Vol. 3

SSIEM

JIMD Reports

Case and Research Reports, 2011/3

Springer

JIMD Reports
Volume 3

SSIEM

JIMD Reports – Case and Research Reports, 2011/3

Springer

SSIEM

Editor
Society for the Study of Inborn
Errors of Metabolism
c/o ACB
Tooley St 130-132
SE1 2TU London
United Kingdom

ISSN 2192-8304 e-ISSN 2192-8312
ISBN 978-3-642-24935-8 e-ISBN 978-3-642-24936-5
DOI 10.1007/978-3-642-24936-5
Springer Heidelberg Dordrecht London New York

Contents

JIMD Reports
DOI 10.1007/8904_2011_70

CASE REPORT

Liver Failure with Coagulopathy, Hyperammonemia and Cyclic Vomiting in a Toddler Revealed to Have Combined Heterozygosity for Genes Involved with Ornithine Transcarbamylase Deficiency and Wilson Disease

Valerie Mira · Richard G. Boles

Received: 6 May 2010 / Revised: 8 June 2011 / Accepted: 10 June 2011 / Published online: 22 September 2011

Abstract A girl with a 2 month history of cyclic episodes of vomiting, diarrhea, and lethargy lasting 2–3 days each presented with acute hepatopathy (ALT 3,500 IU/L) with coagulopathy (PT 55 s) and hyperammonemia (207 μmol/L) at age 1½ years. Biochemical and molecular analyzes revealed ornithine transcarbamylase (OTC) deficiency. While laboratory signs of mild hepatocellular dysfunction are common in OTC deficiency, substantial liver failure with coagulopathy is generally not seen, although four others cases have been reported, three of which presented with cyclic vomiting. Further evaluation in our case revealed elevated urine (198.8 μg/g creatinine) and liver (103 μg/g dry weight) copper content, and a heterozygous mutation in the Wilson disease gene, *ATP7B*. Our patient, now aged 5 years, has remained in excellent health with normal growth and development on fasting avoidance, a modified vegan diet, and sodium phenylbutyrate.

Communicated by: Jean-Marie Saudubray.

Competing interests: None declared.

V. Mira · R.G. Boles (✉)
Division of Medical Genetics, Box 90, Children's Hospital Los Angeles, Los Angeles, CA 90027, USA
e-mail: rboles@chla.usc.edu

V. Mira
Department of Preventive Medicine, Keck School of Medicine at the University of Southern California, Los Angeles, CA, USA

R.G. Boles
Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, CA, USA

R.G. Boles
Department of Pediatrics, Keck School of Medicine at the University of Southern California, Los Angeles, CA, USA

These five cases demonstrate that generalized liver dysfunction/failure is a potential serious complication of OTC deficiency, although not a common one, and suggests that an ALT and PT should be obtained in OTC patients during episodes of hyperammonemia. Cyclic vomiting is a known presentation of OTC deficiency; it is not known if comorbid liver failure predisposes toward this phenotype. We propose that the heterozygote state in *ATP7B* increases the liver copper content, thus predisposing our patient with OTC deficiency to develop liver failure during a hyperammonemic episode. Our present case is an example of the opportunity of molecular diagnostics to identify putative modifier genes in patients with atypical presentations of genetic disorders.

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CMV	Cytomegalovirus
EBV	Epstein–Barr virus
HSV	Herpes simplex virus
INR	International normalized ratio
OTC	Ornithine transcarbamylase
PT	Plasma thromboplastin
PTT	Partial thromboplastin time

Introduction

Ornithine transcarbamylase (OTC) deficiency, an inborn error of the urea cycle inherited in an X-linked manner, is the most common inherited cause of hyperammonemia (Pridmore et al. 1995). While hemizygous males generally suffer from life-threatening early-onset disease, disease manifestations in heterozygous females with unfavorable

X-inactivation are generally milder and of later-onset, often presenting in the first few years. OTC deficiency in females is frequently under-diagnosed (Pridmore et al. 1995), yet potentially fatal. Clinical presentation generally is of acute, often episodic, altered mental status in an otherwise normal young girl, although cyclic vomiting can also occur (Li et al. 2008). Diagnosis is suspected based on high blood ammonia, high serum glutamine, low serum citrulline, and high urine orotate, and confirmed by sequencing the OTC gene. While laboratory signs of mild hepatocellular dysfunction is common in OTC deficiency, substantial liver failure with coagulopathy is not commonly seen, although a few cases have been reported (Mustafa and Clarke 2006; Zammarchi et al. 1996).

Case Report

We report a Hispanic female with normal growth and development who first presented at age 20 months with intermittent vomiting, diarrhea, and lethargy over the preceding 2 months. Episodes were stereotypical, commencing always at 22:30 in the evening, lasting for 2–3 days each, and separated by a few days before the next episode occurred. Symptoms resolved between episodes. On presentation, laboratory studies revealed acute liver failure with peak values of PT 55 s, PTT 68 s, INR 4.3, ALT 3,500 IU/L, AST 2,500 IU/L, and ammonia 207 μmol/L (normal 22–48). Hepatomegaly and jaundice were absent. All symptoms and laboratory anomalies resolved on supportive therapy.

Biochemical analysis 9 days later revealed high plasma glutamine (1,512 μmol/L), low plasma citrulline (17 μmol/L), and high urine orotate (60 mmol/mol creatinine, normal < 1), consistent with OTC deficiency. Genetic evaluation revealed she is a heterozygote for the OTC mutation c.602 T>C, with an amino acid change at L201P. This is a known mutation associated with neonatal-onset disease (Shimadzu et al. 1998). Due to elevations in liver copper (103 μg/g dry weight, normal 10–35) and urine copper (198.8 μg/g creatinine, normal 6.7–18.6), DNA testing was also performed on the *ATP7B* gene, whose dysfunction causes Wilson disease. Results revealed a heterozygote state for a mutation in exon 6, 1934 T>G, with an amino acid change of M645R. Kayser–Fleischer rings were absent. Many other potential causes of liver failure were absent on testing, including hepatitis A, B, and C, adenovirus, CMV, EBV, HSV, antinuclear antibody, alpha-1-antitrypsin, and acetaminophen and salicylate levels.

Shortly after first presentation, the patient was placed on a low-protein (modified vegan) and low-copper diet, fasting avoidance, and sodium phenylbutyrate as a nitrogen-conjugating agent. On this therapy, she has been asymptomatic up to the present age of 5 years, except for a single episode of vomiting during an upper respiratory infection, with ALT elevated at 529, but normal PT (12) and borderline-elevated ammonia (52). Growth and development have remained normal.

Molecular evaluation on the mother revealed carrier status for both the OTC and Wilson disease mutations, although she never had any symptomatology consistent with hyperammonemia or episodes of liver failure. She has always been on a varied diet, including meat and dairy, without protein aversion. Testing of the mother revealed an elevated urine orotate (39), with normal levels for blood glutamine (559), ammonia (< 9), and ceruloplasmin (35), and for urine copper (15.6) (units and normal ranges essentially as listed above). Unfortunately, X-inactivation studies on both mother and child were not interpretable, with two possibilities in each, one essentially normal (83:17 mom, 68:32 child), and one highly skewed (100:1 in both).

Discussion

A literature search revealed four cases with OTC deficiency presenting with acute liver dysfunction with coagulopathy, presenting at ages 3, 10, 14, and 44 months (Mustafa and Clarke 2006; Zammarchi et al. 1996). These cases demonstrate that generalized liver dysfunction/failure is a potential serious complication of OTC deficiency, although likely not a common one, and suggests that an ALT and PT should be obtained in OTC patients during episodes of hyperammonemia.

Wilson disease is an inborn error of copper transport in which reduced excretion of copper into bile leads to copper accumulation in many tissues. It generally presents with neurological, psychiatric, and/or hepatic disease. Liver dysfunction, including liver failure, is the presenting feature in over 80% of cases presenting in the first decade (Gollan and Gollan 1998). Heterozygous carriers for Wilson disease are not known to develop liver failure, although copper content of the liver can be elevated in some (Hoogenraad 1997). We propose that the heterozygote state in *ATP7B* increases the liver copper content, thus acting as a modifier gene by predisposing our patient with OTC deficiency to develop liver failure during a hyperammonemic episode. Finding a heterozygous mutation in the Wilson gene, or in another gene involved in copper metabolism, in other patients with OTC deficiency, and episodic acute liver failure would support our hypothesis. The four above-mentioned cases with OTC deficiency and liver failure reported normal plasma ceruloplasmin (Mustafa and Clarke 2006; Zammarchi et al. 1996), and blood copper was reported as normal in one of those cases (Zammarchi et al. 1996). However, blood copper was also normal in our

patient (105 mcg/dl, normal 76–193), and our patient's ceruloplasmin was only mildly low (16 mg/dl, normal 24–71), which is a common finding in infants with liver dysfunction of varied etiologies.

Our patient's mother is heterozygous for mutations in both genes but, unlike her daughter, the mother never presented with clinical disease. These differences parallel the differences in body fluid metabolite testing, in that the child had elevated blood glutamine and urine copper, but not the mother. The reasons are not clear, but may be due to differences in additional modifying genes and/or X-inactivation proportions for the OTC gene (lyonization). Unfortunately, we were unable to test the latter possibility.

Cyclic vomiting refers to repetitive stereotypical episodes of vomiting separated by intervals without vomiting. While most cases meet criteria for cyclic vomiting syndrome (Li et al. 2008), a condition associated with combinations of specific mtDNA polymorphisms (Zaki et al. 2009), another definable etiology is identified in a substantial minority of cases with a cyclic vomiting pattern. Among the etiologies that should be considered in cases with cyclic vomiting is deficiency of the intra-mitochondrial enzyme OTC (Li et al. 2008). In the four above-mentioned cases with OTC deficiency and liver failure, vomiting was present in three cases that were described as "intermittent" (Mustafa and Clarke 2006), in "recurrent episodes" (Zammarchi et al. 1996), and "persistent" (Zammarchi et al. 1996). OTC can be distinguished from cyclic vomiting syndrome by the presence in the former of true altered mental status and/or aversion to high-protein foods (Hoogenraad 1997). We do not know if the presence of comorbid liver failure predisposes OTC-deficient girls to present with a cyclic vomiting presentation, but the limited number of case reports suggest this as a possibility. Another condition that presents as altered mental status, vomiting and hyperammonemic liver failure is Reye syndrome. However, the presentation associated with that historical term is now known to include cases with many clinically definable metabolic disorders, including urea cycle defects and mitochondrial disorders. Interestingly, liver dysfunction is the most prominent effect of copper toxicity, and this toxicity is thought to at least in part involve mitochondrial dysfunction (Mehta et al. 2006).

Our present case is an example of the opportunity of molecular diagnostics to identify putative modifier genes in patients with atypical presentations of genetic disorders.

Acknowledgment X-inactivation studies were kindly performed by the Greenwood Genetics Center.

One Sentence Take-Home Message

Liver failure is an occasional complication of partial OTC deficiency; potential associations may include cyclic vomiting and/or heterozygosity for the Wilson gene.

References

Gollan JL, Gollan TJ (1998) Wilson disease in 1998: genetic, diagnostic and therapeutic aspects. J Hepatol 28:28–36

Hoogenraad T (1997) Wilson's disease. In: Major problems in neurology. WB Saunders, London, p 209

Li BU, Lefevre F, Chelimsky GG et al (2008) NASPGHAN consensus statement on the diagnosis and management of cyclic vomiting syndrome. J Pediatr Gastroenterol Nutr 47:379–393

Mehta R, Templeton DM, O'brien PJ (2006) Mitochondrial involvement in genetically determined transition metal toxicity II. copper toxicity. Chem Biol Interact 163:77–85

Mustafa A, Clarke JTR (2006) Ornithine transcarbamylase deficiency presenting with acute liver failure. J Inherit Metab Dis 29:586

Pridmore CL, Clarke JT, Blaser S (1995) Ornithine transcarbamylase deficiency in females: an often overlooked cause of treatable encephalopathy. J Child Neurol 10:369–374

Shimadzu M, Matsumoto H, Matsuura T et al (1998) Ten novel mutations of the ornithine transcarbamylase (OTC) gene in OTC deficiency. Hum Mutat 1:S5–S7

Zaki EA, Freilinger T, Klopstock T et al (2009) Two common mitochondrial DNA polymorphisms are highly associated with migraine headache and cyclic vomiting syndrome. Cephalalgia 7:719–728

Zammarchi E, Donati MA, Filippi L, Resti M (1996) Cryptogenic hepatitis masking the diagnosis of ornithine transcarbamylase deficiency. J Pediatr Gastroenterol Nutr 22:380–383

JIMD Reports
DOI 10.1007/8904_2011_33

CASE REPORT

Large Mitochondrial DNA Deletion in an Infant with Addison Disease

Gloria P. Duran • A. Martinez-Aguayo • H. Poggi • M. Lagos • D. Gutierrez • P.R. Harris

Received: 18 October 2010 / Revised: 25 April 2011 / Accepted: 2 May 2011 / Published online: 22 September 2011

Abstract *Background*: Mitochondrial diseases are a group of disorders caused by mutations in nuclear DNA or mitochondrial DNA, usually involving multiple organ systems. Primary adrenal insufficiency due to mitochondrial disease is extremely infrequent and has been reported in association with mitochondrial DNA deletion syndromes such as Kearns–Sayre syndrome.

Aim: To report a 3-year-old boy with Addison disease, congenital glaucoma, chronic pancreatitis, and mitochondrial myopathy due to large mitochondrial DNA deletion.

Method: Molecular analysis of mitochondrial DNA samples obtained from peripheral blood, oral mucosa, and muscle tissue.

Results: A novel large mitochondrial DNA deletion of 7,372 bp was identified involving almost all genes on the big arch of mtDNA.

Conclusions: This case reaffirms the association of adrenal insufficiency and mitochondrial DNA deletions and presents new evidence that glaucoma is another manifestation of mitochondrial diseases. Due to the genetic and clinical heterogeneity of mitochondrial disorders, molecular analysis is crucial to confirm diagnosis and to allow accurate genetic counseling.

Communicated by: Carlo Dionisi-Vici.

Competing interests: None declared.

G.P. Duran (✉) · A. Martinez-Aguayo · P.R. Harris
Department of Pediatrics, School of Medicine, Pontificia Universidad Católica de Chile, Lira 85, 5 piso, ZIP code 833-0074, Santiago, Chile
e-mail: gduran@med.puc.cl

H. Poggi · M. Lagos · D. Gutierrez
Department of Clinical Laboratories, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

Introduction

Primary adrenal insufficiency, commonly known as Addison disease, is uncommon in the Western population and is estimated to affect 90–140 per 1 million people (Arlt and Allollo 2003). The spectrum of adrenal disorders differs in childhood and adult patients. Primary adrenal insufficiency in childhood and adolescence is due to abnormalities of gland development, gland responsiveness, and either to defects of steroid biosynthesis or target organ response.

Primary adrenal dysfunction associated with mitochondrial disease has been reported extremely infrequently (Boles et al. 1998) and is generally associated with mitochondrial DNA deletion syndromes such as Kearns–Sayre Syndrome (KSS).

Clinical features typical of mitochondrial diseases include ptosis, progressive external ophthalmoplegia (PEO), proximal myopathy and exercise intolerance, cardiomyopathy, sensorineural deafness, optic atrophy, pigmentary retinopathy, and diabetes mellitus. KSS has also been associated with a variety of endocrine disorders such as short stature (38%), gonadal dysfunction (20%), and diabetes mellitus (13%) (Harvey and Barnett 1992).

We report the case of a boy with Addison disease associated with congenital glaucoma, chronic pancreatitis, and mitochondrial myopathy due to a large mtDNA deletion.

Methods

Clinical Case

We present a 3-year-old male, who was the second child of healthy and nonconsanguineous white parents, born after

uneventful pregnancy and delivery. He was a 3.2 kg and 49 cm term newborn without a family history of hereditary diseases, and who showed normal growth and development.

At 2 years of age, he was admitted to the hospital for abdominal pain, vomiting, and lethargy of unknown etiology; during that episode, he also had hyponatremia, elevated blood lactate of 6.6 mmol (normal < 2.2 mmol), and normal abdominal ultrasound.

Three months later, he presented with acute bilateral photophobia. The ophthalmologic examination revealed megalocornea and increased intraocular pressure. Congenital glaucoma was diagnosed and the patient underwent trabeculectomy.

At the age of $2^{10/12}$ years, endocrinological examination showed relative short stature (−1.7 SD), increased pigmentation of skin and mucosa, and normal development of genitals for age without pubarche. These findings and the history of hyponatremia suggested adrenal insufficiency, which was confirmed by normal cortisol (7.6 μg/dL) but markedly elevated serum ACTH (911 pg/mL) levels. After the intravenous administration of 250 μg ACTH, the cortisol level was 7.9 μg/dL, 17OH-progesterone was 0.6 ng/dL, and testosterone was < 10 ng/dL. Initially, plasma renin activity (PRA) was in the normal range. Very long chain fatty acid and phytanic acid were normal, antibodies to the adrenal cortex were not detected and brain MRI was normal.

Hydrocortisone replacement therapy was introduced (10 mg/m^2/day) leading to normalization of ACTH and skin pigmentation. During follow-up, PRA increased to 12 ng/ml/h (normal 1.6–5.0 ng/ml/h), so Florinef ™ was added at a dose of 0.1 mg/day.

Abdominal ultrasound showed a calcified pancreas and parenchymal changes consistent with chronic pancreatitis, although the patient had no history of recurrent abdominal pain, anorexia, diarrhea, or cyclic vomiting, nor was there any history of abdominal trauma or family pancreatic disease. The abdomen was soft, and there was no hepatosplenomegaly. Liver function, amylase, lipase, and blood cell count were normal; antinuclear and anti-DNA antibodies were negative. Extensive absorption work-up was negative. The magnetic resonance cholangiopancreatography (MRCP) revealed diffuse pancreatic calcification and decreased pancreatic size with abnormal limits and fatty infiltration. In addition, the main pancreatic duct was diffusely dilated with multiple ductal filling defects, probably due to intraductal pancreatic stones. Based on this evidence, the patient was diagnosed with chronic pancreatitis, and he was started with pancreatic enzyme supplementation (Creon®).

The multisystem involvement, characterized by congenital glaucoma, chronic pancreatitis, and Addison disease, suggested a mitochondrial disease. Echocardiogram, electrocardiogram, and neurologic examination were normal, but muscle biopsy showed typical ragged-red fibers, confirming the diagnosis of mitochondrial disease.

Molecular Studies

DNA was extracted from peripheral blood lymphocytes, oral mucosa cells, and muscle tissue by a commercial method (QIAgen). Mitochondrial DNA deletion analysis was performed by amplifying the entire mitochondrial genome (16,569 bp) in a single reaction, as well as seven smaller fragments in separate reactions, thus encompassing the big arc of the mitochondrial genome. For whole-genome analysis, the D1A and D1B primers were used (Kleinle et al. 1997). For the smaller fragments, we used the following primers: mtF5317 (5317–5333), mtF7392 (7392–7410), mtF8196 (8196–8215), mtF11632 (11632–11651), mtR13832 (13832–13812), mtR15613 (15613–15594), and mtR16134 (16134–16115), as described by Ferlin et al. (1997). The primers were used according to the following combinations: mtF5317/mtR13832 (fragment 1), mtF5317/mtR16134 (fragment 2), mtF7392/mtR15613 (fragment 3), mtF8196/mtR13832 (fragment 4), mtF8196/mtR16134 (fragment 5), mtF8196/mtR15613 (fragment 6), and mtF11632/mtR16134 (fragment 7). All fragments were amplified by long PCR under the same conditions, using a long PCR enzyme mix (Fermentas Life Sciences) in a final volume of 25 μL, and the cycling program used by Kleinle et al. (1997). Five μl of the products were separated on a 0.8% agarose gel and visualized under UV. The deletion breakpoints were studied by sequencing the fragments encompassing the deletion using the amplification primers.

Results

Molecular Studies

Long PCR analysis of the whole mitochondrial genome and of mtDNA segments with the first and second primer pairs showed shorter amplification fragments than those obtained with the wild-type sample. The deletion in peripheral blood, oral mucosa, and muscle had a size of ~7 kb as estimated by gel analysis (Fig. 1). After sequencing fragment 1, the exact deletion size was determined to encompass 7,372 bp, including 16 genes and flanked by direct repeats. Deletion size, breakpoints, the repeat sequence, and the genes abrogated by the deletion are shown in Fig. 2.

Discussion

Mitochondrial disorders are often considered rare conditions seen only in children with severe neurological

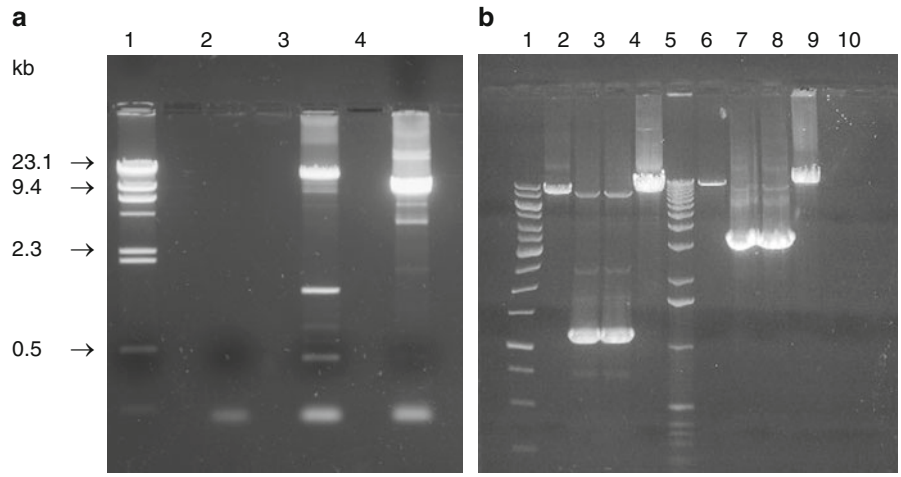


Fig. 1 (**a**): PCR products after whole mitochondrial genome amplification. Lane 1: molecular weight standard (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.5 kb), Lane 2: negative control, Lane 3: wild-type sample with a major fragment (16 kb), Lane 4: patient sample with a smaller fragment (9 kb). (**b**) PCR products after amplification of fragments 1 (lanes 2–5) and 2 (lanes 7–10). Lane 1 and 6: molecular weight standard (10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25 kb), Lanes 2, 5, 7 and 10: wild-type sample. Lanes 3, 4, 8 and 9: patient samples (blood and mucosal cells, respectively). All other amplicons (fragments 3, 4, 5, 6 and 7) from the patient had normal-sized bands

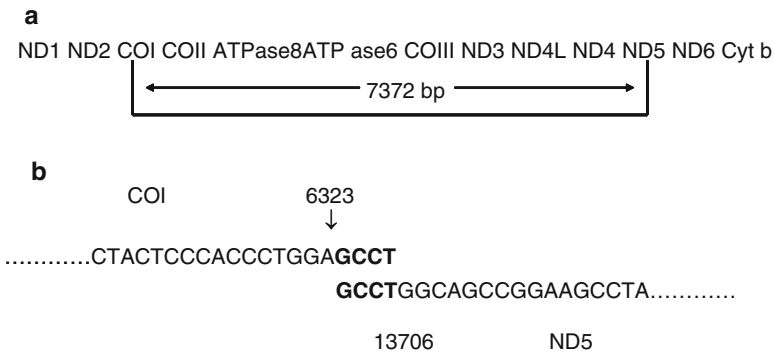


Fig. 2 (**a**): schematic of the deletion size and genes involved. (**b**): Nucleotide sequence and mitochondrial DNA position flanking the repeat region (repeat sequence in *bold*)

impairment and multisystem failure. Here, we describe a 3-year-old boy with Addison disease, congenital glaucoma, chronic pancreatitis, and mitochondrial myopathy, but without central nervous system involvement who harbored a single large mtDNA deletion.

Mitochondrial DNA deletion syndromes included three phenotypes: KSS (OMIM, # 530000), Pearson syndrome (OMIM, # 557000), and PEO. KSS is characterized by the triad of onset before age 20, PEO, pigmentary retinopathy, and at least one of the following features: heart block, ataxia, and cerebrospinal fluid (CSF) protein $>$ 100 mg/dl. Frequent additional signs include short stature, microcephaly, sensorineural hearing loss, cardiomyopathy, renal tubular acidosis and Fanconi syndrome, muscle weakness, basal ganglia calcifications, diffuse signal abnormality of central white matter, dementia, and sensory or motor neuropathy.

Pearson syndrome is characterized by refractory sideroblastic anemia and vacuolization of marrow precursors, as well as exocrine pancreatic dysfunction with malabsorption and pancreatic fibrosis.

Abnormalities of the adrenal axis have been found in patients with KSS. Boles et al. (1998) published a case with mtDNA deletion and Addison disease as a prominent clinical feature. Sanaker et al. (2007) reported a young woman who developed Addison disease, hypothyroidism, and glucose intolerance associated with thyroid peroxidase antibodies and adrenal 21-hydroxylase antibodies. Other endocrinopathies such as early-onset diabetes mellitus (Rötig et al. 1993), growth hormone deficiency (Gücüyener et al. 1998), and hypoparathyroidism (Cassandrini et al. 2006) have also been report in patients with single mtDNA deletions.

Congenital glaucoma was described in a child with KSS and has been reported in two adult patients as a rare

coincidence (Simaan et al. 1999). Normally, the ciliary muscles supplies some force to the trabecular meshwork, increasing the absorption of the aqueous humor. Thus, a decrease in outflow might be secondary to the ciliary mitochondrial myopathy, leading to reduction in aqueous humor absorption, increased ocular pressure, and glaucoma.

Acute or chronic pancreatitis associated with mitochondrial diseases is rare in childhood (Kishnani et al. 1996a). The incidence of pancreatic calcification varies from 19.9% to 83.1% in adults with chronic pancreatitis (Stobbe et al. 1970) and the relationship between calcification and exocrine pancreatic function is not linear. Pancreatic calcifications are virtually pathognomonic of chronic pancreatitis and develop in up to 90% of adult patients with alcoholic chronic pancreatitis (Ammann et al. 1988), but no information is available for younger patients. We could not document pancreatic insufficiency in this patient, but it is likely that the calcifications characterize more severe forms of chronic pancreatitis even in the early phases of the disease (Scuro et al. 1990).

Pancreatic dysfunction has been described in Pearson's disease associated with diabetes mellitus. There have been only a few reports of mitochondrial myopathy associated with pancreatitis (Kato et al. 1990; Toyono et al. 2001). Tsao et al. (2000) reported an infant with chemical pancreatitis due to mtDNA depletion. In addition, pancreatitis and pancreatic calcifications have been reported in patients with the m.3243A > G mutation in mitochondrial DNA, the "MELAS mutation" (Kishnani et al. 1996b; Schleiffer et al. 2000). In our patient, the abdominal ultrasound and MRCP suggested the diagnosis of chronic pancreatitis, although he was asymptomatic and had no steatorrhea. Fintesterer (2000) recommends that, when a mitochondrial disease is suspected, the history should always include queries on the presence or absence of pancreatitis (Finsterer 2007).

In this case, the clinical suspicion of a mitochondrial deletion was very strong. Using this methodological approach, we were able to describe a novel large mitochondrial DNA deletion involving almost all genes on the big arc, flanked by short direct repeats, as often described for mtDNA deletions (Kleinle et al. 1997; Yamashita et al. 2008). We demonstrated this large deletion of mitochondrial DNA in at least three different tissues of this patient. Given the clinical manifestations, we may assume that it is present in other cell types, but we could not prove it, as well as the heteroplasmic mutation level. The patient reported by Boles et al. (1998), who also presented with Addison disease, had a smaller deletion. On the other hand, there are cases with similar deletion sizes but without Addison disease, reinforcing the concept that genotype does not always or exactly correlate with phenotype (Yamashita et al. 2008). Due to the genetic and clinical heterogeneity of mitochondrial disorders, it is especially important to have access to molecular analysis to confirm diagnoses and to achieve more precise genetic counseling (Wong 2007).

Acknowledgments This work was supported by a grant from the Departamento de Laboratorios Clínicos de la Pontificia Universidad Católica de Chile.

References

Ammann RW, Muench R, Otto R, Buehler H, Freiburghaus AU, Siegenthaler W (1988) Evolution and regression of pancreatic calcification in chronic pancreatitis. A prospective long-term study of 107 patients. Gastroenterology 95:1018–1028

Arlt W, Allollo B (2003) Adrenal insufficiency. Lancet 361:1881–1893

Boles RG, Roe T, Senadheera D, Mahnovski V, Wong LJ (1998) Mitochondrial DNA deletion with Kearns Sayre syndrome in a child with Addison disease. Eur J Pediatr 157(8):643–647

Cassandrini D, Savasta S, Bozzola M, Tessa A, Pedemonte M, Assereto S, Stringara S, Minetti C, Santorelli FM, Bruno C (2006) Mitochondrial DNA deletion in a child with mitochondrial encephalomyopathy, growth hormone deficiency, and hypoparathyroidism. J Child Neurol 21:983–985

Ferlin T, Guironnet G, Barnoux MC, Dumoulin R, Stepien G, Mousson B (1997) Detection of mitochondrial DNA deletions by a screening procedure using the polymerase chain reaction. Mol Cell Biochem 174:221–225

Finsterer J (2000) 2007 Pancreatitis as a manifestation of mitochondrial disorder. Am J Med Genet A 143:632–633

Finsterer J (2007) Recurrent pancreatitis as a manifestation of multisystem mitochondrial disorder. Minerva Gastroenterol Dietol 53:285–289

Gücüyener K, Seyrantepe V, Topaloğlu H, Ozgüç (1998) Mitochondrial deletion in a boy with growth hormone deficiency mimicking cerebral palsy. J Inherit Metab Dis 21:173–174

Harvey JN, Barnett D (1992) Endocrine dysfunction in Kearns–Sayre syndrome. Clin Endocrinol (Oxf) 37(1):97–103

Kato S, Miyabayashi S, Ohi R, Nakagawa H, Abe J, Yamamoto K, Watanabe S, Tada K (1990) Chronic pancreatitis in muscular cytochrome c oxidase deficiency. J Pediatr Gastroenterol Nutr 11:549–552

Kleinle S, Wiesmann U, Superti-Furga A, Krähenbühl S, Boltshauser E, Reichen J, Liechti-Gallati S (1997) Detection and characterization of mitochondrial DNA rearrangements in Pearson and Kearns–-Sayre syndromes by long PCR. Hum Genet 100:643–650

Kishnani PS, Van Hove JLK, Shoffner JS, Kaufman A, Bossen EH, Kahler SG (1996) Acute pancreatitis in an infant with lactic acidosis and a mutation at nucleotide 3243 in the mitochondrial DNA tRNALeu(UUR) gene. Eur J Pediatr 155:898–903

Rötig A, Cormier V, Chatelain P, Francois R, Saudubray JM, Rustin P, Munnich A (1993) Deletion of mitochondrial DNA in a case of early-onset diabetes mellitus, optic atrophy and deafness (DIDMOAD, Wolfram syndrome). J Inherit Metab Dis 16:527–530

Sanaker PS, Husebye ES, Fondenes O, Bindoff LA (2007) Clinical evolution of Kearns–Sayre syndrome with polyendocrinopathy and respiratory failure. Acta Neurol Scand Suppl 187:64–67

Schleiffer T,'t Hart LM, Schürfeld C, Kraatz K, Riemann JF (2000) Maternally inherited diabetes and deafness (MIDD): unusual occult exocrine pancreatic manifestation in an affected German family. Exp Clin Endocrinol Diabetes 108:81–85

Simaan EM, Mikati MA, Touma EH, Rötig A (1999) Unusual presentation of Kearns–Sayre syndrome in early childhood. Pediatr Neurol 21:830–831

Stobbe KC, ReMine WH, Baggenstoss AH (1970) Pancreatic lithiasis. Surg Gynecol Obstet 131:1090–1099

Scuro LA, Cavallini G, Benini L, Brocco G, Bovo P, Riela A, Togni M, Cataudella G, Bassi C, Pederzoli P (1990) Pancreatic calcifications in patients with chronic pancreatitis. A sign of long-lasting or severe disease? Int J Pancreatol 6:139–150

Toyono M, Nakano K, Kiuchi M, Imai K, Suzuki H, Shishikura K, Osawa M, Shiratori K, Goto Y, Nonaka I, Sugie H (2001) A case of MERRF associated with chronic pancreatitis. Neuromuscul Disord 11:300–304

Tsao CY, Mendell JR, Luquette M, Dixon B, Morrow G 3rd (2000) Mitochondrial DNA depletion in children. J Child Neurol 15:822–824

Yamashita S, Nishino I, Nonaka I, Goto Y (2008) Genotype and phenotype analyses in 136 patients with single large-scale mitochondrial DNA deletions. J Hum Genet 53:598–606

Wong LJ (2007) Diagnostic challenges of mitochondrial DNA disorders. Mitochondrion 7:45–52

JIMD Reports
DOI 10.1007/8904_2011_35

CASE REPORT

Normal Levels of Plasma Free Carnitine and Acylcarnitines in Follow-Up Samples from a Presymptomatic Case of Carnitine Palmitoyl Transferase 1 (CPT1) Deficiency Detected Through Newborn Screening in Denmark

Luise Borch · Allan Meldgaard Lund · Flemming Wibrand · Ernst Christensen · Charlotte Søndergaard · Birthe Gahrn · David Michael Hougaard · Brage Storstein Andresen · Niels Gregersen · Rikke Katrine Jentoft Olsen

Received: 30 November 2010 / Revised: 19 May 2011 / Accepted: 23 May 2011 / Published online: 22 September 2011

Abstract Carnitine palmitoyl transferase (CPT) 1 A deficiency is a rare disorder of hepatic long-chain fatty acid oxidation. CPT1 deficiency is included in newborn screening programs in a number of countries to allow presymptomatic detection and early treatment of affected patients.

We present a case of presymptomatic CPT1A deficiency detected through newborn screening in Denmark with diagnostic levels of carnitine and acylcarnitines in the initial dried blood spot. Levels of plasma-free carnitine and acylcarnitines in follow-up samples were normal, but reverted to diagnostic levels when the patient developed clinical symptoms at the age of 8 months. At that time, a diagnosis of CPT1A deficiency was confirmed by sequence analysis of the *CPT1A* gene revealing homozygosity for a novel c.167C>T variation in exon 3. Enzyme activity measurements showed a relatively mild enzyme defect with a decreased residual enzyme activity of 17–25%. We conclude that *CPT1A* gene testing and/or enzyme assay is mandatory to confirm an abnormal newborn screen suggesting CPT1A deficiency to avoid delayed diagnoses.

Communicated by: Bridget Wilcken.

Competing interests None declared.

L. Borch (✉)
Paediatric Department, Aarhus University Hospital Skejby, Aarhus, Denmark
e-mail: lborch@live.dk

A.M. Lund · F. Wibrand · E. Christensen
Department of Clinical Genetics, Rigshospitalet, Copenhagen, Denmark

C. Søndergaard
Paediatric Department, Regionshospitalet Herning Hospital, Herning, Denmark

B. Gahrn · N. Gregersen · R.K.J. Olsen
Research Unit for Molecular Medicine, Aarhus University Hospital and Faculty of Health Sciences, Aarhus, Denmark

D.M. Hougaard
Department of Clinical Biochemistry and Immunology, Statens Serum Institut, Copenhagen, Denmark

B.S. Andresen
Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Introduction

Carnitine palmitoyl transferase (CPT) 1A deficiency (MIM ID #255120) is a disorder of long-chain fatty acid beta-oxidation. It is a rare disorder with only approximately 30 cases in the published literature (Bennett 2010). CPT1A deficiency is inherited in an autosomal recessive manner.

The CPT1 enzyme exists in three tissue specific isoforms: the liver isoform (CPT1A), the muscle isoform (CPT1B), and the brain isoform (CPT1C). The three isoforms are encoded by three different genes. CPT1A deficiency is caused by mutations in the *CPT1A* gene, which is localized on the long arm of chromosome 11(11q13.1–q13.2), and codes for the liver enzyme carnitine palmitoyl transferase 1A (CPT1A) (EC 2.3.1.21). CPT1A is localized in the outer mitochondrial membrane and is mainly expressed in liver, kidney, leukocytes, and fibroblasts. The enzyme converts the long-chain fatty acyl-CoA molecules to their respective acylcarnitine molecules, which are then transported across the

inner mitochondrial membrane via the carnitine acylcarnitine translocase. In the mitochondria, they undergo fatty acid beta-oxidation (Bonnefont et al. 2004).

Mitochondrial fatty acid oxidation by the liver provides an alternative source of energy when glycogen reserves are depleted by the increased energy demand during fasting or during a concurrent illness. The clinical symptoms are caused by the reduced ability to use long-chain fatty acids for ketogenesis.

The patients present with hepatomegaly and hypoketotic hypoglycaemia, which can cause seizures and coma (Bennett 2010).

To prevent symptoms, the long-term treatment is to prevent hypoglycaemia. This is accomplished by providing a constant supply of a low-fat, high-carbohydrate diet, and a supply of medium-chain fatty acids. Fasting periods should be kept at a minimum. During acute episodes when the child is unwilling to eat or during surgery, intravenous 10% glucose should be given (Bonnefont et al. 2004).

Because prevention of hypoglycaemia reduces the risk of neurologic damage, it is important to diagnose these patients early. CPT1A deficiency has been included in newborn screening programs world-wide using tandem mass spectrometry to detect abnormal and diagnostic carnitine to long-chain acylcarnitine ratios in dried whole blood spots (Fingerhut et al. 2001; Sim et al. 2001).

Here, we report a newborn diagnosed with CPT1A deficiency through newborn screening in Denmark. The initial newborn screen and a repeat blood spot was consistent with CPT1A deficiency but a plasma sample taken when the patient was stable came out normal reflecting that genetic testing and/or enzyme assay is mandatory to avoid delayed or false-negative diagnoses.

Clinical Case History

Patient M.G is a girl of Turkish descent and she is the second child of consanguineous parents (3rd–4th degree cousins). After a normal pregnancy, she was delivered by caesarean section because of prolonged labour. Her gestational age was 38 + 0, Apgar score 10/1, 10/5, 10/10, birth weight: 3,880 g. She was admitted to the neonatal unit age 3 days under suspicion of a bacterial infection because of respiratory problems, and increased C-reactive protein 50 mg/L (0.0–5.0 mg/L). Further laboratory findings in her blood were normal.

On admission, she suffered from tachypnoea, had very loose stools and a fever of 38.7°C. A white blood cell count was normal and blood cultures were negative. Chest X-ray was normal. The patient was discharged after 24 h in good condition.

When the girl was 16 days old, a newborn screening sample obtained at 7 days of age showed increased blood free carnitine 133 (ref <66.9 μmol/L) and a slightly increased C0/(C16 + C18) ratio of 51 (ref <30). The abnormal results were confirmed in a new blood spot sample obtained when the girl was 20 days old, showing free carnitine of 75 (ref <66.9 μmol/L) and a C0/(C16 + C18) ratio of 68 (ref <30), which is consistent with CPT1A deficiency (Fingerhut et al. 2001; Sim et al. 2001). For further confirmation of the diagnosis, a blood sample for plasma-free carnitine of 35 (ref 24–64 μmol/L) and a C0/(C16 + C18) ratio of 700 (ref <750) were done. These tests showed no signs of CPT1A deficiency. At this point, no *CPT1A* gene sequencing or CPT1 enzyme activity measurements were done. The patient was considered healthy and was discharged from the hospital. The lack of confirmatory diagnostic tests may have led to what later showed to be a premature conclusion.

At age 8 months, the girl was admitted to the paediatric department again after 2 days with diarrhoea and fever. She was severely hypotonic and lethargic, and had a heart murmur at the physical examination. Echocardiography was normal. She was diagnosed with an enteroviral infection.

Laboratory findings in her blood were: pH 7.4, base excess – 6.1 mmol/L (ref – 4.0–2.0 mmol/L), HCO_3 19.4 (ref 21.8–26.2 mmol/L), ammonium 74 (ref <48 μmol/L), lactate 2.7 mmol/L (ref 0.5–2.2 mmol/L), thrombocytes 55×10^9/L (ref $160–360 \times 10^9$/L), Hgb 5.8 mmol/L (ref 6.2–9.0 mmol/L), alkaline phosphatase 145 IU/L (ref 55–425 IU/L), alanine aminotransferase 30 IU/L (ref 5–45 IU/L), bilirubin 19 μmol/L (ref 4–17 μmol/L), plasma glucose 8 mmol/L (3.3–5.5 mmol/L). These blood samples were repeated a week later and were unchanged except for lactate and ammonium that had normalized.

A new determination of amino acids and organic acids in urine was normal. Also plasma amino acids were within the control range, but plasma-free carnitine was slightly increased (65 μmol/L, ref. range 24–64 μmol/L) with an increased C0/C16 + C18 ratio of 2,167 (ref. <750). CPT1A deficiency was reconsidered and confirmed by *CPT1A* gene sequencing and CPT1 enzyme activity measurements (see below).

At 12 months of age, the girl was tested by a child physiotherapist to evaluate whether the girl had suffered any neurologic damage due to her possible episodes of hypoketotic hypoglycaemia associated with CPT1A deficiency. The test showed slight motor retardation and hypotonia. At 13 months of age, the girl started to crawl and at 17 months of age she walked.

Molecular Genetic Findings

Sequence analysis of the *CPT1A* gene (Ensemble gene: ENST00000110090) revealed homozygosity for a novel

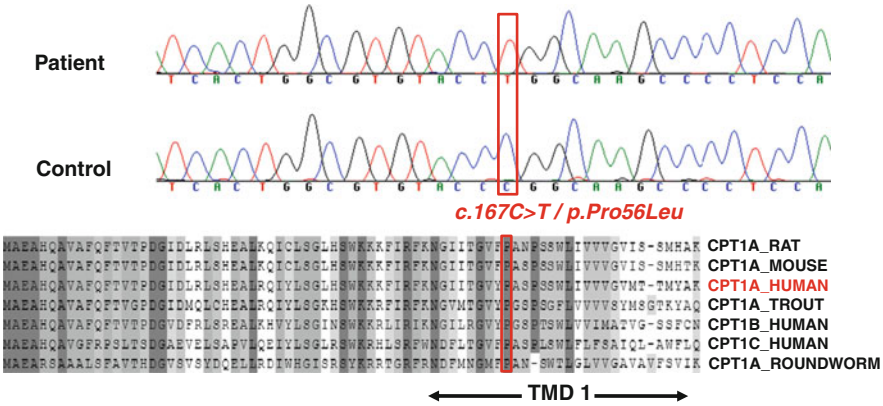


Fig. 1 The *CPT1A* c.167C>T variation. *Upper*: Sequence analysis of genomic DNA from the patient and a control. Shown is position c.152–181 of human *CPT1A*. *Lower*: Amino acid sequence (aa 1–76) alignment of human (*Homo sapiens*) CPT1A (P50416) with rat (*Rattus norvegicus*) CPT1A (P32198), mouse (*Mus musculus*) CPT1A (P97742), trout (*Oncorhynchus mykiss*) CPT1A (D9Z8Q8), roundworm (*Caenorhabditis elegans*) CPT1A (Q9U2F2), human (*Homo sapiens*) CPT1B (Q92523), and human (*Homo sapiens*) CPT1C (Q8TCG5) using protein sequences downloaded from the UniProt database (http://www.ebi.ac.uk/uniprot/index.html) and aligned using the ClustalW alignment tool (http://www.clustal.org/). Conserved residues are marked in *dark grey* and similar residues in *light grey*. Transmembrane domain 1 (TMD 1) is indicated with a *black arrow* and the c.167C>T/p.Pro56Leu change is highlighted in *red*

c.167C>T variation in exon 3. Both parents and a healthy brother with normal plasma carnitine levels were heterozygous for the c.167C>T variation. The variation is not found in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and also not in 114 Danish control alleles (data not shown). We therefore suggest that the c.167C>T variation is responsible for the abnormal biochemical findings observed in the patient. Most likely, the c.167C>T variation results in an abnormal and less active protein in which the highly conserved proline-56 is replaced by a leucine (p.Pro56Leu) (Fig. 1). In addition to the c.167C>T variation we found the patient to be homozygous for three intronic variations (c.556 −16C>T, c.1741 −28G>A and c.2142 +93C>T). These variations are most likely polymorphic and neutral since they are all present in the SNP database with allele frequencies that are more common than the prevalence of CPT1A deficiency.

CPT1 and CPT2 activity in skin fibroblasts was measured essentially as described (Bennett et al. 2004). CPT1 activity was determined as the malonyl-CoA inhibited fraction in the absence of detergent, and CPT2 activity was determined in the presence of malonyl-CoA and *n*-octyl-β-D-glucopyranoside. We found an average CPT1 activity of 0.18 nmol/min/mg protein [ref. (n = 14): 0.40–1.58, mean 0.86, median 0.72] and CPT2 activity of 0.33 (ref.: 0.16–0.57, mean 0.27, median 0.26), with a CPT1/CPT2 ratio of 0.56 (ref.: 1.9–5.1, mean 3.3, median 3.1). These values reflect a relatively mild CPT1A enzyme defect with a decreased residual enzyme activity of 17–25%.

Discussion

Since 2002, the Danish newborn screening program has included screening for disorders of fatty acid oxidation using tandem mass spectrometry, and more than 500.000 newborn children have been tested. Out of these children, one was diagnosed with CPT1A deficiency. According to results from newborn screening programs in Australia, Germany, and the USA the incidence of CPT1A deficiency may be as low as 1:750,000 to 1:2,000,000 (Lindner et al. 2010).

Patients with CPT1A deficiency do not present with symptoms until there is increased energy demands such as during fasting or febrile illness. During acute illness, typical laboratory findings are hypoketotic hypoglycaemia, elevated serum concentrations of liver transaminases, ammonium, and total carnitine (Bennett 2010).

Our patient had clinical symptoms during episodes of acute illness. She had increased plasma carnitine but high plasma glucose, and normal ketones and liver transaminases.

High plasma glucose levels suggest that the acute illness and the slight motor retardation when the patient was stable at age 12 months may have been due to the viral infection, but most likely the reduced CPT1A activity has predisposed to the illness.

She had the newborn screening done at age 7 days and had no symptoms. The test showed increased free carnitine and an increased C0/(C16 + C18) ratio as did a repeat sample at day 20 consistent with a diagnosis of CPT1A deficiency. However, follow-up samples of plasma-free carnitine, acylcarnitines, and of urine organic acids at age 20 days were within the control range. When the patient was 8 months old, acutely ill and laboratory tests once again gave suspicion of CTP1A deficiency with slightly increased plasma-free carnitine and an increased C0/C16 + C18 ratio, the diagnosis of CPT1A deficiency was confirmed by *CPT1A* gene sequence analysis and enzyme

activity measurements revealing homozygosity for a c.167C>T/p.Pro56Leu variation and reduced CPT1A enzyme activity (17–25% of normal controls).

The current case illustrates that also milder cases of CPT1A deficiency are detected through MS/MS-based newborn screening programmes, and that such cases can be difficult to diagnose due to the lack of clear-cut abnormalities on standard metabolic test, particularly during periods when the patient is anabolic and clinically stable.

The reliability of urine organic acids has been discussed. Usually, C12 dicarboxylic acids (Bennett et al. 2004; Brown et al. 2001; Stoler et al. 2004) and according to a single report also 3-OH glutaric acids are elevated in acute cases of CPT1A deficiency (Korman et al. 2005), but as seen in our patient and reported by others urine organic acids may be normal when the patient is well (Bennett 2010; Tsuburaya et al. 2010). Plasma C0/C16 + C18 ratios and to a lesser extent the level of free carnitine are, on the other hand, considered reliable diagnostic markers for CPT1A deficiency even when the patient is stable and biochemical markers of metabolic decompensation are within the control range (Fingerhut et al. 2001; Sim et al. 2001). However, in the current case the C0/C16 + C18 ratios were rather low (two to threefold increase) as compared to the values reported by Fingerhut and co-workers (6–60 fold increases) (Fingerhut et al. 2001) and the repeat C0 values were within the control range or only slightly increased (Fingerhut et al. 2001; Sim et al. 2001). The data most likely reflect the relatively mild enzyme defect in the current case (17–25% of normal controls) as compared to the severe enzyme deficiencies (<7% of normal controls) reported in previously published cases (Fingerhut et al. 2001; Sim et al. 2001).

Our case illustrates that diagnostic levels of plasma-free carnitine and acylcarnitines may depend on the metabolic state of the patient being abnormal only during acute crisis. Newborn screening programs, which allow early detection of metabolic markers in dried whole blood spots when the newborn is catabolic, are therefore very important. However, to avoid delayed or false-negative diagnoses, it is important to supplement biochemical confirmation of an abnormal newborn screen with analysis of the *CPT1A* gene and/or enzyme activity measurements. There is almost complete genetic heterogeneity of disease causing *CPT1A* variations with each affected family demonstrating novel variation(s) of *CPT1A* (Bennett et al. 2004). Therefore, analysis of the entire *CPT1A* gene is required to confirm an abnormal newborn screen and the disease-causing nature of the abnormal genotype needs to be carefully interpreted. The c.167C>T variation is not observed in DNA from healthy control individuals and the variation segregates with the disease in the family. The c.167C>T variation results in replacement of the evolutionary conserved proline-56 with leucine. Proline-56 is located in an α-helical hydrophobic segment (amino acids 48–75), which makes up one of two transmembrane domains that anchor the CPT1A enzyme into the outer mitochondrial membrane (Fraser et al. 1997). It is likely that p.Pro56Leu affects membrane insertion and stability of the variant CPT1A enzyme, which gives rise to the mildly decreased residual enzyme activity and impaired conversion of long-chain acyl-CoAs to their acylcarnitine derivates as reflected in the slightly increased C0/(C16 + C18) ratio in samples from the patient. Excluding the present all disease-causing variations identified to date are located in the C-terminal domain (amino acids 123–773) making up the catalytic core of the protein and most are associated with CPT1A enzyme activities between 0 and 10% and onset of hepatic encephalopathy in infancy or early childhood (Bennett et al. 2004; Brown et al. 2001; Fingerhut et al. 2001; Korman et al. 2005; Sim et al. 2001; Stoler et al. 2004). Exception is the enigmatic p.Pro479Leu Inuit variation, which has residual enzyme activity of 20% (Bennett et al. 2010) and the p.Gly719Asp variant, which was recently identified in a presymptomatic case of CPT1A detected through newborn screening in Japan and with residual activity of 11–26% of controls (Tsuburaya et al. 2010).

The case reported by Tsuburaya et al. and the current case illustrate very well the ethical considerations associated with any screening program for pre-symptomatic genetic diseases, where children with borderline biochemical tests and milder variants of uncertain clinical significance will be identified. Careful follow-up programs and evaluation of new genetic variants are required to establish biochemical cut-off values that allow a reasonable balance between the clinical benefits and the psychosocial risks of screening. Another essential component of neonatal screening for inherited diseases is to have a diagnostic algorithm for each disorder to be able to establish if it is a true or a false positive case. In the present case, the diagnosis relied entirely on the measurement of free carnitine in plasma and to a lesser extent on the ratio between free carnitine to long chain acylcarnitines (C0/C16 + C18). A finding of a carnitine value in plasma within the control range led to a delayed diagnosis. The correct diagnosis was first established when the patient later on developed symptoms that could be consistent with CPT1 deficiency. As a consequence, CPT1 deficiency was excluded from the Danish newborn screening program in 2008. After establishment of *CPT1A* gene analysis and CPT1A enzyme activity measurements, we are currently deliberating on whether to re-include screening for CPT1A deficiency in Denmark.

Acknowledgement We thank the patient and her family for their contribution. This work was supported by grants from the Danish Medical Research Council.

Synopsis

Findings of normal levels of plasma-free carnitine and acylcarnitines in follow-up samples from a presymptomatic case of carnitine palmitoyl transferase 1 (CPT1) deficiency detected through newborn screening in Denmark emphasize the importance of *CPT1A* gene testing and/or enzyme assay to avoid delayed diagnoses.

References

Bennett MJ, Boriack RL, Narayan S, Rutledge SL, Raff ML (2004) Novel mutations in CPT 1A define molecular heterogeneity of hepatic carnitine palmitoyltransferase I deficiency. Mol Genet Metab 82(1):59–63

Bennett MJ, Narayan SB, Santani AB (2010) Carnitine palmitoyltransferase 1A deficiency. In Pagon RA, Bird TD, Dolan CR, et al., editors. GeneReviews. Seattle (WA): University of Washington, Seattle; 1993-. Retrieved from: http://www.ncbi.nlm.nih.gov/books/NBK1527/.

Bonnefont JP, Djouadi F, Prip-Buus C, Gobin S, Munnich A, Bastin J (2004) Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. Mol Aspects Med 25(5–6): 495–520

Brown NF, Mullur RS, Subramanian I, Esser V, Bennett MJ, Saudubray JM, Feigenbaum AS, Kobari JA, Macleod PM, McGarry JD, Cohen JC (2001) Molecular characterization of L-CPT I deficiency in six patients: insights into function of the native enzyme. J Lipid Res 42(7):1134–1142

Fingerhut R, Röschinger W, Muntau AC, Dame T, Kreischer J, Arnecke R, Superti-Furga A, Troxler H, Liebl B, Olgemöller B, Roscher AA (2001) Hepatic carnitine palmitoyltransferase I deficiency: acylcarnitine profiles in blood spots are highly specific. Clin Chem 47(10):1763–1768

Fraser F, Corstorphine CG, Zammit VA (1997) Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane. Biochem J 1;323 (Pt 3):711-718

Korman SH, Waterham HR, Gutman A, Jakobs C, Wanders RJ (2005) Novel metabolic and molecular findings in hepatic carnitine palmitoyltransferase I deficiency. Mol Genet Metab 86(3): 337–343

Lindner M, Hoffmann GF, Matern D (2010) Newborn screening for disorders of fatty-acid oxidation: experience and recommendations from an expert meeting. J Inherit Metab Dis 33(5): 521–526

Sim KG, Wiley V, Carpenter K, Wilcken B (2001) Carnitine palmitoyltransferase I deficiency in neonate identified by dried blood spot free carnitine and acylcarnitine profile. J Inherit Metab Dis 24(1):51–59

Stoler JM, Sabry MA, Hanley C, Hoppel CL, Shih VE (2004) Successful long-term treatment of hepatic carnitine palmitoyltransferase I deficiency and a novel mutation. J Inherit Metab Dis 27(5):679–684

Tsuburaya R, Sakamoto O, Arai N, Kobayashi H, Hasegawa Y, Yamaguchi S, Shigematsu Y, Takayanagi M, Ohura T, Tsuchiya S (2010) Molecular analysis of apresymptomatic case of carnitine palmitoyl transferase I (CPT I) deficiency detected by tandem mass spectrometry newborn screening in Japan. Brain Dev 32(5): 409–411

JIMD Reports
DOI 10.1007/8904_2011_36

RESEARCH REPORT

Identification of Mutations and Evaluation of Cardiomyopathy in Turkish Patients with Primary Carnitine Deficiency

M. Kilic • R.K. Özgül • T. Coşkun • D. Yücel • M. Karaca • H.S. Sivri • A. Tokatli • M. Şahin • T. Karagöz • A. Dursun

Received: 12 December 2010 / Revised: 31 March 2011 / Accepted: 1 April 2011 / Published online: 22 September 2011

Abstract Primary systemic carnitine deficiency (SCD) is an autosomal recessive disorder caused by defective cellular carnitine transport. Patients usually present with predominant metabolic or cardiac manifestations. SCD is caused by mutations in the organic cation/carnitine transporter *OCTN2* (SLC22A5) gene. Mutation analysis of *SLC22A5* gene was carried out in eight Turkish patients from six families. Six patients presented with signs and symptoms of heart failure, cardiomyopathy, and low plasma carnitine levels, five of them with concurrent anemia. A patient with dilated cardiomyopathy had also facial dysmorphia, microcephaly, and developmental delay. Tandem MS analyses in siblings of the patients revealed two more cases with low plasma carnitine levels. SCD diagnosis was confirmed in these two cases by mutation screening. These two cases were asymptomatic but echocardiography revealed left ventricular dilatation in one of them. Carnitine treatment was started before the systemic signs and symptoms developed in these patients. Mean value of serum carnitine levels of the patients was 2.63 ± 1.92 μmol/L at the time of diagnosis. After 1 year of treatment, carnitine values increased to 16.62 ± 5.11 ($p < 0.001$) and all responded to carnitine supplementation clinically. Mutation screening of the *OCTN2* gene study in the patients revealed two novel (p.G411V, p.G152R), and four previously identified mutations (p.R254X, p.R282X, p.R289X, p.T337Pfs12X). Early recognition and carnitine supplementation can be lifesaving in this inborn error of fatty acid oxidation.

Communicated by: Ertan Mayatepek.

Competing interests: None declared.

M. Kilic (✉) · R.K. Özgül · T. Coşkun · D. Yücel · M. Karaca · H.S. Sivri · A. Tokatli · A. Dursun
Department of Pediatrics, Metabolism and Nutrition Unit, Hacettepe University, Ankara, Turkey
e-mail: kilickorkmaz@yahoo.com.tr

R.K. Özgül
Institute of Child Health, Hacettepe University, Ankara, Turkey

M. Karaca
Department of Biology, Aksaray University, Aksaray, Turkey

M. Şahin · T. Karagöz
Department of Pediatrics, Cardiology Unit, Hacettepe University, Ankara, Turkey

Abbreviations

BNP	Brain natriuretic peptide
DC	Dilated cardiomyopathy
EF	Ejection fraction
HC	Hypertrophic cardiomyopathy
OCTN2	Organic cation/carnitine transporter
SCD	Primary systemic carnitine deficiency
SLC22A5	Solute carrier family 22

Introduction

Primary systemic carnitine deficiency (SCD; MIM# 212140) is a potentially lethal, autosomal recessive disorder characterized by progressive cardiomyopathy, skeletal myopathy, hypoketotic hypoglycemia, and hyperammonemia

(Karpati et al. 1975; Mayatepek et al. 2000; Treem et al. 1988; Roe and Coates 1995). Although most of the fatty acid oxidation disorders affect heart and skeletal muscles and liver, cardiac failure is seen as the major presenting manifestation only in carnitine transporter deficiency (Stanley et al. 2006). The disease frequency is ranging from 1:40,000 to 1:120,000 newborns in different parts of the world and is possibly the second most frequent disorder of fatty oxidation after medium chain acyl CoA dehydrogenase deficiency (Koizumi et al. 1999; Wilcken et al. 2001, 2003). Studies in cultured fibroblasts from affected patients established that the primary defect in SCD involves the sodium-dependent high-affinity transporter situated in the plasmalemmal membrane (Tein et al. 1996; Treem et al. 1988). The same transporter is also involved in the renal reabsorption of carnitine, thus explaining the excessive renal waste of carnitine in SCD patients.

Primary carnitine deficiency is caused by mutations in *OCTN2* gene (SLC22A5), which encodes a plasma membrane carnitine transporter. *OCTN2*, transfers carnitine (3-hydroxy-4-trimethylaminobutyric acid) across cell membrane as Na+ dependent and other organic cations such as tetraethylammonium (TEA) as Na+− independent (Ohashi et al. 2001; Scaglia and Longo 1999; Scaglia et al. 1998, 1999). *OCTN2* gene is located on chromosome 5q31 with ten exons encoding a 557-amino acid transmembrane protein consisting of 12 transmembrane domains and one ATP-binding domain and predicted molecular mass of 63 kDa (Saito et al. 2002; Wu et al. 1999). More than 90 mutations have been identified up to date (Amat di San Filippo C et al. 2006a, b, 2008; Burwinkel et al. 1999; Cederbaum et al. 2002; Dobrowolski et al. 2005; Koizumi et al. 1999; Lamhonwah et al. 2002; Li et al. 2010; Makhseed et al. 2004; Mayatepek et al. 2000; Nezu et al. 1999; Rahbeeni et al. 2002; Spiekerkoetter et al. 2003; Tang et al. 1999; Vaz et al. 1999; Wang et al. 1999, 2000a, b, 2001).

In patients with SCD, the key to the diagnosis is the measurement of plasma carnitine levels that are extremely decreased (free carnitine <5 μM, controls 25–50 μM), and low renal uptake of carnitine (Cano et al. 2008; Scaglia et al. 1998; Treem et al. 1988). Diagnosis can be confirmed by demonstrating reduced carnitine transport in skin fibroblasts from the patient or by mutation analyses of the *SLC22A5* gene (Cano et al. 2008).

Since oral L-carnitine supplementation makes a significant improvement in clinical symptoms in patients, early diagnosis is of utmost importance. Therefore, molecular genetic analyses are essential and useful diagnostic tool for patients with asymptomatic SCD.

This study discusses the clinical and molecular features of patients who were being followed with a diagnosis of SCD.

Materials and Methods

Six patients presented with signs and symptoms of heart failure, cardiomyopathy and low plasma carnitine levels, and two siblings of the patients were enrolled in the study. The research protocol for genetic analysis was approved by the Hacettepe University, Ethical Board. Informed consent was obtained from all patients or their parents prior to genetic analyses. Genomic DNA was isolated from peripheral blood. Mutation screening of ten exons and their flanking intronic sequences of the *OCTN2* gene was performed in eight Turkish patients with SCD. PCRs were performed for 32 cycles, with each cycle consisting of denaturation at 95 °C for 4 min, annealing at 56–60 °C for 30 s, and extension at 72 °C for 30 s and then a final extension at 72 °C for 5 min. PCR products were purified with Qiagen MinElute 96 UF PCR purification plates. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit (version 3.1) and analyzed on an ABI3130 automated DNA sequencer (Applied Biosystems, CA). A novel nucleotide changes resulting in missense changes were also analyzed in 100 control chromosomes and computer-based algorithms, PolyPhen (http://genetics.bwh.harvard.edu/pph/) was used for the prediction of the pathogenicity of novel missense variants.

Results

Mutation screening of the *SLC22A5* gene was performed in eight Turkish patients from six unrelated families. Six patients presented with symptoms of heart failure, cardiomyopathy by echocardiography, and low plasma carnitine levels, five of them were diagnosed with concurrent anemia. A patient (patient 4) with dilated cardiomyopathy had also facial dysmorphia, microcephaly, and developmental delay. Tandem MS analyses in siblings of the patients revealed two more patients with a low plasma carnitine levels and diagnosis was confirmed in these patients by mutation screening (patient 6, 8). These two patients were asymptomatic but echocardiography revealed left ventricular dilatation in one of them (patient 6). Treatment was started before the systemic signs and symptoms developed in these patients. We did not see early onset hypoketotic hypoglycemic encephalopathy in our cases. All of the affected patients had later age of presentation with progressive cardiomyopathy. The median age of the patients was 91.5 (57–145) months. Median age at onset was 30 (1–96) months whereas age at diagnosis was 71 (9–125) months. M/F ratio was 3/5 and all had consanguinity. Six patients had dilated cardiomyopathy, one had hypertrophic cardiomyopathy, and one was normal. In five patients, brain natriuretic peptide (BNP) levels were

higher than 100 pg/ml and in six patients EF was detected lower than 65% before carnitine treatment. Mean value of serum carnitine levels of the patients was 2.63 ± 1.92 μmol/L (N: 10–60) at the time of diagnosis. After 1 year of carnitine treatment, carnitine plasma values increased to 16.62 ± 5.11 (N: 10–60) ($p < 0.001$) and all responded to carnitine supplementation clinically. In four of the cases, the urinary carnitine levels were found to be raised. With carnitine supplementation, six of the cases were found to have an increase in the urinary carnitine levels (Table 1).

Three different types of pathogenic mutations (two missense, three nonsense, and one deletion) were detected in *OCTN2* gene. Mutation analysis has harbored for two novel mutations including p.G411V, p.G152R and four known mutations p.R254X, p.R282X, p.R289X, p.T337Pfs12X (Table 1). Two novel missense mutations (p.G411V, p.G152R) were screened in 100 control chromosomes and not detected. The locations of the five non-synonymous variants in the predicted secondary structure of OCTN2 protein are shown in Fig. 1. Carnitine transporter activity was measured only in one case and carnitine uptake: 0.0 (controls: 1.05 ± 0.27) was found (patient 3).

Discussion

Primary carnitine deficiency is caused by defective activity of the organic cation/carnitine transporter *OCTN2*, resulting in urinary carnitine wasting, low serum carnitine levels, and decreased intracellular carnitine accumulation (Scaglia et al. 1998; Tamai et al. 1998; Wu et al. 1998). Carnitine is essential for the transfer of long-chain fatty acids from the cytosol to mitochondria for subsequent β-oxidation and lack of it impairs the ability to use fat as fuel during periods of fasting or stress. This can result in an acute metabolic decompensation, most often early in life, with hepatic encephalopathy, hypoketotic hypoglycemia, Reye syndrome, and sudden infant death, or in a more insidious presentation, which may be very early in childhood but is more often of later onset, with cardiomyopathy (Scaglia and Longo 1999; Longo et al. 2006).

