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Severe Neonatal Holocarboxylase Synthetase Deficiency in West African Siblings

Mauricio De Castro · Dina J. Zand ·
Uta Lichter-Konecki · Brian Kirmse

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Abstract In multiple carboxylase deficiency (MCD), the biotin-dependent carboxylases have decreased activity due to either biotinidase deficiency or holocarboxylase synthetase (HS) deficiency. We report the case of two siblings from Ghana, the first of which presented shortly after birth with profound lactic acidosis and a urine organic acid profile consistent with MCD. In the first sibling, treatment with pulverized biotin tablets (20 mg) was begun immediately, but the patient died at 10 days of age from cardiac arrest secondary to refractory metabolic acidosis. Autopsy revealed a biotin bezoar. Sequencing of *HCLS* showed homozygosity for a novel missense variant (p.G241W). The second sibling had a similar presentation at birth: severe metabolic acidosis and respiratory distress. A urine organic acid profile was consistent with HS deficiency; he was treated with biotin powder (20 mg), and after 24 h, the lactate decreased significantly; by day 5 of life, the patient was tolerating 40 mg of biotin, feeding by mouth and off all other medications and support. This is the first report of the p.G241W mutation. To our knowledge, this is also the first mutation described in West African patients with HS deficiency and the cases demonstrate that it is biotin responsive. Additionally, our experience suggests that the

powdered form of biotin supplementation may be more digestible than tablets for the treatment of severe neonatal HS deficiency.

Introduction

Multiple carboxylase deficiency (MCD) (OMIM#253260, #253270) is a disorder of organic acid metabolism caused by a defect in the biotin pathway. Signs and symptoms include severe, refractory metabolic/lactic acidosis, seizures, hypotonia, respiratory distress, ataxia, impaired consciousness, skin rash, and alopecia (Wolf 2000). Biochemical findings include hyperammonemia, elevated lactic acid, and a specific pattern in urine organic acids including elevations of 3-OH-propionic acid, lactate, and 3-methylcrotonylglycine (Suormala et al. 1998). Clinically, MCD can be divided into an infantile/juvenile form, caused by the inability to recycle endogenous biotin due to biotinidase deficiency (*BTD*) (EC 3.5.1.12), and a neonatal form, caused by holocarboxylase synthetase deficiency (*HLCS*) (EC 6.3.4.11). This distinction is not always clear-cut as patients with HS deficiency can present later in childhood (Sakamoto et al. 2000).

Biotin is covalently bound through its carboxyl group to an inactive apocarboxylase forming in the process the active holoenzyme (Moss 1971); this process is catalyzed by *HLCS*. Additionally, biotin has been found to regulate gene expression (Rodriguez-Melendez and Zempleni 2003), biotinylate histones (Narang et al. 2004), and play a role in systemic processes such as development and immunity (Baez-Saldaña et al. 1998). In the USA, biotin is available in tablet and capsule form. No intravenous form of the drug is available.

We report the case of two West African siblings that presented with severe neonatal HS deficiency and had

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divergent outcomes. They were found to have a novel missense mutation in the *HLCS* gene.

Subjects, Methods, and Results

Case 1

The first patient is a 39-week-old male born to a 36-year-old mother and a 34-year-old father; both are originally from Ghana. The patient was 3,850 g at birth and had a crown-heel length of 53 cm.

Thick, meconium stained fluid was noted during delivery; deep suctioning was not performed, as the baby appeared vigorously active immediately. Apgars at 1 and 5 min were 8 and 9, respectively. The patient was transferred from the delivery room to the newborn nursery for routine care. In the nursery, bi-basilar crackles and increased respiratory effort were noted, a chest X-ray at the time showed patchy infiltrates. On DOL 2, the patient was promptly transferred to the Intensive Care Unit where an arterial blood gas showed a pH of 7.0 and a base deficit of -26 ; in light of the profound acidosis, metabolic testing was performed (acylcarnitine profile, free/total carnitine, urine organic acids, and plasma amino acids).

The patient continued to deteriorate requiring ventilatory support, total parenteral nutrition, and antibiotics for suspected sepsis. The acylcarnitine profile and urine organic acids showed elevations in C-3 and C5-OH and propionate and 3-MCC, respectively; the presumptive diagnosis of MCD was made. The patient was started on biotin supplementation. The form of biotin available in the hospital at the time was tablets, which were crushed at the bedside using a pill crusher and administered via NG tube (20 mg QID). Over the next 3 days, the patient's acidosis failed to improve and seizures ensued. He was placed on several antiepileptics without improvement; a bicarbonate infusion and vasopressors were started as well. Over the next 24 h, the patient developed profound encephalopathy (EEG showed burst suppression pattern with 4–6 s bursts of medium to high-voltage sharp activity interspersed with 10 or more second interval of background suppression) and liver and kidney failure. On DOL 9, he developed bradycardia, followed by pulseless electrical activity; CPR was performed for 10 min, but the patient died.

Autopsy revealed several changes consistent with MCD including changes in skeletal muscle resembling ragged red fibers (observed in metabolic disorders), cystic changes in the brain, and fatty infiltration of the liver. Other gross pathologic findings included multiple areas of liquefactive necrosis in the brain indicating previous infarctions, an enlarged heart weighing 24.6 g (vs. expected 19 g) and a thrombus in a medium-sized pulmonary artery with an area

of infarction. In the stomach, a 69 g bezoar of yellow material was found. The mass was soft and gritty, with no specific odor; there were similar findings in the lung, which showed areas in which the airways were filled with a nonstaining material similar to the material in the stomach. Microscopic analysis of the mass revealed a faintly basophilic material with the appearance of folded string; the same substance was found lining the gastric and intestinal mucosa and to be the main component of the large mass found in the stomach. The identification of the bezoar was made by a pediatric forensic pathologist at Children's National Medical Center; based on the macroscopic (the color was similar to the tablets) and histological appearance, the pathologist felt certain the bezoar was made of biotin. The material was not only found in the stomach but also in the airways, suggesting aspiration of the material as well. The child had been on NPO; otherwise, the only enteral medication he received was the biotin.

A homozygous mutation in *HLCS* was found in exon 5: c.721G>T (p.G241W) by Sanger sequencing from the Emory Genetics Laboratory (Atlanta, GA).

Case 2

The second patient is a 39-week-old male born via vaginal delivery 8 years after the birth of his deceased brother. The parents had 2 healthy, non-affected children in the intervening years. The patient weighed 2,640 g and had Apgars of 4 and 8 at 1 and 5 min, respectively.

The delivery was complicated by presence of meconium and a nuchal cord, the patient was grunting and tachypneic in the nursery, and he was transferred to the NICU on DOL 1 shortly after birth. An arterial stick showed significant metabolic acidosis (pH = 7.26 and base deficit of -20); basic laboratory work showed hyperammonemia, elevated CK, and hyperlactatemia. Due to his brother's diagnosis 8 years before, a diagnosis of *HLCS* deficiency was suspected. The patient was started on a bicarbonate infusion and biotin supplementation at 5 mg PO QID (20 mg daily). Over the next 24 h (DOL 2), the patient's clinical course worsened to include seizures and worsening acidosis. He required CPAP and dopamine infusion.

Preliminary results of the acylcarnitine profile showed severe elevations of C3 and C5-OH; the urine organic acid panel revealed large peaks of propionyl and methylcrotonyl metabolites; all findings consistent with MCD. Since this particular mutation had not been described in the literature, we had presumed this to be a biotin-unresponsive case (Mayende et al. 2012). Because of his brother's history of bezoar, we treated with powdered biotin, 20 mg divided QID. Six hours later, the lactate had decreased from the maximum linearity of the instrument (>20 mg/dL) to 17 mg/dL. Over the next 24 h (DOL 3), the patient was

weaned off hemodynamic support and the lactate level dropped below 10 mg/dL, he was taken off CPAP, and oral feedings were begun. Given his response to biotin, we decided to increase his dose from 20 to 40 mg/day. On DOL 4, the patient was completely off all support; lactate was 1.5 mg/dL and was vigorously bottle-feeding. Brain MRI showed changes consistent with MCD: bilateral caudothalamic and intraventricular germinal matrix hemorrhages, bilateral frontal and anterior temporal horn subependymal cystic lesions and generalized signal abnormalities throughout the white matter. The patient was discharged from the hospital 10 days after birth and is currently 9 months old. On follow-up, he has continued to do well on 40 mg of biotin and 150 mg/kg/day of levocarnitine supplementation; he is closely followed by Medical Genetics and Neurology. His mutation was confirmed via clinical Sanger sequencing, homozygous for c.721G>T, p.G241W.

Discussion

To our knowledge, this is the first time that the p.G241W mutation has been reported and the first mutation described in West African patients. The mutation appears to be biotin responsive.

The exact incidence of HS deficiency is not known and varies between populations. It has been estimated to be less than 1:100,000 in Japan (Narisawa et al. 1982) and as high as 1:10,000 in the Faroe Islands. Studies describing mutations in African populations are limited (Yang et al. 2001). Although the age of presentation can be variable, more than half of cases of HS deficiency manifest in the newborn period with the rest of the cases being reported in children months after birth and in rare cases even up to age 8 (Sakamoto et al. 2000). Most cases respond to oral biotin therapy; in some patients, however, there seems to be progression of the disease even with doses of biotin as high as 200 mg/day (Baumgartner and Suormala 1997; Wilson et al. 2005). These particular cases, dubbed “biotin unresponsive,” are limited to a handful of mutations known to affect the N-terminal domain of the protein (Mayende et al. 2012).

Some mutations are limited to specific ethnic groups. IVS10+5G>A, a common western European mutation, has been determined via polymorphic microsatellite marker identification to come from the Faroe Islands (Yang et al. 2001). The mutations p.L237P and c.782delG are founder mutations in the Japanese (Sakamoto et al. 1998). Other mutations such as p.R508W and p.V550M have been seen across different ethnic groups and belonging to different haplotypes hinting to a recurrent mutation mechanism for these particular changes (Yang et al. 2000).

In these two cases, the mutation p.G241W replaces a highly conserved (through Baker’s yeast) glycine with a tryptophan in position 241; the Grantham score (a quantitative measure of the chemical dissimilarity between two aminoacids) between them is high at 184 (0–215), representing a large physicochemical difference. Of the multiple available transcripts for the *HLCS* gene, we used NM_000411.6 for our analysis, used as the reference transcript in HGMD. The p.G241W mutation localizes to exon 5 which is the largest and one of the most commonly affected exons; other well-characterized exon 5 mutations include p.L237P, c.782delG, and c.655dupA. It is interesting to note that p.L237P and c.782delG are the most common mutations in the Japanese population and as such have been well characterized; these mutations are known to abolish enzyme activity (Yang et al. 2001). The p.L237P mutation is described in the literature as a non-Km mutant as it does not affect the putative biotin binding domain, and recent research showed that mutations at this site (and more generally between amino acids 159–314) affect a structured domain present in the N-terminal portion of the protein abolishing *HLCS* activity by affecting the rate of dissociation from its substrate (Mayende et al. 2012). The clinical presentation in our patients was similar to that observed in Japanese patients with the p.L237P mutation, that of severe, neonatal form responsive to biotin.

Finally, we do not have a satisfactory explanation as to why the first patient had a fatal outcome or whether the bezoar played a role. There are no reports in the literature of a biotin bezoar in general, let alone in MCD. Pharmacobezoars are unusual entities and have been described for a number of drugs; their formation is usually associated with enteric-coated drugs or presence of binding agents (Stack and Eapen 1995). The available literature on the subject states that the cause for the majority of cases of pharmacobezoars is GI obstruction and/or decreased motility. Many factors are known to affect the rate of absorption of a drug: dissolution rate, particle size, solvates, ionization state, and drug pK_a . Tablets are more likely to form bezoars owing to their more difficult disintegration and absorption, while finely granulated powder has improved dissolution and absorption (Shargel and Andrew 1999). In the first sibling, we suspect some degree of gastroparesis owing to the critical severity of his illness in addition to the intrinsic characteristics of the tablet (presence of binding) contributed to the formation of the bezoar.

It is also possible that the first sibling’s more refractory clinical course was due to a relative delay in the initiation of biotin. Biotin was started as soon as the presumptive diagnosis was made in both cases but was on DOL 2 for the first sibling and on DOL 1 for the second sibling. This 24 h delay could have theoretically played a role in the first

siblings having been farther along the course of multisystem critical illness and thus less responsive to therapy.

In conclusion, we present the case of two brothers from West Africa with HS deficiency caused by a novel missense mutation that is responsive to biotin clinically. Whenever available, we suggest using the powdered (capsule) form of biotin for the treatment of neonatal HS deficiency.

Synopsis

The *HLCS* mutation G241W is biotin responsive, and powdered biotin may be more bioavailable than crushed biotin tablets for the treatment of neonatal HS deficiency.

Compliance with Ethics Guidelines

Conflict of Interest Statements

Mauricio De Castro, Dina J. Zand, Uta Lichter-Konecki, and Brian Kirmse declare that they have no conflict of interest.

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

Details of the Contributions of Individual Authors

Mauricio De Castro was involved in the management of the second patient in the report and wrote the main manuscript.

Dina J. Zand was involved in the management of the first patient in the report and contributed to the manuscript.

Uta Lichter-Konecki was involved in the management of the first patient in the report and contributed to the manuscript.

Brian Kirmse was involved in the management of the second patient in the report and contributed to the manuscript.

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Expanding Our Understanding of Lower Urinary Tract Symptoms and Incontinence in Adults with Pompe Disease

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Abstract Objective: To study the prevalence of lower urinary tract symptoms (LUTS) and incontinence in late-onset Pompe disease (LOPD)

Methods: Adult LOPD patients seen at the Duke Pompe Clinic were prospectively recruited and asked to complete validated questionnaires on LUTS and incontinence as part of an IRB-approved study. Patient demographics as well as previous urologic history were reviewed.

Results: 35 patients with LOPD were included in the study (17 males and 18 females). The median age was 51.8 (range 18–72 years of age). Of these patients, 27/35 were receiving enzyme replacement therapy (ERT) with median duration of 54 months (range 5–88 months). In the male patients, 9/17 (53%) described their stream as dribbling, weak, or intermittent, and 9/17 (53%) complained of post-void dribbling. In addition 38% of the men were unable to stop their urination midstream. In the female patients, the most common complaint was urinary incontinence, reported in 14/18 (78%). In addition, 7/18 (39%) com-

plained of post-void dribbling, and 47% were unable to stop their urination midstream. Bowel incontinence was reported in 45% of patients. There was a significant association between urinary symptoms and lower extremity function scores and duration of ERT ($p = 0.005$ and $p = 0.04$, respectively)

Conclusions: This is the first study in a large cohort of LOPD patients that demonstrates LUTS and incontinence occur at a high rate. This study emphasizes the spectrum of LOPD is beyond isolated gross motor and pulmonary involvement and has a significant effect on the lower urinary tract.

Introduction

Pompe disease (glycogen storage disease type II) is an autosomal recessive disorder caused by mutations in the gene that encodes alpha-1,4-glucosidase (GAA). The incidence of Pompe disease across the disease spectrum has been reported to be 1 in 40,000 (Martiniuk et al. 1998). Late-onset Pompe disease (LOPD) can present anytime from after the first year of life to as late as the sixth decade, with considerable phenotypic heterogeneity. The most common complaints are progressive weakness in a limb-girdle distribution and up to 30% may present with respiratory issues (Hagemans et al. 2005).

Glycogen accumulation within the skeletal muscles as well as the organs containing the smooth muscle (the bladder, intestine, and esophagus) has been demonstrated in autopsy reports (Hobson-Webb et al. 2012). These findings support the various clinical symptoms of the disease seen in this population such as urinary and fecal incontinence, dysphagia, gastroesophageal reflux, and gastrointestinal dysmotility. Urinary incontinence has been previously

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described in other neuromuscular and neurological disorders but has not yet been studied within the LOPD population (van der Walt et al. 1987; Kobayashi et al. 2010; Fidzianska et al. 2011). There are case reports that look at incontinence, mainly fecal incontinence, with relationship to enzyme replacement therapy (ERT) (Remiche et al. 2012).

The goal of this study was to systematically collect validated patient questionnaires that evaluated lower urinary tract symptoms (LUTS), urinary and fecal incontinence, as well as patient demographics and disease severity to better understand the prevalence of LUTS and incontinence within this population. We hypothesized that the glycogen accumulation in this disease process has a deleterious effect on urinary function in adults with LOPD.

Methods

Patient Population

Adult patients with LOPD seen as a part of the Duke Pompe Clinic at Duke University Medical Center were asked to fill out validated questionnaires on LUTS and incontinence. This study was IRB approved, and all patients signed consent prior to data collection.

Questionnaire Details

The following questionnaires were given to each patient according to gender: (1) Male/Female Incontinence Impact Questionnaire, (2) Male/Female Urogenital Distress Inventory, and (3) AUASS (American Urologic Association Symptom Score and Quality of Life). These are validated questionnaires used in the urology and urogynecology literature to evaluate lower urinary tract symptoms and urinary incontinence as well as bother scores. These questionnaires focus on frequency and description of urination, stress and urgency incontinence, and other associated symptoms. They also address impact of urinary symptoms on activities and quality of life. There are questions that ask about previous urologic and incontinence history as well as relevant medications used for LUTS and incontinence. To address other etiologies of bladder complaints, questions were added that assessed urinary tract infections, number of infections, and presence of hematuria. In addition to bladder function, these questionnaires obtain data on bowel function, including the presence of constipation or diarrhea, pain with bowel movements, and problems with losing gas, loss of loose or solid stool, and number of episodes per week.

Medical records of all patients were reviewed for patient demographics, including age and gender as well as urologic history. With regard to Pompe disease status, we reviewed the age at onset of LOPD, the disease duration, treatment with ERT or not, and ERT duration. In order to analyze functional status with respect to urinary continence, the 6-minute walk test (6MWT) results were reviewed for each patient as well as the use of ambulatory assistive devices and upper and lower extremity functional scores rated by a licensed physical therapist (LC) (Personius et al. 1994; Laboratories 2002). We determined pulmonary status of patients by their current use of BIPAP.

Statistical Analysis

We used descriptive statistics to define the study population. Bivariate associations between potentially important clinical factors and the presence of urinary symptoms were evaluated by using Mann–Whitney test for continuous variables and Fisher's exact test for binary variables, and statistical significance was set at $p < 0.05$ on two-tailed analysis (GraphPad Prism 6[®]).

Results

A total of 35 adult patients seen at the Duke Pompe Clinic completed the validated questionnaires in the period April 2012–July 2013. Only three patients with LOPD seen at our clinic did not consent to the study and were not included. Demographics and disease characteristics by gender are presented in Table 1. Of the 35 patients, 17 were male and 18 were female. For the overall cohort, the median age was 51.8 years of age (range 18–72 years old), the median duration of disease was 69.6 months (range 1–244 months), 77% were on ERT, and median duration of ERT was 54 months (range 5–88 months).

In our adult LOPD cohort, 23/35 (65.7%) reported at least one lower urinary tract symptom on the questionnaires. The most frequently mentioned symptoms were urinary incontinence, weak stream, post-void dribbling, and inability to stop urinary stream. The symptoms varied by gender. These results of the questionnaires by gender are shown in Table 2. Males were more likely to report weak stream, post-void dribbling, and inability to stop stream. Females were more likely to report urinary incontinence and fecal incontinence. For the overall cohort, 14/35 (40%) reported weak stream, 16/35 (45.7%) reported post-void dribbling, 12/28 (42.9%) reported inability to stop urinary stream, 19/35 (54%) reported urinary incontinence, 8/35 (22.9%) reported incomplete emptying, and 14/31 (45.2%) reported fecal incontinence. There were no patients in our

Table 1 Demographic characteristics of cohort by gender

Characteristics	Male (<i>N</i> = 17)	Female (<i>N</i> = 18)
Age in years, median (IQR)	50 (20.8)	53.3 (14.2)
Duration of disease, months median (IQR)	54 (68.4)	82.8 (172.8)
ERT, <i>N</i> (%)	16 (94)	11 (61)
Duration of ERT, months median (IQR)	39.6 (44.4)	61.2 (48)
Ambulatory assistance, <i>N</i> (%)	6 (35)	12 (67)
Upper extremity functional score, <i>N</i> (%)		
1	12 (71)	13 (72)
2	2 (12)	3 (17)
3	2 (12)	1 (5.5)
Missing	1	1
Lower extremity functional score, <i>N</i> (%)		
1	9 (53)	4 (22)
2	5 (29)	4 (22)
3	0 (0)	4 (22)
6	2 (12)	5 (28)
Missing	1	1
6 MWT in meters median (IQR)	410.8 (111.4)	320.2 (175.2)
Use of Bipap, <i>N</i> (%)	11 (64.7)	5 (27.8)

Table 2 Results from questionnaire by gender

Questionnaire results	Male, <i>N</i> (%)	Female, <i>N</i> (%)
Weak, intermittent, dribbling urinary stream	9/17 (53)	5/18 (28)
Post-void dribbling	9/17 (53)	7/18 (39)
Inability to stop stream	5/13 (38)	7/15 (47)
Urinary incontinence	5/17 (29)	14/18 (78)
Stress	0 (0)	4 (28.6)
Urge	5 (100)	6 (42.8)
Both	0 (0)	4 (28.6)
Fecal incontinence (loose stool at least 2 times/week)	3/16 (19)	11/15 (73)
QOL: frustration, interference with daily activities, overall bother	7/17 (41)	8/18 (44)

cohort that reported hematuria, and only one patient reported pain with urination. Bladder infections were reported in 5/35 (14.2%) with an average of 2.4 infections/year. Only one patient was on an anticholinergic medication for lower urinary tract symptoms, and no patients reported any surgery related to urinary symptoms. While obstetric history was not obtained, no female patients reported vaginal symptoms. When asked about quality-of-life parameters, 15/35 (42.9%) reported frustration, interference with activities, or overall bother from their urinary symptoms.

On bivariate analysis, factors significantly associated with lower urinary tract symptoms included the following: duration of ERT ($p = 0.04$), lower extremity functional

scores ($p = 0.005$), and the 6MWT ($p = 0.003$). Age, gender, duration of disease, treatment with ERT vs. not on ERT, the use of ambulatory assistance device, and the use of BIPAP were not significantly associated with urinary symptoms on bivariate analysis. The odds ratio of having bowel incontinence in patients with bladder incontinence was 4.1 (95% CI 0.8–20.3, $p = 0.07$). See Table 3.

Discussion

In our cohort, LUTS and urinary and fecal incontinence were seen in a large percentage of adult LOPD patients. Age, gender, duration of disease, treatment with ERT, need

Table 3 Bivariate association of clinical characteristics and urinary symptoms

Characteristics	No urinary symptoms (<i>N</i> = 12)	Urinary symptoms (<i>N</i> = 23)	<i>p</i> -value
Age			
<50	8 (66.7)	7 (30.4)	0.07 ^a
≥50	4 (33.3)	16 (69.6)	
Gender, <i>N</i> (%)			
Male	8 (66.7)	9 (39.1)	0.16 ^a
Female	4 (33.3)	14 (60.9)	
Duration of disease in months, median (IQR)	37.5 (97.5)	87 (111)	0.38 ^b
Use of ERT, <i>N</i> (%)	11 (91.7)	16 (69.6)	0.22 ^a
Duration of ERT in months, median (IQR)	33 (31)	59 (42)	0.04 ^b
Use of ambulatory assistance, <i>N</i> (%)	3 (25)	15 (65.2)	0.07 ^a
LE functional scores ≥3	0 (0)	11 (47.8)	0.005 ^a
6 MWT in meters, median (IQR)	415.8 (124.4)	335 (188.5)	0.003 ^b
Use of BIPAP, <i>N</i> (%)	5 (41.7)	11 (47.8)	1 ^a

^a Fisher's exact test, two tailed

^b Mann–Whitney test, two tailed

for ambulatory assistance, and the use of BIPAP were not significantly associated with the presence of these symptoms. However, duration of ERT treatment, lower extremity functional scores, and the 6MWT were significantly associated with symptoms, and patients with bladder incontinence had higher odds of having bowel incontinence.

To date, there has only been one other study looking at urinary incontinence in this patient population. Remiche et al. described five patients, four of which had fecal incontinence and one that had both urinary and fecal incontinence. In their study, they concluded that patients with LOPD had a higher prevalence of incontinence and that there may be improvement with ERT (Remiche et al. 2012). Our study findings expand the knowledge of the prevalence of urinary symptoms as our data indicates a high prevalence of symptoms across all ages and stages of disease.

The prevalence of urinary incontinence in our cohort was much higher and occurred at a younger age than in the general population. In healthy men, urinary incontinence has been reported in 17% of the population with the prevalence being lowest in men under 69 years of age (11%) and highest in men older than 85 (31%) (Anger et al. 2006). In our study, all five of the men (29.4%) that reported urinary incontinence were 62 years old or younger. Other studies show prevalence of female incontinence to range from 15 to 50% within the community (Minassian et al. 2003; Wallner et al. 2009; Cameron et al. 2013; Matthews et al. 2013). The incontinence rate in our population was much higher at 78%. Lower urinary tract

symptoms (LUTS) are also seen at much lower rates within the community compared to our LOPD population. A large population-based study reported the 5-year incidence of LUTS to be 11.4% (8.5% in men and 13.9% in women) and a prevalence of 20% (Maserejian et al. 2013a, b). In our cohort we saw a prevalence of 52.9% in the men and 77.8% in women. Fecal incontinence has also been shown within the population to have a low prevalence of 4–25% (Halland et al. 2013; Matthews et al. 2013). The prevalence of fecal incontinence in the adult LOPD cohort was much higher at 45.2% overall and 73% when we look just at the females.

Our results indicate that this problem is significantly underdiagnosed and possibly undertreated in this population. The difference in urinary symptoms between genders can be explained by the anatomy of the urinary tract. More men have post-void dribbling and weak stream secondary to the length of the urethra and the involvement of the bulbospongiosus muscle in voiding. There is also the issue of the prostatic enlargement that may lead to urinary symptoms. Women, in general, have higher rates of urinary incontinence and have both stress and urge incontinence as they age, related to the pelvic floor inadequacy. In our group we looked at age and prevalence of urinary symptoms, and this did not differ between those older than 50 years of age and younger than 50.

While this study cannot answer the question of pathophysiology of LUTS and incontinence, we hypothesize that these symptoms are most likely related to the underlying disease process of glycogen deposition in the skeletal and smooth muscles. Autopsy reports have shown glycogen deposition within the smooth muscle of bladder

(Hobson-Webb et al. 2012). This is also demonstrated in the animal model of infantile Pompe disease (Bijvoet et al. 1998). An alternative etiology could be the effect on the autonomic nervous system and peripheral nerves that innervate the lower urinary tract. Pompe disease does affect the peripheral nerves, and glycogen may deposit in the axons, although there is conflicting data (van der Walt et al. 1987; Kobayashi et al. 2010; Fidzianska et al. 2011). The symptomatology may direct us to study the detrusor smooth muscle of the bladder, and skeletal muscles of the internal and external sphincter and the pelvic floor in LOPD, all of which play key roles in voiding and continence. The first step is identifying patients who have urinary symptoms and referring them to the appropriate specialist, urology or urogynecology, and sometimes gastroenterology for fecal incontinence. There are treatment options for the myriad of urinary symptoms that are seen in our LOPD patient cohort.

In addition to describing LUTS and incontinence in this population, we were also able to look at the impact of these symptoms on quality of life. Several studies have looked at QOL in LOPD and have found that it is greatly affected by the disease state (Hagemans et al. 2004, 2005; Wokke et al. 2008). There is also literature that shows a decrease in QOL secondary to urinary symptoms within other populations (Naughton et al. 2004; Basra and Kelleher 2007; Howard and Steggall 2010). This has not been studied before in LOPD, but in our cohort, greater than 40% of patients reported frustration, interference, or bother secondary to urinary symptoms. This is an important measure that can be monitored as patients are treated for LOPD and possibly treated for urinary symptoms. As ERT has been successful in improving survival in this patient population, QOL should be an additional primary end point for treatment. Since the advent of ERT, natural history is emerging and new aspects of the disease are being recognized, including other smooth muscle and skeletal muscle involvement and peripheral nerve involvement.

These data must be interpreted within the context of the study design. This is a small patient cohort; however, it is the largest study looking at LUTS and incontinence in the LOPD population. We believe that this data is generalizable to this specific patient population. Because this is a cross-sectional study, we are unable to make any conclusions about the initiation and role of ERT and relation to urinary symptoms and incontinence. Further study needs to be done to collect longitudinal data to study this relationship. More in-depth evaluation of urinary symptoms by urodynamic and urologic/urogynecologic evaluation may elucidate causes of urinary symptoms and incontinence and may be important in developing treatment options.

Conclusion

This study emphasizes that the spectrum of LOPD is beyond isolated gross motor involvement with pulmonary involvement. The prevalence of LUTS and incontinence are significantly higher in patients with LOPD. Our data indicates that these problems may be significantly underdiagnosed and therefore undertreated. The recognition of these issues in adults with LOPD should be emphasized as treatments for these problems can significantly improve quality of life. Further emphasis on the study of the etiology of LUTS and incontinence in this population is needed in order to direct future treatment.

Synopsis

This study highlights an underdiagnosed problem in a large cohort of LOPD patients and describes the prevalence of urinary symptoms and urinary and fecal incontinence.

Compliance with Ethics Guidelines

Funding source: No external funding was secured for this study.

Conflict of Interest

- Dr. McNamara reports no disclosures.
- Stephanie Austin reports no disclosures.
- Lauren Case has received honoraria from Genzyme Corporation of Sanofi; has participated in research supported by Genzyme Corporation of Sanofi, PTC Therapeutics, the Leal Foundation, Families of SMA, Enobia Pharma Inc./Alexion, the Robertson Foundation, and GlaxoSmithKline; has been awarded grant support from the National Skeletal Muscle Research Center; and is a member of the Pompe Registry Board of Advisors for Genzyme Corporation of Sanofi.
- Dr. Wiener has served as a consultant to Eli Lilly and Company.
- Dr. Peterson has no disclosures.
- Dr. Kishnani has received research/grant support and honoraria from Genzyme Corporation and is a member of the Pompe and Gaucher Disease Registry Advisory Board for Genzyme Corporation.

All procedures followed were in accordance with the ethical standards of the responsible committee on human

experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Authorship: All authors have given final approval of the version to be published.

Erin R. McNamara – Dr. McNamara contributed to collection, analysis, or interpretation of the data as well as drafting the manuscript. This author completed all statistical analysis.

Stephanie Austin – Contributed to collection and interpretation of the data as well as revising the manuscript.

Laura Case – Contributed to interpretation of the data as well as revising the manuscript.

John S. Wiener – Contributed to design and conceptualization of the study and interpretation of the data as well as revising the manuscript.

Andrew C. Peterson – Contributed to design and conceptualization of the study and interpretation of the data as well as revising the manuscript.

Priya S. Kishnani – Contributed to design and conceptualization of the study and interpretation of the data as well as revising the manuscript.

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Carnitine-Acylcarnitine Translocase Deficiency: Experience with Four Cases in Spain and Review of the Literature

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Abstract Background: Carnitine-acylcarnitine translocase (CACT) deficiency is a rare autosomal recessive disease in the mitochondrial transport of long-chain fatty acids. Despite early diagnosis and treatment, the disease still has a high mortality rate.

Methods: Clinical symptoms, long-term follow-up, and biochemical and molecular results of four cases are described and compared with the reviewed literature data of 55 cases.

Results: Two cases with neonatal onset, carrying in homozygosity the novel variant sequences p.Gly20Asp (c.59G>A) and p.Arg179Gly (c.536A>G), died during an intercurrent infectious process in the first year of life despite adequate dietetic treatment (frequent feeding, high-carbohydrate/low-fat diet, MCT, carnitine). The other two cases, one with infantile onset and the other diagnosed in the newborn period after a previous affected sibling, show excellent development at 4 and 16 years of age under

treatment. The review shows that the most frequent presenting symptoms of CACT deficiency are hypoketotic hypoglycemia, hyperammonemia, hepatomegaly, cardiomyopathy and/or arrhythmia, and respiratory distress. The onset of symptoms is predominantly neonatal in 82% and infantile in 18%. The mortality rate is high (65%), most in the first year of life due to myocardopathy or sudden death. Outcomes seem to correlate better with the absence of cardiac disease and with a higher long-chain fatty acid oxidation rate in cultured fibroblasts than with residual enzyme activity.

Conclusion: Diagnosis before the occurrence of clinical symptoms by tandem MS–MS and very early therapeutic intervention together with good dietary compliance could lead to a better prognosis, especially in milder clinical cases.

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Introduction

Carnitine-acylcarnitine translocase (CACT) belongs to the family of SLC25 mitochondrial carriers and catalyzes both unidirectional transport of carnitine and carnitine/acylcarnitine exchange in the inner mitochondrial membrane, allowing the import of long-chain fatty acids into the mitochondria where they are oxidized by the β -oxidation pathway (Palmieri 2008). The mitochondrial oxidation of fatty acids is an important source of energy for humans during prolonged fasting and long-term exercise in the skeletal and cardiac muscles.

