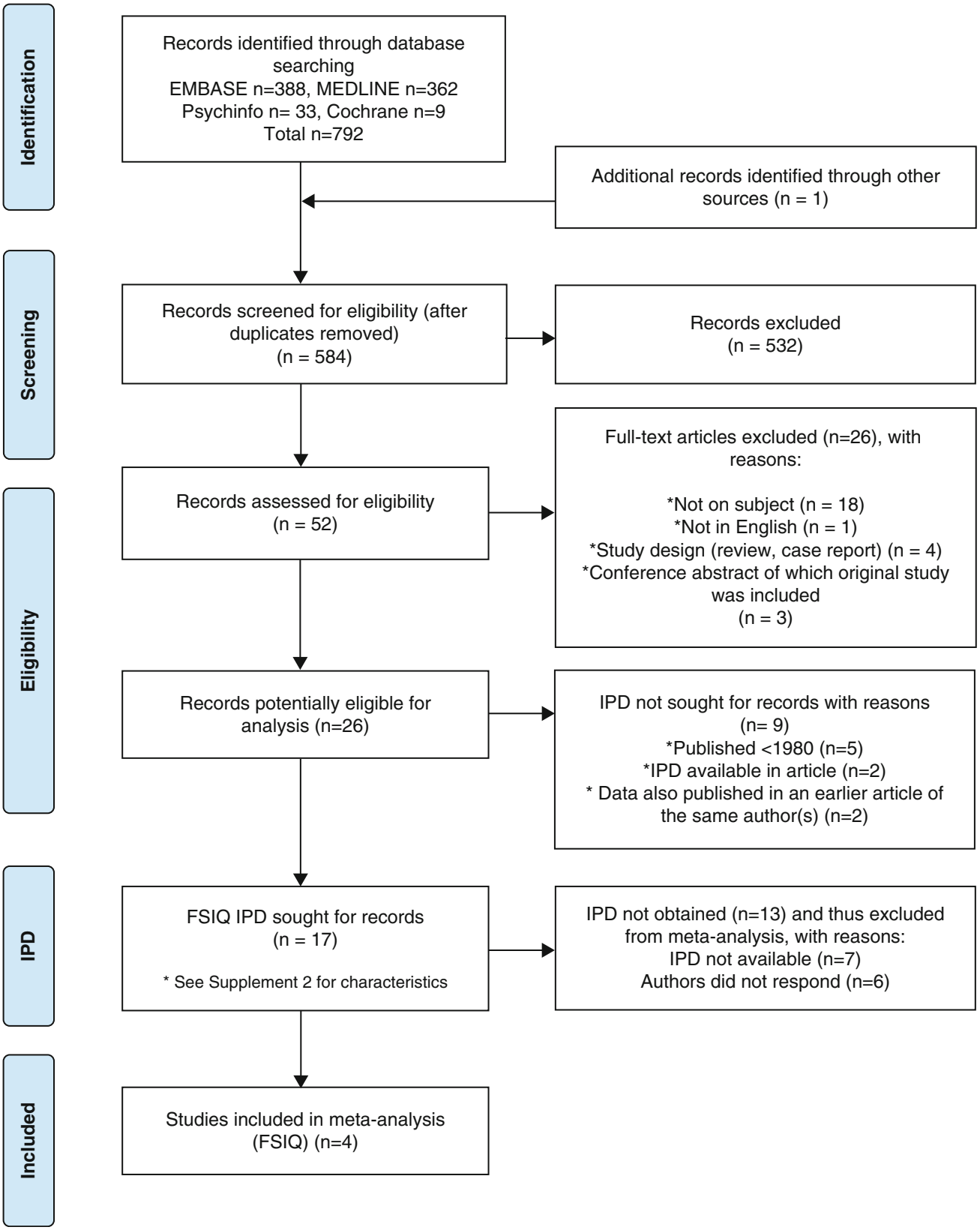

Fig. 1 Flow diagram of study selection process for the meta-analysis of full-scale IQ. *From*: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6 (6): e1000097. doi:10.1371/journal.pmed1000097

an univariate test for equal distributions (Rizzo and Székely 2010). We used linear mixed models (Zuur et al. 2009) with study as a random intercept, to obtain estimates of overall mean FSIQ with 95% confidence intervals (CI) for the means and *p*-values of differences between subgroups based on dichotomized age and sex. We used similar models to assess the association between FSIQ and age in years as a continuous variable. We considered *p*-values less than 0.05 statistically significant. We used R version 3.2.2015-12-04 (R Core Team 2014) and the packages lme4 (Bates et al. 2014) and energy (Rizzo and Székely 2014) to perform the meta-analysis and IBM SPSS Statistics version 22 for descriptive analyses.