Primary carnitine deficiency impairs the accumulation of carnitine within organs and tissues (Longo et al. 2006). In the heart, carnitine is essential for normal fatty acid β-oxidation and even partial deficiency could lead to organ dysfunction (Koizumi et al. 1999). In this study, six patients with the symptoms of heart failure, five had dilated cardiomyopathy, whereas one had hypertrophic cardiomyopathy. Our study showed that the incidence of dilated cardiomyopathy was higher than hypertrophic cardiomyopathy as has been indicated in the literature

Table 1 Clinical, biochemical, and molecular analysis of the patients with *OCTN2* gene defect

Patient no	Age (months)/ sex	Cardiomyopathy type	Age at onset/age at diagnosis (months)	Hemoglobin levels (before and after treatment) (gr/dl)	Plasma free carnitine levels (before and after treatment) (μmol/L)	Urine carnitine levels (before and after treatment) (μmol/l)	BNP levels (before and after treatment) (pg/ml)	EF % (before and after treatment)	[a]Nucleotide change	Protein effect	Consanguinity	Mental retardation (mild)
1	69/F	DC	54/59	7,4/11,7	1,36/10,17	941/535	986/50	37/69	c.1009delA	p.T337Pfs12X	+	–
2	57/M	HC	1/9	10,3/12,2	3,96/9,26	400/726	–/–	49/79	**c.1232G > T**	**p.G411V**	+	+
3	77/F	DC	6/10	10,9/13,8	1,19/19,89	–/849	175/33.5	46/85	c.844C > T	p.R282X	+	–
4	130/M	DC	15/119	11/14.6	2,22/24,81	223/223	134/60	34/72	c.865C > T	p.R289X	+	+
[b]5	145/F	DC	4/124	5,6/10,9	1,37/15,74	614/2820	340/12,6	22/67	**c.454G > C**	**p.G152R**	+	–
[b]6	101/F	DC	75/75	9,8/10,4	2,75/19,12	190/735	21,7/35	63/63	**c.454G > C**	**p.G152R**	+	–
[c]7	104/F	DC	24/89	10,8/12,7	1,45/16	31,7/1386	986/13,5	37/71	c.760C > T	p.R254X	+	–
[c]8	82/M	N	66/67	–/–	6,76/17,94	24,6/94	28,3/90	76/76	c.760C > T	p.R254X	+	+

DC dilated cardiomyopathy, *HC* hypertrophic cardiomyopathy, *N* normal, *BNP* brain natriuretic peptide, *EF%* ejection fraction

[a] All patients are homozygous for the detected mutations

[b,c] Sibling

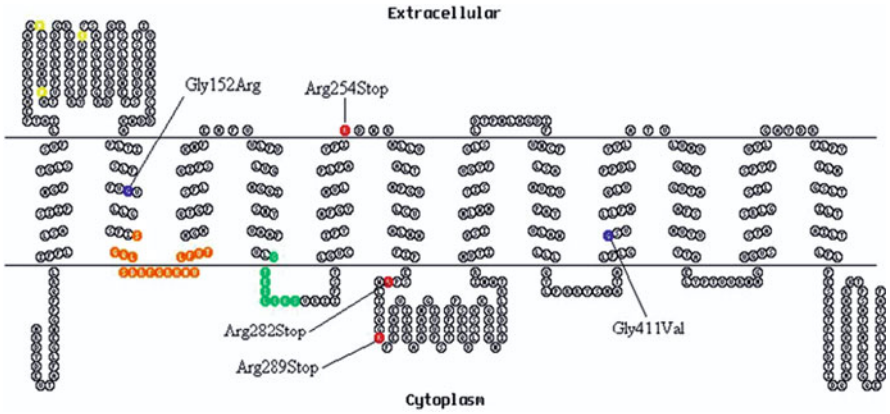


Fig. 1 Location of the five nonsynonymous variants in the predicted secondary structure of OCTN2 protein. The transmembrane topology diagram was rendered using TOPO2 transmembrane protein display software (http://www.sacs.ucsf.edu/TOPO/topo.html). Novel amino-acid substitutions are shown as *blue circles*, previously reported stop mutations that were also found in our patients are shown as *red circles*. Putative N-glycosylation sites are shown in *yellow*, ATP-binding motif is circled in *orange* and "sugar transporter sequence signature" is shown as *green circles*

(Longo et al. 2006; Wang et al. 2000a, b). In the literature, no correlation between the types of mutation and cardiomyopathy was found. In our case with a diagnosis of hypertrophic cardiomyopathy, the clinical findings were observed earlier (patient 2). A new homozygous mutation of c.1232G > T was identified in this patient. As in other previous studies, no genotype–phenotype relationship was observed. (Garavaglia et al. 1991; Lamhonwah et al. 2002; Li et al. 2010; Longo et al. 2006; Stanley et al. 1991; Wang et al. 2000a, b, 2001). Even siblings with the same mutation have different ages of onset and different progressions of disease pointing to the presence of clinical heterogeneity (Table 1). It was observed that the sibling with minor clinical symptoms had higher plasma levels of carnitine. This therefore suggests that the wide variability in phenotypic expression in carnitine transporter defect is most likely related to exogenous stressors that exacerbate the carnitine deficiency. These could include decreased intake due to dietary carnitine deficiency (vegetarian diets), drugs that increase the elimination of carnitine (valproic acid, pivalic acid) or inhibitors of carnitine transport (verapamil, pyrilamine, β-lactam antibiotics), and conditions such as fasting or infection, which would increase the demands on carnitine-dependent fatty acid oxidation (Holme et al. 1989; Lamhonwah et al. 2002; Li et al. 2010; Spiekerkoetter et al. 2003; Tein et al. 1993; Toh et al. 2010). Lack of genotype/phenotype correlation could also be influenced by polygenic factors.

Since newborn screening with tandem mass spectrometry is not a common practise in our country, the cases are usually diagnosed late. The median (min–max) age of disease onset findings was 30 (1–96) months, and age at diagnosis is 71 (9–125) months. The cases presented with cardinal symptoms of malaise, easy fatigability, and anorexia along with findings of heart failure and anemia. Physical examination revealed tachycardia, tachypnea, and palor. Telecardiography revealed increased cardiothoracic ratio and by echocardiography five dilated and one hypertrophic type of cardiomyopathy were detected. The patient's siblings were screened by tandem mass spectrometry revealed two asymptomatic cases and echocardiography revealed one case of cardiomyopathy (patient 6). The echocardiography of other case was normal (patient 8). This case's serum carnitine level was higher than other cases (6.76 μmol/L). Identification of mutations in siblings is critical because of the progressive and lethal nature of this disorder and the high incidence of sudden unexpected infant deaths unless there is early diagnosis and prompt therapeutic intervention (Stanley et al. 1991; Tein et al. 1990). Free carnitine levels in the plasma before initiation of therapy were 2.63 ± 1.92 μmol/L (N: 10–60), after a year of therapy free carnitine levels in the plasma raised to 16.62 ± 5.11 (N: 10–60) ($p < 0.001$). The increase in free carnitine levels was slow and never reached the upper limit. Almost in all cases urinary carnitine levels increased and they increased furthermore with carnitine supplementation. Carnitine supplementation with a daily dosage of 100 mg/kg was started per oral along with cardiac inotropic and diuretics agents. During the follow-ups, carnitine supplementations were adjusted according to serum carnitine levels. Only in one case, a dosage of 300 mg/kg/day was given. Adverse reactions to therapy were seen only in one patient as a putrefied fish smell of the feces and body of the patient and carnitine dosage was lowered subsequently. No side effect was seen in other cases. All cases showed clinical improvement. BNP levels which reflect the degree of heart failure decreased and ejection fractions were increased. Also five cases had moderate to mild anemia

and after therapy hemoglobin levels returned to normal. Three cases had iron deficiency (patient 1,5,6) and one case had both iron and vitamin B_{12} deficiencies (patient 2), these cases were given necessary supplements. The other case had no underlying cause for his mild anemia (patient 4). Carnitine deficiency could be the cause for his anemia. After therapy all cases had increase in their hemoglobin levels. In the literature, anemia was noted in some cases of primary carnitine transporter deficiency (Cano et al. 2008; Komlósi et al. 2009; Lamhonwah et al. 2002; Melegh et al. 2004; Tein and Di Mauro 1992). Carnitine is known to have a role in red blood cell metabolism: it stabilizes the cellular membrane and raises the red blood cell osmotic resistance (Evangeliou and Vlassopoulos 2003). Iron metabolism is also linked with carnitine because various authors showed low serum carnitine concentration in healthy children with iron deficiency anemia (Cemeroglu et al. 2001; Tanzer et al. 2001). Thus, anemia may be present in carnitine transporter deficiency and an iron deficiency may worsen anemia in this context. Moreover, iron deficiency may be a cause of secondary carnitine deficiency (Cano et al. 2008). One of the our cases had facial dysmorphia, microcephaly, mild mental retardation (patient 4). The chromosomal analysis of this case was normal and this case could not be related to another dysmorphic syndrome. The additional features observed in this patient may not be caused by this defect. Two other cases also had mild mental retardation (patient 2 and 8). No such relation between carnitine deficiency and mild mental retardation has been reported in the literature. Deficiency of carnitine and iron might have contributed to the development of mild mental retardation in these two patients. One of the cases identified by mass spectrometry screening of siblings revealed a history of recent story of muscle weakness such as difficulty in walking, climbing stairs (patient 8). Two other cases had similar myopathic findings (patient 2 and 5).

Using the PolyPhen program (http://genetics.bwh.harvard.edu/pph/), the potential functional effects of the novel p.G411V and p.G152R mutations were evaluated. The Gly411Val and Gly152Arg missense mutations were predicted to be possibly damaging according to position-specific independent count (PSIC) score differences derived from multiple alignment around substitution position. In this improper substitution, G411, in the nineth transmembrane domain (TMD9), the nonpolar glycine residue is replaced by a more nonpolar hydrophobic valine amino acid. In G152R residue in TMD2, the polar hydrophilic glycine residue is replaced by a nonpolar hydrophobic asparagine. Because of the localization of these two novel missense mutations in the transmembrane domain of the protein, they are functionally crucial. These variants may affect substrate specificity of OCTN2 protein.

Other known mutations namely p.R254X, p.R282X, p.R289X, p.T337Pfs12X detected by present study resulted in a premature insertion of a stop codon. The premature insertion of a stop codon can produce a truncated protein, result in unstable RNA, or cause exon skipping (Wang et al. 1999). These mutations would result in the production of a truncated membrane transporter with 5 (R254X), 6 (R282X, R289X, T337Pfs12X) transmembrane domains instead of the normal 12. The lack of several transmembrane domains may cause degraded or nonfunctional protein product. R254X, previously described mutation, was reported in Chinese and Saudi Arabians, whereas R282X was a common mutation in Caucasians (Burwinkel et al. 1999; Lamhonwah et al. 2004; Tang et al. 2002; Vaz et al. 1999; Wang et al. 1999). Other 1-bp deletion c.1009delA (T337Pfs12X) results in a frameshift with Thr337Pro leading to a predicted truncated protein of 347 aa length (Lamhonwah et al. 2002).

Conclusion

In conclusion, a total of six genetic mutations in the *OCTN2* gene were identified in this study, which of two were described as novel. Carnitine membrane transporter deficiency is one of the rare treatable etiologies of metabolic cardiomyopathies. It should be suspected and searched for by measuring the levels of free and total carnitine in plasma and urine from such patients. The clinical phenotype of OCTN2 deficiency may include anemia and mild mental retardation when other causes have been searched for and eliminated. Like biotinidase deficiency, primary carnitine deficiency is one of the metabolic disorders that is easy to treat disease with very good prognosis in early diagnosis.

Acknowledgement This study was supported by the State Planning Organization of Turkey (project No: DPT 2006 K120640).

Synopsis

The article describes novel and previously detected mutations in *OCTN2* gene and their clinical consequences in patients with primary carnitine deficiency.

References

Amat di San Filippo C, Wang Y, Longo N (2006) Functional domains in the carnitine transporter OCTN2, defective in primary carnitine deficiency. J Biol Chem 278:47776–47784

Amat di San Filippo C, Pasquali M, Longo N (2006) Pharmacological rescue of carnitine transport in primary carnitine deficiency. Hum Mutat 27:513–523

Amat di San Filippo C, Taylor MR, Mestroni L et al (2008) Cardiomyopathy and carnitine deficiency. Mol Genet Metab 94:162–166

Burwinkel B, Kreuder J, Schweitzer S et al (1999) Carnitine transporter OCTN2 mutations in systemic primary carnitine deficiency: a novel Arg169Gln mutation and a recurrent Arg282ter mutation associated with an unconventional splicing abnormality. Biochem Biophys Res Commun 261:484–487

Cano A, Ovaert C, Vianey-Saban C et al (2008) Carnitine membrane transporter deficiency: a rare treatable cause of cardiomyopathy and anemia. Pediatr Cardiol 29:163–165

Cederbaum SD, Koo-McCoy S, Tein I et al (2002) Carnitine membrane transporter deficiency: a long-term follow up and OCTN2 mutation in the first documented case of primary carnitine deficiency. Mol Genet Metab 77:195–201

Cemeroglu AP, Kocabaş CN, Coşkun T, Gürgey A (2001) Low serum carnitine concentrations in healthy children with iron deficiency anemia. Pediatr Hematol Oncol 18:491–495

Dobrowolski SF, McKinney JT, di San A, Filippo C, Giak Sim K, Wilcken B, Longo N (2005) Validation of dye-binding/high-resolution thermal denaturation for the identification of mutations in the SLC22A5 gene. Hum Mutat 25:306–313

Evangeliou A, Vlassopoulos D (2003) Carnitine metabolism and deficit–when supplementation is necessary? Curr Pharm Biotechnol 4:211–219

Garavaglia B, Uziel G, Dworzak F, Carrara F, DiDonato S (1991) Primary carnitine deficiency: heterozygote and intrafamilial phenotypic variation. Neurology 41:1691–1693

Holme E, Greter J, Jacobson CE et al (1989) Carnitine deficiency induced by pivampicillin and pivmecillinam therapy. Lancet 2:469–473

Karpati G, Carpenter S, Engel AG et al (1975) The syndrome of systemic carnitine deficiency. Clinical, morphologic, biochemical, and pathophysiologic features. Neurology 25:16–24

Koizumi A, Nozaki J, Ohura T et al (1999) Genetic epidemiology of the carnitine transporter OCTN2 gene in a Japanese population and phenotypic characterization in Japanese pedigrees with primary systemic carnitine deficiency. Hum Mol Genet 8:2247–2254

Komlósi K, Magyari L, TaliÄn GC et al (2009) Plasma carnitine ester profile in homozygous and heterozygous OCTN2 deficiency. J Inherit Metab Dis. doi:10.1007/s10545-009-0926-1

Lamhonwah AM, Olpin SE, Pollitt RJ et al (2002) Novel OCTN2 mutations: no genotype-phenotype correlations: early carnitine therapy prevents cardiomyopathy. Am J Med Genet 111:271–284

Lamhonwah AM, Onzuka R, Olpn SE et al (2004) OCTN2 mutation (R254X) found in Saudi Arabian kindred: recurrent mutation or ancient founder mutation? J Inherit Metab Dis 27:473–6

Li FY, El-Hattab AW, Bawle EV et al (2010) Molecular spectrum of SLC22A5 (OCTN2) gene mutations detected in 143 subjects evaluated for systemic carnitine deficiency. Hum Mutat 31:1632–1651

Longo N, di San A, Filippo C, Pasquali M (2006) Disorders of carnitine transport and the carnitine cycle. Am J Med Genet C Semin Med Genet 142:77–85

Makhseed N, Vallance HD, Potter M et al (2004) Carnitine transporter defect due to a novel mutation in the SLC22A5 gene presenting with peripheral neuropathy. J Inherit Metab Dis 7:778–780

Mayatepek E, Nezu J, Tamai I et al (2000) Two novel missense mutations of the OCTN2 gene (W283R and V446F) in a patient with primary systemic carnitine deficiency. Hum Mutat 15:118

Melegh B, Bene J, Mogyorósy G et al (2004) Phenotypic manifestations of the OCTN2 V295X mutation: sudden infant death and carnitine-responsive cardiomyopathy in Roma families. Am J Med Genet A 131:121–126

Nezu J, Tamai I, Oku A et al (1999) Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. Nat Genet 21:91–94

Ohashi R, Tamai I, Nezu Ji J et al (2001) Molecular and physiological evidence for multifunctionality of carnitine/organic cation transporter OCTN2. Mol Pharmacol 59:358–366

Rahbeeni Z, Vaz FM, Al-Hussein K et al (2002) Identification of two novel mutations in OCTN2 from two Saudi patients with systemic carnitine deficiency. J Inherit Metab Dis 25:363–369

Roe CR, Coates PM (1995) Mitochondrial fatty acid oxidation disorder. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited diseases. McGraw-Hill, Inc., New York, pp 1508–1509

Saito S, Iida A, Sekine A et al (2002) Catalog of 238 variations among six human genes encoding solute carriers (hSLCs) in the Japanese population. J Hum Genet 47: 576–584

Scaglia F, Longo N (1999) Primary and secondary alterations of neonatal carnitine metabolism. Semin Perinatol 23:152–161

Scaglia F, Wang Y, Singh RH et al (1998) Defective urinary carnitine transport in heterozygotes for primary carnitine deficiency. Genet Med 1:34–39

Scaglia F, Wang Y, Longo N (1999) Functional characterization of the carnitine transporter defective in primary carnitine deficiency. Arch Biochem Biophys 364:99–106

Spiekerkoetter U, Huener G, Baykal T et al (2003) Silent and symptomatic primary carnitine deficiency within the same family due to identical mutations in the organic cation/carnitine transporter OCTN2. J Inherit Metab Dis 26:613–615

Stanley CA, DeLeeuw S, Coates PM et al (1991) Chronic cardiomyopathy and weakness or acute coma in children with a defect in carnitine uptake. Ann Neurol 30:709–716

Stanley CA, Bennett MJ, Mayatepek E (2006) Disorders of fatty acid oxidation and related metabolic pathways. In: Fernandes J, Saudubray JM, Van Den Berghe G, Walter JH (eds) Inborn metabolic disease diagnostic and treatment, 4th edn. Springer, Heidelberg, pp 177–188

Tamai I, Ohashi R, Nezu J et al (1998) Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. J Biol Chem 273:20378–20382

Tang NL, Ganapathy V, Wu X et al (1999) Mutations of OCTN2, an organic cation/carnitine transporter, lead to deficient cellular carnitine uptake in primary carnitine deficiency. Hum Mol Genet 8:655–660

Tang NL, Hwu WL, Chan RT et al (2002) A founder mutation (R254X) of SLC22A5 (OCTN2) in Chinese primary carnitine deficiency patients. Hum Mutat 20:232

Tanzer F, Hizel S, Cetinkaya O, Sekreter E (2001) Serum free carnitine and total triglyceride levels in children with iron deficiency anemia. Int J Vitam Nutr Res 71:66–69

Tein I, Di Mauro S (1992) Primary systemic carnitine deficiency manifested by carnitine-responsive cardiomyopathy. In: Ferrari R, Di Mauro S, Sherwood G (eds) L-carnitine and its role in medicine: from function to therapy Academic Press, San Diego, pp. 155–183

Tein I, De Vivo DC, Bierman F et al (1990) Impaired skin fibroblast carnitine uptake in primary systemic carnitine deficiency manifested by childhood carnitine-responsive cardiomyopathy. Pediatr Res 28:247–255

Tein I, DiMauro S, Xie ZW, De Vivo DC (1993) Valproic acid impairs carnitine uptake in cultured human skin fibroblasts. An in vitro model for the pathogenesis of valproic acid-associated carnitine deficiency Pediatr Res 34:281–287

Tein I, Bukovac SW, Xie ZW (1996) Characterization of the human plasmalemmal carnitine transporter in cultured skin fibroblasts. Arch Biochem Biophys 329:145–155

Toh DS, Yee JY, Koo SH et al (2010) Genetic variations of the SLC22A5 gene in the Chinese and Indian populations of Singapore. Drug Metab Pharmacokinet 25:112–119

Treem WR, Stanley CA, Finegold DN, Hale DE, Coates PM (1988) Primary carnitine deficiency due to a failure of carnitine transport in kidney, muscle, and fibroblasts. N Engl J Med 319:1331–1336

Vaz FM, Scholte HR, Ruiter J et al (1999) Identification of two novel mutations in OCTN2 of three patients with systemic carnitine deficiency. Hum Genet 105:157–161

Wang Y, Ye J, Ganapathy V et al (1999) Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency. Proc Natl Acad Sci USA 96:2356–2360

Wang Y, Taroni F, Garavaglia B et al (2000a) Functional analysis of mutations in the OCTN2 transporter causing primary carnitine deficiency: lack of genotype-phenotype correlation. Hum Mutat 16:401–407

Wang Y, Kelly MA, Cowan TM, Longo N (2000b) A missense mutation in the OCTN2 gene associated with residual carnitine transport activity. Hum Mutat 15:238–245

Wang Y, Korman SH, Ye J, Gargus JJ et al (2001) Phenotype and genotype variation in primary carnitine deficiency. Genet Med 3:387–392

Wilcken B, Wiley V, Sim KG, Carpenter K (2001) Carnitine transporter defect diagnosed by newborn screening with electrospray tandem mass spectrometry. J Pediatr 138:581–584

Wilcken B, Wiley V, Hammond J, Carpenter K (2003) Screening newborns for inborn errors of metabolism by tandem mass spectrometry. N Engl J Med 348:2304–2312

Wu X, Prasad PD, Leibach FH, Ganapathy V (1998) cDNA sequence, transport function, and genomic organization of human OCTN2, a new member of the organic cation transporter family. Biochem Biophys Res Commun 246:589–595

Wu X, Huang W, Prasad PD et al (1999) Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. J Pharmacol Exp Ther 290:1482–1492

JIMD Reports
DOI 10.1007/8904_2011_43

RESEARCH REPORT

Kinetic Analyses Guide the Therapeutic Decision in a Novel Form of Moderate Aromatic Acid Decarboxylase Deficiency

M. Barth • V. Serre • L. Hubert • Y. Chaabouni • N. Bahi-Buisson • M. Cadoudal • D. Rabier • S. Nguyen The Tich • M. Ribeiro • D. Ricquier • A. Munnich • D. Bonneau • P. de Lonlay • L. Christa

Received: 29 December 2010 / Revised: 29 April 2011 / Accepted: 2 May 2011 / Published online: 16 September 2011

Abstract *Background*: Aromatic amino acid decarboxylase (AADC) deficiency is a rare autosomal recessive disorder resulting in a combined dopamine and serotonin deficiency. About 50% of the cases set in the neonatal period. Here, we report an atypical clinical presentation with moderate symptoms.

Patient: At 10 months old, the patient presented paroxysmal eye movements without seizures, and feeding difficulties which were attributed to gastroesophageal reflux. She was investigated at the age of 7 years, because of orofacial dyspraxia, hypomimie, axial hypotonia and focal segmental dystonia, bilateral ptosis, without evidence for cognitive impairment.

Results: HVA [110 nM; (reference value (rv): 202–596)] and HIAA (12 nM; rv: 87–366) decreased, OMD (520 nM; rv: 5–60) and 5-HTP (56 nM; rv: 2–16) increased in CSF. We confirmed the diagnosis of AADC deficiency because the activity in plasma was low: 4 pmol/min/ml; rv: 16–137. The kinetic analysis revealed a sixfold increase in the apparent affinity for L-dopa (4.26 mM; control = 0.71), but the V_{max} was unchanged (37.5 pmol dopamine/min/ml; control = 39.1), suggesting a modification in the substrate binding-site. Molecular analysis revealed two heterozygous mutations in the DDC gene: c1040G > A; pR347Q already described, and a novel mutation c478C > T, pR160W.

Conclusion: (1) CSF neurotransmitters metabolites suggested a moderate AADC deficiency; (2) The initial velocity saturation curve for L-dopa displayed a cooperative ligand binding behavior, in keeping with the modifications of the three-dimensional structure, induced by the amino acid substitutions (3) The treatment combination of L-dopa with pyridoxine dramatically improved the quality of life, the fatigability, and the paroxysmal eye movements.

Abbreviations

3D	Three dimensional
3-OMD	3-Ortho-methyldopa
5-HIAA	5-Hydroxyindolacetic acid
5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
AADC	Aromatic L-amino acid decarboxylase
CSF	Cerebral spinal fluid
DA	Dopamine

Communicated by: Nenad Blau.

Competing interests: None declared.

These authors equally contributed to this work.

M. Barth (✉) · M. Ribeiro · D. Bonneau
Service de Génétique, CHU Angers, Angers, France
e-mail: magaliesylvie@hotmail.com

Y. Chaabouni · M. Cadoudal · D. Rabier · D. Ricquier · L. Christa
Service de Biochimie Métabolique, Hôpital Necker – Enfants Malades, Paris, France

V. Serre · L. Hubert · A. Munnich · P. de Lonlay
Unité INSERM 781, Université Paris Descartes, Paris, France

N. Bahi-Buisson
Service de Neurologie Pédiatrique, Hôpital Necker – Enfants Malades, Paris, France

S. Nguyen The Tich
Service de Neurologie pédiatrique, CHU Angers, Angers, France

N. Bahi-Buisson · D. Rabier · D. Ricquier · L. Christa
Université Paris Descartes, Paris, France

V. Serre
Université Denis Diderot, Paris, France

D. Rabier · D. Ricquier · P. de Lonlay · L. Christa
Reference center for inherited metabolic diseases, Hôpital Necker – Enfants Malades, Paris, France

DDC	Aromatic L-amino acid decarboxylase gene
HVA	Homovanillic acid
L-dopa	3,4-Dihydroxyphenylalanine
MTHF	Methyltetrahydrofolate
PDB	Protein database
RV	Reference value

Introduction

Aromatic L-amino acid decarboxylase (AADC, EC4.1.128) is an essential enzyme in the metabolism of the monoamine neurotransmitters serotonin and dopamine. AADC converts both 5-hydroxytryptophan (5-HTP) into serotonin (5-hydroxytryptamine, 5-HT) and 3,4-dihydroxyphenylalanine (L-dopa) into dopamine (DA). AADC is a 54 kDa homodimeric protein, with two catalytic pockets located at the dimer interface. The AADC enzyme requires pyridoxal-5′-phosphate which is the cofactor covalently linked with the K303 residue in the active site, and subsequently bound to the substrate (Burkhard et al. 2001). The recessively inherited deficiency of AADC induces a severe neurometabolic disorder with developmental delay, abnormal movements, oculogyric crises, and vegetative symptoms (Brun et al. 2010; Hyland et al. 1992). The symptoms typically appear in the first months of life. The investigation of neurotransmitters in cerebral spinal fluid (CSF) normally leads to the diagnosis. Patients display a typical pattern with reduction of catabolites of the dopamine and serotonin pathways, homovanillic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA), as well as an elevation of the precursors of dopamine and serotonin, L-dopa and 5-HTP. In addition, 3-ortho-methyldopa (3-OMD) is clearly elevated resulting from methylation of accumulating L-dopa. It was considered that this pattern was unique but a similar pattern had been described in patients with mutations in the pyridoxamine 5′-phosphate oxidase gene (Mills et al. 2005). These patients had a secondary AADC deficiency due to a defect in the synthesis of the pyridoxal phosphate cofactor. Elevated concentration of vanillactic acid in urine was detected in primary and secondary AADC deficiency. Vanillactic acid accumulated after transamination of 3-OMD. The diagnosis was confirmed by the enzyme activity of AADC measured in plasma. Null or very low AADC activity had been attributed to more than 30 different mutations identified in the aromatic L-amino acid decarboxylase gene (*DDC*) (Chang et al. 2004; Haavik et al. 2008; Verbeek et al. 2007). Therapeutic management was challenging. Pyridoxine, as the precursor of the AADC cofactor, monoamine oxidase inhibitors, dopamine agonists, anticholinergics, melatonin, L-dopa, and other treatments has been used. The response to treatment was variable, but overall the outcome remains poor (Brun et al. 2010; Manegold et al. 2009).

We report a new atypical clinical presentation with moderate symptoms of AADC deficiency. We have investigated the kinetics of the enzyme in plasma. We have shown that the combined R347Q and R160W substitutions induced a low L-dopa substrate binding affinity with a normal V_{max}. L-dopa has been shown to have a positive cooperative effect, in relation to the changes in the 3D-dimer structure. Consequently, L-dopa has been introduced to the initial therapy with pyridoxine and dopaminergic agonist. An excellent response to the treatment has been observed.

Patient and Methods

Biochemical Analysis

Biogenic amines pterins and methyl tetrahydrofolate were analyzed as previously described (Ormazabal et al. 2005). AADC activity was measured as previously reported (Hyland et al. 1992; Blau et al. 2002).

Molecular Analysis of the DDC Gene

Genomic DNA was extracted using the GE Healhcare Illustra DNA extraction kit. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing kit v.3.1 (Applied Biosystems), analyzed by capillary electrophoresis on an ABI 3100 sequencer (Applied Biosystems) and sequence analyses were performed using the Seqscape software v2.5 (Applied Biosystems). The informed consent was obtained from the parents according to the Necker Hospital ethics board committee.

Computer Analysis

The kinetic parameters were determined following the selection of an appropriate curve with Kaleidagraph software. Data were fitted to the Michaelis–Menten equation (hyperbolic saturation curve when $n_H = 1$) or to the Hill equation (sigmoid saturation curve when $1 < n_H <$ protomer number). The equation was: $V_i = \frac{V_{max} \times [L-Dopa]^{n_H}}{K_{\frac{1}{2}} + [L-Dopa]^{n_H}}$ V_i = initial velocity, V_{max} = maximum velocity, $K_{1/2}$ = apparent affinity, [L-Dopa] = L-dopa concentration, n_H = Hill coefficient.

A correlation coefficient (R^2) is displayed with the curve fit's equation and coefficients. The correlation coefficient indicates how well the calculated curve fits the original data.

The three-dimensional structure of the human AADC (residues 1–475) was modeled by comparative protein modeling methods and energy minimization, using the Swiss-Model program in the automated mode (Arnold et al. 2006; Kiefer et al. 2009; Peitsch 1995). The Sus Scrofa dopa decarboxylase (protein data base (pdb) code: 1js3) was used as a template for modeling the human AADC protein with the coordinates set at 2.25 Å. Swiss-Pdb Viewer 3.7 (http://www.expasy.org/spdbv) was used to analyze the structural insight into AADC mutations and to visualize the structures.

Case History

This girl was born after two healthy children to unrelated parents. Birth weight, height, and head circumference were in the normal range. She showed feeding difficulties during the first 6 months of life. Hypotonia was noticed and motor development was also delayed. She was able to sit at the age of 10 months and to walk at the age of 22 months. Speech was also delayed because of buccofacial apraxia. Since the age of 10 months, her mother described attacks of tiredness associated with paroxysmal eye movements which exhibited a diurnal variation. She was referred at 7 years old, because of the persistence of the eye movements. She followed mainstream schooling. Her composite IQ was 85 (WISC III). Bilateral ptosis and buccofacial apraxia with permanent open mouth, hypersalivation and permanent nasal obstruction were observed. A fine motor examination showed dyspraxia and dystonia triggered by movement repetition. There was no hypotonia or spontaneous abnormal movements, at this period. Clinical features were not specific for AADC deficiency. Routine plasma analysis was normal. Video EEG confirmed the absence of seizure during oculogyric crises. Glucose was 3.7 and 4.2 mmol/l in CSF and plasma respectively, with a normal ratio of 0.88, thus eliminating Glut1 deficiency. Prolactin was slightly elevated (509 mUi/l, rv $<$ 496). Vanillactic acid (10 µmol/mmol creatinine, rv $<$ 2) and vanilpyruvic acid (48.4 µmol/mmol creatinine, rv $=$ 0) were detected in urines.

Results

CSF Neurotransmitters Analysis

Results of CSF neurotransmitters were reported in Table 1. The catabolites of DA and HT pathways, HVA and HIAA decreased whereas the precursors increased. Neopterin, Biopterin, and methyltetrahydrofolate remained in the normal ranges. These results suggested a deficiency in AADC.

AADC Enzyme Activity and Kinetic Parameters

AADC plasma activity was measured with L-dopa concentrations saturating the normal enzyme. Results were presented in Table 1. Patient plasma activity represented 8% of the mean of the child reference range, confirming the AADC deficiency. The AADC activity was, respectively, for the father and the mother, 56% and 70% of the mean of the adult reference range. The plasma was used to measure the rate of dopamine production by increasing the concentrations of the substrate L-dopa (Fig. 1). The curves were hyperbolic for the control, the mother and the father, suggesting kinetics of Michaelis–Menten type. We have determined apparent affinity and V_{max}, which were similar to those calculated by the Linewaeaver–Burk plots used by Verbeek et al. (2007). In contrast, the plot of reaction velocity versus L-dopa concentration was sigmoid for the patient, revealing a cooperative ligand binding behavior and an allosteric positive homotropic effect conforming to the Hill equation. In these conditions, the patient kinetic analysis revealed a sixfold decrease of the L-dopa apparent affinity, but the V_{max} value was not decreased, suggesting an alteration of the substrate binding-site. The sigmoid curve allowed us to determine a Hill coefficient n_H of 1.78. The Hill coefficient quantifies the cooperativity, with a range between 1 and the number of protomers ($1 < n_H <$ number of protomer). For control and parents, there was no cooperativity because $n_H = 1$. For the patient, the value of 1.78 indicated a positive cooperative fixation of the substrate L-dopa into the active site, in keeping with $n_H < 2$ because the number of protomers or subunits for AADC is 2.

DDC Gene Analysis

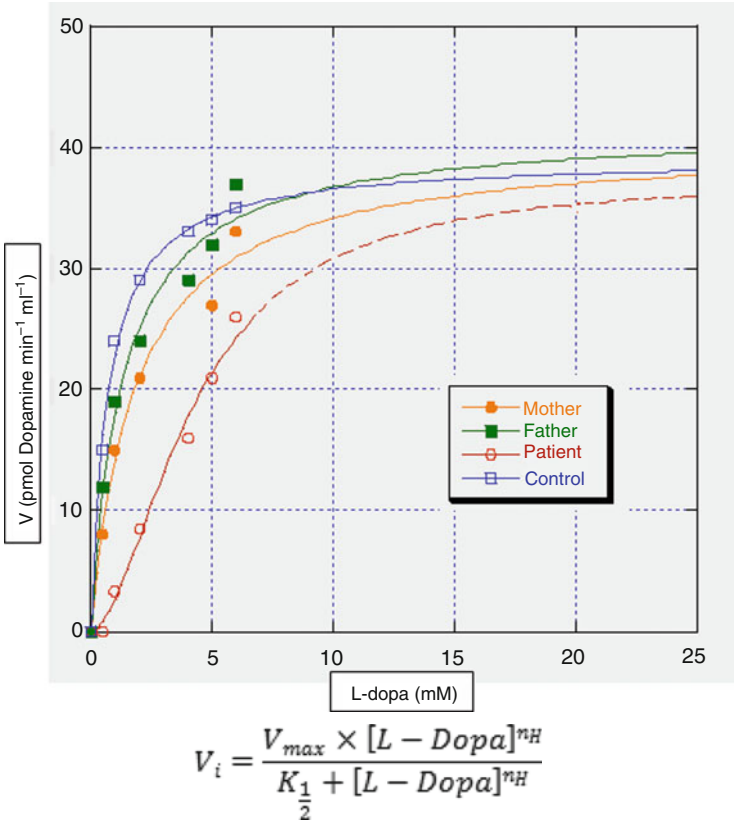
Molecular analysis of the DDC gene identified two heterozygous mutations: c1040G $>$ A; pR347Q already described, and a novel mutation c478C $>$ T, pR160W. The father was heterozygous for the pR347Q mutation and the mother was heterozygous for the pR160W mutation.

In Silico Analysis of the AADC Dimer 3D-Structure

The 87% homology between human and pig AADC allowed us to use the pig protein database 1JS3 as a matrix to model the human AADC, with a good precision. The mis-sense mutations pR347Q and pR160W and the interacted amino acids were conserved in the related species. In Fig. 2, the two peptide chains of the homodimer were shown as strands of ribbons for the α-helix, but only one active site has been indicated to clarify the figure. Both

Table 1 (A) CSF neurotransmitter analysis before and after 1 month treatment with L-Dopa + bromocriptine + pyridoxal phosphate treatment (nmol/L). (B) Plasma AADC enzyme activity. Before treatment CSF neurotransmitters analysis has oriented and the low plasma AADC activity has confirmed the diagnosis of AADC. Plasma AADC activity was measured with saturating L-dopa concentrations for normal enzyme

A: Patient CSF analysis (nmol/L)	Before treatment	After treatment	Reference values
5-HTP	56	24	2-16
HIAA	12	18	87-366
3-OMD	520	794	5-60
HVA	110	123	202-596
MHPG	36	35	13-68
MTHF	100	57	>44
B: AADC activity (pmol/min/ml)			Reference values
Patient	4		16-130 < 15 y
Mother	15		14-41 > 15 y
Father	19		14-41 > 15 y
Control	24		14-41 > 15 y



$$V_i = \frac{V_{max} \times [L-Dopa]^{nH}}{K_{\frac{1}{2}} + [L-Dopa]^{nH}}$$

Fig. 1 Plot of reaction velocity versus L-dopa concentrations. Saturation curves were fitted to the Michaelis–Menten equation or to the Hill equation, by using the equation below. Curves were hyperbolic for control, mother and father conforming to $n_H = 1$. Curve was sigmoid for the patient, conforming to $n_H = 1.78$ (n_H = Hill coefficient). The kinetic parameters were derived by nonlinear least square analysis by using the program kaleidagraph

mutations led to the loss of an arginine residue, both these arginines were located at the interface of the monomers. We analyzed how the mutations could induce modifications in salt-bridge, hydrogen, and Van der Waals contacts, with the environmental amino acids.

We showed that the loss of an arginine (mutation pR347Q) might have relaxed the catalytic pocket. The NH1 primary amine group in arginine 347 was 3.12 Å away from the oxygen of the phenylalanine 103 located into the opposite monomer. The phenylalanine 103 was in

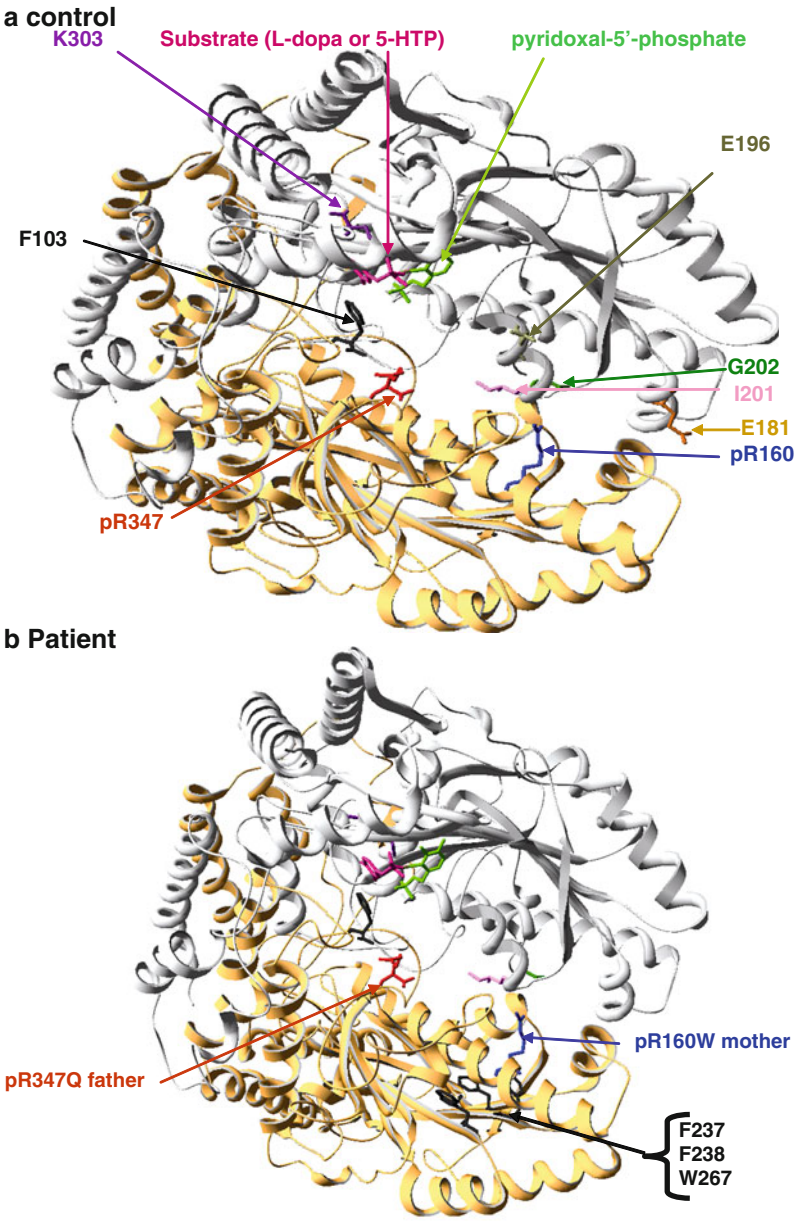


Fig. 2 Structural impact of the AADC mutations. AADC was a homodimer with two catalytic pockets lining at the dimer interface. The human AADC was represented in *yellow* and superimposed with the pig dimeric enzyme in *gray* (pdb code 1JS3). Only one active site was shown with the substrate (L-dopa or 5-HTP in fuchsia), bound to the cofactor pyridoxal-5′-phosphate (in *pea green*), itself covalently linked with K303 (in *purple*). (**a**): control; (**b**): patient with the two substitutions located at the interface of the monomers. (**a**): the substitution of R347 (pR347Q) is shown in *red* and led to a relaxation of the catalytic pocket by the loss of the interaction between F103 in black and R347. The substitution of the R160 (pR160W shown in *blue*) suppressed one or another hydrogen contact and salt–bridge interaction, respectively, with G202 (in *green*) or I201 (in *pink*) and E181 (in *amber*) or E196 (in *khaki*). The interacting amino acids were located into the opposite monomer of R160. (**b**): the introduction of W160 reinforced the hydrophobic patch shown in *black* (F237, F238, and W267) by stronger inter chains Van der Waals contacts, rendering this region less flexible. Thus, both mutations have locally altered the protein dimerization and have modified the kinetic character for its substrate L-dopa

Van der Waals contact with the catechol ring of the substrate, as reported by Burkhard et al. (2001). Thus, arginine 347 participated to the substrate fixation via its interaction with Phe103. In the mutated AADC, the catalytic pocket might be relaxed by the loss of the Phe103/R347 interaction. Furthermore, the introduction of a tryptophane in position 160, instead of an arginine (pR160W) modified the 3D-structure. First, the NH1 primary amine group in arginine 160 was 2.85 Å away from the oxygen in glycine 202. The NH2 primary amine group in arginine 160 was 3.04 Å away from the oxygen in isoleucine 201 (Fig. 2a). The loss of arginine 160

suppressed one or another hydrogen contact with glycine or isoleucine located into the opposite monomer, resulting in a local destabilization of the dimer structure.

Second, the distances from arginine160 to glutamate181 and to glutamate 196 were 14 Å and 15 Å, respectively. They exceeded over the limit authorized to establish salt–bridge interaction in the fixed crystallized structure. However, one could postulate that the movements of the monomers reduced the distance, allowing temporary interactions for local stabilization of the dimer structure. The loss of arginine160 suppressed the salt–bridge interaction with glutamate 181 or glutamate 196, increasing the local destabilization of the dimer structure. Third, the mutation pR160W introduced the highly hydrophobic aromatic tryptophane which can establish new Van der Waals contacts. We have identified three other hydrophobic aromatic amino acids in the region, phenylalanine 237 and 238, and tryptophane 267, each being located less than 7 Å from the mutation pR160W. These three aromatic amino acids constituted a patch of hydrophobic aromatic residues between the two subunits. The introduction of the tryptophane 160 reinforced the hydrophobic patch and may have closed the access to the active site by strong inter-chains Van der Waals contacts.

Drug Therapy

As soon as the AADC deficiency was confirmed, the patient was treated two times a day with pyridoxine 125 mg and bromocriptine (D1 and D2 dopamine agonist) to a total of 0.45 mg/kg/day (7.5 mg/day). After 48 h, improvement was observed with a reduction of oculogyric crises as shown in Table 2. Dyspraxia, fatigability, and quality of life were also improved after the first month of therapy. Based on in vitro kinetic analysis, L-dopa (4 mg/kg/day) was progressively added to a dose of 10 mg/kg/day, and the patient showed an excellent response. After 6 months of combined therapy, Bromocriptine with L-dopa and pyridoxine, Bromocriptine was stopped and L-dopa was only combined with pyridoxine. Clinical improvements still increased: oculogyric crises and dystonia disappeared, and fatigability decreased, as described in Table 2. After 3 months of treatment (2 months after the initial therapy and 1 month after L-dopa supplementation), CSF neurotransmitters were analyzed again (Table 1). HIAA and HVA levels slightly increased, although the clinical response was excellent.

Discussion

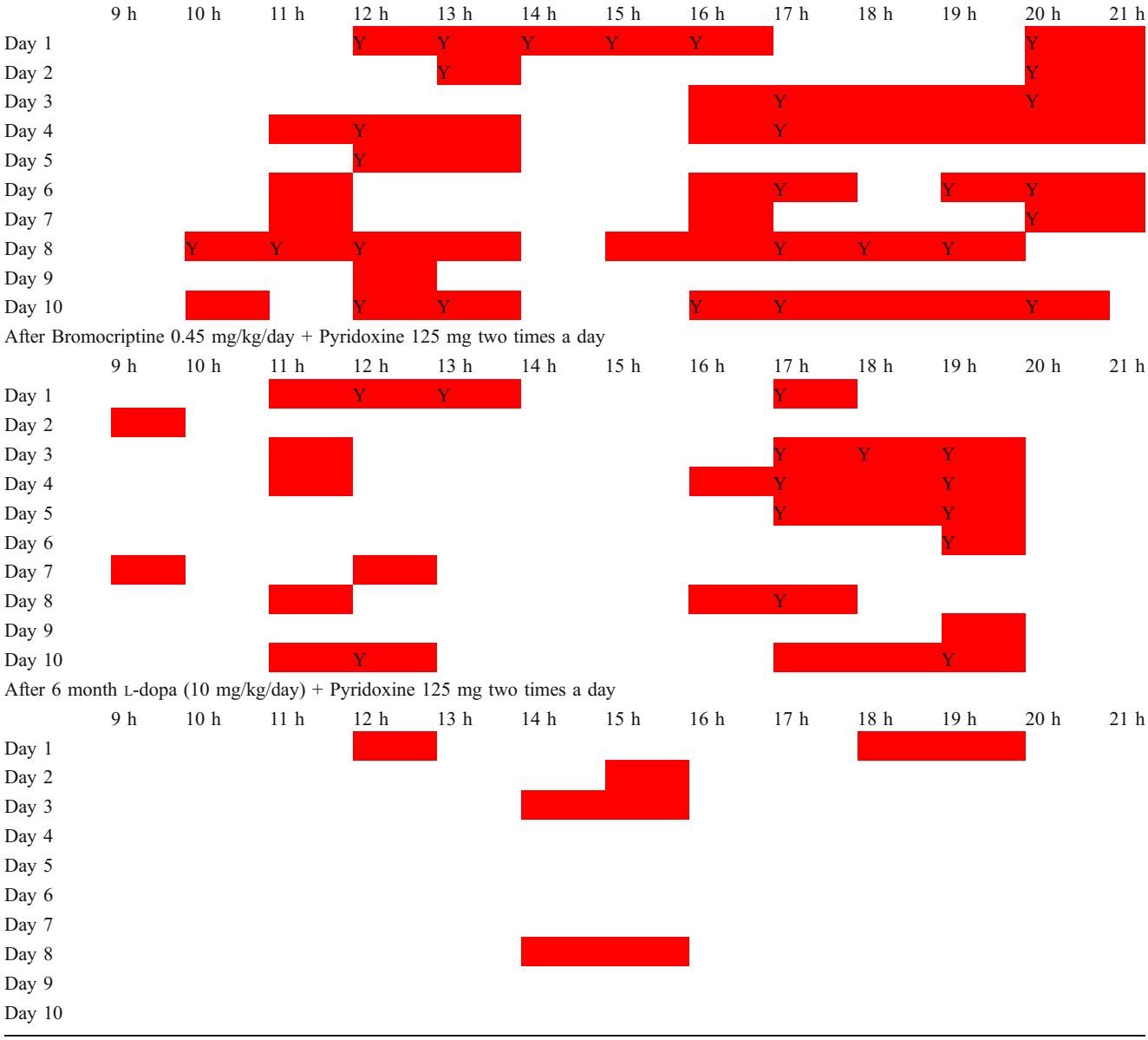
AADC converts L-dopa to DA and 5-HTP to 5-HT and deficiency of this enzyme leads to profound alterations in central and peripheral nervous system homeostasis. Here, we report an atypical patient with moderate clinical features diagnosed at 7 years old, by the analysis of neurotransmitters in CSF and the AADC enzyme activity in plasma. The patient was heterozygous for a mis-sense mutation pR347Q already described by Pons et al. (2004) and a novel mis-sense mutation pR160W.

CSF HVA level in the index patient was only 50% of the lower limit of the reference range and AADC activity using L-dopa as substrate was higher than activities reported in previous AADC deficiencies (Verbeek et al. 2007). These results were in accordance with the moderate phenotype observed in the patient. The pR347Q mutation led to a severe phenotype with null plasma AADC activity, when it was associated with a mutation pR358H in one patient or with a single base deletion in exon2 (delC209-211) in another patient as previously reported by Pons et al. (2004). In contrast, the mutations pR347Q in combination with the novel pR160W mutations led to a moderate phenotype. CSF HIAA level represented 12% of the lower limit of the reference range. It would have been of great interest to determine whether the discrepancy was also reflected in the enzyme activity toward 5-HTP as a substrate. Unfortunately, the whole plasma sample available before therapy was used for three independent kinetic analyses toward L-dopa as a substrate. Both substrates competed for the same active site, and AADC activity was 8–12 lower with 5-HTP than with L-dopa (Verbeek et al. 2007). Consequently, we postulate that the enzyme activity should be undetectable with 5-HTP as a substrate, keeping in mind the low level of HIAA.

We demonstrated a Michaelis–Menten-type kinetic with a hyperbolic curve for enzyme activity using L-dopa as a substrate in plasma of control and parents. The apparent affinity and V_{max} were in the same order of magnitude as previously described (Verbeek et al. 2007). Thus, one heterozygous mutation, either pR347Q or pR160W did not induce any modification in the kinetic parameters. In contrast, the plot of reaction velocity versus L-dopa was sigmoid for the patient, conforming to the Hill equation with $n_h = 1.78$. Thus, we revealed a cooperative ligand binding behavior and its allosteric positive homotropic effect. L-dopa facilitated its own fixation, by inducing a trans-conformation of the enzyme. The L-dopa apparent affinity decreased by sixfold and the V_{max} was similar to the control at saturating concentrations. The homodimeric structure was compatible with the value of 1.78 for n_h for allosteric enzyme. There was no change after dialysis, eliminating the hypothesis of an allosteric inhibitor in the plasma patient (data not shown). In the plasma, the cooperative effect resulted from a mix of three types of dimer: 50% with the combined mutations, one on each monomer, 25% with the pR347Q mutation on each monomer and 25% with the pR160W mutation on each monomer. The influence of each substrate and of the cofactor pyridoxal-5′-phosphate could be determined in

Table 2 Follow-up of the AADC therapy. Timing of tiredness attack and ocular revulsion before, after bromocriptine (0,45 mg/kg/day) + pyridoxine (125 mg two times a day) and after L-dopa (10 mg/kg/day) + pyridoxine (125 mg two times a day)

Before treatment



Y = ocular revulsion and red squares = tiredness periods

a future study by producing recombinant wild-type and mutated AADC proteins.The combination of the three subunit types should allow to simulate the kinetic observed in the patient's plasma.

The active site is located at the monomer–monomer interface. The combination of the two mutations lowered the substrate binding affinity: the pR347Q mutation relaxed the catalytic pocket by the loss of the F103/R347 interaction, and the pR160W mutation introduced a highly hydrophobic aromatic amino acid reinforcing a strong hydrophobic patch already present at the interface of the two monomers. Both mutations destabilized the local 3D-structure and locked the accessibility of the substrate into the binding pocket by inter chains Van der Waals contacts. Increasing the substrate concentrations resulted in a homotropic cooperative effect conforming to allosteric enzymes.

Treatment was initiated with the D1 and D2 dopamine agonist bromocriptine together with pyridoxine. Pyridoxine is the most common drug used in AADC deficiency (71% of the patients) (Brun et al. 2010), because it is the

precursor of the cofactor pyridoxal 5′-phosphate, which may play a role of chaperone molecule and stabilizes the mutated enzyme. Since we had identified a homotropic cooperative effect in vitro, we have introduced L-dopa in the therapy. Clinical improvements still increased even after stopping bromocriptine. The therapeutic use of the substrate L-dopa is less frequent (in only 13% of the patients), and the majority of cases showed none or poor response, probably because the mutations removed most AADC enzyme activity. Three siblings with the homozygous pG102S responded on L-dopa + pyridoxine (Chang et al. 2004). The mutation lied very close to the substrate binding pocket between Isoleucine 101 and phenylalanine 103. In our index case, the mutation pR347Q suppressed the R347/F103 interaction. Moreover, the second pR160W mutation introduced a strong hydrophobic patch at the interface of the two monomers reducing the accessibility of the substrate into the binding pocket. Although the homozygous mutation pG102S clearly reduced the L-dopa substrate binding affinity (apparent affinity was about 20-fold higher than the wild-type apparent affinity in plasma), there was, however, no cooperative effect and the V_{max} still remained substantially below the control level. In contrast, the V_{max} was restored with saturating amounts of L-dopa for our patient, providing the best clinical response to a mixture of L-dopa + pyridoxine. Taking into account the role of the two heterozygous mutations on the 3D-structure, it was the mix of both L-dopa and pyridoxine who probably led to an excellent clinical response and to a drop of CSF 5-HTP after therapy, since they were tested all together. However, we still have to compare the long-term follow-up therapy by L-dopa and pyridoxine for these patients.

In conclusion, we reported a moderate AADC deficiency. The clinical features were nonspecific, indicating that neurotransmitter disorders should be considered in any patients presenting abnormal ocular movements associated with autonomic dysfunction. CSF neurotransmitter analysis has led to the diagnosis. Then, AADC enzyme activity confirmed the diagnosis for a mild clinical presentation with a residual plasma activity. The two amino acid substitutions located at the interface of the monomers induce the L-dopa allosteric positive homotropic effects in keeping with the Hill equation and the modifications of the 3D-structure. The decision to supplement the treatment with L-dopa + pyridoxine led to an excellent clinical response.

Acknowledgements We are grateful to Dr. A Ormazabal and Dr. R Artuch (Hospital San Joan de Déu, Barcelona, Spain) for their help in the development of neurotransmitter analysis in Necker hospital, Paris.

We thank C Robin for reviewing the English language.

References

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195–201

Blau N, Duran M, Blaskovics ME, Gibson KM (2002) Physician's guide to the laboratory diagnosis of metabolic diseases, 2nd edn. Springer, Heidelberg

Brun L, Ngu LH, Keng WT et al (2010) Clinical and biochemical features of aromatic L-amino acid decarboxylase deficiency. Neurology 75(1):64–71, Epub 2010 May 26

Burkhard P, Dominici P, Borri-Voltattorni C, Jansonius JN, Malashkevich VN (2001) Structural insight into Parkinson's disease treatment from drug-inhibited DOPA decarboxylase. Nat Struct Biol 8(11):963–967

Chang YT, Radhakant S, Marsh JL et al (2004) Levodopa responsive aromatic L-amino acid decarboxylase deficiency. Ann Neurol 55:435–438

Haavik J, Blau N, Thöny B (2008) Mutations in human monoamine-related neurotransmitter pathway genes. Hum Mutat 29:891–902

Hyland K, Clayton PT (1992) Aromatic L-amino acid decarboxylase deficiency: diagnostic methodology. Clin Chem 38:2405–2410

Hyland K, Surtees RAH, Rodeck C, Clayton PT (1992) Aromatic L-amino acid decarboxylase: deficiency: clinical features, diagnosis and treatment of a new inborn error of neurotransmitter amine synthesis. Neurology 42:1980–1988

Kiefer F, Arnold K, Künzli M, Bordoli L, Schwede T (2009) The SWISS-MODEL repository and associated resources. Nucleic Acids Res 37:D387–D392

Manegold C, Hoffmann GF, Degen I et al (2009) Aromatic L-amino acid decarboxylase deficiency: clinical features, drug therapy and follow-up. J Inherit Metab Dis 32(3):371–380, Epub 2009 Jan 28

Mills PB, Surtees RA, Champion MP et al (2005) Neonatal epileptic encephalopathy caused by mutations in the PNPO gene encoding pyridox(am)ine 5′-phosphate oxidase. Hum Mol Genet 14:1077–1086

Ormazabal A, García-Cazorla A, FernÄndez Y, FernÄndez-Alvarez E, Campistol J, Artuch R (2005) HPLC with electrochemical and fluorescence detection procedures for the diagnosis of inborn errors of biogenic amines and pterins. J Neurosci Methods 142(1):153–158

Peitsch MC (1995) Protein modeling by E-mail. Bio/Technology 13:658–660

Pons R, Ford B, Chiriboga CA et al (2004) Aromatic L-amino acid decarboxylase deficiency: clinical features, treatment and prognosis. Neurology 62:1058–1065

Verbeek MM, Geurtz PB, Willemsen MA, Wevers RA (2007) Aromatic L-amino acid decarboxylase enzyme activity in deficient patients and heterozygotes. Mol Genet Metab 90(4):363–369, Epub 2007 Jan 19

JIMD Reports
DOI 10.1007/8904_2011_44

RESEARCH REPORT

Effect of Reduced Agalsidase Beta Dosage in Fabry Patients: The Australian Experience

Joanna Ghali • Kathy Nicholls • Charles Denaro • David Sillence • Ian Chapman • Jack Goldblatt • Mark Thomas • Janice Fletcher • On behalf of the Australian Life Saving Drugs Program, the Australian Fabry Disease Advisory Committee and Australian State Fabry Disease Treatment Centres

Received: 29 December 2010 / Revised: 20 May 2011 / Accepted: 23 May 2011 / Published online: 15 September 2011

Communicated by: Frits Wijburg.

Competing interests: None declared.

J. Ghali (✉) · K. Nicholls
Nephrology Department, Royal Melbourne Hospital, Grattan St, Parkville, VIC 3050, Australia
e-mail: joanna.ghali@monash.edu

C. Denaro
Royal Brisbane and Women's Hospital, University of Queensland, Herston, QLD 4029, Australia

D. Sillence
Department of Genetic Medicine, Westmead Hospital, University of Sydney, Wentworthville, NSW 2145, Australia

I. Chapman
Royal Adelaide Hospital, University of Adelaide, Adelaide 5000, SA, Australia

J. Goldblatt
Genetic Services of WA, King Edward Memorial Hospital, Subiaco, WA 6008, Australia

J. Goldblatt
School of Paediatrics and Child Health, University of Western Australia, Perth, WA, Australia

M. Thomas
Royal Perth Hospital, East Perth, WA 6001, Australia

J. Fletcher
Royal Adelaide Hospital, Adelaide 5000, SA, Australia

J. Fletcher
SA Pathology, Adelaide, SA 5000, Australia

Abstract *Background*: In Australia, enzyme replacement therapy (ERT) for Fabry Disease (FD), both Agalsidase alfa (Replagal, Shire HGT) and beta (Fabrazyme, Genzyme), is funded and monitored through a specific government program. Agalsidase beta supply has been rationed by Genzyme since 2009 due to manufacturing issues. Consequently, the Australian Fabry Disease Advisory Committee has treated patients on Agalsidase beta at 50% of their usual dose from mid-2009, with a further reduction to 30% for some patients from late 2009.

Aim: To determine the clinical effect of Agalsidase beta dose reduction in the Australian FD patient cohort.

Methods: A questionnaire assessing FD symptoms was administered to 40 patients on long-term ERT. Clinical data from The Fabry Registry for patients receiving Agalsidase alfa or beta, for at least 2 years prior to the time of enforced Agalsidase beta dose reduction, were reviewed. Disease burden and quality of life (QOL) were graded using the Disease Severity Scoring System, Mainz Severity Score Index, Brief Pain Inventory and Short Form 36 Health Survey at 2 years before dose reduction, at the time of dose reduction and at the most recent clinical review following dose reduction.

Results: Disease severity and QOL scores did not change between the ERT groups. Males on Agalsidase beta reported lower energy levels after dose reduction, while no change was reported by females on either product or by males on a stable dose of Agalsidase alfa.

Conclusion: This study suggests that energy levels in male patients worsen after dose reduction of Agalsidase beta.

Introduction

Fabry Disease (FD; MIM 301500) is associated with reduced quality of life (QOL) compared to the general population, particularly in affected males (Wilcox et al. 2004; Hoffmann et al. 2005). Consistent with the natural history of the disease, QOL continues to deteriorate in untreated FD patients with advancing age, independent of normal aging effect (Miners et al. 2002). We report on the course of FD symptoms and disease burden following an enforced dose reduction in Agalsidase beta therapy.

Two commercially available forms of human enzyme replacement therapy (ERT) are approved for use in FD in Australia: Agalsidase beta (Fabrazyme, Genzyme) at the approved dose of 1 mg/kg/2 weeks and Agalsidase alfa (Replagal, Shire HGT) at 0.2 mg/kg/2 weeks. In Australia, access to either Agalsidase alfa or beta is federally subsidized through the Life Saving Drugs Program (LSDP). Under this program, all patients have a structured review by a Fabry physician and undergo prescribed medical tests and QOL assessments every 6 months (LSDP 2010). This information is reviewed at least annually by the Fabry Disease Advisory Committee as part of the process for ongoing funding.

Since June 2009, a number of manufacturing problems have resulted in a worldwide shortage of Agalsidase beta. After Genzyme introduced dose-rationing measures in mid-2009, a decision was made by the Australian Fabry Disease Advisory Committee to initially continue treating FD patients with Agalsidase beta at 50% of their usual dose, with a further reduction in some patients to 30% from late 2009 until late 2010. The situation presented a unique opportunity to evaluate the impact of reduced Agalsidase beta dose on FD patients.

Systematic review of short-term randomized placebo controlled trials suggest benefit from treatment of FD with either Agalsidase alfa (improvement in pain and QOL scores) or Agalsidase beta (improved GL3 clearance from renal, cardiac, and skin biopsies) (El Dib and Pastores 2010). In uncontrolled studies, Agalsidase alfa improved QOL and pain, produced sustained reduction in left ventricular hypertrophy (LVH) and reduced the rate of decline of estimated glomerular filtration rate (eGFR) (Hoffmann et al. 2005; Mehta et al. 2009). Efficacy in improving pain, disease burden scores, and LVH reduction has been shown in female patients (Whybra et al. 2009). Agalsidase beta has been shown to improve QOL in male and female patients (Watt et al. 2010) and to slow progression to the combined outcome of death, renal, cardiac, and cerebrovascular events in patients with mild-to-moderate renal disease (Banikazemi et al. 2007).

Agalsidase alfa and beta differ only in glycosylation pattern: Agalsidase beta contains more mannose-6-phosphate (M6P), allowing greater M6P receptor mediated uptake by cultured human Fabry fibroblasts and in the kidney, spleen and heart cells of Fabry mice (Lee et al. 2003; Sakuraba et al. 2006). In vivo studies demonstrate similar antigenic profiles and the potency of both enzymes is dose-dependent (Lee et al. 2003; Sakuraba et al. 2006). It is unclear whether the two products differ in clinical efficacy or whether the approved dose of either product is optimal. One systematic review has suggested that Agalsidase beta at a dose of 1 mg/kg/2 weeks has more robust evidence for efficacy than Agalsidase alfa at 0.2 mg/kg/2 weeks, however, head-to-head studies between the two are lacking (Schaefer et al. 2009). Direct comparison of both ERT products at the same dose (0.2 mg/kg/2 weeks) in 34 patients over 24 months failed to show any difference, although the clinical benefit of either product was less than anticipated (Vedder et al. 2007). In 11 male FD patients with declining renal function on Agalsidase alfa 0.2 mg/kg/2 weeks, Agalsidase alfa at 0.2 mg/kg delivered weekly for 24 months significantly slowed the rate of eGFR decline (Schiffmann et al. 2007). Reduced proteinuria in patients receiving high dose (0.4 mg/kg/2 weeks) Agalsidase alfa has also been reported (Torra et al. 2008). Eighteen months of Agalsidase beta therapy at a dose of 0.3 mg/kg/2 weeks, after patients had received 1 mg/kg/2 weeks for 6 months, did not result in an increase in mean plasma globotriaosylceramide (GL3) levels, although some patients in whom plasma GL3 levels normalized recorded elevated levels after dose reduction (Lubanda et al. 2009). Clearance of GL3 from renal capillary endothelium was seen in 100% of patients after 6 months of ERT, and while no significant increase in GL3 accumulation was evident after 18 months at 0.3 mg/kg/2 weeks, small patient numbers limit the conclusions that can be made from this study (Lubanda et al. 2009). Thus, uncertainty remains as to the optimal dose of ERT relevant to clinical outcomes in FD.

Methods

This study utilized The Fabry Registry data of all Australian FD patients receiving ERT, plus a brief custom-designed questionnaire regarding FD symptoms and attitude to changing ERT product. Specific data used in this study, prospectively collated in The Fabry Registry, are listed in Table 1.

Questionnaire

One year after the dose reduction of Agalsidase beta, all Australian patients who had already received Agalsidase beta for a minimum of 18 months, including 6 months of standard dose ERT before 12 months of reduced dose

Table 1 Clinical information from The Fabry Registry used in this study

Medical history, family pedigree, standardized neurological examination
Patient reports of sweating, gastrointestinal symptoms, pain
Pain and Quality of Life scores (Brief Pain Inventory-short form, Short Form-36 Health Survey)
Electrocardiogram, echocardiogram (angiogram and cardiac MRI results if available)
MRI/CT brain scan
Pulmonary function tests
Creatinine and estimated glomerular filtration (eGFR), timed urine collection (creatinine clearance, proteinuria, albumin excretion rate) or spot urine (albumin:creatinine ratio or protein:creatinine ratio), end stage renal failure requiring dialysis or renal transplantation
Genotype
ERT commencement date, current and previous administered doses Plasma GL3 levels and anti-α-galactosidase antibody titre (if available) Plasma GL3 levels (if available)

Table 2 Questionnaire administered to all ERT patients

Are you receiving Fabrazyme or Replagal?
For patients on Agalsidase beta (Fabrazyme): We would like to find out about your attitude to changing to a different type of enzyme replacement therapy, Agalsidase alfa (Replagal). Do you wish to change from Fabrazyme to Replagal or do you wish to remain on Fabrazyme? What is the reason for wishing to change therapy/stay on your current therapy?
For all patients: Compared to this time last year, have you noticed a change in your:
Sweating?
Cold tolerance?
Heat tolerance?
Pain?
(If yes to any of these, please describe your symptoms).
On a scale of 0–10, (with 0 representing no energy and 10 representing feeling full of energy), how would you grade your energy at the moment?
On a scale of 0–10, how would you grade your energy this time last year?
Do you experience diarrhea?
If yes, how many bouts per month?
How many motions per day at the most?
Do you experience abdominal pain or cramping?
If yes, how many days per week?
Are you working? How many hours per week? What does your work involve?
Do you do any regular exercise? How many times per week? How long each time?
Do you notice any difference in how you feel before and after the infusions each fortnight? If yes, what have you noticed?
Have you ever had any infusion reactions? If yes, can you describe them?

therapy, were invited to complete the questionnaire (Table 2).

Serial Assessment of Disease Burden and Quality of Life

Data were collated for all Australian patients who had been on ERT, either Agalsidase alfa or beta, for at least 2 years before dose reduction and for whom Registry data from clinical review in 2010 was available. This data was graded using two tools (Table 3): the Mainz Severity Score Index and the Disease Severity Scoring System (DS3), validated to assess FD burden in classically affected male patients and in the general FD population, respectively (Whybra et al. 2004; Giannini et al. 2010). Disease severity was graded at 3 time points for each patient: 24 months prior to dose reduction, at the time of dose reduction and at the most recent clinical review. To assess the impact of Agalsidase beta dose reduction on patient QOL over this period, results of QOL assessment tools were collated at each of these 3 time points. These included the Brief Pain Inventory (BPI) "worst pain in the last 24 h" and "average pain" scores and Short Form 36 Health Survey (SF36) Mental Component Summary (MCS) and Physical Component Summary (PCS) scores. These tools and their domains have been previously validated and utilized to assess FD symptoms and response to therapy (Schiffmann et al. 2001; Cleeland 2002; Gold et al. 2002).

The New York Heart Association (NYHA) class and the SF36 are valid criteria of heart failure symptoms (Garin et al. 2009). As neither NYHA symptom class nor specific patient symptoms (such as wellbeing, fatigue, and activity level) are currently collected by The Fabry Registry, surrogate measures for these parameters were used in estimating MSSI and DS3 scores. NYHA class I–IV was inferred from the SF36 Physical Functioning (PF) score (quartile 4 = NYHA I, 3 = NYHA II, 2 = NYHA III, 1 = NYHA IV). Reduced activity and fatigue levels (used in MSSI) were inferred if the SF36 PF and Vitality (VT) scores, respectively, were <50%. Assessment of pain in DS3 scoring covers 6 grades and was inferred from the SF36 Bodily Pain (BP) scores. Gastrointestinal (GI) symptoms were graded for the DS3 as shown in Table 4. The DS3 patient "self-report of wellbeing" (a domain of the DS3, scored 0–4) was inferred by averaging the SF36 PCS and MCS scores. These surrogate measurements represent 8% and 24% of the total MSSI and DS3 scores, respectively. The validity of these surrogate markers was confirmed not to affect the total MSSI or DS3 results, as scored using the primary data from 17 patients treated at a single centre where data for NYHA, activity and fatigue scores were all available. Data for all other parameters required by the MSSI and DS3 were obtained directly from the Registry.