CACT is present in the mitochondria of all tissues, particularly in the heart, liver, and skeletal muscle. The CACT protein is encoded by the *SLC25A20* (solute carrier family 25, member 20) gene which has been mapped to

chromosome 3p21.31 and consists of nine exons (Huizing et al. 1997). CACT is the key component of the carnitine cycle which consists of four steps: uptake of carnitine across the plasma membrane by the organic cation carnitine transporter OCTN2, transfer of acyl groups from acyl CoA to carnitine through CPTI in the outer mitochondrial membrane, CACT-mediated transport of acylcarnitines across the inner mitochondrial membrane, and transfer of acyl groups from acylcarnitines to coenzyme A inside the mitochondria through CPTII. Since CACT-mediated transport is an essential step in the long-chain fatty acid β -oxidation pathway, CACT deficiency results in the unavailability of long-chain fatty acids for mitochondrial β -oxidation and ketogenesis.

CACT deficiency (OMIM 212138), first recognized in 1992 (Stanley et al. 1992), is an autosomal recessive disorder presenting in the neonatal period or early infancy with heart problems (cardiac arrhythmia, cardiomyopathy, heart block), muscle weakness, seizures, abnormal liver function, and severe episodes of hypoglycemia and hyperammonemia triggered by fasting or infections. It is one of the more uncommon defects among the fatty acid oxidation disorders; so far, only 55 cases have been reported.

The management of patients consists of fasting prevention with frequent meals, high-carbohydrate intake especially during illness or fever, supplementation of lipids as medium-chain triglycerides (MCT) and essential polyunsaturated fatty acids, and administration of carnitine (Spiekerkoetter et al. 2009).

Here, we report four cases from Spanish pediatric hospitals, two of them with severe neonatal presentation and a fatal outcome, each carrying novel mutations in homozygosity and the remaining two with a good clinical evolution. We have reviewed the clinical, biochemical, and/or molecular data of 55 previously reported cases to better understand the natural history of the disease, focusing on long-term outcome. Despite the improvement in early diagnosis and management in the last decade, CACT deficiency still has a high mortality rate (Spiekerkoetter et al. 2009; Baruteau et al. 2013).

Case Reports

P1, a male born to unrelated parents of sub-Saharan African origin, after normal gestation and delivery (BW 3,050 g), presented at 12 h of life with symptomatic hypoglycemia and hypothermia. Hypotonia, hepatomegaly, biventricular hypertrophic cardiomyopathy, hyperammonemia (312 $\mu\text{mol/L}$), cholestasis and hepatic steatosis (Table 1), increased alpha-fetoprotein (18,000 $\mu\text{g/L}$; normal value <10), and tubular dysfunction with glucosuria and protein-

uria were noted. Plasma amino acids and coagulation studies were normal. Plasma acylcarnitine (ACC) profile showed marked hypocarnitinemia and high C12–C18 species, especially C16 and C18:1. GC-MS analysis of urine organic acids showed increased medium-chain dicarboxylic aciduria. CPT II activity in fibroblasts was normal. Mutation analysis of *SLC25A20* gene revealed homozygosity for a novel mutation, c.59G>A (p.Gly20Asp). Both parents were carriers of the mutation. Feeding was initiated with a medium-chain fatty acid-enriched formula (Monogen©) (90% as MCT and 10% as LCT), carnitine (30 mg/kg/day), and sunflower oil (4 mL/day). Due to persistent free hypocarnitinemia (8.5 $\mu\text{mol/L}$), carnitine was increased to 100 mg/kg/day that normalized the levels. At 100 days due to acute metabolic decompensation. ECG and EcoCG showed no worsening of the cardiomyopathy. Between 5 and 8 months of age, the diet was diversified (fruits, vegetables, meat, and cereals) and supplemented with docosahexaenoic acid (DHA) and soybean oil. The patient died at home at 8 months of age, coinciding with febrile process. Postmortem examination showed pneumonia, cardiomyopathy, and hepatic steatosis.

P2, a female born after normal gestation and delivery (BW 3,100 g) to unrelated Spanish parents, presented with transient tachypnea of the newborn and cutaneous candidiasis in the newborn period. She was asymptomatic until 8 months of age when she was admitted to the hospital after a catarrhal process with duration of 48 h and fever. She presented with generalized tonic–clonic convulsions, hypotonia, irritability, somnolence, hepatosplenomegaly, metabolic acidosis, hypoglycemia, liver dysfunction, high CK (Table 1) and a severe neurological alteration (Glasgow index 10). The cardiologic study was normal. Ophthalmologic examination revealed salt-and-pepper retinopathy. Liver biopsy showed hepatic steatosis. The process slowly reverted after supportive measures with i.v. carnitine administration (150 mg/kg/day) and feeding with a high-carbohydrate, low-fat diet and Monogen©. Plasma ACC profile showed marked hypocarnitinemia and an increase in C14–C18:1. CPT II activity in fibroblasts was normal. The patient was compound heterozygote for two previously described mutations, c.160_163del4ins4 (p.Gly54_Thr55-delinsTrpAla) on the maternal allele and c.532C>T (p.Arg178Term) on the paternal allele. She continues to be treated with a special diet with 30% fat (20% as MCT and 10% as LCT, linoleic acid 3.5%, and linolenic acid 1%), multivitamins, oral carnitine 30 mg/kg/day (persisting deficient plasma free carnitine levels (below 10 $\mu\text{mol/L}$), and avoidance of fasting. Now, at 4 years of age, she is asymptomatic with normal psychomotor and growth development (weight 25th percentile, height 90th percentile, BMI 10–25th percentile).

Table 1 Clinical presentation and biochemical findings at diagnosis in the four CACT patients

	P1	P2	P3	P4**
Age at onset	1 day	8 months	2 days	Asymptomatic
Family history	Unrelated parents Sub-Saharan One healthy sister	Unrelated parents Caucasian	Unrelated parents Moroccan Two healthy brothers Two sisters died at 24 and 48 h of sudden death	Unrelated parents Caucasian One sister died at 5 days
Hypoglycemia	+	+	+	-
Myocardiorhathy	Hypertrophic	No	No	No
Hepatopathy	Cholestasis and hepatic steatosis	Cholestasis and hepatic steatosis	No	No
Additional symptoms		Metabolic acidosis Convulsion Renal failure	Lactic acidosis	
Glucose (mg/dL)	10	54	-	-
AST/ALT/GGT(IU)	346/219/872	1,530/1,021/880	-	-
Bilirubin (mg/dL)	2.1	10.4	-	-
CPK (IU/L)	1,138	1,126	-	-
Ammonia (μmol/L)	312	109	400	-
<i>Free carnitine</i> (μmol/L)				
<1 month (N	7.63	3.59	5	5.7
19.4 ± 7.5)				
>1 month (N 36 ± 8)				
<i>Plasma acylcarnitines</i> (μmol/L)				
C16 (N <0.28)	5.92	11.91	5.35	
C18:1 (N <0.19)	2.89	9.73	1.58	
<i>Urine Organic acids</i> (mmol/mol creatinine)				
Adipic acid (N 2-47)	84	-	-	
Suberic acid (N 1-34)	111	28	81	
Sebacic acid (N 1-22)	152	9	-	
4-OH-phenyllactic acid (N < 14)	105	12	27	
Genotype	p.Gly20Asp*/p.Gly20Asp*	p.Gly54_Thr55delinsTrpAla/p.Arg178Term	p.Asp179Gly*/Falta la p.p.Asp179Gly	p.Gly54_Thr55delinsTrpAla/p.Arg178Term
Follow-up	Exitus 8 months Pneumonia, myocardiorhathy, hepatic steatosis Severe	Alive 4 years	Exitus 12 months Severe gastroenteritis	Alive 16 years
Clinical phenotype	Severe	Mild	Severe	Mild

*Novel mutation

**Patient 4 has been described as patient 3 by Iacobazzi et al. (2004a)

P3 was the fifth child born to unrelated Moroccan parents after a normal gestation and delivery (BW 3,475 g). Two sisters experienced sudden death at 24 and 48 h of life. No studies were performed for the first sister. Postmortem studies in the second sister did not reveal significant abnormalities. At 33 h of life, the proband showed hypoketotic hypoglycemia, lactic acidosis, and hyperammonemia (400 $\mu\text{mol/L}$) (Table 1). Administration of glucose, carnitine, phenylbutyrate, and carbamylglutamate decreased the ammonia levels. The patient was transferred to a neonatal intensive care unit in a referral hospital. The plasma acylcarnitine profile showed marked hypocarnitinemia and increased C12–C18 species, especially C16 and C18:1, and a slight increase in urinary excretion of suberic acid. Sequencing of CPTII and *SLC25A20* genes identified homozygosity for a new *SLC25A20* mutation, c.536A>G (p. Asp179Gly). Both parents were carriers of the mutation. The child was fed orally every 3 h during the day and by a nocturnal continuous nasogastric tube, with a normocaloric, low-fat diet, with medium-chain fatty acid-enriched formula (Monogen©), supplemented with carnitine 30 mg/kg/day. At 2 months of age, his weight was 6.4 kg (p25), length 65 cm (p75), and head circumference 42.5 cm (p50). Psychomotor development and cardiologic study were normal. Due to marked hypocarnitinemia (C0 7.96 $\mu\text{mol/L}$) and a deficiency of PUFAS in the plasma and erythrocytes, carnitine was increased to 80 mg/kg/day and a supplement of DHA (63 mg/day) was added. At 3 months of age, nocturnal enteral feeding was changed to two separate meals, at midnight and 4 a.m., as the parents requested. Between 5 and 9 months, the patient presented with several infections in which an emergency protocol was used. This protocol consisted of i.v. infusion of glucose at a rate of 10 mg/kg/min in order to prevent lipolysis. By that time, a failure to thrive was observed. At 9 months of age, the child was admitted to hospital with cardiac arrest coinciding with respiratory syncytial virus pneumonia, from which he recovered after resuscitation. A port-a-cath catheter was installed at that moment for easy access in case of emergency. Despite this, he died of severe gastroenteritis in a Moroccan hospital at 12 months of age.

P4 has already been described (Iacobazzi et al. 2004a). She is reported here in order to provide updated information regarding her clinical status. In brief, she is a female born after normal gestation and delivery (BW 3,300 g) to unrelated Spanish parents. Her sibling had died at 5 days of life after developing renal failure, hepatosplenomegaly, episodes of ventricular fibrillation and hyperCKemia. Necropsy revealed lipid deposits in different tissues. No further biochemical study was performed. So, this subsequent pregnancy was monitored, and glucose infusion was started soon after birth. The newborn was normal and healthy. Urine organic acid analysis showed mild dicarbox-

ylic aciduria and normal levels of plasma C10:1n-6 and C14:1n-9 fatty acids. However, plasma free carnitine was very low (5.7 $\mu\text{mol/L}$; normal value $36 \pm 7 \mu\text{mol/L}$) with elevated long-chain acylcarnitines (15.9 $\mu\text{mol/L}$; normal value $1.1 \pm 0.35 \mu\text{mol/L}$). CACT enzyme activity in fibroblasts was reduced (6.8% of control). The patient was compound heterozygous for c.160_163del4ins4 (p. Gly54_Thr55delinsTrpAla) and c.532C>T (p.Arg178-Term). Mutations were confirmed in the parents. A low-fat/high-carbohydrate diet with frequent feedings, MCT supplementation, and carnitine (100 mg/kg/day) was started. At 2 years of age, the dose was increased to 150 mg/kg/day, and plasma free carnitine was 19.34 $\mu\text{mol/L}$. She has never had an episode of metabolic decompensation. She had menarche at 11.5 years of age. Now, at 16 years of age, she is completely asymptomatic with normal psychomotor and growth development (weight and height 97th percentile) and excellent school performance.

Results and Discussion

Thirty-one articles describing clinical reports of CACT deficiency were found by searching the PubMed database (Al Aqeel et al. 2003; Al-Sannaa and Cheriyan 2010; Brivet et al. 1996; Chalmers et al. 1997; Costa et al. 1999; Costa et al. 2003; Fearing et al. 2011; Fukushima et al. 2013; Galron et al. 2004; Geven et al. 2007; Huizing et al. 1997, 1998; Iacobazzi et al. 2004a; Iacobazzi et al. 2004b; Korman et al. 2006; Lee et al. 2007; Lopriore et al. 2001; Morris et al. 1998; Niezen-Koning et al. 1995; Nuoffer et al. 2000; Ogawa et al. 2000; Ogier de Baulny et al. 1995; Olpin et al. 1997; Pande et al. 1993; Parini et al. 1999; Pierre et al. 2007; Roschinger et al. 2000; Rubio-Gozalbo et al. 2003; Rubio-Gozalbo et al. 2004; Stanley et al. 1992; Wang et al. 2011; Yang et al. 2001). Data from the literature were compiled, compared with the cases reported here, and discussed.

Clinical and Laboratory Presenting Findings

The literature group consisted of 55 patients from 53 families (Table 2). Consanguinity was reported in 58% and death of siblings, before the diagnosis of the proband, in 60% of cases.

The onset of clinical symptoms was in the first days of life in 28 out of 34 cases, 24 of them in the first week, and only in 6 out of 34 cases symptoms appeared later than 1 month of age. The most frequent initial symptoms reported were hepatomegaly (34%), arrhythmia and/or bradycardia (32%), and respiratory distress (30%). Hypoketotic hypoglycemia (68%) and hyperammonemia (54%) were the most frequent laboratory findings. The suggested mechanism leading to hyperammonemia is urea cycle dysfunction

Table 2 Clinical and laboratory features of 55 CACT reported patients

	Frequency	%
<i>Family history</i>		
Consanguinity	15/26	58
Caucasian	18/43	42
Arab origin	14/43	33
Asian origin	9/43	21
<i>Deceased siblings</i>		
First child	8/20	40
Previous deceased sibling	12/20	60
<i>Age at onset</i>		
Neonatal (<30 day)	28/34	82
>1 month	6/34	18
<i>Presenting symptoms</i>		
Hepatomegaly	17/50	34
Arrhythmia/bradycardia	16/50	32
Apnea/respiratory distress	15/50	30
Myocardopathy	13/50	26
Lethargy/coma	10/50	20
Seizures	7/50	14
Hypotonia	5/50	10
<i>Laboratory findings</i>		
Hypoglycemia	34/50	68
Hyperammonemia	27/50	54
Increased CPK	16/50	32
Increased transaminases	15/50	30
Metabolic acidosis	11/50	22
<i>Outcome (survival) (information only from 43/55)</i>		
Alive 15/43 (35%)		
(*) Cases before 2004: alive 17%	12/15: <5 years	80
(*) Cases since 2004: alive 58%	3/15: 5–9 years	20
Deceased 28/43 (65%)	14/28: <6 months	50
	10/28: 6–12 months	36
	4/28: >12 months	14

(*) In 2004 Rubio-Gozalbo published a review with 25 cases

due to defective *N*-acetyl-glutamate synthesis secondary to an insufficient supply of mitochondrial acetyl CoA and enzymatic inhibition of CPS1 (Brivet 2004; Haberle 2013).

Our four cases were born to unrelated parents of North African or Spanish origin. Two cases (P3–P4) had previous siblings that died in the newborn period due to sudden death or multiorgan failure. Cases P1–P3 presented in the first hours of life with severe hypoglycemia. Although the onset of symptoms began later, at 8 months in P2, she had severe multisystem involvement. P4 never presented symptoms and has been treated since the newborn period due to a previous affected sibling. Hepatopathy was present in cases P1–P2, and myocardopathy only in P1.

Biochemical Findings

In the literature, patients were identified by high levels of plasma long-chain acylcarnitines (C16:0, C18:0, C18:1, and C18:2) and hypocarnitinemia, although this profile is indistinguishable from CPT II deficiency. C6–C10 dicarboxylic aciduria might also be present during decompensation episodes. Our cases (P1–P2–P3) were also identified by deficient plasma carnitine levels and increased C16 and C18:1 acylcarnitines. P4 was diagnosed after a previous affected sibling with a severe hypocarnitinemia and increased long-chain acylcarnitines.

The abnormal blood acylcarnitine profile can be detected by tandem mass spectrometry (MS–MS), allowing the early recognition of this severe disease in the neonatal period. To the best of our knowledge, until now, only two cases have been identified by positive expanded newborn screening (NBS) (Wang et al. 2011; Chien et al. 2013). No clinical data, only the genotype, are available from the first case (Wang et al. 2011). The second case was already symptomatic at the time of diagnosis through NBS (he presented with hyperammonemia and hypoglycemia after birth), and no further data about long-term outcome have been described (Chien et al. 2013).

To confirm the CACT defect, CACT activity was performed in fibroblasts from 31 out of 55 cases. Cases with residual CACT activity (<5%) seem to have a worse outcome than those with >5% activity (Table 3). Oxidation rates of [³H]myristate, [³H]palmitate, and/or [³H]oleate in cultured fibroblasts have also been determined in a few cases in the literature (Table 3). It seems that the surviving cases had a significantly higher oxidation rate of myristate, palmitate, and/or oleate than the deceased cases ($p < 0.002$).

In our cohort, CACT activity was only determined in P4 (residual activity 6.8% of control). In the remaining three cases, CPTII deficiency was first ruled out by enzyme determination in fibroblasts or by gene sequencing; the final diagnosis was achieved by *SLC25A20* gene analysis.

Mutational Findings

A total of 39 mutations have been reported so far in the *SLC25A20* gene which seem to be pan-ethnic (Wang et al. 2011; Indiveri et al. 2011); (HGMD Professional Release 2013.4 www.biobase-international.com). Functional characterization has been performed in very few of them. Mutations are summarized in Table 4, including the two novel changes described in the present study. The most frequently found were the splicing mutation c.199-10T>G in patients from East Asia (Japan, China, Vietnam), and the missense mutation c.713A>G (p.Gln238Arg) in patients of Arab origin, both associated in homozygosity to a severe clinical phenotype. Meaningful phenotype–genotype corre-

Table 3 CACT activity and fatty acid oxidation rates in fibroblasts from CACT patients in relation to the outcome

Outcome (number)	CACT <5% residual activity	Fatty acid oxidation rates (%)		
		[³ H]-myristate*	[³ H]-palmitate*	[³ H]-oleate*
Alive	7/10	15.9 (15.0–37.5)	12.1 (5.70–24.0)	35.0 (25.0–50.5)
<i>n</i> :15		<i>n</i> :8	<i>n</i> :6	<i>n</i> :3
Deceased	20/21	4.0 (2.2–4.4)	4.0 (3.1–5.0)	1.5 (0.6–16.0)
<i>n</i> :28		<i>n</i> :10	<i>n</i> :13	<i>n</i> :5
Statistical test	Fisher; <i>p</i> = 0.086 (NS)	** <i>U</i> = 2; <i>p</i> < 0.002	** <i>U</i> = 5; <i>p</i> < 0.002	** <i>U</i> = 3.5; <i>p</i> < 0.002

*Median (interquartile range)

**Mann–Whitney *U*-test*n* number of cases

lations were limited because of the fact that many mutations were private. Nevertheless clinical phenotype could be elicited from the homozygous and functionally hemizygous patients (Table 4).

We have identified two new homozygous missense mutations, c.59G>A (p.Gly20Asp) in P1 and c.536A>G (p.Asp179Gly) in P3, both of them with a severe clinical phenotype. Both residues of the CACT protein, glycine at position 20, and arginine at position 179 are evolutionarily conserved from frog to human. Both changes were predicted to be damaging by the algorithms Polyphen (<http://genetics.bwh.harvard.edu/pph/>) and SIFT (<http://sift.jcvi.org/>). P2 and P4, two unrelated Spanish cases, both of them with a milder clinical phenotype, presented the same genotype (p.Gly54_Thr55delinsTrpAla/p.Arg178Term). The mutation p.Gly54_Thr55delinsTrpAla has been identified so far in homozygosity in one other Spanish patient with a severe clinical phenotype (Iacobazzi et al. 2004a) and in heterozygosity with c.804delG (p.Phe269Serfs*) in one Cape Indian patient with a late onset of symptoms (Wang et al. 2011; Fearing et al. 2011). The mutation p.Arg178Term has been previously reported in patients of Turkish and North African origin (Rubio-Gozalbo et al. 2003; Costa et al. 2003; Iacobazzi et al. 2004a).

Long-Term Prognosis

In the literature, information about the survival rate was provided for 43 out of 55 cases (Table 2). The mortality rate was high (65%), with most deaths in the first year of life. In a French retrospective multicenter study of fatty acid oxidation (FAO) patients, lethality was even higher (92%) (Baruteau et al. 2013). Reported causes of death were cardiomyopathy, intercurrent infectious processes, or sudden death, as occurred in P1 and P3. About 80% of the surviving reported patients were less than 12 years of age. Very few of the surviving patients presented with a milder clinical

phenotype (normal physical development with no evidence of cardiac disease or myopathy) and showed homozygosity for private mutations (Morris et al. 1998; Iacobazzi et al. 2004a; Parini et al. 1999; Ijlst et al. 2001). Outcomes seem to correlate better with the absence of cardiac disease and a higher long-chain fatty acid oxidation rate in cultured fibroblasts than with residual enzyme activity. Cases P2 and P4 are at present in good clinical condition at 4 and 16 years of age; to the best of our knowledge, P4 is the oldest surviving case of CACT deficiency.

Dietary Treatment and Monitoring

The dietary treatment basically consists of frequent meals, avoiding fasting, restricting fat, and increasing carbohydrates. MCT supplementation is a critical component in the management of these patients. There is no consensus on fasting, changing from every 3 h in neonates to 8–10 h in infancy (Spiekerkoetter et al. 2009). Fat intake should be 30% or less of the total caloric value, with 20% as low C10 MCT that do not need the carnitine system to enter mitochondria and 10% as LCT (Iacobazzi et al. 2004b; Spiekerkoetter et al. 2009; Parini et al. 1999). Low C10 MCT is recommended due to the evidence that C10 fats require CACT for oxidation (Rubio-Gozalbo et al. 2004). Some cases with long-chain FAO disorders (LCHAD, VLCAD, CPT1, CPT2, and CACT) have been treated with triheptanoin, a fatty acid with seven carbon acids, with good results, especially with respect to cardiomyopathy and hypoglycemia (Roe and Mochel 2006). The essential fatty acids linoleic acid (3–4%) and linolenic acid (0.5–1%) should be provided, in a ratio from 5:1 to 10:1, with walnut, canola, linseed, sunflower, wheat germ, or soy oil; this has been used with our patients (Spiekerkoetter et al. 2009). Our cases P1 and P3 required supplements of DHA to normalize plasma levels.

Emergency treatment of CACT deficiency in case of vomiting, diarrhea, fever, or other triggers of catabolism

Table 4 Mutations in the *SLC25A20* gene in CACT patients

Nucleotide change	Effect on protein	Phenotype	Geographical region	Reference
c.59G>A	p.Gly20Asp	Severe (Hmz)	Sub-Saharan Africa	Present study (case P1)
c.65_69dup5	p.Leu24Cysfs*107		Germany	Costa et al. (2003)
c.67 T>C	p.Cys23Arg	Severe (Hmz)	Iran	Wang et al. (2011)
c.82G>T	p.Gly28Cys	Mild (Hmz)	Pakistan	Costa et al. (2003)
c.84delT	p.His29Thrfs*100		China/Europe	Ogawa et al. (2000); Hsu et al. (2001)
c.94G>A	p.Asp32Asn		North Africa	Costa et al. (2003)
c.106-2A>T	p.?		Japan	Fukushima et al. (2013)
c.160_163del4ins4	p.Gly54_Thr55delinsTrpAla	Severe (Hmz)	Spain, Cape Indian	Iacobazzi et al. (2004a); Wang et al. (2011); Fearing et al. (2011)
[c.164C>A; c.260C>T]	p.Thr55Asn; p.Ala87Val			Wang et al. (2011)
c.168delT	p.Phe56Leufs*73			Wang et al. (2011)
c.180delG	p.Lys61Argfs*68		Egypt	Costa et al. (2003)
c.199-10T>G	p.?	Severe (Hmz)	Japan, East Asia	Ogawa et al. (2000); Hsu et al. (2001); Costa et al. (2003); Lee et al. (2007); Fukushima et al. (2013)
c.262_389del128	p.Val188Trpfs*53		Turkey	Huizing et al. (1998)
c.241G>A	p.Gly81Arg		Netherlands	Ijlst et al. (2001)
c.270delC	p.Phe91Leufs*38		Turkey	Hsu et al. (2001)
c.326+1delG	p.?		East Asia, Latin America	Hsu et al. (2001); Yang et al. (2001); Korman et al. (2006)
c.362delG	p.Gly121Alafs*8		North America	Iacobazzi et al. (2004a); Korman et al. (2006)
c.397C>T	p.Arg133Trp		Italy	Costa et al. (2003); Iacobazzi et al. (2004a); Wang et al. (2011)
c.496C>T	p.Arg166Term		France	Costa et al. (1999); Costa et al. (2003) Wang et al. (2011)
c.528delT	p.Met177Cysfs*12			Wang et al. (2011)
c.532C>T	p.Arg178Term	Severe (Hmz)	Spain, North Africa, Turkey	Costa et al. (2003); Rubio-Gozalbo et al. (2003); Iacobazzi et al. (2004a); Present study
c.533G>A	p.Arg178Gln		North Africa	Costa et al. (2003)
c.536A>G	p.Asp179Gly	Severe (Hmz)	North Africa	Present study (case P3)
c.576G>A	p.Trp192Term		Japan	Fukushima et al. (2013)
c.609-1G>A	p.?		France	Costa et al. (2003)
c.609-3C>G	p.?	Severe (Hmz)	Middle East	Korman et al. (2006)
c.673_780del110	p.Ala225Serfs*27		Turkey	Huizing et al. (1998)
c.689C>G	p.Pro230Arg		France	Costa et al. (2003)
c.691G>C	p.Asp231His		Germany	Costa et al. (2003); Iacobazzi et al. (2004a)
c.713A>G	p.Gln238Arg	Severe (Hmz)	Saudi Arabia, Bedouin	Al Aqeel et al. (2003); Iacobazzi et al. (2004b); Galron et al. (2004)

(continued)

Table 4 (continued)

Nucleotide change	Effect on protein	Phenotype	Geographical region	Reference
c.718+1G>C	p.?		Italy	Iacobazzi et al. (2004a)
c.752_761del10	p.Asp251Glyfs*3			Wang et al. (2011)
c.779_781del3	p.Glu260del			Wang et al. (2011)
c.804delG	p.Phe269Serfs*4	Severe (Hmz)	New Zealand	Costa et al. (2003); Wang et al. (2011)
c.823C>T	p.Arg275Term		Caucasian	Wang et al. (2011)
c.842C>T	p.Ala281Val	Mild (Hmz)	Italy	Iacobazzi et al. (2004a)
c.843+4_+50del	p.?	Mild (Hmz)	Italy	Iacobazzi et al. (2004a)
c.897dupC	p.Asn300Glnfs*24	Mild (Hmz)	Netherlands	Huizing et al. (1997); Ijlst et al. (2001)
c.418-?-906+? (25.9 kb del)	p.?		Caucasian	Wang et al. (2011)

The table lists the two novel mutations in bold from this study and the 39 reported mutations (HGMD Professional Release 2013.4: www.biobase-international.com)

Severe phenotype: severe clinical symptoms (hypoglycemia, acidosis, hyperammonemia ± cardiac involvement) before 1 week of age, torpid follow-up (recurrent decompensation), and fatal outcome <12 months of age

Mild phenotype: survival >12 months of age with normal physical development and no evidence of cardiac disease or myopathy

Hmz mutation in homozygosity

should include oral glucose solutions, glucose polymers or increased intake of carbohydrates, as well as maintaining the MCT and carnitine treatment. If the patient does not improve, 10% i.v. glucose (7–12 mg/kg/min), with insulin if required and MCT dripped enterally or in several doses, should be administered (Spiekerkoetter and Duran 2014). In the case of hyperammonemia, *N*-carbamylglutamate that replaces the CPS1 activator *N*-acetylglutamate can be used, as we did in P3 with a good response. A central line catheter for easy access in case of an emergency must be assessed in each case.

There is no consensus regarding the use of carnitine administration in CACT. Some authors do not recommend it because an accumulation of long-chain acylcarnitines may be toxic for cardiac rhythm (Rubio-Gozalbo et al. 2004). However, other authors recommend its use, even with high doses of carnitine in decompensation episodes, in order to restore the mitochondrial pool of coenzyme A (Brivet 2004). In all of our four cases, carnitine was administered (30–150 mg/kg/day) in order to maintain free carnitine in the low-normal level.

Other therapeutic possibilities for milder phenotypes with some residual CACT activity may be the administration of statins and fibrates that have been shown to enhance transcription of the human CACT gene via the peroxisome proliferator response element site, both separately and synergistically (Iacobazzi et al. 2009). This approach has already been used successfully in other FAO defects such as CPTII or VLCAD (Djouadi et al. 2003; Gobin-Limballe et al. 2007; Bonnefont et al. 2009).

Evidence-based guidelines for the treatment and monitoring of this specific disease are lacking. Only recommendations based on the experience of experts on clinical and biochemical monitoring of patients with FAO disorders have been published (Spiekerkoetter et al. 2009; Lund et al. 2010). Follow-up studies have to be performed in metabolic centers. Clinical evaluation, normalization of transaminases and creatine kinase, low-normal plasma free carnitine levels, and decreased long-chain acylcarnitines are considered ideal markers of treatment. Dietary compliance should be evaluated in each checkup. On the other hand, the liposoluble vitamins and polyunsaturated fatty acid (PUFAS) plasma levels should be monitored regularly along with annual cardiologic and ophthalmologic control. However, the best monitoring of treatment is the clinical evaluation. P2 always maintains deficient plasma free carnitine levels (<10 µmol/L), high-normal levels of C16 and C18:1 acylcarnitines, and mild dicarboxylic aciduria. P4 also had deficient plasma free carnitine levels up to 7 years of age and thereafter low-normal levels (15–20 µmol/L). Plasma long-chain acylcarnitines have always been increased in this patient.

Final Considerations

CACT deficiency is a very rare FAO disorder; no more than 60 patients have been reported worldwide in the last two decades. Although significant progress in early recognition has been made according to clinical symptoms in the neonatal period, even with expanded newborn screening,

i.e., detecting C16 and C18:1 acylcarnitines in dried blood spots, the disease still has a high mortality rate in the first year of life. Prognosis seems to be better in presymptomatic individuals identified after a previous affected sibling like P4 or by NBS. It also depends on good compliance with the diet as well as urgent adequate medical intervention in metabolic decompensation episodes. Sometimes, gastrostomy or a feeding tube has to be prescribed to ensure that the energy requirements are met. In cases with frequent decompensations, a port-a-cath catheter should be installed for easy access in case of emergency.

There are few data in the literature about the long-term prognosis of milder clinical cases. The excellent outcome of P4, now 16 years old, who has never presented with metabolic decompensation and shows good compliance with the diet, supports the importance of very early therapeutic intervention.

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Take-Home Message

Long-term clinical outcome in carnitine-acylcarnitine translocase deficiency seems to be better in those cases with a very early therapeutic intervention and a good dietary compliance with diet.

Compliance with Ethics Guidelines

Conflict of Interest

I Vitoria, E Martín-Hernández, L Peña-Quintana, M Bueno, P Quijada-Fraile, J Dalmau, S Molina-Marrero, B Pérez, and B Merinero declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

IV: clinical data of case 1, conception and design, drafting, and coordination of the manuscript

EM-H: clinical data of case 3, analysis of the data, and critical reading of the manuscript

LP-Q: clinical data of case 2, analysis of the data, and critical reading of the manuscript

MB: clinical care of case 4 and critical reading of the manuscript

PQF: critical reading of the manuscript

JD: clinical data of case 1 and critical reading of the manuscript

SM-M: clinical data of case 2 and critical reading of the manuscript

BP: molecular genetic analysis and interpretation of cases 1, 2, and 3

BM: biochemical diagnosis and interpretation of cases 1, 2, and 3, conception and design, and drafting of the manuscript

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Lack of Glibenclamide Response in a Case of Permanent Neonatal Diabetes Caused by Incomplete Inactivation of Glucokinase

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Abstract Background: Hypoglycaemic drugs that close the K_{ATP} channel have been tested in patients with permanent neonatal diabetes due to glucokinase mutations (PNDM-GCK). From the results obtained, it has been suggested that this treatment may be beneficial in patients carrying GCK mutations with mild kinetic defects. The aim of this study was to evaluate the kinetic analysis of glucokinase activity

as a predictive factor for response to sulphonylureas in PNDM-GCK.

Methods: The clinical characteristics of two siblings with PNDM born to non-consanguineous parents are described. Mutation analysis of *KCNJ11*, *INS* and *GCK* genes was done by sequencing. A comprehensive functional characterisation of *GCK* mutation was undertaken. Glibenclamide treatment was assayed for 16 weeks in one child. Response to treatment was evaluated by means of fasting glycaemia, C-peptide and HbA1c levels.

Results: Compound heterozygous *GCK* mutations (p.Ile19Asn and p.Ser441Trp) were identified. Functional analysis of GCK(p.Ile19Asn) indicated that this mutant retained more than 70% of wild-type catalytic activity in vitro, with a slight increase of thermolability. This mutation did not impair the interaction with the glucokinase regulatory protein, and the enzymatic activity of the GCK(p.Ile19Asn) mutant is restored to wild-type levels in the presence of GCK allosteric activator LY2121260. However, glibenclamide treatment of the patient on a reduced dose of insulin did not reduce HbA1c levels, and C-peptide increased only very slightly.