Results

Study Selection Process

A total of 584 articles were identified by our search strategies (without doubles), of which 532 were discarded because they did not address our research question.

Meta-analysis

The study selection process for the meta-analysis is presented in Fig. 1. Of the 26 potentially eligible articles, IPD were sought for a total of 17 articles potentially eligible, and authors of these articles were contacted (see Supplementary Data 2 Data Retrieval, for characteristics of these studies). IPD were not sought for five articles published before 1980 (Donnell et al. 1961; Fishler et al. 1966, 1972; Komrower and Lee 1970; Lee 1972), for two articles in which IPD was already available (Hansen et al. 1996; Rasmussen et al. 1996) and for two articles that reported data also published in an earlier article of the same author(s) (Waggoner and Buist 1993; Hughes et al. 2009). Finally, IPD from four of these 17 articles were included in the meta-analysis (Waisbren et al. 1983, 2012; Antshel et al. 2004; Coss et al. 2013). Due to our inclusion and exclusion criteria, IPD of 87/199 individuals could be included in this meta-analysis. The first and/or last author of each article ascertained that the reported patients were not a selection of individuals tested because of (suspected) cognitive impairment, but were a good representation of the group of patients followed at that time.

Secondary Outcomes

No articles addressing PIQ and VIQ ($n = 5$) met all inclusion criteria, and therefore zero studies were included.

Risk of Bias Within Studies and Quality of Evidence

None of the selected studies had a study design for which a risk of bias checklist from SIGN was available.

Meta-analysis Full-Scale Intelligence Quotient

Four studies were included in our meta-analysis (Waisbren et al. 1983, 2012; Antshel et al. 2004; Coss et al. 2013), with relatively small patient sample sizes in the original studies (varying from 8 to 85 patients tested). Data of 87 individuals were pooled in the meta-analysis, 54 males and 33 females, with a median age of 13 years (range 3–38 years).

Antshel et al. assessed the neuropsychological profile (including FSIQ) in a cross-sectional study in 25 children and adolescents, homozygous for the c.563A>G (p.Gln188Arg) variation in the *GALT* gene (NM_000155.3), and tested with the WISC-III (Antshel et al. 2004). FSIQ scores of two originally tested patients (age 11 and 13 years) were not reported in the article (because the patients were excluded for further neuropsychological testing in that study), but FSIQ scores were provided for the meta-analysis. Therefore a total of 27 children from this study were included, 17 males and 10 females, median age 11 years (range 8–14 years).

In a retrospective study, Coss et al. assessed long-term outcome in 130 individuals (Coss et al. 2013). A total of 85 individuals, aged 6–39 years, were administered IQ tests (32/63 Travellers and 53/67 non-Travellers). Tests used were the WPPSI-III (Third UK Edition) for children aged 2 years and 6 months to 7 years and 3 months, the WISC-IV generally used for children aged 6–16 years and the WAIS-IV for individuals aged 16 years onward. All Travellers were excluded for reasons described in the Methods section. Furthermore, we excluded six non-Traveller individuals who had been diagnosed >4 weeks of life and/or had a variant form of galactosemia. Nine non-Traveller individuals not included in the original article were tested under the same protocol after the study was finished, and one female patient (15 years, FSIQ 90) meeting the inclusion criteria was included in this meta-analysis. A total of 48 non-Traveller individuals were thus included in our meta-analysis, and their median age was 19 years (range 6–38 years). All but three of these 48 individuals were homozygous for the c.563A>G (p.Gln188Arg) variation, two individuals were heterozygous for the known pathologic c.563A>G (p.Gln188Arg)/c.997C>T (p.Arg333Trp), and one individual was heterozygous for c.563A>G (p.Gln188Arg)/c.598delC (p.Gln200Serfs).