Table 3 Disease burden scoring systems utilized in this study

Scoring System		Score
The Mainz Severity Score Index (MSSI)		
General score	Characteristic facies, angiokeratoma, edema, musculoskeletal, cornea verticillata, diaphoresis, abdominal pain, diarrhea/constipation, hemorrhoids, pulmonary, NYHA	18
Neurological	Tinnitus, vertigo, acroparesthesia, fever pain crisis, cerebrovascular (ischemic lesions in MRI/CT, TIA, migraine, stroke), psychiatric/psychosocial (depression, fatigue, reduced activity level)	20
Cardiovascular	Changes in cardiac muscle thickness, valve insufficiency, ECG abnormalities, pacemaker, hypertension	20
Renal	Evidence of renal dysfunction (proteinuria, tubular dysfunction, serum creatinine levels > 3.5 mg/dL, dialysis)	18
	Maximum score	76
Disease severity: mild (<20), moderate (20–40) or severe (>40)		
The Disease Severity Scoring System (DS3)		
PNS	Sweating, gastrointestinal, pain	4
Renal	eGFR, proteinuria, eGFR slope	8
Cardiac	LVH, arrhythmia, NYHA score	8
CNS	White matter lesions, TIA/stroke	8
Self report of wellbeing		4
	Maximum score	32

CNS central nervous system, eGFR estimated glomerular filtration rate, LVH left ventricular hypertrophy, NYHA New York Heart Association score, PNS peripheral nervous system, TIA transient ischemic attack

Statistical analyses were conducted using Graphpad Prism v5.03 (1992–2010 GraphPad Software Inc) and included Mann Whitney tests, Fisher's exact t-test, Wilcoxon matched pairs signed rank tests for nonparametric data and one- and two-way ANOVA for group comparisons over time.

Results

Questionnaire

Of 43, 40 eligible patients completed the questionnaire (93%): 28 Agalsidase beta patients (23 males, 5 females) and 12 Agalsidase alfa patients (9 males, 3 females). Table 5 indicates the mutation class responsible for FD in the patients completing the questionnaire. At the time of the questionnaire, the median age of Agalsidase beta and alfa patients was 46 years (range 25–71 years) and 40 years (range 22–64 years), respectively; this difference was not statistically significant (Mann Whitney $p = 0.29$). Median duration of ERT prior to the time of Agalsidase beta dose reduction was 73.5 months (range 16–138) for Agalsidase beta patients and 100 months (range 38–118) for Agalsidase alfa patients (Mann Whitney $p = 0.06$). Median doses of Agalsidase beta, administered every 2 weeks, before and after the initial 50% dose reduction were 70 mg (range 50–90 mg) and 35 mg (range 15–65 mg), respectively. Of the 28, 14 Agalsidase beta patients completing the questionnaire had a second dose reduction to 0.3 mg/kg, but this did not significantly affect the final median dose in the whole group of 28 patients (35 mg, range 10–65 mg).

Table 4 Scoring of gastrointestinal symptoms (including abdominal pain and diarrhea) for the DS3

Score	Abdominal pain	Diarrhea
0	None	None
1	None	Monthly or weekly
2	None	Daily
	OR	
	Monthly	None or monthly
3	Monthly	Weekly or daily
	OR	
	Weekly	None or monthly
4	Weekly	Daily or Weekly
5	Weekly or daily	Daily

Table 5 Mutations for male and female FD patients on ERT

Mutation	Agalsidase beta		Agalsidase alfa	
	Males ($n = 23$)	Females ($n = 5$)	Males ($n = 9$)	Females ($n = 3$)
Missense	15	5	4	1
Intronic splice site	1	0	0	0
Nonsense	5	0	0	0
Deletion	2	0	4	2
Insertion	0	0	1	0

Self-Reported Energy Levels

Of the 28, 27 Agalsidase beta patients were able to describe their energy levels at the time of the questionnaire compared to 12 months earlier. There was a significant change in energy levels reported in Agalsidase beta patients between 2009 and 2010 (ANOVA $p = 0.03$; see Fig. 1). The decrease in energy levels (scored 0–10) over this

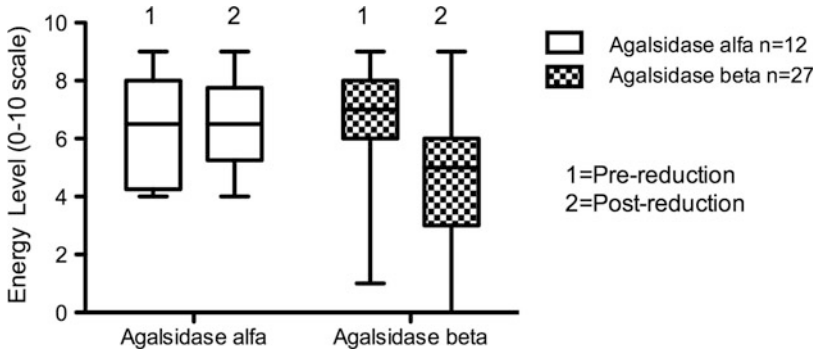


Fig. 1 Energy level for patients receiving enzyme replacement therapy. Anova $p = 0.03$. Note: One patient in the Agalsidase beta group has been excluded from this analysis, as this patient was unable to recall their energy levels 12 months earlier

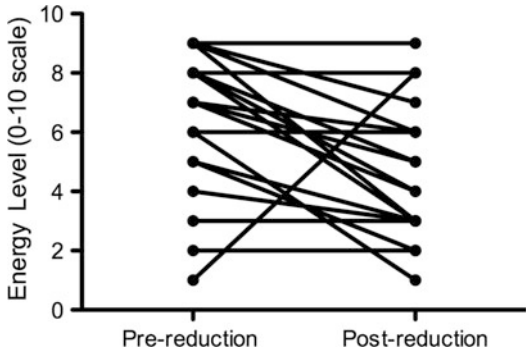


Fig. 2 Energy level in male Agalsidase beta patients ($n = 22$). Wilcoxon matched-pairs signed rank test $p = 0.007$

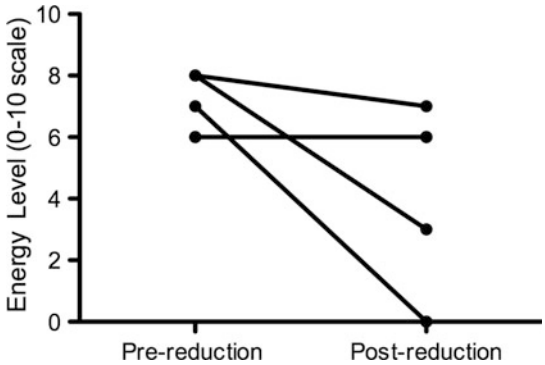


Fig. 3 Energy level in female Agalsidase beta patients ($n = 5$). Wilcoxon matched-pairs signed rank test. $p = 0.25$. Note: 2 patients recorded the same energy level scores pre- and postreduction

12-month period was significant in males receiving Agalsidase beta (median scores of 7 in 2009 and 5 in 2010, range 1–9 for both; Wilcoxon signed rank test $p = 0.007$), but not in females (median scores of 7 in 2009 and 6 in 2010, ranges 6–8 and 0–9, respectively; Wilcoxon signed rank test $p = 0.25$; Figs. 2 and 3). There was no significant change in reported energy score in patients on Agalsidase alfa: male (median scores of 8 in 2009 and 7 in 2010, ranges 4–9 and 5–9, respectively, Wilcoxon signed rank test $p = 0.77$) and female (median scores of 4 in 2009 and 2010, ranges 4–6 and 4–7, respectively, Wilcoxon signed rank test $p = 1.0$).

Impact on Symptoms of Sweating, Heat and Cold Tolerance, Pain, Diarrhea and Abdominal Pain and Differences in Wellbeing After Each Infusion

No significant changes in sweating, heat, or cold tolerance, pain or post-infusion symptoms were identified in any patient group during this period. At the time of the questionnaire, there was no difference in diarrhea or abdominal pain between those on reduced dose Agalsidase beta and standard dose Agalsidase alfa (Fisher's exact t-test $p = 0.16$ and $p = 0.49$, respectively).

Attitude to Changing ERT to Agalsidase Alfa

At the time of questionnaire, 8 of the 23 male Agalsidase beta patients were considering changing product, 1 was uncertain and 14 wished to remain on Agalsidase beta. Two of the 5 female patients were considering changing and 3 did not wish to change. Reasons given for wishing to change ERT from Agalsidase beta to alfa included concern over ongoing supply issues ($n = 5$), perceived worsening of symptoms or change in test results since dose reduction ($n = 3$), immediate home infusion availability ($n = 1$) and because a relative was changing ($n = 1$).

Of the 17 patients who did not wish to change product, a dominant reason was provided by 13:8 were content with current therapy, 3 expressed concerns of infusion reactions if therapy was changed, 1 perceived the alternative therapy as inferior, and 1 wished to defer the decision pending physician's review of test results.

Self-Report of Infusion Reactions

More patients on Agalsidase beta (14 of 28) recalled having infusion reactions at the start of their ERT compared to none of the 12 patients on Agalsidase alfa (Fisher's exact

t-test $p = 0.003$), but there was no statistically significant difference in reported infusion reactions between male and female patients receiving Agalsidase beta (13 of 23 Vs 1 of 5, Fisher's exact t-test $p = 0.33$).

Disease Burden and Quality of Life

Data were available to allow DS3 and MSSI scoring at all time points in 26 Agalsidase beta patients (23 males, 3 females) and 7 Agalsidase alfa patients (6 males, 1 female), all of whom had received full-dose ERT for at least 2 years prior to any dose reduction. Table 6 reports the baseline characteristics of these patients; 79% of patients on Agalsidase beta who had antibody testing performed were seropositive. As the 6-month interval data was provided to the Registry asynchronously for each patient, it was not possible to grade disease severity at the same time point for each patient in relation to dose reduction. Follow-up data was graded at a median of 7.5 months (range 3–12) post-dose reduction for Agalsidase beta patients and at a median of 9 months (range 8–12) follow-up for Agalsidase alfa patients.

Disease Burden

Two years prior to dose reduction, median MSSI scores between ERT groups were similar: 26 (range 12–49) in Agalsidase beta patients and 25 (range 20–44) in Agalsidase alfa patients (Mann Whitney $p = 0.89$). This represents a "moderate" burden of disease in both groups of patients. Similarly, median DS3 scores of Agalsidase beta patients 2 years before dose reduction (12, range 5–22) were not different from those of Agalsidase alfa patients (8, range 5–18; Mann Whitney $p = 0.42$). There was no significant change in DS3 or MSSI scores (Figs. 4 and 5), or in any of the individual domain scores on either tool, between patients receiving either ERT over the period of the study. The "peripheral nervous system" domain of the DS3 includes a score for GI symptoms. The median score for GI symptoms in those receiving Agalsidase beta after dose reduction was 2 (range 0–5), compared to a median score of 1 (range 0–4) for Agalsidase alfa patients; however, this failed to reach statistical significance (ANOVA $p = 0.55$). There was no difference in GI symptom score between males receiving either form of ERT or between males and females treated with Agalsidase beta.

Quality of Life

Average and worst pain BPI scores 24 months before dose reduction were 1 (range 0–7) and 2 (range 0–8), respectively, for Agalsidase beta patients, and 1 (range 0–4) and 2 (range 0–8), respectively, for Agalsidase alfa patients. Over the study period, no significant change between the

Table 6 Baseline characteristics of patients receiving Agalsidase alfa or beta included in the assessment of disease burden and quality of life

	Agalsidase alfa	Agalsidase beta	Mann Whitney test (*p* value)
Males	$n = 6$	$n = 23$	
Age at ERT commencement (yrs)	30 (14–54)	38 (21–69)	0.12
ERT duration (months)	114 (22–118)	87 (20–138)	0.03
LVH (mm)	12.5 (9–20)	14 (7.1–27)	0.64
Proteinuria (g/24 h)	0.1 (0.06–0.63)	0.23 (0–3.1)	0.51[a]
eGFR (mL/min/1.73 m^2)	84 (49–137)	69 (40–125)	0.68[a]
Number with ESRF requiring RRT (%)	1	6	1.00[b]
Number with antibody testing performed	1	14	
Number who were antibody positive (%)	0	11	
Peak antibody titre	–	1:600 (0-1:3200)	
Females	$n = 1$	$n = 3$	
Age at ERT commencement (yrs)	54	49 (43–62)	
ERT duration (months)	114	36 (16–74)	
LVH (mm)	11	16 (11–19)	
Proteinuria (g/24 h)	1.2	0.27 (0.11–0.69)	
eGFR (mL/min/1.73 m^2)	63	76 (73–85)	

eGFR estimated glomerular filtration rate, *ESRF* end-stage renal failure, *LVH* left ventricular hypertrophy, *RRT* renal replacement therapy

Note: Results are presented as median with range in parentheses. No female patient had ESRF requiring RRT or assessment of their antibody status performed.

[a] Patients with ESRF were excluded from proteinuria and eGFR comparisons

[b] Assessed by Fisher's exact t-test

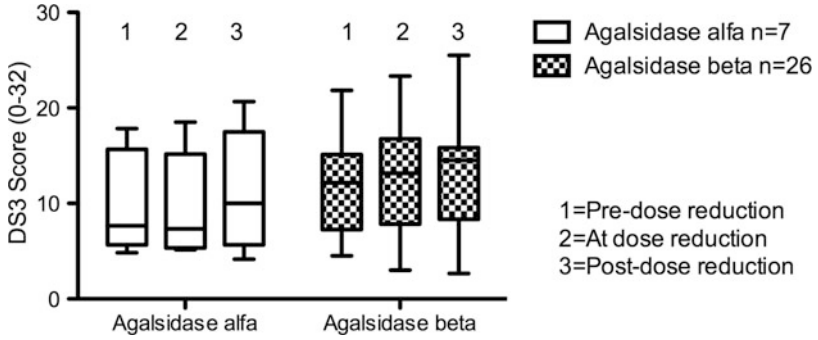


Fig. 4 Disease Severity Scoring System (DS3) total scores over time for patients receiving enzyme replacement therapy. ANOVA $p = 0.8$

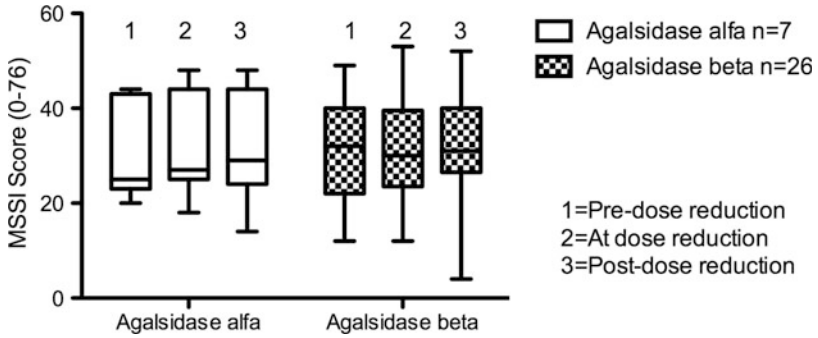


Fig. 5 Mainz Severity Score Index (MSSI) total scores over time for patients receiving enzyme replacement therapy. ANOVA $p = 0.92$

ERT groups was found in average or worst pain scores (ANOVA $p = 0.27$ and $p = 0.05$, respectively). There was no significant change in worst pain scores for male and female patients receiving Agalsidase beta (ANOVA $p = 0.69$ and $p = 0.65$ respectively), or in the six male Agalsidase alfa patients (ANOVA $p = 0.46$). Using the MCS and PCS results of the SF36, neither score changed significantly over the study period for patients receiving Agalsidase beta and alfa (MCS: ANOVA $p = 0.3$; PCS: ANOVA $p = 0.22$).

Plasma GL3

Levels of GL3 were measured before commencement of ERT in 10 male and 2 female patients receiving Agalsidase beta. No GL3 levels prior to ERT commencement for patients receiving Agalsidase alfa were recorded in The Fabry Registry. The pre-ERT median GL3 level for males on Agalsidase beta was 6.37 μg/mL (range 2.7–10.3); in the case of the 2 female patients, pre-ERT levels were 3 and 3.5 μg/mL. There was no correlation between pre-ERT baseline GL3 levels and change in energy level after dose reduction in the 10 males on Agalsidase beta therapy (Spearman correlation $r = 0.2$). The change in energy level reported by males on Agalsidase beta did not differ between those with a GL3 measurement prior to ERT commencement and those without a baseline GL3 level (Mann Whitney $p = 0.97$).

Discussion

Our male FD patients reported reduced energy levels 1 year after reduction in Agalsidase beta dose. The five female patients treated with Agalsidase beta did not report decreased energy levels after dose reduction, but female FD patients might be expected to be less severely affected by ERT dose reduction, given their higher level of endogenous α-galactosidase. It is certainly possible that the psychological effects of dose reduction impact on subjective symptoms, although we could not detect changes in mental health scores within the SF36. “Energy level” is not captured in either of the disease severity scores used in this study, only in our questionnaire. “Self report of wellbeing” in the DS3 did not change over the study period, suggesting that short-term dose reduction does not affect patient well-being, or that the surrogate used to measure well-being (average of the SF36 MCS and PCS results) inadequately assesses this parameter. Neither MCS nor PCS scores changed in either ERT group over the study period. Our patients reported no significant change in “average pain” or higher “worst pain scores” after Agalsidase beta dose was reduced. Ongoing clinical evaluation will be important to verify these effects, as there is considerable variability in pain patterns between patients and over time for a single FD patient, whether or not on ERT.

The unusual supply problem of Agalsidase beta for FD patients on long-term ERT has presented a unique opportu-

nity to assess clinical efficacy at reduced dose. While the glycosylation pattern of the two ERT products varies, their amino acid sequences are identical. If the products are equipotent, it is interesting that Australian patients receiving Agalsidase beta at 0.5 mg/kg/2 weeks report worse energy levels over the study period compared to patients receiving a stable dose of Agalsidase alfa at 0.2 mg/kg/2 weeks. Various factors determine the product choice of individual Australian physicians, including previous patient participation in clinical trials, unit workload, disease phenotype in relation to published data, and perceptions by patients, families, or physicians that the chosen product, at its approved dose, is superior. There are limitations and biases within this study. Agalsidase beta patients were not blinded to their ERT dose, which may induce anxiety and influence symptom perception. The retrospective nature of the questionnaire has relied on patient's recall of energy levels, which may not be accurate. The SF36, DS3, and MSSI may not be sensitive tools to allow accurate longitudinal assessment of energy levels. Other variables affecting perceived energy levels, such as concurrent illness or depression (possibly more common in male FD patients than females) (Cole et al. 2007), have not been evaluated in this study.

To assess the impact of a reduction in Agalsidase beta dose on clinical disease state, we have relied on clinical tools to assess disease load. The size of the patient cohort in this study and the slow, progressive nature of disease manifestations in FD means detecting early progression (or regression) of disease in response to changes in therapy is challenging and unlikely to be identified on studies such as this one. Ideally, a biomarker correlating with clinical disease state or overall disease burden would be helpful for the assessment of therapies and the prediction of prognosis. Elevated plasma and urinary GL3 typically reduce to normal levels after initiation of ERT (Young et al. 2005) and urinary GL3 levels increased when FD patients (already treated with 1 year of standard dose Agalsidase beta therapy) were given only 0.3 mg/kg/2 weeks of Agalsidase beta (Lubanda et al. 2009). However, urinary and plasma GL3 have not been shown to be optimal biomarkers; indeed, there is no validated biomarker to monitor response to therapy in this disease at present (Aerts et al. 2011). Plasma globotriaosylsphingosine (lysoGb3) is elevated in male and female FD patients (Aerts et al. 2008; Togawa et al. 2010). van Breemen et al. have demonstrated that plasma lysoGb3 levels decrease after 3 months of ERT (and these lower levels are sustained after 12 months of ERT) and that a dose-response exists, as patients given Agalsidase beta 1 mg/kg/2 weeks had a greater reduction in lysoGb3 levels than those treated with Agalsidase alfa or beta at 0.2 mg/kg/2 weeks (van Breemen et al. 2011). Plasma lysoGb3 has also been identified as an independent risk factor for white matter lesions in males and LVH in females, with lifetime exposure to plasma lysoGb3 correlating with MSSI score, and may therefore prove to be a clinically useful biomarker in FD (Rombach et al. 2010).

Patients treated with ERT frequently develop IgG antibodies to the recombinant α-galactosidase protein, especially male FD patients who have little or no endogenous production of this enzyme. The delivered dose of enzyme and the type of recombinant enzyme may be important factors in the generation of antibodies, with a number of studies showing a higher prevalence of seropositivity among patients treated with Agalsidase beta (in the order of 80–90%) compared with Agalsidase alfa (~40–60%) (Eng et al. 2001; Linthorst et al. 2004; Schiffmann et al. 2006; Vedder et al. 2008; van Breemen et al. 2011). The prevalence of seropositivity for anti-α-galactosidase antibodies within our Australian cohort is consistent with that described. When Agalsidase alfa and beta are administered at 0.2 mg/kg/2 weeks, some authors still found a higher prevalence of anti-α-galactosidase IgG antibodies in patients treated with Agalsidase beta (van Breemen et al. 2011), citing differences in the cell lines used in the production of these recombinant enzymes as a possible explanation (Pastores and Thadhani 2001; Beck 2002). However, others have not demonstrated this finding (Vedder et al. 2008). The presence of antibodies has been shown to bind circulating enzyme, inhibiting its enzymatic activity in vitro and in vivo, with the neutralizing capacity of antibodies to one recombinant form of α-galactosidase being cross-reactive toward the alternative form of ERT (Linthorst et al. 2004; Ohashi et al. 2008). Therefore, concern exists that these antibodies may interfere with the clinical benefit that is hoped to be gained through ERT; the antibody level and the dose of ERT administered to seropositive patients may be important factors to consider in treatment. Increased urinary GL3 levels have been demonstrated in seropositive patients compared to antibody negative patients, although some seropositive patients do demonstrate normalization of urinary GL3 levels after 12 months of ERT (Linthorst et al. 2004; Ohashi et al. 2007). Fabry mice had reduced α-galactosidase activity within their major organs (including heart and kidneys) after infusion of Agalsidase beta incubated with serum from seropositive patients, compared to those treated with an equal dose of enzyme pre-incubated with serum from seronegative patients (Ohashi et al. 2008). However, a tenfold increase in enzyme dose resulted in increased enzyme activity in all tissues and enzyme activity was similar between seropositive and seronegative patients (Ohashi et al. 2008). Anti-α-galactosidase IgG titer was not found to correlate with a rate of change in eGFR, onset of clinical events or plasma GL3 elevations after 5 years of Agalsidase beta therapy in 134 male and female FD patients, although patients with a high antibody titer were

found to have significantly increased GL3 deposition within dermal capillary endothelial cells during this study, suggesting that plasma GL3 clearance is impaired in the presence of a high antibody titer (Benichou et al., Mol Genet Metab 2009). The shortage of Agalsidase beta has raised practical questions regarding dose of ERT in patients who have been recipients of long-term therapy, the majority of whom have antibodies to the enzyme. What is the clinical implication of a reduction in ERT dose after a patient has received standard dose ERT for many years? Is the background burden of disease an important factor in determining suitability for dose reduction? Should males receiving Agalsidase beta with anti-α-galactosidase antibodies be excluded from a reduction in ERT dose? When supply is restored, should they be given a higher dose of ERT than the "standard" therapeutic dose? Will there be an increase in antibody titer when full dose Agalsidase beta therapy is reinstituted? Are they at greater risk of disease deterioration with prolonged dose reduction? These questions draw attention to the concept of individualized ERT for FD patients to ensure they obtain maximal clinical benefit from this treatment, and warrants ongoing research.

Consistent supply of ERT was a concern for Agalsidase beta patients and a reason for consideration of changing product. However, most patients on Agalsidase beta preferred to continue at the reduced dose, 12 months after dose reduction. A minority feared infusion reactions if their ERT product was changed. The incidence of infusion reactions with Agalsidase beta recalled by patients in this study was consistent with that reported in other work (Wilcox et al. 2004; Keating and Simpson 2007) and lower than in other reports of Agalsidase beta therapy (Ries et al. 2006; Pastores et al. 2007).

Notwithstanding its limitations, our study found that male Australian FD patients report lower energy levels on reduced dose Agalsidase beta therapy. Clinical disease burden and QOL, as measured by the SF36 Health Survey, did not change during the study period. Close monitoring of clinical parameters will be required to identify if QOL deteriorates further and if disease progression has been affected. Currently, patients on either product are administered a single dose irrespective of the phase or severity of their condition or duration of ERT. The analysis of FD patients on lower doses due to supply problems, together with data from long-term trials directly comparing Agalsidase alfa and beta therapy, may help define optimal ERT doses in patients receiving ERT over many years.

Acknowledgements We thank our Fabry disease coordinators and nurse specialists at all Australian treatment centers for their commitment to retrieving data, in maintaining The Fabry Registry and in assisting with this study.

Synopsis

Australian male Fabry disease patients receiving long-term enzyme replacement therapy (ERT) with Agalsidase beta report reduced energy levels (without a change in disease severity or other quality of life measures) following a 50% reduction in Agalsidase beta dose, as a consequence of the global shortage of this product.

Declarations

All authors have received travel support from Genzyme and/or Shire HGT. Dr Nicholls has received research support from Genzyme and Shire HGT. Dr Fletcher and Prof Sillence have received speaker honoraria from Genzyme. Dr Denaro and Prof Sillence have received coordinator support from Genzyme. Genzyme supports Registry data entry for all centers.

Disclaimer

This paper has been produced with the cooperation of the Australian Government Department of Health and Ageing, which funds and administers the Life Saving Drugs Program (LSDP). The analysis which forms the basis of this paper was conducted by the authors without the use or disclosure of personal information of any patient of the LSDP. Any views expressed or conclusions drawn in the paper are entirely those of the authors and do not reflect any views of the Department or the Australian Government.

References

Aerts JM, Groener JE, Kuiper S, Donker-Koopman WE, Strijland A, Ottenhoff R, van Roomen C, Mirzaian M, Wijburg FA, Linthorst GE, Vedder AC, Rombach SM, Cox-Brinkman J, Somerharju P, Boot RG, Hollak CE, Brady RO, Poorthuis BJ (2008) Elevated globotriaosylsphingosine is a hallmark of Fabry disease. Proc Natl Acad Sci U S A 105(8):2812–2817

Aerts JM, Kallemeijn WW, Wegdam W, Joao Ferraz M, van Breemen MJ, Dekker N, Kramer G, Poorthuis BJ, Groener JE, Cox-Brinkman J, Rombach SM, Hollak CE, Linthorst GE, Witte MD, Gold H, van der Marel GA, Overkleeft HS, Boot RG (2011) Biomarkers in the diagnosis of lysosomal storage disorders: proteins, lipids, and inhibodies. J Inherit Metab Dis 34:605–619

Banikazemi M, Bultas J, Waldek S, Wilcox WR, Whitley CB, McDonald M, Finkel R, Packman S, Bichet DG, Warnock DG, Desnick RJ (2007) Agalsidase-beta therapy for advanced Fabry disease: a randomized trial. Ann Intern Med 146(2):77–86

Beck M (2002) Agalsidase alfa–a preparation for enzyme replacement therapy in Anderson-Fabry disease. Expert Opin Investig Drugs 11(6):851–858

Benichou B, Goyal S, Sung C, Norfleet AM, O'Brien F (2009) A retrospective analysis of the potential impact of IgG antibodies to agalsidase beta on efficacy during enzyme replacement therapy for Fabry disease. Mol Genet Metab 96(1):4–12

Cleeland CS (2002) Pain assessment: the advantages of using pain scales in lysosomal storage diseases. Acta Paediatr Suppl 91(439):43–47

Cole AL, Lee PJ, Hughes DA, Deegan PB, Waldek S, Lachmann RH (2007) Depression in adults with Fabry disease: a common and under-diagnosed problem. J Inherit Metab Dis 30(6):943–951

El Dib RP, Pastores GM (2010) Enzyme replacement therapy for Anderson-Fabry disease. Cochrane Database Syst Rev 5:CD006663

Eng CM, Guffon N, Wilcox WR, Germain DP, Lee P, Waldek S, Caplan L, Linthorst GE, Desnick RJ (2001) Safety and efficacy of recombinant human alpha-galactosidase A–replacement therapy in Fabry's disease. N Engl J Med 345(1):9–16

Garin O, Ferrer M, Pont A, Rue M, Kotzeva A, Wiklund I, Van Ganse E, Alonso J (2009) Disease-specific health-related quality of life questionnaires for heart failure: a systematic review with meta-analyses. Qual Life Res 18(1):71–85

Giannini EH, Mehta AB, Hilz MJ, Beck M, Bichet DG, Brady RO, West M, Germain DP, Wanner C, Waldek S, Clarke JT, Mengel E, Strotmann JM, Warnock DG, Linhart A (2010) A validated disease severity scoring system for Fabry disease. Mol Genet Metab 99(3):283–290

Gold KF, Pastores GM, Botteman MF, Yeh JM, Sweeney S, Aliski W, Pashos CL (2002) Quality of life of patients with Fabry disease. Qual Life Res 11(4):317–327

Hoffmann B, Garcia de Lorenzo A, Mehta A, Beck M, Widmer U, Ricci R (2005) Effects of enzyme replacement therapy on pain and health related quality of life in patients with Fabry disease: data from FOS (Fabry Outcome Survey). J Med Genet 42(3):247–252

Keating GM, Simpson D (2007) Agalsidase Beta: a review of its use in the management of Fabry disease. Drugs 67(3):435–455

Lee K, Jin X, Zhang K, Copertino L, Andrews L, Baker-Malcolm J, Geagan L, Qiu H, Seiger K, Barngrover D, McPherson JM, Edmunds T (2003) A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. Glycobiology 13(4):305–313

Linthorst GE, Hollak CE, Donker-Koopman WE, Strijland A, Aerts JM (2004) Enzyme therapy for Fabry disease: neutralizing antibodies toward agalsidase alpha and beta. Kidney Int 66(4):1589–1595

LSDP (2010) Guidelines for the treatment of Fabry Disease through the Life Saving Drugs Program, August 2010

Lubanda JC, Anijalg E, Bzduch V, Thurberg BL, Benichou B, Tylki-Szymanska A (2009) Evaluation of low dose, after a standard therapeutic dose, of agalsidase beta during enzyme replacement therapy in patients with Fabry disease. Genet Med 11(4): 256–264

Mehta A, Beck M, Elliott P, Giugliani R, Linhart A, Sunder-Plassmann G, Schiffmann R, Barbey F, Ries M, Clarke JT (2009) Enzyme replacement therapy with agalsidase alfa in patients with Fabry's disease: an analysis of registry data. Lancet 374(9706):1986–1996

Miners AH, Holmes A, Sherr L, Jenkinson C, MacDermot KD (2002) Assessment of health-related quality-of-life in males with Anderson Fabry Disease before therapeutic intervention. Qual Life Res 11(2):127–133

Ohashi T, Sakuma M, Kitagawa T, Suzuki K, Ishige N, Eto Y (2007) Influence of antibody formation on reduction of globotriaosylceramide (GL-3) in urine from Fabry patients during agalsidase beta therapy. Mol Genet Metab 92(3):271–273

Ohashi T, Iizuka S, Ida H, Eto Y (2008) Reduced alpha-Gal A enzyme activity in Fabry fibroblast cells and Fabry mice tissues induced by serum from antibody positive patients with Fabry disease. Mol Genet Metab 94(3):313–318

Pastores GM, Thadhani R (2001) Enzyme-replacement therapy for Anderson-Fabry disease. Lancet 358(9282):601–603

Pastores GM, Boyd E, Crandall K, Whelan A, Piersall L, Barnett N (2007) Safety and pharmacokinetics of agalsidase alfa in patients with Fabry disease and end-stage renal disease. Nephrol Dial Transplant 22(7):1920–1925

Ries M, Clarke JT, Whybra C, Timmons M, Robinson C, Schlaggar BL, Pastores G, Lien YH, Kampmann C, Brady RO, Beck M, Schiffmann R (2006) Enzyme-replacement therapy with agalsidase alfa in children with Fabry disease. Pediatrics 118(3): 924–932

Rombach SM, Dekker N, Bouwman MG, Linthorst GE, Zwinderman AH, Wijburg FA, Kuiper S, Vd Bergh Weerman MA, Groener JE, Poorthuis BJ, Hollak CE, Aerts JM (2010) Plasma globotriaosylsphingosine: diagnostic value and relation to clinical manifestations of Fabry disease. Biochim Biophys Acta 1802(9):741–748

Sakuraba H, Murata-Ohsawa M, Kawashima I, Tajima Y, Kotani M, Ohshima T, Chiba Y, Takashiba M, Jigami Y, Fukushige T, Kanzaki T, Itoh K (2006) Comparison of the effects of agalsidase alfa and agalsidase beta on cultured human Fabry fibroblasts and Fabry mice. J Hum Genet 51(3):180–188

Schaefer RM, Tylki-Szymanska A, Hilz MJ (2009) Enzyme replacement therapy for Fabry disease: a systematic review of available evidence. Drugs 69(16):2179–2205

Schiffmann R, Kopp JB, Austin HA III, Sabnis S, Moore DF, Weibel T, Balow JE, Brady RO (2001) Enzyme replacement therapy in Fabry disease: a randomized controlled trial. JAMA 285(21):2743–2749

Schiffmann R, Ries M, Timmons M, Flaherty JT, Brady RO (2006) Long-term therapy with agalsidase alfa for Fabry disease: safety and effects on renal function in a home infusion setting. Nephrol Dial Transplant 21(2):345–354

Schiffmann R, Askari H, Timmons M, Robinson C, Benko W, Brady RO, Ries M (2007) Weekly enzyme replacement therapy may slow decline of renal function in patients with Fabry disease who are on long-term biweekly dosing. J Am Soc Nephrol 18(5): 1576–1583

Togawa T, Kodama T, Suzuki T, Sugawara K, Tsukimura T, Ohashi T, Ishige N, Suzuki K, Kitagawa T, Sakuraba H (2010) Plasma globotriaosylsphingosine as a biomarker of Fabry disease. Mol Genet Metab 100(3):257–261

Torra R, Algaba F, Ars E, Santin S, Fernandez-Llama P, Ballarin J (2008) Preservation of renal function in a patient with Fabry nephropathy on enzyme replacement therapy. Clin Nephrol 69(6): 445–449

van Breemen MJ, Rombach SM, Dekker N, Poorthuis BJ, Linthorst GE, Zwinderman AH, Breunig F, Wanner C, Aerts JM, Hollak CE (2011) Reduction of elevated plasma globotriaosylsphingosine in patients with classic Fabry disease following enzyme replacement therapy. Biochim Biophys Acta 1812(1):70–76

Vedder AC, Linthorst GE, Houge G, Groener JE, Ormel EE, Bouma BJ, Aerts JM, Hirth A, Hollak CE (2007) Treatment of Fabry disease: outcome of a comparative trial with agalsidase alfa or beta at a dose of 0.2 mg/kg. PLoS One 2(7):e598

Vedder AC, Breunig F, Donker-Koopman WE, Mills K, Young E, Winchester B, Ten Berge IJ, Groener JE, Aerts JM, Wanner C, Hollak CE (2008) Treatment of Fabry disease with different dosing regimens of agalsidase: effects on antibody formation and GL-3. Mol Genet Metab 94(3):319–325

Watt T, Burlina AP, Cazzorla C, Schonfeld D, Banikazemi M, Hopkin RJ, Martins AM, Sims K, Beitner-Johnson D, O'Brien F, Feldt-Rasmussen U (2010) Agalsidase beta treatment is associated with improved quality of life in patients with Fabry

disease: findings from the Fabry Registry. Genet Med 12(11): 703–712

Whybra C, Kampmann C, Krummenauer F, Ries M, Mengel E, Miebach E, Baehner F, Kim K, Bajbouj M, Schwarting A, Gal A, Beck M (2004) The Mainz Severity Score Index: a new instrument for quantifying the Anderson-Fabry disease phenotype, and the response of patients to enzyme replacement therapy. Clin Genet 65(4):299–307

Whybra C, Miebach E, Mengel E, Gal A, Baron K, Beck M, Kampmann C (2009) A 4-year study of the efficacy and tolerability of enzyme replacement therapy with agalsidase alfa in 36 women with Fabry disease. Genet Med 11(6):441–449

Wilcox WR, Banikazemi M, Guffon N, Waldek S, Lee P, Linthorst GE, Desnick RJ, Germain DP (2004) Long-term safety and efficacy of enzyme replacement therapy for Fabry disease. Am J Hum Genet 75(1):65–74

Young E, Mills K, Morris P, Vellodi A, Lee P, Waldek S, Winchester B (2005) Is globotriaosylceramide a useful biomarker in Fabry disease? Acta Paediatr Suppl 94(447):51–54, discussion 37–58

JIMD Reports
DOI 10.1007/8904_2011_47

RESEARCH REPORT

Miglustat Treatment May Reduce Cerebrospinal Fluid Levels of the Axonal Degeneration Marker Tau in Niemann–Pick Type C

Niklas Mattsson • Henrik Zetterberg • Simona Bianconi • Nicole M. Yanjanin • Rao Fu • Jan-Eric Månsson • Forbes D. Porter • Kaj Blennow

Received: 10 January 2011 / Revised: 14 March 2011 / Accepted: 17 March 2011 / Published online: 28 September 2011

Abstract *Introduction*: Niemann–Pick disease type C (NPC) is a lysosomal storage disorder that leads to progressive neurodegeneration. The glucosylceramide synthase blocker miglustat is being used to treat NPC, but monitoring of disease progression and treatment response is difficult. NPC patients have elevated cerebrospinal fluid (CSF) levels of total-tau (T-tau) indicating axonal degeneration, and increased CSF amyloid β (Aβ) indicating abnormal brain amyloid metabolism, but it is unknown if start of miglustat treatment affects these biomarker levels.

Methods: Biomarkers were measured in serial CSF samples from NPC patients who started miglustat between samplings ($N = 5$), were untreated at both samplings ($N = 5$) or received treatment during the whole study ($N = 6$) (median time between samplings 309 days [range 175–644]). CSF was analyzed for $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, α-cleaved soluble APP, β-cleaved soluble APP, T-tau and phospho-tau.

Results: T-tau levels decreased in patients who started miglustat treatment (median 955 [range 338–1,271] ng/L at baseline vs. 382 [187–736] ng/L at follow-up, $p = 0.043$). Untreated patients and continuously treated patients had stable levels ($p > 0.05$). No changes were seen in the other biomarkers.

Conclusion: Reduced CSF T-tau suggests that miglustat treatment might affect axonal degeneration in NPC. However, the results must be interpreted with caution and verified in future studies, since this pilot study was small, treatment was not randomized, and patients starting treatment had higher baseline CSF T-tau than untreated patients.

Communicated by: Robert Steiner.

Competing interests: None declared.

N. Mattsson (✉) · H. Zetterberg · J.-E. Månsson · K. Blennow
Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy, University of Gothenburg, Mölndal, Sweden

H. Zetterberg
e-mail: Henrik.zetterberg@clinchem.gu.se

J.-E. Månsson
e-mail: jan-eric.mansson@vgregion.se

K. Blennow
e-mail: kaj.blennow@neuro.gu.se

S. Bianconi · N.M. Yanjanin · R. Fu · F.D. Porter
Program in Developmental Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, DHHS, Bethesda, MD, USA

S. Bianconi
e-mail: biancons@mail.nih.gov

N.M. Yanjanin
e-mail: nyanjanin@mail.nih.gov

R. Fu
e-mail: fur@mail.nih.gov

F.D. Porter
e-mail: fdporter@mail.nih.gov

Introduction

Niemann–Pick type C disease (NPC) is a rare autosomal recessive lysosomal storage disorder with accumulation of cholesterol and glycosphingolipids in late endosomes and lysosomes (Pentchev et al. 1987; Zervas et al. 2001a, b). The incidence is about 1/150,000 live births (Vanier and Millat 2003). The clinical spectrum is broad, with presenting symptoms ranging from lethal fetal ascites to psychiatric disease in adults, but progressive neurodegeneration is

the major problem (Imrie et al. 2007; Sevin et al. 2007; Yerushalmi et al. 2002). Most patients have mutations in the *NPC1* gene that encodes a membrane protein involved in intracellular cholesterol transport (Ory 2004). Other patients have mutations in the *NPC2* gene that encodes a soluble lysosomal cholesterol-binding protein (Naureckiene et al. 2000). These mutations point to a defect in cholesterol transport in NPC, but the lipid accumulation is complex and the primary offending metabolite in the brain is disputed (Lloyd-Evans and Platt 2010).

Substrate reduction therapy is available with the glucosylceramide synthase inhibitor miglustat (*N*-butyldeoxynojirimycin, Zavesca®, Actelion Inc., Switzerland). Miglustat treatment seems to stabilize the neurological disease in a majority of NPC patients (Patterson et al. 2007, 2010; Pineda et al. 2009; Wraith et al. 2010). However, disease-monitoring may be difficult, and there is a need for improved biomarkers to identify treatment responders, and monitor progression and treatment effect (Galanaud et al. 2009; Platt and Lachmann 2009).

We recently showed that cerebrospinal fluid (CSF) levels of total-tau (T-tau) are elevated in NPC patients (Mattsson et al. 2011). Tau is a microtubule-stabilizing protein abundant in cortical axons, and increased CSF T-tau is considered a marker of axonal degeneration (Hampel et al. 2009). NPC patients also have increased CSF levels of β amyloid (Aβ) peptides (Mattsson et al. 2011). Aβ is formed through processing of the transmembranous amyloid precursor protein (APP) (Andreasson et al. 2007). Aberrant Aβ metabolism is at the core of pathological events in Alzheimer's disease (AD), where small Aβ oligomers are believed to exert synaptotoxicity and insoluble Aβ fibrils are deposited in extracellular plaques (Querfurth and LaFerla 2010; Zetterberg et al. 2010). In accordance with our recent in vivo patient data, experimental studies have indicated altered amyloid metabolism in NPC (Jin et al. 2004), which is influenced by cholesterol accumulation (Kosicek et al. 2010; Malnar et al. 2010), and might be related to development of pathology (Kodam et al. 2010). The increased CSF Aβ concentration is a unique finding, which has not been described in any other disease. The exact mechanism behind the increase remains unknown, but it might involve altered activity of Aβ-generating enzymes, impaired vesicular trafficking, lysosomal dysfunction, or combinations of these. Further links between NPC and AD are provided by accumulating evidence of lysosomal dysfunction in AD (Lee et al. 2010; Liu et al. 2010; Lorenzen et al. 2010) and AD patients have increased levels of NPC1 in degenerated brain regions (Kagedal et al. 2010).

In the previous cross-sectional study (Mattsson et al. 2011), NPC patients treated with miglustat had lower CSF T-tau than untreated patients, suggesting that miglustat therapy may reduce axonal degeneration. In addition, treated patients had lower CSF levels of the Aβ peptide Aβ1-42, and the APP-derived peptides sAPP-α and sAPP-β, suggesting that treatment may alter amyloid metabolism. Longitudinal studies are needed to verify treatment effects on these biomarkers in NPC. A subset of NPC patients were followed with serial CSF collections and we could therefore conduct a small observational longitudinal study on biomarkers in relation to treatment (in this article "treatment" refers to miglustat treatment). Patients who started treatment between two consecutive CSF samplings were compared with patients who remained untreated, and with patients who received treatment both at baseline and at follow-up ("continuous treatment"). We tested the specific hypothesis that miglustat treatment would result in altered CSF biomarker levels, with the most direct effects seen after start of treatment.

Methods

Standard Protocol Approvals and Patient Consent

All subjects or guardians of subjects provided written informed consent, and assent when appropriate. The study was approved by the National Institute of Child Health and Development (NICHD) Institutional Review Board.

Subjects

As previously described, NPC1 patients were enrolled in an ongoing longitudinal observational trial at the National Institutes of Health between August 2006 and April 2009. The study was made known to the NPC community and all patients or guardians of patients who expressed interest in participating were invited. The inclusion criterion was NPC diagnosis, established by biochemical testing or mutation analysis. Forty patients were eligible. One was excluded from CSF collection due to warfarin treatment, which was a contraindication to lumbar puncture. One was under 1 year of age at sampling and excluded from analyses due to strong post-natal effects on CSF biomarkers for Aβ metabolism and axonal degeneration during the first months of life (Mattsson et al. 2010). Sixteen patients underwent serial lumbar punctures. Six of these were already on miglustat treatment at the first CSF sampling and remained on treatment during the study (continuous treatment). Remaining patients either began miglustat therapy between two CSF collections or were untreated throughout the study. Investigators in this observational trial neither provided nor prescribed miglustat. However, the NICHD IRB specifically approved monitoring patients who were prescribed miglustat by other physicians. Miglustat use was off-label (usage without indication approved

by the United States Food and Drug Administration). Treatment was therefore primarily determined by insurance coverage but not formally randomized. Disease severity was scored as described previously (Yanjanin et al. 2010). This phenotyping index ascertains neurological signs and symptoms in nine major (ambulation, cognition, eye movement, fine motor, hearing, memory, seizures, speech, and swallowing) and eight minor (auditory brainstem response, behavior, gelastic cataplexy, hyperreflexia, incontinence, narcolepsy, psychiatric and respiratory problems) domains. The total possible score ranges from 0 to 61, with a higher score indicating more severe clinical impairment. Demographic data is available in Table 1.

Variables

The endpoints of the study were differences in longitudinal CSF biomarker changes between groups. The main predictor was start of miglustat treatment. We expected to find the strongest biomarker responses directly after start of treatment, since biomarker levels may change and stabilize after start of treatment, reflecting the effect of treatment on the disease process. This may attenuate further biomarker changes.

CSF Sampling

All CSF samples were collected in the morning by lumbar puncture in the L4/L5 interspace, after an age-appropriate overnight fast. The lumbar puncture was done under anesthesia and concurrent with MRI. CSF was collected in a polystyrene tube, and immediately transported to a local laboratory where it was aliquoted into polypropylene tubes. Samples were frozen on dry ice and stored at −80°C prior to assay. Samples were coded prior to sending to the Clinical Neurochemistry Laboratory in Mölndal, Sweden.

CSF Biomarkers of Amyloid Metabolism and Neuronal Cell Damage

CSF levels of $A\beta_{1-42}$, the axonal damage marker T-tau and tau phosphorylated at threonine 181 (P-tau) were

Table 1 Demographics[a]

Group	Patient	Age at first symptoms	Age at baseline (y)	Duration (y)	Sex	*APOE* genotype	Severity at baseline	Severity at follow-up	Time between samplings (d)
Untreated at baseline, treated at follow-up	NPC5	1 y	11.4	10.4	M	E4/E4	25	26	196
	NPC19	2 y	3.8	1.8	F	E3/E3	3	7	268
	NPC20	2 y	3.8	1.8	F	E2/E3	5	9	268
	NPC17	3 w	6.1	6.1	M	E3/E3	7	15	456
	NPC23	6 m	7.7	7.2	F	E3/E3	1	3	357
	Sum	2 y(3 w-2 y)	6.1[b](3.8–11.4)	6.1[b](1.8–10.4)	2 M/3 F		5[b](1–25)	9(3–26)	268 (196–456)
Treated at baseline and at follow-up	NPC16	Neonate	4.7	4.7	M	E3/E3	2	3	366
	NPC2	6 m	7.7	7.2	M	E3/E3	5	5	196
	NPC9	2 w	8.3	8.3	M	ND	8	9	434
	NPC11	3 m	5.4	5.2	M	E3/E4	8	9	349
	NPC12	Neonate	4.7	4.7	F	E3/E3	12	13	175
	NPC3	8 m	13.5	12.8	F	E3/E3	33	35	196
	Sum	7 w(0–8 m)	6.6[c](4.7–13.5)	6.2[d](4.7–12.8)	4 M/2 F		8[e](2–33)	9[f](3–35)	273 (196–434)
Untreated at baseline and at follow-up	NPC6	1.5 y	16.8	15.3	M	ND	18	25	189
	NPC15	39 y	51.3	12.3	F	E3/E3	24	24	183
	NPC24	5 y	21.5	16.5	F	E3/E3	35	40	357
	NPC13	8 y	32.1	24.1	M	E3/E3	39	43	644
	NPC4	Neonate	5.5	5.5	M	E3/E3	12	14	427
	Sum	5 y(0–39 y)	21.5 (5.5–52.3)	12.3 (5.5–24.1)	3 M/2 F		24 (12–39)	25 (14–43)	357 (183–644)

[a] Data for sum are median(range); [b] $p = 0.047$ vs. untreated; [c] $p = 0.030$ vs. untreated; [d] $p = 0.052$ vs. untreated;[e] $p = 0.035$ vs. untreated; [f] $p = 0.028$ vs. untreated

determined using xMAP technology, as previously described (Olsson et al. 2005). CSF sAPP-α and sAPP-β levels were determined using the MSD® sAPPα/sAPPβ Multiplex Assay as described by the manufacturer (Meso Scale Discovery, Gaithersburg, MD, USA). This assay employs the 6E10 antibody to capture sAPP-α and a neoepitope-specific antibody to capture sAPP-β. Both isoforms are detected by SULFO-TAG™-labeled anti-APP antibody p2–1. CSF $A\beta_{x\text{-}38}$, $A\beta_{x-40}$ and $A\beta_{x-42}$ were measured using the MSD® Human/Rodent (4G8) Abeta Triplex Assay as described by the manufacturer. This assay employs C-terminal specific antibodies to specifically capture $A\beta_{x-38}$, $A\beta_{x-40}$ and $A\beta_{x-42}$. All isoforms are detected by SULFO-TAG™-labeled 4G8 detection antibody. Intra-assay coefficients of variation (CVs) were <5% for all analyses, except for $A\beta_{38}$ (11.7%), sAPP-β (10.9%) and one kit of P-tau (5.13%). $A\beta_{42}$ measured by MSD correlated to $A\beta_{1-42}$ measured by Luminex in the total study population ($R = 0.93$, $p < 0.001$) and $A\beta_{1-42}$ and $A\beta_{x-42}$ behaved similar in all statistical analyses. If not stated otherwise, results below are for $A\beta_{x-42}$. All biochemical analyses were performed at the Clinical Neurochemistry Laboratory in Mölndal, Sweden, by experienced and certified laboratory technicians who were blinded to diagnoses and clinical data. Two internal control samples (aliquots of pooled CSF) were run on each plate, and strict acceptance criteria were used for approval of each assay.

Statistics

Statistical calculations were performed using PASW 18.0 (SPSS Inc., Chicago, USA). As the distribution of quantitative measures was significantly skewed, statistical tests involving these variables were conducted using the nonparametric Mann–Whitney *U* test for pair-wise comparisons between groups. Wilcoxon Signed Ranks test was used for pair-wise comparisons of two related samples of quantitative data. The Spearman correlation coefficient was used for analyses of correlation between variables. Quantitative variables are presented as median (range). To investigate potential confounding factors, we examined correlations between biomarkers and age and disease duration. Subgroup analyses were done on patients without treatment, patients starting treatment, and patients on continuous treatment. The significance level threshold was set to $p < 0.05$.

Results

Group Characteristics

Treatment was not formally randomized in this observational trial, and unfortunately the groups of untreated patients, patients starting treatment and patients on continuous treatment were not optimally matched. The untreated group had latest onset of neurological disease, were older at CSF sampling, and had longer disease duration and higher baseline disease severity score than the other two groups (Table 1). Patients starting treatment had higher baseline CSF T-tau levels than the other patients (Fig. 1).

Longitudinal Change in T-Tau

Baseline data have previously been reported for a larger set of NPC patients, including the patients presented here (Mattsson et al. 2011). In that study, patients on miglustat had lower CSF T-tau than patients without miglustat treatment. Five patients in this longitudinal study started miglustat therapy within 1 week after baseline, and decreased in T-tau levels between samplings (955 [338–1,271] vs. 382 [187–736] ng/L, $p = 0.043$, Fig. 1a). The decrease in T-Tau appears to be associated with initiation of miglustat therapy, since T-tau levels were stable in the untreated patients (245 [84–429] vs. 298 [79–429] ng/L, $p = 0.69$, Fig. 1b) and the patients on

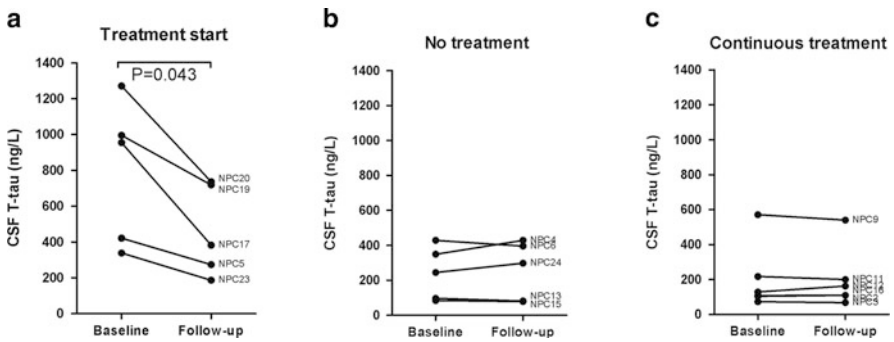


Fig. 1 CSF T-tau and miglustat treatment. Follow-up sample was the next collected consecutive sample after basal sample. At baseline, patients starting treatment (**a**) had higher T-tau than patients without treatment (**b**) ($p = 0.047$) and patients with continuous treatment (**c**) ($p = 0.018$), but there was no significant difference between patients without treatment and patients on continuous treatment

continuous treatment (119 [73–571] vs. 137 [68–540] ng/L, $p = 0.83$, Fig. 1c). In these 16 patients, there was no significant difference in T-tau levels between treated and untreated patients at the follow-up sampling (p = N.S). Compared to previously published control data (CSF T-tau median 79 ng/L, range 23–186 ng/L) (Mattsson et al. 2011), all groups in this study had higher CSF T-tau at baseline ($p = 0.014$ for untreated patients; $p = 0.001$ for patients starting treatment; $p = 0.026$ for patients on continuous treatment) and at follow-up ($p = 0.042$ for untreated patients; $p = 0.001$ for patients starting treatment; $p = 0.029$ for patients on continuous treatment). This suggests that miglustat treatment did not fully stop the axonal neurodegeneration in these patients.

P-Tau

P-tau levels did not change significantly over time in any of the three groups (Fig. 2). This is consistent with the previous study, where there were no differences between patients and controls, or between treated and untreated patients.

Amyloid Markers

All Aβ and sAPP markers correlated between baseline and follow-up measurements, indicating low analytical and biological variability for these measurements (Fig. 3). There were no changes over time in Aβ or sAPP measurements in any of the groups ($p > 0.05$)

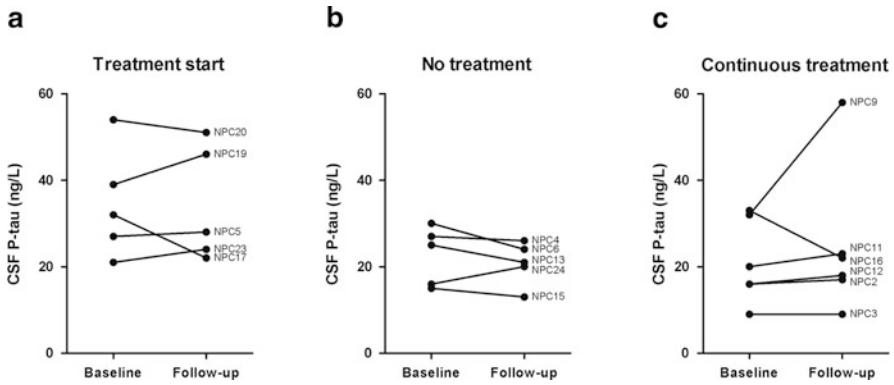


Fig. 2 CSF P-tau and miglustat treatment. Follow-up sample was the next collected consecutive sample after basal sample

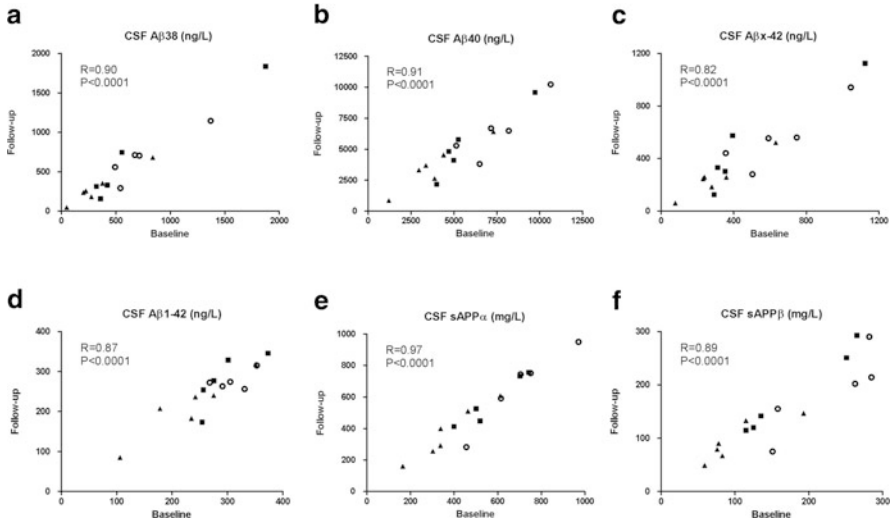


Fig. 3 Correlation of CSF amyloid markers at baseline and follow-up. The Spearman correlation coefficient was used for analyses of correlations. Follow-up sample was the next collected consecutive sample after basal sample. Patients starting miglustat treatment (*circles*), patients on continuous treatment (*triangles*), and untreated patients (*boxes*). As previously reported, NPC patients treated with miglustat had lower CSF Aβ1-42, sAPP-α and sAPP-β, (Mattsson et al. 2011), but no effects of treatment start could be seen in this study

Discussion

CSF T-tau levels decreased after start of miglustat treatment, while untreated and continuously treated patients had stable levels. This might represent a dynamic decrease in axonal degeneration following start of treatment (Hampel et al. 2009). The finding was in accordance with our hypothesis, which was based on a previous cross-sectional study where patients on miglustat had lower T-tau than untreated patients (Mattsson et al. 2011). Miglustat inhibits glucosylceramide synthase, which is believed to reduce the brain load of GM2 and GM3 gangliosides in NPC. Since miglustat treatment affects cognitive symptoms, interference with the pathological lipid metabolism could reduce axonal degeneration, and thus decrease T-tau levels (Patterson et al. 2007). There are at least three possible explanations as to why T-tau decreased in patients starting treatment but not in continuously treated patients. First, T-tau is believed to correlate to the current rate of axonal loss, but not to the total sum of neurons that have been lost during the course of the disease. Levels may therefore be stable despite clinical progression, such as in AD (Blennow et al. 2007; Zetterberg et al. 2007). If a treatment reduces neurodegeneration, T-tau will be expected to decrease corresponding to the treatment-induced lower rate of axonal loss, but not further. Second, if there would be a continuous decrease in T-tau, extended follow-up times might be needed to detect further changes after an initial drop. Third, T-tau levels in continuously treated patients were closer to levels in controls, which intrinsically makes further decrease less likely [control levels published in (Mattsson et al. 2011)].

This study exemplifies how small pilot studies may give information about biochemical drug effects in vivo in humans. Since miglustat may stabilize neurological symptoms in NPC (Patterson et al. 2010; Wraith et al. 2010), these data are encouraging and support the use of T-tau to detect effects on axonal degeneration in clinical trials for neurodegenerative diseases. The results may be interpreted in relation to prior observations on longitudinal T-tau measurements in other brain diseases. In the AN1792 trial reduced levels of T-tau were seen in AD patients immunized against Aβ and interpreted as a possible reduction of cellular degeneration (Gilman et al. 2005). Patients with stroke initially have increased T-tau levels, which normalize after a few months (Hesse et al. 2001). In amateur boxers, head trauma leads to increased T-tau levels, which normalize after a few months of rest (Zetterberg et al. 2006). In patients treated with electroconvulsive therapy, T-tau levels are stable after up to six treatment sessions, indicating that such treatment does not induce neuronal damage (Zachrisson et al. 2000).

NPC and Amyloid Metabolism

Treatment did not affect amyloid markers, suggesting that miglustat did not significantly alter brain amyloid metabolism during the study. Amyloid metabolism is disturbed in NPC, but it is unknown if this plays a role in neurodegeneration (Jin et al. 2004). Larger studies are needed to explore effects of treatment on NPC amyloid metabolism. Such studies might elucidate the links between amyloid metabolism, lipid homeostasis and vesicular trafficking, which are receiving increasing attention in different neurodegenerative diseases, including AD (Grimm et al. 2007; Hirsch-Reinshagen et al. 2009; Lloyd-Evans and Platt 2010). The longitudinal stability of Aβ biomarkers suggests low analytical and biological variability of these parameters in NPC, opening for Aβ measurements to study biochemical effects from amyloid-targeting drugs in NPC. However, since Aβ levels are related to disease severity (Mattsson et al. 2011), these parameters might change over longer time periods.

Limitations of the Study

This is the first longitudinal study of these biomarkers in NPC patients, generating data on in vivo human properties that are unobtainable from animal or cell studies. The major limitation of the study is its design as an observational trial without formal randomization of treatment, and the resulting poor group matching. Patients starting treatment were younger at disease onset and time of sampling, and had higher T-tau at baseline than the other patients, which introduces the risk that T-tau might decrease more in younger patients independently of treatment. However, this hypothetical effect was contradicted by the stable T-tau levels in three of the five youngest patients, who were untreated or continuously treated (NPC4, NPC12, NPC16, Fig. 1). Another possibility is that the decrease in T-tau was influenced by regression to the mean, but this would not explain the decrease in T-tau after start of treatment in patients with baseline T-tau well in the range of the other groups (NPC5, 35% decrease from baseline; NPC23, 45% decrease from baseline). In patients not starting treatment, the largest relative T-tau decrease was 15% (NPC13) and the largest absolute decrease was from 429 to 396 ng/L (NPC6), which was markedly less than for any of the patients starting treatment (Fig. 1). Finally, the stability of amyloid markers and P-tau increases the likelihood that the change in T-tau represents a genuine effect in the nervous system, rather than a measurement artifact.

Future Prospects of Biomarkers in NPC

The efficiency of miglustat therapy on neurological symptoms varies between patients, and it has been

suggested that treatment might be more successful in less-advanced disease stages, and in patients with early-infantile onset (Pineda et al. 2009; Wraith et al. 2009). Since miglustat therapy has side-effects and is economically costly, biomarkers identifying patients most likely to benefit from treatment would be valuable. Such biomarkers for patient stratification could be discovered using post hoc analyses in treatment studies collecting biological samples at baseline and follow-up.

Efficacy biomarkers for NPC treatment would be valuable both in clinical practice and in research. Such biomarkers may be either primary or secondary. Primary treatment biomarkers monitor the main targets of a drug, while secondary biomarkers monitor downstream effects. We propose that T-tau might be a secondary biomarker for axonal degeneration in miglustat treatment of NPC. To verify this, the biomarker should be investigated in relation to other measures of axonal degeneration in larger controlled clinical trials. T-tau should also be examined in relation to clinical outcome. We refrained from such analysis here, due to the small number of participants and the nonrandomized treatment.

The longitudinal response of T-tau has implications for its usability in clinical practice and in research. The decreased levels in patients starting treatment suggest that T-tau may identify short-term changes after start of treatment, but the stable levels in patients on continuous treatment raise concerns about the biomarker's usability for long-term follow-up. Larger studies are needed to clarify this. T-tau might be useful to identify responses to start of treatment or change of dose, but not disease progression, at least over the follow-up time in this study.

Conclusions

CSF biomarkers of axonal degeneration and amyloid metabolism might be useful in clinical settings, clinical research and for drug development in NPC. Although this small pilot study must be interpreted with caution, and validated in larger studies, the results add support to the use of CSF T-tau as a biomarker for treatment effects on axonal degeneration.

Acknowledgments We thank Åsa Källén, Monica Christiansson, Sara Hullberg and Dzemila Secic for excellent technical assistance. We are grateful to Mrs. Chris Hempel for her support of this study. We wish to thank the caretakers and patients for their participation.

Synopsis

Cerebrospinal fluid levels of the axonal degeneration marker tau may decrease after start of miglustat treatment in Niemann–Pick type C.

Disclosures

This study was funded with grants from the Swedish Research Council (projects 14002, 2006–6227, 2006–2740 and 2006–3505), the Alzheimer's Association (NIRG-08-90356), cNEUPRO, the Royal Swedish Academy of Sciences, Sahlgrenska University Hospital, the Söderberg Foundation, the Lundbeck Foundation, the Gothenburg Medical Society, the Swedish Medical Society, Swedish Brain Power, Stiftelsen Gamla Tjänarinnor, Gun och Bertil Stohnes stiftelse, Åhlén-stiftelsen, Alzheimer Foundation, Sweden, The Dementia Association, Sweden, a Bench to Bedside grant from the NIH Office of Rare Diseases, Therapeutics for Rare and Neglected Diseases program, and the intramural research program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

Dr Henrik Zetterberg has participated in an advisory board for GlaxoSmithKline.

Nicole M Yanjanin's position has been supported by the Ara Parseghian Medical Research Foundation and Dana's Angels Research Trust.

Dr Kaj Blennow has participated in an advisory board for Innogenetics.

Dr Niklas Mattsson has participated in an advisory board for Actelion Inc.

Dr Simona Bianconi reports no disclosures.

Dr Jan-Eric Månsson reports no disclosures.

Rao Fu reports no disclosures.

Dr Forbes D. Porter reports no disclosures.

Author Contributions

NM, KB, HZ and FP designed the study. SB, NY and FP established the clinical protocol, managed patients and collected samples. RF performed genotyping. NM analyzed the data and performed the statistical analysis. All authors participated in the interpretation of the data. NM drafted the manuscript and all other authors revised the manuscript.