Conclusion: Hypoglycaemic drugs acting on the K_{ATP} channel might not be useful in the treatment of PNDM-GCK, even in patients carrying GCK mutations with mild kinetic defects.

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Introduction

Inactivating mutations in both alleles of the glucokinase gene (*GCK*) are a cause of permanent neonatal diabetes mellitus (PNDM-GCK), which is generally characterised by onset within the first months of life, low birth weight and lifelong

insulin therapy (Rubio-Cabezas et al. 2011). So far, 38 PNDM-GCK cases have been reported in 28 families (Njølstad et al. 2001; Njølstad et al. 2003; Porter et al. 2005; Turkkahraman et al. 2008; Rubio-Cabezas et al. 2008; Bennett et al. 2011; Wajda-Cuszlag et al. 2012; Durmaz et al. 2012; Raimondo et al. 2014). Most of them are homozygous due to consanguinity. Only two cases show compound heterozygous mutations (Njølstad et al. 2003; Wajda-Cuszlag et al. 2012). PNDM-GCK patients lack the glucose sensor that integrates blood glucose levels and insulin secretion, since glucokinase activity is drastically reduced or completely absent. The glycolytic enzyme glucokinase has a major role in the body glucose homeostasis. GCK activity controls the glycolytic flux necessary for insulin secretion in the pancreatic β -cells and also regulates glucose metabolism in the liver. The specific role of GCK results from the particular kinetic characteristics of this enzyme (i.e., low affinity and cooperativity for glucose) and its unique regulation through protein interactions with other protein partners (mainly the inhibitory glucokinase regulatory protein – GCKRP – in hepatocytes) (Matschinsky 2009).

Although insulin is the treatment of choice in patients with PNDM-GCK, hypoglycaemic drugs have been tried in some cases with varied results (Bakri et al. 2004; Turkkahraman et al. 2008; Bennett et al. 2011; Durmaz et al. 2012). It has been suggested that the relative success of these treatments depends on the functional severity of the GCK mutations carried out by the patients (Bakri et al. 2004; Turkkahraman et al. 2008). In this study, we aimed to test the hypothesis that functional *in vitro* GCK analysis can be used as a predictive factor for responsiveness of PNDM-GCK patients to sulphonylurea treatment. We describe two siblings affected by PNDM that bear compound heterozygous mutations in the *GCK* gene (p.Ile19Asn and p.Ser441Trp). We report the functional characterisation of mutation p.Ile19Asn and the clinical response of one of these patients to glibenclamide therapy. We found that while mutation p.Ile19Asn has mild effects on GCK kinetics, this patient did not respond to treatment. Thus, we provide evidence that sulphonylurea might not be useful in the treatment of PNDM-GCK even in patients with GCK mutations resulting in mild kinetic defects.

Case History and Glibenclamide Treatment

A male baby of Caucasian ancestry was born at 39 weeks' gestation from non-consanguineous parents. Birth weight was 2,260 g. During pregnancy, his mother showed altered oral glucose tolerance test (OGTT) and was treated with diet. His father was not known to have diabetes. Within the first day of life, the patient presented with nausea, vomiting and hyperglycaemia (13.4 mmol/L). Treatment with insulin was initiated

(0.25 U/kg/day). Serum C-peptide was 0.44 μ g/L, and diabetes-associated autoantibodies were negative. One month later, the baby was referred to the *Hospital Universitario La Fe*. Baby's weight was 3,100 g, glycaemia 19.2 mmol/L and plasma insulin 0.8 mg/L, with no acidosis or ketosis. After 3 months, a genetic test identified compound heterozygous *GCK* mutations. Biochemical analysis of parents revealed impaired fasting glucose. Mother's fasting plasma glucose (FPG) was 7.5 mmol/L, HbA1c 6.4% and OGTT at 120 min 5.8 mmol/L. Father's FPG was 7.9 mmol/L, HbA1c 6.2% and OGTT 7.1 mmol/L. Two years later, his sister was born at 39 weeks' gestation by caesarean section due to poor foetal growth and oligohydramnios. Her birth weight was 2,350 g and plasma glucose 14.4 mmol/L. Requirements of insulin were 0.02–0.04 U/kg/h, but with large glucose level fluctuations.

At age of 4 years, the boy was admitted for a trial of glibenclamide therapy. Before the treatment, OGTT values were 7.8 and 19.4 mmol/L at 0 and 120 min, respectively, and C-peptide <0.1 μ g/L. At that time he was treated with subcutaneous insulin (1U/kg/day) and HbA1c was 8.8%. Oral glibenclamide was initiated at 0.1 mg/kg/day split into two equal doses and increased gradually up to 0.75 mg/kg/day over a week. This value is within the same range as that used in a similar case (Turkkahraman et al. 2008) and above the median dose administered in patients with PNDM due to K_{ATP} channel mutations (<http://www.diabetesgenes.org>). Plasma biochemical analysis and liver function tests showed no side effects or hypoglycaemia. Insulin doses were gradually reduced until reaching a dose of 0.6 U/kg/day. C-peptide was measured at 1 and 2 months after initiating the treatment. Fasting capillary blood glucose levels were measured daily during the trial.

Methods

Genetic Studies

Genomic DNA was extracted by standard procedures. Exons and flanking introns of *KCNJ11* (*NM_000525.3*), *INS* (*NM_000207.2*) and *GCK* (*NM_000162.3*) genes were amplified and sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA). PCR products were purified and run on ABI Prism Genetic Analyzer 3130.xl (Applied Biosystems).

Enzymatic Analysis of Wild-Type and Mutant GCK

Recombinant human wild-type β -cell GCK fused to glutathionyl-S-transferase (GST-GCK) was prepared as described previously (Galán et al. 2006). Mutation p.Ile19Asn was produced using the oligonucleotides 5'-gtagagcagaacctggcagagtccaactgcaggag-3' and 5'-ctcctgcagttggaactctgcagggtctgctctac-3' and checked by sequencing and digestion

with PvuII. Determination of kinetic parameters and thermal inactivation were done as previously described (Galán et al. 2006). The relative activity index was normalised to 5 mmol/L basal blood glucose (Christesen et al. 2002). GCK activator LY2121260 (Elli Lilly and Co) was prepared as described (García-Herrero et al. 2012).

Statistical Analysis

Normality of distributions was tested by Shapiro–Wilk test. Normally distributed parameters were compared by two-tailed Student *t*-test in combination with Levene’s test for equality of variances. Mann–Whitney *U* test was used for nonnormally distributed variables. *P* < 0.05 was considered statistically significant.

Results

Identification of GCK Mutations

Sequence analysis of the proband did not reveal any mutation in *KCNJ11* and *INS* genes. Two mutations, both in heterozygosis, were identified in the *GCK* gene: c.56T>A (p.Ile19Asn) in exon 2 and c.1322C>G (p.Ser441Trp) in exon 10. Both were previously described as cause of familiar hyperglycaemia (MODY-GCK) in the heterozygous state (Massa et al. 2001; Estalella et al. 2007). Mutation analysis confirmed that the father carried the MODY-GCK(p.Ser441Trp) mutation, the mother carried the MODY-GCK(p.Ile19Asn) mutation and the sister was also a compound heterozygous for both mutations.

Functional Characterisation of p.Ile19Asn Mutation

Kinetic analysis of the GCK(p.Ser441Trp) mutant, reported by Barbetti et al. (2009) and reflected in Table 1, showed low enzymatic activity compared to the wild-type GCK. However, no functional data was available for the p.Ile19Asn mutation. Kinetic parameters of recombinant wild-type GST-GCK and mutant GST-GCK(p.Ile19Asn) are shown in Table 1. Mutation p.Ile19Asn produced a slight but significant decrease in the catalytic constant, cooperativity and affinity for glucose (as shown by reduced Kcat and n_H and increased S_{0.5} values, respectively) and a small increase in ATP affinity (reduced ATP Km value) that results in a slight reduction of the calculated relative activity index (*I*_{ar} = 0.72; *p* = 0.073). We also observed that the GCK synthetic allosteric activator LY2121260 activates the mutant protein as much as the wild type (Table 1) and that this mutation does not impair the interaction of GCK with GCKRP in the two hybrid assay (supplementary Fig. 1). To ascertain whether protein instability could contribute to reduce activity

Table 1 Kinetic parameters of wild-type GST-GCK and mutant GST-GCK(p.Ile19Asn) and effect of GCK activator LY2121260

Protein	Yield (mg/L) (n = 3)	Kinetic parameters					Effect of LY2121260				
		K _{cat} (s ⁻¹)	Glucose S _{0.5} (mmol/L)	n _H	ATP Km (mmol/L)	I _{ar}	Fold increased K _{cat}	Fold decreased Glucose S _{0.5}	Fold decreased n _H	Fold increased ATP Km	Fold increased I _a
GST-GCK (n = 6)	4.79 ± 0.73	51.29 ± 2.40	7.85 ± 0.68	1.53 ± 0.03	0.49 ± 0.04	1.00 ± 0.19	1.41 ± 0.09	5.09 ± 0.86	1.5 ± 0.14	1.54 ± 0.27	50 ± 16
GST-GCK (p.Ile19Asn) (n = 6)	3.79 ± 0.95	37.22 ± 3.1**	10.68 ± 0.8*	1.30 ± 0.08*	0.33 ± 0.04*	0.72 ± 0.28	1.34 ± 0.16	8.05 ± 1.44*	1.4 ± 0.15	1.44 ± 0.50	78 ± 19
WT/p.Ser441Trp (Barbetti et al. 2009)		65.57 ± 7.19/ 20.72 ± 4.56	7.97 ± 0.31/ 15.89 ± 2.49	1.71 ± 0.07/ 1.38 ± 0.07	0.45 ± 0.05/ 1.12 ± 0.14	1/0.11					

Activity data are shown as mean ± SD for six independent experiments (n = 6) using three separate enzyme preparations, for wild-type and mutant GST-GCK. The Km for ATP was measured at a glucose concentration of 7.5 and 11 mmol/L for wild type and mutant, respectively. The Hill coefficient (n_H) and the relative activity index (I_{ar}) are unit less. For comparison, means of GST-GSK wild-type and p.Ser441Trp mutant kinetic parameter data from Barbetti et al. (2009) are included in the table. To assay the effect of LY2121260, GCK activity was measured in the absence and presence of 10 μmol/L activator. Since LY2121260 was dissolved in a buffer containing 0.8% DMSO, all assays contained the same final concentration of DMSO. Fold variation has been calculated dividing values in the presence of LY2121260 by their corresponding values in its absence. (*) *p* < 0.005, Student’s *t*-test with Levene’s test for equality of variances. (***) *p* < 0.005, non-parametric Mann–Whitney *U* test

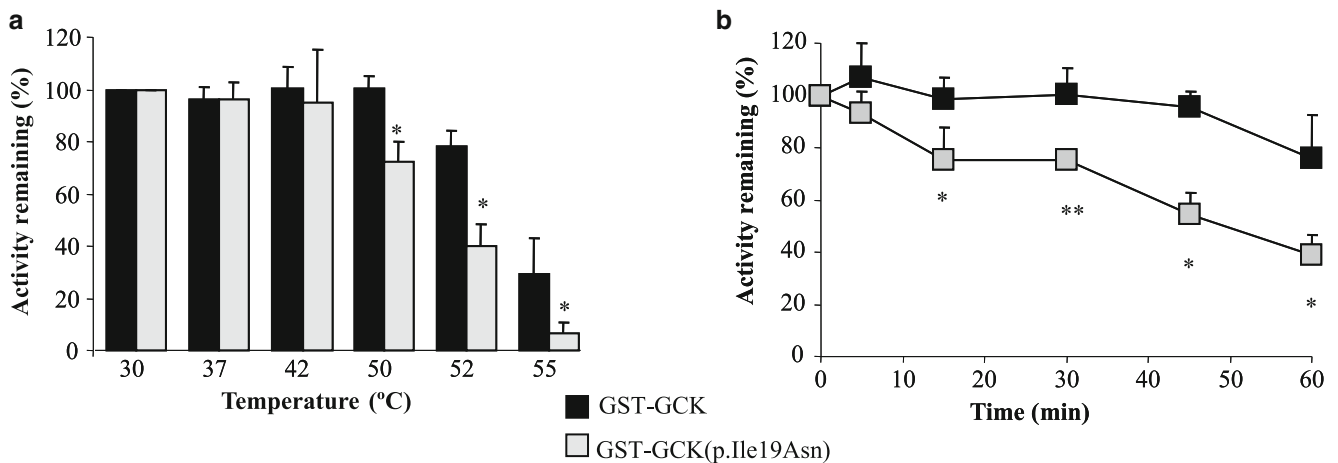


Fig. 1 Assessment of thermostability for wild-type GST-GCK and mutant GST-GCK(p.Ile19Asn) proteins. Enzyme solutions were diluted to 250 mg/L as described in Galán et al. (2006). (a) The enzyme solution was incubated for 30 min at different temperatures ranging from 30 to 55°C and then assayed at 30°C as described in Methods. (b) The enzyme solution was incubated for different periods

of time from 5 to 60 min at 50°C. After incubation, GCK activity was assayed at a glucose concentration of 100 mmol/L. Means and SD of six independent experiments ($n = 6$), from three enzyme preparations, are shown. (*) $p < 0.005$, Student's t -test with Levene's test for equality of variances. (**) $p < 0.005$, non-parametric Mann-Whitney U test

Table 2 Glibenclamide treatment and blood biochemical levels

	Time of treatment				
	Before treatment	1 month	2 months	3 months	4 months
Insulin dose (U/kg/day)	1	0.6	0.6	0.6	0.9
glibenclamide dose (mg/kg/day)	–	0.75	0.5	0.5	0.5
C-peptide ($\mu\text{g/L}$)	< 0.1	0.33	0.2	ND	ND
HbA1c (%)	8.8	10.2	10	10	8.7
FPG (mmol/L)	7.8	12.3	14.8	ND	ND
Fasting capillary blood glucose (mmol/L)	–4 w: 8.5 ± 4.9	1 w: 13.9 ± 2.6	5 w: 8.0 ± 3.2	9 w: 8.3 ± 4.8	13 w: 6.6 ± 2.5
	–3 w: 12.4 ± 6.7	2 w: 8.5 ± 2.7	6 w: 9.5 ± 2.7	10 w: 8.8 ± 3.6	14 w: 7.8 ± 3.0
	–2 w: 9.4 ± 4.1	3 w: 7.1 ± 3.7	7 w: 10.7 ± 2.2	11 w: 9.8 ± 3.5	15 w: 9.3 ± 4.5
	–1 w: 10.6 ± 2.8	4 w: 9.1 ± 3.2	8 w: 12.8 ± 2.8	12 w: 9.4 ± 3.8	16 w: 11.9 ± 4.2
	Mean: 10.2 ± 4.8	Mean: 9.7 ± 3.9	Mean: 10.2 ± 3.1	Mean: 8.9 ± 4.0	Mean: 8.9 ± 4.0

FPG fasting plasma glucose, ND not determined

Fasting capillary blood glucose data are means \pm SD of one measurement per day, 7 days per week (w) of treatment

of GCK(p.Ile19Asn), we tested the activity of the purified fusion proteins at different temperatures. Our results indicate that mutation p.Ile19Asn confers a mild thermal instability to the GCK protein (Fig. 1).

Glibenclamide Treatment

Insulin doses were reduced to 0.6 U/Kg/day but were increased back because HbA1c levels were rising (Table 2). After 1 and 2 months of treatment, C-peptide levels were of 0.33 and 0.20 $\mu\text{g/L}$, respectively. Insulin injections could not be stopped, and after 1 month, because of poor response and in order to avoid secondary effects, glibenclamide dose

was reduced to 0.5 mg/kg/day and maintained 3 months more. After 16 weeks, the boy needed insulin doses similar to those dispensed previously. Then the treatment was discontinued, and patient was kept on continuous subcutaneous insulin infusion (1 U/kg/day), obtaining a better metabolic control, although glycaemia was not optimally controlled (HbA1c: 7.7%).

Discussion

Most neonatal diabetic patients with undetectable insulin secretion need lifelong insulin therapy. Insulin secretion by the pancreatic β -cell occurs in response to high blood glucose

concentration, which is detected by the glucose sensor glucokinase. Increased glucokinase activity stimulates the glycolytic flux rate and increases ATP levels, which in turn induces the closure of the K_{ATP} channel, thus resulting in membrane depolarization, calcium influx and insulin release.

Hypoglycaemic drugs closing the K_{ATP} channel have been tested in PNDM-GCK patients. Insulin treatment was replaced by repaglinide in two patients, a homozygous (IV8+2/IV8+2) and a compound heterozygous (IV8+2/p.Gly264Ser), with a good response only in the latter (Bakri et al. 2004). Interestingly, mutant GCK(p.Gly264Ser) showed near normal kinetics (Njølstad et al. 2003). Glibenclamide was administered to homozygous patients for mutations p.Thr168Ala (Turkkahraman et al. 2008; Durmaz et al. 2012) and p.Gln98X (Bennett et al. 2011). Although sulphonylurea treatment resulted in HbA1c reduction and increased C-peptide, insulin administration could not be stopped. For such severe GCK mutations, it was proposed that the total absence of GCK activity could limit the ATP production to restore insulin secretion, even in conditions in which sulphonylureas would close the K_{ATP} channel (Turkkahraman et al. 2008). From these studies it was inferred that sulphonylurea treatment could be tried in PNDM patients with less severe GCK mutations.

Here we described two PNDM-GCK siblings carrying compound heterozygous mutations. Mutation p.Ser441Trp, inherited from the father, retains about 11% of wild-type GCK activity (Barbetti et al. 2009). Mutation p.Ile19Asn, inherited from the mother, has a much lighter effect on glucokinase kinetics. Although this mutation produces small but significant defects in the kinetic parameters, the mutant enzyme retains more than 70% of wild-type catalytic activity *in vitro*. Thus, our kinetic results suggest that these PNDM patients might keep a reduced but significant level of GCK activity *in vivo*. Moreover, the relative mild intrauterine growth retardation, shown by a birth weight above 2,200 g, suggests that insulin secretion during that period was not as severely impaired as in the majority of the PNDM-GCK, which is also consistent with the remaining GCK activity detected *in vitro*.

However, despite the apparent mild effect of mutation p.Ile19Asn on GCK kinetics, the patient did not respond to the glibenclamide treatment. It is known that GCK mutations may cause other defects that cannot be detected by kinetic assays *in vitro* and result in a stronger reduction of activity *in vivo*, such as protein instability or impairment of interaction with other cellular partners (Osbaek et al. 2009). Actually, it has been recently reported that protein instability is a major determinant of the clinical severity of PNDM-GCK (Raimondo et al. 2014). However, it might not be a general rule since the homozygous p.Arg275Cys mutation, which confers protein instability but has no significant effect on GCK kinetics, causes a MODY-like

clinical phenotype, but not PNDM (Negahdar et al. 2014). We found a very mild effect of mutation p.Ile19Asn on the thermal stability of GCK if compared to those caused by some other mutations previously described (Raimondo et al. 2014). Furthermore, mutation p.Ile19Asn does not appear to impair other regulatory mechanisms of GCK activity, such as allosteric regulation, as shown by the normal response of the mutant enzyme to allosteric activator LY2121260 or the interaction of GCK with GCKRP. From our results, we conclude that hypoglycaemic drugs acting on the K_{ATP} channel might not be useful in the treatment of PNDM-GCK even in patients carrying mutations that result in mild kinetic defects. Although sulphonylurea treatment enables K_{ATP} channel closure, the lack of glucose sensor integrity may still prevent normal insulin secretion and improvement of glycaemic control. Synthetic glucokinase activators are also being developed as potential antidiabetic drugs in the treatment of type 2 diabetes (Matschinsky 2013). We have found that the enzymatic activity of the GCK(p.Ile19Asn) mutant is restored to wild-type levels in the presence of the allosteric activator LY2121260 (Table 1). Therefore, this opens the possibility that GCK allosteric activators could provide a more physiological approach in the treatment of such PNDM-GCK cases.

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Synopsis

This is the first report providing evidence that sulphonylureas might not be useful in the treatment of PNDM-GCK even in patients carrying glucokinase mutations that result in mild kinetic defects.

Compliance with Ethics Guidelines

Conflict of Interest

Josep Oriola, Francisca Moreno, Angel Gutiérrez-Nogués, Sara León, Carmen-María García-Herrero, Olivier Vincent and María-Angeles Navas declare that they have no conflict of interest.

Informed Consent

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

Animal Rights

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

Contributions of Individual Authors

Josep Oriola, Francisca Moreno and Maria-Angeles Navas conceived and designed the experiments. Francisca Moreno and Sara Leon collected the clinical information and conducted the clinical treatment. Josep Oriola, Angel Gutierrez-Nogués, Carmen-Maria Garcia-Herrero and Olivier Vincent performed genetics and biochemical experiments. Josep Oriola, Francisca Moreno, Angel Gutierrez-Nogués, Olivier Vincent and Maria-Angeles Navas analysed the data. Josep Oriola, Francisca Moreno, Olivier Vincent and Maria-Angeles Navas wrote the manuscript.

All authors contributed to and have approved the final manuscript.

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Morphology and Function of Cerebral Arteries in Adults with Pompe Disease

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Abstract Objective: Cerebrovascular abnormalities have been reported in adult patients with Pompe disease. The objective was to study these abnormalities by (1) determining the diameter and mean flow velocity (MFV) of large cerebral arteries and (2) estimating cerebral blood flow (CBF), resistance index (RI) and cerebrovascular reactivity (CVR) as functions of resistance vessels.

Methods: In ten adults with Pompe disease and twenty controls, the diameter, peak systolic (PSV) and end-diastolic velocities (EDV) of arteries supplying the brain were quantified by MR angiography and sonography. MFV, RI and CBF were calculated. CVR in the middle cerebral artery (MCA) was determined by hyperventilation and acetazolamide injection.

Results: MR angiography revealed dilation of cerebral arteries predominantly in the posterior circulation. Dilative arteriopathy was found in three patients; two of them showed vertebrobasilar dolichoectasia. Despite of the dilative arteriopathy, the MFV was normal, indicating increased CBF and dilated resistance vessels. RI of all examined arteries and CVR of MCA were normal.

Conclusion: The data suggest that dilation of small and large cerebral arteries is a common feature in adults with Pompe disease. Increased CBF might be the consequence

of dilated resistance vessels. However, dysfunction of resistance vessels was rarely found.

Synopsis: In adults with Pompe disease, dilation of small and large cerebral arteries is a common feature and might be associated with increased cerebral blood flow.

Introduction

Pompe disease (glycogenosis type II, MIM #232300) is a rare autosomal-recessive lysosomal storage disease caused by mutations in the *GAA* gene encoding the enzyme acid α -glucosidase. This results in decreased enzyme activity and intracellular glycogen accumulation (Pompe 1932; Joshi et al. 2008; van der Ploeg et al. 2010). Glycogen accumulation was found within endothelial cells and smooth muscle cells of cerebral arteries. Additionally, aneurysmal dilations of small cerebral arteries predominantly in the cerebellar cortex were reported in an adult with Pompe disease (Kretzschmar et al. 1990). Otherwise, in three children excessive accumulation of glycogen within endothelial cells was shown to reduce the lumen in cerebral arteries (Garancis 1968). Both abnormalities could disturb the cerebral blood flow regulation by small arteries, arterioles and capillaries (resistance vessels). Dilation, elongation and tortuosity (dolichoectasia) of large cerebral arteries were also reported. Cerebrovascular symptoms in adults with Pompe disease are headache, cerebral compression symptoms, ischemia or haemorrhage (Matsuoka et al. 1988; Kretzschmar et al. 1990; Laforêt et al. 2008; Sacconi et al. 2010).

The objective of this study was to evaluate these cerebrovascular abnormalities by (1) determining the diameter and mean flow velocity (MFV) of large cerebral

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arteries and (2) estimating cerebral blood flow (CBF), resistance index (RI) and cerebrovascular reactivity (CVR) as functions of resistance vessels.

Patients and Methods

Patients

We prospectively investigated ten adult Pompe patients of our outpatient department (age 46 ± 12 years, five females). Diagnosis of Pompe disease was confirmed by deficiency of α -glucosidase and corresponding gene mutations in the *GAA*. With the exception of the siblings P5 and P8, all patients received enzyme replacement therapy (ERT). All measurements were compared to age- and sex-matched control groups. MR and duplex sonography control values were taken from historic age- and sex-matched patients ($n = 20$, age 50 ± 14 years, ten females) with normal MR and sonography findings. In nine additional subjects, CVR was determined by Doppler sonography (age 46 ± 12 years, four females). Informed consent was received from all patients and CVR controls. The local ethics committee approved the study.

Gd-MRA

Imaging was performed using a 1.5-Tesla MR machine (Magnetom Sonata Vision, Siemens, Erlangen, Germany). MRA images were obtained after the injection of gadolinium contrast agent with an axial FLASH 3D sequence (TR 3.7 ms, TE 1.4 ms, flip angle 30° and a spatial resolution of 0.8 mm with isometric voxels). In addition a time-of-flight angiography (TR 37 ms, TE 7 ms, flip angle 25° , a spatial resolution of 0.5 mm with isometric voxels) was performed. Due to claustrophobia, patient P5 was examined by CT angiography (SOMATOM Sensation 64, Siemens). The diameter of the distal internal carotid artery (distal ICA, 1 cm proximal of the terminal end), the middle cerebral artery (MCA at M1 segment, 1 cm distal of its origin), the anterior cerebral artery (ACA at A1 segment, 1 cm distal of its origin), the vertebral artery (at V4 segment, 1 cm proximal of the basilar artery origin), and basilar artery (BA, 1 cm distal of the BA origin) were measured, blinded by a neuroradiologist (Fig. 1b). In both MRA sequences, the diameters were determined in the raw images. A diameter over three times the standard deviation (SD) of control values was defined as dilative arteriopathy. The elongation and tortuosity of cerebral arteries were visually assessed. The terms dolichoectasia and fusiform aneurysm

were used to describe dilated arteries with elongation and tortuosity. White matter lesions were analysed by fluid-attenuated inversion recovery (FLAIR) images (TR 8,000 ms, TE 129 ms, TI 2,500 ms, slice thickness 5 mm) and classified by Fazekas score (grade 0 = absence; grade 1 = punctate; grade 2 = early confluent; or grade 3 = confluent lesions).

Sonography

In the MCA, ACA, V4 and BA, at the same position as the MR diameter measurements, the peak systolic velocity (PSV) and the end-diastolic velocity (EDV) were angle-corrected and quantified by transcranial Doppler sonography (3-MHz probe, Acuson Sequoia™ 512, Siemens). Additionally, the diameter, the angle-corrected PSV and EDV in the vertebral artery at the proximal V2 segment (V2), the proximal ICA (proximal ICA, 1–2 cm after origin) and the common carotid artery (CCA, 2 cm before bifurcation) were measured with a 6-MHz probe. The MFV (in cm/s), the RI and the CBF (in mL/min) were calculated [$MFV = (PSV + 2 * EDV)/3$; $RI = (PSV - EDV)/PSV$; $CBF = \pi * diameter^2 * MFV/4$]. The intima–media complex was determined at the distal CCA.

By head-mounted 2-MHz Doppler probes (Multi-Dop X4, DWL, Sipplingen, Germany), the function of resistance vessels in the MCA vascular bed was quantified in normocapnia and in a standardised setting by hyperventilation-induced vasoconstriction ($CVR_{hyperventilation}$) and acetazolamide-induced vasodilation ($CVR_{acetazolamide}$) (Eicke et al. 1999). CVR was calculated following 1 min of hyperventilation and 10 min after intravenous 1 g acetazolamide administration ($CVR = 100 \times [MFV_{stimulation} - MFV_{baseline}]/MFV_{baseline}$). A CVR outside the threefold standard deviation (SD) of control values was considered to be pathological.

Statistics

Values were given in mean \pm SD; group differences were assessed with Mann–Whitney U tests. For Pearson correlations, the largest diameters were used.

Informed Consent and Ethics

Informed consent was obtained from all patients prior to inclusion. The study was approved by the local ethics committee of the Martin-Luther-University Halle-Wittenberg and followed the ethical standards of the Helsinki Declaration of 1975, as revised in 2000.



Fig. 1 Gadolinium-enhanced MR angiography of three adults with Pompe disease, (a) normal vertebrobasilar arteries in P7, (b) tortuous but not significantly enlarged cerebral arteries in P2 (black markers

present position of diameter and velocity measurements), (c) verte-brobasilar dolichoectasia in P10

Results

Our adult patients with Pompe disease had mild to severe myopathy, and two of them (P3, P7) were on noninvasive ventilation support (Table 1). All patients were ambulatory, normocapnic (arterial pCO₂ 5.0 ± 0.5, range 4.1–5.7) and had normal kidney function. The incidence of cardiovascular risk factors was comparable to that of our control group (hypertension 40 vs. 60%, smoking 30 vs. 10%, hyperlipidaemia 40 vs. 50%, diabetes 20 vs. 40%, coronary heart disease 15 vs. 10%). None of the patients reported headache or symptoms related to cerebrovascular disease. FLAIR–MRI revealed punctate white matter lesions in 3/9 patients (P4, P8 and P9, each with Fazekas score 1). The duration between MR imaging and Duplex sonography was 49.7 ± 46.3 days in patients and 1.4 ± 1.4 days in controls.

The *diameter* of extra- and intracranial arteries (MCA, distal ICA, BA, V4 and V2) was enlarged compared to age- and sex-matched controls (Table 2). Dilative arteriopathy was shown in three patients mostly affecting the vertebrobasilar arteries (P4 at BA, P9 at V4, P10 at V4, BA, distal ICA and MCA). Vertebrobasilar dolichoectasia was apparent in two of these patients (P9, P10) (Fig. 1c). The diameter of distal ICA, BA and V4 increased with the duration of disease (distal ICA, Pearson $r = 0.65$, $p < 0.05$; BA, Pearson $r = 0.91$, $p < 0.0005$; V4, Pearson $r = 0.77$, $p < 0.05$). MRA and sonography of the cerebral arteries showed no stenosis. The intima–media complex of the distal CCA was elevated in three patients (P2 1.4 mm, P5 1.1 mm and P7 1.5 mm).

The *MFV* was elevated in the CCA and decreased in V4 (Table 2). The *CBF* in the CCA, BA, V4 and V2 were elevated; proximal ICA, MCA and ACA revealed a trend towards increased CBF as well (Table 2). The *RI* was normal in all arteries but showed a trend towards decreased values in MCA, ACA and BA ($0.09 > p > 0.06$). *CVR* was not different between patients and controls ($CVR_{\text{hyperventilation}} -30.3 \pm 19.8$ vs. $-27.2 \pm 8.3\%$, $CVR_{\text{acetazolamide}} 36.5 \pm 22.0$ vs $37.9 \pm 14.1\%$). $CVR_{\text{hyperventilation}}$ was unilaterally decreased in patient P9 and elevated in patient P5.