Waisbren et al. (1983) reported IQ scores in eight individuals, two males and six females, in a cross-sectional study (Waisbren et al. 1983). This was the complete group of children, identified at an early age (by NBS) and followed in their hospital at that time. Tests used were the McCarthy Scales of Children's Abilities, the Stanford–Binet (ages 3–6 years) and the WISC (ages 7 years and older). One patient was excluded because of a 5% residual GALT activity compared to virtually no residual activity in the other individuals. Thus, seven individuals (with a median age of 6 years, range 3–11 years) were included in our meta-analysis.

Waisbren et al. (2012) assessed the adult phenotype, including FSIQ, in a cross-sectional study including 33 individuals aged 18–59 years. All had absent GALT activity and two severe or null variations in the *GALT* gene and were administered the WAIS. Of these patients, only five could be included in this meta-analysis, because age at diagnosis was not known for the others. Their median age at testing was 20 years (range 18–23 years).

Full-Scale Intelligence Quotient in CG

The mean FSIQ in the four separate studies, calculated with the obtained IPD, were Antshel et al. (2004) mean 82 (SD 11.6), Coss et al. (2013) mean 83 (SD 18.0), Waisbren et al. (1983) mean 95 (SD 14.5) and Waisbren et al. (2012) mean 100 (SD 20.6). The univariate test of equal distributions with FSIQ data of all individuals included from the four studies indicated that the distribution of FSIQ scores across the four studies was not homogeneous ($p = 0.02$), justifying the use of linear mixed models to obtain mean FSIQ scores.

Following correction for the grouping of patients within studies, data from 87 individuals (median age 13 years, range 3–38 years) from these four studies revealed FSIQ range from 47 to 122 and a group mean of 87 (95% CI, 81–94). In all, 13 individuals (15%) attained scores lower than two standard deviations below the general population mean (FSIQ <70), 27 individuals (31%) performed between one and two standard deviations below the population mean (FSIQ 70–84), 34 individuals (39%) attained scores within one standard deviation below the population mean (FSIQ 85–100), and 13 individuals (15%) attained scores above 100.

Males and Females

The mean FSIQ score of 54 males (median age 13 years, range 3–35 years) was 92 (95% CI, 81–103). The mean FSIQ score of 33 females (median age 16 years, range 8–38 years) was 81 (95% CI, 77–86). The difference in mean FSIQ between males and females was not statistically significant (5.5 points, 95% CI −1.4 to 12.5, $p = 0.1241$). The estimate of the difference between mean FSIQ in males and females is different from the difference between the estimates for males and females, because of the correction for the difference between the studies in the meta-analysis.

FSIQ Score and Age

There was no statistically significant association between FSIQ and age (increase of 0.4 points/year, 95% CI −0.1 to 0.9, $p = 0.1146$).

Performance and Verbal IQ Scores in Early-Treated CG Individuals

No studies addressing PIQ and VIQ scores that met all our inclusion criteria were identified.

Discussion

In this systematic review and meta-analysis, we demonstrate, to our knowledge in the largest and most homogeneous sample with early-treated individuals with a confirmed diagnosis, that individuals with the classical form of galactosemia are at risk for cognitive impairment. The mean FSIQ in pooled data of 87 children and adults with CG was found to be 87, which is at the 19th percentile of the general population and substantially lower compared to the general population mean. Compared to the general population, a large percentage of individuals perform in the borderline to low average intellectual range (FSIQ 70–85) and in the range of intellectual disability (FSIQ <70). Slightly more individuals perform within one SD below the general population mean (FSIQ 85–100), and fewer individuals attained a FSIQ score above 100. The range of FSIQ in patients is remarkably wide. Thus, developmental and neuropsychological assessments are important in clinical follow-up to identify individuals in need of early intervention, special education or other supportive services. No statistical difference was found between mean FSIQ in males and females (5.5 points, 95% CI −1.4 to 12.5, $p = 0.1241$). However, the sample sizes were small, consequently the confidence interval was relatively wide, and therefore such a difference between males and females cannot be ruled out. Biological factors (hereditary traits) and environmental factors may influence cognitive development, such as perinatal factors, individual and parental educational opportunities and socioeconomic status. One very important factor was taken into account in this study, by excluding patients who started treatment after the first

month of life. Because the other environmental factors were not reported in the original studies, these could not be accounted for in this meta-analysis.