References

Andreasson U, Portelius E, Andersson ME, Blennow K, Zetterberg H (2007) Aspects of beta-amyloid as a biomarker for Alzheimer's disease. Biomarkers Med 1:59–78

Blennow K, Zetterberg H, Minthon L et al (2007) Longitudinal stability of CSF biomarkers in Alzheimer's disease. Neurosci Lett 419:18–22

Galanaud D, Tourbah A, Lehericy S et al (2009) 24 month-treatment with miglustat of three patients with Niemann-Pick disease type C: follow up using brain spectroscopy. Mol Genet Metab 96:55–8

Gilman S, Koller M, Black RS et al (2005) Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. Neurology 64:1553–62

Grimm MO, Grimm HS, Hartmann T (2007) Amyloid beta as a regulator of lipid homeostasis. Trends Mol Med 13:337–44

Hampel H, Blennow K, Shaw LM, Hoessler YC, Zetterberg H, Trojanowski JQ (2009) Total and phosphorylated tau protein as biological markers of Alzheimer's disease. Exp Gerontol 45:30–40

Hesse C, Rosengren L, Andreasen N et al (2001) Transient increase in total tau but not phospho-tau in human cerebrospinal fluid after acute stroke. Neurosci Lett 297:187–90

Hirsch-Reinshagen V, Burgess BL, Wellington CL (2009) Why lipids are important for Alzheimer disease? Mol Cell Biochem 326:121–9

Imrie J, Dasgupta S, Besley GT et al (2007) The natural history of Niemann-Pick disease type C in the UK. J Inherit Metab Dis 30:51–9

Jin LW, Shie FS, Maezawa I, Vincent I, Bird T (2004) Intracellular accumulation of amyloidogenic fragments of amyloid-beta precursor protein in neurons with Niemann-Pick type C defects is associated with endosomal abnormalities. Am J Pathol 164:975–85

Kagedal K, Kim WS, Appelqvist H et al (2010) Increased expression of the lysosomal cholesterol transporter NPC1 in Alzheimer's disease. Biochim Biophys Acta 1801:831–8

Kodam A, Maulik M, Peake K et al (2010) Altered levels and distribution of amyloid precursor protein and its processing enzymes in Niemann-Pick type C1-deficient mouse brains. Glia 58:1267–81

Kosicek M, Malnar M, Goate A, Hecimovic S (2010) Cholesterol accumulation in Niemann Pick type C (NPC) model cells causes a shift in APP localization to lipid rafts. Biochem Biophys Res Commun 393:404–9

Lee JH, Yu WH, Kumar A et al (2010) Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell 141:1146–58

Liu RQ, Zhou QH, Ji SR et al (2010) Membrane localization of beta-amyloid 1–42 in lysosomes: a possible mechanism for lysosome labilization. J Biol Chem 285:19986–96

Lloyd-Evans E, Platt FM (2010) Lipids on trial: the search for the offending metabolite in Niemann-Pick type C disease. Traffic 11:419–28

Lorenzen A, Samosh J, Vandewark K et al (2010) Rapid and direct transport of cell surface APP to the lysosome defines a novel selective pathway. Mol Brain 3:11

Malnar M, Kosicek M, Mitterreiter S et al (2010) Niemann-Pick type C cells show cholesterol dependent decrease of APP expression at the cell surface and its increased processing through the beta-secretase pathway. Biochim Biophys Acta 1802:682–91

Mattsson N, Savman K, Osterlundh G, Blennow K, Zetterberg H (2010) Converging molecular pathways in human neural development and degeneration. Neurosci Res 66:330–2

Mattsson N, Zetterberg H, Bianconi S et al (2011) Gamma-secretase-dependent amyloid-beta is increased in Niemann-Pick type C: a cross-sectional study. Neurology 76:366–72

Naureckiene S, Sleat DE, Lackland H et al (2000) Identification of HE1 as the second gene of Niemann-Pick C disease. Science 290:2298–301

Olsson A, Vanderstichele H, Andreasen N et al (2005) Simultaneous measurement of beta-amyloid(1–42), total tau, and phosphorylated tau (Thr181) in cerebrospinal fluid by the xMAP technology. Clin Chem 51:336–45

Ory DS (2004) The niemann-pick disease genes; regulators of cellular cholesterol homeostasis. Trends Cardiovasc Med 14:66–72

Patterson MC, Vecchio D, Prady H, Abel L, Wraith JE (2007) Miglustat for treatment of Niemann-Pick C disease: a randomised controlled study. Lancet Neurol 6:765–72

Patterson MC, Vecchio D, Jacklin E et al (2010) Long-term miglustat therapy in children with Niemann-Pick disease type C. J Child Neurol 25:300–5

Pentchev PG, Comly ME, Kruth HS et al (1987) Group C Niemann-Pick disease: faulty regulation of low-density lipoprotein uptake and cholesterol storage in cultured fibroblasts. FASEB J 1:40–5

Pineda M, Wraith JE, Mengel E et al (2009) Miglustat in patients with Niemann-Pick disease Type C (NP-C): a multicenter observational retrospective cohort study. Mol Genet Metab 98:243–9

Platt FM, Lachmann RH (2009) A new surrogate marker for CNS pathology in Niemann-Pick disease type C? Mol Genet Metab 96:53–4

Querfurth HW, LaFerla FM (2010) Alzheimer's disease. N Engl J Med 362:329–44

Sevin M, Lesca G, Baumann N et al (2007) The adult form of Niemann-Pick disease type C. Brain 130:120–33

Vanier MT, Millat G (2003) Niemann-Pick disease type C. Clin Genet 64:269–81

Wraith JE, Baumgartner MR, Bembi B et al (2009) Recommendations on the diagnosis and management of Niemann-Pick disease type C. Mol Genet Metab 98:152–65

Wraith JE, Vecchio D, Jacklin E et al (2010) Miglustat in adult and juvenile patients with Niemann-Pick disease type C: long-term data from a clinical trial. Mol Genet Metab 99:351–7

Yanjanin NM, Velez JI, Gropman A et al (2010) Linear clinical progression, independent of age of onset, in Niemann-Pick disease, type C. Am J Med Genet B Neuropsychiatr Genet 153B:132–40

Yerushalmi B, Sokol RJ, Narkewicz MR, Smith D, Ashmead JW, Wenger DA (2002) Niemann-pick disease type C in neonatal cholestasis at a North American Center. J Pediatr Gastroenterol Nutr 35:44–50

Zachrisson OC, Balldin J, Ekman R et al (2000) No evident neuronal damage after electroconvulsive therapy. Psychiatry Res 96:157–65

Zervas M, Dobrenis K, Walkley SU (2001a) Neurons in Niemann-Pick disease type C accumulate gangliosides as well as unesterified cholesterol and undergo dendritic and axonal alterations. J Neuropathol Exp Neurol 60:49–64

Zervas M, Somers KL, Thrall MA, Walkley SU (2001b) Critical role for glycosphingolipids in Niemann-Pick disease type C. Curr Biol 11:1283–7

Zetterberg H, Hietala MA, Jonsson M et al (2006) Neurochemical aftermath of amateur boxing. Arch Neurol 63:1277–80

Zetterberg H, Pedersen M, Lind K et al (2007) Intra-individual stability of CSF biomarkers for Alzheimer's disease over two years. J Alzheimers Dis 12:255–60

Zetterberg H, Blennow K, Hanse E (2010) Amyloid beta and APP as biomarkers for Alzheimer's disease. Exp Gerontol 45:23–9

JIMD Reports
DOI 10.1007/8904_2011_48

RESEARCH REPORT

A Twelve-Year Follow-Up Study on a Case of Early-Onset Parkinsonism Preceding Clinical Manifestation of Gaucher Disease

Maciej Machaczka • Martin Paucar Arce • Malgorzata Rucinska • Takashi Yoshitake • Jan Kehr • Wojciech Jurczak • Aleksander B. Skotnicki • Jan-Erik Månsson • Anna Tylki-Szymanska • Per Svenningsson

Received: 13 January 2011 / Revised: 21 May 2011 / Accepted: 24 May 2011 / Published online: 20 September 2011

Abstract Mutations in the glucocerebrosidase gene (*GBA1*) cause Gaucher disease (GD) and are the most common genetic risk factor for the development of Parkinson's disease (PD). Here, we present a 12-year follow-up study of a male with GD and PD (diagnosed 24 years ago), which PD preceded the clinical manifestation of GD by 12 years. The patient is a compound heterozygote for mutations c.115 + 1G > A and c.1226A > G (IVS2 + 1/N370S) in the *GBA1* gene. Imiglucerase had a beneficial effect on GD, but not on PD. Treatment with L-dopa and other PD drugs showed temporary efficacy but 2 years later significant wearing-off phenomenon and dyskinesias appeared. Unilateral pallidotomy was performed with transient benefit. Cognitive decline appeared later and developed in to akinetic mutism. A lumbar puncture was performed to characterize the biochemical profile of cerebrospinal fluid (CSF). Analyses of monoamine metabolites levels in the CSF, determined by reverse-phase high-performance liquid chromatography, revealed remarkably low levels of all studied monoamine metabolites (HVA, DOPAC, 5-HIAA, MHPG). These data indicate that PD associated with *GBA1* mutations may not only affect dopaminergic neurons, but also noradrenergic and serotonergic neurons. Of note, normal levels of P-tau, total tau and β-amyloid (1–42) were detected on ELISA assay. Thus, the cognitive decline, akinetic mutism and moderate cortical atrophy found on the CT scan were not paralleled by any changes of dementia markers in CSF. This single case study extends the follow-up period and adds novel CSF information; however additional data on other patients with both PD and GD may help put our observations in its ultimate proper context.

Key Words Gaucher disease · Glucocerebrosidase · Movement disorders · Parkinson's disease · Synucleinopathies

Abbreviations

ADL	Activities of daily living
CSF	Cerebrospinal fluid

Communicated by: Gregory M. Pastores.

Competing interests: None declared.

M. Machaczka (✉)
Hematology Center Karolinska, M54, Karolinska University Hospital Huddinge, 141 86 Stockholm, Sweden
e-mail: maciej.machaczka@ki.se

M. Paucar Arce · P. Svenningsson
Department of Neurology, Karolinska University Hospital Huddinge, 141 86 Stockholm, Sweden

M. Rucinska · W. Jurczak · A.B. Skotnicki
Department of Hematology, Collegium Medicum of the Jagiellonian University, Krakow, Poland

M. Paucar Arce · P. Svenningsson
Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

T. Yoshitake · J. Kehr
Department of Pharmacology and Physiology, Karolinska Institutet, Stockholm, Sweden

J.-E. Månsson
Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy, University of Gothenburg, Mölndal, Sweden

A. Tylki-Szymanska
Department of Metabolic Diseases, Endocrinology and Diabetology, The Children's Memorial Health Institute, Warszawa, Poland

DBS	Deep brain stimulation
ERT	Enzyme replacement therapy
GBA	Glucocerebrosidase
GD	Gaucher disease
GD1	Gaucher disease type 1
LSD	Lysosomal storage disorder
PD	Parkinson's disease
P-tau	Phosphorylated tau protein (181P)
RBD	REM-sleep behavioral disorder
Tau	Total tau protein
UPDRS	Unified Parkinson's disease rating scale

Introduction

Gaucher disease, the most common LSD, is caused by deficient activity of the lysosomal enzyme, glucocerebrosidase (glucosylceramidase, E.C.3.2.1.45), arising from autosomal recessive mutations in the *GBA1* gene (1q21) (Hruska et al. 2008). Recent studies have shown that *GBA1* mutations are the most common genetic risk factor for Parkinson's disease (Bultron et al. 2010; Neumann et al. 2009; Sidransky et al. 2009). One of the early reports on association between parkinsonism and GD1 was published by our group in 1999, describing a Polish patient who developed parkinsonism preceding clinical manifestation of GD by about 12 years (Machaczka et al. 1999). Here, we provide an update on the patients' current status, 12 years after the initial report and 24 years after the onset of parkinsonism. To our knowledge, this is the longest published follow-up of early-onset PD associated with *GBA1* mutations. Furthermore, we provide data on GD biomarkers in blood and cerebrospinal fluid namely analyses of monoamine metabolites, total tau protein, phosphorylated tau protein (181P) and β-amyloid (1–42).

Material and Methods

Patient and Previous Clinical Outcome

A 63-year-old right-handed non-Jewish Caucasian male with no family history of movement disorders developed a shuffling gait, rigidity, bradykinesia and resting tremor confined to the left side of his body at the age of 39 (in 1986). Shortly after, symptoms resembling RBD have been reported. L-dopa therapy started 2 years later in 1988 with initially beneficial effect. Bilateral symptoms were evident in 1992. In 1997, the patient developed notorious wearing-off and disabling dyskinesias. Then also thrombocytopenia and splenomegaly were noticed and further investigations disclosed Gaucher disease type 1 with the heterozygous mutations c.115 + 1G > A (IVS2 + 1) and c.1226A > G (N370S) in the *GBA1* gene (Machaczka et al. 1999). Enzyme replacement therapy with imiglucerase was started in 1999, 40 U/kg (2,600 U, later reduced to 1,200 U) every other week. The treatment has led to prompt normalization of spleen size and thrombocytopenia.

At the age of 54, the patient became wheelchair bound due to severe akinesia and stiffness despite treatment with 1,000 mg L-dopa/day. This deterioration motivated a right-sided posteroventrolateral pallidotomy in 2001, which resulted in significant improvement that lasted for 3 years (Sobstyl et al. 2009). The patient became ambulatory and L-dopa was reduced to 300 mg/day; he was able to perform all ADL independently. Before pallidotomy his mini mental test score was 27/30 but a neuropsychological evaluation was not performed.

Methods

In 2010, the patient's symptoms were reported by his wife and confirmed by revision of chart notes. Detailed neurological examination and lumbar puncture (performed at the level L3–4) were performed. The biochemical profile of CSF was characterized by measurements of monoamine metabolites by reverse phase high-performance liquid chromatography, as previously described (Weikop et al. 2007). P-tau, tau and β-amyloid (1–42) were measured using ELISA assay by Innogentics N.V., Ghent, Belgium (Mattsson et al. 2009). The patient's clinical condition made it impossible to perform new imaging examinations, but we report the results of CT scan of his brain taken in 2009.

The study was approved by the Regional Ethical Review Board in Krakow, Poland (KBET/62/B/2010).

Results

Outcome After Pallidotomy

Four years after pallidotomy (in 2005) antecollis and frequent falls were reported. One year later increasing memory loss was noticed. He became again wheel chair-bound in 2007 and decided then to discontinue both ERT (due to deterioration of general and neurological status) and L-dopa (due to loss of response and occurrence of benign visual hallucinations). He has since progressed into a state of akinetic mutism. Currently, he requires complete assistance with all ADLs. Pronounced daytime sleepiness and a new pattern of breathing apnea resembling Cheyne-Stokes breathing have developed in 2010. He is still able to swallow and drink, but has gradually lost his weight from 80 to 50 kg. Symptom fluctuations are denied.

On clinical examination in February 2010, the patient is confined to a wheel chair, somnolent and in a state of akinetic mutism but reacts to simpler commands. Clinical examinations yielded a total UPDRS score of 101 (9 + 41 + 45 + 6); a Hoehn and Yahr score of 5; and a Schwab and England score of 10%. He is unable to stand up with support; while sitting, his feet are in a supine position without signs of resting tremor or myoclonus. Rigidity and Gegenhaltung in the forearms and legs are present bilaterally. Marked axial neck dystonia with antecollis, an intermittent startle reaction and a limited range of horizontal and especially vertical ocular pursuit are found. Of the primitive reflexes, only grasping reaction is evident. Bilateral plantar signs are present but tendon reflexes are otherwise normal. L-dopa test was performed (with 100 mg) but did not yield any improvement 1 h after drug intake.

Biochemical and Radiological Results

Significant decreases of monoamine metabolites (HVA, DOPAC, 5-HIAA, MHPG) were found in the CSF (Table 1). In contrast, normal levels of P-tau, tau and β-amyloid (1–42) were found in the CSF. Analyses of GD biomarkers in blood are summarized in Table 1.

A CT scan of the brain, taken approximately 1 year earlier, showed status post right-sided pallidotomy, mild-to-moderate bilateral subcortical atrophy, and moderate bilateral cortical atrophy of the frontal and temporal lobes with enlargement of the subarachnoidal spaces (Fig. 1).

Discussion

Recent studies indicate that both GD patients and single *GBA1* mutation carriers face an increased risk for PD (Bultron et al. 2010; Neumann et al. 2009; Sidransky et al. 2009). Common *GBA1* mutations such as c.1226A > G (N370S) and c.1448T > C (L444P) have been reported as the most frequent among PD patients of non-Jewish ancestry (Neumann et al. 2009; Sidransky et al. 2009). An open question remains whether the presence of the heterozygous *GBA1* gene mutation c.115 + 1G > A (IVS2 + 1) in the reported patient, uncommonly seen among GD patients and *GBA1* mutations carriers suffering from PD, influenced the reported course of PD.

The parkinsonian phenotypes reported among *GBA1* mutation carriers are variable and their response to L-dopa treatment has yielded conflicting reports (Toft et al. 2006; Velayati et al. 2010). Visual hallucinations and varying degrees of cognitive decline have been described among individuals with parkinsonism associated with *GBA1* mutations (Neumann et al. 2009). Interestingly, some

Table 1 Laboratory data on Gaucher disease biomarkers in blood and monoamine metabolites and dementia proteins in cerebrospinal fluid

Variable		Results (units)	Control ranges
Gaucher disease biomarkers measured in peripheral blood	Hemoglobin	133 (g/L)	120–180
	Platelets	63 ($\times 10^9$/L)	150–450
	Chitotriosidase	2,010 (nkat/L)[a]	<40
	CCL18	2,070 (μg/L)	<100
	Glucocerebroside (RBC)	9.6 (μmol/L)	1.8–6.0
	Glucocerebroside (plasma)	13.9 (μmol/L)	4.7–9.7
Monoamine metabolites and dementia proteins measured in cerebrospinal fluid	HVA	18.7 (nmol/L)	140–360
	DOPAC	8.9 (nmol/L)	20–50
	5-HIAA	29.6 (nmol/L)	70–180
	MHPG	1.6 (nmol/L)	35–60
	HVA_{corr}	6.6	40–170
	$5\text{-}HIAA_{corr}$	21.4	50–170
	$MHPG_{corr}$	3.3	65–140
	P-tau (181P)	31 ng/L	<80
	Total tau	128 ng/L	<400
	β-amyloid (1–42)	624 ng/L	>450

CCL18 chemokine (C–C motif) ligand 18 (pulmonary and activation-regulated), *RBC* red blood cells, *HVA* homovanillic acid, *DOPAC* 3,4-dihydroxyphenylacetic acid, *5-HIAA* 5-hydroxyindole-3-acetic acid, *MHPG* 3-methoxy-4-hydroxyphenylethylenglycol

[a] The patient is a heterozygote for polymorphism in chitotriosidase gene and the theoretical activity of the chitotriosidase is doubled (4,020 nkat/L). The activity of chitotriosidase is expressed in the international unit (SI) as nkat/L. 1 nkat/L = 3.6 nmol/mL/h

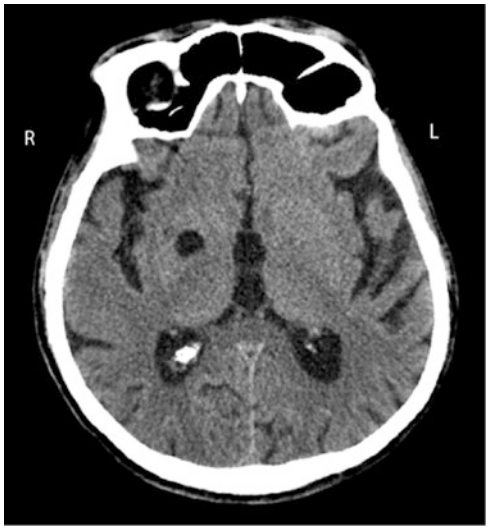


Fig. 1 Horizontal CT scan of brain showing status post right-sided pallidotomy and mild-to-moderate bilateral subcortical atrophy as well as moderate bilateral cortical atrophy in frontal and temporal lobes with associated increases of subarachnoidal spaces. *R* – right hemisphere; *L* – left hemisphere

patients with parkinsonism associated with *GBA1* mutations had partial (Neudorfer et al. 1996) or excellent (Toft et al. 2006) responses to neurosurgical treatment by means of thalamotomy or DBS of the subthalamicus nucleus. In accordance with previous studies (Neumann et al. 2009; Tayebi et al. 2003), optimal ERT did not influence the course of PD in our patient. The relatively early loss of L-dopa responsiveness in this case was interpreted as atypical parkinsonism in previous reports; however, the patient did not display autonomic dysfunction, apraxia, early falls or ataxia during the course of the disease. Moreover, in contrast to the present case, survival is significantly reduced in atypical parkinsonism.

Recent studies have demonstrated the presence of Lewy bodies in the brains of patients with PD associated with *GBA1* mutations (Neumann et al. 2009). Lewy bodies consist of α-synuclein inclusions associated with other proteins. Glucosylceramide has been found in α-synuclein inclusions in the brains of individuals with parkinsonism associated with *GBA1* mutations and less frequently in individuals without *GBA1* mutations (Goker-Alpan et al. 2010).

The role of tau in PD associated with GBA mutations has been less studied than that of α-synuclein. Several atypical forms of parkinsonism including progressive supranuclear palsy, corticobasal degeneration, and frontotemporal dementia show increased levels of P-tau, tau and/or altered splicing of this protein (Eller and Williams 2009). Our patient demonstrated normal concentrations of P-tau and tau in his CSF. In contrast to previous case study (Alonso-Canovas et al. 2010), reporting lowered β-amyloid (1–42) levels in CSF, our patient showed normal CSF concentrations of β-amyloid (1–42). Thus, the cognitive decline, akinetic mutism, and moderate cortical atrophy found in the reported patient are not paralleled by any changes of dementia markers in CSF. These findings are in contrast with those found in Niemann–Pick disease type C (neuronopathic LSD), where tau and β-amyloid (1–42) levels in CSF are increased (Mattsson et al. 2011). Further CSF analyses revealed that our patient had remarkably low levels of all studied monoamine metabolites. These data indicate that parkinsonism associated with *GBA1* mutations may not only affect dopaminergic neurons, but also noradrenergic and serotonergic neurons.

Both the gain- and loss-of-function of GBA have been proposed as mechanisms of disease leading to possible ceramide accumulation (Bras et al. 2008), disturbances in lysosomal functioning/trafficking in microglia and/or neurons, due to a membrane imbalance of recycled glucosylceramide as well as enhanced α-synuclein aggregation (Goldin 2010). One possibility is that mutated GBA may act as a "second hit" in some individuals genetically predisposed to develop PD. Further studies on how *GBA1* mutations predispose to Lewy bodies pathology, neuronal vulnerability and parkinsonism are needed. This report extends current knowledge on PD associated with *GBA1* mutations and suggests that parkinsonian symptoms in such cases can develop over many years and be treated relatively successfully.

Acknowledgments This study was supported by The Swedish Research Council (PS) and Karolinska University Hospital (MM, MPA, PS). We thank the patient and his wife for participation in this study.

Synopsis

The article reports the longest follow-up of Parkinson's disease associated with Gaucher disease and provides the clinical and biochemical data on this association.

Financial Disclosures

MM: Advisor/honoraria/financial support to attend meetings from Genzyme Corporation, Actelion Pharmaceuticals, Shire HGT and BMS. MPA: none. MR: none. TY: none. YK: none. WJ: Advisor/honoraria/financial support to attend meetings from Novartis and Roche. ABS: Advisor/honoraria/financial support to attend meetings from BMS. JEM: none. ATS: Advisor/honoraria/financial support to attend meetings from Genzyme Corporation. PS: none.

References

Alonso-Canovas A, Katschnig P, Tucci A et al (2010) Atypical parkinsonism with apraxia and supranuclear gaze abnormalities in type 1 Gaucher disease. Expanding the spectrum: case report and literature review. Mov Disord 25:1506–1509

Bras J, Singleton A, Cookson MR, Hardy J (2008) Emerging pathways in genetic Parkinson's disease: potential role of ceramide metabolism in Lewy body disease. FEBS J 275:5767–5773

Bultron G, Kacena K, Pearson D et al (2010) The risk of Parkinson's disease in type 1 Gaucher disease. J Inherit Metab Dis 33 (2):167–173

Eller M, Williams DR (2009) Biological fluid biomarkers in neurodegenerative parkinsonism. Nat Rev Neurol 5:561–570

Goker-Alpan O, Stubblefield BK, Giasson BI, Sidransky E (2010) Glucocerebrosidase is present in α- synuclein inclusions in Lewy body disorders. Acta Neuropathol 120:641–649

Goldin E (2010) Gaucher disease and parkinsonism, a molecular link theory. Mol Genet Metab 101:307–310

Hruska KS, LaMarca ME, Scott CR, Sidransky E (2008) Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). Hum Mutat 29:567–583

Machaczka M, Rucinska M, Skotnicki AB, Jurczak W (1999) Parkinson's syndrome preceding clinical manifestation of Gaucher's disease. Am J Hematol 61:216–217

Mattsson N, Zetterberg H, Hansson O et al (2009) CSF biomarkers and incipient Alzheimer disease in patients with mild cognitive impairment. JAMA 302:385–393

Mattsson N, Zetterberg H, Bianconi S et al (2011) Gamma-secretase-dependent amyloid-beta is increased in Niemann-Pick type C: a cross-sectional study. Neurology 76:366–372

Neudorfer O, Giladi N, Elstein D et al (1996) Occurrence of Parkinson's syndrome in type I Gaucher disease. QJM 89:691–694

Neumann J, Bras J, Deas E et al (2009) Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease. Brain 132:1783–1794

Sidransky E, Nalls MA, Aasly JO et al (2009) Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N Engl J Med 361:1651–1661

Sobstyl M, Zabek M, Koziara H, Dzierzecki S (2009) A patient with parkinsonism related to Gaucher's disease type I successfully treated by unilateral pallidotomy – a 3-year follow-up. Neurol Neurochir Pol 43:293–297

Tayebi N, Walker J, Stubblefield B et al (2003) Gaucher disease with parkinsonian manifestations: does glucocerebrosidase deficiency contribute to a vulnerability to parkinsonism? Mol Genet Metab 79:104–109

Toft M, Pielsticker L, Ross OA, Aasly JO, Farrer MJ (2006) Glucocerebrosidase gene mutations and Parkinson disease in the Norwegian population. Neurology 66:415–417

Velayati A, Yu WH, Sidransky E (2010) The role of glucocerebrosidase mutations in Parkinson disease and Lewy body disorders. Curr Neurol Neurosci Rep 10:190–198

Weikop P, Yoshitake T, Kehr J (2007) Differential effects of adjunctive methylphenidate and citalopram on extracellular levels of serotonin, noradrenaline and dopamine in the rat brain. Eur Neuropsychopharmacol 17:658–671

JIMD Reports
DOI 10.1007/8904_2011_58

RESEARCH REPORT

Differential Expression of Matrix Metalloproteinases in the Serum of Patients with Mucopolysaccharidoses

Spyros P. Batzios • Dimitrios I. Zafeiriou • Euthymia Vargiami • George Karakiulakis • Eleni Papakonstantinou

Received: 15 February 2011 / Revised: 20 May 2011 / Accepted: 23 May 2011 / Published online: 22 September 2011

Abstract Mucopolysaccharidoses (MPS) represent a heterogeneous group of hereditary disorders, characterized by accumulation of glycosaminoglycans within the lysosomes. The objective of this study was to elucidate the expression and activity of matrix metalloproteinases (MMPs) in the serum of pediatric patients with MPS. Serum gelatinase activity was assessed by gelatin zymography and the concentration of circulating MMP-2, MMP-9, and of tissue inhibitors of MMPs (TIMP)-1 and TIMP-2 was measured by ELISA in the serum of seven patients with MPS (five with MPS III, 1 with MPS II and 1 with MPS VI), and healthy age- and sex-matched participants. Serum activity and protein levels of MMP-9 were significantly reduced whereas of MMP-2 were significantly increased in patients with MPS III, as compared to controls. There were no significant alterations in serum protein levels of TIMP-1 and TIMP-2 in patients with MPS III, as compared to controls. In MPS II, proMMP-2 activity and protein levels of MMP-2 were significantly increased, as compared to control. In MPS VI, enzyme replacement therapy reduced the activity and protein levels of MMP-9 up to 4 months after the initiation of treatment. The reported alterations in the expression of MMPs in the serum of patients with MPS suggest that these molecules may be used as potential biomarkers for the diagnosis, follow-up and response to therapy in patients with MPS.

Communicated by: Olaf Bodamer.

Competing interests: None declared.

S.P. Batzios · G. Karakiulakis · E. Papakonstantinou (✉)
2nd Department of Pharmacology, School of Medicine, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
e-mail: epap@med.auth.gr

S.P. Batzios · D.I. Zafeiriou · E. Vargiami
1st Department of Pediatrics, Aristotle University of Thessaloniki, Hippokration General Hospital, Thessaloniki, Greece

Introduction

Mucopolysaccharidoses (MPS) represent a heterogeneous group of hereditary disorders characterized by the accumulation of glycosaminoglycans (GAGs) within the lysosomes (Neufeld and Muenzer 2001). To date, 11 distinct types of MPS have been described, each one resulting from the deficient activity of a specific lysosomal hydrolase (Clarke 2008). In each disease, the primary enzyme deficiency leads to the accumulation of different types of GAGs resulting in a wide spectrum of clinical features that progress with age. Short stature and skeletal abnormalities, hepatosplenomegaly, hernias, and coarse facial features are prominent in most types of MPS with different involvement of cardiovascular, respiratory, and central nervous system in each syndrome (Muenzer 2004).

Although crucial steps have been made toward understanding the full etiopathogenetic repertoire of MPS, the exact mechanisms by which deficiencies of lysosomal hydrolases ultimately lead to disease manifestations are not clear. Recent findings indicate that the primary accumulation of GAGs within the lysosomes may trigger a cascade of events which influence various biochemical and physiological processes of the cell (Clarke 2008). The introduction of enzyme replacement therapy (ERT) increased the scientific interest in identifying molecular biomarkers of the disease and underlined the need for establishing new methods for rapid and early diagnosis of these disorders. Currently, there are no specific biomarkers for the diagnosis and treatment follow-up, apart from qualitative and quantitative measurement of urinary GAG

excretion (Gallegos-Arreola et al. 2000). Both techniques indicate the likely presence of an MPS disorder, rather than providing a definitive diagnosis or reflecting total body burden of disease.

GAGs accumulate within the lysosomes of various types of cells, including the cells of the immune system, and therefore it is not surprising that in many lysosomal storage disorders, altered immune responses are observed (Castaneda et al. 2008). Furthermore, it is widely accepted that these macromolecules have both pro- and anti-inflammatory properties, play a role as co-receptors for some cytokines (Mulloy and Rider 2006), whereas chemokines exert their biological functions through interactions with proteoglycans (Proudfoot 2006). Thus, there is emerging evidence for the involvement of inflammation in the pathophysiology of MPS. Accordingly, several mediators of the inflammatory response have been tested as possible molecular biomarkers for these disorders (Ohmi et al. 2003; Richard et al. 2008; Villani et al. 2007; Simonaro et al. 2001).

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases classified according to domain structure into collagenases, gelatinases, stromelysins, matrilysines, membrane-types, and others (Nagase and Woessner 1999). They represent key enzymes involved in the dissolution of extracellular matrix (Woessner 1991) and have been implicated in various processes, both normal and pathological, usually related to inflammation and cell apoptosis (Borkakoti 1998; Rydlova et al. 2008). Most MMPs are secreted as zymogens and require proteolytic activation, whereas their transcription, translation and proenzyme activity are regulated by growth factors, cytokines, and tissue inhibitors of metalloproteinases (TIMPs) (Brew et al. 2000; Clark et al. 2008).

In the present prospective case–control study, we examined the enzyme activity and expression of gelatinases, MMP-2 and MMP-9 as well as the expression of TIMP-1 and TIMP-2 in the serum of patients with MPS. The goal of this study was to elucidate the etiopathological mechanisms involved in this group of disorders aiming to provide new insights into the molecular mechanisms of these syndromes and unravel new potential biomarkers for the diagnosis, follow-up and response to therapy in patients with MPS. We demonstrate that MPS are associated with alterations in gelatinase activity and circulating levels of both MMP-2 and MMP-9.

Methods

Participants

Seven patients with MPS, followed up at the outpatient clinic of the 1st Department of Pediatrics of the Aristotle University of Thessaloniki at Hippokration General Hospital formed the study group. Patient's age was between 7 and 26 years old (14.21 ± 2.81). Five out of seven patients were male. Concerning the type of MPS, five out of seven patients suffered from MPS III (Sanfilippo syndrome), one from MPS II (Hunter syndrome) and one from MPS VI (Maroteaux–Lamy syndrome). The last patient is under ERT for the last 9 months. The control group consisted of healthy age- and sex-matched participants, as follows: 5 controls for each patient with MPS III (25 in total) and 10 controls for each patient with MPS II and MPS VI. Subjects' characteristics are presented in Table 1.

Table 1 Characteristics of patients

Patient number	Sex	Age (years)	MPS type
1	Male	26	VI
2	Male	7	III B
3	Female	7	III B
4	Male	16.5	III B
5	Female	21	III B
6	Male	14	III C
7	Male	8	II

Diagnosis of MPS was based in measurement of urine GAG excretion and was confirmed with enzyme activity assays and genetic testing for the identification of the causative mutation. Exclusion criteria in patients diagnosed with MPS were the coexistence of acute or chronic inflammatory disease and the use of drugs known to suppress inflammatory response.

Guardians and parents gave written consent for the participation of their children in the study. Whole blood samples were collected after single venipuncture and centrifuged at 1,750 g, for 10 min, at 4°C (High Speed Refrigerated Bench-top Centrifuge, Shanghai Lishen Scientific Equipment Co., Ltd, China). Serum samples were stored at 80°C until analysis. The study conformed fully with the Declaration of Helsinki for biomedical research on human subjects.

Gelatin Zymography

The gelatinolytic activity of MMPs was determined by gelatin zymography analysis using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing but nonreducing conditions (Papakonstantinou et al. 2005). In brief, 10 μl of serum samples from patients and healthy subjects were diluted 1:100 and 6 μl of the diluted samples were applied on an 8% SDS–PAGE containing 0.1% gelatin (25 mA, 2 h, at room temperature). Gels were then equilibrated in 2.5% Triton X-100 buffer

for 1 h and subsequently incubated in 50 mM Tris-HCl, pH 7.3 buffer containing 200 mM NaCI, 5 mM $CaCl_2$ and 0.1% Triton X-100 for 18 h, at 37°C. Bands of enzymatic activity were visualized by negative staining with standard Coomassie brilliant blue R-250 dye solution. Molecular size of bands displaying enzymatic activity were estimated by comparison to purified proMMP-2 (72 kDa), active MMP-2 (64 kDa), proMMP-9 (92 kDa), and active MMP-9 (78 kDa) (Anawa Trading, Wangen). Prestained standard protein molecular weight markers used were: myosin (250 kDa), phosphorylase (148 kDa), bovine serum albumin (98 kDa), L-glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin, B chain (4 kDa) (SeeBlue Plus2 Prestained, Invitrogen, USA). Gelatinolytic activity was quantified using a computer-assisted image analysis program (1D Image Analysis Software, Kodak Digital Science v.3.0, Eastman Kodak, Rochester, NY, USA). All experiments were performed in triplicate.

Immunoassays

Concentration of MMP-2, MMP-9, TIMP-1, and TIMP-2 (ng/ml) was quantified in duplicate in the serum of patients with MPS and healthy participants, using an ELISA kit (R&D Systems Europe, Abingdon, UK), performed according to manufacturer's instructions. The MMP-2 and MMP-9 assays measure total MMP-2 and MMP-9 (proenzymes and activated forms). Sensitivity of the method employed was: MMP-2: 0.16 ng/ml, MMP-9: 0.156 ng/ml, TIMP-1: 0.08 ng/ml, and TIMP-2: 0.011 ng/ml. Preliminary investigation established the appropriate serum dilution for each MMP and TIMP as follows: MMP-2, 1:10; MMP-9, 1:40; TIMP-1, 1:100; TIMP-2, 1:50.

Statistical Analysis

Normal distribution of data was checked using Shapiro–Wilk analysis for all dependent variables. Student's t test was used for variables following normal distribution, whereas Mann–Whitney U test was used to compare differences between the mean values of continuous variables violating the assumption of normality. Two-tailed levels of significance were used in all statistical calculations. All data are expressed as mean ± SEM. Difference was considered to be statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$. The relationship between continuous variables was investigated using Pearson product-moment correlation coefficient and Spearman's Rank Order Correlation (rho). The computer software SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical calculations and analyses.

Results

Differential Expression of Gelatinase Activity in Serum of Patients with MPS III and MPS II

Gelatin zymography analysis revealed that serum from patients with MPS III, as well as from control subjects express gelatinase activity of variable molecular mass (Fig. 1a). The two gelatin lysis bands with the lower molecular mass migrated as purified proMMP-2 (72 kDa) and MMP-2 (64 kDa) with proMMP-2 being the most prominent (Fig. 1a). The other two lysis bands of higher molecular mass, which exhibited the same intensity, correspond to the proform of MMP-9 (92 kDa) and the activated MMP-9 (78 kDa) (Fig. 1a). Quantitation of the lysis bands, using a computer-assisted image analysis program revealed that the activity of proMMP-9 and MMP-9, was significantly reduced in Sanfilippo patients, as compared to controls ($p = 0.016$ and 0.028, respectively, for proMMP-9 and MMP-9) (Fig. 1b). In the same group of patients, enzyme activity of MMP-2 was significantly increased compared to healthy controls ($p = 0.046$), whereas no difference was observed concerning proMMP-2 ($p = 0.917$) (Fig. 1b).

Furthermore, we observed that the ratio of latent (proMMP-2) to activated MMP-2 in the serum of Sanfilippo patients was 5.5:1, whereas in healthy subjects the same ratio was 11:1, indicating a higher degree of MMP-2 activation in the serum of Sanfilippo patients. We found no differences in the ratio of proMMP-9 to MMP-9 between Sanfilippo patients and controls (1:1).

Finally, we found a strong negative correlation between activated MMP-9 and age ($r = -0.926$, $p = 0.024$) in MPS III patients, as opposed to controls, where no correlation was observed ($r = -0.414$, $p = 0.441$) (Table 2). No significant correlation was found between the activity of proMMP-9, proMMP-2, and MMP-2 and the age of patients with MPS III (Table 2).

Gelatin zymography analysis revealed that the serum of the patient with MPS II exhibited no lysis bands corresponding to proMMP-9, MMP-9, and MMP-2, but only one band, migrating as proMMP-2 (Fig. 2a). Quantification of the gelatinolytic activity revealed a 1.6-fold increase in proMMP-2 activity in MPS II, as compared to healthy controls (Fig. 2b).

Enzyme Replacement Treatment Alters Gelatinase Activity in Serum of Patients with MPS VI

The patient with MPS VI is under ERT and thus, we obtained consecutive blood samples before the initiation of treatment, as well as, 1, 2, 3, 4 and 6 months following the administration of the enzyme. Gelatin zymography analysis

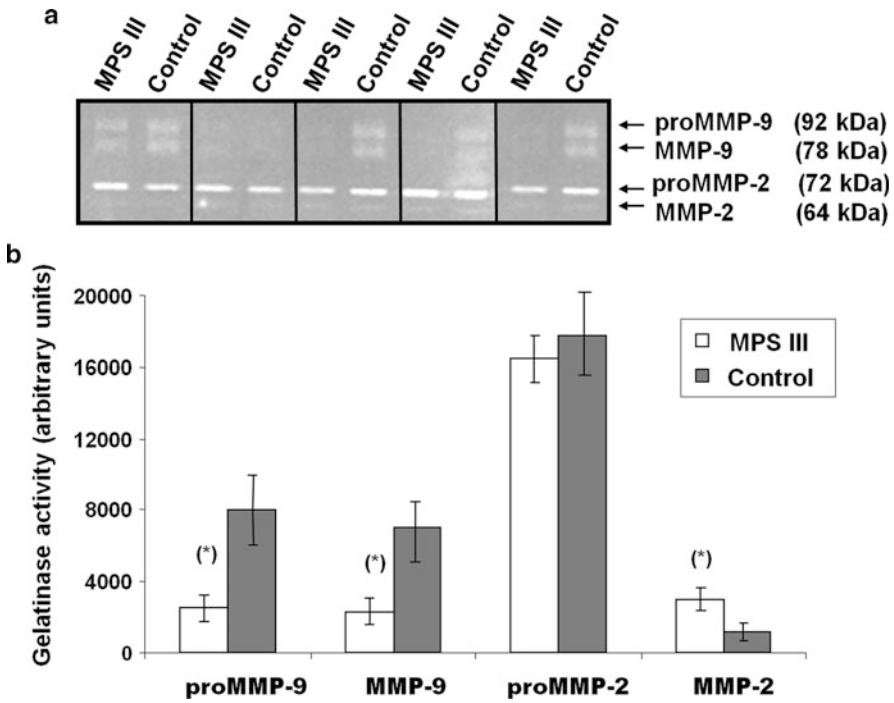


Fig. 1 Gelatinase activity is altered in patients with Sanfilippo disease. (**a**) Representative gelatine zymography in the serum of patients with MPS III ($n = 5$) and age- and sex-matched controls ($n = 5$, for each MPS III patient). (**b**) Quantitative analysis of gelatinolytic activity in serum of patients with MPS III and healthy controls using a computer-supported image analysis program. Each *bar* represents the mean ± SED from zymographies which were performed in triplicate for each patient. Statistical significance: (*) $p < 0.05$

Table 2 Correlation analysis between age and gelatin zymography values

Variable	Patients with MPS III ($n = 5$)	Controls ($n = 25$)
proMMP-9	$r = -0.876$, $p = 0.052$	$r = -0.149$, $p = 0.791$
MMP-9	$r = -0.926$, $p = 0.024$*	$r = -0.414$, $p = 0.441$
proMMP-2	$r = 0.154$, $p = 0.805$	$r = 0.723$, $p = 0.189$
MMP-2	$r = -0.333$, $p = 0.584$	$r = -0.678$, $p = 0.199$

*Statistically significant

revealed that the serum of the patient with MPS VI exhibited no lysis bands corresponding to MMP-9 and MMP-2, but only for proMMP-9 and proMMP-2 (Fig. 3a). Quantification of zymography lysis bands revealed that before treatment, proMMP-9 activity was decreased by 30% (Fig. 3b), whereas proMMP-2 activity was increased by 146%, (Fig. 3c), as compared to healthy controls.

ERT altered gelatinase activity in the serum of the patient with MPS VI. As shown in Fig. 3b, proMMP-9 was significantly reduced 1 month after the initiation of ERT. Thereafter, proMMP-9 activity was increased to reach, 6 months after treatment, the same level as observed in the age- and sex-matched controls.

The activity of proMMP-2 was initially decreased to control levels, 1 month after the initiation of ERT (Fig. 3c). However, 2 months after treatment, proMMP-2 activity was increased to the same level as before ERT and remained at this increased level up to 6 months following ERT (Fig 3c).

Immunoassays of MMPs and TIMPs

The concentration of MMP-2, MMP-9, TIMP-1, and TIMP-2 in serum of patients with MPS was assessed by ELISA. In Sanfilippo patients, circulating levels of MMP-2 were significantly increased ($p = 0.028$) (Fig. 4a), whereas serum concentration of MMP-9 was significantly decreased ($p = 0.047$) (Fig. 4b), as compared to sex- and age-matched controls. Regarding TIMP-1 and TIMP-2, no significant differences were found in circulating levels of both TIMPs between Sanfilippo patients and controls ($p = 0.095$ and 1.0, respectively, for TIMP-1 and TIMP-2) (Fig. 4c, d).

No significant correlations were found in patients with MPS III between all measured parameters and age, as revealed by statistical analysis (Table 3).

Regarding the MPS II patient, we observed a 2.6-fold increase in serum concentration of MMP-2, a 0.25-fold

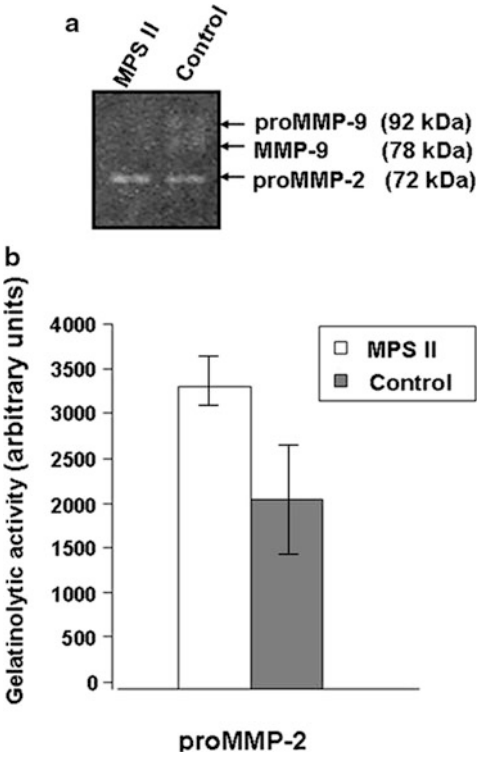


Fig. 2 Gelatinase activity is increased in MPS II. (**a**) Representative gelatine zymography in the serum of a patient with MPS III and age- and sex-matched controls ($n = 10$). (**b**) Quantitative analysis of gelatinolytic activity in serum of a patient with MPS II and healthy controls using a computer-supported image analysis program. Each *bar* represents the mean ± SED from zymographies, which were performed in triplicate for each patient

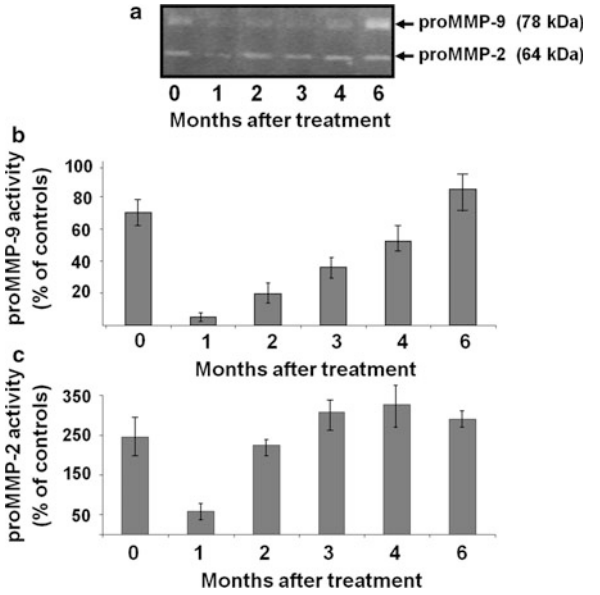


Fig. 3 Enzyme replacement therapy alters gelatinase activity in MPS VI. (**a**) Representative gelatine zymography in the serum of a patient with MPS VI before the initiation of treatment (0), as well as 1, 2, 3, 4, and 6 months after treatment. (**b**–**c**) Quantitative analysis of gelatinolytic activity using a computer-supported image analysis program. Each bar represents the mean ± SED from zymographies which were performed in triplicate and are presented as % of the gelatinolytic activity of age- and sex-matched controls ($n = 10$)

decrease in MMP-9, a 1.6-fold increase in TIMP-1 levels, and a twofold increase in circulating levels of TIMP-2, as compared to controls (Fig. 5).

Circulating levels of gelatinases and TIMPs were also assessed in consecutive blood samples taken from the patient with Maroteaux–Lamy syndrome, before treatment, as well as 1, 2, 3, 4, and 6 months following the initiation of ERT. All concentrations are expressed as % of serum levels of healthy subjects. As shown in Fig. 6, the administration of the enzyme alters serum concentration

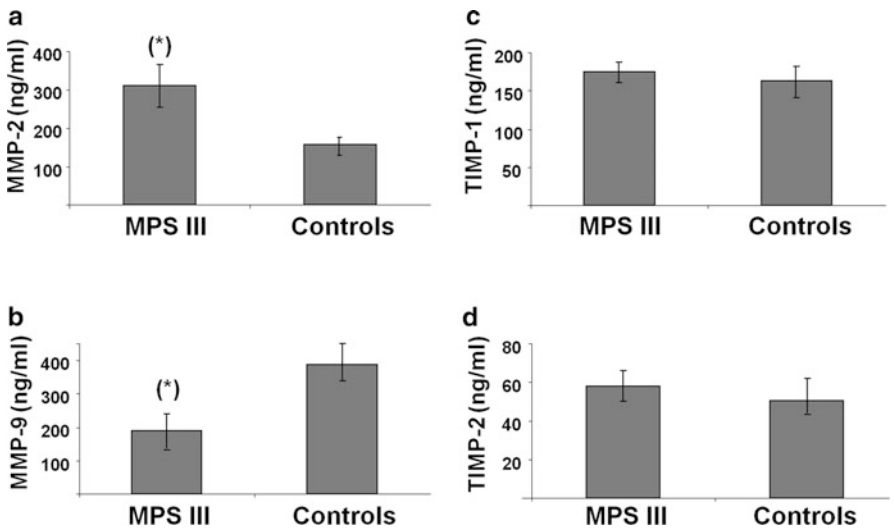


Fig. 4 Concentration of circulating MMP-2 (**a**), MMP-9 (**b**), TIMP-1 (**c**), TIMP-2 (**d**), in the serum of patients with MPS III ($n = 5$) and age- and sex-matched controls ($n = 5$, for each MPS III patient) measured by ELISA. Each bar represents mean ± SED from experiments performed in duplicate. Statistical significance: (*) $p < 0.05$

Table 3 Correlation analysis between age and immunoassays values

Variable	Patients with MPS III ($n = 5$)	Controls ($n = 25$)
MMP-9	$r = -0.344$, $p = 0.517$	$r = 0.112$, $p = 0.873$
MMP-2	$r = 0.107$, $p = 0.864$	$r = 0.668$, $p = 0.291$
TIMP-1	$r = 0.813$, $p = 0.187$	$r = -0.031$, $p = 0.955$
TIMP-2	$r = 0.812$, $p = 0.188$	$r = -0.282$, $p = 0.632$

of MMP-2, MMP-9, TIMP-1 and TIMP-2, but no stable pattern was observed. MMP-2 showed a 1.5-fold increase before treatment with no significant alterations during ERT (Fig. 6a). MMP-9 concentration exhibited an almost 60% decrease, 2 and 3 months following the initiation of ERT, and thereafter its concentration increased to reach, 6 months after the initiation of treatment, the same levels as before treatment (Fig. 6b). Concerning circulating levels of TIMPs before treatment, TIMP-2 showed a 1.6-fold increase and TIMP-1 a slight decrease in comparison with healthy subjects, with those levels showing no significant alterations during the administration of ERT (Fig. 6c, d).

Discussion

Very few analytical tools are at present available to diagnose MPS disorders, as well as to predict disease

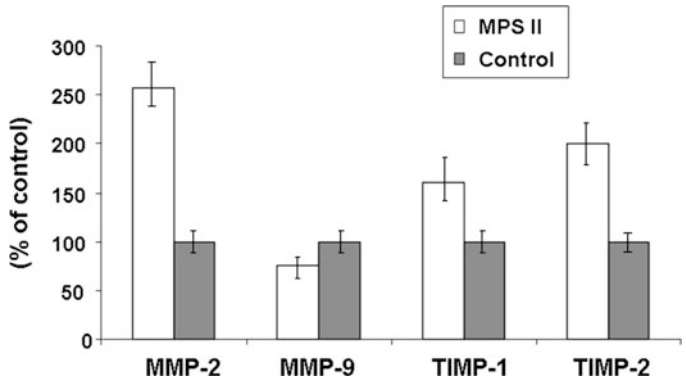


Fig. 5 Levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 measured by ELISA in the serum of one patient with MPS II. Each *bar* represents the mean ± SED from experiments performed in duplicate and are presented as % of values of age- and sex-matched controls ($n = 10$)

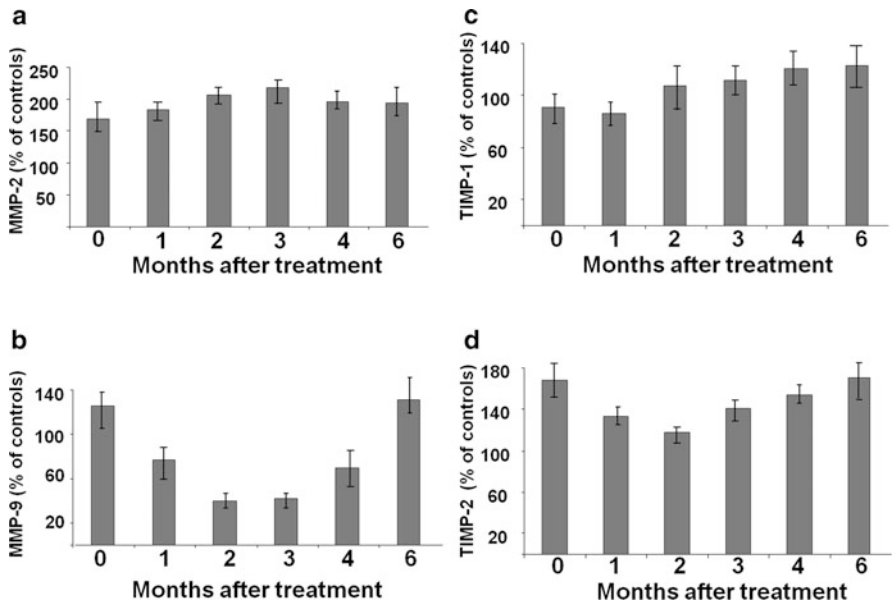


Fig. 6 Enzyme replacement therapy alters protein levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the serum of patients with MPS VI. The protein levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 were measured in the serum of a patient with MPS VI before the initiation of treatment (0), as well as 1, 2, 3, 4, and 6 months after treatment by ELISA. Each bar represents the mean ± SED from experiments performed in duplicate and are presented as % of values of age- and sex-matched controls ($n = 10$)

severity and responsiveness to therapy. Randall et al., have shown a significant increase of heparin cofactor II-thrombin complex in the serum of patients with MPS I in relation to healthy subjects, with its levels showing responsiveness to various treatment regimens (Randall et al. 2008). In addition, with the use of SELDI-TOF mass spectrometry, it has been found that patients with MPS show an increase in the ratio of the two forms of apolipoprotein ApoCI in plasma, which was associated with an increase in the activity of dipeptidyl peptidase IV (Beesley et al. 2009). These molecules were reported to be good biomarkers both for the diagnosis and for the therapy follow-up in MPS patients.

This study includes a small population of patients, most of them suffering from subtype B of Sanfilippo disease, which is the most common type of MPS disorders in our country (Michelakakis et al. 1995). Aiming to elucidate the mechanisms underlying the etiopathogenesis of these syndromes and unravel potential biomarkers, we examined the enzyme activity and protein levels of MMP-2 and MMP-9 in the serum of MPS patients. Additionally, we studied circulating levels of TIMP-1 and TIMP-2.

Many members of the MMP family represent research targets and were examined in tissues of animal models suffering from MPS during the past years. Common characteristic of all these studies is the altered expression of enzymes studied, such as MMP-1 and MMP-13 (Simonaro et al. 2008), MMP-12 (Ma et al. 2008), as well as the two members of gelatinase family, MMP-2 and MMP-9 (Richard et al. 2008; Simonaro et al. 2005). Despite all this work, MMPs have not been sufficiently studied in human tissues, with the exception of one study, conducted by Di Natale et al., which revealed a down-regulation of MMP-9 in patients with MPS VI (Di Natale et al. 2008). Apart from the expression of MMPs, enzyme activity of these proteins is also a promising study field. Simonaro et al., have found a significant increase in enzyme activity of gelatinases in synovial membranes of animals with MPS VI and VII (Simonaro et al. 2005). Thus, this study represents the first work investigating enzyme activity and expression of MMP-2 and MMP-9 in a human population with MPS. Concerning MMP-2, we found a significant increase both in enzyme activity and serum concentration, whereas a decrease in gelatinolytic activity and circulating levels of MMP-9 was observed. Those differences were even more obvious in the Sanfilippo group of patients. Given the fact that MMPs seem to be involved in numerous neuroinflammatory diseases and an overproduction of these enzymes is coupled with a disruption of blood–brain barrier (Mun-Bryce and Rosenberg 1998), MMP-2 and MMP-9 might represent suitable biomarkers demonstrating CNS involvement. In relation to treatment responsiveness, the alteration in enzyme activity and serum expression of gelatinases following the initiation of ERT classifies these molecules as possible candidates for follow-up markers.

In conclusion, the data presented in this study provide an important baseline for the differential expression of MMPs and TIMPs in MPS. A limitation of our study is the low number of MPS patients, and especially those undergoing ERT. Thus, our results need to be reconfirmed in studies employing a larger population of MPS patients of all types, in order to define more accurately the implication of the studied molecules, in the etiopathogenesis of these disorders. However, even with the limited number of patients, our results indicate that gelatinases are potential biomarkers for the diagnosis, follow-up and response to therapy in patients with MPS and should be further examined at a genetic level.

Take-Home Message

MMPs represent potential biomarkers for the diagnosis, follow-up, and response to therapy in patients with MPS.

References

Beesley CE, Young EP, Finnegan N et al (2009) Discovery of a new biomarker for the mucopolysaccharidoses (MPS), dipeptidyl peptidase IV (DPP-IV; CD26), by SELDI-TOF mass spectrometry. Mol Genet Metab 96(4):218–224

Borkakoti N (1998) Matrix metalloproteases: variations on a theme. Prog Biophys Mol Biol 70(1):73–94

Brew K, Dinakarpandian D, Nagase H (2000) Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 1477(1–2):267–283

Castaneda JA, Lim MJ, Cooper JD, Pearce DA (2008) Immune system irregularities in lysosomal storage disorders. Acta Neuropathol 115(2):159–174

Clark IM, Swingler TE, Sampieri CL, Edwards DR (2008) The regulation of matrix metalloproteinases and their inhibitors. Int J Biochem Cell Biol 40(6–7):1362–1378

Clarke LA (2008) The mucopolysaccharidoses: a success of molecular medicine. Expert Rev Mol Med 10:e1

Di Natale P, Villani GR, Parini R et al (2008) Molecular markers for the follow-up of enzyme-replacement therapy in mucopolysaccharidosis type VI disease. Biotechnol Appl Biochem 49 (3):219–223

Gallegos-Arreola MP, Machorro-Lazo MV, Flores-Martínez SE et al (2000) Urinary glycosaminoglycan excretion in healthy subjects and in patients with mucopolysaccharidoses. Arch Med Res 31(5):505–510

Ma X, Tittiger M, Knutsen RH et al (2008) Upregulation of elastase proteins results in aortic dilatation in mucopolysaccharidosis I mice. Mol Genet Metab 94(3):298–304

Michelakakis H, Dimitriou E, Tsagaraki S, Giouroukos S, Schulpis K, Bartsocas CS (1995) Lysosomal storage diseases in Greece. Genet Couns 6(1):43–47

Muenzer J (2004) The mucopolysaccharidoses: a heterogeneous group of disorders with variable pediatric presentations. J Pediatr 144 (5):27–34

Mulloy B, Rider CC (2006) Cytokines and proteoglycans: an introductory overview. Biochem Soc Trans 34(3):409–413

Mun-Bryce S, Rosenberg GA (1998) Gelatinase B modulates selective opening of the blood–brain barrier during inflammation. Am J Physiol 274(5 Pt 2):1203–1211

Nagase H, Woessner JF Jr (1999) Matrix metalloproteinases. J Biol Chem 274:21491–21494

Neufeld EF, Muenzer J (2001) The mucopolysaccharidoses. In: Scriver CR, Beaudet AL, Sly WS (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 3421–3452

Ohmi K, Greenberg DS, Rajavel KS, Ryazantsev S, Li HH, Neufeld EF (2003) Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. Proc Natl Acad Sci USA 100 (4):1902–1907

Papakonstantinou E, Aletras AJ, Glass E et al (2005) Matrix metalloproteinases of epithelial origin in facial sebum of patients with acne and their regulation by isotretinoin. J Invest Dermatol 125(4):673–684

Proudfoot AE (2006) The biological relevance of chemokine-proteoglycan interactions. Biochem Soc Trans 34(3):422–426

Randall DR, Colobong KE, Hemmelgarn H et al (2008) Heparin cofactor II-thrombin complex: a biomarker of MPS disease. Mol Genet Metab 94(4):456–461

Richard M, Arfi A, Rhinn H, Gandolphe C, Scherman D (2008) Identification of new markers for neurodegeneration process in the mouse model of Sly disease as revealed by expression profiling of selected genes. J Neurosci Res 86(15):3285–3294

Rydlova M, Holubec L Jr, Ludvikova M Jr et al (2008) Biological activity and clinical implications of the matrix metalloproteinases. Anticancer Res 28(2B):1389–1397

Simonaro CM, Haskins ME, Schuchman EH (2001) Articular chondrocytes from animals with a dermatan sulfate storage disease undergo a high rate of apoptosis and release nitric oxide and inflammatory cytokines: a possible mechanism underlying degenerative joint disease in the mucopolysaccharidoses. Lab Invest 81(9):1319–1328

Simonaro CM, D'Angelo M, Haskins ME, Schuchman EH (2005) Joint and bone disease in mucopolysaccharidoses VI and VII: identification of new therapeutic targets and biomarkers using animal models. Pediatr Res 57:701–707

Simonaro CM, D'Angelo M, He X et al (2008) Mechanism of glycosaminoglycan-mediated bone and joint disease: implications for the mucopolysaccharidoses and other connective tissue diseases. Am J Pathol 172(1):112–122

Villani GR, Gargiulo N, Faraonio R, Castaldo S, Gonzalez Y, Reyero E, Di Natale P (2007) Cytokines, neurotrophins, and oxidative stress in brain disease from mucopolysaccharidosis IIIB. J Neurosci Res 85(3):612–622

Woessner JF Jr (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5(8): 2145–2154

JIMD Reports
DOI 10.1007/8904_2011_61

CASE REPORT

COG5-CDG with a Mild Neurohepatic Presentation

C.W. Fung • G. Matthijs • L. Sturiale • D. Garozzo • K.Y. Wong • R. Wong • V. Wong • J. Jaeken

Received: 23 February 2011 / Revised: 23 February 2011 / Accepted: 4 April 2011 / Published online: 22 September 2011

Abstract The conserved oligomeric Golgi (COG) complex is an eight subunit protein involved in the retrograde transport of Golgi components. It affects the localization of several Golgi glycosyltransferases and hence is involved in N- and O-glycosylation. Genetic defects in this complex belong to the rapidly expanding family of congenital disorders of glycosylation (CDG). Patients have been reported with defects of subunit 1 (CDG1-CDG), subunit 4 (CDG4-CDG), subunit 5 (CDG5-CDG), subunit 6 (CDG6-CDG), subunit 7 (CDG7-CDG), and subunit 8 (CDG8-CDG). This paper is on the second reported patient with COG5-CDG. She showed a mild neurohepatic disease with central as well as peripheral neurological involvement while in the first reported patient (with a different mutation) only mild central neurological involvement was reported.

Communicated by: Eva Morava.

Competing interests: None declared.

C.W. Fung · K.Y. Wong · R. Wong · V. Wong
Department of Pediatrics and Adolescent Medicine, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong

C.W. Fung · K.Y. Wong · R. Wong · V. Wong
Duchess of Kent Children's Hospital, The University of Hong Kong, Pokfulam, Hong Kong

G. Matthijs
Centre for Human Genetics, Katholieke Universiteit Leuven, Leuven, Belgium

L. Sturiale · D. Garozzo
CNR-Istituto di Chimica e Tecnologia dei Polimeri, Catania, Italy

J. Jaeken (✉)
Centre for Metabolic Diseases, Katholieke Universiteit Leuven, Leuven, Belgium
e-mail: jaak.jaeken@uzleuven.be

Abbreviations

CDG	Congenital disorder(s) of glycosylation
COG	Conserved oligomeric GoLgi complex
IEF	Isoelectrofocusing

Introduction

Congenital disorders of glycosylation (CDG) (Freeze 2006; Jaeken and Matthijs 2007; Jaeken et al. 2009) are mostly due to defects in the specific glycosylation machinery but a growing group of CDG is caused by disordered multifunctional proteins e.g., ATP6V0A2-CDG (Guillard et al. 2009) and the COG-CDG (Foulquier 2009; Zeevaert et al. 2008). The cytosolic COG complex is made up of eight subunits and involved in retrograde vesicular Golgi trafficking (reviews in Lees et al. 2010 and in Reynders et al. 2011). Patients have been reported with defects in subunits 1, 4, 5, 6, 7 and 8. The first report on a patient with a defective subunit 5 was by Paesold-Burda et al. (2009). This is the second report on a patient with COG5-CDG.

Patient and Methods

Patient

The patient was the second child of a nonconsanguineous Chinese couple. Intrauterine growth retardation was noted since 20 weeks of gestation with decreased liquor at 30 weeks. She was born at 35 weeks of gestation by Cesarean section with a birth weight of 2.1 kg. Neonatal jaundice was treated with phototherapy. At 1 month, she

developed abdominal distension due to dilated bowel, and jaundice. Contrast enema showed a short segment of persistent narrowing in the most distal part of the rectum and anus but rectal biopsy excluded Hirschsprung disease. On liver function testing there was an increase of serum bilirubin [total bilirubin 206 μmol/L (normal: 10–24), unconjugated bilirubin 172 μmol/L (normal 3–17), conjugated bilirubin 13 (normal: 0–10)], alkaline phosphatase [891 U/L (normal: 145–420)], alanine aminotransferase [67 U/L (normal: 5–35)], aspartate aminotransferase [281 U/L (normal: 15–60)] and gamma-glutamyl transpeptidase [149 U/L (normal: 6–22)], with normal albumin. Scintigraphy excluded biliary atresia. Ultrasonography of the abdomen was normal, and the abdominal distension resolved.

Physical examination at 8 months showed microcephaly (39 cm, 2 cm < 3rd percentile). Weight was 6.3 kg (3rd percentile) and height 65.7 cm (10th–25th percentile). There was no dysmorphy. She had global developmental delay (mental age of 3–4 months), hypotonia, fixed flexion contractures of all fingers and mild hepatosplenomegaly. Liver dysfunction persisted except for normalization of bilirubin [alkaline phosphatase 737 U/L (normal: 145–420)], alanine aminotransferase 123 U/L (normal: 5–35), aspartate aminotransferase 271 U/L (normal: 15–60), gamma-glutamyl transpeptidase 41 U/L (normal: 6–22), fasting bile acids 11.4 μM (normal: <7), prothrombin time 13.4 s (normal: 11.3–13.2), activated partial thromboplastin time 40.6 s (normal: 27.6–37.6)). The renal function, blood hemoglobin, and total white cells were normal but there was a mild thrombocytopenia [135×10^9/L (normal: $150–400 \times 10^9$)].

A repeat ultrasonography of the abdomen showed liver cirrhosis with regeneration nodules and splenomegaly. There was no significant portal hypertension. A liver biopsy at 12 months confirmed cirrhotic changes. There was no decrease in the number of bile ducts in comparison with the hepatic arterioles, no increased copper or copper-associated protein nor immunohistochemical evidence of alpha-1 antitrypsin deficiency, and the liver cells were negative for hepatitis B surface antigens. No infectious, autoimmune, or metabolic cause of the liver disease was found.

Serum alpha-fetoprotein was increased [at 9 months: 4,178 ng/mL (normal: <50); at 16 months: 72 ng/mL (normal: <20)] with persistent mild hyperlactatemia [2.2/2.3/2.9/3.9 mmol/L (normal: 0.7–2.1)].

Magnetic resonance imaging (MRI) of the brain at 13 months showed delayed myelination (corresponding to 7–9 months) without other structural abnormalities.

Griffiths Mental Developmental Scale performed at 14 months showed an overall mental age of 9.5 months (9 months for locomotor, 12 months for personal–social, 8.75 months for hearing and speech, 10.5 months for eye and hand coordination, and 7 months for performance domains). At 20 months, there was persistent failure to thrive and generalized hypotonia as well as evidence of peripheral neuropathy including muscle wasting, weakness of both hands and feet (with contractures in the digits), and depressed deep tendon reflexes especially over the lower limbs. There was no cerebellar ataxia or sensory disturbances. In view of the multisystem involvement and hyperlactatemia, a mitochondrial oxidative phosphorylation defect or a congenital disorder of glycosylation was suspected.

She was started on ursodeoxycholic acid at 36 months. At 4 years 5 months, serum transferrin isoelectrofocusing (University Hospital Leuven, Belgium) revealed a type 2 pattern. Additional investigations in the context of a possible glycosylation defect showed normal serum haptoglobin, cholesterol, antithrombin III, factor IX, and factor XI. Echocardiography at 6 years was normal.

Ultrasonography of the abdomen at 9 years showed cirrhotic liver with a normal size without evidence of portal hypertension. The spleen was enlarged to 12 cm. Serial monitoring of liver function showed gradual improvement [alkaline phosphatase 468 U/L (normal: 145–420), aspartate aminotransferase 48 U/L (normal: 15–40), and normalization of all other parameters]. Platelet count went down to 63×10^9/L (normal: $150–400 \times 10^9$).

There was catching up of her developmental milestones. She was studying in a mainstream school with mild learning difficulty. Intellectual assessment (Hong Kong Wechsler Intelligence Scale for Children) at 8 years 8 months showed a verbal intelligent quotient of 99 (in normal range) and a performance intelligent quotient of 62 (mild mental retardation range). She showed a mild attention deficit disorder but hearing and visual assessments were normal. Repeat brain MRI at 4 years showed an improvement in myelination corresponding to about 24 months. Brain proton magnetic resonance spectroscopy was normal.