Discussion

We prospectively investigated the abnormalities of small and large cerebral arteries in adults with Pompe disease but without any cerebrovascular symptoms. We showed that the diameter of large cerebral arteries including the distal ICA and the MCA was enlarged leading to dilative arteriopathy in three patients. Vertebrobasilar dolichoectasia was found in the two oldest patients. This is a higher incidence that is reported for the general population, where the incidence of vertebrobasilar dolichoectasia was between 0.3 and 4.4% (Yu et al. 1982; Ubogu and Zaidat 2004). The predominance of dolichoectasia in the vertebrobasilar circulation has already been reported in adult Pompe patients (Laforêt et al. 2008; Sacconi et al. 2010). In our patients, however, the MCA and the distal ICA were also enlarged although the diameters of the proximal ICA and CCA were normal, implicating a more widespread, but not generalised, arterial

Table 1 Clinical characteristic from ten adult patients with Pompe disease

Patient	P1	P2	P3	P4 ^{a,b}	P5	P6	P7	P8 ^b	P9 ^{a,b}	P10 ^{a,b}
Age (years)	21	37	44	44	46	47	54	56	64	71
Sex	F	F	M	M	M	F	M	F	M	F
Duration of disease (years)	10	10	4	12	10	12	9	16	11	30
Duration of ERT (months)	3	33	41	54	0	33	48	0	14	47
GAA genotype	IVS1-13T>G	p.Leu552Pro	IVS1-13T>G	IVS1-13T>G	p.Pro493Leu	IVS1-13T>G	IVS1-13T>G	p.Pro493Leu	IVS1-13T>G	IVS1-13T>G
	IVS9-1G>C	p.Pro493Leu	c.2136-74delGT	p.Trp499Arg	p.Cys103Gly	p.Leu552Pro	p.Cys103Gly	p.Cys103Gly	p.Gly309Arg	c.2481+102_2646+31del
6-minute walk test [m]	510	295	380	130	420	375	380	420	120	60
WGMS	3	3	5	6	4	6	5	3	5	6
Slow vital capacity (%)	60	110	38, NVS	53	94	65	50, NVS	91	40	83
Cardiovascular risk factors		HL, O	HT, HL	DM, HL		HT, DM, HL, O, S	HT, DM, HL, O	HT	HT, DM, O, CHD	HT

^a Patient with dilative arteriopathy (* and vertebrobasilar dolichoectasia)

^b Patient with punctate white matter lesions

CHD coronary heart disease, DM diabetes mellitus, ERT enzyme replacement therapy, F female, HT arterial hypertension, HL hyperlipidaemia, M male, NVS noninvasive ventilation support, O obesity, S smoking, WGMS Walton Gardner Medwin scale

Table 2 Diameter, mean flow velocities and cerebral blood flow in adult patients with Pompe disease ($n = 10$) and controls ($n = 20$)

	Pompe patients Mean \pm SD	Control group Mean \pm SD (+3SD)	Mann–Whitney Test
<i>Diameter (D) in mm</i>			
MCA ^a	2.3 \pm 0.5	1.9 \pm 0.4 (3.2)	$p < 0.005$
ACA ^a	1.6 \pm 0.3	1.4 \pm 0.3 (2.3)	n.s. ($p = 0.07$)
Distal ICA ^a	3.4 \pm 0.6	2.7 \pm 0.5 (4.1)	$p < 0.00005$
BA ^a	3.7 \pm 0.9	2.7 \pm 0.5 (4.2)	$p < 0.005$
V4 ^a	2.7 \pm 1.0	1.8 \pm 0.4 (3.1)	$p < 0.0005$
V2 ^b	3.8 \pm 0.7	3.1 \pm 0.5 (4.6)	$p < 0.005$
Proximal ICA ^b	5.1 \pm 0.6	4.9 \pm 0.6 (6.6)	n.s. ($p = 0.07$)
CCA ^b	6.2 \pm 0.6	6.2 \pm 0.8 (8.5)	n.s. ($p = 0.89$)
<i>Mean flow velocity (MFV) in cm/s</i>			
MCA ^c	50 \pm 12	53 \pm 16	n.s. ($p = 0.54$)
ACA ^c	43 \pm 19	38 \pm 16	n.s. ($p = 0.29$)
BA ^c	31 \pm 8	36 \pm 10	n.s. ($p = 0.73$)
V4 ^c	27 \pm 11	32 \pm 7	$p < 0.05$
V2 ^b	14 \pm 6	13 \pm 6	n.s. ($p = 0.62$)
Proximal ICA ^b	30 \pm 10	28 \pm 11	n.s. ($p = 0.47$)
CCA ^b	30 \pm 10	23 \pm 8	$p < 0.05$
<i>Cerebral blood flow (CBF) in mL/min</i>			
MCA	120 \pm 49	98 \pm 51	n.s. ($p = 0.14$)
ACA	50 \pm 27	39 \pm 23	n.s. ($p = 0.18$)
BA	201 \pm 57	127 \pm 59	$p < 0.05$
V4	94 \pm 57	52 \pm 24	$p < 0.05$
V2	97 \pm 66	60 \pm 30	$p < 0.05$
Proximal ICA	364 \pm 111	311 \pm 119	n.s. ($p = 0.06$)
CCA	516 \pm 150	417 \pm 142	$p < 0.05$

^a Measured by gadolinium-enhanced MR angiography

^b Measured by extracranial duplex sonography

^c Measured by transcranial duplex sonography

ACA anterior cerebral artery, BA basilar artery, CCA common carotid artery, Distal ICA distal internal carotid artery, MCA middle cerebral artery, n.s. not significant, Proximal ICA proximal internal carotid artery, SD standard deviation, V2 vertebral artery at proximal V2 segment, V4 vertebral artery at distal V4 segment

dilation. Previous studies revealed similar findings: in adults with Pompe disease, the ascending aorta was found to be enlarged (El-Gharbawy et al. 2011), and recently a reduced diameter in the CCA was reported (Wens et al. 2014).

The pathological basis of dilative arteriopathy and dolichoectasia in the general population is unknown, but both conditions are usually associated with severe atherosclerosis. Dilative arteriopathy is combined with chronic kidney dysfunction, old age, male sex and cardiovascular risk factors as hypertension, smoking and coronary heart disease (Yu et al. 1982; Ichikawa et al. 2009). Old age and hypertension could have caused the dolichoectasia in P9 and P10. However, our adult Pompe patients had no to mild atherosclerosis and normal kidney function, and, as

reported, most were younger (Yu et al. 1982; Ubogu and Zaidat 2004; Ichikawa et al. 2009). In some families with Pompe disease, the dilative arteriopathy accumulates at a premature age (Makos et al. 1987; Matsuoka et al. 1988; Cipullo et al. 2013), indicating the importance of hereditary factors. The presence of cardiovascular risk factors was distributed evenly among patients and controls. Compared with age- and sex-matched controls, the adult Pompe patients had substantially enlarged cerebral arteries. The diameters of distal ICA, BA and V4 increase with the duration of disease. In adult Pompe patients, dilative arteriopathy was histopathologically associated with glycogen accumulations, extensive vacuolar degeneration and necrosis within the vessel wall (Makos et al. 1987; Matsuoka et al. 1988; Kretzschmar et al. 1990).

Additionally, vasodilation, elongation and tortuosity of arteries supplying the brain are reported as consequences of increased CBF (Sho et al. 2004; Hoi et al. 2008). CBF is strongly related to the cerebral metabolism and controlled by the cross-sectional area of resistance vessels (Heistad and Kontos 1983): an increase of cross-sectional area due to dilation of resistance vessels leads to increased CBF. In the present study, CBF was increased in CCA, BA, V4 and V2. Therefore, temporarily or permanently dilated resistance vessels could cause dilative arteriopathy and dolichoectasia in adult Pompe patients. Histopathologically, dilation of numerous resistance vessels has been described in an adult with Pompe disease (Kretzschmar et al. 1990). Furthermore, dilation of resistance vessels would decrease the RI. In our patients, a clear trend towards reduced RI in MCA, ACA and BA was shown as compared to controls.

In patients with Pompe disease, two additional factors may lead to dilation of resistance vessels: partial pressure of blood carbon dioxide and glycogen-associated changes of vessel walls. The common occurrence of respiratory insufficiency leads to elevated partial pressures of carbon dioxide, causing vasodilation. Glycogen-filled vacuoles in the wall of resistance vessels (Kretzschmar et al. 1990) could disturb the production of extracellular matrix proteins like collagen and elastin (Dobrin 1978), of matrix metalloproteinases (Loftus and Thompson 2002), or of vasoactive substances like nitric oxide (McCarron et al. 2006). We suppose that confounding cardiovascular risk factors leading to cerebral microangiopathy diminish the effect of dilated resistance vessels on RI. The vasoconstriction and vasodilation function of resistance vessels is thus upheld, as is indicated in our patients by normal CVR.

In adult patients with Pompe disease, the abnormalities of cerebral arteries will probably increase with the duration of disease, resulting in increased risk of adjacent structures compression and cerebral ischemic and hemorrhagic stroke. We visualise the cerebral vessels in a newly diagnosed Pompe patient and suggest a follow-up every 5 years. We screen patients with siblings affected by dilative arteriopathy in shorter intervals. The changes of cerebral arteries and CBF should be examined in further studies.

Our study is limited by the small number of patients, the limited resolution of MR angiography, and the fact that MRA and Doppler examinations occurred at different dates. The cross-sectional area of resistance vessels can't be measured directly; therefore, we had to use the CBF and RI as surrogates.

In conclusion, dilation of small and large cerebral arteries seems to be a common feature in adults with Pompe disease. Our data suggest CBF increase due to dilated resistance vessels. In contrast, dysfunction of resistance vessels was rarely seen.

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Compliance with Ethics Guidelines

Conflict of Interest

- F. Hanisch has received lecturer honoraria from Genzyme Corporation.
- M. Deschauer has received payment for lectures and manuscript preparation and a grant from Genzyme Corporation.
- T. Müller has received speaking fees from Boehringer Ingelheim.
- O. Hensel, D. Stoevesandt and K. Stock report no disclosure.

Informed Consent

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

Individual Contributions

- O. Hensel: acquisition, analysis and interpretation of sonography data; drafting and revising the manuscript content, including the medical content; corresponding author
- F. Hanisch: study concept and design; recruitment of patients; drafting and revising the manuscript content, including the medical content; interpretation of data
- K. Stock: acquisition, analysis and interpretation of radiological data, drafting and revising the manuscript content
- D. Stoevesandt: acquisition, analysis and interpretation of radiological data, drafting and revising the manuscript content
- M. Deschauer: study concept and design, revising the manuscript content
- T. Müller: study concept and design; drafting and revising the manuscript content, including medical writing of content; interpretation of data; study supervision and coordination; principal investigator

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Parkinsonism in Phenylketonuria: A Consequence of Dopamine Depletion?

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Abstract Phenylketonuria (PKU) is caused by a deficiency or inactivity of the enzyme phenylalanine hydroxylase that converts phenylalanine (Phe) to tyrosine (Tyr). It has been proposed that a reduction of brain Tyr levels, as well as reduced activity of the key regulatory enzyme of dopamine (DA) synthesis tyrosine hydroxylase, leads to a depletion in DA activity in patients with PKU. We report a case of a 56-year-old woman with an intellectual disability due to late diagnosis of PKU and parkinsonism, with a modest clinical response to levodopa therapy.

We hypothesize that the signs of parkinsonism might be caused by the depletion of DA activity in the brain. Clinicians should be alert on parkinsonian symptoms in patients with PKU, particularly in those treated with agents that negatively influence DA transmission.

Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder of phenylalanine metabolism caused by a deficiency or inactivity of phenylalanine hydroxylase. This enzyme is responsible for conversion of the amino acid phenylalanine (Phe) to the amino acid tyrosine (Tyr). If left untreated, high levels of Phe cause intellectual disability, microcephalia, behavioral disturbances, dermatopathy, and epilepsy (Blau et al. 2010; Williams et al. 2008).

The biochemical consequences of PKU include accumulation of Phe and a deficiency of Tyr in the plasma and brain (Paans et al. 1996; Hanley et al. 2000). Tyr depletion in the brain is aggravated by a competitive mechanism through which Phe and Tyr cross the blood–brain barrier. The movement of large neutral amino acids (LNAA) across this barrier is mediated by a high-affinity, low-capacity transport system: if high levels of one of these amino acids are present in the plasma, the transport system becomes saturated, and the migration of other LNAA into the brain will be inhibited. Elevated levels of Phe in plasma thereby reduce cerebral Tyr brain influx (de Groot et al. 2013; Pietz et al. 1998).

Tyr is a precursor for dopamine (DA), a neurotransmitter that is involved in several functions in the brain, including specific cognitive functions, mood and movement. The biochemical pathway of DA is summarized in Fig. 1. The rate of synthesis of DA may be modified by the brain levels of Tyr. Furthermore, Phe is a competitive inhibitor of the enzyme tyrosine hydroxylase, which catalyzes hydroxylation of Tyr to L-3,4-dihydroxyphenylalanine (L-DOPA).

The relationship between these biochemical disturbances and the clinical outcome in patients with PKU remains to be clearly established.

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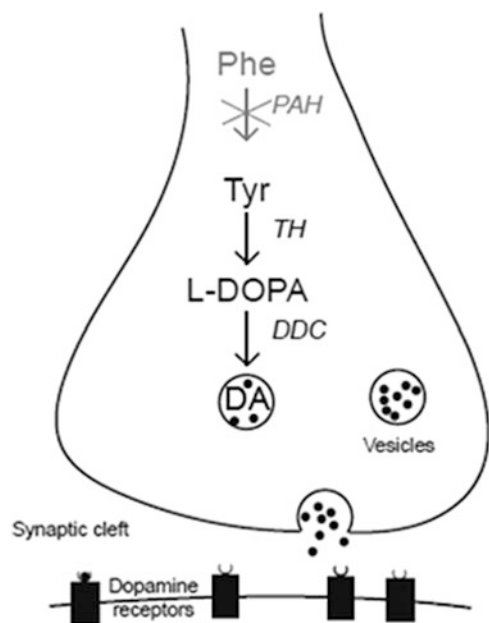


Fig. 1 Schematic model of a central dopaminergic nerve terminal illustrating dopamine (DA) synthesis, storage, release, and postsynaptic receptor binding. DA synthesis originates from the amino acid tyrosine (Tyr), and its rate-limiting step is the conversion of Tyr to levodopa (L-DOPA) by the enzyme tyrosine hydroxylase (TH). Consequently, L-DOPA is converted to DA by the enzyme dopa decarboxylase (DDC)

We present a case of an adult patient suffering from PKU and parkinsonism (a neurological syndrome characterized by tremor, hypokinesia, rigidity, and postural instability), with a modest clinical response on levodopa therapy. We hypothesize that signs of parkinsonism may be caused by the depletion of DA activity in the brain of PKU patients.

Patients and Methods

A 56-year-old woman, treated at our outpatient clinic for adults with inherited metabolic diseases, was diagnosed with PKU at the age of one year, after psychomotor developmental delay had occurred. A Phe-restricted diet was immediately started, which partially improved speech and language skills. During childhood and adolescence until she was 30 years of age, her Phe levels were usually high. It was difficult for her to adhere to the diet. Her cognitive abilities appeared to be severely impaired.

The Phe-restricted diet was discontinued between the age of 30 and 46 for unknown reasons. At the age of 46, the diet was reintroduced because of mood swings. She was first seen at our adult clinic at the age of 52. Her main complaints were tremor and mood swings. The diet

appeared to be only mildly Phe restricted. An attempt to reintroduce a stricter diet, including adequate supplementation with amino acids, caused severe distress, resulting in an inability to comply fully with protein restriction. Plasma Phe levels remained typically around 1,500 $\mu\text{mol/L}$, and the diet was discontinued. At 53 years of age, valproic acid was started for aggressive behavior, with limited improvement. On examination there was an action and resting tremor of the head, hands, and arms. This tremor was more pronounced on the right side and impaired drinking and eating. There was hypomimia (masked facies). There was no rigidity, shuffling gait, or decreased arm swing. Reintroduction of a Phe-restricted diet was not considered attainable, and because of the parkinsonian features (resting tremor and hypomimia), the patient was referred to the neurologist at 55 years of age. The signs of parkinsonism (most notably hypomimia and tremor) were confirmed. Imaging of the central dopaminergic system to confirm or exclude loss of nigrostriatal DA neurons could not be performed because of the lack of cooperation from the patient. She was started on levodopa/carbidopa 50/12.5 mg three times daily. The caretakers initially reported a good response, with decreased tremor. Most notably, eating and drinking became much easier with less spill. On follow-up after about 6 months, it appeared that this response was only temporary, and the dose of the medication was gradually increased to 250/25 mg three times daily over a period of about 3 months, with again a temporary effect. Eventually she used levodopa/carbidopa 250/25 mg six times daily and the DA agonist pramipexole 0.125 mg three times daily. At this dose there was a suspicion the patient had visual hallucinations that improved after pramipexole was discontinued. An “On-Off scoring” by a specialized Parkinson nurse was performed while the patient was still using levodopa/carbidopa. The scoring was done using the Unified Parkinson’s Disease Rating Scale part III (UPDRS part III, Ramaker et al. 2002), a systematic motor observation, where a lower score corresponds to better motor skills. This was done before (“off”) and after medication (“on”). The scores were 32 and 28, respectively. The on score was better because there seemed to be a slight decrease in tremor after administration of levodopa/carbidopa 250/25 mg, and drinking was easier. After this test the medication was discontinued because of the small clinical effect. On follow-up caregivers did complain that tremor was worse without treatment, and levodopa was restarted in a low dose (125/12.5 mg thrice daily).

Currently, she lives in an assisted care facility and continuous supervision is required. She is completely ADL (activities of daily living) dependent. She is able to

understand some simple verbal commands, and her expressive communicative capacities are limited to brief monosyllabic answers.

Discussion

We described a case of a 56-year-old female PKU patient with severe intellectual disability and inadequate restriction of dietary Phe, who presented with symptoms of parkinsonism at age 52. A modest clinical response to levodopa treatment was observed. Although a chance association between PKU and parkinsonism, and a diagnosis of “true Parkinson’s disease” is possible, the identification of this and previous cases, in combination with a well-defined disorder of brain catecholamine biosynthesis, suggests a possible etiologic association.

The prevalence of neurological symptoms in PKU patients is high: an action or postural tremor is estimated to occur in 5–32% of early-treated adult PKU patients (Cleary et al. 1994; McDonnell et al. 1998; Pietz et al. 1996; Weglage et al. 1995). Hand tremor is present in a third of those afflicted with late diagnosis, and generalized dystonia has also been reported (Brenton and Pietz 2000; Paine 1957). Late motor and cognitive decline may occur in adults who have relaxed their diet (Brenton and Pietz 2000; Thompson et al. 1990). The clinical deterioration is often reversible on resumption of dietary therapy (Thompson et al. 1990).

Several previous studies suggest DA deficiency in PKU patients. For example, using a genetic murine model of PKU, reduced levels of L-DOPA were demonstrated in the medial prefrontal cortex (Pascucci et al. 2012). Furthermore, a positron emission tomography study was performed to measure the utilization of 6-[¹⁸F]fluoro-L-dopamine (FDOPA) in the brain of seven adults suffering from PKU with elevated Phe levels, but lacking neurological deficits, compared to healthy subjects (Landvogt et al. 2008). A reduction of the rate of utilization of FDOPA in the striatum of adult PKU patients was found, suggesting reduced DA synthesis. Another study reported a reduction of homovanillic acid (HVA, the main metabolite of DA) levels in cerebrospinal fluid (CSF) in 8 early-treated on-diet PKU adolescents, with Phe levels between 708 and 1,161 $\mu\text{mol/L}$, in comparison with a control group (Burlina et al. 2000). Six of these patients had brisk deep tendon reflexes, intentional tremor, and/or ankle clonus. McKean found low levels of DA in brain tissue at autopsy. An inverse relation between plasma levels of Phe and HVA in the CSF was also found (McKean 1972). However, a DA deficiency in PKU, as shown above, does not lead to overt parkinsonism in all cases, and reports of clear parkinsonism in PKU

are rare. We are aware of one case of levodopa-responsive parkinsonism reported in an adult with PKU (Evans et al. 2004).

Considering the biochemical changes in PKU and the knowledge from abovementioned studies, PKU patients might be more susceptible for neurological symptoms caused by cerebral DA deficiency, especially those with high Phe–Tyr ratios (who relaxed their diet). DA plays a crucial role in voluntary movement. For example, Parkinson’s disease, the most common neurodegenerative cause of parkinsonism, is associated with degeneration of dopaminergic neurons. Therefore clinicians should be alert on possible parkinsonian symptoms in patients with PKU. Notably, a substantial part of the PKU patients with an intellectual disability is prescribed DA antagonists that are associated with extrapyramidal symptoms such as parkinsonism. Other agents that negatively influence DA transmission include the calcium channel blockers flunarizine and cinnarizine (Brücke et al. 1995) and the antiemetic drug metoclopramide. Although no systematic studies exist, and motor symptoms emerge only after pronounced cerebral DA dysfunction (Cummings et al. 2014), prescription of medication that negatively influences DA transmission might carry a risk to cause or aggravate parkinsonian symptoms in PKU patients. Also diet should be optimized whenever possible. If parkinsonism persists, levodopa therapy should be considered. We suggest that this issue should be further explored.

Synopsis

Our case illustrates a slight decrease of symptoms of parkinsonism after introduction of levodopa in a patient suffering from PKU. Signs of parkinsonism might be caused by DA depletion in the brain. Symptoms might be aggravated or provoked by agents that negatively influence DA transmission.

Compliance with Ethics Guidelines

Conflict of Interest

Marieke Velema, Erik Boot, Marc Engelen, and Carla Hollak declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human

experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the legal representative of the patient for being included in the study.

Contribution of the Authors

Marieke Velema: involved in the conception and design and drafting the article

Erik Boot: involved in the conception and design and revising the article critically

Marc Engelen: involved in the conception and design and revising the article critically

Carla Hollak: involved in the conception and design and revising the article critically. Guarantor

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Characterization of Variegate Porphyria Mutations Using a Minigene Approach

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Abstract Porphyrrias are a group of metabolic diseases that affect the skin and/or nervous system. In 2008, three unrelated patients were diagnosed with variegate porphyria at the CIPYP (Centro de Investigaciones sobre Porfirinas y Porfirias). Sequencing of the protoporphyrinogen oxidase gene, the gene altered in this type of porphyria, revealed three previously undescribed mutations: c.338+3insT, c.807G>A, and c.808-1G>C. As these mutations do not affect the protein sequence, we hypothesized that they might be splicing mutations. RT-PCRs performed on the patient's mRNAs showed normal mRNA or no amplification at all. This result indicated that the aberrant spliced transcript is possibly being degraded. In order to establish whether they were responsible or not for the patient's disease by causing aberrant splicing, we utilized a minigene approach. We found that the three mutations lead to exon skipping; therefore, the abnormal mRNAs are most likely degraded by a mechanism such as nonsense-mediated decay. In conclusion, these mutations are responsible for the disease because they alter the normal splicing pathway, thus providing a functional explanation for the appearance of disease and highlighting the use of minigene assays to complement transcript analysis.

Introduction

Porphyrias are a group of metabolic diseases that arise from deficiencies in the heme biosynthetic pathway. There are seven different types of porphyria, depending on which enzyme is affected, after the first one, in this pathway. They are categorized in terms of the main tissue where the enzyme deficiency is expressed or by the clinical symptoms. Specific patterns of accumulation of the heme precursors δ -aminolevulinic acid (ALA), porphobilinogen (PBG), and porphyrins are associated with characteristic clinical features such as acute neurovisceral attacks, skin lesions, or both (Pietrangelo 2010; Puy et al. 2010).

Protoporphyrinogen oxidase (PPOX; EC 1.3.3.4) is the seventh enzyme involved in this pathway, and a partial deficiency leads to variegate porphyria (VP; OMIM 176200). This is a hepatic porphyria and can present itself with skin lesions, acute attacks, or both. Cutaneous photosensitivity is characterized by skin fragility, erosions, blisters, milia, and pigmentary changes in sun-exposed areas. Neurological symptoms include intermittent attacks of abdominal pain, constipation, vomiting, hypertension, tachycardia, fever, and various peripheral and central nervous system manifestations. Acute attacks may frequently result from exposure to diverse porphyrinogenic drugs, alcohol ingestion, reduced calories intake due to fasting or dieting, infections, and hormones which stimulate heme synthesis by delta-aminolevulinic acid synthase (ALA-S) induction thereby increasing the production of the porphyrin precursors ALA and PBG (Batlle 1997; Anderson et al. 2001; Kauppinen 2005; Rossetti et al. 2008).

VP is an autosomal dominant disorder with incomplete penetrance, associated to a 50% decrease of enzymatic activity in heterozygous individuals (Deybach et al. 1981).

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Table 1 Specific primers design for each PCR

Mutation	Construct	Forward primer	Reverse primer
c.338+3insT	pTB E4	<i>ggaattccCATATGgtgggatgtctaggagaggtt</i>	<i>tggaaggCATATGaggatgag</i>
c.807G>A	pTB E7	<i>ggaattcCATATGttcaagcaattctctgcct</i>	<i>ggaattcCATATGggcctaggattctgggtag</i>
c.807G>A	U1snRNA	<i>atggtatctcccctgcaaagtaggggagagatcttggcctctgcccca</i>	<i>tcgggagcagagcccaagatctcccctactttgcaggggagataccat</i>
c.808-1G>C	pTB E8	<i>ggaattcCATATGgcctgggaaactgagagtga</i>	<i>ggaattcCATATGcttctactgttaggggttg</i>

Capital letters indicate restriction sites. Italics indicate nucleotide changes. If not indicated, the primers were used for both WT and mutated constructs

Nonetheless, there are reports about homozygous, either compound heterozygous or true homozygous, in which the PPOX activity is even lower than 50% (Hift et al. 1993; Frank et al. 1998; Roberts et al. 1998; Corrigall et al. 2000; Kauppinen et al. 2001; Palmer et al. 2001; Poblete-Gutiérrez et al. 2005; Poblete-Gutiérrez et al. 2006; Pinder et al. 2013).

The PPOX gene is located at chromosome 1 (1q22-23), spans a 5.5 kb genomic region, and contains one noncoding and 12 coding exons (Nishimura et al. 1995; Roberts et al. 1995; Taketani et al. 1995; Puy et al. 1996). Its mRNA is 1.8 kb and encodes a 477 amino acid polypeptide with a molecular weight of 50.8 kDa (Roberts et al. 1995).

To date more than 140 different mutations have been identified in the PPOX gene causing VP (Human Gene Mutation Database HGMD, <http://www.hgmd.cf.ac.uk/ac/index.php>).

Previously, we have reported three mutations, two of them are intronic (c.338+3insT and c.808-1G>C) and a missense mutation (c.807G>A) in the PPOX gene of three unrelated patients suffering from porphyria (Rossetti et al. 2008). It was hypothesized that these mutations may affect the splicing process as the coding sequence of the protein would be unaffected. However, the RT-PCR assay performed with each patient's mRNA showed either the correctly processed mRNA or no amplification at all. This may be due to the fact that the abnormal mRNAs can be degraded by a mechanism such as nonsense-mediated decay (NMD), a mechanism of degradation that occurs when a premature stop codon is generated within the transcribed sequence (Maquat 2004). If this were to occur, the transcripts are undetectable in normal RNA analysis (Baralle et al. 2009). The aim of the present work was to study the effect of these mutations using functional minigene splicing assays that would permit us to observe the effect on splicing caused by the mutations. This strategy allowed us to confirm that the three mutations are responsible for the symptoms observed in the patients who carry them.

Materials and Methods

Patients

Written informed consent was obtained from all patients prior to their inclusion in the study. The study protocol was approved by the Ethical Committee of the Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP – Hospital de Clínicas, CONICET) and was performed in accordance with the 1964 Declaration of Helsinki and its modifications. Three patients were included in this study with biochemical and molecular VP diagnosis, as described previously (Rossetti et al. 2008).

Constructs

Hybrid minigenes were made by amplifying WT and mutated exons of interest together with flanking introns of the PPOX gene. In order to obtain this from patient's genomic DNA, a PCR reaction was carried out with specific primers (Table 1) for each region of the PPOX gene (RefSeq NM_001122764.1) to be studied, flanked by NdeI sites (New England Biolabs). Each amplicon was inserted into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions, and then subcloned into the previously described pTB minigene vector (Baralle and Baralle 2005) using the NdeI restriction sites (Fig. 1a).

The U1 snRNA complementary to the nucleotide change c.807G>A was obtained by means of the QuickChange Site-Directed Mutagenesis Kit (Stratagene), using specific primers (Table 1). The U1 snRNA plasmid has been previously described (Pagani et al. 2002).

The integrity of every clone was confirmed by means of automatic sequencing (Macrogen, ABI3730XL).

Minigenes Expression Assays

The splicing assays were performed by transfecting 0.5 μ g of each minigene construct into 2.5×10^5 HeLa cells using

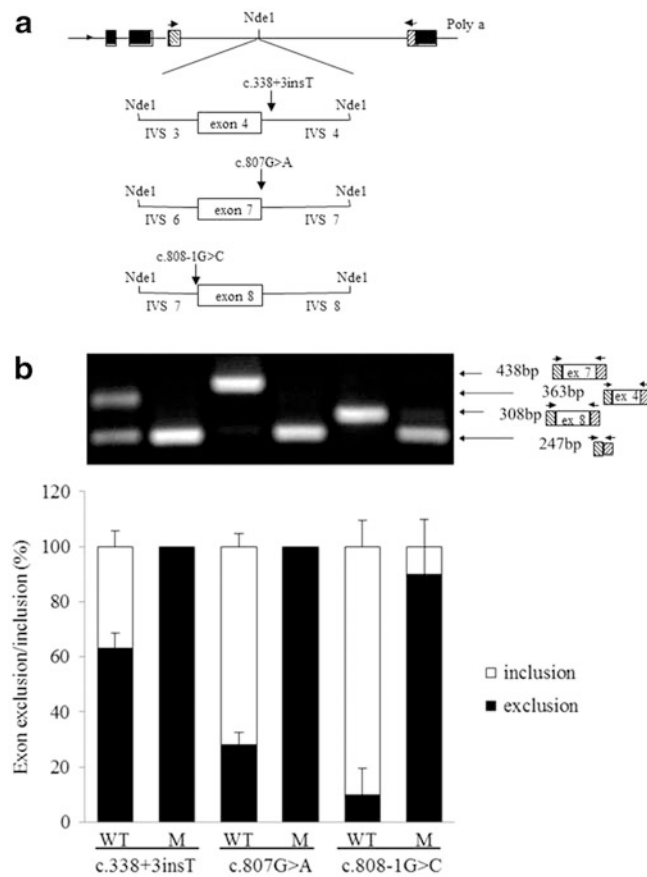


Fig. 1 pTB minigene splicing assay. **(a)** Representation of the three hybrid minigenes designed for each mutation. The vector comprises the β-globin promoter and SV40 enhancer (arrow at the start of the gene), β-globin polyadenylation site, and a series of exonic sequences corresponding to the β-globin (black boxes) and fibronectin (striped boxes). Each hybrid minigene is composed of the exon under study (white boxes) inserted into the NdeI site with and without the mutation together with the flanking introns (IVS). The black arrows above the vector indicate that the specific primers, the position of the mutations, and NdeI site are shown. **(b)** Transfection with pTB constructs. WT = wild-type construct, M = mutated construct. 247 bp = exon exclusion, 308 bp = inclusion of exon 8 (c.808-1G>C), 363 bp = inclusion of exon 4 (c.338+3insT), 438 bp = inclusion of exon 7 (c.807G>A)

Effectene Transfection Reagent (Qiagen). RNA extraction was performed using TriFast Reagent (peqGOLD). RT-PCR analysis was carried out with primers complementary to sequences present in the pTB minigene vector.

The U1 snRNA constructs were co-transfected along with pTB E7, WT or mutated, following the same procedure described above.

Statistical Analysis

Three independent transfections were made for each experiment, the expression profiles were analyzed using Scion Image Software (Scion Corporation, USA), and the results are shown as mean ± SD.

Bioinformatic Tools

NNSplice (Reese et al. 1997) was used to evaluate the 5' and 3' splice site strength of the sequence involved in the mutations. This tool compares a set of consensus splicing sites with the input sequence giving a score from 0 (weak site) to 1 (strong site), using a threshold score of 0.1 for donor sites and 0.4 for acceptor sites. ESE Finder (Cartegni et al. 2003; Smith et al. 2006) and Rescue ESE (Fairbrother et al. 2002) served to analyze the possible effect of the mutation on exonic enhancer elements. ESE Finder searches for binding sites for specific serine-/arginine-rich (SR) proteins within the given sequence, using the following threshold scores: SF2/ASF, 1.956; SC35, 2.383; SRp40, 2.670; and SRp55, 2.676. Rescue ESE finds these putative ESEs by comparing the input sequence to a large database of hexamers previously identified as such elements.

SeqBuilder (Laser Gene, DNA star) was used to predict the formation of the premature stop codons generated by the frameshift due to the exon skipping.

Results

As a first approach, bioinformatic tools were used to evaluate the possible effects of these mutations on the mRNA. The exonic mutation (c.807G>A) was observed through the use of ESE Finder and Rescue ESE not to affect possible splicing enhancer elements found in exon 7. The most striking outcome was the decrease of the strength assigned to the 5' splice site of exons 4 and 7. In the case of the mutation c.338+3insT in exon 4, this was reduced from 0.96 to 0.25 and in the case of c.807G>A in exon 7 from 0.85 to 0. The 3' splice site of exon 8 in the case of the c.808-1G>C mutation changed from 0.93 to 0.

In order to investigate if the lack of any affect on mRNA in the patients carrying the mutations was indeed being masked by degradation of the mRNA, we created a series of wild-type (WT) and mutated minigenes for each of the exons (Fig. 1a). HeLa cells were transfected with the six pTB minigene constructs, and the mRNA processing analyzed after RT-PCR on RNA extracted from the cells. Figure 1b shows quite clearly the deleterious effect on exon inclusion of all three mutations.

While the WT minigenes used to study the mutations involved with exons 7 and 8 showed a strong inclusion of the corresponding exon (72 ± 4.7% and 90 ± 9.6%, respectively), the WT minigene made to study the effect of the mutation c.338+3insT showed only 37 ± 5.8% exon inclusion. Notwithstanding, the effect the mutation has on the processing of this exon is still evident, as its introduction results in 100 ± 0% exon exclusion (Fig. 1b).