To date, no consensus exists regarding whether CG is a progressive disorder, with deteriorating cognitive abilities. This systematic review and meta-analysis provided no indication of deterioration of FSIQ over time. However, information about FSIQ after the fourth decade of life is lacking. Our finding contradicts results from other cross-sectional studies, which reported a correlation between age and cognition, and supports results from longtinal studies demonstrating that FSIQ does not correlate with age (Fishler et al. 1966; Waggoner et al. 1990; Manis et al. 1997; Schadewaldt et al. 2010). This difference may be explained by a cohort effect in these cross-sectional studies, whereby patients born before a certain date did not have access to the modern standard of care and diagnosis techniques. This may have resulted in later diagnosis and start of treatment in the elder patients in these studies, possibly negatively affecting cognitive outcome. In these studies, age at diagnosis and start of treatment was frequently not taken into account or not reported. This may falsely result in the impression that cognitive abilities regress over time. In our study, this effect was eliminated by applying strict inclusion criteria, with all patients being treated soon after birth. Furthermore, additional studies are needed to assess potential differences in VIQ and PIQ.

This study has several strengths and limitations. Strengths are the large number of patients included compared to previous studies assessing cognitive abilities and the homogeneous sample of early-diagnosed individuals with a confirmed diagnosis of CG, increasing power and precision of the results. Our results confirm and, importantly, fortify the conclusions of previously published studies, settling controversies arising from these previous studies. By only including studies that did not test patients because of (suspected) cognitive impairment, the risk of sampling bias was kept as small as possible. The results of this study are likely the most representative published to date. Limitations are the small patient sample sizes of the included studies, and in some cases not all patients reported in the original paper could be included in our review. We used mixed-effects models to estimate group mean FSIQ. Occasionally, these models may produce biased parameter estimates when the number of groups or individuals within groups is very small (Maas and Hox 2005). However, we feel that using random effects to account for the group structure is more appropriate than using fixed effects, as the patient selections may have been subtly and functionally different between studies, though studies that tested patients on indication were excluded (Borenstein et al. 2009). The small patient sample in Waisbren et al. (1983) is most likely the cause of the smaller range of FSIQ demonstrated in that group of patients and it is unlikely that this is caused by selection bias because all patients met our definition of CG. Since only cross-sectional data were used in this review and the majority of adult FSIQ scores were from one study, conclusions about FSIQ scores over time must be interpreted with caution.

Future research is needed to determine the factors that contribute to the broad variability in cognitive functioning in CG, predicting which patients are at risk for a low FSIQ (Coss et al. 2014). This could lead to interventions or modifications in treatment that may allow each individual to reach his/her full cognitive potential. Neuropsychological testing incorporated in regular clinical follow-up will identify infants and toddlers in need of early intervention and school-age children in need of special education or other supportive services.

Conclusions

Early-treated individuals with confirmed classical galactosemia, defined by a profound impairment of GALT enzyme activity (absent or barely detectable) and/or the presence of two null or severe missense variations, with age at diagnosis/start of dietary treatment $\leq$4 weeks of age, are at risk for having impaired cognitive abilities, with almost two-thirds of individuals performing more than a standard deviation below the normative mean of 100 (FSIQ range $\leq$85), a quarter performing in the low average to average range (FSIQ 85–100) and a minority (15%) attaining scores $\geq$100. These results confirm the wide range in cognitive functioning in individual patients. There is no indication that FSIQ deteriorates over time, as assessed in patients up to 38 years of age, but prospective longitudinal studies following FSIQ in individual patients, also after the third decade of life, are needed to confirm this impression.

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Take-Home Message

Early-treated individuals with classical galactosemia are at risk for having impaired cognitive abilities, but FSIQ scores vary considerably between affected individuals.

Contributions of Individual Authors

All authors participated in analysis and interpretation of data and drafting the article and revising it critically for important intellectual content. Annet M. Bosch, Johanna H. van der Lee, Susan E. Waisbren and Lindsey Welling

participated additionally in conception and design of the study.

Guarantor

Annet M. Bosch.

Conflict of Interest

Annet M. Bosch is in receipt of research grants from Nutricia and was a member of an advisory board for Nutricia, Merck Serono and BioMarin.

Kevin M. Antshel declares that he has no conflict of interest.

Hugh-Owen Colhoun declares that he has no conflict of interest.

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