At the age of 9, her microcephaly was nonprogressive and the peripheral neuropathy was stable. Because of the functional limitation, she underwent a corrective surgery for the finger contractures and was considering surgery also for the toes.

Methods

Serum transferrin IEF (Jaeken et al. 1984), serum apolipoprotein C-III IEF (Wopereis et al. 2003), and serum transferrin glycan MALDI TOF analysis (Sturiale et al. 2005) were performed as described.

Mutation analysis of the eight COG subunit genes was performed by direct sequencing.

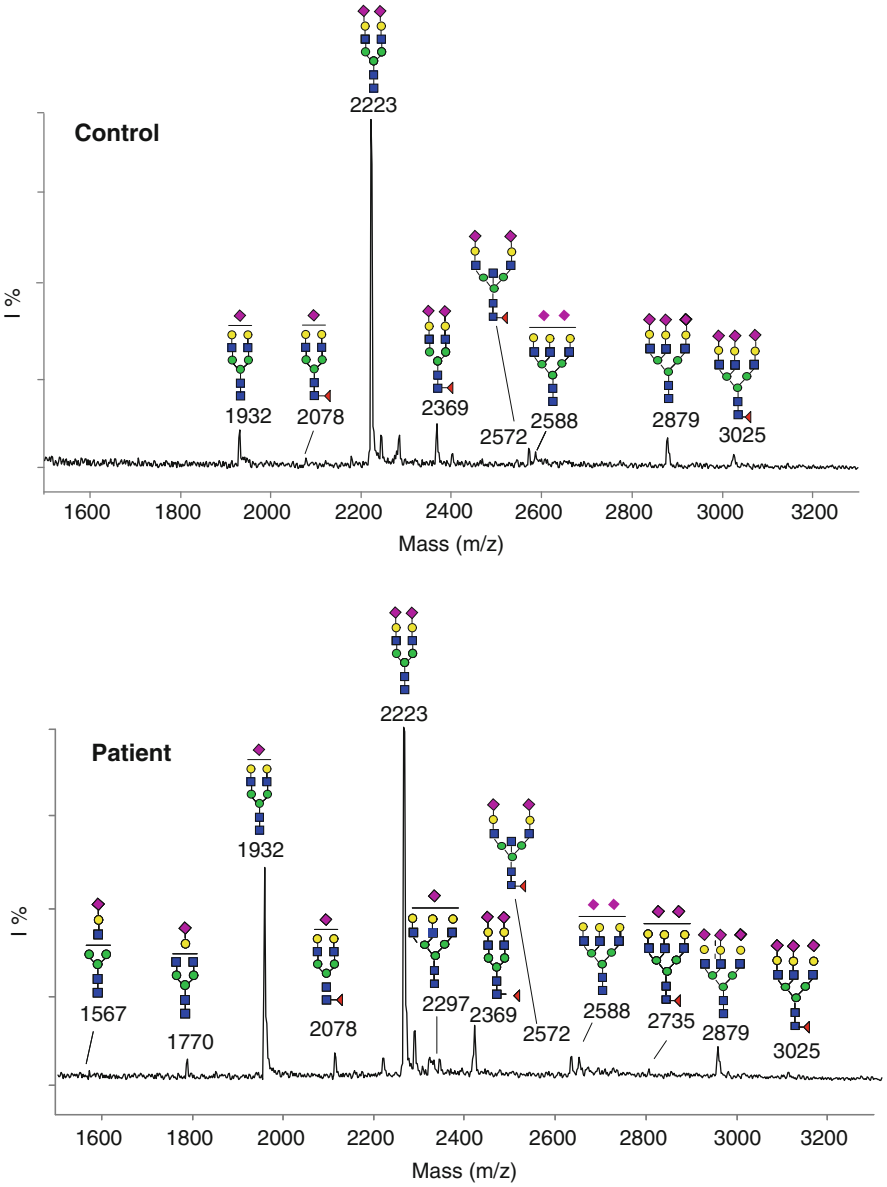


Fig. 1 MALDI TOF analysis of the acidic N-glycans from serum transferrin shows a remarkable underglycosylation mainly due to the lack of a terminal sialic acid (increased peaks at *m/z* 1932, *m/z* 2078, *m/z* 2588, *m/z* 2735) and, to a much lesser extent, also due to the presence of truncated biantennary glycoforms at *m/z* 1770 (lacking a terminal sialic acid and a galactose unit) and at *m/z* 1567 (trace amount of a species lacking a terminal trisaccharide composed of a sialic acid, a galactose and an *N*-acetylglucosamine residue). *Blue squares N*-acetylglucosamine; *green circles* mannose; *yellow circles* galactose; *red diamonds* sialic acid

Results

Serum transferrin IEF showed a type 2 pattern and serum apolipoprotein C-III a decrease of the disialo isoform and an increase of the asialo isoform. MALDI TOF analysis of serum transferrin glycans showed mainly evidence of hyposialylation (Fig. 1).

Mutation analysis of *COG5* showed two novel mutations: c.556–560delAGTAAinsCT (maternal) and c.1856T > C (p.I619T) (paternal).

Discussion

Genetic defects in subunits of the COG complex belong to a peculiar subgroup of the CDG family since these disorders affect proteins that are not specifically involved in the glycosylation machinery. Patients have been reported with defects in subunit 1 (COG1-CDG; three patients from different families), in subunit 4 (COG4-CDG; one patient), in subunit 5 (COG5-CDG; one patient), in subunit 6 (COG6-CDG; two siblings), in subunit 7 (COG7-CDG; 7

patients from 5 families), and in subunit 8 (COG8-CDG; 2 unrelated patients) (reviews in Foulquier 2009 and in Zeevaert et al. 2008; Lübbehusen et al. 2010; Paesold-Burda et al. 2009; Reynders et al. 2009). Their clinical presentations are variable (mild as well as severe) combinations mainly of neurological, morphological, and hepatic abnormalities. COG7-CDG is actually the largest group and, interestingly, six of the seven reported patients showed the same homozygous mutation and a fairly homogenous phenotype. They were from Moroccan or Tunesian descent and their disease was characterized by prenatal growth retardation, prenatal microcephaly with postnatal progression, feeding problems, severe psychomotor retardation, epilepsy, dysmorphy of face and hands (retro/micrognathy, small mouth, short neck, wrinkled skin, overlapping fingers), episodes of hyperthermia, and liver dysfunction. The number of patients with the other COG-CDG is too small to associate them with a consistent phenotype except for the fact that two (unrelated) patients with the same homozygous, intronic mutation in COG subunit 1 showed a cerebrocostomandibular-like syndrome (Zeevaert et al. 2009).

The one reported patient with COG5-CDG showed a mild neurological phenotype with psychomotor retardation and delayed motor and language development, and hyposialylation of N- and O-glycans. Mutation analysis showed a homozygous intronic substitution (c.1669−15T > C) leading to exon skipping and severely reduced expression of the COG5 protein. The present patient also shows a mild phenotype but her neurological involvement is central as well as peripheral and there is also a mild, stable hepatic phenotype. She has two mutations, different from the one found in the other reported patient.

We propose to perform mutation analysis for the COG subunit genes in patients with a type 2 serum transferrin IEF pattern and aspecific serum transferrin glycan abnormalities on MALDI TOF (hyposialylation and/or hypogalactosylation) unless there is a typical clinical cutis laxa type II syndrome (ATP6V0A2-CDG).

References

Foulquier F (2009) COG defects, birth and rise! Biochim Biophys Acta 1792:896–902

Freeze HH (2006) Genetic defects in the human glycome. Nat Rev Genet 7:537–551

Guillard M, Dimopovlov A, Fischer B et al (2009) Vacuolar H^+−AT Pase meets glycosylation in patients with cutis laxa. Biochim Biophys Acta 1792: 903–914

Jaeken J, van Eijk HG, van der Heul C et al (1984) Sialic acid-deficient serum and cerebrospinal fluid transferrin in a newly recognized syndrome. Clin Chim Acta 144:245–247

Jacken J, Matthys G (2007) Congenital disorders of glycosylation: a rapidly expanding disease family. Ann Rev Genomics Atom Genet 8:261–278

Jaeken J, Hennet T, Matthijs G, Freeze HH (2009) CDG nomenclature: time for a change! Biochim Biophys Acta 1792:825–826

Lees AJ, Yip CK, Walz T, Hughson FM (2010) Molecular organization of the COG vesicle tethering complex. Nat Struct Mol Biol 17:1292–1297

Lübbehusen J, Thiel C, Rind N et al (2010) Fatal outcome due to deficiency of subunit 6 of the conserved oligomeric Golgi complex leading to a new type of congenital disorders of glycosylation. Hum Mol Genet 19:3623–3633

Paesold-Burda P, Maag C, Troxler H et al (2009) Deficiency in COG5 causes a moderate form of congenital disorders of glycosylation. Hum Mol Genet 18:4350–4356

Reynders E, Foulquier F, Leão TE et al (2009) Golgi function and dysfunction in the first COG4-deficient CDG type II patient. Hum Mol Genet 18:3244–3256

Reynders E, Foulquier F, Annaert W, Matthijs G (2011) How Golgi glycosylation meets and needs trafficking: the case of the COG complex. Glycobiology 21:853–863

Sturiale L, Barone R, Fiumara A et al (2005) Hypoglycosylation with increased fucosylation and branching of serum transferrin N-glycans in untreated galactosemia. Glycobiology 15:1268–1276

Wopereis S, Grünewald S, Morava E et al (2003) Apolipoprotein C-III isofocusing in the diagnosis of genetic defects in O-glycan biosynthesis. Clin Chem 49:1839–1845

Zeevaert R, Foulquier F, Jaeken J, Matthijs G (2008) Deficiencies in subunits of the conserved oligomeric Golgi (COG) complex define a novel group of congenital disorders of glycosylation. Mol Genet Metab 93:261–278

Zeevaert R, Foulquier F, Dimitrov B et al (2009) Cerebrocostomandibular-like syndrome and a mutation in the conserved oligomeric Golgi complex subunit 1. Hum Mol Genet 18:517–524

JIMD Reports
DOI 10.1007/8904_2011_62

RESEARCH REPORT

Partial Rescue of Biochemical Parameters After Hematopoietic Stem Cell Transplantation in a Patient with Prolidase Deficiency Due to Two Novel *PEPD* Mutations

Désirée Caselli • Rolando Cimaz • Roberta Besio • Antonio Rossi • Ersilia De Lorenzi • Raffaella Colombo • Luca Cantarini • Silvia Riva • Marco Spada • Antonella Forlino • Maurizio Aricò

Received: 1 March 2011 / Revised: 2 May 2011 / Accepted: 3 May 2011 / Published online: 27 September 2011

Abstract Prolidase deficiency (PD) is a rare recessive disorder resulting from mutations in the prolidase gene (*PEPD*); only 17 causative mutant alleles had been so far characterized. Prolidase is a ubiquitous enzyme that hydrolyses dipeptides with C-terminal proline or hydroxyproline residues and indeed, lack of this enzyme activity causes massive urine excretion of undigested iminodipeptides. The clinical manifestations of PD are widely variable, and include intractable skin ulcers, unusual face, different degree of mental retardation, and recurrent infections. No definitive treatment is at present available.

We report an 8-year girl with a typical PD facies, normal intelligence, and recurrent deep ulcerations complicated by infections. She was found to be compound heterozygous for two novel mutations in *PEPD*, c.1133delACG and c.1301delT, affecting the C-terminal end of the enzyme where the active site is located. Given her life-threatening course, she underwent allogeneic hematopoietic stem cell transplantation (HSCT) from her HLA-identical brother, confirmed heterozygous for the c.1133delACG allele. Successful engraftment was documented by full-donor chimerism. Posttransplant monitoring of erythrocyte prolidase activity showed that the child had converted to a heterozygous pattern. Reduction of excreted urine dipeptides, evaluated by capillary electrophoresis, supported the effectiveness of the treatment. Unfortunately the patient died on day +92 of invasive fungal infection.

Despite the unfavorable outcome, we provide the first evidence that HSCT has the potential to reverse some of the biochemical features of PD patients. The indication to transplant must be balanced against the clinical manifestation of individual patients.

Communicated by: Jean-Marie Saudubray.

Competing interests: None declared.

Electronic supplementary material The online version of this article (doi:10.1007/8904_2011_62) contains supplementary material, which is available to authorized users.

D. Caselli · M. Aricò (✉)
Department Pediatric Hematology Oncology, Azienda Ospedaliero-Universitaria Meyer, Viale Pieraccini, 24, 50139, Florence, Italy
e-mail: m.arico@meyer.it

R. Cimaz
Rheumatology, AOU Meyer and University of Florence, Florence, Italy

L. Cantarini
Rheumatology Department, University of Siena, Siena, Italy

S. Riva · M. Spada
Istituto Mediterraneo Trapianti e Terapie ad Alta Specializzazione (IsMeTT), Palermo, Italy

R. Besio · A. Rossi · A. Forlino
Department of Biochemistry, University of Pavia, Pavia, Italy

A. Forlino
e-mail: aforlino@unipv.it

E. De Lorenzi · R. Colombo
Department of Drug Sciences, University of Pavia, Pavia, Italy

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
PRBC	Packed red blood cells

Introduction

Prolidase deficiency (PD; MIM 170100) is a rare disorder with recessive inheritance caused by mutations in the prolidase gene (*PEPD*, 19cen-q13.11). The prolidase enzyme hydrolyzes dipeptides containing C-terminal proline and hydroxyproline residues, and PD causes massive excretion of urinary iminodipeptides.

The clinical manifestations of PD are widely variable: affected patients show mainly intractable skin ulcers of lower extremities, unusual facial, ocular abnormalities, deafness, splenomegaly, obesity, often with mental retardation, and a history of recurrent infections (Royce and Steinman 2002). The molecular basis of such phenotype is still unknown.

Point mutations causing single amino acid substitutions, premature stop codon or exon splicing, small and large deletions, and a small duplication had so far been reported as causative mutations for PD (Lupi et al. 2008). Due to the limited number of patients investigated, the genotype– phenotype correlations are still poorly understood. Thus, the characterization of new mutant alleles in PD patients, together with their complete clinical description, will be important to better define the pathophysiology of this disease and to help develop appropriate treatments.

No definitive therapy is so far available for PD, although different therapeutic approaches had been attempted, mainly with no or partial rescue of the disease. Oral supplementation with manganese, a cofactor of prolidase, and vitamin C, acting on collagen synthesis, have been used as well as blood transfusions and apheresis, corticosteroid treatment, oral supplementation with antioxidants and topical antibiotics for the skin lesions (Lupi et al. 2008).

Prolidase is a ubiquitous enzyme highly expressed in circulating cells (erythrocytes and leukocytes) which are the preferred target for cell replacement therapy using hematopoietic stem cell transplantation (HSCT). Thus considering the recent advance in the treatment of cancer as well as inborn errors with HSCT, it seemed promising to apply such approach to PD patients (Archuleta et al. 2004; MacMillan et al. 2008; Trounson 2009).

Materials and Methods

Molecular Study

Genomic DNA was extracted from patient, donor and controls peripheral blood by standard techniques. The 14 exons and exons boundary of prolidase gene were amplified by PCR using 0.2 μg of gDNA and the primer pairs and annealing temperature listed in Supplementary Table 1. The PCR conditions were as follow: an initial 3 min denaturation at 94°C, followed by 35 PCR amplification cycles (1 min 94°C, 1 min at the specific annealing temperature and 1 min at 72°C) and a final 10 min extension at 72°C. The amplicons were run on agarose gel, gel purified and directly sequenced. The sequences obtained were compared with the reported PEPD gene sequence (MN_000285.3).

Determination of Prolidase Activity

Blood samples from the patient and healthy age-matched controls were collected in heparinized tubes before and at day 7, 14, 21, 28, 35, and 58 posttransplant. The donor, patient's brother, blood was also collected before the treatment. Prolidase activity in red blood cells was evaluated. Briefly, whole blood samples were fractionated by centrifugation at 1,200 g for 5–10 min at room temperature. The pelleted red blood cells were resuspended in 2 volumes of phosphate buffer saline (PBS, Sigma), centrifuged again at 1,200 g for 5 min at room temperature and washed 3 times with PBS. The cells were finally resuspended in 4 volumes of water and freeze-thawed twice. The solutions were then dialyzed overnight at 4°C against 50 mM Tris pH8.0, 1 mM $MnCl_2$. The prolidase activity was evaluated according to Myara procedure (Myara et al. 1982), which is based on measurement of proline by Chinard's reagent (Chinard 1952). Hemoglobin concentration was determined by spectrophotometric measurement (Davis and Sheard 1927). Results were expressed as μmol of proline released in 1 h per g of hemoglobin and presented as percentage with respect the activity of the healthy controls, considered as 100%.

Determination of Dipeptides in Urine

Urine samples from the patient were collected at the same time points than blood samples, before and at day 7, 14, 21, 28, 35, and 58 posttransplant. Urines from the donor patient's brother and a healthy age/gender-matched control were also collected. The samples were centrifuged (5 min at 1,600 g, at 4°C) and the supernatants were analyzed by capillary electrophoresis (CE).

The experiments were performed on a P/ACE MDQ capillary electrophoresis (Beckman, Fullerton, CA, USA) with built-in diode-array detector. The uncoated fused-silica capillaries (50 μm ID, 360 μm OD, effective length 46.8 cm, total length 57 cm) were from Polymicro Technologies (Phoenix, Arizona, USA). Before use, the capillaries were pretreated with 1 M NaOH for 60 min and 50 mM sodium tetraborate buffer pH 9.2 (Merck, Darmstadt, Germany) for 75 min, by applying a pressure of

14.5 psi. Before each run the capillary was rinsed with 0.1 M NaOH for 3 min and background electrolyte (sodium tetraborate buffer pH 9.2) for 3 min at 14.5 psi. Buffer solutions were prepared daily using deionized water filtered through 0.45 μm membrane filters (Millipore, Bedford, MA, USA) and degassed by sonication. The deionized water was produced using a Millipore Direct-Q TM.

The samples were hydrodynamically injected at 1.6 psi for 7 s. The electrophoretic run was carried out by applying a voltage of 25 kV (current 65–70 μA). Capillary cartridge and autosampler were thermostatted at 25°C. The acquisition wavelength was 200 nm.

Standard dipeptides mixture was prepared by mixing four dipeptides (Sigma) in different proportions (final concentrations were 2 mg/ml Gly-Pro, 1 mg/ml Ala-Pro, 1 mg/ml Phe-Pro, 1 mg/ml Leu-Pro). The samples were normalized to the creatinine concentration (Lupi et al. 2005).

Results were expressed ($n = 3$) as percentage of undigested dipeptides, namely (dipeptide area urine sample/dipeptide area standard mixture)/(creatinine area urine sample/creatinine area healthy age-matched control sample) × 100.

Results

Clinical Features

A 7-year-old female came to our attention because of multiple necrotic skin lesions on the lower limbs and buttocks; the first episode had occurred at the age of 17 months, then recurring at 5, and 6 years, but no diagnosis had been made. There was no significant family history, and past medical history was unremarkable. Physical examination showed dysmorphic features with hypertelorism and splenomegaly, with slight speech delay. A skin biopsy was performed, and showed leukocytoclastic vasculitis involving the venules of dermis and subcutaneous tissues, with extensive parietal fibrinoid necrosis, occlusion of vascular lumen, spongiosis, and ischemic necrosis of the superficial portion of malpighian layer. Clinical finding were suggestive of prolidase deficiency, and the diagnosis was confirmed by enzymatic activity evaluation in erythrocytes (22.41 ± 0.9 μmol/h per g in the patient, 514.14 ± 15.1 μmol/h per g in controls). Initial treatment consisted in low dose systemic steroids, topical preparations with proline and glycine, a proline-free diet, and erythrocytoapheresis. However, treatment had been unsuccessful, and the child still suffered from deep and very painful skin lesions. Infectious complications of the skin lesions occurred repeatedly; in particular, she developed one episode of septicemia that required treatment in the ICU of our children's hospital. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were isolated from blood culture; after a life-threatening course, she progressively recovered.

Molecular Characterization

The sequence of all prolidase-coding region and intronic boundaries revealed that the patient was a compound heterozygous for two novel mutations: c.1133delACG, causing the deletion of Asp378 in exon 13, and c.1301delT, causing a frameshift and a premature stop codon in exon 14 (Fig. 1 and Supplementary Fig. 1). Both mutations affect the terminal end of the prolidase, where the active site is known to be located (Lupi et al. 2008; Besio et al. 2009). The parents were carriers, the mother of the mutation in exon 13 (c.1133delACG) and the father of the mutation in exon 14 (c.1301delT); the donor, patient's brother, was carrier for the maternal c.1133delACG allele (data not shown).

Hematopoietic Stem Cell Transplantation

Given her clinical course, characterized by not only significant morbidity but also by one life-threatening infectious complication, the hypothesis of performing an allogeneic HSCT was considered. Based on the HLA typing, her brother turned to be HLA-identical. He was then studied under the metabolic point of view, and confirmed to be heterozygous for PD. Thus, after a thorough discussion with the parents, indication to HSCT was defined. The diagnostic pretransplant work-up showed that her liver and pulmonary function were normal. After a conditioning regimen with Busulfan 16/mg per kg + cyclofosfamide 200 mg/kg, we infused bone marrow HSC with

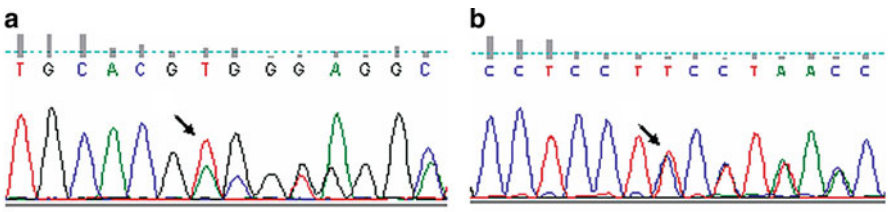


Fig. 1 Determination of the two novel PEPD mutations in patient gDNA. (**a**) 1131delACG, causing the deletion of Asp378 in exon 13. (**b**) 1301delT, causing a frameshift in exon 14 and the creation of a premature stop codon. The *arrows* indicate the position of the mutations

3.52 millions CD34+/donor kg. Defibrotide 10 mg/Kg was given for veno-occlusive disease (VOD) prophylaxis from day +1; Graft versus host disease prophylaxis consisted of methotrexate 10 mg/sqm i.v. on day +3, +6, +11, and cyclosporin 3 mg/Kg from day −1. Prophylaxis of infection consisted of: fluconazole 6 mg/kg per day i.v., and ayclovir 10 mg/kg per die i.v. q 8 h since day +1, and cefepime 100 mg/kg per day i.v. since day +4. The neutrophil take was recorded on day +16, while no platelet take was obtained; full-donor chimerism was repeatedly documented. On day +16 she developed spiking AST/ALT levels, up to 10,460/4,507 IU/L on +19, with jaundice, hepatomegaly and weight gain. Liver biopsy confirmed the histological diagnosis of VOD. The child died on +92 of invasive by Geotrichum Capitatum infection. A list of PRBC transfusion as well as time of urine and blood collection is listed in Table 1.

Enzyme Study and Urine Dipeptide Analysis

The patient's prolidase activity prior to transplantation was 4.36 ± 0.22% compared to age-matched controls. The sibling donor showed a prolidase activity ~50% of the control, confirming his carrier status for the disease also at the biochemical level. The patient's activity at d7 posttransplant was unchanged, whereas an increase to 50.51 ± 7.74% was detected 2 weeks after the transplant, to remain stable over the following 8 weeks (Fig. 2a).

Pretransplant dipeptides measured by CE was assumed to be 100% with respect to an age-matched control (0%), which, as expected, did not reveal any peak in the imidodipeptides chromatographic region (Fig. 3). The urine from the donor showed a modest percentage of undigested peptides of 8.46% ± 1.20 (Fig. 2b). As shown in Fig. 2b, the patient's percentage of undigested dipeptides declined after transplant down to 34.66 ± 1.19 at d35. The increment to 52.58 ± 3.51 at d58 was not due to exogenous administration of glutamine, which was ruled out; whether it may depend on liver alteration, remains unclear.

Table 1 Time of red blood cell transfusion, prolidase activity evaluation and urine dipeptide excretion measurement from the transplantation

Transfusion[a]	Transfusion volume (Units)	Activity measurement[a]	Dipeptide analysis[a]
7	1	7	7
12	1.5		
		14	14
18	1		
21	1	21	21
22	1		
		28	28
		35	35
37	1		
51	0.8		
		58	58

[a] Days from HSCT considered as d1

Discussion

Up to now only 17 *PEPD* pathogenic mutations had been identified in patients with PD; thus, the genotype–phenotype correlations remain largely unclear (Lupi et al. 2008). The wide range of clinical severity and age of onset in patients with the same molecular defects, leaves open the possibility that modifier genes could play a role in PD phenotype and outcome; thus, reporting new mutations remains relevant.

Here, we describe the third *PEPD* allele carrying a three base pairs deletion causing the lack of a single amino acid. Both mutations, c.691delTAC, resulting in p.231delTyr (Lupi et al. 2004), and c.1354delGAG, resulting in p.452delGlu (Ledoux et al. 1994), had been previously reported in patients with PD. The deletion of Tyr231 was hypothesized to be responsible for the disruption of the prolidase structure, whereas the deletion of Glu452 impaired the Mn2+ binding at the enzyme active site. In our patient, one of the *PEPD* allele carried c.1133delACG, determining the absence of the Asp378 that, although not directly involved in the catalytic active metal-binding site, is located in that region; furthermore, the lack of a negative-charged residue could probably alter its structure (Besio et al. 2009).

The second mutation documented in our patient, c.1301delT, is a novel one. It is responsible for a frameshift in exon 14 and the consequent creation of a premature stop codon altering the prolidase sequence from residue 434. This caused, in the mutant allele, the absence of two relevant residues, Glu412 and Glu452, known to be involved in Mn^{2+} binding.

To improve the quality of life of patients with PD, and to prevent fatal complications, several therapeutic approaches have been applied. Interestingly, the use of systemic therapy induced a partial phenotype amelioration. Berardesca et al. reported a 15-year-old boy with PD and defective erythrocyte prolidase activity, which, after blood transfusions, increased to 15.7% of donor activity, then declining to 12% and 3.4% of normal activity after 8 and 45 days, respectively (Berardesca et al. 1992). Apheresis exchanges were repeated monthly for four consecutive months, in parallel, on two patients, replacing prolidase-deficient red blood cells with normal filtered cells. This allowed the constant presence of active prolidase inside cells leading to a

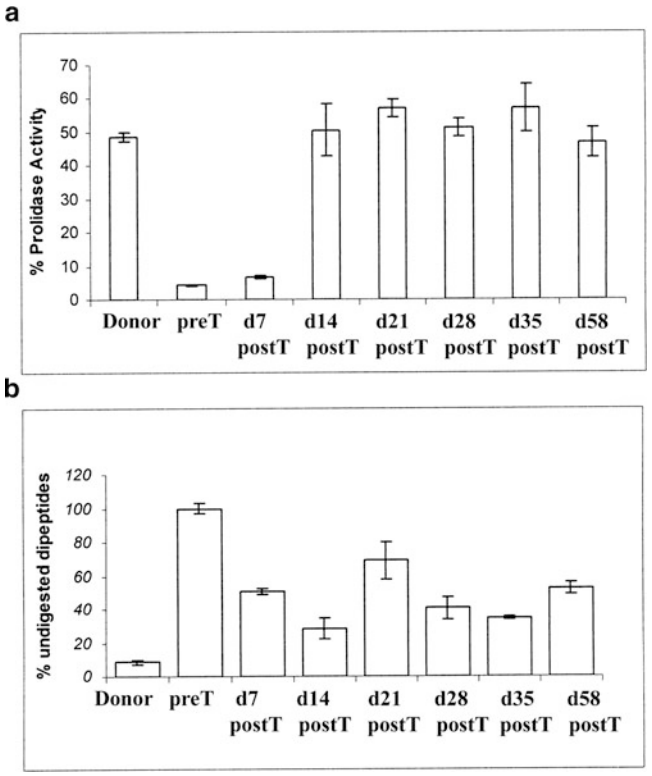


Fig. 2 Prolidase activity measurement and urinary iminodipeptides analysis. (**a**) Prolidase activity of the donor brother's patient and of the patient pretransplant and 7, 14, 21, 28, 35 and 58 days posttransplant are expressed as percentage with respect to age-matched control. (**b**) Percentage of donor and patient's undigested peptides, pretransplant and 7, 14, 21, 28, 35 and 58 days posttransplant, as quantified by capillary electrophoresis. Controls did not reveal any measurable urinary dipeptides

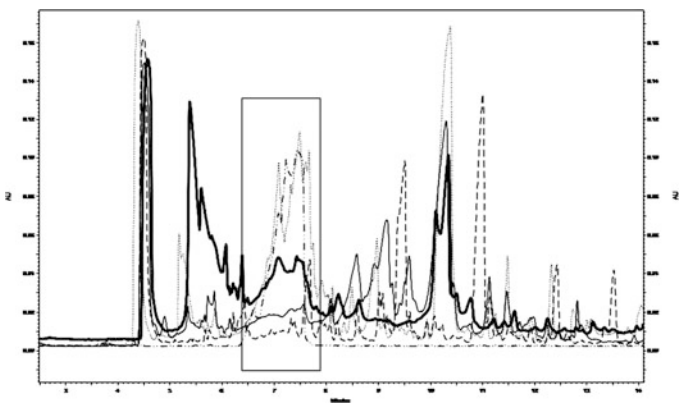


Fig. 3 Representative overlapped CE electropherograms of urine samples; the frame represents the time window where dipeptides migrate. From *bottom to top*: healthy age/gender-matched control, donor patient's brother urine, posttransplant patient's urine (corresponding to the second collection posttransplant), standard dipeptide mixture, pretransplant patient's urine (*dotted line*)

continuous, although partial, degradation of imidodipeptides, with a concomitant improvement of skin ulceration (Lupi et al. 2002). The efficacy of such approaches is still unclear. Since prolidase is a cytosolic enzyme, the effectiveness of blood transfusion could be either determined by enzyme release due to cell lysis or by normal red blood cell undigested dipeptides uptake through simple diffusion or low affinity carrier and their subsequent intracellular hydrolysis (Lochs et al. 1990; Odoom et al. 1990).

Prolidase is an ubiquitously expressed enzyme, and HSCT is expected to replace the host hematopoietic cells with donor-derived ones. This might end up in rescuing enzyme activity in only a few cell types, not in other

tissues. Yet, the high prolidase expression in blood cells, and the reported evidence that repeated blood transfusions contributed to reduce the clinical manifestations in patients with severe PD, suggest that the systemic presence of this enzyme could contribute to dispose dipeptide accumulation, contributing to the disease phenotype (Forlino et al. 2002). These observations pointed to HSCT as a potentially valuable therapeutic approach.

In the present case, the parents of a child with a long-lasting history of skin ulcers and infectious complications, which turned to be life-threatening in at least one event, asked us to apply any novel therapeutic approach, in the search for a cure for PD. The availability of an HLA-identical sibling raised the discussion on the possible indication to HSCT, a long-time recognized therapeutic option for selected inborn errors in which the source of the defective molecule can be replaced by stem-cell derived cells (Prasad and Kurtzberg 2008; Massberg and von Andrian 2009).

Successful engraftment of the transplant was documented by full-donor chimerism. In parallel, posttransplant monitoring of blood prolidase activity showed that, in keeping with the full-donor chimerism, the child had converted to a heterozygous pattern.

We are aware that RBC independence posttransplantation is extremely variable and that by evaluating prolidase activity from red blood cells we could have simply measured the donor cell activity, but the very low prolidase activity at d7 posttransplant allowed us to exclude this possibility. During the follow-up the patient was also transfused with PRBC (see Table 1), but her enzymatic activity remained stable, independently from the time between each transfusion and the activity measurement. This strongly suggest that the prolidase activity detected was mainly due to donor cells engrafted in the patient, although we cannot exclude a partial contribution by the transfused erythrocytes. It is important to remember that prolidase activity in PD patients after PRBC transfusion was previously reported to decline already after 5 days (Lupi et al. 2002); thus the stable prolidase activity documented on days +28 and +35 posttransplant, respectively 7 and 14 days following any transfusion, supports our conclusion.

CE analysis of the patient's urines also showed a reduction of iminodipeptides peaks. Whether incomplete elimination in the urine of the secreted iminodipeptides may depend on the patient's multiorgan failure remains to be assessed.

The main limitation of the present study was the relatively short follow-up that made us impossible to clearly determine the clinical effects of HSCT in the patient, thus forcing us to consider only the surrogate, biochemical parameters.

With the aim of preventing transplant rejection, an event that may be more frequent in patients with some inborn errors such as mucopolysaccaridosis (Boelens et al. 2009), we decided to apply a myeloablative conditioning regimen. VOD is one possible complication of HSCT. To address this issue, the child received specific prophylaxis, which yet turned to be insufficient to prevent the complication. Whether or not underlying PD may have been responsible for any predisposing condition to liver VOD, remains questionable at present.

In conclusion, despite the unfavorable outcome in this case, we provide the first evidence that PD may be at least partially reversed by HSCT. The indication to transplant must be balanced against the clinical manifestation of individual patients.

Acknowledgments This work was supported by MIUR 2008 (2008XA48SC), by Fondazione Cariplo 2007 to A.F. and by Progetto Regione Lombardia (cod. SAL/45) "Dalla scienza dei materiali alla medicina molecolare" to A.F., A.R., and E.D.L.

Short Summary

Two novel causative mutations were documented in a patient with Prolidase Deficiency (PD), a rare autosomal recessive disease of connective tissue for which only 17 mutant causative alleles had been reported. No therapies with curative potential are available for PD patients; this is the first report of HSCT with at least partial rescue of this disease. Reversal of enzyme activity and urine dipeptides excretion suggested that PD could be added to the disorders treatable by transplantation.

Accession Codes: OMIM: 170100 (Prolidase Deficiency); E.C.: 3.4.13.9 (Prolidase); HUGO: PEPD (Proliase); GeneBank: NM_000285.3 (Prolidase mRNA)

Contributions of Individual Authors

- Rolando Cimaz made initial diagnosis and designed therapeutic approach
- Maurizio Aricò Désirée Caselli: took care of the evaluation of the feasibility and then of performing HSCT transplantation
- Silvia Riva Marco Spada: provided expert clinical care during the phase of severe liver failure
- Luca Cantarini: performed the molecular characterization of the patient and her family
- Antonella Forlino Roberta Besio, Antonio Rossi performed the molecular and biochemical characterization of the patient and determined prolidase activity at the different pre and posttransplantation time

- Ersilia De Lorenzi Raffaella Colombo: performed the capillary electrophoresis experiments to evaluate urine dipeptide excretion following transplantation
- Maurizio Aricò and Antonella Forlino: planned the study analyzed the data and wrote the manuscript

The guarantor for the present article is Maurizio Aricò.

All authors declare that the answers to all questions on the JMDI competing interest questionnaire are No and therefore they have nothing to declare.

Funding

This work was supported by MIUR 2008 (2008XA48SC) by Fondazione Cariplo 2007 to A.F. and by Progetto Regione Lombardia (cod. SAL/45) "Dalla scienza dei materiali alla medicina molecolare" to A.F., A.R. and E.D.L.

The authors confirm independence from the sponsors the content of the article has not been influenced by the sponsor. Informed consent for all the laboratory and genetic studies, and for treatment including HSCT was obtained from both parents.

References

Archuleta TD, Devetten MP, Armitage JO (2004) Hematopoietic stem cell transplantation in hematologic malignancy. Panminerva Med 46(1):61–74

Berardesca E, Fideli D, Bellosta M, Dyne KM, Zanaboni G, Cetta G (1992) Blood transfusions in the therapy of a case of prolidase deficiency. Br J Dermatol 126(2):193–195

Besio R, Alleva S, Forlino A et al (2009) Identifying the structure of the active sites of human recombinant prolidase. Eur Biophys J 5:5

Boelens JJ, Rocha V, Aldenhoven M, et al. on behalf of EUROCORD, Inborn Error Working Party of EBMT and Duke University (2009). Risk Factor Analysis of Outcomes after Unrelated Cord Blood Transplantation in Patients with Hurler Syndrome. Biol Blood Marrow Transplant; 15:618–625

Chinard FP (1952) Photometric estimation of proline and ornithine. J Biol Chem 199(1):91–95

Davis GE, Sheard C (1927) The spectrophotometric determination of hemoglobin. Arch Intern Med 40:226–236

Forlino A, Lupi A, Vaghi P et al (2002) Mutation analysis of five new patients affected by prolidase deficiency: the lack of enzyme activity causes necrosis-like cell death in cultured fibroblasts. Hum Genet 111(4–5):314–322

Ledoux P, Scriver C, Hechtman P (1994) Four novel PEPD alleles causing prolidase deficiency. Am J Hum Genet 54 (6):1014–1021

Lochs H, Morse EL, Adibi SA (1990) Uptake and metabolism of dipeptides by human red blood cells. Biochem J 271(1):133–137

Lupi A, Casado B, Soli M et al (2002) Therapeutic apheresis exchange in two patients with prolidase deficiency. Br J Dermatol 147(6):1237–1240

Lupi A, De Riso A, Torre SD et al (2004) Characterization of a new PEPD allele causing prolidase deficiency in two unrelated patients: natural-occurrent mutations as a tool to investigate structure-function relationship. J Hum Genet 49(9):500–506

Lupi A, Rossi A, Vaghi P, Gallanti A, Cetta G, Forlino A (2005) N-benzyloxycarbonyl-L-proline: an in vitro and in vivo inhibitor of prolidase. Biochim Biophys Acta 1744(2):157–163

Lupi A, Tenni R, Rossi A, Cetta G, Forlino A (2008) Human prolidase and prolidase deficiency: an overview on the characterization of the enzyme involved in proline recycling and on the effects of its mutations. Amino Acids 35(4):739–752

MacMillan ML, Davies SM, Nelson GO et al (2008) Twenty years of unrelated donor bone marrow transplantation for pediatric acute leukemia facilitated by the national marrow donor program. Biol Blood Marrow Transplant 14(9 Suppl):16–22

Massberg S, von Andrian UH (2009) Novel trafficking routes for hematopoietic stem and progenitor cells. Ann N Y Acad Sci 1176:87–93

Myara I, Charpentier C, Lemonnier A (1982) Optimal conditions for prolidase assay by proline colorimetric determination: application to iminodipeptiduria. Clin Chim Acta 125(2):193–205

Odoom JE, Campbell ID, Ellory JC, King GF (1990) Characterization of peptide fluxes into human erythrocytes. A proton-n.m.r. study. Biochem J 267(1):141–147

Prasad VK, Kurtzberg J (2008) Emerging trends in transplantation of inherited metabolic diseases. Bone Marrow Transplant 41 (2):99–108

Royce PM, Steinman B (2002) Prolidase deficiency. In: Royce PM, Steinman B (eds) Connective tissue and its heritable disorders. Wiley-Liss, New York

Trounson A (2009) New perspectives in human stem cell therapeutic research. BMC Med 7(29):29

JIMD Reports
DOI 10.1007/8904_2011_65

CASE REPORT

Levodopa Response Reveals Sepiapterin Reductase Deficiency in a Female Heterozygote with Adrenoleukodystrophy

Ronald Thibert • Keith Hyland • Joe Chiles • Steven Steinberg • Florian Eichler

Received: 14 March 2011 / Revised: 16 May 2011 / Accepted: 23 May 2011 / Published online: 16 September 2011

Abstract We report a 4-year-old girl heterozygous for X-linked adrenoleukodystrophy (ALD) who displayed dopa-responsive motor symptoms and was subsequently diagnosed with sepiapterin reductase deficiency (*SPR*; OMIM 182125). Her father and paternal uncle had known ALD, and she was found to have elevated plasma very long chain fatty acids and a mutation in the *ABCD1* gene. She had language delay, severe hypotonia and abnormal eye movements and responded dramatically to a trial of levodopa (4 mg/kg per day). Two mutations within the gene for sepiapterin reductase were found and cultured skin fibroblasts demonstrated near zero activity of the enzyme. This case illustrates the importance of treatment trials that may give rise to biochemical clues to the underlying diagnosis, and the need to continue to search for diagnoses despite a misleading family history.

Communicated by: Georg Hoffmann.

Competing interests: None declared.

R. Thibert · F. Eichler (✉)
Department of Neurology, Massachusetts General Hospital,
55 Fruit Street, ACC 708, Boston, MA 02114, USA
e-mail: feichler@partners.org; rthibert@partners.org

K. Hyland · J. Chiles
Medical Neurogenetics, Atlanta, GA, USA
e-mail: khyland@mnglab.com; jchiles@mnglab.com

S. Steinberg
Kennedy Krieger Institute/Johns Hopkins, Baltimore, MD, USA
e-mail: steinbergs@kennedykrieger.org

Introduction

For patients with suspected inherited disorders of metabolism the family history will often guide the initial laboratory evaluation but it can also introduce bias that delays recognition of symptom mismatch and subsequent diagnosis. Treatment trials that assess the levodopa response reveal important biochemical clues even though the patient may not display the classic symptom constellation.

Defects in monoamine biosynthesis have been associated with developmental delay, diurnal variation in symptom severity and dystonia that may improve with levodopa (Hyland 1999, 2008). Collectively, the disorders that respond to levodopa are termed dopa-responsive dystonias (DRD), the best known being GTP cyclohydrolase deficiency (Segawa's disease). In addition, defects in tyrosine hydroxylase, aromatic L-amino acid decarboxylase and sepiapterin reductase (SR) can also lead to DRD (Zorzi et al. 2002; Friedman et al. 2006; Thöny and Blau 2006; Abeling et al. 2006; Neville et al. 2005). No association with peroxisomal disorders has ever been described. We present a patient who is a female heterozygote for ALD but also carries bi-allelic alterations in the sepiapterin reductase (SPR) transcript and exhibits a syndrome of dopa-responsive neurological symptoms.

Clinical Report

The female patient was the offspring of a gravida 2, para 2 mother. Pregnancy and delivery were uneventful. The patient was born at term via elective repeat cesarian section. She was initially evaluated at 7 months of age for axial hypotonia and eye movements which proved to be

nonepileptic on video-EEG. Her family history was significant for her father and paternal uncle having adrenomyeloneuropathy confirmed by elevated plasma very long chain fatty acids (VLCFA) and the *ABCD1* mutation c.692_694delGGGinsC.

At the time of first evaluation, she had not achieved sitting position and had poor head control. Tests of peroxisomal function, including plasma VLCFAs, phytanic acid, plasmalogens, and bile acid intermediates, along with other metabolic tests such as urine organic acids, serum amino acids, lactate, pyruvate, and biotinidase were performed. All test results were normal except for elevated plasma VLCFA, consistent with obligate carrier status. Magnetic resonance imaging (MRI) and spectroscopy (MRS) of her brain was normal as was a lumbar puncture, which was sent only for routine studies.

Over the next few years, she continued to show significant motor delay with low muscle tone and inability to sit independently. Interestingly, there were some elements of activated tone with diurnal variation. Further metabolic testing, including serum carnitine levels and acylcarnitines, and urine acylglycines, were normal. Karyotype with subtelomeric FISH was also normal.

On examination at 4 years of age, she had significant axial and appendicular hypotonia, dysarthria and preserved reflexes. Language was not well articulated due to reduced oral motor control. Her tone would become activated with excitement and continued to show a diurnal variation. A muscle, nerve, and skin biopsy was performed which was normal with no pathologic findings on electron microscopy. Nerve conduction studies performed prior to biopsy were also normal. Southern blot analysis was performed to rule out the possibility of a second *ABCD1* mutation, but revealed no large deletions.

Due to the fluctuation in her muscle tone and the diurnal nature of her symptoms, she was started on a trial of levodopa (4 mg/kg per day) at 4 years of age. Carbidopa was given together with levodopa at a ratio of 1:4. She showed a dramatic response to treatment with improved energy and tone and improved sleep. In the first weeks after initiation of treatment she developed dyskinesias. Four months after initiating treatment, the patient ambulated without assistance.

Results

Given her dramatic response to treatment, a lumbar puncture for diagnostic neurotransmitter metabolite and pterin analysis was considered but forfeited while she was on L-Dopa. We proceeded to sequence analysis of genes encoding enzymes responsible for the DRDs. No mutations were found in the GTP cyclohydrolase, tyrosine hydroxylase or aromatic L-amino acid decarboxylase genes; however, two mutations were found within the sepiapterin reductase (*SPR*) gene. Both were in exon 2: one novel mutation (c.407 C>T; p.S136F) and one known pathogenic mutation (c.448 A>G; p.R150G) (Thöny and Blau 2006). The mother was heterozygous for the p.S136F mutation. The father was heterozygous for the p.R150G mutation. Measurement of SR activity in cultured skin fibroblasts showed a severe deficiency with activity being less than 1% of the control mean.

A year after commencing Sinemet, she had made further gains in motor function but only modest gains in expressive language skills. We increased levodopa (5 mg/kg per day) and began the serotonin precursor 5-hydroxytryptophan (5-HTP) (0.5 mg/kg per day). Within 4 months of these changes, she was communicating in full sentences despite continued articulation difficulties.

Discussion

This case illustrates the importance of persistence in the diagnostic evaluation of patients with a known genetic disorder whose symptoms fall outside the expected disease spectrum. Although female heterozygotes with X-ALD can show a spastic paraparesis in adulthood, severe symptoms in early childhood have not been reported in carriers (Moser et al. 2001, Maier et al. 2002, Heffungs et al. 1980). Furthermore, by testing of the healthy ABCD1 allele we have reduced the chance of a second mutation on the opposing allele.

More importantly, our patient displayed prominent motor delay with subtle but reproducible diurnal variation. This raised the suspicion for an aberration in monoamine metabolism. The monoamine neurotransmitters include the catecholamines (dopamine, norepinephrine, and epinephrine) and serotonin. Figure 1 illustrates the key pathways involved in their synthesis and degradation. Deficiencies in the primary enzymes involved in monoamine synthesis and degradation have been described at the level of tyrosine hydroxylase, aromatic L-amino acid decarboxylase, monoamine oxidase, and dopamine beta-hydroxylase. Tetrahydrobiopterin is a necessary cofactor for both tryptophan hydroxylase and tyrosine hydroxylase. Therefore, enzymatic deficiencies that lead to reduced levels of tetrahydrobiopterin can interfere with the synthesis of the monoamine neurotransmitters. Deficiencies have been identified in each of the synthetic enzymes in the tetrahydrobiopterin pathway: guanosine triphosphate cyclohydrolase I, 6- pyruvoyl-tetrahydropterin synthase, and SR. The finding of biallelic alterations in the *SPR* gene and the near zero fibroblast SR enzyme activity demonstrates SR deficiency as the primary cause of our patient's symptoms.

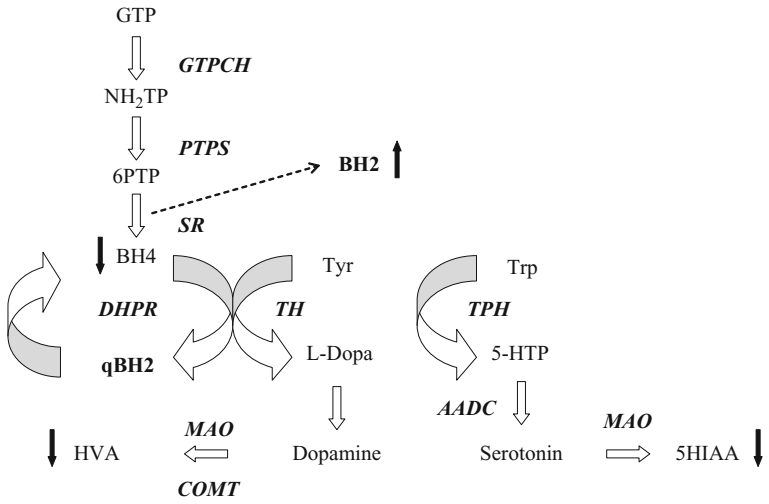


Fig. 1 Pathway for synthesis and catabolism of dopamine and serotonin. Dopamine and serotonin are formed from tyrosine (Tyr) and tryptophan (Trp) respectively. The rate limiting enzymes are tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) and these enzymes require the cofactor tetrahydrobiopterin (BH4). In brain, BH4 is synthesized from GTP in a three step pathway with the final step being catalyzed by sepiapterin reductase (SR). SR deficiency leads to conversion of 6-pyruvoyltetrahydropterin (6PTP) to sepiapterin which is in turn converted to 7,8-dihydrobiopterin (BH2) (*dashed line*). SR deficiency causes decreased levels of BH4, and consequently low levels of dopamine, serotonin and their metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (HVA). *GTP* GTP cyclohydrolase; *NH2TP* dihydroneopterin triphosphate; *PTPS* 6-pyruvoyltetrahydropterin synthase; *DHPR* dihydropteridine reductase; *5-HTP* 5-hydroxytryptophan; *AADC* aromatic L-amino acid decarboxylase; *MAO* monoamine oxidase; *COMT* catechol-O-methyltransferase

The *SPR* gene is composed of 3 exons on 2p13, and more than 19 different mutations have been identified in affected patients (Thöny and Blau 2006). Patients exhibit psychomotor retardation, inconsolable crying, neurological abnormalities (hypotonia, dystonic posturing, oculogyric crises, spasticity, tremor, ataxia, gait disorder, chorea, Parkinsonism, seizure-like movements), psychiatric symptoms (depressed affect, aggressive behavior, hypersomnolence), and occasional physical findings (microcephaly, growth deficiency) (Abeling et al. 2006, Neville et al. 2005). As with other dopa-responsive disorders, symptoms are characteristically worse in the evenings, so-called diurnal variation. The disease is hard to diagnose due to lack of peripheral markers and brain MRI is normal in most cases. CSF reveals low HVA and 5-HIAA and high levels of biopterin and dihydrobiopterin with the presence of sepiapterin (Zorzi et al. 2002). As in our case the diagnosis can be also be established by mutation analysis and confirmed by documenting low SR activity in skin fibroblast cultures.

Our patient also showed several unique features for SRD. Dystonia and oculogyric crises, in contrast to most previously reported cases, were absent. Table 1 summarizes and contrasts the core clinical features of SRD and X-ALD. As the core features of SR deficiency are nonspecific and dystonia may not always be present, it is crucial to be cognizant of subtle signs that may suggest monoamine dysfunction. Although abnormal muscle tone and motor development improved dramatically with L-dopa/carbidopa, language skills appeared to benefit most following 5-HTP administration.

Further investigation is needed to define genotype/phenotype relationships, optimize pharmacologic therapy, understand pathogenesis of tone fluctuation and clarify the role of individual monoamines in language disturbance. SRD and other disorders of monoamine metabolism should be considered in all cases of abnormal eye movements in the setting of significant motor delay. Diurnal fluctuation is often difficult to discern in cases of infantile onset as well as severe affected individuals, as in our patient. Therapies should be directed at not only dopamine deficiency, but also other biochemical deficiencies present in this disorder.

Acknowledgment We want to thank for support by NIH grant K08NS52550 (F.E.).

Synopsis

Levodopa response reveals sepiapterin reductase deficiency in a female heterozygote with adrenoleukodystrophy.

Details of the Contributions of Individual Authors

Thibert and Eichler performed the clinical evaluation of the patient and wrote up the report. Hyland and Chiles performed the sepiapterine reductase gene sequencing and enzyme assay.

Table 1 Signs and symptoms in patients with sepiapterin reductase deficiency (SRD) versus X-linked adrenoleukodystrophy (X-ALD)

	This case	SR	X-ALD	
		Patients	Hemizygotes	Heterozygotes
Age at diagnosis	4 years	0.5–26 years	4–65 years	8–65 years
Age at first symptom	6 months	6 months	4 years	8 years
Sex	F	6 F/7 M	M	F
Clinical symptoms				
Motor delay	+++	+++	–	–
Motor regression	–	–	++	+
Speech delay	++	++	–	–
Speech regression	–	–	++	–
Diurnal fluctuation	+	++	–	–
Abnormal eye movements +	++	–	–	
Tremor		–	+	+
Pain	–	–	+	++
Dystonia	–	++	++	–
Axial hypotonia	+++	++	+	–
Limb hypertonia	–	+	++	+
Weakness	+	+	+	+

– absent, + uncommon, ++ common, +++ very common

The frequencies are derived from published reports (Friedman et al. 2006; Moser et al. 2001; Maier et al. 2002)

Steinberg performed the lipid assay and gene sequencing for adrenoleukodystrophy and contributed to write-up.

Name of One Author Who Serves as Guarantor

Florian Eichler accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

A Competing Interest Statement

Dr. Keith Hyland is co-owner of Medical Neurogenetics, a company that provides genetic and metabolite testing for sepiapterin reductase deficiency. Mr. Joe Chiles is an employee of Medical Neurogenetics, a company that provides molecular and metabolite testing for sepiapterin reductase deficiency. None of the other authors report disclosures.

The authors confirm independence from any potential sponsors; the content of the article has not been influenced by potential sponsors.

Details of Ethics Approval

Consent was obtained from the parents to disclose the video material.

References

Abeling NG, Duran M, Bakker HD et al (2006) Sepiapterin reductase deficiency an autosomal recessive DOPA-responsive dystonia. Mol Genet Metab 89:116–120

Friedman J, Hyland K, Blau N, MacCollin M (2006) Dopa-responsive hypersomnia and mixed movement disorder due to sepiapterin reductase deficiency. Neurology 67:2032–2035

Heffungs W, Hameister H, Ropers HH (1980) Addison disease and cerebral sclerosis in an apparently heterozygous girl: evidence for inactivation of the adrenoleukodystrophy locus. Clin Genet 18:184–188

Hyland K (1999) Presentation, diagnosis, and treatment of the disorders of monoamine neurotransmitter metabolism. Semin Perinatol 23:194–203

Hyland K (2008) Clinical utility of monoamine neurotransmitter metabolite analysis in cerebrospinal fluid. Clin Chem 54:633–641

Maier EM, Kammerer S, Muntau AC, Wichers M, Braun A, Rocher AA (2002) Symptoms in carriers of adrenoleukodystrophy relate to skewed X inactivation. Ann Neurol 52:683–688

Moser HW, Smith KD, Watkins P, Powers J, Moser AB. X-linked adrenoleukodystrophy, in The metabolic and molecular bases of inherited disease, C. Scriver, et al., Editors. 2001, McGraw Hill: New York. 3257–3301.

Neville BG, Parascandalo R, Farrugia R, Felice A (2005) Sepiapterin reductase deficiency: a congenital dopa-responsive motor and cognitive disorder. Brain 128:2291–2296

Thöny B, Blau N (2006) Mutations in the BH4-metabolizing genes GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, sepiapterin reductase, carbinolamine-4a-dehydratase, and dihydropteridine reductase. Hum Mutat 27(9):870–878

Zorzi G, Redweik U, Trippe H, Penzien JM, Thöny B, Blau N (2002) Detection of sepiapterin in CSF of patients with sepiapterin reductase deficiency. Mol Genet Metab 75:174–177

JIMD Reports
DOI 10.1007/8904_2011_66

CASE REPORT

Biochemical Monitoring and Management During Pregnancy in Patients with Isovaleric Acidaemia is Helpful to Prevent Metabolic Decompensation

D.D.J. Habets • N.C. Schaper • H. Rogozinski • F.J. van Spronsen • M. van Rijn • J. Bierau • J.A. Bakker

Received: 18 May 2011 / Revised: 18 May 2011 / Accepted: 23 May 2011 / Published online: 27 September 2011

Abstract The facilities for neonatal screening, early diagnosis, and effective treatment of isovaleric acidaemia (IVA) have improved greatly over the past decades. Accordingly, IVA patients reach adolescence and may consider having children. The maintenance of a stable metabolic condition is a challenge to both the patients and their multidisciplinary team of care providers. This report presents three women with IVA during their five single or twin pregnancies, whose clinical condition were monitored with contrasting approaches. Metabolic profiles were determined and compared in these pregnancies. In one case, two pregnancies were strictly managed and monitored by measuring plasma acylcarnitine and amino acid profiles, together with adjustment of the diet and/or supplementation of L-carnitine and/or glycine. In addition, complications were prevented by intravenous glucose and L-carnitine during labor and postpartum. In two other cases, the metabolic condition of patients was less frequently monitored and additional treatment with intravenous L-carnitine and intravenous glucose/dextrose was only prescribed during periods of hyperemesis gravidarum. With respect to the differences in management and monitoring of maternal IVA all pregnancies were without complications for mother and child. Despite the favorable outcome in uncontrolled pregnancies in IVA, careful monitoring and management during pregnancy is helpful to prevent life-threatening conditions like metabolic decompensation.

Communicated by: Jerry Vockley.

Competing interests: None declared.

Electronic supplementary material The online version of this article (doi:10.1007/8904_2011_66) contains supplementary material, which is available to authorized users.

D.D.J. Habets (✉) · J. Bierau · J.A. Bakker
Laboratory of Biochemical Genetics, Department of Clinical Genetics, Maastricht University Medical Centre, Noordbuilding, P. Debyelaan 25, 6229 HX, Maastricht, The Netherlands
e-mail: d.habets@mumc.nl

N.C. Schaper
Department of Medicine and Endocrinology, Maastricht University Medical Centre, Maastricht, The Netherlands

H. Rogozinski
Department of Pediatrics, Bradford Royal Infirmary, Bradford BD9 6RJ, UK

F.J. van Spronsen · M. van Rijn
Section of Metabolic Diseases, Beatrix Children's Hospital, Department of Facilities, University Medical Center of Groningen, University of Groningen, Groningen, The Netherlands

Abbreviations

IVA Isovaleric acidaemia
IVD Isovaleryl-CoA dehydrogenase

Introduction

Isovaleric acidaemia (IVA; OMIM 243500) is an autosomal recessive form of a branched-chain organic aciduria. IVA can present in the neonatal period with an acute episode of severe metabolic acidosis with vomiting, secondary hyperammonemia, and moderate ketosis, potentially resulting in coma/death when left untreated. Patients with IVA can suffer episodes of acute metabolic decompensation precipitated by intercurrent stressing events. IVA is caused by deficiency of isovaleryl-CoA dehydrogenase (IVD; OMIM 607036), the enzyme which catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA. This results in

the accumulation of isovaleryl-CoA derivates, which are detected in urine and blood as the conjugation products isovalerylglycine and isovalerylcarnitine (Vockley and Ensenauer 2006). The main goal of treatment is to achieve a state of anabolism, reducing formation of isovaleryl-CoA formation from leucine catabolism. Treatment strategy for patients with IVA may include a leucine-free formula, protein-restricted diet, L-carnitine or glycine supplementation or a combination thereof. The effects of treatment on long-term neurological development in IVA patients have not yet been completely clarified (Martin-Hernandez et al. 2009). By adhering to effective treatment, patients reach adolescence in a healthy condition and may consider having children.

Pregnancies and the fetal outcome have been described in a number of patients with amino acidopathies and disorders of intermediate metabolism. Most pregnancies were successful despite poor biochemical monitoring. Only a small number of maternal inborn errors have been shown to affect fetal health (Lee 2006; Rubio-Gozalbo et al. 2010; van Spronsen et al. 2003). The effects of treatment also need consideration; however, no pregnancy safety profiles are available for the majority of therapeutic drugs (Preece and Green 2002; Walter 2000).

Sharing experiences in this field will lead to improved therapy during pregnancy for patients with an inborn error of metabolism and expands the knowledge of adverse effects of therapy for the fetus. Two previous cases with a favorable outcome of two pregnancies in patients with IVA have been described (Shih et al. 1984; Spinty et al. 2002), to which we now add the clinical history of five more pregnancies with favorable outcomes.

Case Report

First Case

This woman with IVA had two successful pregnancies as described below. She has always been compliant to a combined therapy of protein-restricted diet, a leucine-free formula (30 g/day), and supplementation of L-carnitine (2,970 mg/day) and was reasonably well controlled with a normal development. Biochemical follow-up during the past years was performed in plasma taken randomly during the day. In the preconception period of both pregnancies her treatment was intensified in order to optimize metabolic control. Before her first pregnancy the free carnitine concentration was 8.7 μmol/l (total carnitine: 33.1 μmol/l, isolalerylcarnitine: 20.1 μmol/l), this concentration is below the reference range for healthy individuals (Blau et al. 2008). Increasing L-carnitine supplementation (three doses of 1,980 mg L-carnitine/day) marginally increased the free carnitine concentration (11.3 μmol/l). A combination of three doses of 1,320 mg L-carnitine supplementation with three doses of 6 g glycine/day resulted in 23.6 μmol/l free carnitine. The patient stopped her anticonception and became pregnant quickly thereafter. Also in the pre-conception period of the second pregnancy she was treated with the L-carnitine/glycine combination.

During pregnancy, supplementation (Supplementary Table) was adjusted by biochemical monitoring and not by weight gain. In the first months of her first pregnancy the free carnitine concentration decreased from 36.3 μmol/l to 10.5 μmol/l (Table 1A). Increasing the supplementation to three doses of 10 g glycine/day in the fifth month was not effective; increasing supplementation to 15 g glycine in the sixth month resulted in a concentration of 24.5 μmol/l free carnitine (Table 1A). The concentrations of the branched-chain amino acids (Table 1B) and the other amino acids (data not shown) were normal compared to the reference range during the first pregnancy. During the second pregnancy, already in the third month low concentrations of free carnitine levels (13.1 μmol/l; Table 1A) were measured. Glycine and L-carnitine supplementation were ultimately increased to 100 g glycine/day and 10 g L-carnitine/day with only a transient increase of free carnitine (47.3 μmol/l; Table 1A) in the eighth month. The management of the amino acid levels was a challenge in the second pregnancy in which the concentrations of branched-chain amino acids were lower than in the previous pregnancy (Table 1B). The leucine-free formula intake was increased to 80 g/day during the 2 months. The overall increase in maternal weight was normal during both pregnancies and the patient had no periods of hyperemesis gravidarum. Subsequently, growth and development of both fetuses were normal.

At the first signs of labor, intravenous glucose (10%; 2.5 l/day) and intravenous L-carnitine (200 mg/kg per day) were started and oral glycine supplementation (three doses of 15 g glycine/day) was continued during both pregnancies. After additional supplementation during labor of the first pregnancy, free carnitine and total carnitine increased to 435.6 μmol/l and 467.8 μmol/l, respectively (Table 1A). The amino acid profile (Table 1B) and acid–base balance (pH: 7.46, pCO_2: 4.6 kPa, HCO_3^-: 24.9 mmol/l) were normal. During labor of the second pregnancy, free carnitine was 127.7 μmol/l and total carnitine was 175.3 μmol/l (Table 1A) after additional supplementation. Lower levels of glycine and the branched-chain amino acids (leucine and valine; Table 1B) were measured and blood-gas analysis was normal (pH: 7.36, pCO_2: 5.3, HCO_3^-: 21.6 mmol/l). In both pregnancies, intravenous therapy was stopped after 4 h postpartum and acylcarnitine and amino acid profiles normalized within 3 days (Table 1A, B: first day post partum). The acylcarnitine and amino acid

Table 1 Acylcarnitine (A) and amino acid (B) concentrations (μmol/l) in the plasma of a patient with isovaleric acidaemia (case 1) during the first pregnancy, the second pregnancy, labor of both pregnancies, and reference range in healthy individuals (Blau et al. 2008). Free carnitine (C0), total carnitine (Total), isovalerylcarnitine and 2-methylbutyrylcarnitine (C5-iso)

A

Pregnancy(month)	C0		Total		C5-iso	
	First	Second	First	Second	First	Second
1st	36.3	–	52.3	–	11	–
2nd	29.2	28.9	45.2	48.8	10.8	12.2
3rd	–	13.1	–	24.3	–	7.2
4th	20.4	13.9	32.8	26.2	8.1	7.5
5th	10.5	13.1	21.9	20.8	7.7	4.1
6th	24.5	13.5	37.1	21.8	7.2	3
7th	27.7	–	40.6	–	7	–
8th	–	47.3	–	59.1	–	5.8
9th	11.9	12.9	25	26.7	6.7	8.6
Labor	435.6	127.7	467.8	175.3	19.7	30.7
Maternal cord blood	240.9	233.1	276.7	266.3	14	18.3
Post partum	288.4	935.7	334.9	991.6	27.9	37.8
Day 1 after labor	225.9	22.9	279.6	41	36	10.3
Reference	22.3–54.8				0.03–0.23	

B

Pregnancy(month)	Glycine		Leucine		Isoleucine		Valine	
	First	Second	First	Second	First	Second	First	Second
1st	541	–	45	–	67	–	214	–
2nd	570	317	64	131	95	68	253	193
3rd	–	223	–	198	–	65	–	202
4th	320	225	61	85	70	49	202	160
5th	313	221	78	105	48	55	182	151
6th	815	377	39	65	71	38	214	122
7th	614	–	47	–	87	–	249	–
8th	–	1,446	–	24	–	83	–	220
9th	338	345	130	66	67	38	234	106
Labor	391	110	74	54	68	72	226	87
Maternal cord blood	389	248	102	85	72	45	300	144
Post partum	887	154	66	69	87	46	253	154
Day 1 after labor	410	320	100	113	59	68	208	205
Reference	147–321		73–160		34–84		145–283	

profiles in cord blood reflected the maternal metabolic status (Table 1A, B).

From both pregnancies, healthy infants were born at term with a normal weight (3,980 g and 4,200 g, respectively) and Apgar score. Isovalerylcarnitine levels (first child: 0.3 μmol/l, second child: 0.2 μmol/l; Table 2A; the cut-off value is 1.0 μmol/l for isovalerylcarnitine in the newborn screening in The Netherlands) and the concentrations of the branched-chain amino acids (Table 2B) were normal in plasma taken in the first hour after birth of both infants. The free- and total carnitine concentrations (Table 2A) were increased due to maternal supplementation but these levels resolved within a few days without treatment or complications.

Second Case

This woman was diagnosed with IVA immediately after birth. At 25 years of age, she had a normal development and had a twin pregnancy. She was treated with a protein-

Table 2 Acylcarnitine (A) and amino acid (B) concentrations (μmol/l) in plasma of two children immediately after birth from a mother with isovaleric acidaemia (case 1), and reference range in healthy individuals (Blau et al. 2008). Free carnitine (C0), total carnitine (Total), isovalerylcarnitine and 2-methylbutyrylcarnitine (C5-iso)

A

Carnitine	First child	Second child	Reference
C0	79.9	58.9	6.1–22.2
Total	113.4	85.9	
C5-iso	0.3	0.2	0.06–0.37

B

Amino acid	First child	Second child	Reference
Glycine	524	238	101–317
Leucine	88	54	47–175
Isoleucine	38	35	22–82
Valine	135	90	65–201

restricted diet, a leucine-free formula, and L-carnitine (9 g/day). Prior to pregnancy, the plasma concentrations of free carnitine (5 μmol/l) and total carnitine (12 μmol/l) were in the low range compared to the reference range of healthy individuals, and plasma isovalerylcarnitine was 5.7 μmol/l. No additional therapy was prescribed to increase the free carnitine levels. During twin pregnancy, the free and total carnitine concentrations in plasma were 7 μmol/l and 14 μmol/l in the first month and 5 μmol/l and 8 μmol/l in the eighth month, respectively. The amino acid profile was determined in the eighth month of pregnancy: glycine (153 μmol/l), valine (100 μmol/l), isoleucine (35 μmol/l), and leucine (61 μmol/l). She solely received additional treatment (intravenous 10% glucose and 100 mg/kg per day L-carnitine) during periods of hyperemesis gravidarum in the fourth and fifth month of the pregnancy. Blood-gas analyses revealed no metabolic acidosis during these periods (pH: 7.45 and 7.39, pCO_2: 4.1 and 5.2 and HCO_3^-: 21 and 23 mmol/l, respectively), and glucose was within the normal range. A low-to-normal growth of the fetuses was observed when compared to single pregnancies, and protein intake was monitored and controlled during the last trimester of the pregnancy. Without additional medical care during labor and the postpartum period, there was a favorable outcome for mother and twins.