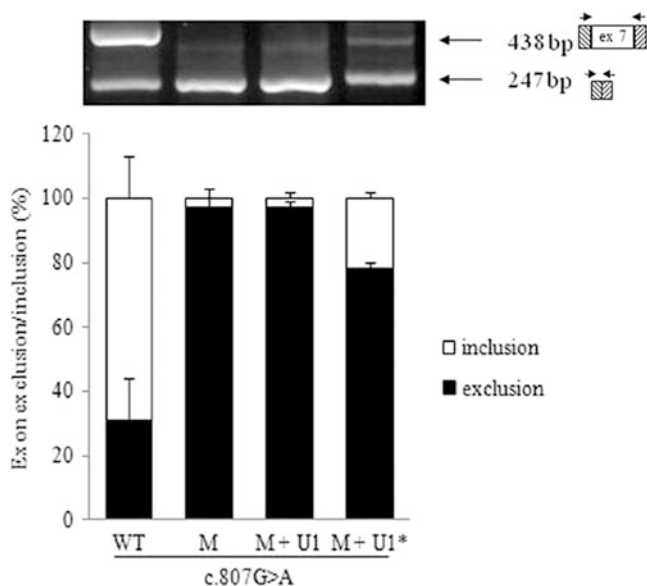


Fig. 2 Splicing rescue assay with U1 snRNA. WT = wild type pTB construct for c.807G>A, M = mutated pTB construct, M + U1 = mutated pTB construct and U1 snRNA, M + U1* = mutated pTB construct and snRNA U1 complementary to the c.807G>A mutation. 247 bp = exon exclusion, 438 bp = inclusion of exon 7

However, to elucidate if there was something in the flanking intron sequence at either site of exon 4 that would be rendering the wild-type construct less efficient at including the exon, we redesigned a new larger minigene including exon 3 to exon 5 and the corresponding introns. It was inserted in the eukaryotic vector pcDNA 3, PCR amplified and digested, as well as the vector, with XpnI and XhoI restriction enzymes. The ligated product was used to transform *E. coli* competent cells. Then, following the same protocols described in Materials and Methods for the other minigenes, we again obtained an important exclusion of exon 4 ($98.5 \pm 1.5\%$) in the presence of the mutation. However, despite using a more extended minigene, the wild-type version did not improve the inclusion of this exon ($43.5 \pm 6.5\%$) (data not shown).

In silico analysis of the mutation c.807G>A showed that no ESE is affected, while the analysis of the effect on the splice site was to reduce the strength from 0.85 to 0. However, to further demonstrate that the cause of aberrant splicing in this case is due to the weakening of the complementarity of the 5' splice site to U1snRNA, we created an U1snRNA complementary to the mutation. Co-transfection of this construct with the pTB minigenes containing the exon 7 of the PPOX gene, carrying the c.807G>A mutation, resulted in partial rescue of exon inclusion ($22 \pm 2\%$). This was not observed when WT U1 snRNA was co-transfected along with the mutated pTB minigene ($3 \pm 2\%$ inclusion) (Fig. 2).

Discussion

Previously, the PPOX gene from the three unrelated patients presenting VP symptoms was screened for mutations, finding a single base insertion at the exon 4 (c.338+3insT), a transition in the last base of exon 7 (c.807G>A), and a transversion in the last base of intron 7 (c.808-1G>C) (Rossetti et al. 2008). These mutations were thought to affect splicing as c.338+3insT could affect 5' ss definition, c.808-1G>C affect one of the universally conserved dinucleotides of the 3' ss, and c.807 G>A, the only exonic alteration, does not lead to an amino acid change. To verify this, these alterations were studied by RT-PCR using the patient's mRNA; however, we found only the normal transcript or no amplification occurred (Rossetti et al. 2008). A possible explanation why we were not able to find the mutant transcript would be that the mRNA was being degraded through a process such as nonsense-mediated decay (NMD), as in all three cases skipping of the exon leads to a premature stop codon. In order to test whether these mutations are affecting splicing or not, we used a minigene approach.

The mutation c.808-1G>C was shown to result in the skipping of the exon 8 of the PPOX gene. Considering that this alteration is affecting directly the 100% conserved AG dinucleotide present at the intron-exon junction (Zhang 1998), that NNSplice program predicts a total loss of the acceptor site, and that the experimental evidence shows that in fact the exon is being skipped, there is no doubt that the effect of this mutation on the PPOX gene is exon skipping.

With regard to the mutation c.338+3insT, although the WT minigene shows only $37 \pm 5.8\%$ and $43.5 \pm 6.5\%$ inclusion of the exon for the short and large versions, respectively, as opposed to the 100% expected under normal conditions, the effect the mutation has on this sequence is clear as the mutant constructs show nearly $100 \pm 0\%$ exon exclusion.

In the case of the mutation c.807G>A, the results show quite clearly that its effect is to cause exon skipping. Taking into account that there is no ESE affected by this mutation, as predicted by Rescue ESE and ESE Finder, the mutation might be disrupting U1 snRNA binding site. We decided to further investigate this latter hypothesis by making an U1snRNA complementary to the mutation, which lead to a partial rescue of the exon. Taken together, the bioinformatics and experimental data indicate that the mutation is disrupting the U1 snRNA binding site.

In the cases of the three mutations resulted in exon skipping, a premature stop codon is generated in the following exon. As previously described (Maquat 2004), a premature stop codon leads the mRNA to NMD, a mechanism which selectively degrades them explaining the difficulty experienced in obtaining an amplicon

corresponding to these alleles from the patient's mRNA. Through the use of minigenes, we have now however demonstrated that these three mutations are responsible for VP in the patients who carry them through alterations in the normal splicing pathway.

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Synopsis

Characterization of splicing mutations by a minigene approach

Details of Contribution of Individual Authors

Granata BX: carried out the experimental studies and analysis of data and draft the manuscript
 Baralle M: supervision of experimental studies in Italy and revising the manuscript
 De Conti L: helped in experimental studies
 Parera VE: supervision of biochemical studies in Argentina
 Rossetti MV: guarantor; conception, design, and supervision of the whole work; and revising the manuscript

Conflict of Interest

Bárabra X Granata, Marco Baralle, Laura De Conti, Victoria E Parera, and María V Rossetti declare that they have no conflict of interest.

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

Details of Ethic Approval

Written informed consent was obtained from all patients prior to their inclusion in the study. The study protocol was approved by the Ethical Committee of the Centro de

Investigaciones sobre Porfirinas y Porfirias (CIPYP – Hospital de Clínicas, CONICET) and was performed in accordance with the Helsinki Declaration of 1964 and its modifications (Tokio, Japón 1975; Venecia, Italia, 1983; Hong Kong, 1989; SudÁfrica, 1996; Edimburgo, Escocia, 2000; Washington 2002; Tokio, 2004; Seúl, Corea, 2008; Fortaleza, Brasil, 2013).

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Homozygous Truncating Intragenic Duplication in *TUSC3* Responsible for Rare Autosomal Recessive Nonsyndromic Intellectual Disability with No Clinical or Biochemical Metabolic Markers

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Abstract Intellectual disability (ID), which affects around 2–3% of the general population, is classically divided into syndromic and nonsyndromic forms, with several modes of inheritance. Nonsyndromic autosomal recessive ID (NS-ARID) appears extremely heterogeneous with numerous genes identified to date, including inborn errors of

metabolism. The *TUSC3* gene encodes a subunit of the endoplasmic reticulum (ER)-bound oligosaccharyltransferase complex, which mediates a key step of N-glycosylation. To date, only five families with NS-ARID and *TUSC3* mutations or rearrangements have been reported in the literature. All patients had speech delay, moderate-to-severe ID, and moderate facial dysmorphism. Microcephaly was noted in one third of patients, as was short stature. No patients had congenital malformation except one patient with unilateral cryptorchidism. Glycosylation analyses of patients' fibroblasts showed normal N-glycan synthesis and transfer. We present a review of the 19 patients previously described in the literature and report on a sixth consanguineous family including two affected sibs, with intellectual disability, unspecific dysmorphic features, and no additional malformations identified by high-resolution array-CGH. A homozygous truncating intragenic duplication of the *TUSC3* gene leading to an aberrant transcript was detected in two siblings. This observation, which is the first reported case of *TUSC3* homozygous duplication, confirms the implication of *TUSC3* in NS-ARID and the power of the high-resolution array-CGH in identifying intragenic rearrangements of genes implicated in nonsyndromic ID and rare diseases.

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Introduction

Intellectual disability (ID), defined as an intelligence quotient below 70, is the most frequent handicap in children. ID affects 2–3% of the general population and is classically divided into syndromic (S-ID) and nonsyndromic (NS-ID) forms, with

several modes of inheritance (Ropers 2008). The vast majority of cases with moderate-to-severe ID results from chromosomal aberrations, rearrangements, submicroscopic deletions or duplications, and point mutations in numerous genes or candidate regions (Ropers 2008). Array-based comparative genomic hybridization (array-CGH) allows the detection of a de novo genomic abnormality in 15–20% of patients with major multiple malformations and intellectual disability (Miller et al. 2010; Jaillard et al. 2010; Cooper et al. 2011). Until 2011, a genetic cause was identified in only 10% of NS-ID and no more than 8 genes were implicated in non-syndromic autosomal recessive ID (NS-ARID) (Çalışkan et al. 2011; Musante and Ropers 2014). This number has increased significantly since the emergence of new-generation sequencing strategies (Rauch et al. 2012; Musante and Ropers 2014). These genes are involved in different pathways, thus showing the extreme functional diversity of pathological mechanisms involved in NS-ARID: synaptic proteolysis, regulation of mitochondrial energy metabolism, regulation of the I- κ B kinase/NF- κ B cascade, induction of long-term potentiation, GABAB receptor-mediated spikes and wave discharges in the thalamocortical pathway, regulation of polymerase II genes, mRNA metabolism, and N-glycosylation (Garshasbi et al. 2008, 2011; Musante and Ropers 2014). However, the genetic basis of ID is still unclear in a large number of affected patients, especially those with nonsyndromic intellectual disability, in whom there are no physical signs (Rauch et al. 2012).

Glycosylation is an important posttranslational modification in eukaryotic cells and has a significant impact on numerous biological processes. About half of the body's proteins contain carbohydrate chains essential for protein structure and function, and about 200–300 genes (\approx 1% of the human genome) have been found to be involved in glycosylation processes (Varki 1993; Morava et al. 2008). Many defects in asparagine-linked glycosylation (N-glycosylation) have been identified in congenital disorders of glycosylation (CDG) syndromes, often described as multi-systemic diseases. To date, more than 70 different genetic disorders of glycosylation have been described, mostly during the past 15 years, and this number is still growing (Freeze et al. 2012).

TUSC3, the *Ost3 Saccharomyces cerevisiae* human ortholog, was initially identified as a 34 kD subunit in the yeast oligosaccharyltransferase complex (Kelleher and Gilmore 2006). The *TUSC3* gene, composed of 11 exons spanning \sim 224 Kbp of the genomic DNA on chromosome 8p22, encodes a predicted 348-amino-acid protein with five potential transmembrane domains. *TUSC3* is thought to encode a subunit of the ER-bound oligosaccharyltransferase (OTase) complex that mediates the central step in the N-linked protein glycosylation pathway (Garshasbi et al. 2008). Indeed, OTase is an oligomeric membrane-protein

complex that mediates the transfer of a preassembled high-mannose oligosaccharide onto asparagine residues of nascent polypeptides entering the lumen of the endoplasmic reticulum. Two mammalian OTase complexes composed of seven proteins differ by the presence of *TUSC3* or IAP proteins (Kelleher and Gilmore 2006). *TUSC3* appears ubiquitously expressed with greater expression in the fetal brain (Molinari et al. 2008) and interacts with the alpha isoform of the protein phosphatase 1 (PPPC1A; OMIM176875) catalytic subunit, which is involved in the modulation of synaptic plasticity, and in memory and learning processes in mice (Garshasbi et al. 2008). *TUSC3* has also been implicated in the Mg²⁺ membrane transport system with a possible role in learning capacities, working memory, and short- and long-term memory. Indeed, it has been shown that *TUSC3* and/or IAP's KO leads to a marked decrease in total and free magnesium concentration in human cells and that morpholino knockdown of IAP and *TUSC3* protein expression in zebra fish embryos results in early developmental arrest (Zhou and Clapham 2009). Interestingly, it has recently been proven that an increased concentration of magnesium in the brain leads to enhanced learning capacity, working memory, and short-term and long-term memories in rats (Slutsky et al. 2010). It has been suggested that disturbed Mg²⁺ levels caused by *TUSC3* impairment are in part responsible for the ID phenotype observed in patients carrying homozygous mutations in *TUSC3* (Garshasbi et al. 2011).

Reported patients with *TUSC3* mutations have normal results of glycosylation analyses, which could be explained by the compensation, at least partially in a tissue-specific manner, of *TUSC3* by *IAP*, the *TUSC3* paralog, coding for an OTase subunit and known to be involved in the N-glycosylation process (Kelleher et al. 2003; Garshasbi et al. 2008; Molinari et al. 2008). Another hypothesis would be that *TUSC3* and IAP protein function is essential for the glycosylation of proteins belonging to cellular subgroups in a specific tissue, mainly the central nervous system, which cannot be explored by usual serum glycosylation screening techniques. Normal glycosylation profiles in patients carrying a homozygous *TUSC3* deletion could also signify that *TUSC3* has no major role in the extracerebral N-glycosylation process (Garshasbi et al. 2008).

TUSC3 mutations or rearrangements have previously been reported in only five families, which were all but one consanguineous. The mutations lead not to aberrant DNA but to a reduced amount of transcript (Molinari et al. 2008; Garshasbi et al. 2008, 2011; Khan et al. 2011; Loddo et al. 2013).

We report a new family with NS-ARID secondary to a *TUSC3* rearrangement leading to an aberrant transcript and discuss the power of high-resolution array-CGH in

identifying intragenic rearrangements in genes implicated in nonsyndromic ID and rare diseases.

Clinical Report

Two siblings were referred to our unit for genetic investigations of ID. They were born to healthy consanguineous parents (Fig. 1a). The family history was unremarkable. In both cases, pregnancy and delivery were uneventful, and no postnatal infection, toxic exposure, or significant head trauma was noted.

Patient 1

Patient 1 was born at term with normal anthropometry. He presented significant developmental disability with independent sitting at 15 months, independent walking after 2 years, and disabled speech with very poor language. He was oriented towards a school for special needs. At 36 years of age, the physical examination was unremarkable with normal height, weight, and head circumference, except for facial dysmorphism, which included a long face with small deep set eyes (Fig. 1b). ID was estimated as severe with insufficient speech and behavioral disturbances but appeared to be nonprogressive. Neurological examinations were normal. Cardiac, abdominal, and renal ultrasound scans, skeletal X-rays, and brain MRI were normal as were funduscopy and electroencephalography.

Patient 2

Patient 2 was born at term with length 48 cm, weight 3,390 g, and occipitofrontal circumference (OFC) 33 cm. She presented developmental disability with sitting from 10 months and speech delay. She was oriented towards a school for special needs. At 29 years of age, physical examination was unremarkable with normal height, weight, and head circumference, except for facial dysmorphism, which included a long face, wide mouth, deep set eyes, and bulbous nose (Fig. 1c). Her gait was normal without ataxia. ID was estimated as moderate with insufficient speech; she was more autonomous than her brother and was able to hold a limited conversation, but never learned to read or write. Neurological examinations were normal. Cardiac, abdominal, and pelvic ultrasound scans, skeletal X-rays, brain MRI, and funduscopy were normal. She presented an epileptic stroke with an epileptic electroencephalogram. Ophthalmologic examinations were normal.

Standard metabolic screening and chromosomal analyses were normal. Molecular testing ruled out fragile X syndrome.

Methods

Genomic DNA of the affected siblings, their unaffected sister, and both parents was extracted from blood samples by conventional methods after informed consent had been provided.

Array-CGH

Array-CGH experiments were performed using the Agilent Human Genome CGH 105A oligonucleotide arrays (Agilent Technologies, Santa Clara, CA, USA; www.agilent.com), following the protocols provided by Agilent. The 105 K array has a genome-wide resolution of one probe each 21.7 kb on average. The array was analyzed with the Agilent scanner and the Feature Extraction software (v.9.5.1.3). A graphical overview was obtained using the DNA Analytics software (v4.0), using the statistical algorithms ADM-2 according to a sensitivity threshold at 6.0 and a moving average window of 5 pt. Mapping data were analyzed on the human genome sequence using ensemble (www.ensembl.org).

Molecular Investigations

Genomic Real-Time Quantitative PCR

Quantitative PCR (qPCR) was performed on genomic DNA, using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). We designed 5 primer sets within *TUSC3*. All primer sequences used in this study are shown in Table 1. qPCR was carried out in a total volume of 20 μ l containing 10 μ l of SYBR Green Master Mix (Applied Biosystems), 0.4 mM of each primer, and 10 ng of genomic DNA. Thermal cycling conditions were 95°C for 20 s, followed by 40 cycles with 95°C for 3 s and 60°C for 30 s. The *RPPHI* gene was selected as the control amplicon. Validation experiments demonstrated that amplification efficiencies of the control and all target amplicons were approximately equal. All analyses were done in triplicate. The dosage of each amplicon relative to *RPPHI* and normalized to control male DNA was determined using the $2^{-\Delta\Delta C_t}$ method.

RNA Isolation and RT-qPCR

Total RNAs were isolated from PAXgene blood RNA tubes for patient 1 and a male control using RNeasy[®] mini kit (Qiagen). RNA was reverse transcribed through the use of random primers (Superscript, Invitrogen). Reverse transcriptase quantitative real-time PCR (RT-qPCR) was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). We designed three primer

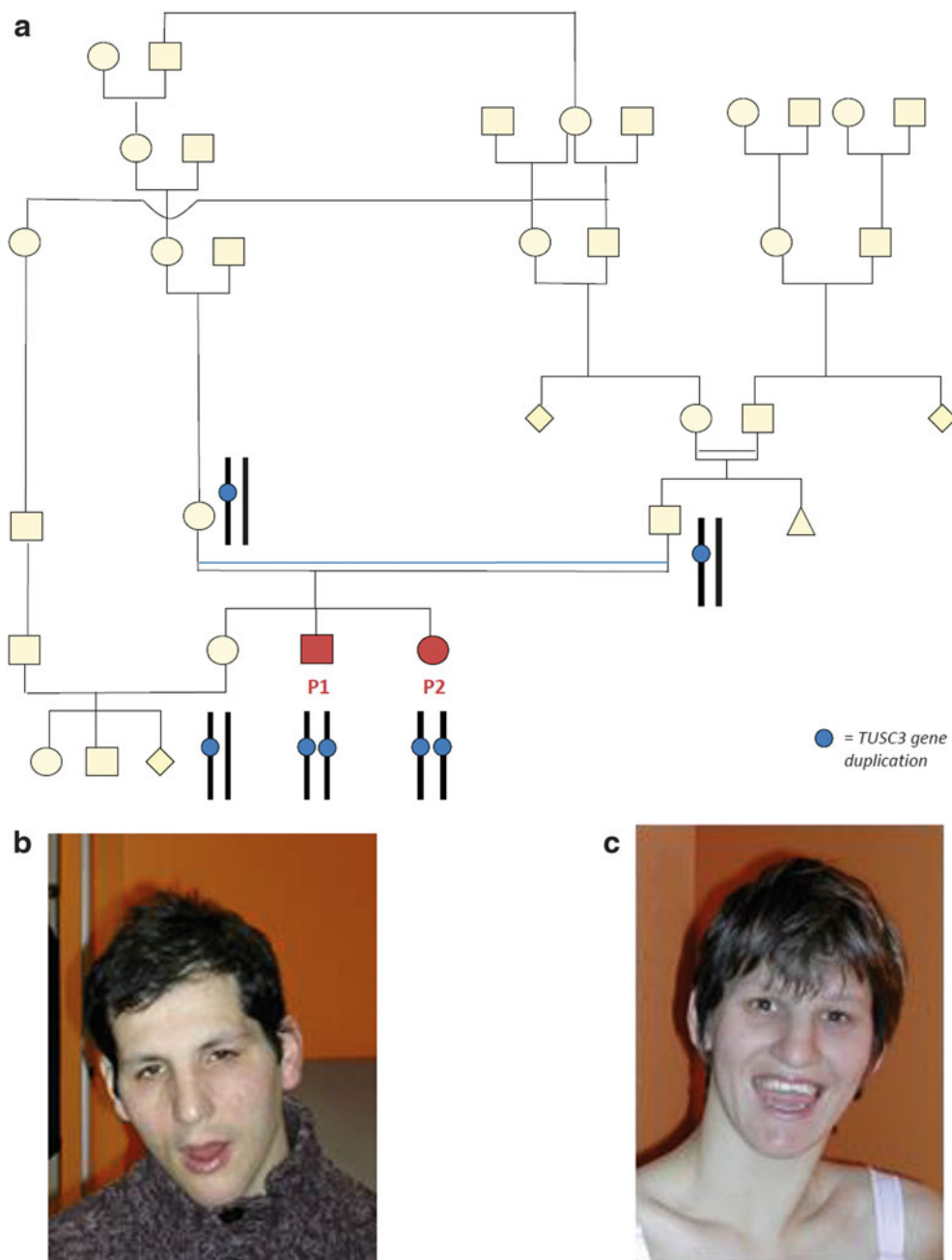


Fig. 1 Consanguineous pedigree (a). Pictures of patient 1 (b) and patient 2 (c) showing similar facial dysmorphism

sets within *TUSC3* (Table 1). RT-qPCR was carried out in a total volume of 20 μ l containing 10 μ l of SYBR Green Master Mix (Applied Biosystems), 0.4 mM of each primer, and 5 μ l of cDNA. The thermal cycling conditions were 95°C for 20 s, followed by 40 cycles with 95°C for 3 s and 60°C for 30 s. The *ESD* and *ABL* genes were selected as control amplicons. Validation experiments demonstrated that amplification efficiencies of control and all target amplicons were approximately equal. All analyses were

done in triplicate. The dosage of each amplicon relative to *ESD* and *ABL* and normalized to control male cDNA was determined using the $2^{-\Delta\Delta C_t}$ method.

Reverse transcription PCR (RT-PCR) was performed using a forward primer in exon 7 of *TUSC3* and a reverse primer in exon 2 of *TUSC3*. RT-PCR was carried out in a total volume of 25 μ l containing 0.4 mM of each primer and 5 μ l of cDNA. RT-PCR products were electrophoresed on agarose gels, purified with NucleoSpin[®] Extract II kit

Table 1 Primer sequences

Primer set	Forward primer	Reverse primer	PCR product genomic localization (hg18)	PCR product size (bp)	Gene
<i>qPCR primers</i>					
1	TCTGTGCTAGGCCCCAGGTA	CCATCCTGCTACTGAACCCAAT	chr8:15441901+15441962	62	<i>TUSC3</i> (5'UTR)
2	GCAGCAGTAAAGAAACAGCTATGCA	AAATGCCGGGAAGTCTTATCTC	chr8:15463964+15464032	69	<i>TUSC3</i> (intron 1)
3	ACTCCTGGCGCTATTCATCTG	CCCCTCATCATAGTCCACCATAAC	chr8:15552609+15552676	68	<i>TUSC3</i> (exon 3)
4	GGAGCTGCTAGCCCACCAT	TATTATAGGAAGAGAGAAACT GAGCCTTT	chr8:15624330+15624395	66	<i>TUSC3</i> (intron 6)
5	GTTTGTGGCAGAAATCACACATTATT	CCCTTTTCAAATTCGTGGTATC	chr8:15632575+15632643	69	<i>TUSC3</i> (exon 7)
<i>RT-qPCR primers</i>					
6	TCAGCGGCAGTGTTCGTGT	AGATGAATAGCGCCAGGAGTTC		80	<i>TUSC3</i> (exons 2–3)
7	AGATGAATAGCGCCAGGAGTTC	CAGGGCCAAAAGCAATGGTA		81	<i>TUSC3</i> (exons 4–5)
8	AGACTGGTTGGCCCATGGT	CACGGATATGGTTCCACATCTG		79	<i>TUSC3</i> (exons 5–6)
Primer name	Forward primer	Gene	Primer name	Reverse primer	Gene
<i>RT-PCR primer</i>					
TUSC3-7F	GGCTCAGTTTGTGGCAGAAT	<i>TUSC3</i> (exon 7)	TUSC3-2R	GTTTCGAGGT GGTGCCTTIA	<i>TUSC3</i> (exon 2)

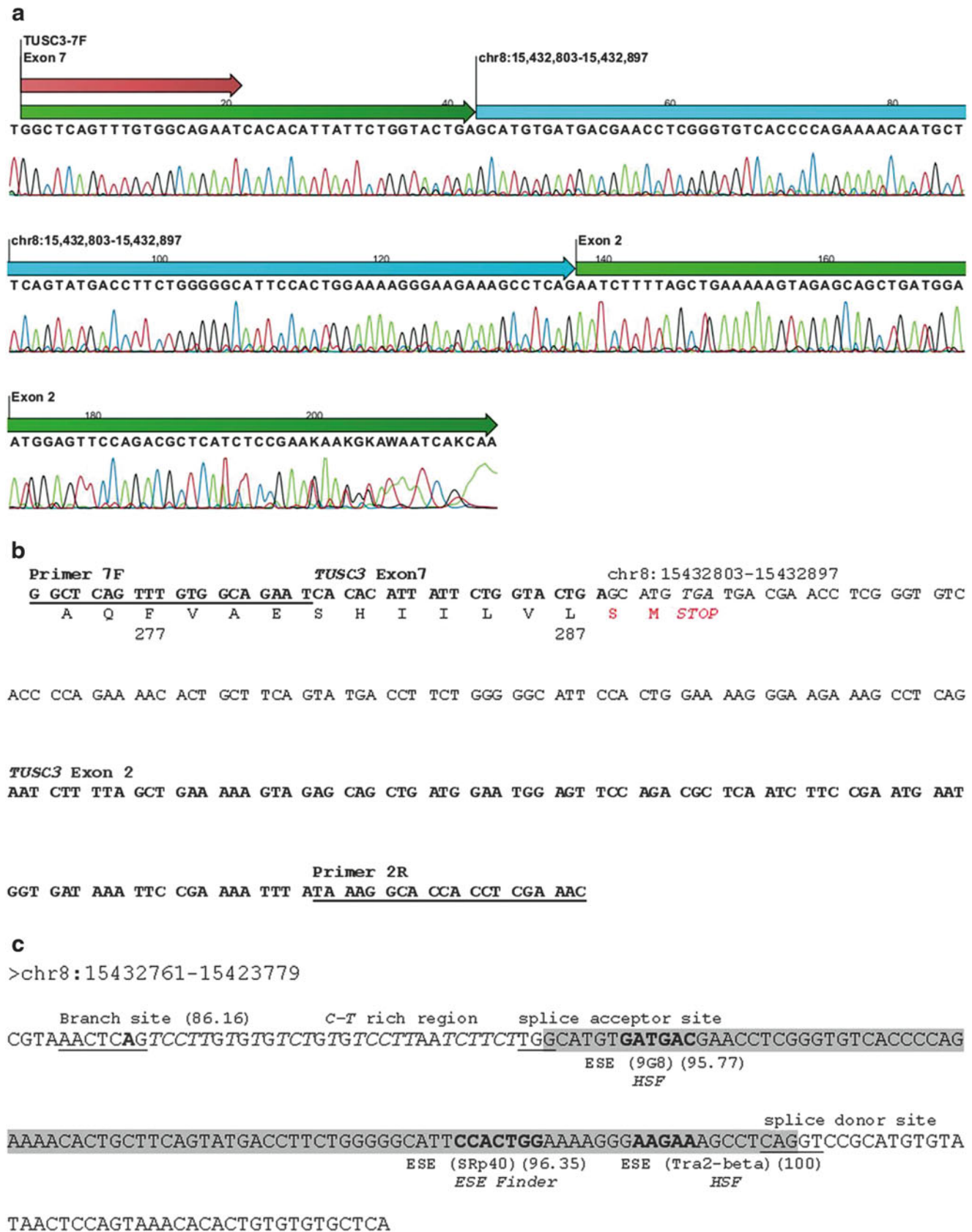


Fig. 2 (a) Sequencing results of the RT-PCR product showing an aberrant *N33/TUSC3* transcript. (b) Structure of the aberrant *N33/*

TUSC3 transcript—underlined, primer sequences; in bold, exon 7 and exon 2 sequences; third line, amino-acid translation; in red, amino-acid

(Macherey Nagel, SARL), and sequenced using the BigDye Terminator kit (Applied Biosystems).

Cell Culture

Primary fibroblasts from both patients, their parents, and controls were cultured at 37°C under 5% CO₂ in RPMI 1640/Glutamax (Invitrogen) supplemented with 10% of fetal calf serum (Invitrogen).

Biochemical Studies of the N-glycosylation Pathway

Transferrin Glycoform Study

Western blot analyses of serum transferrin, orosomucoid, alpha-antitrypsin, and haptoglobin were used to identify the loss of glycan chains leading to a decrease in the molecular weight. Polyacrylamide gel electrophoresis (SDS-PAGE) was done and followed by transfer onto nitrocellulose and revealed by a rabbit polyclonal anti-transferrin antibody as described previously (Seta et al. 1996).

Results

Array-CGH

An interstitial homozygous 178 kb amplification in the chromosome 8p22 locus was identified in both patients (Chr8:15403439–15581139, Hg19). The minimal region included exons 2 to 6 of the *TUSC3* gene. The mean log₂ratio at +0.86 was compatible with homozygous duplication or triplication.

Two other amplifications located at 4q31.3 (Chr4: 151898224–152284954) and 9p24.1 (Chr9:7598025–8106493), with a minimal size of 386 and 508 kb, respectively, were detected in patient 1. The 4q31.3 and 9p24.1 duplications were inherited from the father and were absent in patient 2. Therefore, the patient's phenotype did not appear to be explained by these two genomic imbalances. Except for polymorphic regions, no other copy-number alterations were observed (data not shown).

Genomic Quantitative Real-Time PCR

By quantitative PCR, the homozygous 8p22 duplication was confirmed in both patients, and the parents turned out to be heterozygous. We performed qPCR on genomic DNA

in order to determine more precisely the extent of the duplication within the *TUSC3* gene. It appeared to start before the 5'UTR region and to end after exon 7.

Functional Analysis of the N33 Intragenic Duplication

RT-qPCR revealed a very low expression of *TUSC3* cDNA in peripheral blood. The method was therefore not sensitive enough to determine a difference of expression level between heterozygous, homozygous and normal individuals.

To find the intragenic duplication at the RNA level, RT-PCR using a forward primer in exon 7 and a reverse primer in exon 2 of *TUSC3* was performed and amplified one fragment of approximately 250 bp in patient 1 and no specific fragment in a male control. This fragment was sequenced. It corresponded to exon 7, an inclusion of 95 bases 9,414 bp on the 3' side of *TUSC3* and exon 2 (Fig. 2a, b). Input of the chr8:15432761–15423779 region in the Human Splicing Finder (<http://www.umd.be/HSF/>) revealed a cryptic splicing site, a branch site, a CT-rich region, and an Exon Splicing Enhancer (ESE) as shown in Fig. 2c. Inclusion of this intergenic region leads to an aberrant transcript carrying the intragenic exon 2–7 duplication. Translation of this aberrant transcript leads to a premature termination codon (TGA) at position 289 as shown in Fig. 2b. Amino acids 277 to 297 are predicted to code for a transmembrane domain.

In summary, this intragenic duplication in *TUSC3* led to a premature termination codon and truncated protein with interruption in a transmembrane domain.

Glycosylation Analysis

Analysis of transferrin glycoforms by Western blot did not show any abnormal profiles in patient 1 and patient 2 compared with controls. The N-glycan structure of other serum glycoproteins was analyzed, but did not reveal any significant difference between patients and controls.

Discussion

We describe here a cohort of 21 patients harboring a homozygous *TUSC3* mutation, 19 affected members from 5 families previously described, and 2 new cases in the family we report here. All but one family were consanguineous. All of the reported 21 patients with homozygous *TUSC3* mutations/rearrangements, including our two patients, had speech delay, moderate-to-severe ID, and no

Fig. 2 (continued) translation of the intergenic included region leading to a premature stop codon; 4th line, amino-acid position. **(c)** Nucleotide sequence of the chr8:15432761–15423779 region analyzed with Human Splicing Finder—*underlined*, branch site, splice acceptor

site, and splice donor site consensus sequences; in *italics*, CT-rich region; in *bold*, predicted ESE; in *gray*, 95 bp intergenic included region

Table 2 Reported patients and methods of detection

	Molinari et al. (2008)	Garshasbi et al. (2008)	Garshasbi et al. (2011)	Khan et al. (2011)	Loddo et al. (2013)	Present study
Number of patients	2	7	3	6	1	2
Consanguinity	Yes	Yes	Yes	Yes	No	Yes
Main clinical features	ID (severe) Speech delay	Moderate facial dysmorphism ID (moderate) Speech delay Short stature (4/6) Microcephaly (1/6)	Moderate facial dysmorphism ID (severe) Speech delay Ankyloglossia Short stature (1/3) Microcephaly (2/3) NA	Moderate facial dysmorphism ID (severe) Speech delay Short stature (1/6) Microcephaly (3/6) Normal (2/2) (CT scan) Homozygous deletion (170.673 kb)	Moderate facial dysmorphism ID (moderate) Speech delay Motor instability, anxiety, oppositional-defiant disorder Hypermetropia Left cryptorchidism Hypertensive hydrocephalus (MRI) Homozygous deletion (203 kb)	Moderate facial dysmorphism ID (severe) Speech delay
Brain imaging data	Normal (2/2) (MRI)	Normal (2/2) (MRI)	NA	Normal (2/2) (CT scan)	Left cryptorchidism Hypertensive hydrocephalus (MRI)	Normal (2/2) (MRI)
Molecular defect	Mutation c.787_788insC (exon 6)	Homozygous deletion (120–150 kb)	Mutation c.163C>T (exon 2)	Homozygous deletion (170.673 kb)	Homozygous deletion (203 kb)	Homozygous duplication (238 kb)
Array	SNP array Affymetrix GeneChip Human Mapping 10 K	SNP array Affymetrix Human Mapping 250 K (Nsp)	SNP array Illumina Linkage Panel IV (6 K)	SNP array Affymetrix 500 K NspI	SNP array Affymetrix GeneChip 6.0	Array-CGH Agilent Human Genome 105A 105 K
Strategy	Autozygosity mapping and sequencing analysis	Autozygosity mapping and CNV analysis	Autozygosity mapping and sequencing analysis	Autozygosity mapping and CNV analysis	Autozygosity mapping and CNV analysis	CNV analysis

ID intellectual disability, CT computed tomography, MRI magnetic resonance imaging, NA not available

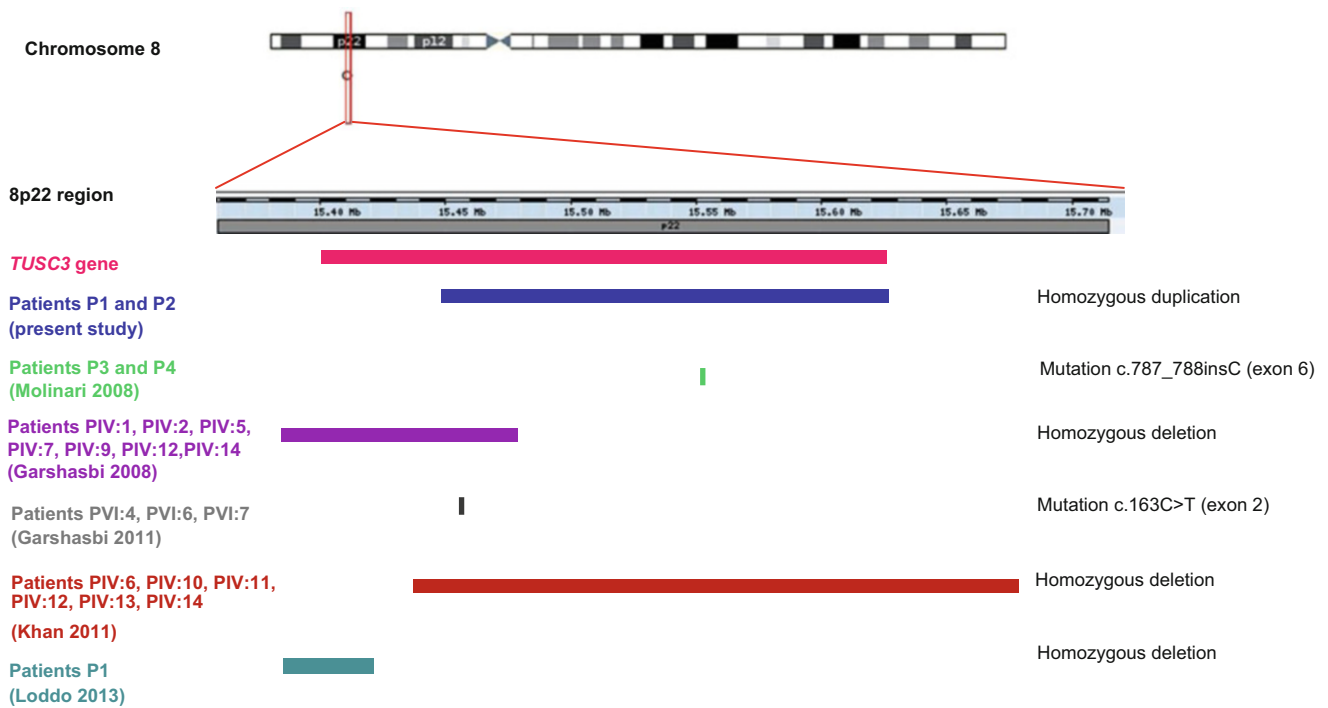


Fig. 3 Schematic representation of the mutations in *TUSC3* reported in the literature and in our patients

or moderate facial dysmorphism. Among the 20 patients for whom the values were available, 6 (30%) had microcephaly, 6 (30%) had short stature, and none of them had congenital malformation except one patient who presented left cryptorchidism. Clinical features are summarized in Table 2. Among the patients who had brain imaging, only one had brain abnormalities (hypertensive hydrocephalus, surgically treated at age 3 months, and mild enlargement of the frontal subarachnoid spaces and ventricles on the follow-up brain MRI) and none of them had the cerebellar abnormalities classically described in CDG (Table 2) (Molinari et al. 2008; Garshasbi et al. 2008, 2011; Khan et al. 2011; Loddo et al. 2013). The majority of patients (16 patients, 4 families) were identified by array-CGH analysis (Table 2). Other families were identified by homozygosity mapping and direct sequencing of *TUSC3* (Molinari et al. 2008; Garshasbi et al. 2008, 2011; Khan et al. 2011; Loddo et al. 2013) (Fig. 3).

The family we describe here is the sixth with mutations identified in the *TUSC3* gene. Since more than two thirds of the genes responsible for NS-ARID were reported in only one family, *TUSC3* appears to be one of the genes most frequently implicated in NS-ARID, (Table 2) (Musante and Ropers 2014). Interestingly, the present *TUSC3* mutation was an intragenic duplication leading to genomic imbalance identified by high-resolution 105 K

array-CGH. It is the first reported case of *TUSC3* duplication, which was found in the homozygous state, due to parental consanguinity. Because of the absence of specific signs of inborn errors of metabolism and syndromic ID, next-generation technologies including array-CGH and exome sequencing will be the only way to detect such patients and expand the clinical and biological spectrum of such pathologies. Indeed, our patients, like the other patients previously described, had normal glycosylation analyses using Western blot and isoelectric focusing, which classically permit the detection of type Ia and II CDG syndromes (Molinari et al. 2008).

Since its development, high-resolution array-CGH has shown its ability to detect intragenic copy-number variations (CNVs) and has highlighted the implication of new genes in ID, in particular in autosomal dominant familial ID (Thevenon et al. 2012). Differentiating between benign and pathogenic CNVs could be a challenge, as it depends on the size, the genes involved, and the mode of inheritance. The constitution of databases will be of help (Cooper et al. 2011). Intragenic rearrangements have been shown to contribute enormously to allelic heterogeneity in certain diseases and have been, for example, implicated in one third of patients with Cohen syndrome in the *VPS13B* gene (Knijnenburg et al. 2009; Balikova et al. 2009; El Chehadeh-Djebbar et al. 2011).

The identification of a sixth family with mutations in the *N33/TUSC3* gene not only supports the hypothesis that mutations leading to an abnormal OTase are responsible for NS-ARID but also adds to the list of N-glycosylation disorders with a normal transferrin isoelectric focusing (IEF) profile (GCS1-CDG (IIb), SLC35C1-CDG (IIc) et SLC35A1-CDG (IIf) (Lefeber et al. 2011; Freeze et al. 2012).

In conclusion, this report confirms that patients carrying *TUSC3* mutations/rearrangements cannot be diagnosed by classical CDG screening analyses and emphasizes the power of high-resolution array-CGH in identifying intragenic rearrangements in genes implicated in nonsyndromic ID, in particular when diagnosis using usual metabolic screening and gene-by-gene sequencing analysis methods is difficult.

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Conflict of Interest

S. El Chehadeh, C. Bonnet, P. Callier, M. Béri, T. Dupré, M. Payet, C. Ragon, A.L Mosca-Boidron, N. Marle, F. Mugneret, A. Masurel-Paulet, J. Thevenon, N. Seta, L. Duplomb, P. Jonveaux, L. Faivre, and C. Thauvin-Robinet declare that they have no conflict of interest.

Informed Consent

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

The study was performed according to ethical recommendations of the French National Ethics Committee.

Additional informed consent was obtained from all patients for whom identifying information is included in this article.

Contributions of Individual Authors

Conception and design, conduct, report and review, and clinical follow-up of patients: S. El Chehadeh, L. Faivre, C. Thauvin-Robinet.

Technical aspects (array-CGH, RT-PCR, functional analyses, metabolic analyses): C. Bonnet, M. Béri, M. Payet, C. Ragon, P. Jonveaux, L. Duplomb, P. Callier, A.L Mosca-Boidron, N. Marle, F. Mugneret, A. Masurel-Paulet, J. Thevenon, T. Dupré, N. Seta.

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Tandem Duplication of Exons 1–7 Neither Impairs *ATP7A* Expression Nor Causes a Menkes Disease Phenotype

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Abstract *ATP7A* duplications are estimated to represent the molecular cause of Menkes disease in 4–10% of affected patients. We identified a novel duplication of *ATP7A* exons 1–7 discovered in the context of a challenging prenatal diagnostic situation. All other reported *ATP7A* duplications ($n = 24$) involved intragenic tandem duplications, predicted to disrupt the normal translational reading frame and produce nonfunctional *ATP7A* proteins. In contrast, the exon 1–7 duplication occurred at the 5' end of the *ATP7A* gene rather than within the gene and did not correspond to any known copy number variants. We hypothesized that, if the exon 1–7 duplication was in tandem, functional *ATP7A* molecules could be generated depending on promoter selection, mRNA splicing, and the proximal and distal duplication breakpoints and that Menkes disease would be averted. Here, we present detailed molecular characterization of this novel duplication, as well as 2-year postnatal clinical and biochemical

correlations. The case highlights the ongoing need for cautious interpretation of prenatal genetic test results.

Introduction

Menkes disease (MIM# 309400) is a lethal infantile X-linked recessive disorder of copper metabolism caused by mutations in *ATP7A* (NCBI accession number: NM_000052.5), which is located at Xq21.1 and encodes a copper-transporting ATPase (Kaler and Packman 2013). This condition is characterized by male gender, early-onset cerebral and cerebellar neurodegeneration, failure to thrive, seizures, hypotonia, coarse hair, and connective tissue abnormalities. Death typically occurs by 3 years of age. Biochemical features include decreased activities of copper-dependent enzymes such as dopamine-beta-hydroxylase, cytochrome c oxidase, and lysyl oxidase (Kaler 2011). Affected individuals manifest low copper and ceruloplasmin levels in plasma or serum, as well as in cerebrospinal fluid (Donsante et al. 2010). Even in healthy newborns, serum copper and ceruloplasmin levels remain low for several weeks and thus are not reliable for diagnosis of the illness until at least 6–8 weeks of age (Kaler et al. 1993a, b, c). Prenatally, chorionic villus and amniocyte copper accumulation offer useful biochemical markers of the disease (Kaler and Tumer 1998).

On a molecular basis, the spectrum of *ATP7A* mutations causing the Menkes disease clinical and biochemical phenotype includes gene deletions and duplications, as well as missense and splice junction alterations (Moizard et al. 2011; Mogensen et al. 2011; Tümer 2013). While *ATP7A* genotype is generally predictive of response to early copper replacement therapy (Kaler 1996; Kaler et al. 2008), ambiguous situations involving novel molecular alterations in this gene may occur, as we recently reported (Schoonveld

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et al. 2013). Specifically, the latter case involved an unborn fetus with a novel duplication (exons 1–7) of the *ATP7A* gene detected prenatally. Based on the molecular context, we posited that the alteration did not preclude transcription and translation of functional *ATP7A* species and that the fetus would likely not be affected with Menkes disease (Schoonveld et al. 2013). Here, we present molecular, biochemical, and 2-year clinical follow-up data for this infant.

Materials and Methods

Human Subjects Protection: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of the patient included in this study.

Fibroblast Cell Culture: We cultured fibroblasts from the subject, from a known Menkes disease patient, and from a normal control CRL-2076 (ATCC, Manassas, VA, USA) in Dulbecco's Modified Eagles Media (DMEM) with 10% fetal calf serum and antibiotics in a 5% CO₂ incubator at 37°C.

Total RNA Preparation: The PureLink RNA mini-kit (Invitrogen) and DNase I (Qiagen) were used to isolate fibroblast total RNA from the subject, from a known Menkes disease patient, and from a normal control.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): RT-PCR was performed on fibroblast total RNA using the Enhanced Avian RT First Strand Synthesis Kit (Sigma #STR-1) with random nonamer primers, Platinum Taq DNA Polymerase High Fidelity (Invitrogen #11304-011), and *ATP7A*-specific primers 7eF:GAATGACGTGT

GCCTCCTGCGTACATA; 1eR, GAGCTACGCA-GACCGTGGCAGCGAT; 3bF, AAAATTTACCTCA-GAAAAGAACTGTA; and 4aR, CAATGCATGCCATCAAT. GATG, as described in the text.

Western Blotting: Western blots were prepared by electrophoresis of fibroblast proteins, transferring to PVDF membranes and probing with a reliable carboxyl-terminus anti-*ATP7A* antibody or an anti-beta-actin antibody, as previously described (Haddad et al. 2012).

Immunofluorescence Confocal Microscopy: The patient's fibroblasts were examined by confocal microscopy (Zeiss 510) and images captured using META software, as previously described (Yi et al. 2012).

Fluorescent in situ Hybridization: Metaphase spreads from fibroblast cell lines were prepared by standard air-drying technique, and FISH (fluorescent in situ hybridization) performed with labeled DNA BAC clones, essentially as described (Dutra et al. 1996). We chose two

BAC clones predicted to encompass both duplication breakpoints using the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly. To cover the proximal breakpoint, we used labeled BAC clone RP11-637B20 (chrX: 77,067,633-77,221,610), which covers the genomic region upstream of *ATP7A*, *ATP7A* exon 1, and most of *ATP7A* intron 1 (size of BAC clone: 153,978 bp). For the distal breakpoint, we concurrently used labeled BAC clone RP11-776014 (chrX: 77,242,535-77,414,058), which extends from *ATP7A* intron 2 until the end of the *ATP7A* locus (size of BAC clone: 171,524 bp).

On each slide, 50 ng of labeled probe was applied. Repeat sequences were blocked with Cot-1 (10X excess). A 10 µL hybridization mixture containing the labeled DNA in 50% formamide, 2x SSC, and 10% dextran sulfate were denatured at 75°C for 10 min and then incubated at 37°C for 30 min for pre-annealing. Slides were then denatured and hybridized for at least 18 h and counterstained with DAPI-Antifade.

Results

Clinical and Biochemical Findings

When examined at 7 months of age, the infant was well nourished and well developed. He weighed 8.85 kg (50–75th percentile) and his head circumference was 45.3 cm (75th percentile). His hair was normal in color and texture and his skin showed no excess laxity. Neurologically, he smiled, had excellent head control, rolled from front to back and back to front, sat independently, and transferred objects. His overall muscle tone was normal and there were no focal neurological deficits. Serum copper and ceruloplasmin levels were normal (Table 1). His plasma catechol levels (Kaler et al. 1993a, b) also were normal at 7 months, as at birth (Table 1). Microscopic examination of 25 hair shafts showed no *pili torti*. He walked independently at 13 months of age, and at 2 years of age, his neurodevelopment was entirely age appropriate.

Molecular Analysis

The patient had been diagnosed prenatally as having a duplication of exons 1–7 of the *ATP7A* gene. We hypothesized that, if the *ATP7A* promoter region had not been interrupted by meiotic crossover, at least three potential *ATP7A* molecules could be generated depending on promoter choice, mRNA splicing, and position of the 5' breakpoint and inferred that at least one would be functional (Schoonveld et al. 2013). Potential transcripts were calculated to encode a 623 amino acid *ATP7A*

Table 1 Blood biochemical data

Age	DOPA pg/mL	DOPAC pg/mL	DA pg/mL	NE pg/ml	DHPG pg/mL	DOPA:DHPG	DOPAC:DHPG	DA:NE	Cu µg/dL	Cp mg/L
1 day	4474	4120	29	428	1903	2.35	2.17	0.068	NA	NA
7 months	2499	941	10	215	901	2.77	1.04	0.047	148	344
Normal values ^{a, b}	2488 ± 526	2144 ± 319	4 ± 4	748 ± 106	1021 ± 100	2.36 ± 0.25	2.17 ± 0.38	0.04 ± 0.03	40–170	190–420
Menkes disease ^{a, b}	5346 ± 468	4832 ± 1528	199 ± 45	341 ± 92	458 ± 71	14.28 ± 2.59	11.73 ± 1.74	0.83 ± 0.71	10–53	NA

± values represent standard deviation

^a Kaler et al. (1993b)

^b Kaler et al. 2008

DOPA plasma dihydroxyphenylalanine, *DOPAC* dihydroxyphenylacetic acid, *DA* dopamine, *NE* norepinephrine, *DHPG* dihydroxyphenylglycol, *Cu* serum copper, *Cp* ceruloplasmin, *NA* not available

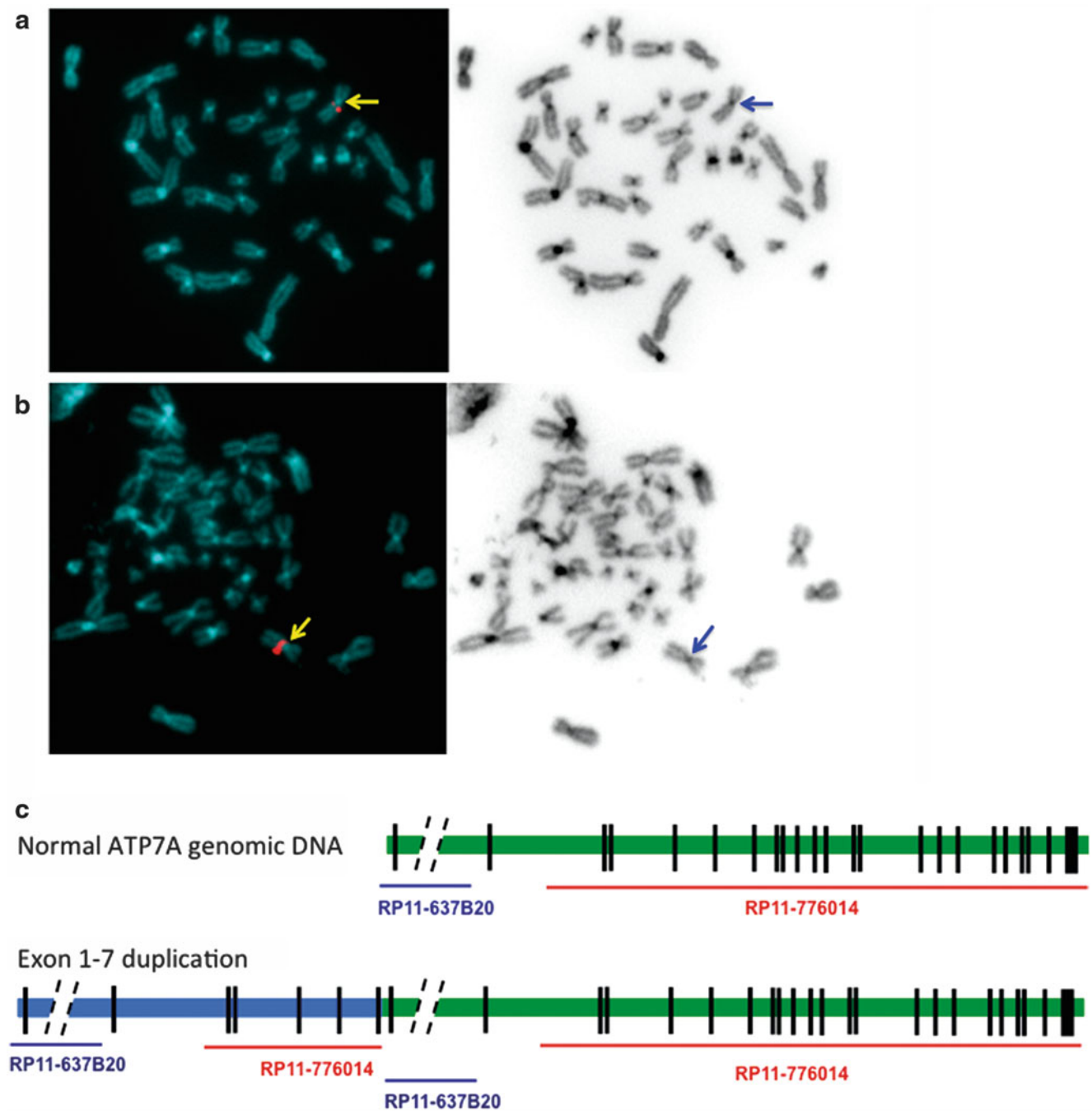


Fig. 1 FISH analysis indicates X chromosomal localization of *ATP7A* exon 1–7 duplication. Metaphase spread of chromosomes from a normal male control fibroblast cell line (**a**) and from the patient's fibroblasts (**b**) hybridized with DNA BAC clones containing segments of the *ATP7A* exon 1–7 region. The patient's metaphase (**b**) shows a more intense signal on the long arm of the X chromosome compared to normal, consistent with Xq21.1 cytogenetic localization, and no autosome signal is evident. Arrows indicate X chromosome centro-

mers. (**c**) Diagram of BAC clones RP11-637B20 (*lavender*) and RP11-776014 (*red*) employed, depicting coverage in relation to the *ATP7A* locus (*green*) and in the context of an exon 1–7 tandem duplication (*light blue*). Vertical black lines denote approximate locations of the 23 exons within the 140 kB *ATP7A* gene. Intron 1 is large (≈ 60 kB) and not drawn to scale. Please see “Materials and Methods” for detailed probe descriptions

fragment (nonfunctional), the normal 1,500 amino acid *ATP7A*, and a 2,176 amino acid version of *ATP7A*, requiring activation of a cryptic splice acceptor site at the downstream (3') exon 1 (see Figure 1, Schoonveld et al.

2013). A fourth product, 2,130 residues in length, was also theoretically possible, if exon skipping were to join exon 7 of the duplicated segment to the downstream exon 2, given the weak exon 1 splice acceptor. These larger versions

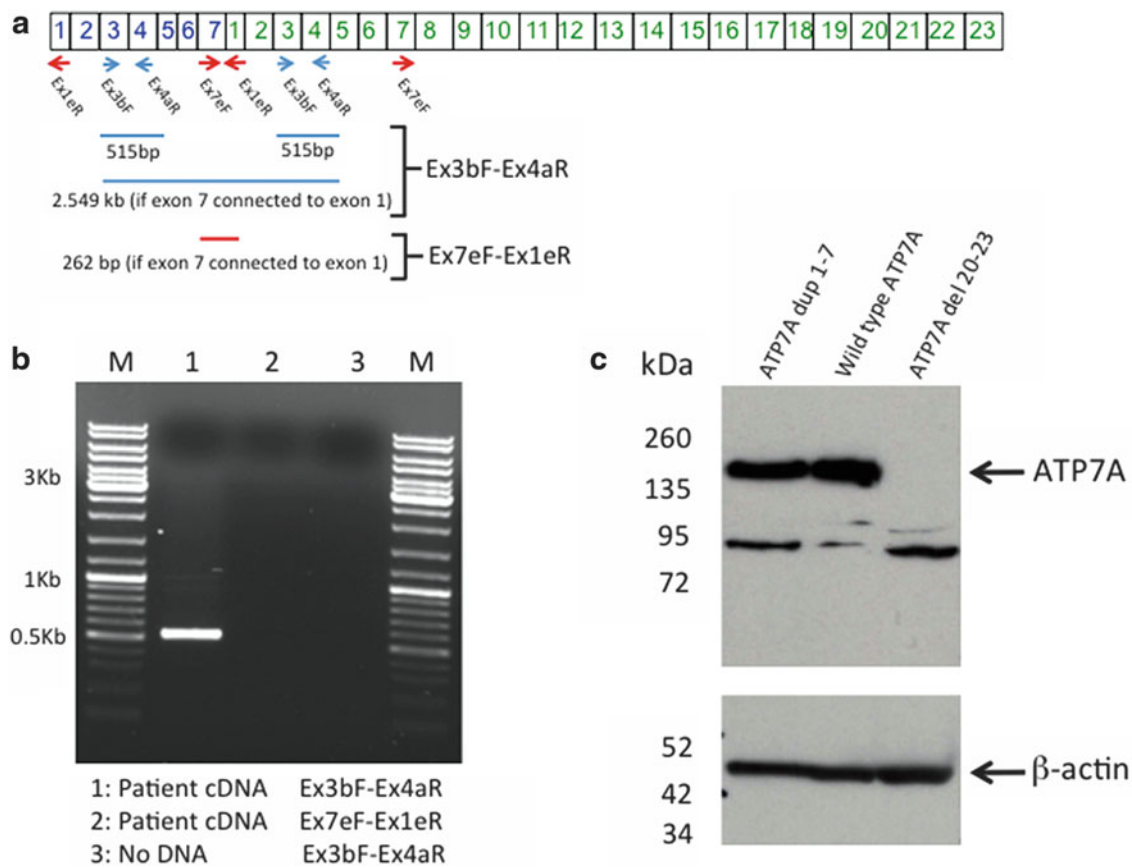


Fig. 2 (continued)

would retain the proper reading frames for ATP7A but would include 53 and 7 extraneous amino acids, respectively, between the duplicated and parent segments.

To investigate the chromosomal localization of the duplicated ATP7A fragment, we performed FISH analysis on the patient’s fibroblasts. Compared to a normal male control (Fig. 1a), the patient’s metaphase showed increased signal on the long arm of the X chromosome using DNA BAC probes encompassing the duplicated segment (Fig. 1b, c). No signal was detected on any other chromosome(s). These data indicate that the exon 1–7 duplication occurred adjacent to the *ATP7A* locus on the X chromosome.

Also utilizing the patient’s cultured fibroblasts, we evaluated *ATP7A* transcripts in this infant. We sought to determine the presence of cDNA species predicted by mRNA transcripts containing the tandem duplication (Fig. 2a). We purified total RNA from cultured fibroblasts and generated cDNA using reverse transcription-polymerase chain reaction (RT-PCR). These RT-PCR assays failed to detect any RNA transcripts that supported inclusion of the duplicated segment (Fig. 2b). Western blots of the patient’s fibroblast protein showed ATP7A protein of the normal size and amount, with no larger version(s) evident (Fig. 2c). Immunofluorescence confocal microscopy of the

patient’s fibroblasts revealed normal ATP7A quantity and *trans*-Golgi localization, as well as normal intracellular trafficking in response to increased copper concentration (Fig. 2d).

Discussion

All prior reported ATP7A duplications ($n = 24$) involved intragenic tandem duplications predicted to disrupt the normal translational reading frame and produce nonfunctional ATP7A proteins (Moizard et al. 2011; Mogensen et al. 2011; Tümer 2013). In contrast, the exon 1–7 duplication occurred at the 5’ end of ATP7A rather than within the gene. While the parents considered pregnancy termination following the prenatal genetic diagnosis, they elected to continue after careful consideration of the risks and the unknown genotype-phenotype correlation (Schoonveld et al. 2013). An apparently healthy male infant was delivered at 36 weeks gestation and showed neither biochemical nor clinical evidence of disturbed copper metabolism (Kaler et al. 1993a, b, c) (Table 1). He has achieved normal neurodevelopment throughout infancy up to his current age (24 months),

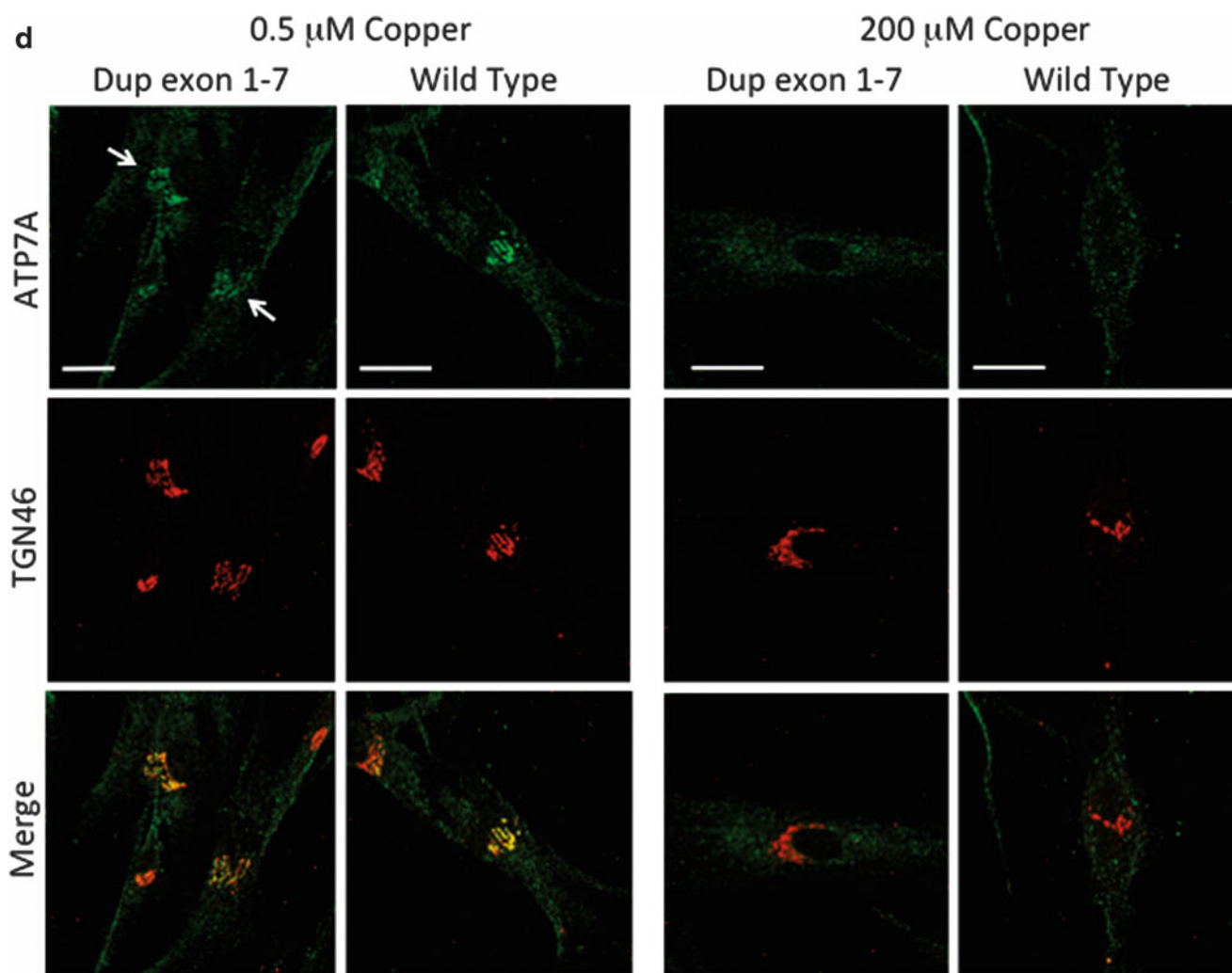


Fig. 2 Normal *ATP7A* transcript and protein in subject with duplication of *ATP7A* exons 1–7. **(a)** If the patient's cells produced a messenger RNA containing the tandem duplication (exons 1–7 + exons 1–23), we predicted amplification of a 262 bp product (red) by RT-PCR using the primer pair Exon7eF/Exon1eR and a 2.549 kb product (blue) with the primer pair Exon3bF/Exon4aR. The latter primers would also produce a 515 bp product, both from the putative mutant transcript and the normal transcript (blue). **(b)** RT-PCR resulted in amplification of only the 515 bp fragment (lane 1) and neither of the specific products predicted from a mutant transcript with the duplication (262 bp, 2.549 kb) were detected (lanes 2 and 1, respectively). The absence of a PCR product in lane 2 also excluded an inverted duplication. **(c)** Western blot of protein extracted from patient's fibroblasts shows only the normal-sized *ATP7A*. (Extra

bands of approximate size 95 kDa represent nonspecific interaction with this antibody that we have observed previously.) A well-characterized fibroblast cell line from a Menkes disease patient with deletion of *ATP7A* exons 20–23 showed no *ATP7A*, as expected. **(d)** Confocal imaging of fibroblasts from the patient (dup exon 1–7) and a normal control (wild type) illustrates normal quantity, *trans*-Golgi localization, and intracellular trafficking of *ATP7A*. Arrows indicate intense perinuclear signal in the patient's cells after staining with anti-*ATP7A* (green) under basal copper concentration (0.5 μ M). Middle panels show staining with the *trans*-Golgi marker, TGN46 (red). Merged images illustrate co-localization (yellow signal). Under exposure to elevated copper (200 μ M), the *ATP7A* signal is no longer evident in the *trans*-Golgi, consistent with intracellular trafficking to the periphery, as expected. Scale bars = 10 μ m

without copper replacement treatment (Sheela et al. 2005), and his biochemical phenotype has remained normal (Table 1).

Postnatally, we evaluated whether this patient's fibroblasts produced *ATP7A* mRNA and protein that included the exon 1–7 duplication and did not find evidence to support these theoretical possibilities. Instead, we found only normal results in our molecular and cellular functional analyses, implying that this duplication is a benign copy

number variant, presumably due to its position at the 5' region of *ATP7A* rather than at an intragenic location. We have submitted this apparently benign copy number variant to the *ClinVar* database (http://www.ncbi.nlm.nih.gov/clinvar/docs/submit/#min_content).

This case highlights the ongoing need for cautious interpretation of prenatal molecular genetic test results that involve previously uncharacterized alterations, including copy number variants.

Acknowledgments We thank the patient's parents for their kind cooperation in these studies.