Third Case

This woman with IVA has been very stable with a normal development in spite of minimal therapy compliancy for many years. She has had three pregnancies, the details on the first pregnancy were published previously (Spinty et al. 2002). In this pregnancy, the monitoring and management was carefully planned, with additional treatment of intravenous L-carnitine (100 mg/kg per day), sodium benzoate (250 mg/kg per day), and a standard electrolyte/glucose solution during labor. There were no maternal complications and a healthy child was born.

The prescribed therapy was a protein-restricted diet combined with L-carnitine supplementation (2 doses of 3 g L-carnitine/day). In general, the patient's compliance to therapy was limited with metabolic decompensation during the second and third pregnancy. In the second month of both pregnancies, periods of hyperemesis gravidarum were treated with intravenous 10% dextrose and antiemetics. In addition, intravenous L-carnitine (100 mg/kg) was started because oral L-carnitine was refused. During the second pregnancy, amino acids (branched-chain amino acids: low, glycine: 210 μmol/l) and acylcarnitine profiles (free carnitine: 5.5 μmol/l) in plasma were measured once in the fourth month. During the third pregnancy, the metabolic condition of the patient was checked four times (branched-chain amino acids: low-to-normal, glycine: between 175 and 305 μmol/l plasma). Free carnitine concentrations in plasma were found to be in the range of 6.8–12.1 μmol/l with the isovalerylcarnitine concentration ranging from 3.6 to 7.6 μmol/l. Labor and the postpartum period of the second and third pregnancy were without complications and no additional therapy was given. The second pregnancy and third pregnancy resulted in healthy children with a normal birth weight (2,920 g and 3,940 g, respectively).

Discussion

With neonatal screening, early diagnosis, and effective treatment life expectancy of patients with IVA has considerately improved (van Spronsen et al. 2003). When these patients become pregnant, it is a challenge to both the patients and the multidisciplinary team to maintain metabolic stability. Regarding the changes in maternal metabolism, a planned monitoring and management needs to be considered preconceptionally, as well as during pregnancy, labor, and the postpartum period (Lee 2006). There is limited information about reference ranges for metabolite concentrations in pregnancy (Ghadimi and Pecora 1964; Talian et al. 2007; Winter et al. 1995) and no information about target values for pregnant patients with IVA or other inborn errors of metabolism. By sharing our experience, we hope to improve the management of pregnant patient with IVA and their infants.

It is recommended to monitor the metabolic state of a woman with an inborn error of metabolism before conception (Lee 2006). The metabolic profile of the patient presented in case 1 was strictly managed with different supplements and dosages during preconception. L-carnitine

and glycine supplementation shunts isovaleryl-CoA towards the nontoxic metabolites isovalerylcarnitine and isovalerylglycine, respectively. This combination is especially effective during metabolic stress (Fries et al. 1996). With the addition of glycine supplementation L-carnitine is spared, resulting in higher free carnitine levels. Therefore, a combined L-carnitine and glycine supplementation was prescribed in the patient of case 1. In contrast, in case 2 and 3, the metabolic profile of the patients was not monitored nor intensively managed in the period preceding conception.

The main goals of therapy in IVA are to prevent metabolic decompensation and to prevent accumulation of isovaleryl-CoA. Regarding the first goal, awareness of the anabolic and catabolic periods is required in maternal IVA. The effects of endocrine changes (increased circulation hormones and growth factors) are anabolic and can be beneficial in maternal IVA. In addition, growth development during pregnancy enhances the maternal protein requirements resulting in increased protein tolerance. Therefore, the metabolic condition of the patient needs to be monitored and can be managed by adequate leucine and/or caloric intake. There is a risk of catabolism during periods of hyperemesis gravidarum, during the labor/delivery and the postpartum period in healthy woman but especially in a pregnant woman with IVA. Metabolic decompensation during these periods can be prevented by intravenous glucose/dextrose. The second goal was to enhance the conjugation of isovaleryl-CoA to less toxic compounds; therefore, optimal dosages of L-carnitine and glycine supplementation are required. It is known that free carnitine concentrations reduce during pregnancy in healthy controls (Talian et al. 2007). However, in all three cases there was a difference in the free carnitine levels and reductions (Table 3). In the first pregnancy of case 1, treatment of the patient with L-carnitine and glycine supplementation resulted in a free carnitine level comparable to the reference range of healthy controls. In the second pregnancy of this patient, the same supplementation resulted in lower free carnitine concentrations compared to the first pregnancy. Even a higher L-carnitine supplementation (10 g/day) did not result in a normal concentration of free carnitine. This can be explained by the fact that efficacy of L-carnitine supplementation is limited since there is a maximal dose absorbance of 2 g L-carnitine per administration (Harper et al. 1988). In the case that L-carnitine supplementation is maximal, it is advised to prescribe a combined supplementation of L-carnitine and glycine. Although this combined therapy increased the free carnitine concentration in plasma during the preconception period and the first pregnancy, it was not effective during the second pregnancy. The difference between patients and pregnancies should be evaluated and treated in its own right. In cases 2 and 3 there was no adjustment of therapy, resulting in low carnitine and amino acid concentrations together with periods of hyperemesis gravidarum. The question remains whether a more strict management would have prevented this complication during the pregnancy. The labor and postpartum period has an enormous catabolic effect and it is a time of considerable risk of metabolic decompensation. In two previously published cases (Shih et al. 1984; Spinty et al. 2002) and in case 1 of this report, catabolism was prevented by intravenous glucose and L-carnitine during these periods. In the twin pregnancy of case 2 without additional care, in which the risk for complications is expected to be higher, and in the two pregnancies of case 3 without additional care, however, no complications were observed. Although the postpartum period is mainly recognized as a catabolic event, all patients were metabolically stable within the first days after delivery.

Table 3 Overview of the monitoring and management of three patients with isovaleric acidaemia during five pregnancies

	Case 1	Case 2	Case 3
Total pregnancies	2	1	3(First pregnancy Spinty et al. 2002)
Biochemical follow-up during the pregnancy	Intense	Limited	Limited
Therapy during pregnancy	First pregnancy: increased glycine supplementation	During hyperemesis gravidarum: intravenous glucose and L-carnitine	During hyperemesis gravidarum: intravenous
	Second pregnancy: increased glycine, L-carnitine supplementation and increased protein intake	In the last part of the pregnancy: increased protein intake	Dextrose, L-carnitine, and antimetics
Complications during pregnancy	Both pregnancies: none	Hyperemesis gravidarum	Hyperemesis gravidarum
Therapy during labor	Both pregnancies: glucose and L-carnitine intravenous and glycine per os	None	None
Outcome	Two healthy infants	Healthy twin	Two healthy infants

Another aspect of pregnancies, is the possible effect of maternal IVA on the fetus and safety of medication used during pregnancy. Fetal carnitine is derived from two sources; from the mother via transplacental transfer (El-Hattab et al. 2010) and to a lesser extent from in utero synthesis (Oey et al. 2006). Therefore, immediately after birth the plasma-free carnitine level of the neonate reflects that of the mother. Carnitine in the fetus and placenta is biosynthesized from lysine and methionine involving four enzymatic steps and may be highly relevant in situations where the maternal carnitine supply is limited, like in maternal IVA (Oey et al. 2006). The safety of medications during pregnancy is known for some drugs, but the list of agents without safety information is still long (Preece and Green 2002; Walter 2000). The effects of L-carnitine during pregnancy have been studied in sows, but unfortunately no information is available in humans. In pregnant sows, it has been shown that L-carnitine has limited influence on lipid metabolism and utilization of nitrogen, but increases the plasma concentrations of insulin-like growth factors. This in turn may stimulate placental development and intrauterine nutrition, resulting in increased birth weight (Doberenz et al. 2006). However, L-carnitine supplementation during the pregnancies of the reported cases did not result in an increased birth weight of the infants. The safety of glycine and L-carnitine supplementation in pregnancies is unknown. Transplacental transfer of amino acids is an active process, with fetal plasma amino acids being higher than maternal plasma levels (Cleal et al. 2007). The concentrations of glycine in the fetus and their effects during maternal glycine supplementation are unknown and need further investigation. The previously described cases (Shih et al. 1984; Spinty et al. 2002) and the patients described in this report suggest that L-carnitine and glycine supplements were not harmful for the developing fetus although the long-term outcomes are not known. If this supplementation could have potentially beneficial effects for the fetus, e.g., by preventing maternally L-carnitine deficiency, remains to be proven. Finally, it is recommended to screen the offspring for IVA. In parents with an autosomal recessive inherited metabolic disorder, the risk of having an affected offspring is low, but is nevertheless considerably higher depending on the allele frequency of carrier in the population.

There is very limited knowledge about metabolic changes in pregnancies. Reference values for acylcarnitine and amino acid concentrations in pregnant patients with IVA are lacking and cannot be extracted from the results of the three cases presented in this report. This suggests that a carefully planned biochemical follow-up and additional management during the pregnancy period can be helpful. We conclude that patients with IVA may have uncomplicated pregnancies, regardless of their metabolic condition or differences in therapeutic strategies (Table 3). The pregnancy, the labor, and the postpartum period were successful in all reported IVA patients with or without additional monitoring and management. A well-established protocol for pregnant patients with IVA has not been established, but managing a well-controlled metabolic condition of the patient with IVA is the main goal in general and should be especially so during pregnancy.

Synopsis

Managing a well-controlled metabolic condition of the patient with IVA is the main goal in general and should be especially so during pregnancy.

Consent

Written informed consent was obtained from the patient for publication of this case report.

Author's Contribution

All authors have contributed to the work presented in this manuscript.

References

Blau N, Duran M, Gibson KM (2008) Laboratory guide of the methods in biochemical genetics. Springer, Berlin

Cleal JK, Brownbill P, Godfrey KM, Jackson JM, Jackson AA, Sibley CP, Hanson MA, Lewis RM (2007) Modification of fetal plasma amino acid composition by placental amino acid exchangers in vitro. J Physiol 582:871–882

Doberenz J, Birkenfeld C, Kluge H, Eder K (2006) Effects of L-carnitine supplementation in pregnant sows on plasma concentrations of insulin-like growth factors, various hormones and metabolites and chorion characteristics. J Anim Physiol Anim Nutr (Berl) 90:487–499

El-Hattab AW, Li FY, Shen J, Powell BR, Bawle EV, Adams DJ, Wahl E, Kobori JA, Graham B, Scaglia F, Wong LJ (2010) Maternal systemic primary carnitine deficiency uncovered by newborn screening: clinical, biochemical, and molecular aspects. Genet Med 12:19–24

Fries MH, Rinaldo P, Schmidt-Sommerfeld E, Jurecki E, Packman S (1996) Isovaleric acidemia: response to a leucine load after three weeks of supplementation with glycine, L-carnitine, and combined glycine-carnitine therapy. J Pediatr 129:449–452

Ghadimi H, Pecora P (1964) Free amino acids of cord plasma as compared with maternal plasma during pregnancy. Pediatrics 33:500–506

Harper P, Elwin CE, Cederblad G (1988) Pharmacokinetics of bolus intravenous and oral doses of L-carnitine in healthy subjects. Eur J Clin Pharmacol 35:69–75

Lee PJ (2006) Pregnancy issues in inherited metabolic disorders. J Inherit Metab Dis 29:311–316

Martin-Hernandez E, Lee PJ, Micciche A, Grunewald S, Lachmann RH (2009) Long-term needs of adult patients with organic acidaemias: outcome and prognostic factors. J Inherit Metab Dis 32:523–533

Oey NA, van Vlies N, Wijburg FA, Wanders RJ, Attie-Bitach T, Vaz FM (2006) L-carnitine is synthesized in the human fetal-placental unit: potential roles in placental and fetal metabolism. Placenta 27:841–846

Preece MA, Green A (2002) Pregnancy and inherited metabolic disorders: maternal and fetal complications. Ann Clin Biochem 39:444–455

Rubio-Gozalbo ME, Gubbels CS, Bakker JA, Menheere PP, Wodzig WK, Land JA (2010) Gonadal function in male and female patients with classic galactosemia. Hum Reprod Update 16:177–188

Shih VE, Aubry RH, DeGrande G, Gursky SF, Tanaka K (1984) Maternal isovaleric acidemia. J Pediatr 105:77–78

Spinty S, Rogozinski H, Lealman GT, Wraith JE (2002) Second case of a successful pregnancy in maternal isovaleric acidaemia. J Inherit Metab Dis 25:697–698

Talian GC, Komlosi K, Decsi T, Koletzko B, Melegh B (2007) Determination of carnitine ester patterns during the second half of pregnancy, at delivery, and in neonatal cord blood by tandem mass spectrometry: complex and dynamic involvement of carnitine in the intermediary metabolism. Pediatr Res 62:88–92

van Spronsen FJ, Molendijk H, Erwich JJ, Smit GP (2003) Inherited metabolic diseases and pregnancy: consequences for mother and child. Ned Tijdschr Geneeskd 147:235–240

Vockley J, Ensenauer R (2006) Isovaleric acidemia: new aspects of genetic and phenotypic heterogeneity. Am J Med Genet C Semin Med Genet 142C:95–103

Walter JH (2000) Inborn errors of metabolism and pregnancy. J Inherit Metab Dis 23:229–236

Winter SC, Linn LS, Helton E (1995) Plasma carnitine concentrations in pregnancy, cord blood, and neonates and children. Clin Chim Acta 243:87–93

JIMD Reports
DOI 10.1007/8904_2011_67

CASE REPORT

Hyperoxaluria and Rapid Development of Renal Failure Following a Combined Liver and Kidney Transplantation: Emphasis on Sequential Transplantation

Ahmed M. Alkhunaizi · Nouriya A. Al-Sannaa · Wasim F. Raslan

Received: 20 April 2011 / Revised: 30 May 2011 / Accepted: 31 May 2011 / Published online: 6 September 2011

Abstract Primary hyperoxaluria type I (PH I) is a rare genetic disorder that leads to end stage renal disease (ESRD) at an early age due to excessive deposition of calcium oxalate in the kidney. Combined liver-kidney transplantation (LKTx) has been advocated as the treatment of choice for patients with PH I who have progressive renal disease. With combined LKTx the risk of early renal failure secondary to oxalate deposition is anticipated. Here we report a patient with PH I who developed ESRD and underwent a combined LKTx. He lost the kidney graft secondary to early recurrence of oxalosis. Repeat kidney transplantation 13 months after the initial procedure was successful. Elevated plasma oxalate levels persisted for a long time following LKTx and lead to further deposition of oxalate in the second kidney graft. Combined LKTx for patients with PH I requires meticulous preparation and very careful post operative management. Sequential liver transplantation followed by kidney transplantation is to be considered for PH I patients who have ESRD and very high oxalate load.

Communicated by: Verena Peters.

Competing interests: None declared.

A.M. Alkhunaizi (✉)
Internal Medicine Services Division, Dhahran Health Center, Saudi Aramco, Box 10955, Dhahran 31311, Saudi Arabia
e-mail: ahmed.khunaizi@aramco.com

N.A. Al-Sannaa
Pediatric Services Division, Dhahran Health Center, Dhahran, Saudi Arabia

W.F. Raslan
Pathology Services Division, Dhahran Health Center, Dhahran, Saudi Arabia

Introduction

Primary hyperoxaluria (PH) is a rare autosomal recessive disorder caused by distinct enzyme deficiencies that lead to an accumulation of the poorly soluble oxalate in various tissues, especially the kidney. PH I which is more common and more severe than type II is caused by deficiency of the hepatic enzyme alanine: glyoxalate aminotransferase (AGT), which is encoded by the AGXT gene. This deficiency will result in failure to transaminate glyoxylate, which will accumulate as glyoxylate and in turn will be oxidized to oxalate. A third type of PH has been recently identified (Belostostky et al. 2010). Excessive deposition of oxalate in the kidney will lead to the formation of oxalate stones and severe interstitial nephritis. Unlike PH II and III, patients with PH I have a high risk of developing end stage renal disease (ESRD) at an early age or during early adulthood. Since the enzyme exists within the hepatic peroxisomes, liver transplantation is hoped to provide the cure by replacing the missing enzyme and correcting the underlying biochemical defect. A combined liver and kidney transplantation has been advocated in many centers as the main approach to patients with PH I who have ESRD. The outcome of this procedure has been variable. Here we report on a patient with PH I complicated by ESRD. He underwent a combined LKTx, which resulted in an early loss of the kidney graft due to excessive deposition of oxalate. The subsequent kidney transplantation was successful.

Case Report

A 20-year-old male was born to a healthy first cousin Saudi couple. His first presentation was at the age of 18 years

with symptoms of renal colics and he was found to have a serum creatinine (Cr) of 765 μmol/L (reference range 62–105) corresponding to an estimated glomerular filtration rate (eGFR) of 8.5 ml/min. Kidney ultrasound confirmed the presence of multiple and bilateral kidney stones. He underwent several urological procedures, including nephrostomies and lithotripsy, with little effect. Stone analysis showed 100% calcium oxalate (CaOx) monohydrate crystals. His kidney biopsy revealed severe CaOx crystal deposition in the proximal and distal tubules (Fig. 1). Chemical analysis of the urine showed the following: glycolate 54 μgm/mg Cr (reference range <9), glycerate 0 μgm/mg Cr (reference range <9), oxalate 169 μgm/mg Cr (reference range <57), and glyoxylate 3.7 μgm/mg Cr (reference range <3.0). The plasma oxalate level was 87 μmol/L (reference range <1.8). The activity of the enzyme AGT in the hepatocytes was 1.9 μmol/h/mg proteins (reference range 19.1–47.9). Genetic study showed a homozygous c.473C>T (p.Ser158Leu) missense mutation at the AGXT gene. Both parents were confirmed to be heterozygous for the same mutation. The patient progressed to ESRD and was started on hemodialysis (HD) in September 2007. He received conventional, 4 h, thrice weekly HD aiming at a KT/V of 1.3 in addition to pyridoxine and multivitamin supplements. In December 2008, 15 months after initiation of dialysis, he underwent a combined liver and kidney transplantation from a deceased donor. The immunosuppression protocol included steroids, mycophenolate mofetil, and tacrolimus. Although the patient had an immediate kidney allograft function, his urine output dropped on the third post operative day despite hydration and he had to go back on HD. He received daily dialysis for 2 months with the hope of restoring the kidney graft function. A kidney allograft biopsy on the 47th day post transplantation showed heavy deposition of CaOx with no evidence of rejection. The liver allograft function remained very good. The patient was maintained on HD three times per week aiming at a KT/V of 1.3. Thirteen months after the initial liver and kidney transplantation, the patient underwent a second kidney transplantation, which was successful. The immunosuppression protocol included steroids, mycophenolate mofetil, and tacrolimus. The patient was placed on a regimen of high fluid intake, magnesium, and citrate replacement. Four months later, his creatinine started to increase and a kidney biopsy showed scattered deposition of oxalate with no evidence of rejection. The plasma oxalate levels after the liver transplantation decreased, but remained above the normal values as shown in Fig. 2. Urinary oxalate values at different time points are listed in Table 1. At 1 year following the second kidney transplantation serum creatinine was 175 μmol/L corresponding to eGFR of 45 ml/min.

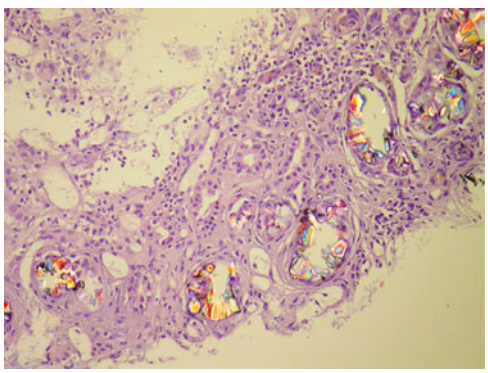


Fig. 1 Light microscopy of the native kidney showing extensive oxalate deposition with advanced interstitial fibrosis

Discussion

PH I is a rare autosomal recessive disorder. It is characterized by deficiency of the hepatic peroxisomal enzyme alanine: glyoxylate aminotransferase (Hepatic AGT) that leads to overproduction and accumulation of CaOx in different tissues (Danpure 2005; Jamieson 2009). The most important affected organ is the kidney, where patients may develop nephrolithiasis, renal dysfunction, and possibly ESRD. The onset of ESRD in PH I patients varies from infancy to adolescence and possibly adulthood (Bobrowski and Langman 2008). Some patients are diagnosed late with advanced renal failure. Others may present with bilateral nephrolithiasis and get subjected to various and futile urological procedures such as lithotripsy and nephrosotomy tube insertions. Supportive therapies to prevent renal failure using pyridoxine supplementation, urinary crystallization inhibitors, and hydration have been used with variable success (Bobrowski and Langman 2008). Oxalate-degrading bacteria (probiotics) have also been tried to prevent oxalate absorption in the gut (Hoppe et al. 2005). Novel approaches using hepatocyte cell transplantation, or enzyme replacement by recombinant gene therapy may emerge in the future as the treatment of choice (Bobrowski and Langman 2006). Until now, organ transplantation of the kidney alone, LK transplantation either combined or sequential or a preemptive liver transplantation, have offered the only potential for cure (Jamieson 2009; Bobrowski and Langman 2008; Kemper 2005; Brinkert et al. 2009; Millan et al. 2003; Gagnadoux et al. 2001; Cochat et al. 2006; Jamieson and European PHI Transplantation Study Group 2005; Saborio and Scheinman 1999). The experience with organ transplantation in PH I has been variable. Recurrence of oxalosis following kidney transplantation alone is anticipated since the underlying biochemical defect is not corrected (Broyer et al. 1990). Combined LK transplantation has been advocated for patients with PH I by the European transplant registry

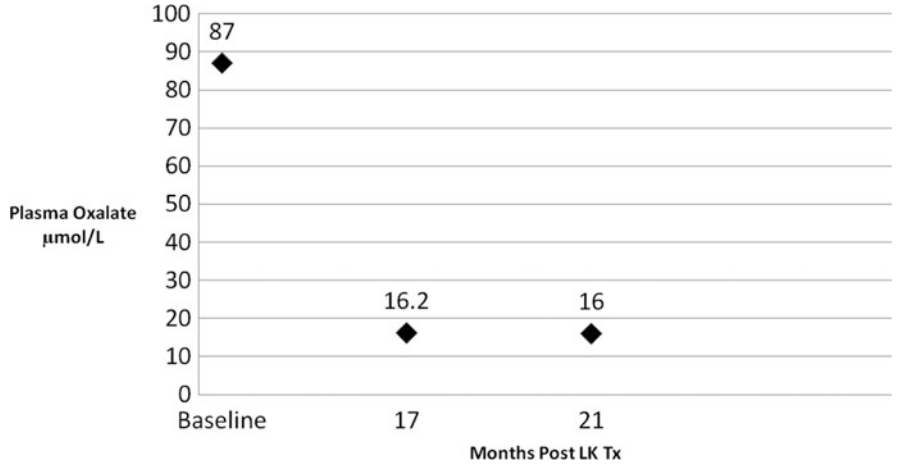


Fig. 2 Plasma oxalate levels at different time points in relation to liver/kidney transplantation (LK Tx)

Table 1 Hyperoxaluria urine panel before, and after the transplant procedures

	Pre-transplant (S. Cr 765)	10 months post-LK transplantation (S. Cr 722)	2 weeks post second K transplantation (S. Cr 97)
Glycolate (ref value<79)	54	9	53
Glycerate (ref value<9)	0	6	1
Oxalate (ref value<57)	169	61	342
Glyoxylate (ref value<3.0)	3.7	3.9	1.2

Values are expressed in µgm/mg creatinine

S. Cr serum creatinine expressed in µmol/L, *L* liver, *K* kidney

(Jamieson and European PHI Transplantation Study Group 2005; Broyer et al. 1990). However, a report analyzing data from the United States Renal Data System has recommended that kidney transplantation alone could be a first option for patients with PH I (Saborio and Scheinman 1999). Patients with PH I especially those of pediatric age group have significant morbidity and mortality post organ transplantation and need very careful and expertise post operative care. These patients may have a large load of oxalate that not only affects the kidneys but also other organs such as the cardiovascular system leading to significant morbidity and mortality (Mookadam et al. 2010). The large oxalate load may exert its effect on the newly transplanted kidney even in the setting of a combined LK transplantation subjecting the patients to considerable morbidity and mortality. In one report, two out of six patients with PH I who underwent organ transplantation died (Ellis et al. 2001). Recent reports have shown more favorable results for combined LKTx. For example, Millan et al. from Stanford University have reported 100% patients and organ survival among six patients with infantile PH I who received simultaneous LKTx (Millan et al. 2003). More recently, Brinkert et al. have reported on their experience on 13 patients with PH I at the University of Hamburg, Germany (Brinkert et al. 2009). In this study, two patients who were planned for sequential transplantation died after liver surgery and prior to receiving a kidney transplant. However, those who underwent preemptive liver transplantation (four patients) or received combined LKTx (seven patients) had an excellent long term outcome with 100% patient survival at a median follow-up of 11.6 and 3.3 years, respectively. The organ survival was 86% and 100% for the kidney and liver grafts, respectively.

Combined LK transplantation has some advantages including subjecting patients to one rather than two surgical procedures and limiting the risk of anesthesia and post operative complications. In addition there may be some immunological advantages by transplanting both organs from the same donor. However, rapid recurrence of oxalosis in the transplanted kidney in addition to high postoperative morbidity is of a big concern. In a recent study, Harps et al. have reported that patients with PH I had a more prolonged stay in the intensive care unit following combined LKTx compared with those who had polycystic disease

(Harps et al. 2011). Patients with PH I require careful pre- and postoperative management including intense preoperative dialysis, postoperative hydration, urine alkalinization, vitamin supplementation, and forced diuresis. Continuous veno-venous hemofiltration may be utilized to decrease the oxalate load and was performed routinely by the Hamburg team for the first postoperative days (Harps et al. 2011).

It is, however, important to mention that both hemo- and peritoneal dialysis do not clear oxalic acid efficiently, and secondary oxalosis is not an uncommon finding in patients undergoing dialysis (Hoppe et al. 1996; Ogawa et al. 2004; Alkhunaizi and Chan 1996).

Sequential liver followed by kidney transplantation should theoretically provide better protection of the kidney allograft. Similarly, preemptive liver transplantation has been shown recently to be effective in arresting the long-term deterioration of renal function in patients with PH I (Perera et al. 2011). The best time to do the kidney transplantation is, however, not known since the total body load of oxalate is difficult to quantify. A strategy of daily dialysis for several months using high flux membranes to reduce the oxalate load while enzyme replacement is offered by the liver graft should in theory reduce further oxalate formation and deposition. Serial measurements of plasma oxalate should aid in determining the best time of the procedure. Urinary oxalate excretion on the other hand depends on the GFR and urinary volume and may not provide a clear picture of the status of oxalate load. Due to the rarity of PH I, organ transplantation should ideally take place in a center where there is established expertise in dealing with the disease and where monitoring of both plasma and urinary oxalate levels is available.

Our patient is an example of the detrimental role of the body's oxalate load on the newly transplanted kidney despite having a simultaneous LK transplantation. Although plasma oxalate levels decreased after the initial transplantation, they were still elevated above the normal values at 17 and 21 months later. This has led to further deposition of CaOx in the second kidney graft. In addition, lack of prior experience with this disease may have contributed to the early loss of the first kidney graft.

Our patient was found to be homozygous for c.473C > T (p.Ser158Leu) missense mutation at the AGXT gene. Both of his parents were confirmed to be heterozygous for the same mutation. This mutation is known to be associated with severe enzyme deficiency. Identifying the disease-causing mutation for this family will provide a good tool to screen the remaining asymptomatic family members. Early diagnosis has the advantage of an early medical intervention and brings hope of improving the clinical outcome for those unfortunate individuals.

In conclusion, simultaneous liver and kidney transplantation carries the risk of early renal failure due to extensive oxalate deposition and requires careful pre- and postoperative management. A sequential liver followed by kidney transplantation should be considered for patients with PH I and ESRD who have a very high oxalate load.

Acknowledgment The authors acknowledge the use of Saudi Aramco Medical Services organization (SAMSO) facilities for research data used in this article. Opinions expressed in this article are those of the authors and not necessarily of SAMSO. They also thank Gill Rumsby, PhD, at the University College of London, for measuring the activity of the enzyme alanine: glyoxalate aminotransferase and identifying the mutation of the hyperoxaluria gene.

References

Alkhunaizi AM, Chan L (1996) Secondary oxalosis: a cause of delayed recovery of renal function in the setting of acute renal failure. J Am Soc Nephrol 7:2320–2326

Belostostky R, Seboun E, Idelson GH et al (2010) Mutations in DHDPSL are responsible for primary hyperoxaluria type III. Am J Hum Genet 87:392–399

Bobrowski AE, Langman CB (2006) Hyperoxaluria and systemic oxalosis: current therapy and future directions. Expert Opin Pharmacother 7:1887–1896

Bobrowski AE, Langman CB (2008) The primary hyperoxalurias. Semin Nephrol 28:152–162

Brinkert F, Ganschow R, Helmke K et al (2009) Transplantation procedures in children with primary hyperoxaluria type 1: outcome and longitudinal growth. Transplantation 87:1415–1421

Broyer M, Brunner FP, Brynger H et al (1990) Kidney transplantation in primary oxalosis: data from the EDTA Registry. Nephrol Dial Transplant 5:332–336

Cochat P, Liutkus A, Fargue S et al (2006) Primary hyperoxaluria type 1: still challenging! Pediatr Nephrol 21:1075–1081

Danpure CJ (2005) Primary hyperoxaluria: from gene defects to designer drugs? Nephrol Dial Transplant 20:1525–1529

Ellis SR, Hulton SA, McKiernandt PJ et al (2001) Combined liver-kidney transplantation for primary hyperoxaluria type 1 in young children. Nephrol Dial Transplant 16:348–354

Gagnadoux MF, Lacaille F, Niaudet P et al (2001) Long term results of liver-kidney transplantation in children with primary hyperoxaluria. Pediatr Nephrol 16:946–950

Harps E, Brinkert F, Ganschow R et al (2011) Immediate postoperative intensive care treatment of pediatric combined liver-kidney transplantation: outcome and prognostic factors. Transplantation 91:1127–1131

Hoppe B, Graf D, Offner G et al (1996) Oxalate elimination via hemodialysis or peritoneal dialysis in children with chronic renal failure. Pediatr Nephrol 10:488–492

Hoppe B, von Unruh G, Laube N et al (2005) Oxalate degrading bacteria: new treatment option for patients with primary and secondary hyperoxaluria? Urol Res 33:372–375

Jamieson NV (2009) Primary hyperoxaluria type 1: gene therapy by liver transplantation. Transplantation 87:1273–1274

Jamieson NV, European PHI Transplantation Study Group (2005) A 20-year experience of combined liver/kidney transplantation for primary hyperoxaluria (PH1): the European PH1 transplant registry experience 1984–2004. Am J Nephrol 25:282–289

Kemper MJ (2005) The role of preemptive liver transplantation in primary hyperoxaluria type 1. Urol Res 33:376–379

Millan MT, Berquist WE, So SK et al (2003) One hundred percent patient and kidney allograft survival with simultaneous liver and kidney transplantation in infants with primary hyperoxaluria: a single-center experience. Transplantation 76:1458–1463

Mookadam F, Smith T, Jiamsripong P et al (2010) Cardiac abnormalities in primary hyperoxaluria. Circ J 74:2403–2409

Ogawa Y, Machida N, Jahana M et al (2004) Major factors modulating the serum oxalic acid level in hemodialysis patients. Front Biosci 9:2901–2908

Perera MT, Sharif K, Lloyd C et al (2011) Pre-emptive liver transplantation for primary hyperoxaluria (PH-I) arrests long-term renal function deterioration. Nephrol Dial Transplant 26:354–359

Saborio P, Scheinman JI (1999) Transplantation for primary hyperoxaluria in the United States. Kidney Int 56:1094–1100

JIMD Reports
DOI 10.1007/8904_2011_71

CASE REPORT

Adult-Onset Presentation of a Hyperornithinemia-Hyperammonemia-Homocitrullinuria Patient Without Prior History of Neurological Complications

Kamer Tezcan · Kristal T. Louie · Yong Qu · Jorge Velasquez · Frank Zaldivar · Natalia Rioseco-Camacho · José Angel Camacho

Received: 15 November 2010 / Revised: 16 May 2011 / Accepted: 20 June 2011 / Published online: 22 September 2011

Abstract The Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome is a disorder of the urea cycle and ornithine degradation pathway caused by mutations in the mitochondrial ornithine transporter, ORNT1 (SLC25A15). In general, the majority of patients with HHH syndrome come to medical attention during infancy or early school years with symptoms such as learning disabilities, changes in cognitive development, spasticity, or liver dysfunction. In this report, we describe a 35-year-old male of Indian descent who was diagnosed with HHH syndrome after he presented to the emergency room with gastroenteritis, disorientation, and slurred speech. Molecular analysis revealed that this patient was heterozygous for two ORNT1 mutations, p.[Gly220Arg(+)Arg275X] (c.[658G>A(+)823C>T]) that had been previously reported in homozygous probands who presented during the first year of life. Cellular studies revealed that the ORNT1 p.Gly220Arg mutation was nonfunctional but targeted to the mitochondria. Given that this patient was a successful college graduate on a vegetarian diet without a prior history of learning or neurological impairment, additional factors such as gene redundancy, environmental, and epigenetic factors may have contributed to the delay in onset of presentation and lack of any previous symptoms. To the best of our knowledge, this is the first reported case of an adult-onset HHH syndrome presentation without a prior history of neurological or cognitive deficiency.

Communicated by: Sedel Frederic.

Competing interests: None declared.

K. Tezcan · K.T. Louie
Department of Genetics, Kaiser Permanente, Sacramento, CA, USA

Y. Qu
Department of Genetic Testing, Kaiser Permanente, Los Angeles, CA, USA

J. Velasquez · F. Zaldivar · N. Rioseco-Camacho · J.A. Camacho (✉)
Department of Pediatrics, University of California-Irvine, 326 Sprague Hall, ZOT 3905, Irvine, CA 92697, USA
e-mail: jcamacho@uci.edu

Introduction

The Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome (OMIM # 238970) is an autosomal recessive disorder of the urea cycle and ornithine degradation pathway caused by mutations in the mitochondrial ornithine transporter, ORNT1 (SLC25A15) (Valle and Simell 2001). In spite of the impermeability of the inner mitochondrial membrane to amino acids, the great majority of patients with nonfunctional ORNT1 mutations have a later onset and milder clinical course when compared to patients with ornithine transcarbamylase deficiency with no enzymatic activity (Brusilow and Horwich 2001). Since ORNT1 deficiency does not completely abolish ornithine transport across the inner mitochondrial membrane in HHH patients' liver and fibroblasts, we hypothesized that additional transporters with overlapping function must exist. Our hypothesis was confirmed by the isolation of two additional mitochondrial ornithine transporters, the retroposon ORNT2 (SLC25A2) and, more recently, ORNT3 (SLC25A29), a carrier protein that also functions as a carnitine/acylcarnitine-like transporter (CACT-like) (Camacho and Rioseco-Camacho 2009). A salient feature of HHH syndrome is phenotypic variability in patients with the same mutation and a lack of correlation between ORNT1 mutant genotype and

the clinical phenotype in terms of onset and severity. Presenting symptoms of patients with ORNT1 deficiency include liver dysfunction, coagulopathy, unexplained seizure activity, developmental delay, academic difficulties, cognitive impairment, stroke-like lesions on MRI and/or gait disturbances (Debray et al. 2008; Tessa et al. 2009). The cognitive development of patients with ORNT1 deficiency can range from normal intelligence to severe impairment. In some instances, patients suffer from significant neurological deficits without any evidence of cognitive impairment. Moreover, HHH patients commonly develop spastic paraparesis. Classically, patients are characterized by chronic elevation in plasma ornithine, episodic or postprandial hyperammonemia and urinary excretion of homocitrulline and orotic acid. In general, patients are maintained on a protein-restricted diet and supplemented with special formulas, citrulline and sodium phenylbutyrate.

In this report, we describe the case of a previously asymptomatic 35-year-old male who was diagnosed with HHH syndrome after he presented to the ER with a gastroenteritis, disorientation, slurred speech, and mild elevations in plasma ammonia, glutamine and ornithine. Molecular analysis revealed that the patient carries two ORNT1 mutations, p.[Gly220Arg(+)Arg275X] (c.[658G>A(+)823C>T]), which have been previously described in HHH homozygous probands presenting during infancy and the early childhood period (Al-Hassnan et al. 2008; Torisu et al. 2006).

Methods and Patient Information

Patient Data

All medical records, blood for DNA and lymphocyte cell lines were obtained with institutional IRB approval. For this report, only the proband was available for blood draw since his mom had recently died due to chronic lung disease and his father is frail at the age of 80. RS is a 35-year-old college-educated male of Indian descent who, prior to the onset of symptoms, never had a history of learning disabilities, liver disease, psychiatric illness, or neurological deficits. The patient was born to a previously healthy 29-year-old G1A0P1 after an uncomplicated pregnancy. He had a healthy and active life and always did well in his schoolwork. The patient went to college and started to work in his native country until all of his family moved to the USA 4 years prior to the onset of symptoms. The patient's sister (only sibling) and her two children are all in good health. There is no family history of genetic or metabolic disorders.

The patient states he never smoked, used alcohol or drugs. Nutritional history was remarkable for self-restriction of protein products since childhood (pre-adolescence years) given that they would make him feel sick or induce vomiting. Although, on occasion, he would eat eggs, he avoided milk because of lactose intolerance. The patient also reported avoiding seafood because of a prior history of severe allergic reaction that caused respiratory compromise. The patient's diet consisted mostly of rice, lentils, nuts, fruits, and vegetables. The calculated daily protein intake prior to the onset of symptoms was about 47 g per day (0.48 g/kg/day). Approximately 4 years prior to the onset of symptoms, the patient reported getting overweight and was diagnosed with asthma that required bronchodilator therapy. Currently, at 6 ft tall, he weighs 227 lbs and has a BMI of 30.7. The patient states that for the last 10 years he has slowly been gaining weight.

Five weeks prior to the initial onset of symptoms, his mother died from chronic lung disease. This family loss led RS to start consuming nonhealthy foods in large quantities and, in part, abandon his vegetarian diet. The patient was brought to the ER by his brother-in-law with a 3-day history of vomiting, diarrhea, dizziness, and confusion. Upon examination, the patient was noted to be disoriented, had slurred speech, and could not read or recall his home telephone number. No evidence of GI bleeding was found, and an abdominal CT scan was normal. His plasma ammonia was elevated at 114 μM (normal range 25–50 μM), but the rest of his blood chemistries and blood count were normal. During the patient's brief stay at the ER, he received IV fluids with dextrose and electrolytes that stabilized his condition (ammonia went down to 43 μM). He was discharged home the same day on Lactulose. No specific diagnosis was given.

Two days after discharge from the ER, the patient returned with a similar set of symptoms. On this occasion, his plasma ammonia level was 140 μM. Patient was admitted to the hospital and a genetic consult was called. Although his blood chemistries and blood count were again within normal limits, his biochemical profile was suggestive of HHH syndrome. These values included elevations in plasma ornithine at 292 μM (normal range 30–110 μM) and glutamine at 1,322 μM (normal range 400–900 μM) with normal levels of lysine at 82 μM (normal range 80–250 μM), arginine at 48 μM (normal range 20–120 μM), and citrulline at 20 μM (normal range 13–45 μM). His urine organic were normal and his urine amino acids did not show increased excretion of cationic amino acids (lysine, ornithine, arginine), citrulline or arginosuccinic acid. No orotic aciduria was observed nor increases in methionine (with normal plasma levels) in the urine amino acid profile, thus suggesting no urinary excretion of homocitrulline given that the peaks of these two amino acids overlap. This observation on our patient contrasts sharply with a group of five previously reported Kaiser-Permanente HHH patients, who consistently showed

a urinary pattern suggestive of homocitrullinuria (Camacho et al. 2006). The patient's plasma carnitine profile was normal. CT scan of his brain and liver did not reveal any abnormalities. The patient was subsequently followed 1 month later at the Human Genetics Clinic where he was found to be neurologically asymptomatic and feeling back to normal. After evaluation by a nutritionist, the patient was placed on a protein-restricted diet of 50–60 g/day (~0.5 g/kg/day), citrulline and sodium phenylbutyrate.

In general, this patient's plasma levels of ornithine (327–704 μM) and glutamine (1,043–1,435 μM) have always remained elevated. On several occasions, values for lysine and phenylalanine were decreased in the range of 47–60 μM (normal range 80–250 μM) and 28–30 μM (normal range 40–100 μM), respectively. Finally, the patient reported suffering from a severe asthma attack that required the use of intravenous anti-inflammatory steroids. This treatment modality caused a low-grade episode of hyperammonemia that lasted 4 days and caused mental confusion, forgetfulness, and generalized body weakness. After the patient recovered, he returned to the Human Genetics Clinic where he was found to have a normal physical exam. A follow-up brain MRI and a recent psychometric testing were normal.

Mutation Analysis of Human ORNT1 and ORNT2

Genomic DNA (blood) and total RNA (EBV-transformed lymphocytes) were used for mutation analysis utilizing previously published protocols (Torisu et al. 2006; Camacho et al. 2006). We identified two mutant *ORNT1* alleles: c.658G>A (p.Gly220Arg) in Exon 6 and c.823C>T (p.Arg275X) in Exon 7. To confirm that this patient was heterozygous for the c.823 C > T mutation, we used forward primer 5′-GCATCCTTCTATGACTTGTTG-3′ and backward primer 5′-AATAGAGGTTCTTCCATAAAG-3′ to amplify a 431 bp fragment that when digested with Taq I would generate a wild-type and heterozygous pattern. Finally, we amplified and sequenced human ORNT2 as previously detailed (Camacho et al. 2003).

Expression Constructs, Transfections, Ornithine Transport Assay and Indirect Immunofluorescence

For our studies, we used previously described N-myc–tagged wild-type mouse *Ornt1* (*Slc25a15*) and *Ornt3* (*Slc25a29*) constructs as positive controls (Camacho and Rioseco-Camacho 2009). Using a wild-type ORNT1-N-myc-tagged construct as a backbone, we generated the *ORNT1* mutation p.Gly220Arg as previously reported (Camacho et al. 2006). We performed electroporation (transient transfection), the ornithine transport assay, and immunofluorescence as previously described (Camacho and Rioseco-Camacho 2009). For transient transfection studies, we assayed three to six wells per transfected plasmid and expressed results as mean ± SD. We expressed the ability to incorporate ^{14}C-ornithine into protein as glutamate and proline relative to total protein synthesis (^{3}H-leucine) as a ^{14}C/^{3}H ratio. We analyzed data using the unpaired *t* test and presented our results in graphs as percent increase compared to control cells.

Results

Molecular Studies

Initial amplification by RT-PCR revealed two mutations. The first mutation, c.658G>A (p.Gly220Arg), was previously reported in a homozygous HHH proband who presented with hypotonia, liver dysfunction, and stroke-like lesions on MRI at the age of four (onset of symptoms by 12 mo) (Al-Hassnan et al. 2008). The second mutation, c.823C>T (p.Arg275X), was previously reported in a homozygous HHH proband who became symptomatic at the age of 5 months and who, by the age of 6 years, had a phenotype which included deafness, significant cognitive impairment, and mild spastic paraplegia (Torisu et al. 2006).

Genomic PCR confirmed that our proband was heterozygous for the *ORNT1* c.[658G>A(+)823C>T] alleles (Fig. 1). Given that the stop codon mutation, c.823C>T, eliminated a Taq I site, we amplified a smaller PCR product from genomic DNA and subjected it to enzymatic digestion in order to further demonstrate that this patient was heterozygous for this allele. Results shown in Fig. 1b demonstrate the presence of three fragments in the patient's lane corresponding the proband's wild-type (250-bp and 181-bp fragments) and mutant, undigested (431-bp fragment) alleles.

Finally, we examined the sequence of the second mitochondrial ornithine transporter, ORNT2, for any potential polymorphisms that could affect its function. Results of our search demonstrated that the patient has the c.676G>A (p.Val226Iso) change, which has been annotated in the NCBI-SNP database (RefSNP ID: rs3749780) and which has a frequency that varies from 0.058 to 0.237 in several geographical and ethic groups. Contrary to two previously reported ORNT2 protein polymorphisms, p.Val226Iso does not appear to affect this carrier's mitochondrial targeting or transporting ability (unpublished observations) (Camacho et al. 2003).

Functional Studies

We thought of further investigating the functional consequences of the Gly^{220} to Arg^{220} change given that, in the past, several reports have demonstrated that ORNT1

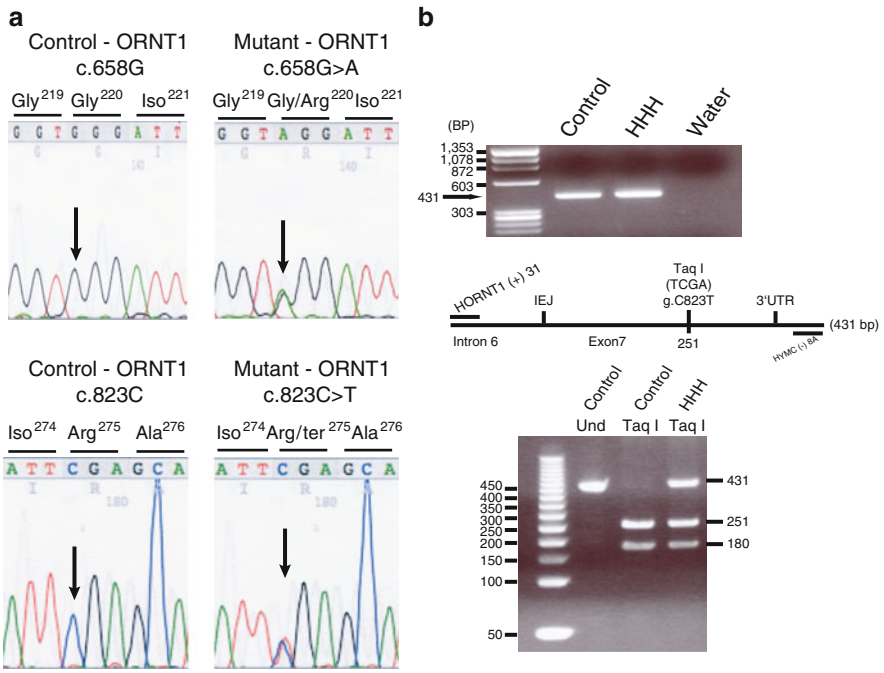


Fig. 1 Human ORNT1 c.[658G>A(+)823C>T] (p.[Gly220Arg(+)Arg275X]) mutations. (**a**) Patient was found to have two previously known ORNT1 mutations, p.[Gly220Arg(+)Arg275X]. *Left* and *right* panel shows the control and mutant sequences, respectively. (**b**) *Top panel*: Undigested PCR products. *Bottom panel*: Genomic PCR amplification of part of intron 6 and exon 7 that contains a single Taq I (TCGA) site that was abolished by the c.823C>T mutation. Approximately 20 μl of Taq I-digested patient genomic PCR product was run on a 7% acrylamide gel

mutations that appeared to be nonfunctional have residual function (Camacho et al. 2006). Overexpression studies shown in Fig. 2a clearly demonstrate that the p.Gly220Arg change completely eliminates ORNT1 transporting capacity when compared to both mouse (*Mm*) Ornt1 and Ornt3. The level of ornithine transporting ability of the cells expressing the human (*Hs*) N-myc-ORNT1-Gly220Arg construct is the same as those expressing only the empty pcDNA3.1 vector and nontransfected HHH fibroblasts expressing the nonfunctional ORNT1 p.Phe188del mutation. We also performed immunofluorescence studies using previously published protocols to determine whether the p.Gly220Arg change affected the mitochondrial targeting pattern of ORNT1 (Camacho and Rioseco-Camacho 2009). Our results also demonstrate that the N-myc-*Hs*ORNT1-Gly220Arg transporter has a normal mitochondrial-targeting pattern similar to the wild-type ORNT1 construct (Fig. 2b).

Discussion

In this report, we describe a patient with HHH syndrome who was initially diagnosed at the age of 35, although he carries two nonfunctional ORNT1 mutations, p.[Gly220Arg(+)Arg275X]. This late-onset presentation and lack of any prior neurological or hepatic clinical findings is in sharp contrast to two previously reported HHH probands who were homozygous for the same mutations and who presented at the age of 5 (p.Arg275X) and 12 (p.132Gly220Arg) months (Al-Hassnan et al. 2008; Torisu et al. 2006). The lack of changes in MRI and CT scan and a negative medical history of cognitive or neurological impairment suggest that this patient had not suffered from the chronic effects of elevated ammonia and glutamine or from severe potential abnormalities in ornithine, lysine, and arginine metabolism. This is the first case, to the best of our knowledge, of an adult onset HHH patient who does not exhibit any previous form of neurological or cognitive deficit. After nearly 35 years without any neurological symptoms or cognitive deficiency, it is difficult to ascertain the triggering event(s) that caused this patient's symptoms, though deviating from a vegetarian diet may certainly have precipitated this crisis.

The incomplete metabolic triad exhibited by this HHH patient, viz. the absence of a urinary amino acid profile suggestive of homocitrullinuria, is particularly interesting. Given that our patient had a very mild phenotype, it is conceivable that his redundant ornithine transport was enough to prevent a significant intramitochondrial carbamylation of lysine. In fact, orotic acid excretion, which in HHH syndrome reflects the accumulation of carbamyl

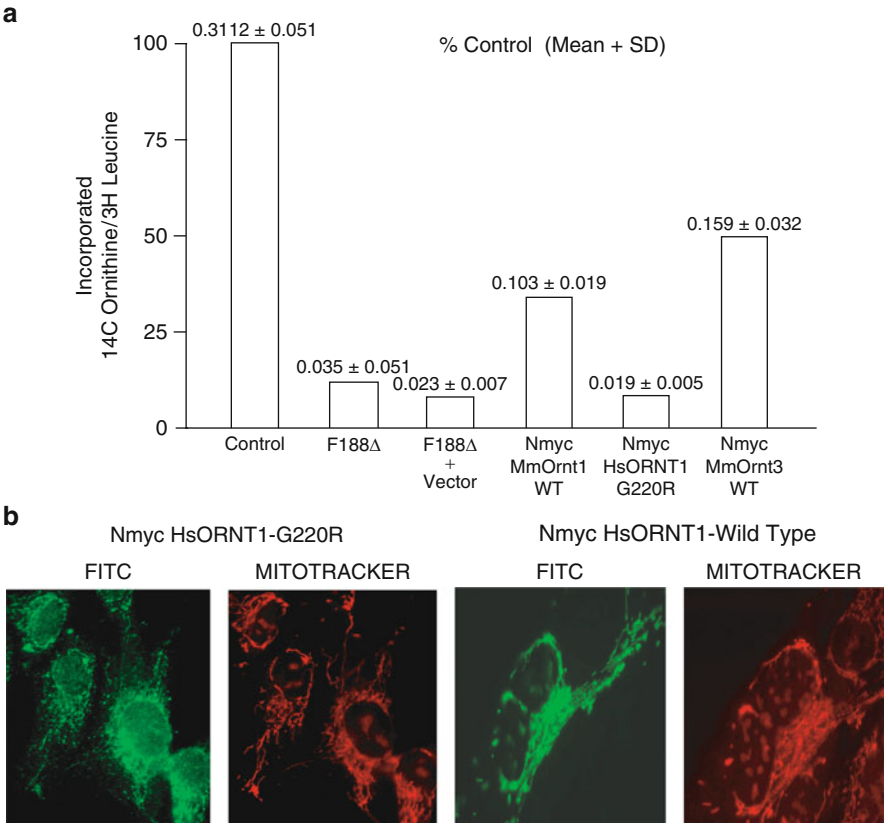


Fig. 2 Transient transfection studies of Nmyc-*Hs*ORNT1-G220R construct does not restore ornithine metabolism in HHH cultured fibroblasts cells. (**a**) Fibroblasts carrying the nonfunctional ORNT1-F188Δ allele were electroporated with an N-myc-*Hs*ORNT1-G220R, N-myc-*Mm*Ornt1, N-myc-*Mm*Ornt3 or empty pcDNA3.0 vector using previously described conditions. The ornithine transport assay is performed at the nonsaturating ornithine concentration of 2.1 μM. *$p < 0.05$ vs. HHH+vector. (**b**) Immunofluorescence studies of wild-type N-myc-Ornt1 and N-myc-ORNT1-G220R mutant constructs. Transfection efficiency for these experiments was ~15–20%. Ornithine transport assays and immunofluorescence studies are representative of three experiments

phosphate and, thus, its intramitochondrial underuse (Valle and Simell 2001), was also absent further validating the lack of homocitrulline in the urine. Moreover, the patient's history of a low protein (vegetarian) diet may have reduced the amount of homocitrulline since there is a correlation between homocitrulinuria and the level of protein intake in ORNT1 deficiency (Valle and Simell 2001).

It is well known that the toxic effects of elevated levels of ammonia and glutamine on the brain astroctye are in part responsible for the symptoms of patients with urea cycle disorders including HHH syndrome (Sofroniew and Vinters 2010). However, given that ORNT1 also functions as a cationic amino acid transporter, it is reasonable to propose that the pathophysiology of HHH syndrome may also be dependent on the interruption of physiological/biological functions of ORNT1, which include a role in mitochondrial protein synthesis, the metabolism of arginine and lysine, plus alterations in polyamine metabolism (Palmieri 2008). Another factor that may also contribute to the mechanisms of disease in HHH syndrome relates to the synthesis of creatine given that ornithine negatively regulates the activity of L-arginine:glycine amidinotransferase, the enzyme that catalyzes the first step in the synthesis of creatine (Valle and Simell 2001; Humm et al. 1997). Interestingly, mitochondrial dysfunction as a putative disease mechanism in HHH syndrome was previously posited in two siblings homozygous for the *ORNT1* c.446delG mutation (Korman et al. 2004). Lastly, recent work suggests that both ornithine and homocitrulline may directly induce oxidative stress, affect cellular bioenergetics and cause mitochondrial dysfunction (Viegas et al. 2011).

Synopsis

Presentation of a 35-year-old HHH patient without prior history of neurological complications.

Authors' Contributions

Kamer Tezcan and Kristal T. Louie – Main persons taking care of the patient and contributed to the writing and editing of the manuscript.

Yong Qu – Performed and interpreted all clinical biochemical analysis and contributed to the writing and editing of the manuscript.

Jorge Velasquez – Performed the site-directed mutagenesis experiments, sequenced and purified all constructs, conducted immunoflourescence studies and editing of the manuscript.

Frank Zaldivar – Responsible for establishing the B-lymphocyte cell lines of the patient and contributed to editing of the manuscript.

Natalia Rioseco-Camacho – Performed DNA and RNA isolation, all tissue culture related experiments, assisted in the design of experiments and contributed to the writing and editing of the manuscript.

José A. Camacho – Contributed to patient care, ORNT1 sequencing, experimental design, main manuscript writing and editing. In addition, will assume all the responsibility as guarantor for this manuscript.

Competing Interest Statement

All authors declare that the answers to all questions on the JIMD competing interest form are No, and therefore they have nothing to declare.

Funding

J.A. Camacho was supported by a Robert Wood Johnson Foundation-Dr. Harold Amos Faculty Development Award and F. Zaldivar by a Public Health Service research grant M01RR00827. Part of this work was previously presented in poster form at the Urea Cycle Disorders Satellite Symposium of the 11th International Congress of Inborn Errors of Metabolism in San Diego, CA (August 27–29, 2009). The authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsors.

Ethics Approval

This study was conducted under the Institutional IRB #2002-2608.

Patient Consent

No patient identifiers were used. Patient consented to the review of his medical records, laboratory studies, scans and the publication of his case.

References

Al-Hassnan ZN, Rashed MS, Al-Dirbashi OY et al (2008) Hyperornithinemia-Hyperammonemia-Homocitrullinuria syndrome with stroke-like imaging presentation: clinical, biochemical and molecular analysis. J Neurol Sci 264:187–194

Brusilow SW, Horwich AL (2001) Urea cycle enzymes. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular basis of inherited disease. McGraw Hill, New York, pp 1909–1964

Camacho JA, Rioseco-Camacho N (2009) The human and mouse SLC25A29 mitochondrial transporters rescue the deficient ornithine metabolism in fibroblasts of patients with the Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome. Pediatr Res 66:35–41

Camacho JA, Rioseco-Camacho N, Andrade D et al (2003) Cloning and characterization of human ORNT2: a second mitochondrial ornithine transporter that can rescue a defective ORNT1 in patients with the Hyperornithinemia-Hyperammonemia-Homocitrullinuria syndrome, a urea cycle disorder. Mol Genet Metab 79:257–271

Camacho JA, Mardach R, Rioseco-Camacho N et al (2006) Clinical and functional characterization of a human ORNT1 mutation (T32R) in the Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome. Pediatr Res 60:423–429

Debray FG, Lambert M, Lemieux B et al (2008) Phenotypic variability among patients with Hyperornithinaemia-Hyperammonaemia-Homocitrullinuria syndrome homozygous for the delF188 mutation in SLC25A15. J Med Genet 45:759–764

Humm A, Fritsche E, Steinbacher S et al (1997) Crystal structure and mechanism of human L-arginine:glycine amidinotransferase: a mitochondrial enzyme involved in creatine biosynthesis. EMBO J 16:3373–3385

Korman SH, Kanazawa N, Abu-Libdeh B et al (2004) Hyperornithinemia-Hyperammonemia-Homocitrullinuria syndrome with evidence of mitochondrial dysfunction due to a novel SLC25A15 (ORNT1) gene mutation in a Palestinian family. J Neurol Sci 218:53–58

Palmieri F (2008) Diseases caused by defects of mitochondrial carriers: a review. Biochim Biophys Acta 1777:564–578

Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. Acta Neuropathol 119:7–35

Tessa A, Fiermonte G, Dionisi-Vici C et al (2009) Identification of novel mutations in the SLC25A15 gene in Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome: a clinical, molecular, and functional study. Hum Mutat 30:741–748

Torisu H, Kira R, Kanazawa N et al (2006) A novel R275X mutation of the SLC25A15 gene in a Japanese patient with the HHH syndrome. Brain Dev 28:332–335

Valle D, Simell O (2001) The hyperornithinemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular basis of inherited disease. McGraw Hill, New York, pp 1857–1896

Viegas CM, Busanello EN, Tonin AM et al (2011) Dual mechanism of brain damage induced in vivo by the major metabolites accumulating in Hyperornithinemia-Hyperammonemia-Homocitrullinuria syndrome. Brain Res 1369:235–244

JIMD Reports
DOI 10.1007/8904_2011_69

CASE REPORT

Cerebral Edema in Maple Syrup Urine Disease Despite Newborn Screening Diagnosis and Early Initiation of Treatment

Kenneth A. Myers • Melanie Reeves • Xing-Chang Wei • Aneal Khan

Received: 2 May 2011 / Revised: 5 June 2011 / Accepted: 8 June 2011 / Published online: 22 September 2011

Abstract A 7-day-old girl had an elevated leucine level on newborn screen drawn at 2 days of age and was suspected of having maple syrup urine disease (MSUD). When reported, the patient was immediately admitted to hospital, and started on a modified diet involving high calories with reduced branched chain amino acid (BCAA) formula. Clinical exam was normal at initial assessment. Despite rapid initiation of treatment, the baby became lethargic and somnolent over the next day. Diet was stopped and infusions of 12.5% dextrose and 20% intravenous lipids at 2 g/kg per day were immediately started. Lethargy improved within 3 h of intravenous therapy initiation. Brain magnetic resonance imaging demonstrated diffuse cerebral edema, and symmetric restricted diffusion in bilateral cerebellar white matter, dorsal brainstem, thalami, globi pallidi, posterior limbs of internal capsules, and corona radiata. Plasma leucine was 1.98 mmol/L on admission (normal 0.05–0.17 mmol/L), decreasing to 1.34 mmol/L with diet, however clinical deterioration occurred despite improving leucine levels.

Cerebral edema in MSUD is thought secondary to a combination of increased cerebral BCAA levels, and depleted levels of other essential amino acids, as well as neurotransmitters. Our case illustrates that newborns can develop encephalopathy with cerebral edema despite treatment with special formula initiated while asymptomatic. These findings suggest decompensation may begin early on, so that early introduction of high dextrose infusion and intravenous lipids, in combination with reduced BCAA formula, should be initiated for any patient with a positive newborn screen for MSUD.

Communicated by: K. Michael Gibson.

Competing interests: None declared.

K.A. Myers (✉)
Department of Pediatrics, Division of Neurology, Alberta Children's Hospital, University of Calgary, 2888 Shaganappi Dr. NW, Calgary, AB, Canada T3B 6A8
e-mail: kmyers@ucalgary.ca

M. Reeves
Alberta Children's Hospital, Metabolic Diseases Clinic, 2888 Shaganappi Dr. NW, Calgary, AB, Canada T3B 6A8

X.-C. Wei
Diagnostic Imaging, Alberta Children's Hospital, 2888 Shaganappi Dr. NW, Calgary, AB, Canada T3B 6A8

A. Khan
Department of Pediatrics and Medical Genetics, Alberta Children's Hospital, University of Calgary, 2888 Shaganappi Dr. NW, Calgary, AB, Canada T3B 6A8

Introduction

Maple syrup urine disease (MSUD; OMIM 248600) results from deficiency of the branched chain ketoacid dehydrogenase complex (BCKD) leading to accumulation of branched chain amino acids (BCAA) leucine, isoleucine and valine and their associated alpha-ketoacids (BCKA). In the classic neonatal presentation, infants are typically asymptomatic at birth, but by 4–7 days of age, can develop feeding intolerance, vomiting, and apneas commensurate with encephalopathy. Untreated, there is rapid progression to seizures, coma, and death. MSUD can be difficult to detect in a newborn infant because there may be little, if any, disturbance in typical biochemical markers for metabolic disease such as lactate, ammonia, acidosis, or hypoglycemia.

Ketosis can be present. Having clinical suspicion for MSUD in a newborn baby with unexplained encephalopathy remains one of the best methods to direct the necessary diagnostic investigations, initiate early treatment and limit the neurological damage (Strauss et al. 2010).

The pathophysiology of neurologic deterioration in MSUD is not precisely understood; however, the radiologic changes are well characterized. The typical pattern begins with a marked, generalized cerebral edema beginning late in the first week of life (Brismar et al. 1990). This progresses to a more severe, localized edema primarily affecting the deep cerebellar white matter, dorsal brainstem, cerebral peduncles, and dorsal limb of the internal capsule (Brismar et al. 1990). With appropriate treatment, the edema gradually resolves by 6–7 weeks of life, at which point well-demarcated periventricular white matter disease is usually apparent, with some loss of brain substance (Brismar et al. 1990).

Expanded newborn screening has the potential to make a huge impact on early diagnosis and successful treatment of children with MSUD. The value of early diagnosis and initiation of treatment in endemic populations has been demonstrated; however, there is still limited data on the most appropriate intervention in asymptomatic infants with a positive newborn screen for MSUD. In some patients, the newborn screen may be falsely positive and no specific treatment indicated. However, if the screen is true positive, delaying treatment until confirmation by diagnostic tests can lead to progression of the disease and neurological injury. Medical centers specialized in the care of patients with metabolic diseases may have the capability to confirm the findings of the newborn screen by performing quantitative plasma amino acids with results within 24 h or less, but there is little data on whether it is safer to start the patient on treatment while awaiting results of confirmatory tests. In this report, we detail a case that was detected by newborn screening and had dietary intervention started during the presymptomatic stage resulting in falling plasma leucine levels. Our case suggests that in a patient with a positive newborn screen for MSUD starting a high rate dextrose infusion may be preferable to initially treating with diet alone, even if the patient is asymptomatic.

Case

A full term newborn baby girl was reported at 7 days of age to have a positive newborn screen for MSUD (leucine/isoleucine 0.373 mM, screening cut-off 0.250 mM) from a blood spot drawn at 2 days of age. The family was contacted by the Metabolic service and advised to bring the child immediately to hospital, a blood sample for plasma amino acids was drawn, the child admitted to hospital and started on an MSUD formula with zero BCAA. The child was completely asymptomatic and feeding well. The parents were both of Nicaraguan ancestry, nonconsanguineous and there was no family history suspicious for MSUD. Twelve hours after start of diet therapy, the baby was noted to be lethargic and feeding poorly. A glucose infusion of 11 mg/kg per day and 20% intravenous lipids at 2 g/kg per day were immediately started and she did not continue to feed. An emergent cranial computed tomography (CT) was performed, which showed diffuse cerebral swelling (Fig. 1). An urgent MRI within 2 h of her deterioration showed symmetric restricted diffusion in bilateral cerebellar white matter, dorsal brainstem, thalami, globi pallidi, posterior limbs of internal capsules, and corona radiata (Fig. 1). Within 3 h, her lethargy noticeably improved and by 5–6 h had returned to baseline. Plasma BCAAs from the first venous blood sample (prior to diet therapy) showed elevations in leucine, isoleucine, valine, and alloisoleucine and confirmed the diagnosis of MSUD. The overall plasma amino acid panel, as well as urine organic acid profile, was in keeping with a classic presentation of MSUD. Review of her plasma amino acid levels showed that during the time she developed cerebral edema, her plasma leucine levels were decreasing with diet therapy. Plasma BCAA levels were all brought within acceptable levels by the next testing and oral feeding was restarted 24 h following the initial deterioration (Fig. 2). A septic workup was negative showing no alternative explanation for her decompensation. At 6 months follow-up, the baby continues to be developing normally.