Compliance with Ethics Guidelines

Informed Consent

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

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

Eun-Young Choi, Keyur Patel, Marie Reine Haddad, and Ling Yi performed the molecular and cell biological experiments described in this article. Courtney Holmes and David S. Goldstein performed the neurochemical analyses. Amalia Dutra and Evgenia Pak performed fluorescence in situ hybridization (FISH) experiments. Eun-Young Choi and Stephen Kaler planned the studies and wrote the manuscript.

All authors (Eun-Young Choi, Keyur Patel, Marie Reine Haddad, Ling Yi, Courtney Holmes, David S. Goldstein, Amalia Dutra, Evgenia Pak, and Stephen Kaler) declare that they have no conflict of interest.

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Outcome of Patients with Classical Infantile Pompe Disease Receiving Enzyme Replacement Therapy in Germany

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Abstract Purpose: Enzyme replacement therapy (ERT) has been shown to improve outcome in classical infantile Pompe disease. The purpose of this study was to assess mortality, morbidity, and shortcomings of ERT in a larger cohort of patients treated outside clinical trials. To accomplish this, we retrospectively analyzed the data of all 23 subjects with classical infantile Pompe disease having started ERT in Germany between January 2003 and December 2010.

Results: Ten patients (43%) deceased and four others (17%) became ventilator dependent. Seven infants (30.5%) made no motor progress at all, while seven (30.5%)

achieved free sitting, and nine (39%) gained free walking. Besides all the seven patients (100%) attaining no improvement of motor functions, four out of the seven (57%) achieving to sit without support, and three out of the nine (33%) being able to walk independently, secondarily deteriorated, and died or became ventilator dependent. Sustained reduction of systolic function despite reversal of cardiac hypertrophy ($n = 3$), gastroesophageal reflux ($n = 5$), swallowing difficulties or failure to thrive ($n = 11$), recurrent pneumonias ($n = 14$), port system complications ($n = 4$), anesthesia-related incidents ($n = 2$), severe allergic reactions ($n = 6$), hearing loss ($n = 3$), and orthopedic deformities ($n = 4$) were problems frequently encountered.

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Conclusion: Although this study has important shortcomings due to its retrospective nature and because important variables potentially influencing outcome were not available for a substantial amount of patients, these data suggest that classical infantile Pompe disease still remains a life-threatening condition associated with high morbidity and often dismal prognosis. Currently, a relevant number of patients do not benefit definitely from ERT.

Introduction

Pompe disease is a rare autosomal recessive disorder caused by deficiency of lysosomal acid α -glucosidase (GAA) (Hirschhorn and Reuser 2001). In classical infantile Pompe disease, virtual absence of enzyme activity leads to marked accumulation of glycogen in the heart, skeletal muscle, and other tissues. Affected patients present with hypertrophic cardiomyopathy (HCM), failure to thrive, and muscular hypotonia and weakness during the first months of life (Hirschhorn and Reuser 2001). The disease is rapidly progressive, and the majority of untreated subjects die within the first year of life without achieving any motor milestone such as turning, sitting, or standing. Survival beyond the age of 18 months is exceptional (van den Hout et al. 2003).

First enzyme replacement therapy (ERT) trials with recombinant human GAA (rhGAA) including small numbers of patients with infantile Pompe disease were conducted at the end of the 1990s and yielded promising results (van den Hout et al. 2000, 2001; Amalfitano et al. 2001). In 2006, a multinational multicenter open-label study was published that had enrolled eight infants ranging in age from 3 to 15 months with HCM and a residual enzyme activity less than 1% (Kishnani et al. 2006). All patients showed a reduction of left ventricular muscle mass reflecting reversal of HCM; five gained motor functions, and three achieved free walking. After 12 months of follow-up, two out of eight patients had died, one had become ventilator dependent, and four patients had died during the extended follow-up phase. Median age of death in the whole group was 21.7 months, being significantly later than in untreated patients (Kishnani et al. 2006).

Since this trial suggested that an early start of ERT may yield better results, a further pivotal study was performed that included 18 infants younger than age 6 months (Kishnani et al. 2007), receiving 20 or 40 mg/kg rhGAA biweekly. After 12 months of ERT, there was a significant reduction of left ventricular muscle mass and all patients were still alive. Thirteen subjects made motor progress, whereas five did not gain motor functions. Seven out of 18

achieved free walking, three were able to stand, and three were sitting without support, while six patients became ventilator dependent. Follow-up of these 18 patients for up to 3 years demonstrated that all of them had survived until age 18 months but that five patients (28%) had died thereafter and that further four subjects (22%) had become ventilator dependent. No significant differences were observed between the groups treated with 20 or 40 mg/kg rhGAA, respectively (Kishnani et al. 2007, 2009). Based on the positive results of these pivotal studies, ERT with rhGAA at a recommended dosage of 20 mg/kg biweekly has been approved by the EMA in 2006 for the treatment of Pompe disease in Europe.

Patients with classical infantile Pompe disease synthesize a nonfunctional form of GAA or are completely unable to form any kind of native enzyme (Kishnani et al. 2010a). Western blot analysis of fibroblasts derived from patients using antibodies directed against GAA can distinguish subjects with a nonfunctional protein and those synthesizing no enzyme at all. The former patients are designated as cross-reactive immunologic material (CRIM) positive, whereas the latter are classified as CRIM negative (Kishnani et al. 2007, 2010a). Alternatively, the CRIM status can be deduced from the results of genotyping, if the effects of a specific mutation on protein synthesis have been characterized (Kishnani et al. 2010a; Banugaria et al. 2011). Recently, it has been demonstrated that CRIM-negative subjects are much more likely to develop high-titer antibodies directed against rhGAA than CRIM-positive individuals and that irrespective of CRIM status, patients with high antibody titers have an attenuated therapeutic response to ERT (Kishnani et al. 2010a; Banugaria et al. 2011).

Since information about mortality and morbidity of larger patient cohorts treated outside clinical trials is yet rare (Chakrapani et al. 2010; van Gelder et al. 2014) and because data about the long-term results of ERT is still limited (Nicolino et al. 2009; Chakrapani et al. 2010; Rohrbach et al. 2010; van Gelder et al. 2014), we retrospectively analyzed outcome as well as problems encountered and mode of treatment in clinical practice of patients with classical infantile Pompe disease, who were born and had started rhGAA (Myozyme[®]) treatment in Germany between 2003 and 2010.

Patients and Methods

Physicians with special expertise potentially treating patients with classical infantile Pompe disease in Germany were contacted and asked to participate in the study. Informed consent to analyze anonymized data was obtained from all parents of patients alive, and the study was

approved by the local ethics committee of the medical faculty of the University of Giessen, Germany.

Criteria for inclusion were a definite diagnosis of classical infantile Pompe disease and treatment with rhGAA (Myozyme[®]) in children born in Germany between January 2003 and December 2010. Data acquisition took place in August 2013.

Definite diagnosis of classical infantile Pompe disease was accepted when patients had onset of clinical symptoms within the first 6 months of life, HCM diagnosed by echocardiography in combination with muscular hypotonia and proximal muscle weakness, and significantly reduced GAA activity in lymphocytes confirmed by determination in fibroblasts and/or by mutational analysis of the *GAA* gene. Twenty-three patients met these criteria. Ten subjects (43%) were female. Twelve children were of German, ten of Turkish, and one of Arabian descent. All subjects were treated with ERT.

The medical records of all subjects were retrospectively analyzed and the following data were extracted by the local physicians: age at onset of symptoms, age at diagnosis, age at start of ERT, age at ventilator dependency, age at death, or current age. We also collected data concerning the mode of initial diagnostics and their results, enzyme dosage applied and other treatment modalities, and problems, complications, surgical interventions, or procedures related to disease or to ERT. Furthermore, we analyzed the results of CRIM-status testing, genotype studies, and antibody titer determination if available (Table 1).

To assess the effects of ERT on motor function, we asked for the best motor milestone ever achieved and for the current or last motor status (i.e., no sitting, sitting without support, free walking), since standardized tests such as the Bailey scales or the Alberta Infant Motor Scale were not applied in a substantial number of patients.

Cardiac function was analyzed in patients for whom reliable data were available at least at start, after 6 months, and after 12 months of ERT. Echocardiographic findings were used for analysis in case examinations were performed by an experienced pediatric cardiologist with a 5-MHz transducer. Standard apical two- and four-chamber views were analyzed for the determination of interventricular septum (IVS) thickness during diastole, left ventricular posterior wall (LVPW) thickness during diastole, and shortening fraction (SF). IVS and LVPW thickness were assumed to reflect cardiac hypertrophy, and SF was supposed to indicate systolic function. The results were related to normal values (Kampmann et al. 2000).

All results are given as median values and range, the Mann–Whitney-rank sum test was used to compare median values between single groups, and *p*-values <0.05 were accepted as significant.

Results

At first presentation, all patients displayed elevated CK values (median 814 U/L, range 512–1,387 U/L; normal range <295 U/L) and signs of HCM, while muscular hypotonia and weakness were documented in 20 subjects (87%). Only one patient was able to turn around with help at age 3 months, whereas no other child had reached a motor milestone appropriate for the age at first presentation (i.e., turning around or sitting with or without support). No patient was ventilator dependent. In all subjects, Pompe disease was assumed after measuring a reduced GAA activity in lymphocytes. Diagnosis was confirmed by the determination of reduced enzyme activity in fibroblasts in 6 (26%) and/or by molecular genetic analysis in 22 patients (96%). Muscle biopsies performed in nine subjects (39%) revealed a vacuolar myopathy with increased glycogen content and reduced GAA activity in all.

Patient demographics and baseline characteristics are summarized in Table 2. Median age at first symptoms was 1.4 months (range 0–5.0), which at diagnosis 2.8 months (range 0–8.4), and which at start of ERT 3.3 months (range 0–9.3) (Table 2). The median time interval between diagnosis and start of ERT was 0.5 months (range 0–2.8). ERT was begun within the first month in four (17%), within the second in two (9%), within the third in five (22%), within the fourth in four (17%), and within the fifth month in four children (17%). One subject each started ERT within the sixth, seventh, eighth, and tenth month of age.

Survival, ventilator-free survival, and motor function represent important outcome measures of ERT in classical infantile Pompe disease. The overall mortality rate in this cohort was 43% (ten subjects). Additionally, four children (17%) became ventilator dependent, and two of them are completely paralyzed. Cause of death was cardiac arrhythmia related to continuously progressive HCM in one subject, whereas lethal tachyarrhythmia was suspected in another. One child died due to severe sepsis in conjunction with respiratory problems. In the remaining seven cases, parents declined start or continuation of invasive ventilation, necessary to treat respiratory insufficiency. Median age at death was 21.1 months (range 8.0–41.8). The current age of the 13 surviving patients at time of data acquisition was 62.8 months (range 43.3–122.1).

To assess whether the effects of ERT on motor development allow estimating survival or ventilator-free survival, we related these two parameters to the best motor milestone achieved (Fig. 1). All seven patients who did not achieve to sit without support (100%) died or became ventilator dependent, while this was the case for four out of seven sitting free (57%) and for three out of nine achieving free walking (33%). To determine whether beginning ERT

Table 1 Outcome measures and variables potentially related to morbidity and mortality in 23 German patients with classical infantile Pompe disease

P	Sex	Start ERT (months)	Genotype		CRIM status	Antibody titer	Survival/death (months)	Ventilated	Motor status			ERT dosage (mg/kg biweekly)	Tube feeding	Complications during ERT
			Maternal allele	Paternal allele					Best	Last/current				
1	M	3.9	c.2078msA	c.2078msA	n.k.	1:800	Alive, 43.3	-	Walking	Walking	20	-	Recurrent pneumonias	
2	F	4.7	c.1799G>A	c.1859G>A	+	1:1,200	Alive, 113.7	-	Sitting	Sitting	40	+	Aspiration pneumonia	
3	M	4.9	c.IVS17 + 102_IVS18 + 31	c.1465G>T	+	1:200	Alive, 122.1	-	Sitting	Sitting	20	-	Chronic diarrhea	
4	M	3.3	c.IVS18-IG>C	c.IVS18-IG>C	+	- ^a	Died, 8.0	+	None	None	20	+	Aspiration pneumonia, AR IV	
5	M	0.9	c.2662G>T	c.2662G>T	n.k.	1:12,000	Alive, 76.9	+	None	None	40	+	Recurrent pneumonias, AR III	
6	M	2.8	c.915G>A	c.915G>A	n.k.	1:84,000	Died, 18.2	+	Sitting	None	30	-	Pneumonia + deterioration, progressive HCM, AR IV	
7	F	2.3	c.1933G>A	c.1933G>A	+	1:800	Alive, 90.6	-	Walking	Sitting	20	-	Femur fracture, port thrombosis	
8	F	3.7	c.266G>A	c.104T>C	+	1:4,800	Died, 36.8	+	Sitting	Sitting	40	+	GER, recurrent pneumonias	
9	M	6.8	c.1157msA	c.1157msA	n.k.	- ^d	Died, 16.1	+	None	None	40	+	Port infection, sepsis	
10	M	7.0	c.2560C>T	c.2560C>T	n.k.	- ^d	Died, 21.1	-	None	None	40	+	Recurrent pneumonias	
11	M	2.6	c.1548G>A	c.1548G>A	-	1:51,200	Died, 14.1	+	None	None	40	+	Aspiration pneumonia, AR IV	
12	F	9.3	c.2431msC	c.2431msC	+	- ^e	Alive, 115.0	+	Sitting	None	80	+	Pneumonia + deterioration	
13	M	0	c.IVS11-2A>G	c.IVS11-2A>G	n.k.	n.k.	Alive, 69.2	-	Walking	Walking	20	-	Hearing impairment	
14	M	1.5	c.1978C>T	c.1978C>T	n.k.	n.k.	Died, 41.8	+	Walking	None	30	-	Pneumonia + deterioration	
15	F	4.7	c.1687C>T	c.1687C>T	-	n.k.	Died, 8.1	-	None	None	20	+	Sepsis, asystolia, port infection, AR IV	
16	F	3.4	c.IVS14 + 20A>G	c.1548G>A	+	1:5,600	Died, 27.0	+	Sitting	None	40	+	Pneumonia + deterioration, AR III	
17	F	5.5	c.1978C>T	c.1978C>T	n.k.	1:800	Alive, 62.8	-	Walking	Walking	20	-	Recurrent pneumonias	
18	M	1.4	c.896T>C	c.896T>C	+	n.k.	Died, 36.4	-	Walking	None	20	-	Recurrent pneumonias	
19	M	2.6	c.12126G>A	c.12126G>A	n.k.	- ^b	Alive, 50.4	-	Walking	Sitting	20	-	Pneumonia + deterioration	
20	F	2.9	c.2740-2742dup	c.2740-2742dup	+	1:6,400	Alive, 50.6	-	Sitting	Sitting	40	-	Recurrent pneumonias	
21	M	0.5	c.IVS07A>G	c.IVS07A>G	n.k.	- ^c	Alive, 49.4	-	Walking	Walking	20	+	Swallowing difficulties	
22	F	0.2	c.2004 C>A	c.541545delTTAC	n.k.	1:1,800	Alive, 47.0	-	Walking	Walking	20	-	Contractures	
23	F	4.7	c.1456G>C	c.1456G>C	n.k.	n.k.	Alive, 45.8	+	None	None	40	+	Scoliosis, recurrent pneumonias	

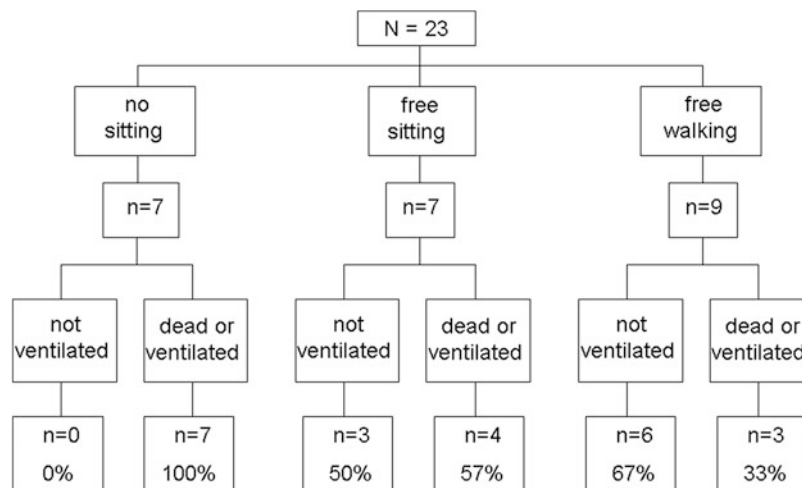
^a At age 4 months^b At age 18 months^c At age 32 months^d At age 12 months^e At age 36 months

n.k. not known, HCM hypertrophic cardiomyopathy, AR allergic reaction

Table 2 Patient demographics and baseline characteristics of 23 German patients with classical infantile Pompe disease

Sex	
Female	10 (43%)
Male	13 (57%)
Age at first symptoms	1.4 (0–5.0) months
Age at diagnosis	2.8 (0–8.4) months
Age at start of ERT	3.3 (0–9.3) months
Outcome	
Deceased	10 (43%)
Alive with ventilatory support	4 (17%)
Alive without ventilatory support	9 (40%)
Age at death	21.1 (8.0–41.8) months
Current age of surviving patients	62.8 (43.3–122.1) months
Best motor milestone achieved	
None	7 (30%)
Sitting without support	7 (30%)
Walking without support	9 (40%)
Secondary loss of motor milestones	5 (22%)
Cardiac function (n = 15)	
Reversal of cardiac hypertrophy	14 (96%)
No reversal of cardiac hypertrophy	1 (7%)
Sustained impairment of contractility	3 (13%)
CRIM status (n = 11)	
Positive	9 (39%)
Negative	2 (9%)
RhGAA dosage	
Standard	11 (48%)
Nonstandard	12 (52%)

Data is presented as median value and range or as absolute and percentage numbers

**Fig. 1** Outcome in relation to the best motor milestone achieved in 23 German patients with classical infantile Pompe disease

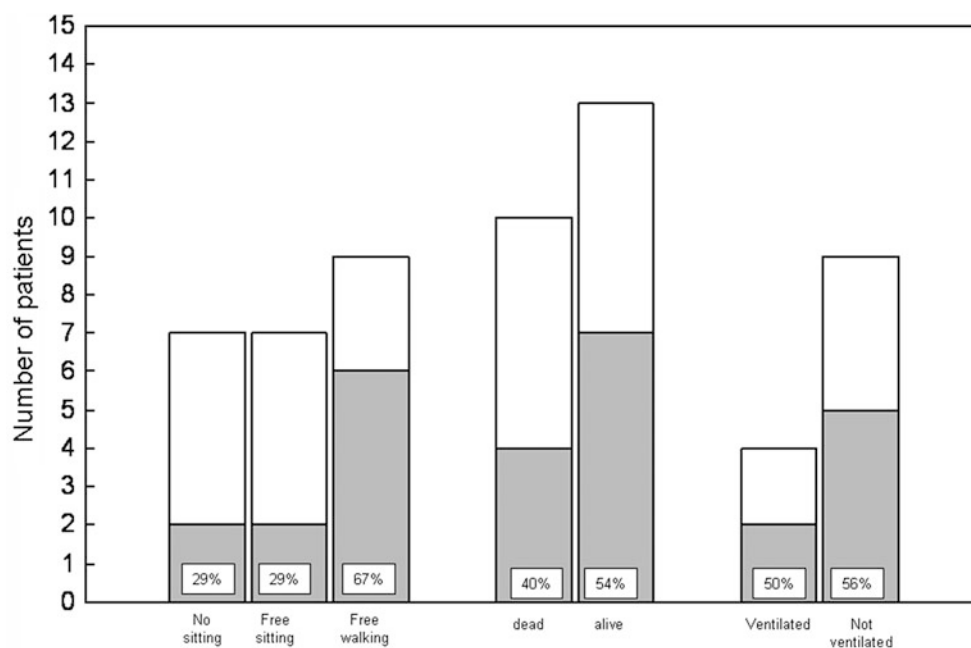


Fig. 2 Best motor milestone achieved, survival and ventilator dependency, in relation to young age at start of ERT in 23 German patients with classical infantile Pompe disease. *Gray areas* correspond to the number of subjects starting ERT within the first 3 months of life

at young age is associated with a better outcome, we depicted the best motor status achieved and the number of patients becoming ventilator dependent or having deceased in relation to start of therapy within the first 3 months of life (Fig. 2). This showed that approximately two thirds of patients starting ERT at young age achieved walking independently and that a further quarter reached the ability to sit without support.

The CRIM status, assumed to present an important predictor of outcome, was known for 11 subjects only. Two of these patients were CRIM negative and died at age 8 and 14 months, respectively (100%), without making any motor progress. Three CRIM-positive patients (37.5%) deceased at age 27, 31, and 36 months, respectively. Two of these subjects were able to sit without support and one individual achieved free walking. Determination of GAA antibody titers, also known to be related to outcome, was not performed on a regular basis in this cohort, but maximum antibody titers determined in 18 patients are presented in Table 1. Seroconversion occurred in 12 of these subjects (67%). Two individuals with very high titers (1:84,000 + 1:51,000) deceased at age 14 and 18 months, respectively. One of them made no motor progress at all, whereas the other subject deceased due to relentlessly progressive HCM. Three out of five subjects with negative antibody titers also died. Two of these patients started ERT beyond the age of 6 months, while in the third one, the antibody titer had been determined only once, shortly after start of ERT.

Problems related to the underlying disease, intercurrent illnesses, and complications of ERT may also change outcome and contribute to morbidity in individual patients. Table 3 summarizes operative procedures, problems, and complications occurring in the patient group. Port implantation was the most frequent operative procedure performed. Problems related to ERT occurred in eight patients (35%). Port complications encompassed infections in two subjects and thrombosis of the catheter in one child. Allergic reactions grade III or IV necessitating immediate interruption of intravenous application of rhGAA were documented in six patients (26%). Orthopedic deformities emerged in four subjects (17%) and included scoliosis in one and talipes in three patients. Beneath swallowing difficulties and gastroesophageal reflux necessitating gastric tube placement and sometimes fundoplication, recurrent pneumonias were a major factor contributing to morbidity in this cohort. Five subjects (22%) experienced a distinct deterioration of their functional status during pneumonias. Three of these patients were able to sit without support but lost this ability and became ventilator dependent, while two subjects, who had walked independently, showed an abrupt and unexpected worsening of their functional status resulting in ventilatory failure at age 3 and 3½ years, respectively.

Augmentation of the enzyme dosage is a further factor that may influence the course of disease. In 12 patients (52%), the recommended dosage of rhGAA (20 mg/kg biweekly) was intermittently or permanently increased. It was doubled in nine and raised to 30 mg/kg weekly in two,

Table 3 Synopsis of surgical interventions performed and problems and complications encountered in 23 German patients with classical infantile Pompe disease

Surgical interventions	N	(%)
Fundoplication	5	22
Tracheostomy	5	22
Muscle biopsy	9	39
Gastric tube placement	11	48
Port implantation	15	65
Problems/complications		
Gastroesophageal reflux	7	30
Failure to thrive/swallowing difficulties	15	65
Recurrent pneumonias	14	61
Sudden deterioration during infection	5	22
Anesthetic complication	2	9
Complication related to port system	3	13
Allergic reaction grade III/IV	6	26
Orthopedic deformities	4	17
Hearing impairment	3	13

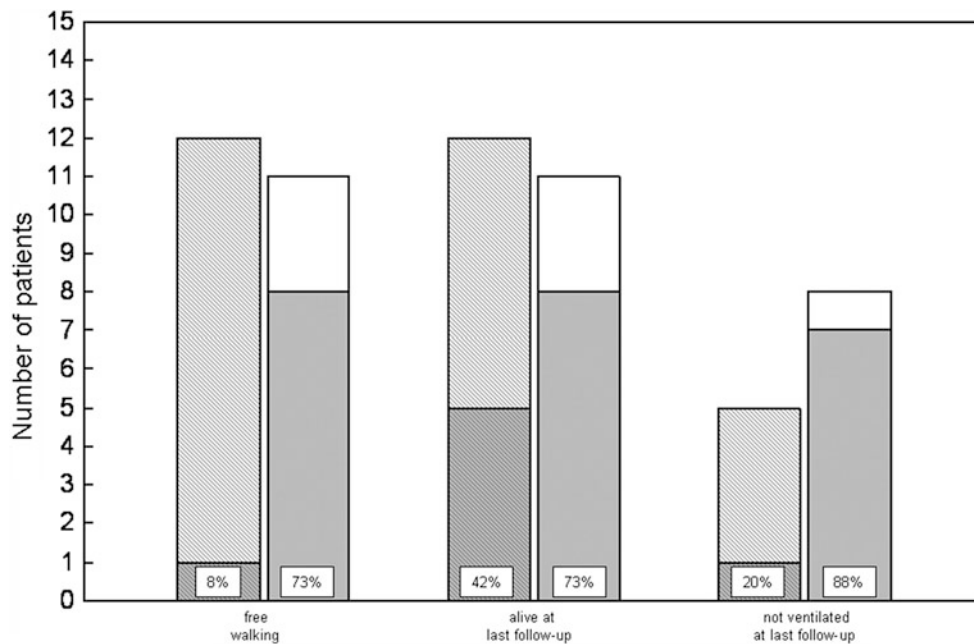


Fig. 3 Achievement of free walking, survival, and ventilator-free survival in relation to the enzyme dosage applied in 23 German patients with classical infantile Pompe disease. *Hatched bars* correspond to subjects treated with nonstandard (higher) dosages,

and *white bars* reflect individuals receiving the standard regimen (20 mg/kg biweekly). *Gray areas* correspond to patients achieving free walking, being alive, and being alive and not ventilated, respectively

while the maximum dosage administered in one child was 40 mg/kg weekly. Reasons to increase enzyme dosage were secondary worsening of motor or pulmonary function in nine, profound muscle weakness at start of therapy in one,

and cardiac insufficiency or protracted improvement of cardiac function in two subjects. Figure 3 shows the outcome of patients treated with the standard regimen and of those receiving higher rhGAA dosages. While only less

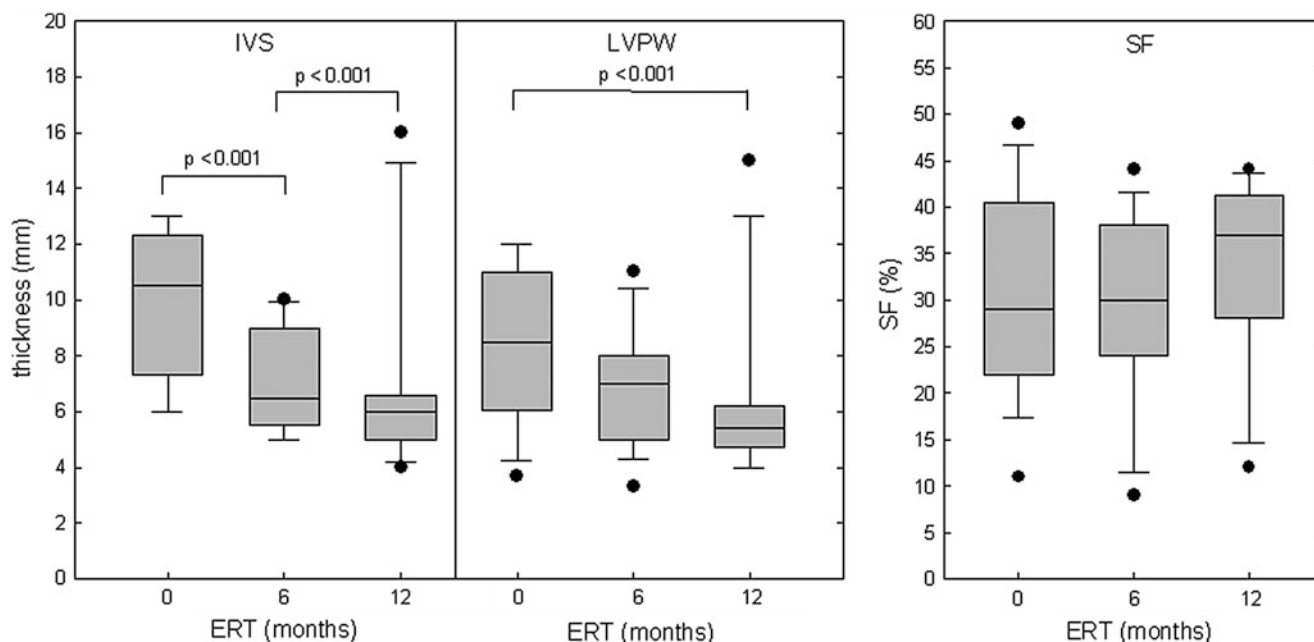


Fig. 4 Interventricular septum (IVS) thickness during diastole, left ventricular posterior wall (LVPW) thickness during diastole, and shortening fraction (SF) in 17 subjects with classical infantile Pompe disease at start, in 15 after 6 months, and in 12 after 12 months of ERT

than 10% of individuals with increased dosages achieved free walking, nearly three quarter of subjects treated with the standard dosage reached this motor milestone. In addition, more than 50% of patients getting higher enzyme dosages had deceased during follow-up, while nearly 75% of patients treated with 20 mg/kg biweekly were still alive. Moreover, 80% of the surviving patients receiving higher rhGAA dosages had to be ventilated, whereas this was the case for about 10% of subjects treated with the standard regimen only.

Clinical, laboratory, or echocardiographic symptoms of cardiac insufficiency prompting treatment with anticongestive medication were present in 17 patients (74%). Medications used included beta-blockers in 11, diuretics in 10, angiotensin-converting enzyme inhibitors in 4, calcium channel blocker in 1, and digoxins in 2 children.

Reliable echocardiographic data were available for 17 subjects (74%) at start, for 15 (65%) after 6, and for 12 (52%) after 12 months of ERT. By definition, IVS and LVPW were thickened in all subjects at the start of ERT, while SF was decreased in 6 out of 17 (35%) patients. IVS thickness had decreased significantly after 6 months and LVPW thickness after 12 months of ERT (Fig. 4). IVS thickness was normalized after 6 months of ERT in seven, had markedly decreased in seven, but had further increased in one subject despite an increase of enzyme dosage. SF remained reduced in 4 out of 15 subjects after 6 ($2 \times < 30\%$ and $2 \times < 25\%$) and in 3 out of 12 after 12 months of ERT (Fig. 4).

Discussion

This study retrospectively assessed the outcome of patients with classical infantile Pompe disease who were born and had started treatment with ERT in Germany between January 2003 and December 2010. Follow-up of the cohort was performed until death or for a period of at least 30 months of ERT. Major findings of this study with longer follow-up were that nearly 40% of patients achieved free walking but that about 60% of the patients died or became ventilator dependent and that approximately 50% of the patients with an initially positive response to ERT showed a secondary loss of acquired motor milestones during the further course of disease. These results are similar to those obtained in the pivotal study analyzing safety and efficacy of ERT in classical infantile Pompe disease (Kishnani et al. 2009), even though these children were selected for the pivotal trial and forming a more homogenous group of patients that were all treated in the same way and received utmost care. Our results are also in-line with the published UK experience on 20 patients treated from 2000 to 2009, which suggested a poorer outcome than initially hoped for (Chakrapani et al. 2010). In the British cohort, 35% of patients (7 out of 20) died, and another 30% (6/20) were alive but ventilator dependent. Moreover, our findings are also consistent with the recently reported outcome of 11 Dutch infants followed up for 0.3–13.7 years. In this cohort that included nine subjects starting ERT within the first 4

months of life, three patients died and another two became ventilator dependent (van Gelder et al. 2014).

The exact number of subjects with classical infantile Pompe disease born and treated with ERT in Germany is not known since an official registry does not exist. Based on annual birth numbers of 665,000 to 707,000 in Germany during the years 2003 to 2010 (Poetsch 2012), the calculated incidence of classical infantile Pompe disease in our study was $\sim 1:208,000$, which is lower than the frequency of $1:140,000$ per year expected according to the literature (Hirschhorn and Reuser 2001). Thus, it cannot be excluded with certainty that some individuals with classical infantile Pompe disease receiving or not receiving ERT have been missed. The high level of cooperation between specialized institutions potentially being in charge of such children, however, makes a substantial bias of data unlikely.

Patients with limited benefit from ERT included infants that did not make any substantial motor progress from the very beginning as well as children with an initial positive response but a secondary loss of functions that untreated patients would not have attained. A decline of motor and respiratory capabilities after initial improvement has also been observed in other studies with longer follow-up periods (Kishnani et al. 2009; Nicolino et al. 2009).

Factors assumed to influence outcome of patients with classical infantile Pompe disease are age at onset of symptoms, age at start of ERT, negative CRIM status due to mutations of the *GAA* gene resulting in complete enzyme absence, and high antibody titers against rhGAA (Kishnani et al. 2010a; van Gelder et al. 2014).