Discussion

Cerebral edema in MSUD is thought secondary to a combination of increased cerebral BCAA/BCKA levels, and depleted cerebral levels of other essential amino acids, as well as neurotransmitter depletion (Strauss et al. 2010). With respect to the latter, mouse model experiments have shown lower CSF levels of glutamate, dopamine, and GABA, as well as lower plasma levels of tryptophan, the precursor to serotonin (Zinnanti et al. 2009). The BCKAs appear to exert effects, at least partially through disruption of pyruvate metabolism (Patel et al. 1973) and respiratory chain function (Sgaravatti et al. 2003). Nevertheless, the exact mechanism by which edema is generated remains unclear, thus designing and evaluating an effective initial management approach for a presymptomatic newborn infant with a positive newborn screen for MSUD is difficult. Our experience with this case suggests to us that dietary management alone, even if plasma leucine levels are falling, may not be sufficient to prevent clinical decompensation. Dextrose infusion may slow conversion of BCAAs to the more toxic BCKAs, a hypothesis supported by the

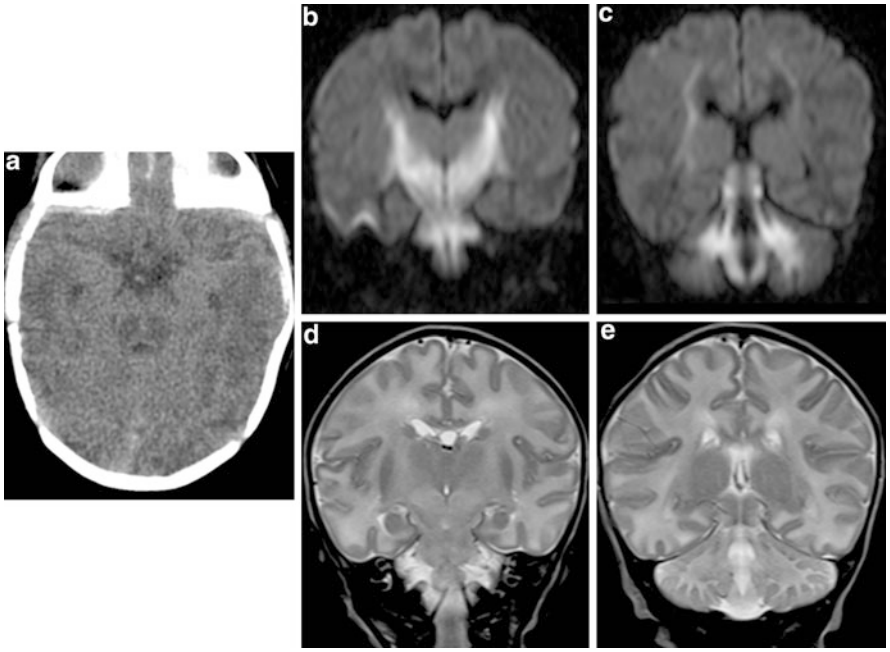


Fig. 1 Noncontrast CT of the brain (**a**) shows diffuse cerebral edema. The ambient cistern is effaced. Focal hypodense areas are seen bilaterally in the posterior aspect of the midbrain. Coronal diffusion-weighted images (**b** and **c**, DWI b factor = 1,000 s/mm^2) show hyperintense signal in white matter of cerebellum, dorsal brainstem, thalami, globi pallidi, internal capsules, and the corona radiata. These changes are hypointense on apparent diffusion coefficient (ADC) map (not shown), which is consistent with restricted diffusion. On T2-weighted images (**d** and **e**), these areas show increased signal, although the changes can be difficult to recognize without comparison to normal subjects of the same age

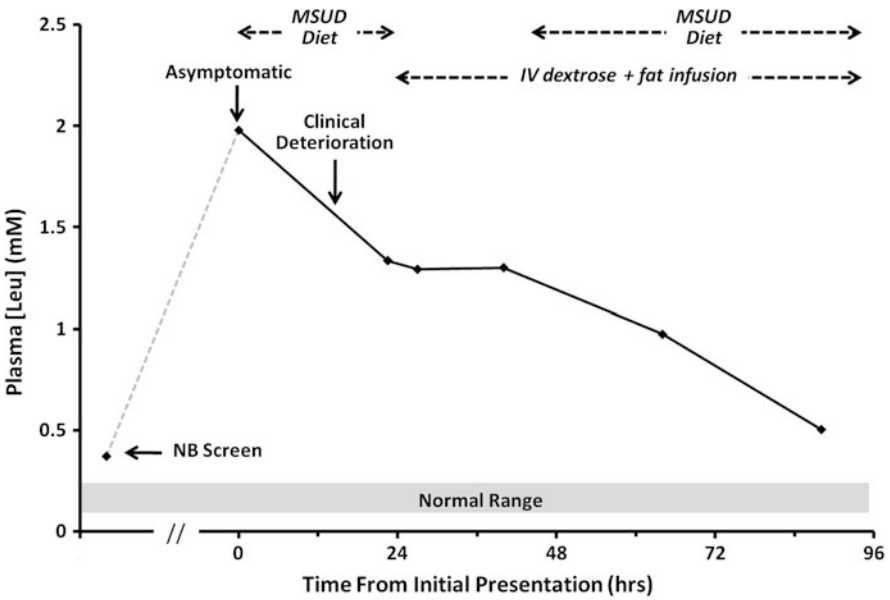


Fig. 2 Serum leucine measurements during the patient's initial presentation. New born screen was done at 2 days of life. The patient presented at 7 days of life, while asymptomatic, and treatment was initiated immediately. Despite improvement in leucine concentration, the patient experienced a clinical deterioration 12 h later

observed clinical improvement with dextrose infusion, over a period when serum BCAA levels had actually plateaued.

We feel this case illustrates that the following actions should be considered for reported positive newborn screens for MSUD:

1. Plasma leucine levels should be obtained immediately in infants with a positive newborn screen for MSUD.
2. Treatment with a high infusion rate of dextrose and intravenous lipids, in addition to BCAA-free formula, should be initiated immediately. If the child is not in a

tertiary center, IV therapy should be started immediately, and maintained during transport to a facility with appropriate dietary and molecular-testing capabilities. When leucine levels can be confirmed to have returned to the normal range, a transition to oral therapy with appropriate low BCAA diet can be made.

More data is needed on whether neuroimaging should be performed to determine whether cerebral edema is a common finding that can be prevented by early treatment or whether the results can guide therapy. If the clinical examination is not reassuring, neuroimaging can help identify evidence of cerebral edema. If an urgent MRI scan is not possible, a brain CT can be performed to evaluate the extent of cerebral swelling. For neonates with positive screen tests for MSUD, an MRI of the brain can assess the extent of brain swelling, the presence of restricted diffusion classically in the myelinated white matter, as well as the level of BCAA on proton spectroscopy (Cavalleri et al. 2002; Jan et al. 2003; Sakai et al. 2005). As most of the neonates with MSUD at presentation are lethargic, an MRI without sedation can be attempted. Close cooperation with the neuroimaging service to avoid any additional sedative or anesthesia in a child already with mild features of encephalopathy would be prudent.

Recent case reports have shown that follow up MRI with DWI may be of predictive value for the efficacy of treatment. In the reported cases, the restricted diffusion predominantly in the areas of myelinated white matter was reversible after treatment, whereas the methyl resonance of BCAAs shown on proton MR spectroscopy persisted even after the acute metabolic crisis had been controlled and the plasma leucine level had reached normal (Sener 2007; Ferraz-Filho et al. 2009).

Synopsis

Cerebral edema and clinical deterioration can occur in the initial presentation of MSUD despite improving serum leucine levels, thus initial therapy for positive newborn screens should include intravenous dextrose and fat infusions, in combination with BCAA-free formula.

Disclosures

None of the authors have any interests or relationships, financial or otherwise, that would be influenced by the findings reported in this paper.

References

Brismar J, Aqeel A, Brismar G et al (1990) Maple syrup urine disease: findings on CT and MR scans of the brain in 10 infants. AJNR Am J Neuroradiol 11:1219–1228

Cavalleri F, Berardi A, Burlina AB et al (2002) Diffusion-weighted MRI of maple syrup urine disease encephalopathy. Neuroradiology 44:499–502

Ferraz-Filho JR, Floriano VH, Quirici MB et al (2009) Contribution of the diffusion-weighted MRI in the diagnosis and follow-up of encephalopathy caused by maple syrup urine disease in a full-term newborn. Arq Neuropsiquiatr 67:719–723

Jan W, Zimmerman RA, Wang ZJ et al (2003) MR diffusion imaging and MR spectroscopy of maple syrup urine disease during acute metabolic decompensation. Neuroradiology 45:393–399

Patel MS, Auerbach VH, Grover WD et al (1973) Effect of the branched-chain alpha-keto acids on pyruvate metabolism by homogenates of human brain. J Neurochem 20:1793–1796

Sakai M, Inoue Y, Oba H et al (2005) Age dependence of diffusion-weighted magnetic resonance imaging findings in maple syrup urine disease encephalopathy. J Comput Assist Tomogr 29:524–527

Sener RN (2007) Maple syrup urine disease: diffusion MRI, and proton MR spectroscopy findings. Comput Med Imaging Graph 31:106–110

Sgaravatti AM, Rosa RB, Schuck PF et al (2003) Inhibition of brain energy metabolism by the alpha-keto acids accumulating in maple syrup urine disease. Biochim Biophys Acta 1639: 232–238

Strauss KA, Wardley B, Robinson D et al (2010) Classical maple syrup urine disease and brain development: principles of management and formula design. Mol Genet Metab 99: 333–345

Zinnanti WJ, Lazovic J, Griffin K et al (2009) Dual mechanism of brain injury and novel treatment strategy in maple syrup urine disease. Brain 132:903–918

JIMD Reports
DOI 10.1007/8904_2011_72

CASE REPORT

Three Japanese Patients with Beta-Ketothiolase Deficiency Who Share a Mutation, c.431A>C (H144P) in *ACAT1*: Subtle Abnormality in Urinary Organic Acid Analysis and Blood Acylcarnitine Analysis Using Tandem Mass Spectrometry

Toshiyuki Fukao • Shinsuke Maruyama • Toshihiro Ohura • Yuki Hasegawa • Mitsuo Toyoshima • Antti M. Haapalainen • Naomi Kuwada • Mari Imamura • Isao Yuasa • Rik K. Wierenga • Seiji Yamaguchi • Naomi Kondo

Received: 25 November 2010 / Revised: 10 June 2011 / Accepted: 20 June 2011 / Published online: 6 September 2011

Communicated by: K. Michael Gibson.

Competing interests: None declared.

T. Fukao (✉) · N. Kondo
Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan
e-mail: toshi-gif@umin.net

T. Fukao
Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1194, Japan

S. Maruyama · M. Toyoshima · N. Kuwada · M. Imamura
Department of Pediatrics, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8520, Japan

T. Ohura
Department of Pediatrics, Sendai City Hospital, Sendai, Miyagi 984-8501, Japan

Y. Hasegawa · S. Yamaguchi
Department of Pediatrics, Shimane University Faculty of Medicine, Izumo, Shimane 693-8501, Japan

A.M. Haapalainen · R.K. Wierenga
Department of Biochemistry and Biocenter Oulu, University of Oulu, Oulu 90014, Finland

M. Imamura
Kagoshima Prefectural Oshima Hospital, Naze, Kagoshima 894-0015, Japan

I. Yuasa
Division of Legal Medicine, Tottori University Faculty of Medicine, Yonago, Tottori 683-8503, Japan

Abstract Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency affects both isoleucine catabolism and ketone body metabolism. The disorder is characterized by intermittent ketoacidotic episodes. We report three Japanese patients. One patient (GK69) experienced two ketoacidotic episodes at the age of 9 months and 3 years, and no further episodes until the age of 25 years. She had two uncomplicated pregnancies. GK69 was a compound heterozygote of the c.431A>C (H144P) and c.1168T>C (S390P) mutations in T2 (*ACAT1*) gene. She was not suspected of having T2 deficiency during her childhood, but she was diagnosed as T2 deficient at the age of 25 years by enzyme assay using fibroblasts. The other two patients were identical twin siblings who presented their first ketoacidotic crisis simultaneously at the age of 3 years 4 months. One of them (GK77b) died during the first crisis and the other (GK77) survived. Even during severe crises, C5-OH and C5:1 were within normal ranges in their blood acylcarnitine profiles and trace amounts of tiglylglycine and small amounts of 2-methyl-3-hydroxybutyrate were detected in their urinary organic acid profiles. They were H144P homozygotes. This H144P mutation has retained the highest residual T2 activity in the transient expression analysis of mutant cDNA thus far, while the S390P mutation did not retain any residual T2 activity. The "mild" H144P mutation may result in subtle profiles in blood acylcarnitine and urinary organic acid analyses. T2-deficient patients with "mild" mutations have severe ketoacidotic crises but their chemical phenotypes may be subtle even during acute crises.

Abbreviations

SCOT Succinyl-CoA:3-ketoacid CoA transferase
T2 Mitochondrial acetoacetyl-CoA thiolase

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2, gene symbol ACAT1) deficiency (OMIM 203750) is an autosomal recessive inborn error of metabolism that affects the catabolism of isoleucine and ketone bodies. This disorder, first described by Daum et al. (1971), is characterized by intermittent episodes of metabolic ketoacidosis associated with vomiting and unconsciousness often triggered by infections (Fukao et al. 2001). There are no clinical symptoms between episodes. Typical T2 deficiency is easily diagnosed by urinary organic acid analysis, characterized by massive excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate both during ketoacidotic episodes and between episodes (Fukao et al. 2001, 2003). Diagnosis is confirmed by measurement of T2 activity on cultured skin fibroblasts (Robinson et al. 1979; Zhang et al. 2004). T2 deficiency is caused by mutations in the *ACAT1 (T2)* gene located on chromosome 11q22.3-q23.1 (Fukao et al. 1990; Kano et al. 1991). T2 deficiency is very heterogeneous at the genotype level, with at least 50 different mutations described (Fukao et al. 1995, 1997, 1998, 2001, 2002, 2003, 2007, 2008, 2010a, b; Wakazono et al. 1995; Nakamura et al. 2001; Zhang et al. 2004, 2006; Sakurai et al. 2007).

Some T2-deficient patients with mutations which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003). We herein describe three Japanese patients with T2 deficiency whose H144P mutation retains significant residual activity. Their urinary organic acid and blood acylcarnitine profiles were atypical and subtle even during severe ketoacidotic crises.

Materials and Methods

Case Reports

GK69

This Japanese woman (GK69), born in 1984, developed severe metabolic acidosis at the age of 9 months. On admission to a third-level hospital, she was semicomatose, polypneic (48/min), and hypotonic. Laboratory values were: blood glucose 6.8 mmol/L, NH_3 92 μmol/L, blood pH 7.225, pCO_2 7.2 mmHg, bicarbonate 3 mmol/L, base excess −21.3, Na 153 mEq/L (normal range: 139–146), BUN 28.5 mg/dL (normal range: 10–18), and creatinine 1.1 mg/dL (normal range: 0.18–0.46). Metabolic acidosis was refractory to sodium bicarbonate therapy. Peritoneal dialysis was performed for 2 days. On the second hospital day, polypnea and unconsciousness disappeared and the blood gas data improved. Urinary organic acid analysis showed massive amounts of acetoacetate and 3-hydroxybutyrate with dicarboxylic aciduria. No increases in 2-methyl-3-hydroxybutyrate or tiglylglycine were noted, although this analysis was performed in an outside laboratory and no urine samples were available for reanalysis. At that time, T2 deficiency was excluded from differential diagnosis based on this organic acid data and the tentative diagnosis was succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. However, an enzyme assay for SCOT was not performed. At the age of 3 years, the patient had a similar but milder episode. Subsequently, she had no further ketoacidotic episodes. Growth and development were normal. She had two uncomplicated pregnancies.

Twin Siblings (GK77b and GK77)

GK77b is a twin Japanese boy. He was born at 36 weeks gestation weighing 2,400 g. His parents had no known consanguinity but both were from a small island in Amami islands in Japan. He experienced several febrile illnesses without ketoacidosis. However, at 3 years 4 months of age, after a 3-day history of fever, cough, and vomiting, he developed anorexia, lethargy, and polypnea. He was admitted to a local hospital. His blood glucose level was 2.3 mmol/L. Blood gas analysis was not performed. Hypoglycemia was corrected with intravenous glucose injection of 20 ml of 20% glucose solution followed by continuous infusion of a 2.6% glucose solution. About 30 h after admission, his condition worsened. Blood gas analysis revealed severe metabolic acidosis showed pH 6.88, pCO_2 6.1 mmHg, and bicarbonate 1.1 mmol/L. He was transferred to a regional hospital. On arrival at the hospital, he was unconscious with a heart rate of 168/min and respiratory rate of 39/min. Blood laboratory data were: WBC 19,050/μL, CRP 0.2 mg/dL (normal values: <0.15), BUN 36.2 mg/dL (normal range: 10–18) creatinine 0.5 mg/dL (normal range: 0.25–0.49), NH3 33.5 μmol/L, glucose 3.8 mmol/L, pH 7.17, pCO_2 20 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 16.3 mmol/L. He received continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.4–0.47 mEq/kg/h. However, unconsciousness and metabolic acidosis did not improve. On the fifth hospital day, he died before being transferred to a third-level hospital.

GK77 is the twin brother of GK77b. Pyloric stenosis was diagnosed at the age of 1 month and corrected surgically;

thereafter, he was well until 3 years 4 months of age. Two days after the onset of his twin brother, he developed frequent repeated vomiting after cough and nasal discharge. Therefore, he was admitted to the regional hospital at the same time as his twin. On admission, he was lethargic. Laboratory findings were: WBC 7,760/μL, CRP 0.5 mg/dL (normal values: <0.15), BUN 20.2 mg/dL (normal range: 10–18), creatinine 0.4 mg/dL (normal range: 0.25–0.49), glucose 3.7 mmol/L, NH3 25 μmol/L, blood pH 7.135, pCO_2 19.5 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 10.1 mmol/L. He received a continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.3 mEq/kg/h. On the third hospital day, his condition worsened and he was transferred to a third-level hospital. On admission, the blood gasses were pH 7.372, pCO_2 21.6 mmHg, bicarbonate 12.2 mmol/L, and base excess −11.2 mmol/L. A glucose infusion rate was further increased to 6.5 mg/kg/min with 10% glucose solution. Acidosis normalized with 9 h (pH 7.399, bicarbonate 21.7 mmol/L, base excess −2.6 mmol/L). Two days later, the urinary ketones became negative and he started eating.

GK77 is now 4 years 8 months and has experienced no further ketoacidotic episodes. The family has been advised to avoid fasting and to come to the local hospital if he has a high fever or appetite loss. His growth and development are within normal ranges.

Urinary Organic Acid Analysis and Acylcarnitine Analysis

Urine samples containing 0.2 mg of creatinine were used for our high risk screening of organic acids. As internal standards, 20 mg each of tropate (TA, C9), margarate (MGA, C17), and tetracosane (C24) were added to these samples. Trimethylsilylated samples were analyzed using capillary gas chromatography-mass spectrometry (QP 5050A, Shimadzu Co. Ltd., Kyoto, Japan), as described earlier (Kimura et al. 1999). The values of organic acids were expressed as the peak area (%) relative to IS-1 (margarate) on the mass chromatogram. Quantification of 2-methyl-3-hydroxybutyrate and tiglylglycine in urine samples from GK77b and GK77 was kindly done by Dr. Sass (Freiburg University) (Lehnert 1994). For comparison, quantification was also done in urine samples from T2-deficient patients whose urinary screening profiles had typical T2 deficient ones. We used urine sample in stable condition from GK01 who is a compound heterozygote of A333P and c.149delC (Fukao et al. 1998) and samples in acute and stable conditions from T2-deficient patients from India (GK(Ind)) in our high-risk screening. Blood spot and serum acylcarnitine analysis using tandem mass spectrometry was also done, as described (Kobayashi et al. 2007), and blood spot samples from GK75 and GK79, who are R208X homozygotes (Fukao et al. 2010b) were used as positive controls.

Enzyme Assay and Immunoblot Analysis Using Fibroblasts

Control and patients' fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase activity was assayed, as described (Robinson et al. 1979; Zhang et al. 2004). We assayed acetoacetyl-CoA thiolase activity in the presence and absence of potassium-ion, since T2 is the only thiolase which is activated by the ion. Immunoblot analysis was done, as described (Fukao et al. 1997). In the cases of the controls, twofold serial dilution samples from 30 to 3.75 μg were electrophoresed together with samples (30 μg) of GK68 and GK77 to determine the amount of T2 protein in the patients' fibroblasts relative to that in the control fibroblasts.

Mutation Analysis

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University. Genomic DNA was extracted from fibroblasts using a SepaGene kit (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed by PCR and direct sequencing of genomic fragments that included each exon and its surrounding intron sequences (Fukao et al. 1998). For GK77b and the parents, exon 5 was amplified from a dried blood spot 1.25 mm in diameter, which was used for tandem mass spectrometry, using Amplidirect Plus (Shimadzu Biotech, Tsukuba, Japan).

Restriction Enzyme Assay to Detect c.431A>C (H144P)

The c.431A>C (H144P) mutation creates a new BmgT120 I site (GGACC). DNAs from 110 Japanese controls were examined using a restriction enzyme assay, as follows.

A fragment (314 bp), including exon 5 and its surrounding introns, was amplified using the following primers:

In4 as (in intron, −69 to −48)5′-CATGCTCTATTAAG-TTCTGCAG-3′

In5 as (in intron, +137 to +119) 5′-ATCCAGACACTCT-TGAGCA-3′

An aliquot of the resulting amplicon was digested with BmgT120 I, then resolved on a 5% polyacrylamide gel. The c.431A fragment (wild-type) is 314-bp long and the c.421C fragment is cut into 162-bp and 152-bp fragments.

Transient Expression Analysis of Mutant cDNAs

Transient expression of T2 cDNAs was performed using a pCAGGS eukaryote expression vector (Niwa et al. 1991), as described (Sakurai et al. 2007). After transfection, cells were cultured at 37°C or 40°C for 48 h, then harvested and kept at −80°C until use. Cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 0.1% Triton X-100. After centrifugation at 10,000 × g for 10 min, the supernatant was used in an enzyme assay for acetoacetyl-CoA thiolase activity and for immunoblot analysis.

Results and Discussion

Confirmation of the Diagnosis

GK69's fibroblasts were assayed for SCOT activity to confirm the diagnosis in 2008, when GK69 was 24 years old. As shown in Table 1, she was diagnosed as having T2 deficiency but not as having SCOT deficiency.

SCOT deficiency was first suspected in GK77 and GK77b, based on the following facts (1) Two of the four SCOT deficient Japanese families were from the Amami islands, the population of which is about 120,000. They were T435N homozygotes (Fukao et al. 2004). (2) The acylcarnitine profiles and urinary organic acid analysis during acute ketoacidotic crisis in both patients had no typical profile for T2 deficiency, as discussed below. As shown in Table 1, GK69's and GK77's fibroblasts had normal SCOT activity and a higher ratio (1.3) of acetoacetyl-CoA thiolase activity in the presence to the absence of potassium ions than typical T2-deficient fibroblasts (the ratio was around 1.0). Immunoblot analysis also showed a clearly detectable amount of T2 protein in GK77's fibroblasts, and a lower amount in GK69's fibroblasts. Densitometric analysis showed that the amounts of T2 protein in GK77 and GK69 were estimated to be 50% and 25% of control, respectively (Fig. 1).

Table 1 Acetoacetyl-CoA thiolase activities in the absence and presence of potassium ions

Fibroblasts	Acetoacetyl-CoA thiolase activity			SCOT activity
	$-K^+$	$+K^+$	$+K^+/-K^+$	
Controls ($n = 5$)	5.0 ± 0.7	10.8 ± 0.9	2.2 ± 0.3	6.7 ± 2.1
GK69	3.6 ± 0.5	4.1 ± 0.9	1.2 ± 0.1	4.7 ± 1.4
GK77	4.2 ± 0.3	5.8 ± 1.5	1.4 ± 0.3	3.9 ± 0.5
T2D	4.5 ± 1.4	4.7 ± 1.6	1.0 ± 0.1	5.6 ± 0.5

Enzyme activity is expressed as nmol/min/mg of protein. In cases of patients, enzyme assay was done three times and shows average ± SD. T2D, A disease control

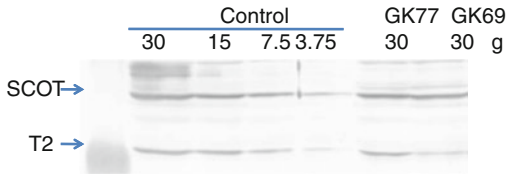


Fig. 1 Immunoblot analysis. In the cases of the controls, serial twofold dilutions from 30 to 3.75 μg were studied together with samples (30 μg) from GK68 and GK77. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

Mutations and Their Effects on T2 Protein

Mutation screening revealed that GK69 was a compound heterozygote of c.431A>C (H144P) and c.1168T>C (S390P). Her mother had S390P heterozygously but did not have H144P. The father's DNA was not available for analysis. GK77 had an H144P mutation homozygously, shown by mutation screening at the genomic level. Their parents and GK77b were heterozygous carriers and a homozygote of H144P, respectively. The c.431A>C (H144P) mutation creates a BmgT120I site (GGACA to GGACC). We could not find c.431A>C (H144P) in the 110 Japanese controls using the restriction enzyme assay with BmgT120I.

We performed transient expression analysis of wild-type and mutant cDNAs in T2-deficient SV40-transformed fibroblasts. Following expression of T2 cDNAs for 48 h at 37°C, an enzyme assay and immunoblots were performed (Fig. 2a,b). The transfection of wild-type T2 cDNA produced high potassium ion-activated acetoacetyl-CoA thiolase activity (T2 activity), whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. The H144P mutant retained a residual T2 activity of ~25% of the wild-type value (Fig. 2a). The S390P mutant did not retain any residual T2 activity. In immunoblot analysis (Fig. 2b), the H144P mutant protein was detected, whereas no S390P protein was detected. The relative amount of the H144P mutant protein, as compared to the wild-type, was estimated to be 50%. Hence, the specific activity (unit/mg of T2 protein) of the H144P mutant protein was estimated to be about 50% of the wild type. Protein-folding and post-folding stability is predicted to vary with the incubation temperature. Hence, we also performed transient expression at 40°C for 48 h. The H144P mutant in expression at 40°C had a similar level of residual activity to that at 37°C.

We reported the tertiary structure of the human T2 tetramer (Haapalainen et al. 2007). Figure 3a shows the positions of the H144P and S390P mutations on the dimer.

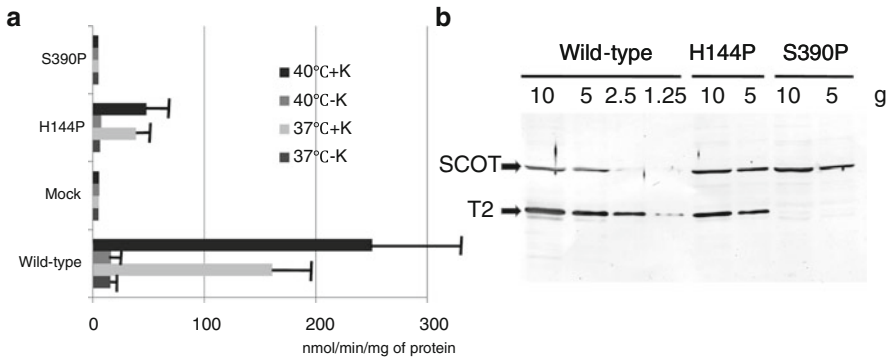


Fig. 2 Transient expression analysis of H144P and S390P mutant cDNAs. Transient expression analysis was performed at 40°C and 37°C. (**a**) Potassium ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence (−K) and presence (+K) of potassium ions are shown together with the SD of three independent experiments. (**b**) Immunoblot analysis. The protein amounts applied are indicated above the lanes. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

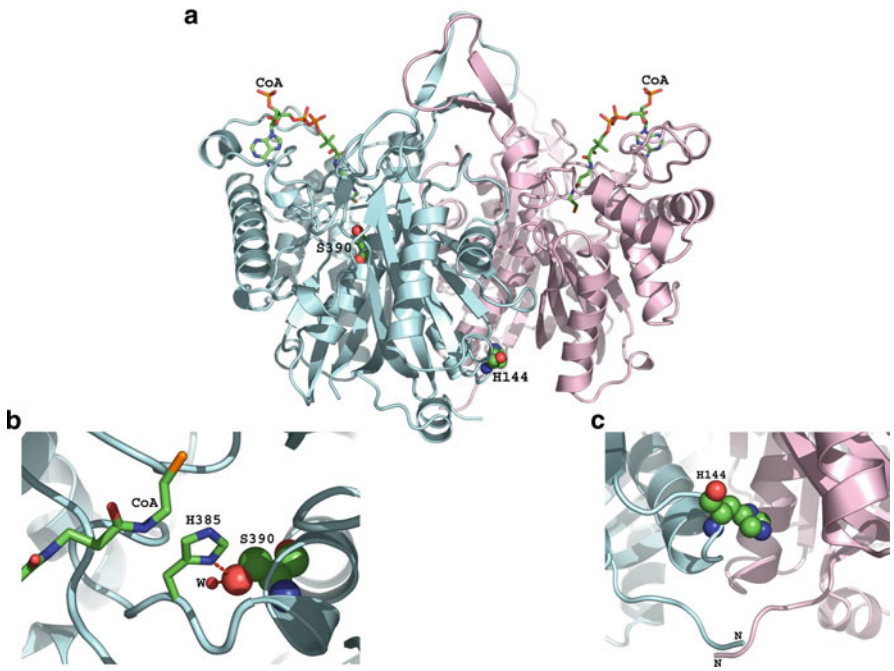


Fig. 3 The positions of H144P and S390P on the tertiary structure of human T2 dimers with substrates of coenzyme A

As seen in the figure, S390 is close to the active site and H144 is at the dimer interface close to the surface of the protein. Figure 3b shows a zoomed-in view around S390. This mutant is located at the active site. S390 is hydrogen-bonded to catalytic histidine, H385; it could be that this serine is needed to orient histidine in a way that the histidine can stabilize the transient negative charge of the substrate optimally. S390 is also hydrogen-bonded to a water molecule that is needed in stabilizing parts of the enzyme. So, if S390 is mutated into proline, these two hydrogen bonds do not exist. Hence, this S390P is expected to bring about a serious change in T2 catalytic cavity. In our expression analysis, this S390P was also too unstable to detect mutant protein. Figure 3c shows a zoomed-in view at the dimer interface. H144 is interacting with the residues of the neighboring subunit. If this residue is mutated into Pro, there is less dimeric interaction, which in turn might destabilize the overall structure. Since this residue is far from the active site and substrate binding site, it is difficult to explain why this H144P mutant had reduced specific activity in transient expression analysis from the viewpoint of structural analysis.

Urinary Organic Acid Analysis

GK69 was first suspected to having T2 deficiency as a probable diagnosis; however, urinary organic acid analysis at the first ketoacidotic crisis indicated no characteristic profile for T2 deficiency such as elevated 2-methyl-3-hydroxybutyrate and tiglylglycine in 1985 (no data was available). The results of the urinary organic acid analysis of our patients are shown in comparison with those of typical T2-deficient patients, GK01 and GK(Ind) (Table 2, Fig. 4). At the age of 24 years when her condition was stable, GK69's urinary organic acid analysis showed that there were only trace amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine (Table 2). In our screening, this low level of tiglylglycine was difficult to detect. Urinary organic acid analysis during the acute crises of GK77 and GK77b showed huge amounts of 3-hydroxybutyrate and acetoacetate with elevated 2-methyl-3-hydroxybutyrate but only trace amounts of tiglylglycine. The levels of 2-methyl-3-hydroxybutyrate and tiglylglycine during a stable condition in GK77 are similar with those in GK69.

In cases of typical T2-deficient patients, it is easy to suspect T2 deficiency based on large amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine as shown in Fig. 4. However, even in cases of trace amounts of tiglylglycine (possibly under the detection limit), T2 deficiency cannot be excluded. An H144P mutation, which retained high residual activity, may contribute to atypical profiles in the presented cases. These findings strengthen our previous observations that some T2-deficient patients with mutations, which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003).

Table 2 Quantitative analysis of urinary organic acid analysis during acute crises and stable conditions

Patients	Acute crises		Stable conditions	
	2M3HB	Tiglylglycine	2M3HB	Tiglylglycine
GK69	NA	NA	14.0	13.3
GK77b	405.7	45.8	NA	NA
GK77	160.2	6.7	27.3	14.8
GK01	NA	NA	399.1	732.1
GK(Ind)	484.6	503.9	195.1	797.6
Controls ($n = 42$)			10.7 ± 7.6	24.6 ± 14.6

Values are expressed as mmol/mol creatinine

NA means that samples were not available for the analysis. GK01 is a compound heterozygote of c.149delC and A333P, which retained no residual activity (Fukao et al. 1998). GK(Ind) indicates a patient with typical T2-deficient profiles of urinary organic acids in our screening

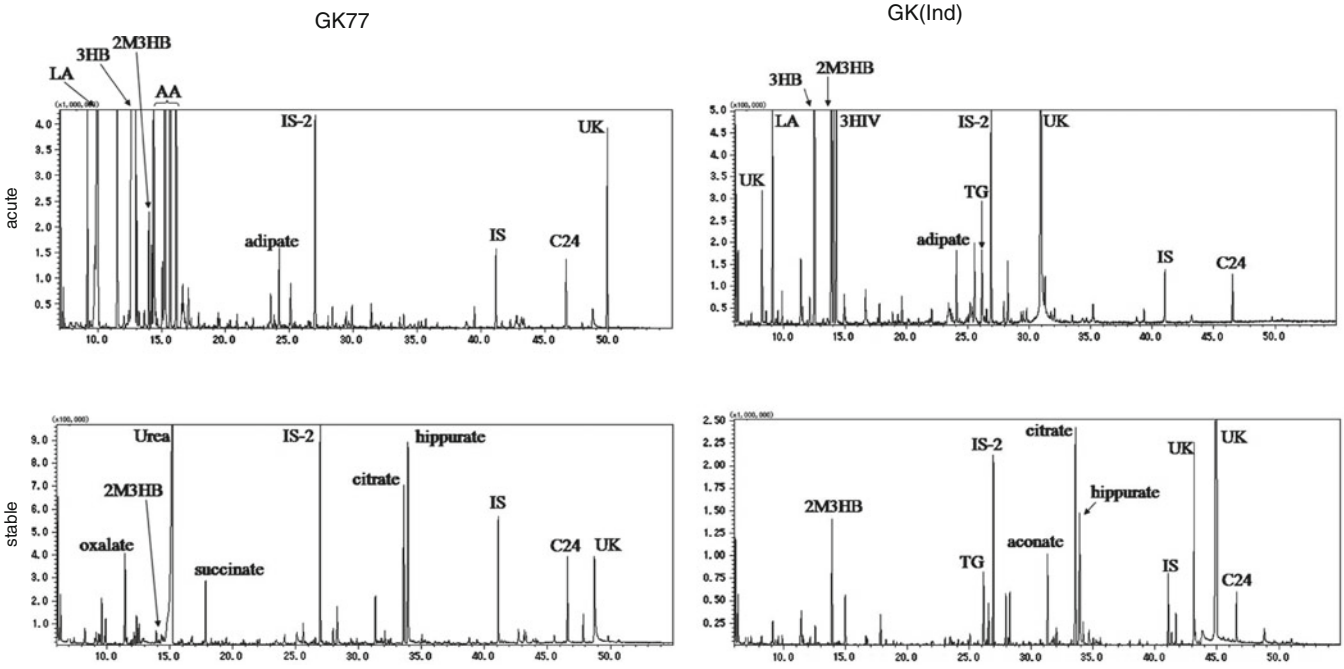


Fig. 4 Urinary organic acid profiles of GK77 during the acute episode and an asymptomatic period in comparison with those of a typical T2-deficient patient (GK(Ind)). *LA* Lactate, *3HB* 3-OH-butyrate, *3HIV* 3-OH-isovalerate, *AA* Acetoacetate, *2M3HB* 2-Methyl-3-OH-butyrate, *TG* Tiglylglycine, *IS-2* and *IS* Internal standards, *UK* Unknown. Since acetoacetate is unstable and samples from GK(Ind) were shipped on filter papers after thoroughly drying, the levels of acetoacetate are likely underestimated

Table 3 C5-OH and C5:1 carnitines in blood filters and serum samples from GK77 and GK77b during acute crises

Patients	Dried blood spots		Serum	
	C5:1	C5-OH	C5:1	C5-OH
GK77b	0.027	0.11	ND	0.12
GK77	0.012	0.11	0.044	0.10
R208X homozygotes				
GK75 (acute)	0.89	2.89	NA	NA
GK79 (stable)	1.20	2.35	NA	NA
Controls ($n = 30$)				
Average ± SD	0.015 ± 0.016	0.26 ± 0.15	0.015 ± 0.013	0.059 ± 0.024

ND not detected, *NA* not applicable

The values are expressed as μmol/L

GK75 and GK79 are positive controls for T2 deficient patients who are R208X homozygotes (Fukao et al. 2010b)

Blood and Serum Acylcarnitine Analyses

Acylcarnitine analysis was done using samples during the acute crises of GK77 and GK77b. Table 3 shows the results in comparison with those of typical T2-deficient patients (R208X homozygotes) (Fukao et al. 2010b). C5:1 and C5OH elevation in blood spots, characteristic for T2 deficiency, was clearly detected in the samples from the typical T2-deficient patients but was absent in samples from GK77 and GK77b. We previously reported that the abnormality of the acylcarnitine profiles in T2-deficient patients with mutations which retain some residual activity is subtle during nonepisodic conditions (Fukao et al. 2003), but the present study clearly showed that it could be also subtle even during severe ketoacidotic episodes. This means that acylcarnitine analysis using blood spots cannot detect some T2-deficient patients like GK77 and GK77b. Serum acylcarnitine analysis might detect elevation of these compounds to some extent, but we need to analyze more cases to clarify the usefulness of serum acylcarnitine analysis in such T2-deficient patients with mutations which retain some residual activity.

T2 deficiency cannot be excluded even if acylcarnitine profiles during acute episodes are within normal ranges. Careful evaluation of urinary organic acids, especially for the presence of 2-methyl-3-hydroxybutyrate, is necessary not to overlook T2 deficiency.

Clinical Issues

Since they were confirmed as identical twins by DNA analysis (data not shown), their genetic backgrounds were identical and most environmental factors were also very similar between them. One died during the ketoacidotic crisis and the other survived.

In Japan, intravenous infusion therapy for vomiting, appetite loss, and dehydration is commonly performed with commercially available initial infusion solution, such as Solita T1 (2.6% glucose) followed by maintenance solution, such as Solita T2 and T3 (4.3% glucose). These solutions are effective for physiological ketosis. However, in the case of T2 deficiency, a higher concentration of glucose may be necessary. Accordingly, we had the impression that GK77 became much better after the glucose concentration was changed from 5% to 10%. In the case of prolonged ketoacidosis, consideration should be given to increasing the infusion rate of glucose to ensure high normal blood glucose level to suppress ketone body synthesis and isoleucine catabolism via insulin secretion.

Acknowledgments We thank professor Jörn Oliver Sass (Freiburg Univ) for quantification of urinary 2-methyl-3-hydroxybutyrate and tiglylglycine, Drs Hironori Kobayashi and Yuichi Mushimoto (Shimane University) for urinary organic acid analysis and tandem mass analysis, Dr Tamayo Ishikawa (Kagoshima University) for patients' care, and Ms Keiko Murase and Ms Naomi Sakaguchi (Gifu University) for technical assistance. We also thank Paul Langman, PhD for his assistance with scientific English usage.

This study was in part supported by Health and Labor Science Research Grants for Research on Intractable Diseases and for Research on Children and Families from The Ministry of Health, Labor and Welfare of Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

Concise One-Sentence Take-Home Message

Patients with beta-ketothiolase deficiency having a mutation which retains some residual activity showed subtle abnormality in urinary organic acid analysis and blood acylcarnitine analysis even during acute ketoacidotic episodes.

Details of the Contributions of Individual Authors

Toshiyuki Fukao and Naomi Kondo performed the enzyme assays, immunoblot/mutation analysis, and expression analysis of cDNAs. Toshiyuki Fukao mainly wrote this manuscript. Shinsuke Maruyama, Toshihiro Ohura, Mitsuo Toyoshima, Naomi Kuwada, and Mari Imamura are the physicians responsible for the patients. Yuki Hasegawa and Seiji Yamaguchi performed gas chromatography-mass spectrometry and tandem mass spectrometry analyses and first suspected the disorder. Isao Yuasa confirmed GK77 and 77b as identical twins by DNA analyses. Antti M Haapalainen and Rik K Wierenga analyzed the tertiary structural effects of the mutations.

References to Electronic Databases

Alpha-methylacetoacetic acidura, mitochondrial acetoacetyl-CoA thiolase deficiency (OMIM 203750, 607809)

Mitochondrial acetoacetyl-CoA thiolase, acetyl-CoA acetyltransferase 1 (EC 2.3.1.9)

ACAT1 gene (gene ID 38, NM_000019.3)

Details of Funding

This study was in part supported by Health and Labor Science Research Grants for Research on Intractable Diseases and Research on Children and Families from the Ministry of Health, Labor and Welfare of Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan

Details of Ethics Approval

This study has been approved by the Ethical Committee of the Graduate School of Medicine, Gifu University.

References

Daum RS, Lamm PH, Mamer OA, Scriver CR (1971) A "new" disorder of isoleucine catabolism. Lancet 2:1289–1290

Fukao T, Yamaguchi S, Kano M et al (1990) Molecular cloning and sequence of the complementary DNA encoding human mitochondrial acetoacetyl-coenzyme A thiolase and study of the variant enzymes in cultured fibroblasts from patients with 3-ketothiolase deficiency. J Clin Invest 86:2086–2092

Fukao T, Yamaguchi S, Orii T, Hashimoto T (1995) Molecular basis of beta-ketothiolase deficiency: mutations and polymorphisms in the human mitochondrial acetoacetyl-coenzyme A thiolase gene. Hum Mutat 5:113–120

Fukao T, Song XQ, Mitchell GA et al (1997) Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases. Pediatr Res 42:498–502

Fukao T, Nakamura H, Song XQ et al (1998) Characterization of N93S, I312T, and A333P missense mutations in two Japanese families with mitochondrial acetoacetyl-CoA thiolase deficiency. Hum Mutat 12:245–254

Fukao T, Scriver CR, Kondo N (2001) The clinical phenotype and outcome of mitochondrial acetoacetyl-CoA thiolase deficiency (beta-ketothiolase or T2 deficiency) in 26 enzymatically proved and mutation-defined patients. Mol Genet Metab 72:109–114

Fukao T, Nakamura H, Nakamura K et al (2002) Characterization of six mutations in five Spanish patients with mitochondrial acetoacetyl-CoA thiolase deficiency: effects of amino acid substitutions on tertiary structure. Mol Genet Metab 75: 235–243

Fukao T, Zhang GX, Sakura N et al (2003) The mitochondrial acetoacetyl-CoA thiolase (T2) deficiency in Japanese patients: urinary organic acid and blood acylcarnitine profiles under stable conditions have subtle abnormalities in T2-deficient patients with some residual T2 activity. J Inherit Metab Dis 26:423–431

Fukao T, Shintaku H, Kusubae R et al (2004) Patients homozygous for the T435N mutation of succinyl-CoA:3-ketoacid CoA Transferase (SCOT) do not show permanent ketosis. Pediatr Res 56:858–863

Fukao T, Zhang G, Rolland MO et al (2007) Identification of an Alu-mediated tandem duplication of exons 8 and 9 in a patient with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency. Mol Genet Metab 92:375–378

Fukao T, Boneh A, Aoki Y, Kondo N (2008) A novel single-base substitution (c.1124A > G) that activates a 5-base upstream cryptic splice donor site within exon 11 in the human mitochondrial acetoacetyl-CoA thiolase gene. Mol Genet Metab 94:417–421

Fukao T, Horikawa R, Naiki Y et al (2010a) A novel mutation (c.951 C > T) in an exonic splicing enhancer results in exon 10 skipping in the human mitochondrial acetoacetyl-CoA thiolase gene. Mol Genet Metab 100:339–344

Fukao T, Nguyen HT, Nguyen NT et al (2010b) A common mutation, R208X, identified in Vietnamese patients with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency. Mol Genet Metab 100:37–41

Haapalainen AM, Merilainen G, Pirila PL, Kondo N, Fukao T, Wierenga RK (2007) Crystallographic and kinetic studies of human mitochondrial acetoacetyl-CoA thiolase: the importance of potassium and chloride ions for its structure and function. Biochemistry 46:4305–4321

Kano M, Fukao T, Yamaguchi S, Orii T, Osumi T, Hashimoto T (1991) Structure and expression of the human mitochondrial acetoacetyl-CoA thiolase-encoding gene. Gene 109:285–290

Kimura M, Yamamoto T, Yamaguchi S (1999) Automated metabolic profiling and interpretation of GC/MS data for organic acidemia screening: a personal computer-based system. Tohoku J Exp Med 188:317–334

Kobayashi H, Hasegawa Y, Endo M, Purevsuren J, Yamaguchi S (2007) A retrospective ESI-MS/MS analysis of newborn blood spots from 18 symptomatic patients with organic acid and fatty acid oxidation disorders diagnosed either in infancy or in childhood. J Inherit Metab Dis 30:606

Lehnert W (1994) Long-term results of selective screening for inborn errors of metabolism. Eur J Pediatr 153:S9–S13

Nakamura K, Fukao T, Perez-Cerda C et al (2001) A novel single-base substitution (380 C > T) that activates a 5-base downstream cryptic splice-acceptor site within exon 5 in almost all transcripts in the human mitochondrial acetoacetyl-CoA thiolase gene. Mol Genet Metab 72:115–121

Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199

Robinson BH, Sherwood WG, Taylor J, Balfe JW, Mamer OA (1979) Acetoacetyl CoA thiolase deficiency: a cause of severe ketoacidosis in infancy simulating salicylism. J Pediatr 95:228–233

Sakurai S, Fukao T, Haapalainen AM et al (2007) Kinetic and expression analyses of seven novel mutations in mitochondrial acetoacetyl-CoA thiolase (T2): identification of a Km mutant and an analysis of the mutational sites in the structure. Mol Genet Metab 90:370–378

Wakazono A, Fukao T, Yamaguchi S et al (1995) Molecular, biochemical, and clinical characterization of mitochondrial acetoacetyl-coenzyme A thiolase deficiency in two further patients. Hum Mutat 5:34–42

Zhang GX, Fukao T, Rolland MO et al (2004) Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency: T2-deficient patients with "mild" mutation(s) were previously misinterpreted as normal by the coupled assay with tiglyl-CoA. Pediatr Res 56:60–64

Zhang G, Fukao T, Sakurai S, Yamada K, Michael Gibson K, Kondo N (2006) Identification of Alu-mediated, large deletion-spanning exons 2–4 in a patient with mitochondrial acetoacetyl-CoA thiolase deficiency. Mol Genet Metab 89:222–226

JIMD Reports
DOI 10.1007/8904_2011_76

RESEARCH REPORT

Altered Carbon Dioxide Metabolism and Creatine Abnormalities in Rett Syndrome

Nicky S.J. Halbach • Eric E.J. Smeets • Jörgen Bierau • Irene M.L.W. Keularts • Guy Plasqui • Peter O.O. Julu • Ingegerd Witt Engerström • Jaap A. Bakker • Leopold M.G. Curfs

Received: 08 November 2010 / Revised: 24 June 2011 / Accepted: 04 July 2011 / Published online: 28 September 2011

Abstract Despite their good appetite, many females with Rett syndrome (RTT) meet the criteria for moderate to severe malnutrition. Although feeding difficulties may play a part in this, other constitutional factors such as altered metabolic processes are suspected. Irregular breathing is a common clinical feature, leading to chronic respiratory alkalosis or acidosis. We assumed that these changes in intracellular pH cause disturbances in the metabolic equilibrium, with important nutritional consequences. The study population consisted of a group of thirteen well-defined RTT girls with extended clinical, molecular and neurophysiological assessments. Despite normal levels of total dietary energy and protein intakes, malnutrition was confirmed based on significantly low fat-free mass index (FFMI) values. Biochemical screening of multiple metabolic pathways showed significantly elevated plasma creatine concentrations and increased urinary creatine/creatinine ratio in five RTT girls. Four girls, 10 years and older, were forceful breathers, one 13-year-old girl had an undetermined cardiorespiratory phenotype. An isolated increase of the urinary creatine/creatinine ratio was seen in two girls, a 9-year old forceful and a 4-year old feeble breather. Given that the young girls are feeble breathers and the older girls are forceful breathers, it is impossible to determine whether the elevated creatine concentrations are due to increasing age or cardiorespiratory phenotype. Furthermore, MeCP2 deficiency may cause epigenetic aberrations affecting the expression of the creatine-transporter gene, which is located at Xq28. Further studies are required to confirm these findings and to provide greater insight into the pathogenesis of the abnormal creatine metabolism in RTT.

Trial registration number (www.clinicaltrials.gov): 00786071

Communicated by: Carlo Dionisi-Vici.

Competing interests: None declared.

N.S.J. Halbach (✉) · E.E.J. Smeets · J. Bierau · I.M.L.W. Keularts · J.A. Bakker · L.M.G. Curfs
Department of Clinical Genetics, Maastricht University Medical Centre, P. Debeyelaan 25, P.O. Box 5800, 6202, AZ Maastricht, The Netherlands
e-mail: nicky.halbach@mumc.nl

N.S.J. Halbach · E.E.J. Smeets · L.M.G. Curfs
Governor Kremers Centre, Maastricht University Medical Centre, Maastricht, The Netherlands

N.S.J. Halbach · L.M.G. Curfs
School for Public Health and Primary Care Caphri, Maastricht University, Maastricht, The Netherlands

G. Plasqui
Department of Human Biology, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands

P.O.O. Julu · I.W. Engerström
Swedish Rett Center, Östersund Hospital, Östersund, Sweden

P.O.O. Julu
The Wingate Institute of Neurogastroenterology, Queen Mary School of Medicine and Dentistry, London, UK

Abbreviations

BMI	Body mass index
FFM	Fat-free mass
FFMI	Fat-free mass index
ID	Intellectual disability
MECP2	Methyl CpG binding protein 2 gene
MeCP2	Methyl CpG binding protein 2
RTT	Rett syndrome
SD	Standard deviation
TBW	Total body water

Introduction

Rett syndrome (RTT; OMIM 312750) is a unique X-linked neurodevelopmental disorder. Affecting 1 in 10,000 females, RTT is a common genetic cause of severe intellectual disability in females (Smeets and Schrander-Stumpel 2005; Williamson and Christodoulou 2006). In up to 95% of all classical cases, RTT is caused by a mutation in the Methyl-CpG-binding protein 2 gene (*MECP2*) located on the long arm of the X chromosome (Xq28) (Amir et al. 1999; Williamson and Christodoulou 2006).

Despite their good appetite, the majority of the females with RTT meet the criteria for moderate to severe malnutrition. However, not all RTT females present in this manner (Rice and Haas 1988). The true prevalence of malnutrition in RTT is unknown, and the pathological mechanism underlying is barely understood. Although feeding difficulties may play a role, other constitutional factors such as altered metabolic processes are suspected (Motil et al. 1998; Oddy et al. 2007; Reilly and Cass 2001).

Irregular breathing is a common clinical feature of RTT, reflecting the immaturity of the brainstem in these females. The underlying pathophysiology involves a defective control mechanism of carbon dioxide exhalation, leading to chronic respiratory alkalosis or acidosis (Julu et al. 2008a; Julu et al. 2001; Smeets and Schrander-Stumpel 2005). This change in pH might cause disturbance of the metabolic equilibrium in RTT females, with potential consequences for their nutritional status.

Hitherto, no systematic studies on altered metabolic processes as a cause of impaired nutritional status in RTT have been carried out. The objective of this study was to examine whether chronic respiratory acidosis or alkalosis leads to disturbed metabolic profiles and enzyme activities in multiple metabolic pathways.

Patients and Methods

Ethical approval was obtained from the Medical Ethical Committee at the Maastricht University Medical Centre, and all parents provided informed written consent. The study was registered at *clinicaltrials.gov*.

Patient Population

The patient population consisted of a group of thirteen well-defined RTT girls with extended clinical, molecular and neurophysiological assessment. This assessment consisted of:

1. Clinical examination comprising a full physical examination including measurement of height, weight and Body Mass Index (BMI). In addition, clinical features were scored uniformly using the International Scoring System (ISS) (Kerr et al. 2001). The ISS is used to determine clinical severity concerning 20 items (maximum score being 40), grouped into five functional domains (growth and development, musculoskeletal, movement, cortical and autonomic features).
2. Mutation analysis of *MECP2* by PCR, sequencing the coding regions and multiplex ligation-dependent probe amplification analysis (nomenclature according to the MECP2A isoform reference sequence AF158180, numbering starting at the A of the ATG translation initiation codon).
3. Detailed neurophysiological assessment using the Neuroscope, a technique for continuous and real-time assessment of brainstem function to define the cardiorespiratory phenotype (Julu and Engerström 2005). The different cardiorespiratory phenotypes (Apneustic, Feeble, and Forceful breathers) in RTT have been described in detail elsewhere (Julu et al. 2008a, b).

Diagnosis of RTT was based on the consensus diagnostic criteria for RTT and confirmed by mutation finding in *MECP2* gene (Hagberg et al. 2002). All girls were diagnosed as classical RTT.

Study Design

The RTT girls were recruited at the Clinical Genetics outpatient clinic at the Maastricht University Medical Centre, the Netherlands. All participants were clinically assessed by the same experienced clinician (Dr. E. Smeets). The nutritional status was assessed by a complete nutrition assessment and measurement of body composition. Blood and urine samples were collected for biochemical analysis and stored at $-20^{\circ}C$ until analysis after the appropriate workup.

Nutritional Status

Dietary intake was assessed using a standardized format including two 3-day food records. Parents were instructed to record all foods and beverages consumed on three subsequent days, including one weekend day. The timing of the first 3-day record period was prior to the blood sampling, and the second period was recorded approximately 6 months later. The amount of liquid and the size of food portions offered and actually consumed at each meal were reported in household measures. Energy and nutrient intake was calculated using the Dutch food composition table (NeVo, version 06) and the software Komeet® (BaS Nutrition Software, Arnhem).

In addition to the food records, a semistructured interview was administered to the parents to specify usual dietary intakes, eating abilities, and food preferences.

Body composition was measured using the Deuterium dilution method according to the Maastricht protocol (Westerterp et al. 1995). A random urine sample was used to determine the background isotope level before the administration of a D_2O mixture. At least 6 h after administration of a D_2O dose (per oz or through a PEG catheter), a urine sample was taken from the second voiding. Fat-free mass (FFM) was then calculated from total body water (TBW) using the child-specific hydration factors published by Lohman (Lohman 1989). Subsequently, FFM was divided by squared height to calculate the fat-free mass index (FFMI; kg/height(m)^2). Malnutrition was defined as an FFMI below the fifth percentile (or −2 SD) for age and gender (VanItallie et al. 1990; Schutz et al. 2002).

Metabolic Measurements

Routine chemistry and hematology were carried out, as proposed by the Frösö Declaration and included full blood count, total protein, albumin, protein electrophoresis, urea, creatinine, electrolytes such as $Na^+/K^+/Ca^{2+}/Cl^-/PO_4$. Liver function tests were only done if specifically indicated (Julu et al. 2008b).

In addition, the metabolic screening program as shown in Table 1 was carried out to identify markers pointing toward possible consequences of an altered carbon dioxide metabolism. In view of the specific breathing irregularities in combination with feeding problems, one would expect changes in the "intermediate metabolism" including metabolism of glucose, glycogen, amino acids/proteins, and fatty acids. Short- and long-term changes due to, for example, a specific nutritional status are reflected in amino acid profiles in urine and plasma, acylcarnitine profiles in plasma (to also exclude a fatty acid oxidation defect or organic acidurias) and excretion of metabolites (organic acids) in urine (organic acid excretion profile).

Statistical Analysis

Using SPSS version 15.0, frequency tables including percentages were obtained for the variables "*MECP2*-mutation," "cardiorespiratory phenotype," and "anthropometry data." Concerning age, mean values and standard deviation were calculated. Regarding ISS scores, both frequency tables and mean scores were calculated.

Results

Patient Characteristics

Patient characteristics are presented in Table 2.

Table 1 General overview of selective metabolic screening including matrix and methodology

Analysis	Matrix	Methodology
Amino acid analysis	Urine, plasma	LC–MS/MS
Organic acid analysis	Urine, plasma	GC–MS, silylated
Purines and pyrimidines	Urine, plasma	LC–MS/MS
Guanidinoacetate and creatine	Urine, plasma	LC–MS/MS
Phenolic compounds	Urine	HPLC-fluorescence
Acylcarnitine profiling	Plasma	MS/MS
Very-long chain fatty acids	Plasma	LC–MS/MS
Sialotransferrines	Plasma	Isoelectric focusing
Methylmalonate	Plasma	LC–MS/MS
Homocysteine	Plasma	LC–MS/MS
Adenosine deaminase activity	Erythrocytes	HPLC–UV
Adenine phosphoribosyltransferase activity	Erythrocytes	HPLC–UV
Hypoxanthine phosphoribosyltransferase activity	Erythrocytes	HPLC–UV
Inosine triphosphatase activity	Erythrocytes	HPLC–UV
Nucleotide profile	Erythrocytes	HPLC–UV
α-Galactosidase A activity	Leukocytes	Fluorescence

LC–MS/MS liquid chromatography with tandem mass spectrometry, *GC–MS* gas chromatography-mass spectrometry, *HPLC* high-performance liquid chromatography, *MS/MS* tandem mass spectrometers, *HPLC–UV* high-pressure liquid chromatography with UV detector

Age

The ages of RTT girls ranged from 2 years and 4 months to 20 years and 3 months (mean age 9 years and 5 months, SD = 5 years and 11 months).

Anthropometry

Mean height, weight, BMI, and head circumference scores of RTT girls were below that of their age group. The height of 54% of the RTT girls was below the fifth percentile, of which 86% were 9 years or older. Regarding BMI, 31% of the girls were below the fifth percentile and two girls had a BMI over the 50th percentile. Regarding age, low BMI was present in 75% of those 9 years and older.

ISS Score

Mean ISS scores for the RTT girls was 18.8 (range: 8–27, SD = 6.78). Severe RTT (score 25–29) was present in two

Table 2 The clinical characteristics of the RTT girls

RTT girl	Age (yr. month)	Height (cm + p)	Weight (kg + p)	Head circumference (cm + p)	BMI (kg/m^2 + p)	ISS	MECP2 mutation[a]	CRPh	tcpCO_2 (mmHg)
1	2.4	85 (p2)	11.3 (p10)	45 (p2)	15.6 (p25)	14	p.R255X	FeB	43.3
2	3.11	98 (p50)	16 (p25)	48.5 (p25)	16.7 (p50)	8	p.T158M	FeB	48.2
3	4.0	107 (p70)	14.5 (p10)	50.5 (p50)	12.7 (p2)	8	p.R294X	FeB	52.3
4	4.3	103 (p50)	20.5 (p98)	47 (p2)	19.3 (p98)	21	fs710dupG	FeB	50.6
5	5.3	112 (p50)	19 (p50)	49 (p25)	15.2 (p50)	20	p.R270X	FeB	40.9
6	6.2	113 (p25)	18 (p25)	49 (p25)	14.3 (p25)	9	p.R306C	FoB	22.3
7	9.8	120 (<p2)	20.5 (p2)	51 (p25)	14.2 (p10)	20	fs705delG	FoB	15.5
8	9.8	132 (p2)	26.4 (p10)	49.5 (p2)	15.2 (p25)	20	p.P152R	FoB	25.8–42.0
9	10.11	127 (<p2)	24.5 (p2)	50.5 (p25)	15.2 (<p2)	26	p.R106W	FoB	15.5
10	13.3	148 (p2)	31.7 (p2)	50 (<p2)	14.5 (p2)	23	p.R294X	FoB	10.5–22.5
11	13.11	148 (<p2)	54 (p75)	54 (p50)	24.7 (p98)	27	c.1158del55	Und	35.2
12	19.3	171 (p50)	65 (p75)	52 (p3)	22.3 (p50)	24	p.R306C	FoB	20.8
13	20.3	150 (<p2)	43 (p2)	53 (p25)	19.1 (p2)	24	p.R168X	FoB	N.A.

p percentile, *ISS* total ISS score, *CRPh* cardiorespiratory phenotype, *FeB* feeble breather, *FoB* forceful breather, *Und* undetermined, *tcpCO_2* transcutaneous pCO_2 (reference values 38–44 mmHg), *N.A.* not acquired

[a] Nomenclature according to the MECP2A isoform reference sequence AF158180, numbering starting at the A of the ATG translation initiation codon

girls, mild to less severe RTT (score 10–24) in eight and very mild RTT (score below 10) in three girls. Severe RTT was only seen in girls 9 years and older and very mild scores only in those younger than 9 years. Mean score of the girls younger than 9 years was 13.3 (range: 8–21, SD = 5.99), compared to 23.4 (range: 20–27, SD = 2.70) in those 9 years and older.

Mutation Analysis

Both missense and nonsense mutations in *MECP2* were each observed in five girls. Of these mutations, four were located in the Methyl-CpG binding domain and six in the transcription repression domain. Two girls had a frameshift mutation and only one girl had a CTS deletion. In view of the variety in mutations, no differences concerning other patient characteristics could be observed.

Cardiorespiratory Phenotype

Of the 13 girls included in this study, five girls were feeble breathers, seven girls were forceful breathers, and one girl had an undetermined cardiorespiratory phenotype. Remarkably, all feeble breathers were below 5 years of age and all forceful breathers were 6 years and older. Furthermore, Table 2 shows the tcpCO_2 values measured during the Neuroscope assessment. As one can see, overall feeble breathers show high tcpCO_2 levels in contrast to forceful breathers who show low tcpCO_2 levels.

Table 3 Nutritional status of the RTT girls including age-specific and lifestyle adjusted recommended/reference values

RTT girl	Dietary energy (kcal/day)	Protein (gm/kg/day)	FFMI (kg/l^2)
1	1,179 (800)	3.0 (>0.9)	11.94 (unknown)
2	1,222 (800)	2.4 (>0.9)	12.71 (unknown)
3	1,920 (1,120)	4.5 (>0.9)	N.A.
4	1,150 (1,120)	2.3 (>0.9)	9.62 (unknown)
5	1,189 (1,120)	2.1 (>0.9)	**9.12**[a] (11.7–15.7)
6	**1,010**[b] (1,120)	1.3 (>0.9)	**11.48**[a] (11.7–15.7)
7	1,137 (1,120)	2.4 (>0.9)	**11.60**[a] (11.7–15.7)
8	1,222 (1,120)	1.9 (>0.9)	**10.34**[a] (11.7–15.7)
9	1,710 (1,680)	2.2 (>0.9)	**12.05**[a] (11.7–15.7)
10	1,828 (1,680)	1.6 (>0.9)	**10.46**[a](13.2–17.2)
11	**895**[b] (1,680)	1.2 (>0.9)	**11.23**[a] (13.2–17.2)
12	1,893 (1,680)	0.9 (>0.8)	**12.29**[a] (13.8–17.6)
13	**1,630**[b] (1,680)	1.5 (>0.8)	**12.18**[a] (13.8–17.6)

FFMI fat-free mass index, *N.A.* not acquired

[a] Low dietary energy compared to the recommended values

[b] FFMI value < 2 SD or p5 (Freedman et al. 2005; Schutz et al. 2002)

Nutritional Status

Results on dietary intakes and body composition are presented in Table 3

Dietary Intakes

Dietary intakes were evaluated and compared to age-specific standards for an inactive lifestyle, by which the normal values were adjusted to 80% of the recommended values for healthy girls. Overall, the total daily dietary energy was reasonably normal in the RTT girls, as the mean dietary intake was 117% of the adjusted recommended values. Almost 77% of the RTT girls met these adjusted recommended values. Only three girls had a dietary energy intake below these values, respectively, of 90%, 53%, and 97% of the recommended values. The RTT girl having a dietary energy intake of only 53% of the recommended values had a BMI at the 98th percentile and she was on a strict diet.

Protein intake in all patients was above the age-specific recommended values.

Prior to blood and urine sampling, no specific nutrients were consumed which could influence the metabolic investigations as has been described by Arias and colleagues (Arias et al. 2007).

Body Composition

As one can see in Table 3, 9 RTT girls confirm the definition of malnutrition, defined as an FFMI below the fifth percentile (or −2 SD) for age and gender. Since reference values for girls younger than 5 years of age are not available, one cannot confirm malnutrition in these younger girls.

Metabolic Investigations

As shown in Table 4, significantly elevated plasma creatine concentrations and increased urinary creatine/creatinine ratios were observed in five RTT girls. An isolated increase of the urinary creatine/creatinine ratio was seen in two girls. The creatinine plasma concentrations were relatively low in all RTT girls. The slightly elevated guanidinoacetate concentrations observed in three females are not clinically relevant, since they do not meet the criteria of a guanidinoacetate methyltransferase deficiency (Stromberger et al. 2003). An elevated CK concentration was seen in a girl with normal creatine concentrations. Other metabolic investigations (Table 1) showed no abnormalities.

Elevated creatine concentrations were only seen in those 9 years and older, except for one 4-year-old girl with an isolated increased urinary creatine/creatinine ratio. Mean age of the girls having an elevated plasma creatine was 14.54 years (range: 9.6–20.3, SD = 4.33), compared to 5.09 years (range: 2.3–9.7, SD = 2.34) of the girls having a normal plasma creatine. Mean age of the girls having an increased urinary creatine/creatinine ratio was 13.07 years (range: 4.3–20.3, SD = 2.34), compared to 5.47 years (range: 2.3–9.7, SD = 2.75) in the normal urinary creatine/creatinine ratio group.

Anthropometric characteristics between the two groups were roughly equal, except for height. Height was below the fifth percentile in 83% of the girls with elevated creatine concentrations, and greater than the 50th percentile in 57% of the girls with normal creatine concentrations.

Table 4 Creatine metabolism in RTT girls including age-specific reference values

RTT girl	Cr plasma (μmol/l)	Cr/Crn urine (mmol/mol)	Crn plasma (μmol/l)	CK (U/L)	GAA plasma (μmol/l)	GAA/Crn urine (mmol/mol)
1	75.7 (*17–109*)	490.6 (*6–1,200*)	**29.7**[a] (*35–62*)	73 (*<140*)	1.1 (*0.35–1.8*)	116.2 (*4–220*)
2	79.5 (*17–109*)	421.3 (*6–1,200*)	**27.0**[a] (*35–62*)	79 (*<140*)	**2.1**[b] (*0.35–1.8*)	102.3 (*4–220*)
3	88.1 (*17–109*)	N.A.	**30.7**[a] (*44–71*)	77 (*<140*)	1.6 (*0.35–1.8*)	N.A.
4	81.0 (*17–109*)	**828.8**[b] (*17–720*)	**28.0**[a] (*44–71*)	49 (*<140*)	1.2 (*0.35–1.8*)	76.8 (*4–220*)
5	83.5 (*17–109*)	28.6 (*17–720*)	**34.4**[a] (*44–71*)	44 (*<140*)	1.4 (*0.35–1.8*)	26.8 (*4–220*)
6	63.0 (*17–109*)	629.7 (*17–720*)	**28.8**[a] (*44–71*)	**285**[b] (*<140*)	1.2 (*0.35–1.8*)	56.7 (*4–220*)
7	52.9 (*17–109*)	**756.2**[b] (*17–720*)	**43.6**[a] (*44–80*)	104 (*<140*)	1.4 (*0.35–1.8*)	73.2 (*4–220*)
8	68.7 (*17–109*)	124.3 (*17–720*)	**29.2**[a] (*44–80*)	66 (*<140*)	0.8 (*0.35–1.8*)	45.0 (*4–220*)
9	**70.4**[b] (*6–50*)	**1,305.3**[b] (*17–720*)	**31.7**[a] (*53–88*)	65 (*<140*)	1.6 (*0.35–1.8*)	72.7 (*4–220*)
10	**73.5**[b] (*6–50*)	**448.0**[b] (*11–240*)	**42.3**[a] (*62–97*)	64 (*<140*)	1.6 (*0.35–1.8*)	64.4 (*4–220*)
11	**101.5**[b] (*6–50*)	**1,040.3**[b] (*11–240*)	**40.3**[a] (*62–97*)	26 (*<140*)	1.4 (*0.35–1.8*)	59.2 (*4–220*)
12	**88.5**[b] (*6–50*)	**403.7**[b] (*11–240*)	**36.7**[a] (*50–100*)	53 (*<140*)	1.3 (*1.0–3.8*)	**106.7**[b] (*3–78*)
13	**65.0**[b] (*6–50*)	**538.3**[b] (*11–240*)	**38.3**[a] (*50–100*)	19 (*<140*)	1.9 (*1.0–3.8*)	**82.7**[b] (*3–78*)

Cr creatine, *Crn* creatinine, *CK* creatine kinase, *GAA* guanidinoacetate, *N.A.* not acquired

[a] Elevated value

[b] Low value of creatinine

Furthermore, only the girls with an elevated creatine concentration in plasma and urine had a severe ISS score, and the girls with a normal creatine concentration in plasma and urine had a very mild ISS score. The mean score of the girls in the elevated plasma creatine group was 24.0 (range: 20–27, SD = 2.45) versus 14.3 (range: 8–21, SD = 6.02) in the normal plasma creatine group. The mean score of the girls in the increased urinary creatine/creatinine ratio group was 23.6 (range: 20–27, SD = 2.51) compared to 14.2 (range: 8–20, SD = 5.76) in the normal urinary creatine/creatinine ratio group. Except for ISS domain four (mental/cortical function), in which the scores were equally distributed in both groups, girls with an elevated creatine showed high scores in all domains.

In view of the variety in mutations, no differences concerning other patient characteristics could be observed.

Concerning the cardiorespiratory phenotype, in the elevated creatine group, four girls were forceful breathers and one girl had an undetermined cardiorespiratory phenotype. The girls with an isolated increased urinary creatine/creatinine ratio were a forceful and a feeble breather.

Finally, all RTT girls wherefore reference values of FFMI were available met the criteria for malnutrition. Therefore, a possible relationship between elevated creatine concentrations and an impaired nutritional status could not be investigated.

Discussion

This study is the first attempt to correlate metabolic alterations as a possible explanation of impaired nutritional status in RTT. Despite normal levels of total dietary energy and protein intakes, malnutrition was confirmed in all RTT girls wherefore reference values of FFMI were available. Although the loss of skeletal muscle mass could be partly due to physical inactivity, these low values are certainly an indication for impaired nutritional status in these RTT girls.

Blood and urine samples were collected for biochemical screening of multiple metabolic pathways. We observed significantly elevated plasma creatine concentrations and increased urinary creatine/creatinine ratios in half of the RTT girls. The creatinine plasma concentrations were relatively low, as one would expect in view of the low FFMI values. Nonetheless, the elevated creatine/creatinine ratios are most likely primarily due to the elevated creatine concentrations, considering the high plasma creatine concentrations at the same time.

Given that in this study the young girls were feeble breathers and the older girls were forceful breathers, it is impossible to determine whether the elevated creatine concentrations are age related or associated with the cardiorespiratory phenotype. This age distribution is likely coincidental since previous studies showed no significant age difference between the different cardiorespiratory phenotypes (Julu and Engerström 2005). At present, it is uncertain whether an altered carbon dioxide metabolism affects the creatine metabolism in females with RTT.

It is difficult to explain why creatine concentrations should increase with age in the RTT cohort. According to the reference values for creatine, creatine concentrations normally decrease with increasing age (Salomons et al. 2003). Furthermore, Horská and colleagues reported stable creatine concentrations with increasing age in RTT girls using proton magnetic resonance spectroscopy (Horská et al. 2009). Consequently, it seems highly unlikely that the altered creatine concentrations can be explained by age.

Regarding the cardiorespiratory phenotype, two possible explanations can be given. First of all, as has been described by Julu and colleagues, a major difference in the cardiorespiratory phenotypes is the blood pH value (Julu et al. 2008a, b). Females with a forceful breathing type tend to be alkalotic due to excessive loss of CO_2 via respiration, in contrast to females with a feeble breathing pattern who tend to be acidotic. The pH differences between these phenotypes can be as much as 0.4–0.5 pH-units (personal observation Dr. P.O.O. Julu). This pH difference may affect the creatine metabolism in RTT girls. Second, it can be speculated that the elevated creatine concentrations might be due to the increased energy expenditure as described in forceful breathers (Julu et al. 2008b). Phospho-creatine (P-creatine) together with ATP makes up the phosphagen energy system. P-creatine contains the high-energy phosphate bond, which is 3–8 times as abundant as ATP. As soon as more ATP is needed, P-creatine transfers its high energy phosphate to ATP (Guyton and Hall 2000). Following this suggestion, it may be speculated that in forceful breathers, the elevated creatine concentrations are indirectly caused by higher P-creatine needs.

Furthermore, as the creatine-transporter gene (*SLC6A8*) is located adjacent to MECP2 at Xq28, MeCP2 deficiency may cause epigenetic aberrations affecting the expression of the *SLC6A8*. The clinical phenotypes of RTT and an X-linked creatine transporter deficiency overlap regarding mental retardation, epilepsy, and language delay (Salomons et al. 2001). However, few studies have examined the brain metabolites of RTT females using proton magnetic resonance spectroscopy (Gökcay et al. 2002; Hashimoto et al. 1998; Horská et al. 2000, 2009; Khong et al. 2002; Pan et al. 1999). Overall, *N*-acetylaspartate/total creatine was significantly decreased compared with age-matched controls, primarily reflecting reduced *N*-acetylaspartate levels. Even so, an 8% greater mean creatine concentration was previously found in the frontal white matter in the RTT group. This difference was not significantly different,

because of a high variability in creatine levels in both RTT and control groups (Horská et al. 2000, 2009). Nevertheless, in case of a decreased expression of *SLC6A8,* less creatine would be present in the brain, making this diagnosis highly unlikely in our patients (Salomons et al. 2001).

Clinically, the girls with an elevated creatine concentration differed from girls with a normal creatine concentration regarding height and ISS score. Girls with an elevated creatine concentration were substantially shorter and a more severe phenotype was observed. This severe phenotype reflected several functional domains, that is growth and development, musculoskeletal, movement, and autonomic features.

In summary, this is the first study to report abnormalities in creatine concentrations in RTT girls. Hitherto, only Freilinger and colleagues performed metabolic screening including urinary creatine/creatinine ratio (Freilinger et al. 2007). They did not report any abnormalities in creatine/creatinine ratios in 29 Rett females with a mean age of 13 years. Reference values were not defined. However, as can clearly be seen in that article, three females have a highly elevated creatine/creatinine ratio. Furthermore, an *MECP2* mutation was identified in 76% of the females, which is rather low compared to previously published data (Williamson and Christodoulou 2006). Cardiorespiratory data were not available. So, the discrepancy may be because of difference in reference values or patient populations, as the diagnosis of RTT in the previous study was mainly based on clinical criteria alone and a possibly lower percentage of forceful breathers. The strength of our study was the use of a well-defined group of RTT girls. Only due to these strict inclusion criteria, our study is hampered by the small sample size, which does not allow us to understand the observed creatine abnormalities fully. At this moment, we are not able to confirm our hypothesis regarding metabolic alterations as a possible explanation of impaired nutritional status in RTT, since impaired nutritional status was seen in all RTT girls wherefore reference values of FFMI were available. Different etiologies can explain the observed elevated creatine concentrations in the RTT girls. Except for creatine transporter defects, no other neurological disease ever showed elevated creatine values. Further studies concerning the creatine metabolism in relation to the nutritional requirements and cardiorespiratory status or phenotype of RTT girls are important in order to provide appropriate and effective management.

Conclusions

Despite normal levels of total dietary energy and protein intakes, malnutrition was confirmed in all RTT girls wherefore reference values of FFMI were available. An important percentage of RTT girls showed creatine concentrations above the reference values. Currently, it is undetermined how the creatine metabolism is affected in females with RTT, and how this may affect their nutritional status. Further studies are required to confirm these findings and to provide greater insight into the pathogenesis of the abnormal creatine metabolism in RTT.

Acknowledgments The authors would like to thank the RTT girls and their parents for participating in this project. Furthermore, we would like to thank the ESRRA group (European Scientific Rett Research Association; Mrs Flora Apartopoulos, Dr Robert S Delamont, Dr Stig Hansen, Mr Elias Keter, Mrs Märith Bergström-Isacsson, Dr Bengt Engerström, Dr Lars Engerström, Mrs Gunilla Larsson, Dr Stefania Bigoni, Dr Giorgio Pini and Dr Sami SF Al-Rawas), Dr. Alison Kerr, and Dr. Dick van Waardenburg for their support and advice.

Synopsis

A striking increase in creatine concentration was observed in the body fluids of females with Rett syndrome, which may reflect the altered carbon dioxide metabolism of these girls or their impaired nutritional status or both.

Contributor's Statement Page

The article has not been and will not be published elsewhere in substantially the same form. The article has been circulated and final approval of the version to be peer-reviewed was secured from all co-authors prior to article submission. This includes confirmation of absence of previous similar or simultaneous publications their inspection of the manuscript, their substantial contribution to the work, and their agreement to submission.

Details of the Contributions of Individual Authors

Nicky S.J. Halbach: involvement in conception and design analysis and interpretation of data and drafting the article.

Eric E.J. Smeets: involvement in conception and design analysis and interpretation of data and revising the article critically for important intellectual content.

Jörgen Bierau: involvement in conception and design analysis and interpretation of data and revising the article critically for important intellectual content.

Irene M.L.W. Keularts: involvement in conception and design analysis and interpretation of data and revising the article critically for important intellectual content.

Guy Plasqui: involvement in conception and design analysis and interpretation of data and revising the article critically for important intellectual content.

Peter O.O. Julu: involvement in analysis and interpretation of data and revising the article critically for important intellectual content.

Ingegerd Witt Engerström: involvement in analysis and interpretation of data and revising the article critically for important intellectual content.

Jaap A. Bakker: involvement in conception and design analysis and interpretation of data and revising the article critically for important intellectual content.

Leopold M.G. Curfs: involvement in conception and design analysis and interpretation of data and revising the article critically for important intellectual content.

Guarantor for the article: Leopold M.G. Curfs.

Details of funding: the authors confirm independence from the sponsors the content of the article has not been influenced by the sponsors.

Details of ethics approval: ethical approval was obtained from the Medical Ethical Committee at the Maastricht University Medical Centre (MEC 08-2-119) and all parents provided informed written consent. The study was registered at clinicaltrials.gov.

References

Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY (1999) Rett syndrome is caused by mutations in X-linked *MECP2*, encoding methyl-CpG-binding protein 2. Nat Genet 23:185–188

Arias A, Corbella M, Fons C et al (2007) Creatine transporter deficiency: prevalence among patients with mental retardation and pitfalls in metabolite screening. Clin Biochem 40:1328–1331

Freedman DS, Wang J, Maynard LM et al (2005) Relation of BMI to fat and fat-free mass among children and adolescents. Int J Obes 29(1):1–8

Freilinger M, Kalisch D, Muehl A, Haas O, Moritz A, Bodamer O (2007) Methylation status in females with Rett syndrome. J Child Neurol 22:635–638

Gökcay A, Kitis O, Ekmekci O, Karasoy H, Sener RN (2002) Proton MR spectroscopy in Rett syndrome. Comput Med Imaging Graph 26:271–275

Guyton AC, Hall JE (2000) Energetics and metabolic rate. In: Textbook of medical physiology. Elsevier Saunders, Philadelphia, pp 815–821

Hagberg B, Hanefeld F, Percy A, Skjeldal O (2002) An update on clinically applicable diagnostic criteria in Rett syndrome. Comments to Rett syndrome Clinical Criteria Consensus Panel Satellite to European Paediatric Neurology Society Meeting, Baden Baden, Germany, 11 September 2001. Eur J Paediatr Neurol 6:293–297

Hashimoto T, Kawano N, Fukuda K et al (1998) Proton magnetic resonance spectroscopy of the brain in three cases of Rett syndrome: comparison with autism and normal controls. Acta Neurol Scand 98:8–14

Horská A, Naidu S, Herskovits EH, Wang PY, Kaufmann WE, Barker PB (2000) Quantitative 1 H MR spectroscopic imaging in early Rett syndrome. Neurology 54(3):715–722

Horská A, Farage L, Bibat G et al (2009) Brain metabolism in Rett syndrome: age, clinical, and genotype correlations. Ann Neurol 65:90–97

Julu POO, Engerström IW (2005) Assessment of the maturity-related brainstem functions reveals the heterogeneous phenotypes and facilitates clinical management of Rett syndrome. Brain Dev 27: S43–S53

Julu PO, Kerr AM, Apartopoulos F et al (2001) Characterisation of breathing and associated central autonomic dysfunction in the Rett disorder. Arch Dis Child 85(1):29–37

Julu PO, Engerström IW, Hansen S et al (2008a) Cardiorespiratory challenges in Rett's syndrome. Lancet 371:1981–1983

Julu POO, Engerström IW, Hansen S et al (2008b) Clinical update: addressing the cardiorespiratory challenges posed by Rett syndrome in medicine. The Frösö decleration

Kerr A, Nomura Y, Armstrong D et al (2001) Guidelines for reporting clinical features in cases with MECP2 mutations. Brain Dev 23:208–211

Khong P, Lam C, Ooi CGC, Ko C, Wong VCN (2002) Magnetic resonance spectroscopy and analysis of MECP2 in Rett syndrome. Pediatr Neurol 26:205–209

Lohman TG (1989) Assessment of body composition in children. Pediatr Exerc Sci 1:19–30

Motil KJ, Schultz RJ, Wong WW, Glaze DG (1998) Increased energy expenditure associated with repetitive involuntary movement does not contribute to growth failure in girls with Rett syndrome. J Pediatr 132(2):228–233

Oddy WH, Webb KG, Baikie G et al (2007) Feeding experiences and growth status in a Rett syndrome population. J Pediatr Gastroenterol Nutr 45(5):582–590

Pan JW, Lane JB, Hetherington H, Percy AK (1999) Rett syndrome: 1H spectroscopic imaging at 4.1 Tesla. J Child Neurol 14:524–528

Reilly S, Cass H (2001) Growth and nutrition in Rett syndrome. Disabil Rehabil 23:118–128

Rice MA, Haas RH (1988) The nutritional aspects of Rett syndrome. J Child Neurol 3:S35–S42

Salomons GS, Van Dooren SJM, Verhoeven NM et al (2001) X-linked creatine-transporter gene (SLC6A8) defect: a new creatine-deficiency syndrome. Am J Hum Genet 68:1497–1500

Salomons GS, Van Dooren SJM, Verhoeven NM et al (2003) X-linked creatine-transporter defect: an overview. J Inherit Metab Dis 26:309–318

Schutz Y, Kyle UU, Pichard C (2002) Fat-free mass index and fat mass index percentiles in Caucasians aged 18–98 y. Int J Obes Relat Metab Disord 26(7):953–960

Smeets EE, Schrander-Stumpel CTRM (2005) Rett syndrome. In: Cassidy SB, Allanson JE (eds) Management of genetic syndromes. Wiley-Liss, New York, pp 457–468

Stromberger C, Bodamer OA, Stöckler-Ipsiroglu S (2003) Clinical characteristics and diagnostic clues in inborn errors of creatine metabolism. J Inherit Metab Dis 26:299–308

VanItallie TB, Yang MU, Heymsfield SB, Funk RC, Boileau RA (1990) Height normalized indices of the body's fat-free mass and fat mass: potentially useful indicators of nutritional status. Am J Clin Nutr 52(6):953–959

Westerterp KR, Wouters L, Van Marken - Lichtenbelt W. 1995. The Maastricht protocol for the measurement of body composition and energy expenditure with labelled water. Obes Res 3:49–57.

Williamson SL, Christodoulou J (2006) Rett syndrome: new clinical and molecular insights. Eur J Hum Genet 14:896–903

JIMD Reports
DOI 10.1007/8904_2011_80

RESEARCH REPORT

Molecular Genetic Characterization of Novel Sphingomyelin Phosphodiesterase 1 Mutations Causing Niemann–Pick Disease

Beata Tóth · Melinda Erdős · Annamária Székely · László Ritli · Péter Bagossi · János Sümegi · László Maródi

Received: 9 April 2011 / Revised: 30 June 2011 / Accepted: 14 July 2011 / Published online: 27 September 2011

Abstract Niemann–Pick disease (NPD) types A and B are autosomal recessive disorders caused by acid sphingomyelinase (ASM) deficiency due to mutation in the sphingomyelin phosphodiesterase 1 gene (*SMPD1*). Although a number of *SMPD1* mutations were reported, expression studies were performed for only a small number of missense mutations. We evaluated three unrelated patients with clinical manifestations of NPD. Sequence analysis revealed two previously described (S248R and W391G) and two novel (G247D and F572L) missense mutations. To analyze the effects of the novel mutations on ASM function, cDNA was generated by site-directed mutagenesis and expressed in COS-7 cells. In vitro biochemical assays revealed marked deficiency of ASM activity consistent with the disease phenotype in cells homoallelic for each mutation. We show that each mutation dramatically reduced half-life and catalytic activity of ASM with more pronounced decrease by the G247D mutation. These data suggest that impaired protein stability and decreased enzyme activity are responsible for the disease in sphingomyelinase-deficient patients carrying the G247D and F572L mutations.

Communicated by: Gregory M. Pastores.

Competing interests: None declared.

Electronic supplementary material The online version of this article (doi:10.1007/8904_2011_80) contains supplementary material, which is available to authorized users.

B. Tóth · M. Erdős · A. Székely · J. Sümegi · L. Maródi (✉)
Lysosomal Storage Disease Unit, Department of Infectious and Pediatric Immunology, Medical and Health Science Center, University of Debrecen, Nagyerdei krt. 98, 4032, Debrecen, Hungary
e-mail: lmarodi@dote.hu

L. Ritli
Oradea Children's Hospital, Oradea, Romania

P. Bagossi
Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

J. Sümegi
Division of Bone Marrow Transplantation and Immunodeficiency, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH, USA

Introduction

Niemann–Pick disease (NPD) types A (NPDA) and B (NPDB) are autosomal recessive lysosomal storage disorders caused by acid sphingomyelinase (ASM; E.C.3.1.4.12) deficiency due to mutations in the sphingomyelin phosphodiesterase 1 gene (*SMPD1*; Supplementary Fig. 1) (Da Veiga et al. 1991). NPDA (MIM #257200) is a severe neurodegenerative disease with early onset and rapidly progressing psychomotor retardation, and death by 2–3 years of age (Da Veiga et al. 1991; Schuchman and Desnick 2001) In contrast, NPDB (MIM #607616) is the non-neuropathic, slowly progressing visceral form with hepatosplenomegaly and pulmonary involvement, and survival to adolescence or adulthood (Dardis et al. 2005; Simonaro et al. 2002). Some NPDB patients may develop mild peripheral neuropathy and cherry-red spot and halo (Mihaylova et al. 2007). Recent studies on intermediate forms (NPDA/B) have somewhat questioned this classification, and suggested a continuous disease spectrum related to the amount of residual ASM activity rather than two well-defined extreme clusters (Harzer et al. 2003; Pavlu-Pereira et al. 2005; Schuchman 2007; Wasserstein et al. 2006).

Although a number of *SMPD1* mutations have been identified, expression studies were performed for only a small number of missense mutations (Rodríguez-Pascau et al. 2009; Desnick et al. 2010). Here we report two novel missense mutations in *SMPD1* and present biochemical and molecular genetic data showing decreased ASM activity and reduced protein stability of the mutant proteins.

Materials and Methods

Patients

Written informed consent was obtained from the parents in accordance with the Declaration of Helsinki. All studies were approved by the Institutional Review Board of the University of Debrecen.

Two Hungarian and one Gypsy patients and their family members were studied (Table 1, Fig. 1). Diagnosis of NPDA and NPDB was made by analysis of clinical and genetic findings, and segregation of *SMPD1* mutations in the families. Patient M1 was born to non-consanguineous Hungarian parents, and there was no history of chronic illness in the family. Rapid deterioration of organ functions led to death at 2 years of age. Patients A1 and S1 have no neurological disease manifestations.

Molecular Genetics

Genomic DNA was isolated from leukocytes. Exons 1–6 of *SMPD1* and the flanking intronic regions were amplified using polymerase chain reaction (PCR) (Erdős et al. 2005; Jiao et al. 2008). Mutational analysis was performed using the BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA), and an ABI 3130 DNA analyzer (Applied Biosystems) (Tóth et al. 2010). Sequence data were compared to the published sequence of *SMPD1* (GeneCards GC11P006368 and HGMD). The DNA mutation numbering was based on the cDNA sequence (Acc. No. NM_000543) and the first nucleotide is A in the translation initiation codon.

Expression Constructs and Site-Directed Mutagenesis

SMPD1 cDNA was reverse transcribed from human peripheral blood mononuclear cells polyA+mRNA and cloned into the pCR4-TOPO vector (Invitrogen Corp. Carlsbad, CA). The coding sequence of the *SMPD1* cDNA was amplified by PCR using primer pairs 5′-GCGATCGC-CATGCCCCGCTACGGAGCGTCACTCCGCC-3′ and 5′-CGTACGCGTTTAAACCTTATCGTCGTCATCCTTG-TAATCGCAAAACAGTGGCCTTG-3′ and cloned into pCMV6-Neo vector (Origene, Rockville, MD). Mutations,

Table 1 Demographics, clinical findings, and *SMPD1* mutations in patients and family members

Patients and family members (NPD type)	Sex	Age (year)	Clinical manifestations	Nucleotide change	Predicted amino acid change	Mutation published
M1, proband (NPDA)	M	2 (died)	Hepatosplenomegaly, lymphadenomegaly, failure to thrive, psychomotor retardation, neurodegeneration	c.740G>A/c.1716C>G	p.G247D/p.F572L	This report
M2, mother of M1	F	30	–	c.740G>A/wt	p.G247D	
M3, father of M1	M	35	–	c.1716C>G/wt	p.F572L	
M4, sister of M1	F	6	–	c.1716C>G/wt	p.F572L	
A1, proband (NPDB)	F	5	Abdominal distension, hepatosplenomegaly	c.742A>C/c.742A>C	p.S248R	(Sikora et al. 2003)
A2, mother of A1	F	32	–	c.742A>C/wt	p.S248R	
A3, father of A1	M	40	–	c.742A>C/wt	p.S248R	
A4, stepbrother of A1	M	10	–	c.742A>C/wt	p.S248R	
S1, proband (NPDB)	M	6	Abdominal distension, hepatosplenomegaly, cherry-red spot	c.1171T>G/c.1171T>G	p.W391G	(Ferlinz et al. 1997)
S2, mother of S1	F	25	–	c.1171T>G/wt	p.W391G	
S3, father of S1	M	35	–	c.1171T>G/wt	p.W391G	
S4, brother of S1	M	9	–	c.1171T>G/wt	p.W391G	

Patient M1 developed clinical manifestations of NPD at age 6 months, whereas Patients A1 and S1 presented with abdominal distension and hepatosplenomegaly at 18 months and 24 months, respectively.

NPD Niemann–Pick disease, *M* male, *F* female

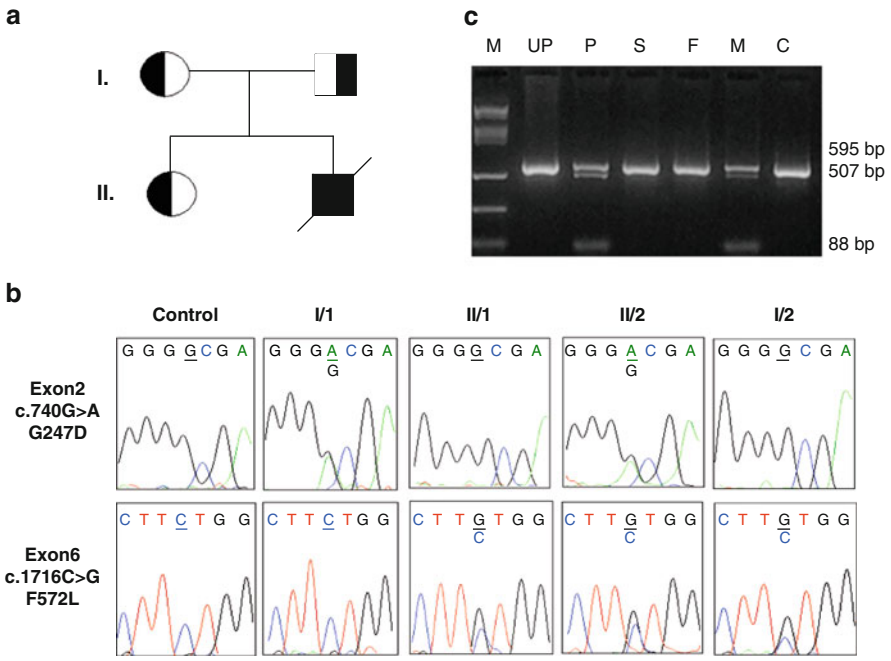


Fig. 1 Pedigree and electropherograms of the patient with NPDA and his family members. (**a**) *Filled square* denotes the male patient (II/2), *half-filled square* (I/2) and *circles* (I/1 and II/1) show heterozygous family members. *Bar* indicates the patient died. (**b**) Electropherograms of G247D and F572L mutations indicating heterozygous (I/1, I/2, II/1) and compound heterozygous (II/2) mutations of *SMPD1*. (**c**) Restriction digestion analysis of the c.740G>A mutation of *SMPD1*. Agarose gel electrophoresis of *Bsm*FI digests of the amplified exon 2 is shown in patient, his family members, and a control. *M* molecular weight marker, *UP* untreated product of the PCR amplification, *P* patient, *S* sister of patient, *F* father of patient, *M* mother of patient, *C* control. In the mutant allele (P, M), *Bsm*FI digestions yielded a 507 and a 88 bp restriction fragments. Such a digestion pattern was not found in the sister and the father, and the control that carried two wild-type alleles at this locus as shown in the figure

to obtain cDNAs coding for G247D or F572L *SMPD1*, were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the supplier's recommendation. All constructs were verified by resequencing with the BigDye Terminator method to ensure that no additional mutation had occurred during the mutagenesis procedure.

Cells and Transfection

COS-7 cells were maintained in tissue culture flasks containing Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO). Transfection was performed with different expression vectors containing the appropriate cDNA insert using FuGENE 6 (Roche, Indianapolis, IN).

Measurement of ASM Activity

COS-7 cells were resuspended in lysis buffer and sonicated for 2 min on ice. Protein concentrations were determined by Lowry method. Protein samples were incubated with 15 mM 2-*N*-hexadecanoylamino-4-nitrophenylphosphorylcholine (Calbiochem, Darmstadt, Germany) as substrate and 250 mM sodium acetate buffer at 37°C for 4 h. A colorimetric method was used to determine the activity of ASM by measuring the absorbance at 410 nm.

Immunoblotting

Protein extracts from transfected and G418-resistant COS-7 cells (50 μg protein/lane) were subjected to sodium dodecyl sulfate denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Corp, Billerica, MA). The blots were blocked in 5% nonfat milk in phosphate buffered saline (PBS) containing 0.2% Tween 20 (PBST) overnight. Subsequently, the membranes were probed with goat antihuman SMPD1 antibody (R&D System, Minneapolis, MN) for 4 h in the presence of 1% nonfat milk in PBST. After incubation, the membranes were washed with PBST four times for 10 min. Rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as secondary antibody. Images were obtained with the enhanced chemiluminescence system (Thermo Scientific, Rockford, IL). For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), goat anti-GAPDH (Santa Cruz) antibody was used. Prestained molecular weight markers (Bio-Rad GmbH, Munich, Germany) were used as the molecular size standard.

Metabolic Labeling Study to Determine SMPD1 Half-Life

Transfected COS-7 cells expressing wild-type or mutant proteins were starved for methionine and cysteine for 1 h and then pulse-labeled for 3 h with Tran^{35}S-label (MP Biomedicals LLC, Santa Ana, CA). Cells were cultured in complete medium containing cycloheximide, and newly synthesized proteins were chased for various times. Cells were lysed, and the fate of radiolabeled ASM was analyzed by immunoprecipitation with the M2 anti-FLAG antibody (Sigma-Aldrich) and gel electrophoresis through a 4–15% SDS-PAGE from Bio-Rad (Hercules, CA). Gels were fixed and incubated with Amersham Amplify™ Fluorographic Reagent (GE Healthcare Biosciences, Piscataway, NJ) before autoradiography. The density of each band was quantified.

Results and Discussion

Genetic Findings

The SMPD1 gene of three unrelated patients with typical clinical manifestations of NPD was analyzed. In two patients (Patients A1 and S1), we found previously described mutations in the *SMPD1* (Table 1). Genetic testing of patient M1 revealed compound heterozygosity for two missense mutations. Both c.740G>A in exon 2 and c.1716C>G in exon 6 were novel. The mutant alleles were confirmed by the analysis of two independent PCR products and were identified in the parents and the sister. The 740G>A mutation introduces a novel *Bsm*FI restriction enzyme recognition site into the DNA sequence. Restriction fragment length polymorphism (RFLP) was performed using PCR products carrying the c.740G>A sequence variant. The presence of the mutation was analyzed using the *Bsm*FI restriction enzyme. Exon 2 of the *SMPD1* gene was amplified in 595 bp products. In individuals with the c.740G>A mutation digestion with *Bsm*FI yielded 507 bp and 88 bp DNA fragments. However, *Bsm*FI did not digest wild-type exon 2 DNA of 595 bp. The novel sequence variants were not found in the control group of 50 healthy Hungarian individuals, representing 100 chromosomes by either RFLP analysis (c.740G>A) or gDNA sequencing (c.1716C>G). The co-segregation of the mutations with the disease in the family further suggested that these were disease-related.

The G247D novel mutation brings the total number of mutations affecting codon 247 to two. In previous studies, the G247S mutation was commonly reported in compound heterozygous patients with the NPDB (Simonaro et al. 2002). The mild course of the disease observed in those patients could be due to the second alleles with neuroprotective mutations. However, when homoallelism was found for the G247S mutation, all patients presented with the clinical course of NPDA. The occurrence of G247D with F572L in our patient presented a classical NPDA phenotype with severe neurological involvement and fatal outcome suggesting that the novel mutation at amino acid position 572 may not exert neuroprotective effect.

Expression and Function of ASM Proteins

Western blot analysis showed that both wild-type and mutant proteins were expressed in COS-7 cells, meaning that the expression was stable for the plasmid constructs (Fig. 2A). We evaluated the functional effect of the G247D and F572L missense mutations on the activity of ASM. We found that ASM activity was severely decreased in cells expressing G247D protein when compared to COS-7 cells expressing wild-type *SMPD1* and reduced in cells transfected with the c.1716C>G mutant allele (Fig. 2B).

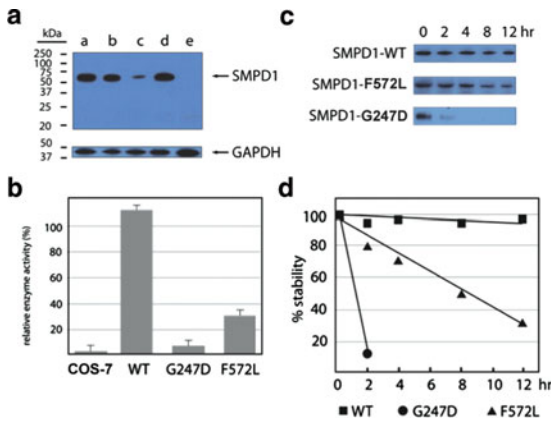


Fig. 2 (**A**) Detection of wild-type and mutant ASM proteins following transfection in COS-7 cells. Wild-type and mutant *SMPD1* cDNAs in pCMV6 were transfected in COS-7 cells and selected in the presence of 400 μg/ml G418. G418-resistant clones expressing the wild-type and mutant *SMPD1* cDNAs were expanded. Lanes contained 50 μg of cell extract of HL-60 (*a*), COS-7 cells expressing wild-type *SMPD1* (*b*), *SMPD1*-G247D (*c*), and *SMPD1*-F572L (*d*) mutant proteins, and COS-7 cells transfected by empty plasmid (*e*). (**B**) Activity of ASM translated from wild-type and mutant SMPD1 alleles in COS-7 cells. The data are shown as mean ± SD of at least three different experiments, each performed in triplicate. *Error bars* correspond to standard deviation. *WT* wild-type, G247D and F572L, mutant ASM. (**C**) Autoradiography demonstrating half-life of [35S] methionine and [35S]cysteine-labeled ASM. Experiments were performed with wild-type and mutant enzymes for 12 h. At different time points (0, 2, 4, 8, and 12 h), excess cold amino acid was added to the culture medium. (**D**) Densitometry demonstrating half-life of ASM. The amount of wild-type and mutant proteins remaining at the indicated time points was also quantified by densitometry, and protein stability was plotted as percentage of the intensity of band compared to wild-type sample

Stability of ASM Proteins

To further evaluate the functional effect of the novel missense mutations on SMPD1 activity, we transfected the wild-type and mutant cDNAs cloned into pCMV6 vector into COS-7 cells. As a negative control, COS-7 cells were transected with pCMV6 vector DNA (Fig. 2B). Cells were selected on 400 μg/ml G418 and G418 resistant cells were used to assay enzyme activity and immunoblotting. The mean wild-type SMPD1 activity was 97.6 ± 18.2 nmol/mg/h, 112-fold higher than the activity measured in COS-7 cells transfected with the empty vector. No SMPD1 protein was detected in the COS-7 cells transfected with pCMV6. Cells transfected with the construct coding for F572L proteins showed comparable level of expression to the wild-type protein. In contrast, cells expressing G247D showed marked reduction in mutant SMPD1 protein expression. To determine whether the SMPD1 mutations may lead to protein instability, we compared the half-life of wild-type and mutant proteins by measuring the protein levels in transected cells after pulse chase and cycloheximide block of protein synthesis (Fig. 2C). According to the cycloheximide/immunoblot analysis, the wild-type SMPD1 protein has a half-life over 12 h. The half-life for the F572L protein was shorter, between 4 h and 8 h. The G247D mutation dramatically altered the stability of the mutant protein. The half-life of G247D SMPD1 protein was 2 h or less. The two substituted amino acids are highly conserved among human, mouse, marmoset, opossum, and *Caenorhabditis elegans*; therefore, it is not surprising that constructs bearing mutations of G247D and F572L expressed proteins severely compromised in function. Both mutations are in the metallophosphoesterase catalytic domain of SMPD1.

In summary, two novel mutations causing NPDA with severe neurological involvement and fatal outcome were identified and characterized. These data indicate that the decreased half-life of the G247D and F572L mutant proteins could be responsible for the low enzyme activity and the severe clinical phenotype in patient M1. This study provides further evidence to the genetic heterogeneity of *SMPD1* mutations causing NPD as well as a solid bases to predict the clinical phenotype in patients with the novel G247D/F572L heterozygous mutations.

Acknowledgments We thank the parents of the patients for helpful collaboration. This work was supported by TÁMOP 4.2.1./B-09/1/KONV-2010-0007 and TÁMOP 4.2.2-08/1-2008-0015 grants to L.M.

References

Da Veiga PL, Desnick RJ, Adler DA, Disteche CM, Schuchman EH (1991) Regional assignment of the human acid sphingomyelinase gene (SMPD1) by PCR analysis of somatic cell hybrids and in situ hybridization to 11p15.1-15.4. Genomics 9:229–234

Dardis A, Zampieri S, Filocamo M, Burlina A, Bembi B, Pittis MG (2005) Functional in vitro characterization of 14 *SMPD1* mutations identified in Italian patients affected by Niemann–Pick type B disease. Hum Mutat 26:164, in Brief

Desnick JP, Kim J, He X, Wasserstein MP, Simonaro CM, Schuchman EH (2010) Identification and characterization of eight novel SMPD1 mutations causing types A and B Niemann-Pick disease. Mol Med 16:316–321

Erdős M, Uzvölgyi E, Nemes Z et al (2005) Characterization of a disease-causing mutation of SH2D1A in a family with X-linked lymphoproliferative disease. Hum Mutat 25:506, in Brief

Ferlinz K, Hurwitz R, Moczall H, Lansmann S, Schuchman EH, Sandhoff K (1997) Functional characterization of the N-glycosylation sites of human acid sphingomyelinase by site-directed mutagenesis. Eur J Biochem 243:511–517

Harzer K, Rolfs A, Bauer P et al (2003) Niemann-Pick disease types A and B are clinically but also enzimatically heterogeneous: pitfall in the laboratory diagnosis of sphingomyelinase deficiency associated with the mutation Q292K. Neuropediatrics 34:301–306

Jiao H, Tóth B, Erdős M et al (2008) Novel and recurrent STAT3 mutations in hyper-IgE syndrome patients from different ethnic groups. Mol Immunol 46:202–206

Mihaylova V, Hantke J, Sinigerska et al (2007) Highly variable neural involvement in sphingomyelinase-deficient Niemann-Pick disease caused by ancestral Gypsy mutation. Brain 130:1050–1061

Pavlu-Pereira H, Asfaw B, Poupctova H et al (2005) Acid sphingomyelinase deficiency. Phenotype variability with prevalence of intermediate phenotype in a series of twenty-five Czech and Slovak patients. A multi-approach study. J Inherit Metab Dis 28:203–227

Rodríguez-Pascau L, Gort L, Schuchman EH, Vilageliu L, Grinberg D, Chabás A (2009) Identification and characterization of SMPD1 mutations causing Niemann-Pick types A and B in Spanish patients. Hum Mutat 30:1117–1122

Schuchman EH (2007) The pathogenesis and treatment of acid sphingomyelinase-deficient Niemann-Pick disease. J Inherit Metab Dis 30:653–663

Schuchman EH, Desnick RJ (2001) Niemann-Pick disease type A and B: acid sphingomyelinase deficiencies. In: Scriver CR, Beaudet AL, Sly WS, Childs B, Valle D, Kinzler KW, Vogelstein B (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 3589–3610

Sikora J, Pavlu-Pereira H, Elleder M, Roelofs H, Wevers RA (2003) Seven novel acid sphingomyelinase gene mutations in Niemann-Pick type A and B patients. Ann Hum Genet 67:63–70

Simonaro CM, Desnick RJ, McGovern MM, Wasserstein MP, Schuchman EH (2002) The demographics and distribution of type B Niemann-Pick disease: novel mutations lead to new genotype/phenotype correlations. Am J Hum Genet 71:1413–1419

Tóth B, Wolff AS, Halász Z et al (2010) Novel sequence variation of AIRE and detection of interferon-omega antibodies in early infancy. Clin Endocrinol (Oxf) 72:641–647

Wasserstein MP, Aron A, Brodie SE, Simonaro C, Desnick RJ, McGovern MM (2006) Acid sphingomyelinase deficiency: prevalence and characterization of an intermediate phenotype of Niemann-Pick disease. J Pediatr 149:554–559

JIMD Reports
DOI 10.1007/8904_2011_31

RESEARCH REPORT

Dietary Protein Counting as an Alternative Way of Maintaining Metabolic Control in Phenylketonuria

A.L. Sweeney • R.M. Roberts • J.M. Fletcher

Received: 6 September 2010 / Revised: 29 March 2011 / Accepted: 16 May 2011 / Published online: 16 September 2011

Abstract *Objective*: To compare a gram protein exchange system (1 g = 50-mg Phenylalanine) with a unit exchange system (1 unit = 15-mg Phenylalanine) and its effect on the blood Phenylalanine (Phe) levels and acceptance in the dietary management for children and adolescents with Phenylketonuria.

Methods: In Phase One, participants were randomised to continue counting Phe unit exchanges ($n = 8$) or changed to counting gram protein exchanges ($n = 10$), using a new diet chart developed in-house. Foods containing less than 20 mg Phe per serve were now considered "free." Interim data analysis confirmed no significant deterioration in Phe levels of the study group and the control group was changed to protein counting.

In Phase Two, 18 participants were educated to use an updated version of the in-house diet chart – in this version foods containing less than 50 mg Phe per serve were considered "free."

In both phases, attitudes to PKU and its management were evaluated at baseline and 6 months. Phenylalanine and tyrosine levels were measured from filter paper blood spots by tandem mass spectrometry.

Results: Phase One: Phe levels over 6 months were comparable to pre-study levels (mean Phe pre 366 μmol/L +/− 169, mean Phe post change = 388 μmol/L+/− 160).

Phase Two: Four participants had a significant improvement in blood Phe levels, nine showed no significant change and one participant's levels were significantly higher. There was incomplete data on four participants. All participants preferred the freer diet chart.

Conclusion: Protein exchanges (foods containing less than 50 mg Phe/serve uncounted) are an alternative method of measuring Phe intake in the dietary management of Phenylketonuria.

Communicated by: Anita MacDonald.

Competing interests: None declared.

A.L. Sweeney (✉)
Department of Nutrition, Children, Youth and Women's Health Service, Women's and Children's Hospital, North Adelaide 5006, Adelaide, SA, Australia
e-mail: annabel.sweeney@health.sa.gov.au

R.M. Roberts
School of Psychology, University of Adelaide, Adelaide, SA, Australia

J.M. Fletcher
Department of Genetic Medicine, Children, Youth and Women's Health Service, Women's and Children's Hospital, North Adelaide 5006, Adelaide, SA, Australia

Introduction

Compliance with dietary treatment for phenylketonuria (PKU; OMIM 261600) is a significant issue (MacDonald 2000) with parents reporting that managing the diet is the most significant problem related to parenting a child with PKU (Awiszus and Unger 1990). Current recommendations are for adherence to dietary treatment for life (Medical Research Council Working Party on PKU 1993). This further compounds the need to review dietary treatment recommendations to ensure that treatment is manageable for life and that optimal dietary control is achieved (Cockburn and Clark 1996). Dietary treatment should aim to be as least restrictive as possible, given that a simple approach is likely to be easier for patients to follow and hence more likely to be complied with (MacDonald 2000).

Traditional Australian dietary management of PKU consists of a medical formula (protein supplement) providing phenylalanine-free amino acids, vitamins, minerals, and trace elements. Special commercial low protein products and foods that contain minimal amounts of Phe such as fats and sugars are uncounted. Phenylalanine (Phe) is provided in carefully measured quantities of low Phe foods, (some cereals and most fruits and vegetables), using a 15 mg Phe unit exchange system. The number of units is based on blood Phe levels and varies between individuals and at different growth times. Our experience shows that this 15 mg Phe unit system enabled normal growth and development and helped achieve acceptable blood Phe levels (Australasian Society for Inborn Errors of Metabolism 1996).

There are, however, variations throughout the world for determining the Phe content of the diet used in the management of PKU, ranging from the 50 mg Phe exchange system in the UK, to the 15 mg Phe exchange system in the USA. (MacDonald 2000). It is acknowledged that the prescriptive nature of the PKU diet is challenging for the child and caregiver and how the family copes with these challenges influence the blood Phe levels (Ievers-Landis et al. 2005; Vetrone et al. 1989). MacDonald (2000) reviewed the current dietary management systems and concluded that "it is important that the diet is fully evaluated to ensure this regime is not stricter than necessary to achieve acceptable blood Phe control" (p.S141). Each dietary counting method has its own limitations and we were unable to locate publications that have compared dietary methods. Our study assessed the impact on metabolic control achieved using the unit exchange system (1 unit = 15 mg Phe) with a gram of protein exchange system (1 g = 50 mg Phe). Perceptions of the children and their parents were also sought. The new Australian food labelling standards provided the opportunity to directly read the protein content from the commercial foods. Our hypothesis was that this less rigid diet, offering "free fruit and vegetables" would improve dietary variety. There was a theoretical concern that the more relaxed diet adversely impact on Phe control.

It was predicted that (1) simplifying the protein counting system for children with PKU would not have an adverse metabolic effect, compared to the standard Australian PKU counting system (15 mg phe exchanges), and (2) parents and participants would find the new method simpler.

Methods

Study Design

The study was conducted in two phases. In the first phase, an interim diet chart based on 1 g of protein = 50 mg Phe was introduced, "free" foods were defined as those with a cut off point less than 20 mg Phe/serve, resulting in a small number of free fruits and vegetables. The second phase diet chart defined foods with less than 50 mg Phe/serve as "free." The new definition resulted in an extensive list of free foods, similar to that already in place in the UK (NSPKU 2005/2006).

Measurement of Blood Phe Levels

Blood Phe levels were measured using a weekly home-collected filter paper blood spot and measured by tandem mass spectrometry. Dietary management aimed for blood Phe levels within the range recommended by the Medical Research Council Working Party on Phenylketonuria (1993).

PKU Diet Attitudes Questionnaire

A questionnaire was designed for this study to evaluate the attitudes of young people with PKU and their parents about the preparation of foods, monitoring of the diet, collecting blood tests, metabolic control, dietary variety, stressfulness of the diet, and quality of life. The main caregiver and school aged children and adolescents completed the PKU Diet Attitudes Questionnaire. The questionnaire comprised 18 questions, answered on a five-point Likert scale and three open-ended questions. A parent version and an equivalent version for young people with PKU were used.

Assessment of Dietary Intake

A 3-day diet diary, with a follow up interview was used to assess dietary and supplement intake in both phases (Hackett et al. 1983). The quality of 3-day dietary information was not of sufficient quality to formally report results; however, it was used during consultations with the dietitian.

Phase One

Diet Chart Development

An interim diet chart was developed, before moving to the final and freer counting system in phase 2, to confirm that metabolic control would not be compromised. The phase 1 diet chart was developed in-house using the basis of 1 g of protein as equivalent to 50 mg Phe and permitted all foods

that contained less than 20 mg Phe/serve to be uncounted. Where the Phe content per serve was greater than 20 mg the protein content was counted using 0.5 g increments. This was in contrast to the usual 15 mg Phe unit system where foods containing 7.5 mg Phe per serve were counted as a 0.5 unit. The change in cut-off value resulted in new foods, particularly fruit and vegetables becoming "free." The new "protein" diet chart was written in the same format as the old unit diet chart. Food was listed in grams of protein per serve of a particular food and amount of the food in grams that contained 1 g of protein. Parents and children were taught to use values directly from the nutritional panel for commercial foods and to round protein values up and down. This differed from the unit system where parents and children had to be taught to multiply the protein value on the nutritional panel by 3 to obtain the number of units in a particular food contained. Free commercial snack foods were limited to <0.3 g of protein/serve, due to the ease of being able to eat large serves of these foods compared to fruit and vegetables. Nutritional information was sourced from food tables with amino acid composition information (NUTTAB 1995; Holland et al. 1991)

Participants

All parents and participants over 1 year of age currently attending the Women's and Children's Hospital Metabolic Unit for early treated PKU, were invited by telephone, letter or personal approach to participate in the study. Eighteen of 21 parents gave informed consent for their child to participate, 5 males and 13 females, with a median age of 10 years 1 month (range 2 years 5 months to 17 years 6 months) (Fig. 1). Sixteen participants had the classic PKU phenotype and two had the moderate PKU phenotype. (Medical Research Council Working Party on PKU 1993). All participants and parents had been previously educated on the 15 mg Phe unit system and were on phe-free protein supplement for the treatment of PKU. The study was approved by the Women's and Children's Hospital Research Ethics Committee.

Study Protocol

Before randomization all participants completed a baseline 3-day diet diary, including protein supplement intake and our PKU Diet Attitudes Questionnaires. Participants with

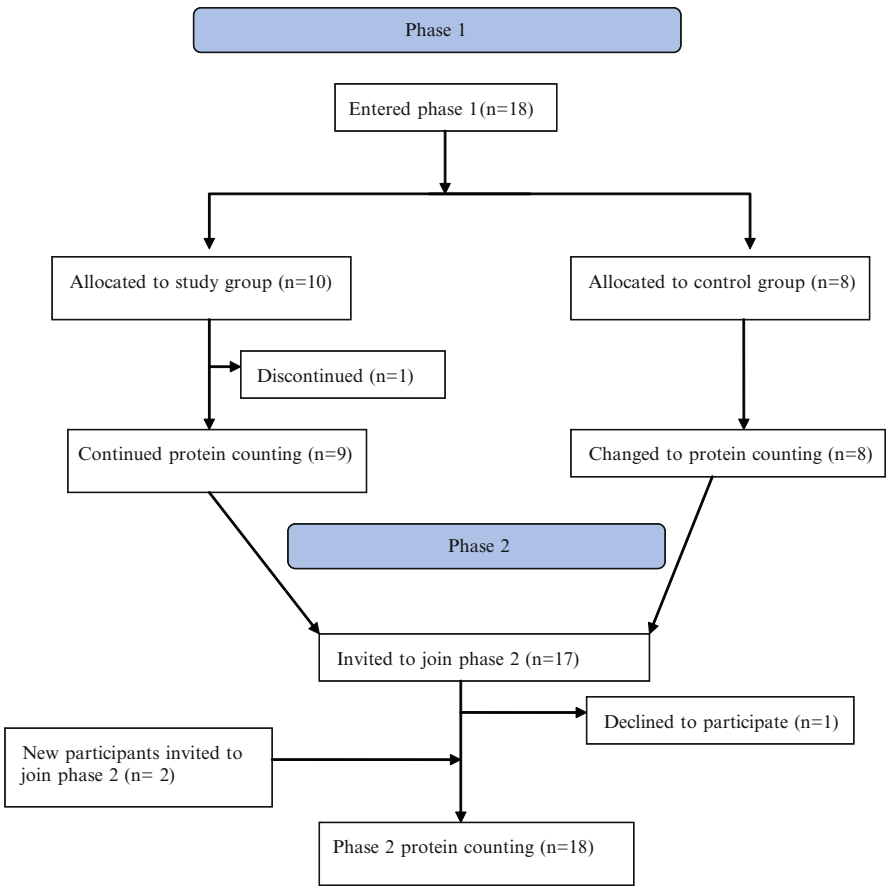


Fig. 1 Methodology of allocation of participants in phases 1 and 2

classic PKU were randomly assigned to two groups using a computer generated randomization schedule with balanced blocks of 4 to two groups: either to continue their current Phe exchange system (control group) or change to counting protein exchanges (study group). Two participants with moderate PKU who had difficulty with the PKU diet were allocated to the study group.

All participants had an individual appointment with the same dietitian, separate from their regular clinic appointment, for re-education on dietary management. The study group were educated on the gram protein counting system at this time.

Clinic visits were unchanged – at these visits weight and height, protein supplement, and dietary information were recorded and appropriate changes made. The protein supplement is recommended to be taken throughout the day, as timing of taking the supplement has been shown to affect plasma Phe (MacDonald et al. 1996; MacDonald et al. 2004). Weekly home blood phe monitoring, at a regular time that suited the family, continued as usual (van Spronsen et al. 1993).

There was no change in the method of reporting blood results (phone call, letter, or email) and no other changes were made to the management in either group of patients.

Six months after the initial appointment, or when families had been using the new counting method for at least 6 months, questionnaires and diet diaries were completed for both groups and were analysed along with blood Phe results. The control group were then educated on the phase 1 diet counting method (Fig. 1). Six months after the control group had used the new counting method, questionnaires and diet diaries were completed.

Phase Two

At the conclusion of phase 1 of the study, the second phase of the study commenced, to assess the impact on blood Phe of a further liberalization of uncounted foods.

Diet Chart Development

The study diet chart used in Phase 1 was modified to test whether a further liberalisation of uncounted foods to 50 mg Phe per serve would have a detrimental effect on blood Phe levels. In addition,

- Foods containing 40–50 mg Phe/serve were considered to be free but were given a serve size limit
- Foods containing more than 50 mg Phe/serve were counted in 0.5 g increments

The Phase 2 diet chart therefore had an extensive list of free foods, particularly for fruits and vegetables. The phase 2 diet chart was written in the same format as the phase one diet chart. Information on reading nutritional panels on commercial foods was given and commercial snack foods were again limited to those <0.3 g of protein/serve considered free. Nutritional information was again sourced from food tables with amino acid composition information, (NUTTAB 1995; Holland et al. 1991).

Participants

Participants ($n = 17$) from phase 1 were invited by telephone, letter or personal approach to participate in phase 2 of the study; one declined. Two participants with early treated classical PKU (previously educated on the 15 mg Phe unit system) were now eligible to participate as they were over 1 year of age. In total, 18 participants (16 classic PKU, 2 moderate PKU) were enrolled in phase 2; 13 females and 5 males with a median age of 11 years, 6 months, (range 1 year 7 months to 20 years 3 months) (Fig. 1).

Study Protocol

All participants completed a baseline 3 day diet diary, including protein supplement intake and the PKU Diet Attitudes Questionnaires prior to re-education on the updated counting method at an individual appointment, separate from their regular clinic appointment, by the same dietician. As in phase 1, no other changes were made to the management of the participants. Six months after the education session questionnaires and diet diaries were completed.

Statistics

For both phases 1 and 2, all blood tests results and questionnaires for the 6 months prior to the education session and the first 6 months after the educations session were analysed using a non-parametric statistic, the Wilcoxon Signed Rank Test. SPSS for WINDOWS version 10.0 (SPSS Inc., Chicago, IL, USA) at a statistical significance of $p < 0.05$.

Results

Phase One

Of the 18 phase 1 participants, 10 were allocated to the study group, [3 males, 7 female, median age 10 years 5 months, (range 2 years 5 months to 15 years)]. One young mother of a toddler declined the allocation to the new diet because of lack of confidence. Data was analysed

Table 1 Blood Phe results for the nine participants, pre and post change to the phase one new counting method

No.	Age	Prescribed units	Prescribed dietary Phe (mg)	Phe pre[a] 6/12 Mean +/− SD	Prescribed protein (g)	Phe post[a] 6/12 Mean +/− SD	Sign
1	9.5	17.0	255	517 +/− 270	5.0	660 +/− 244	0.108
2	12	24.0	360	570 +/− 371	7.0	728 +/− 363	0.149
3	12.5	17.0	255	723 +/− 166	5.0	737 +/− 351	0.776
4	15	25.0	375	463 +/− 136	7.5	516 +/− 136	0.173
5	5	25.0	375	251 +/− 57	7.5	307 +/− 131	0.272
6	7.5	20.0	300	359 +/− 255	6.0	425 +/− 208	0.449
7	2.5	21.0	315	202 +/− 117	6.0	247 +/− 99	0.126
8	12	u/c	u/c	463 +/− 100	28.0	488 +/− 64	0.583
9	11	u/c	u/c	499 +/− 104	25.0	505 +/− 87	0.893

[a] μmol/l

from the remaining 9 study subjects (2 males, 7 females, median age 11 years 5 months, range 2 years 5 months to 15 years) and 8 control subjects. (Fig. 1)

Blood Phe Levels

Table 1 summarises the Phe results for the nine participants in the initial study group. For each participant, there was no significant difference between the Phe levels in the 6 months prior to the study and the 6 months of the new counting method. Participants 8 and 9 had moderate PKU and did not currently use the unit system of counting but avoided animal protein and limited bread and cereal serves. They reported that they found the protein counting system easier than just avoidance of foods as they now had a guideline of the quantity they should be eating of certain foods and felt more confident in their foods choices.

Table 2 summarises the Phe results for the eight participants in the control group for whom there was no change to their dietary counting method in the pre- to post -6 month periods There was no significant difference in blood Phe levels during these two 6 month periods.

As there was no detrimental effect on blood Phe and there was positive acceptance of the new counting method by participants and their families the control group were educated on the new diet counting method. At the time of change from control to phase 1 diet chart the median age of the group was 9 years 11 months (range 4 years to 18 years 2 months) (Fig. 1). Table 3 summarises the Phe levels for these participants, pre and post the change to the protein counting method. One participant had a significant improvement in their Phe levels and the other seven participant's Phe levels were unchanged.

Questionnaire Results

Every participant from the first group of 8 families, children, and parents indicated that they preferred the protein counting method. Written comments from the first group of 8 parents and children to change counting method described the protein counting method as easier, with smaller numbers to count and thus considered easier to track. Comments were also made that they didn't need to be as precise with all foods, that "free" foods were used

Table 2 Blood Phe results for the eight control group participants during the phase one initial study (no change made to diet chart)

No.	Age	Prescribed units	Prescribed Phe (mg)	Phe pre[a] 6/12 Mean +/− SD	Phe post[a] 6/12 Mean =/− SD	Sign
10	11	38.0	570	357 +/− 288	341 +/− 233	0.693
11	12.5	36.0	540	321 +/− 121	270 +/− 132	0.300
12	18.5	20.0	300	450 +/− 189	512 +/− 183	0.249
13	11	26.0	390	552 +/− 151	557 +/− 183	0.460
14	5.5	16.0	240	231 +/− 240	119 +/− 68	0.104
15	3	14.0	210	460 +/− 226	390 +/− 261	0.322
16	4	14.0	210	252 +/− 131	275 +/− 166	0.477
17	6.5	20.0	300	249 +/− 167	254 +/− 94	0.705

[a] μmol/l

Table 3 Blood Phe results for the eight (initial control) participants, pre and post change to the new counting method

No.	Age	Prescribed units	Prescribed Phe (mg)	Phe pre[a] 6/12 Mean +/− SD	Prescribed protein (g)	Phe post[a] 6/12 Mean =/− SD	Sign
10	11	38.0	570	402 +/− 207	11	237 +/− 181	0.008*
11	14	36.0	540	350 +/− 132	11	405 +/− 197	0.300
12	18	20.0	300	561 +/− 158	6	505 +/− 80	0.799
13	12	26.0	390	609 +/− 199	7.5	553 +/− 111	0.056
14	6.5	16.0	240	67 +/− 66	5	81 +/− 73	0.289
15	4	14.0	210	318 +/− 434	4	178 +/− 123	0.465
16	4.5	14.0	210	274 +/− 155	3.5–4	215 +/− 145	0.260
17	8	20.0	300	232 +/− 104	6	275 +/− 88	0.778

*$p = <0.05$

[a] μmol/l

particularly as snacks, with counted protein often kept for meals. Parents noted that commercial products were easier to use and that their children could now understand and read labels now, and were more interested in their diet.

Total scores for all phase one participants on the PKU Diet Attitudes Questionnaire for both parents and children were not significantly different pre and post change to the protein counting method (parents pre change mean = 51.6, SD = 6.5, post change mean = 53.8, SD = 5.6, $Z = -1.1$, $p = 0.25$; children pre change mean = 30.7, SD = 5.8, post change mean = 33.1, SD = 5.9, $Z = -1.3$, $p = 0.20$). Parent responses to the question "to what extent is there variety of foods in your child's diet?" indicated a trend towards reporting more variety in their children's diet post change in counting method, ($Z = 0.48$, $p = 0.06$) while children's own report was of significantly more variety in their diet ($Z = -2.45$, $p = 0.1$). All phase one participants preferred the protein counting method.

Phase Two

There were 18 participants enrolled in this phase, 4 participants (3 classic PKU, 1 moderate PKU) have been excluded as they failed to neither provide regular blood tests nor complete all the questionnaires. Complete data was available on 14 participants (9 females, 5 males; median age 9 years 6 months, range 1 year 7 months to 20 years 3 months).

Both parents and children unanimously preferred the freer diet chart.

Blood Phe Levels

Table 4 summarises the blood Phe results for participants in phase 2. In summary, four participant's blood Phe levels were significantly better following the change in diet chart while nine participants showed no significant difference in blood Phe levels. Participant 18's blood Phe levels were significantly higher in the second 6 months period; he was a young child who had a number of illnesses during this period of time. He has continued with the new counting method and subsequent results have been acceptable.

Discussion

Phase One

Researchers and participants were not prepared to change from a strict 15 mg Phe unit system to a free 50 mg Phe exchange system in one step and a two phase study was designed. There was no significant deterioration in the metabolic control of the interim protein counting group compared to their pre-study levels. Qualitative reports were also positive. The protein-counting group reported ease of dietary management, as the number of exchanges was smaller to count, easier to grasp and made using commercial products easier. In addition, some extended family members reported more confidence in assisting with the management of the child with PKU. During phase 1, the dietitian received feedback from some families who had previously counted all foods very accurately, that they found it hard to try the new system and were very anxious about the possible adverse effect on metabolic control. Other families had already not been counting many of the low Phe foods such as salad vegetables and fruits but had been feeling guilty about this. At the end of phase one, the anxious families were reassured enough to try the more liberal diet.

Phase Two

The change to more "free foods" with a protein prescription resulted in stable blood Phe levels of 13 of the 14 participants compared to their previous levels. One participant,

Table 4 Blood Phe results for all study participants, pre and post change to the phase two new counting method

No.[a]	Age	Prescribed grams of protein	Pre 6/12[b] Mean +/− SD	Post 6/12[b] Mean =/− SD	Sign
4	18	5	604 +/− 133	663 +/− 95	0.262
5	8.5	7.5	279 +/− 101	218 +/− 127	0.114
6	10.5	6	657 +/− 120	546 +/− 238	0.272
7	6	7	370 +/− 124	392 +/− 112	0.889
8	15.5	15	605 +/− 146	503 +/− 108	0.029*a
10	15	11	528 +/− 316	328 +/− 167	0.004*a
11	16	10	493 +/− 118	343 +/− 99	0.002*a
12	20.5	5	550 +/− 128	658 +/− 153	0.401
14	9	5–6	285 +/− 145	185 +/− 234	0.071
15	6.5	5	418 +/− 485	324 +/− 340	0.679
16	7.5	4	327 +/− 298	374 +/− 165	0.477
17	10	6	495 +/− 174	318 +/− 159	0.001*a
18	1.5	6	299 +/− 160	456 +/− 151	0.001*b
19	2.5	6	274 +/− 252	277 +/− 178	0.886

*a = $p < 0.05$, based on positive ranks, *b = $p < 0.05$, bases on negative ranks

[a] Number = participant number in phase one

[b] μmol/l

who was ill frequently during this period, had worsening metabolic control while four participant's levels improved. All reported improved ease of dietary management. Qualitative feedback was also positive. Many parents reported a reduction in the burden of food preparation time, as fewer foods needed to be weighed. Most children were reported to have become more involved in their diets. The older children reported using the extensive list of free fruits and vegetables for their snacks, counting protein only at mealtimes which they reported they did more accurately as there were less foods to measure and count. By allowing "free foods" the children were theoretically consuming more Phe than prescribed in the strict unit system. However, as others have reported (MacDonald et al 2003), we found no negative effect on Phe levels. Some children's blood Phe levels remained above the target range; however, they reported they found this gram protein exchange system easier to use.

We did not find any significant changes in the attitudes of young people with PKU and their parents about the preparation of foods, monitoring of the diet, collecting blood tests, metabolic control, stressfulness of the diet, and quality of life, as a result of changes in dietary management, except for a small increase in reports of dietary variety.

It is possible that the imposition on a child and their family's life of a low Phe diet in an Australian setting is so significant that ratings of the overall impact of the diet did not change, although qualitative comments indicated some acknowledgement that this new counting system represented a small but helpful improvement.

A strength of this research was the high participation rate of the families approached, which may have been the result of the close and longstanding relationship the researchers have with the children and parents with PKU who attend this clinic, as well as the interest families have in simplifying dietary management.

The U.K. clinics have been using the 50 mg Phe exchange system for many years and these research findings have validated their method of counting Phe in PKU diets. Following this study, many PKU treatment centres in Australia have changed to counting protein.

As this research was carried out in only one centre, participant numbers were low. Future work should aim for collaborative work across centres. This would also enable consideration of a possible impact of the age of children on the effect of changes to diet management. Although food diaries were completed, they were not accurate enough for quantitative analysis. Future work will need to consider how food diaries are collected and examined. Further research should aim to establish whether these findings are replicated in other cultures and countries and should also aim to evaluation of longer term changes to blood Phe levels.

Since completion of this study, further information has been published by English researchers on Phe content of foods and the effect on Phe levels of allowing these foods freely in the PKU diet (Weetch and MacDonald 2006). Updated information on the Phe content of foods (NUTTAB 2006) continues to be published and emphasises the need for dietitians to continually update and review diet charts.

In our centre, the dietary management of PKU during pregnancy has been informed by these findings and three recent pregnancies in our Unit, for women with classic PKU, have been managed using the phase two diet chart with good metabolic control achieved (Sweeney et al. 2006).

Conclusion

Protein exchanges (foods containing less than 50 mg Phe/serve uncounted) are an alternative method of measuring Phe intake in the dietary management of PKU. All participants found protein counting easier to manage compared to the traditional Australian 15 mg Phe exchange system and continue to use this method of counting the Phe in the PKU diet. Some participants have continued to count protein for 8 years with good metabolic control, indicating sustainability of this counting method. Counting protein exchanges is now is now used for all PKU patients including maternal PKU in South Australia.

Acknowledgements The authors are very grateful and thank the children and their parents from the W.C.H. Metabolic Clinic for participating in the 2 phases of this study.

We also thank Dr. Maria Makrides (Child Health Research Institute, WCH Adelaide) for advice on study design and for the randomization schedule.

This research was supported by Nutricia Australia; none of the researchers had a financial or personal interest in Nutricia Australia

Take Home Message

Protein exchanges with foods containing less than 50 mg Phe/serve uncounted is an alternative method of measuring Phe intake in the dietary management of Phenylketonuria and is preferred by participants and their families when compared with a 15 mg Phe unit system.

Details of the Contributions of Individual Authors

A. Sweeney: Study design, planning and conduct, analysis and interpretation of data, and writing of manuscript.

R. Roberts: Study planning and conduct, interpretation of data, and writing of manuscript.

J. Fletcher: Study design, planning and conduct, interpretation of results, and writing of manuscript.

Author Serving as Guarantor for Article

A. Sweeney accepts full responsibility for the work, had access to the data, and controlled the decision to publish.

Details of Funding

This research was supported by SHS Nutricia Australia. The authors confirm independence from SHS Nutricia Australia. There was no control over design of study, interpretation or reporting of results. The content of the article has not been influenced by the sponsor.

Details of Ethics Approval

This study was approved by the Women's and Children's Hospital Research Ethics committee. Approval number 1270/12/2007.

Patient Consent Statement

Informed consent to participate in this study was obtained for all participants (or parents of participants if participant was younger than 18 years).

References

Awiszus D, Unger I (1990) Coping with PKU: results of narrative interviews with parents. Eur J Pediatr 149(Suppl 1):S45–51

Australasian Society for Inborn Errors of Metabolism (1996) PKU Handbook. Human Genetic Society of Australasia, Surry Hills, NSW, Australia

Cockburn F, Clark BJ (1996) Recommendations for protein and amino acid intake in PKU patients. Eur J Pediatr 155(Suppl 1):S125–129

Hackett AF, Rugg-Gunn AJ, Appleton DR (1983) Use of dietary diary and interview to estimate the food intake of children. Hum Nutr Appl Nutr 37A:293–300

Holland B, Welch AA, Unwin ID, Buss DH, Paul AA, Southgate DAT (1991) McCance and Widdowson's the composition of foods, 5th edn. Royal Society of Chemistry and Ministry of Agriculture, Fisheries and Food, Cambridge

Ievers-Landis C, Hoff AL, Brez C, Cancilliere MK, McConnell J, Kerr D (2005) Situational analysis of dietary challenges of the treatment regimen for children and adolescents with phenylketonuria and their primary caregivers. J Dev Behav Paediatr 26:186–193

MacDonald A, Rylance G, Hall SK, Asplin A, Booth IW (1996) Factors affecting the variation in plasma phenylanaline in patients with phenylketonuria on diet. Arch Dis Child 74:412–417

MacDonald A (2000) Diet and compliance in phenylketonuria. Eur J Pediatr 159(Suppl 2):S136–S141

MacDonald A, Rylance G, Davies P, Asplin D, Hall SK, Booth IW (2003) Free use of fruit and vegetables in phenylketonuria. J Inherit Metab Dis 26:327–338

MacDonald A, Dayly A, Davies P et al (2004) Protein substitutes for PKU: what's new? J Inherit Metab Dis 27:363–371

Medical Research Council Working Party on PKU (1993) Arch Dis Child 68:426–427

NSPKU (2005/2006) The National Society for Phenylketonuria (United Kingdom) Ltd. Dietary information for the treatment of phenylketonuria

NUTTAB (1995) Nutrient tables for use in Australia. Food Standards Australia and New Zealand (www.foodstandards.gov.au)

NUTTAB (2006) Nutrient tables for use in Australia. Food Standards Australia and New Zealand (www.foodstandards.gov.au)

Sweeney AL, Ketteridge DB, Ranieri E, Fletcher JM (2006) Managing PKU pregnancies using a low protein diet (abstract). J Inherit Metab Dis 29(Suppl 1):39

van Spronsen FJ, van Rijn M, van Dijk T et al (1993) Plasma phenylananine and tyrosine responses to different nutritional conditions (fasting/postprandial) in patients with phenylketonuria: effect of sample timing. Pediatrics 92:570–573

Vetrone P, Leuzzi V, Zazzara V, Antonozzi I (1989) Psychological effects on parents of children with early detected Phenylketonuria. J Inher Metab Dis 12:345–6

Weetch E, MacDonald A (2006) The determination of phenylalanine content of foods suitable for phenylketonuria. J Hum Nutr Diet 19:229–236