In this study, ERT started earlier than in the UK cohort (Chakrapani et al. 2010) and also as compared to the pivotal study (Kishnani et al. 2009) (3.3 vs. 6.5 vs. 5.3 months). This was caused by a younger age at diagnosis (2.8 vs. 4.3 vs. 5.8 months) and by short intervals between diagnosis and start of ERT (0–2.8 months), suggesting that a late diagnosis and a delayed start of therapy were no factors negatively influencing the outcome in our cohort. The observation that two children who began ERT within the first 6 weeks of life gained no motor functions at all underscores that an early start of treatment does not guarantee a positive response (Kishnani et al. 2009; Chakrapani et al. 2010). Nevertheless, subjects starting ERT within the first 3 months of life were more likely to achieve free walking than those beginning later (Fig. 2). This supports the idea that early diagnosis and timely treatment allow a better motor development (Kishnani et al. 2009; van Gelder et al. 2014). These results are in contrast to the unexpected finding of the UK study that children starting ERT later had a better outcome compared to those beginning early (Chakrapani et al. 2010). This discrepancy can be explained by differences in the study populations. All individuals from the present study manifested clinically

within the first 6 months of life as it is characteristic of the classical infantile type (van Gelder et al. 2014). By contrast, the UK cohort included three subjects with onset of clinical symptoms beyond the first year of life, being compatible with a more attenuated phenotype (Hirschhorn and Reuser 2001).

Unfortunately, information about the CRIM status was not available for a substantial amount of our patients, and antibody titers were not assessed systemically, thereby limiting interpretation of our results. This lack of data is mainly explained by difficulties in performing such tests on a routine basis, especially in the first years after the introduction of ERT. However, our findings are also compatible with the assumption that high antibody titers and negative CRIM status are predictors of poorer outcome (Kishnani et al. 2010a; Banugaria et al. 2011).

Notably, the outcome of patients treated with higher enzyme dosages concerning motor function, survival, and ventilator-free survival seemed to be less favorable than that of subjects receiving the standard dosage of 20 mg/kg rhGAA biweekly (Fig. 3). This unsuspected result can be explained by the fact that enzyme dosages were increased mainly in patients showing a deterioration of their functional status or in those responding not well to the standard regimen. These findings may also suggest that starting to treat patients with higher enzyme dosages after deterioration of their functional status is of limited benefit.

In contrast to other European countries such as the Netherlands and the UK, prescription of rhGAA therapy in Germany is not restricted to a single institution or to few designated centers. Experience in treating an extremely rare disorder may also contribute to outcome of affected patients (Kishnani et al. 2010b). In our cohort, we documented a multitude of medical problems and complications related to the underlying disease and to ERT. Therefore, complexity of the disease and high morbidity require a multidisciplinary approach and render supervision right from the start by centers experienced in the care of such patients mandatory. Although international treatment guidelines for Pompe disease are crucial (Kishnani et al. 2010b), national recommendations accommodating the peculiarities of a specific country are also meaningful (Llerena et al. 2009; Hahn et al. 2012).

Severity of HCM is a further factor known to influence the outcome in classical infantile Pompe disease (van den Hout et al. 2003). Several studies have demonstrated normalization of left ventricular muscle mass during ERT even in subjects showing no improvement of motor functions (Klinge et al. 2005; Kishnani et al. 2007, 2009). The information obtained on cardiac function in our retrospective study has to be interpreted cautiously since reliable data were available only for a limited number of patients. Nonetheless and in-line with others, we observed a

marked reduction or even normalization of left ventricular muscle wall thickness in the majority of patients (van Gelder et al. 2014). However, progressive and lethal HCM despite ERT in one subject and suspected malignant tachyarrhythmia in another demonstrate that cardiac dysfunction still contributes to mortality in the era of ERT. Moreover, systolic function, as expressed by reduced shortening fractions, remained permanently impaired despite reversal of cardiac hypertrophy in about 20% of patients with serial echocardiographies.

In summary, rhGAA therapy has dramatically improved the outcome of patients with classical infantile Pompe disease but still raises important ethical problems (Kishnani et al. 2010b). While ERT should start as soon as possible, it is not known in many cases whether the infant will respond well or not. ERT may substantially prolong life by reversing HCM, whereas its inferior effects on skeletal muscles may not prevent complete paralysis and long-term artificial ventilation (Chakrapani et al. 2010). Moreover, no recommendations exist on how to proceed if unfavorable outcome predictors (e.g., negative CRIM status) are identified during course.

Our study has several important shortcomings such as its retrospective nature, data ascertainment by independently acting institutions, and lack of data for factors potentially influencing outcome in a substantial amount of subjects. However, the data compiled in this investigation are of avail, since knowledge about potential problems occurring during ERT in clinical practice and about the limitations of this therapy are essential to provide accurate advice to families.

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Key Sentence

A retrospective analysis of 23 infants with classical infantile Pompe disease starting ERT in Germany between January 2003 and December 2010 confirms that ERT has substantially improved long-term outcome but also demonstrates that this disorder still remains a life-threatening condition associated with high morbidity and often dismal prognosis.

Compliance with Ethics Guidelines

Conflicts of Interest

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Thorsten Marquardt, Martina Huemer, Marianne Rohrbach, Gökce Seyfullah, and Eugen Mengel have received speaker honoraria and/or research grants from Genzyme Corporation, Germany.

Johanna Diebel, Dorle Schmidt, Reinald Motz, Claudia Haase, René Santer, Claudia Thiels, Martin Smitka, and Ann Meyer declare that they have no conflict of interest.

Informed Consent

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

Animal Rights

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

Details of the Contributions of Individual Authors

Andreas Hahn: planning of the study, analysis and interpretation of data, and drafting of the manuscript.

Julia B. Hennermann, Thorsten Marquardt, and Eugen Mengel: planning of the study, analysis and interpretation of data, and critical reading of the manuscript with significant intellectual contribution.

Susanne Praetorius, Nesrin Karabul, Martina Baethmann, Nicole Muschol, Martina Huemer, Marianne Rohrbach, Gökce Seyfullah, Johanna Diebel, Dorle Schmidt, Reinald Motz, Claudia Haase, René Santer, Claudia Thiels, Martin Smitka, and Ann Meyer: acquisition of data, analysis and interpretation of data, and critical reading of the manuscript with significant intellectual contribution.

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Long-Term Functional Outcomes of Children with Hurler Syndrome Treated with Unrelated Umbilical Cord Blood Transplantation

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Abstract Objectives: Hurler syndrome is characterized by progressive multisystem deterioration leading to early death in childhood. This prospective study evaluated the long-term outcomes of patients with Hurler syndrome who underwent umbilical cord blood transplantation from unrelated donors.

Study design: Only patients with Hurler syndrome who underwent umbilical cord blood transplantation between December 1995 and March 2006 ($n = 25$) and who were followed for at least 5 years ($n = 19$) were included in the analysis. The patients were longitudinally evaluated by a multidisciplinary team of specialists following a standardized protocol.

Results: Median age at transplantation was 15.9 months (range 2.1–35), and patients were followed up until a median age of 10.1 years (range 7.2–14.9). Overall survival was 80%. All successfully transplanted patients achieved full donor chimerism and normal enzyme levels, and all

children continue to make gains in development. Gross motor function was the most affected area. Vision and hearing were compromised in a minority of the patients, with some requiring corneal transplant or hearing aids. Cardiopulmonary function improved. Some children required orthopedic surgery, but severe complications were prevented in most patients. Although longitudinal growth was lower than that of unaffected children, it was considerably higher than expected from the natural course of the disease. Head circumference normalized. Hydrocephalus was not observed at longer follow-up, and cerebral atrophy decreased over time.

Conclusions: In this descriptive study of children with Hurler syndrome, unrelated umbilical cord blood transplantation was associated with improved somatic disease and neurodevelopment.

Introduction

Hurler syndrome is the most severe form of mucopolysaccharidosis type I (MPS I), an autosomal recessive lysosomal storage disorder caused by a deficiency of α -L-iduronidase (IDUA). The resulting accumulation of glycosaminoglycans leads to generalized cell, tissue, and organ dysfunction. Without treatment, patients with Hurler syndrome experience multisystem manifestations including mental retardation, skeletal deterioration, severe cardiopulmonary disease, hepatosplenomegaly, visual impairment, and deafness, usually leading to death within the first decade of life (Neufeld and Muenzer 2001).

Allogeneic hematopoietic stem cell (HSC) transplantation was first performed in a patient with Hurler syndrome three decades ago and has been extensively performed since that time (Hobbs et al. 1981). The engrafted donor-derived

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stem cells provide a continuous source of IDUA throughout the body, including the central nervous system. Since enzyme replacement therapy (ERT) is not capable of crossing the blood–brain barrier, HSC transplantation is the treatment of choice for Hurler syndrome. Clinical outcomes include reversal of organomegaly, preservation of neurocognitive development, and improved hearing, vision, and cardiopulmonary function in most transplanted patients (Guffon et al. 1998; Whitley et al. 1993; Shapiro et al. 1995; Peters et al. 1996; Vellodi et al. 1997; Peters et al. 1998; Souillet et al. 2003; Aldenhoven et al. 2008; Malm et al. 2008). However, outcomes are highly variable, and manifestations in certain organ systems continue to progress. These results were often based on small study populations or short follow-up. Additionally, there is significant heterogeneity among the stem cell sources used, conditioning regimens applied, and methods of clinical evaluation. Finally, enzyme levels were suboptimal in a significant percentage of these children because of mixed chimerism or use of a carrier donor.

In addition to bone marrow and peripheral blood, unrelated umbilical cord blood is an effective HSC source. There is evidence of improved neurocognitive outcomes after umbilical cord blood transplantation (UCBT) in children with Hurler syndrome, adrenoleukodystrophy, and Krabbe disease, particularly when performed early in the disease course (Staba et al. 2004; Escolar et al. 2005; Beam et al. 2007; Prasad et al. 2008). Various studies of UCBT in Hurler syndrome patients report high rates of sustained engraftment with full donor chimerism and normal IDUA activity (Staba et al. 2004; Martin et al. 2006; Prasad et al. 2008). Furthermore, umbilical cord blood is readily available, reducing the time between diagnosis and transplantation, which is important for optimal prognosis (Staba et al. 2004; Escolar et al. 2005).

The purpose of this manuscript is to describe the outcomes of 25 patients with Hurler syndrome who underwent UCBT in two institutions and were prospectively followed long-term using a standardized protocol.

Methods

Patient Characteristics

We reviewed the data of patients with Hurler syndrome referred for neurodevelopmental evaluations to the Program for the Study of Neurodevelopment in Rare Disorders (NDRD) at the University of North Carolina at Chapel Hill. The patients underwent UCBT between December 1995 and March 2006 at Duke University Medical Center and the

University of Minnesota and were followed by the NDRD for at least 5 years. In all patients, diagnosis was confirmed by clinical phenotype and low IDUA activity in peripheral blood leukocytes. A standardized protocol was used to assess all patients. Somatic disease was evaluated at various departments at Duke University, University of North Carolina, or University of Minnesota. The transplant data of all the patients were previously reported (Prasad et al. 2008; Orchard et al. 2010). This study was reviewed by the institutional review board of the University of North Carolina at Chapel Hill. Parents of all patients provided written informed consent before enrollment.

Mutation Analysis

Mutation analysis was performed on DNA from skin fibroblasts by the Department of Genetic Medicine of the Government of South Australia Children, Youth and Women's Health Service in North Adelaide, Australia. All patients were initially tested for four common mutations (p.Q70X, p.A327P, p.W402X, p.P533R). If the mutations could not be identified by targeted mutation testing, sequencing of the *IDUA* gene was performed, and mutations were confirmed by restriction fragment length analysis or allele-specific oligonucleotide analysis. The decision to proceed with UCBT was based primarily on clinical phenotype and/or family history and low enzyme activity. The rationale for this was that although some common nonsense mutations are associated with the severe Hurler syndrome phenotype, many MPS I patients have at least one private mutation, which makes phenotype prediction difficult (Beesley et al. 2001; Terlato and Cox 2003).

Transplantation Procedure

All patients underwent conditioning with busulfan, cyclophosphamide, and horse antithymocyte globulin. Prophylaxis against graft-versus-host disease (GvHD) was given using cyclosporine for 9 months and methylprednisolone for 2–3 months. Supportive care was provided as previously described (Staba et al. 2004; Thomas et al. 2006; Prasad et al. 2008). Most patients underwent tonsillectomy, adenoidectomy, and pressure-equalizing tube placement before transplant.

Clinical Follow-Up

Patients were evaluated at baseline and every 6–12 months after transplant by multiple pediatric subspecialists, including a cardiologist, audiologist, ophthalmologist, otolaryngology specialist, orthopedic surgeon, and a neurodevelopmental

pediatrician working with speech therapists, psychologists, and physical therapists. Brain magnetic resonance imaging (MRI) and neurophysiology tests (electromyography, brain-stem auditory evoked responses) were performed at the specified intervals. Serial neuroimaging and neurophysiologic and neurodevelopmental studies were all performed within 1 week at each clinical time point.

Neurodevelopmental Assessment All patients underwent comprehensive neurodevelopmental examinations using standardized and validated neurobehavioral tools, and outcomes were compared to norms of typically developing children (Martin et al. 2006). Age equivalents were used to allow comparison across tests and determine the acquisition of new skills. Gross motor, cognitive, language (receptive and expressive), adaptive behavior, and fine motor skills were longitudinally assessed. Adaptive behavior is a standardized measure of independent functioning and self-help skills based on parents' perceptions of their child's abilities.

Somatic Assessments Patients were assessed for somatic outcomes including:

Cardiopulmonary outcome: Echocardiograms and cardiologic and otolaryngology consultations were performed to assess valvular insufficiency, cardiomyopathy, and upper respiratory obstruction.

Orthopedic outcome: Orthopedic assessments were carried out to determine the need for orthopedic surgery, including those related to kyphoscoliosis, atlantoaxial instability, cord compression, hip dysplasia, genu valgum, or carpal tunnel syndrome.

Audiologic outcome: Behavioral audiometry and brain-stem auditory evoked responses were performed to detect hearing loss and need for hearing aids.

Ophthalmologic outcome: Ophthalmologic examinations were performed at baseline and during follow-up to assess corneal clouding and need for corneal transplantation.

Brain MRI: Brain MRI images were analyzed for abnormalities including hydrocephalus and cerebral atrophy.

Growth: Weight, longitudinal height, and head circumference were compared to gender- and age-specific growth charts from the Centers for Disease Control and Prevention. A subgroup of the patients was tested for growth hormone deficiency and evaluated to determine the need for growth hormone treatment.

All patients were followed at least 5 years; we divided the follow-up before and after 2 years to help identify outcomes that occurred closer to the time of transplantation and those that were at least 2 years from transplantation.

Statistical Analysis

The cumulative incidences of engraftment and GvHD were calculated by standard methods. The probability of event-free survival (survival with durable engraftment of donor cells) was calculated by Kaplan–Meier analysis. The cutoff date for data analysis was December 1, 2011. To evaluate neurodevelopment, a general linear mixed model was fit to the data. Age at evaluation and age-equivalent scores were used to describe and compare development across different domains of function (neurocognitive, language, motor, and adaptive). Descriptive statistics were used to describe somatic outcomes.

Results

Patient Characteristics

Twenty-five children (11 boys, 14 girls) were included in the study, and 19 children were followed up for >5 years after UCBT. Median follow-up time was 13.0 years (range 9.9–18.5) after transplantation, and median age at last follow-up was 10.1 years (range 7.2–14.9). Most (88%) of the children were of European descent (Table 1). Median age at diagnosis was 11.0 months (range 0–28 months), and median age at transplantation was 15.9 months (range 2.1–35). None of the patients received ERT. Only patients transplanted at Duke fit the inclusion criteria and were used for the analysis.

Mutation Analysis

Mutation analysis was performed for 19/25 patients. At least two putative disease-causing *IDUA* mutations were detected in 14 patients, one disease-causing mutation in four patients, and an unknown mutation in one patient (Table 2). Fifteen patients were homozygous or compound heterozygous for mutations associated with the severe phenotype. In four patients an unknown mutation was identified. Targeted mutation analysis for the four common mutations detected two (p.Q70X, p.W402X) *IDUA* mutations in eight patients, and targeted mutation analysis combined with gene sequencing detected both mutations in seven other patients. For four patients targeted mutation analysis detected only one mutation, with no additional mutations identified by gene sequencing, and for two patients gene sequencing identified both mutations. The following six *IDUA* mutations were identified by sequence analysis: p.M133I, p.Y202X, Q400X, p.R628X, c.1614delG, and IVS9-1G>T.

Table 1 Patient and donor graft characteristics, engraftment, graft-versus-host disease, and survival of Hurler syndrome patients who underwent umbilical cord blood transplantation

Patient Characteristics		<i>n</i> (%)	
Total number of patients (<i>N</i> = 25)			
Gender	Male	11 (44)	
	Female	14 (56)	
Race	American Indian/Alaska Native	1 (4)	
	Asian	1 (4)	
	African American	1 (4)	
	White	22 (88)	
Ethnicity	Hispanic	3 (12)	
		Median	Range
Age at diagnosis (months)		11	0–28
Age at UCBT (months)		15.9	2.1–35
Age at last follow-up (years) living and engrafted		10.1	7.2–14.9
Age at last follow-up (years) (<i>n</i> = 6 deceased or graft failure)		1.8	0.9–3.2

Table 2 Mutation analysis for 19 of 25 patients

Patient	Allele 1 mutation	Allele 2 mutation
1	W402X	W402X
2	W402X	M133I/Y202X
3	Q70X	W402X
4	None detected	None detected
5	Missing	Missing
6	Q70X	Q70X
7	W402X	Unknown
8	Missing	Missing
9	W402X	Unknown
10	Missing	Missing
11	Missing	Missing
12	W402X	W402X
13	W402X	W402X
14	W402X	E299X
15	W402X	W402X
16	W402X	c.1614delG
17	Q400X	IVS9-1G>T
18	W402X	Q70X
19	Q400X	IVS9-1G>T
20	W402X	W402X
21	W402X	Unknown
22	W402X	Unknown
23	W402X	R628X
24	Missing	Missing
25	Not performed	Not performed

Engraftment and GvHD

Six children had chronic and extensive GvHD, whereas seven patients had mild cases with only skin involvement.

Survival

As of December 1, 2011, five of the 25 children died post-transplant because of infection (*n* = 2), hepatic/gastrointestinal bleeding (*n* = 1), respiratory failure (*n* = 1), or chronic GvHD (*n* = 1). Graft failure occurred in two patients, both of whom underwent a second transplant. One of these patients died, and the second failed to engraft. Overall survival was 80%. All patients who underwent successful transplantation achieved full donor chimerism (>90%), and all showed IDUA activity within the reference range.

Neurodevelopmental Function

Post-transplantation neurodevelopmental function of children with Hurler syndrome was compared to the neurodevelopmental function of unaffected children. All six areas of development (gross/fine motor, cognitive, receptive/expressive language, adaptive behavior) contribute to overall functioning. Gross and fine motor functions were the most affected, progressing at significantly lower rates compared to those of unaffected children of the same age. On the other hand, after successful UCBT, patients gained

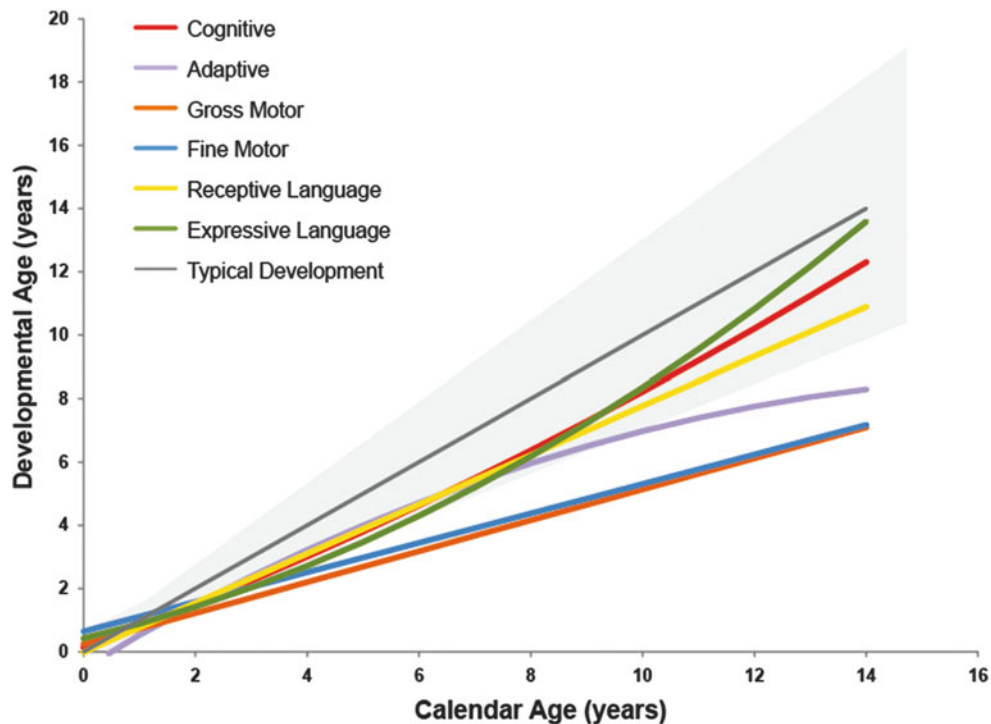


Fig. 1 Neurodevelopmental function of children with Hurler syndrome after umbilical cord blood transplantation compared to that of unaffected children. Age-equivalent scores were used to compare and monitor developmental progress. The *colored lines* depict the mean developmental curves (i.e., cognitive, adaptive, gross motor, fine

motor, receptive language, and expressive language) of the surviving patients. These *lines* were plotted against the mean typical cognitive growth curve (*gray continuous line*) and approximate variability (95%; *gray area*) observed in typically developing children

cognitive skills at a normal rate and maintained the gains over time (Fig. 1). Because of the degree of motor involvement, many activities of daily living were affected, leading to lower adaptive behavior compared to age-matched unaffected children.

Somatic Function

Table 3 outlines somatic manifestations present before treatment, during the initial 2 years after transplantation, and after the subsequent 2-years to understand which outcomes are expected closer and further away from the time of transplant.

Cardiopulmonary Outcome Cardiomyopathy was observed at baseline (15%), during the first 2 years post-transplant (27%), and after the 2-year follow-up (16%). However, cardiomyopathy resolved in all but one patient after longer follow-up. In contrast, cardiac valve insufficiency appeared to progress over time, detected in 29% of patients before UCBT, 25% during the first 2 years post-transplant, and 68% after the 2-year follow-up.

Orthopedic Outcome Orthopedic manifestations requiring surgical intervention included kyphoscoliosis (32%), hip

dysplasia (21%), genu valgum (63%), and carpal tunnel syndrome (47%). Other operations performed were trigger finger surgery, surgery for valgus deformity of the ankles, and Achilles tendon lengthening. All orthopedic interventions were performed more than 2 years after transplantation. No interventions were required for atlantoaxial instability, and none of the patients had cord compression.

Audiologic Outcome Amplification for hearing loss was required for 0% of patients before UCBT, 27% of patients during the first 2 years post-transplant, and 37% of patients after the 2-year follow-up. After 2 years only one patient had abnormal brainstem auditory evoked responses.

Ophthalmologic Outcome After 2 years of follow-up, 95% of the patients had corneal clouding (mild, $n = 8$; moderate, $n = 7$; severe, $n = 3$). Corneal clouding progressed over time in 56% of patients, stabilized in 38% of patients, and improved in 6% of patients. Corneal transplantation was required for severe corneal clouding in 21% of patients >2 years post-transplant.

Neurological Outcome Neurosurgical intervention with a ventriculoperitoneal shunt (VPS) was performed for hydrocephalus in 16% of patients before transplantation, 33% of

Table 3 Somatic manifestations in Hurler syndrome patients who were evaluated more than 5 years after receiving umbilical cord blood transplantation

Organ system	Manifestation	At baseline (pre-UCBT) n (%)	At 0–2 years FU (post-UCBT) n (%)	At >2 years FU (post-UBCT) n (%)
Cardiopulmonary	Valvulopathy: insufficiency	4/14 (29)	4/16 (25)	13/19 (68)
	Cardiomyopathy	2/13 (15)	4/15 (27)	3/19 (16)
	Upper respiratory obstruction	4/13 (31)	0/15 (0)	13/19 (68)
Orthopedic	Thoracolumbar kyphosis: spinal surgery	0/13 (0)	0/15 (0)	6/19 (32)
	Hip dysplasia: surgery	0/13 (0)	0/15 (0)	4/19 (21)
	Genu valgum: surgery	0/13 (0)	0/15 (0)	12/19 (63)
	Carpal tunnel syndrome: surgery	0/13 (0)	0/15 (0)	9/19 (47)
	Cord compression: surgery	0/13 (0)	0/15 (0)	0/19 (0)
	Atlantoaxial instability: surgery	0/13 (0)	0/15 (0)	0/19 (0)
	Other surgery*	0/12 (0)	0/15 (0)	4/19 (21)
	Audiologic	Hearing loss: BAER abnormalities	2/13 (15)	0/15 (0)
	Hearing loss: amplification	0/13 (0)	4/15 (27)	7/19 (37)
Ophthalmologic	Corneal clouding	8/14 (57)	8/16 (50)	18/19 (95)
	Degree	None	6/14 (43)	8/16 (50)
		Mild	5/14 (36)	6/16 (38)
		Moderate	3/14 (21)	2/16 (13)
		Severe	0/14 (0)	0/16 (0)
	Corneal transplant	0/13 (0)	0/15 (0)	4/19 (21)
Cerebral MRI	Hydrocephalus: surgery (VP shunt)**	3/13 (16)	5/15 (33)	6/19 (32)
	Cerebral atrophy	8/16 (50)	5/15 (33)	6/19 (32)
Growth	GH deficiency	0/13 (0)	0/15 (0)	7/19 (37)
	GH treatment	0/13 (0)	0/15 (0)	11/19 (58)

*Other operations: trigger finger release 2/24 (8%), ankle surgery 2/24 (8%), Achilles tendon lengthening 1/24 (4%)

**New cases of VP shunt insertion

the patients required a VPS in the first 2 years post-transplantation, and 32% continued to require a VPS >2 years post-transplant. However, none required additional VPS insertions 2 years after transplantation.

Cerebral MRI Cerebral atrophy was present in 50% of patients at baseline but was detected in only 32% of patients >2 years post-transplant.

Growth Longitudinal height, weight, and head circumference are shown in Fig. 2a–f. After successful transplantation, longitudinal height remains most affected, ranging from <5th to 50th percentile in both boys and girls. Eleven patients received growth hormone treatment. Weight was normal in the vast majority of patients, and head circumference normalized during longer follow-up.

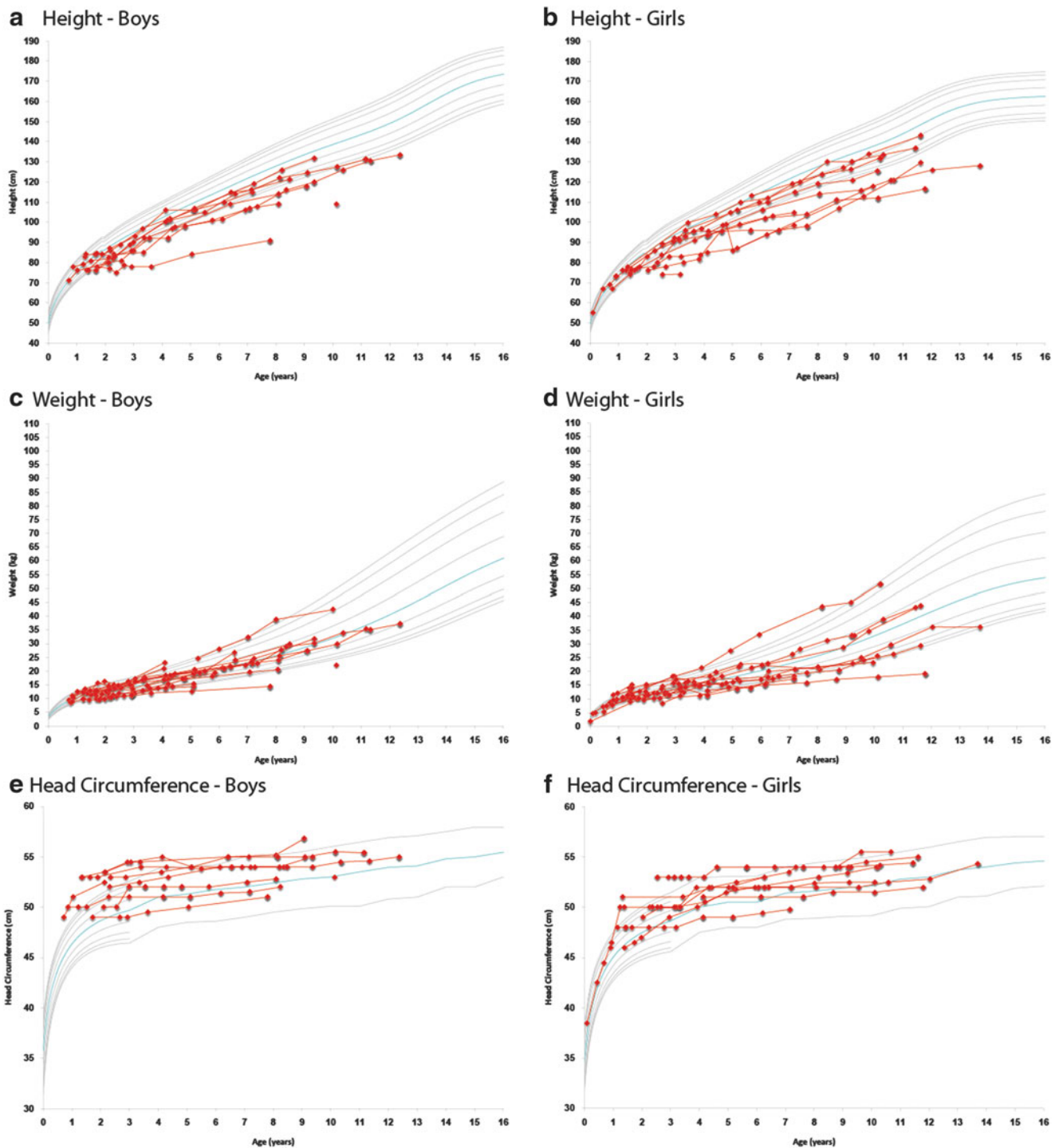


Fig. 2 Longitudinal height, weight, and head circumference in boys and girls from umbilical cord blood transplantation until last follow-up visit. The *red lines* represent individual patients. The *gray curves* represent standard growth curves (3rd, 5th,

10th, 25th, 50th, 75th, 90th, 95th, and 97th percentiles) for (a, b) height, (c, d) weight, and (e, f) head circumference. The *red lines* represent individual patients

Discussion

In this study we evaluated the long-term functional outcomes of 19 children who underwent UCBT for Hurler syndrome. The safety and efficacy of UCBT from unrelated

donors was similar to that reported by previous studies (Staba et al. 2004; Martin et al. 2006; Prasad et al. 2008). However, this is the only comprehensive prospective longitudinal study on a large cohort of patients with Hurler syndrome who were evaluated before and after transplanta-

tion using a standardized protocol. Transplantation was performed using a single graft source (umbilical cord blood) and the same conditioning regimen for all patients. Full donor chimerism and normal enzyme levels were obtained in all successfully transplanted patients. Clinical follow-up consisted of multidisciplinary evaluations performed within a single week and at regular intervals for a mean of 8.6 years (range 0.1–14.9) after transplantation. Neurodevelopmental data provided information in the six areas of functional skills.

Our results show that UCBT successfully provided a means of enzyme replacement and stabilized the disease in these patients. Motor skills were more severely affected than other functional domains and responded less well to treatment, affecting adaptive behavior, self-help skills, and ability to carry out activities of daily living. On the other hand, cognitive development was preserved, with continued gains in cognitive function.

Long-term somatic outcomes were encouraging. Cardio-pulmonary function significantly improved in most patients, with cardiomyopathy or severe respiratory obstruction rarely seen after the 2-year follow-up. Transient cardiomyopathy was observed before or within the first 2 years after transplantation, mainly due to steroid use or as a transplantation-related complication. Valve involvement was still common at longer follow-up but did not appear to significantly interfere with cardiac function. More than 2 years after transplantation, hearing was considerably compromised in some patients, requiring amplification. Although residual corneal clouding was common, progression to an advanced state requiring corneal transplantation was prevented in most patients.

Overall, the skeletal system responded less well to UCBT, as demonstrated by the need for orthopedic interventions. Importantly, severe complications including cord compression and atlantoaxial instability were prevented, and linear growth was significantly higher than would be expected during the natural course of the disease. Without treatment, children with Hurler syndrome show severe growth failure by the age of 2 years, achieving a maximal height of 110 cm (Neufeld and Muenzer 2001). However, this increased height after transplantation may have contributed to the progression of orthopedic complications. Eleven patients received growth hormone treatment; however, whether a defined subgroup of patients will benefit from treatment with growth hormone needs further evaluation. Hydrocephalus did not develop at longer follow-up in most patients, and a VPS was placed mainly before, during, or immediately after transplantation. Furthermore, longitudinal head circumference normalized and subsequently stabilized during long-term follow-up. The presence of cerebral atrophy on MRI also decreased over time.

At present, HSC transplantation is the only treatment able to provide the IDUA enzyme throughout the body, including the central nervous system, making it the treatment of choice for Hurler syndrome patients. For patients who do not undergo transplantation because of delayed diagnosis, lack of a compatible donor, or major concerns of parents, ERT has been used in an attempt to improve quality of life (Thomas et al. 2006; Tokic et al. 2007; Wraith et al. 2007). Although some clinical benefits have been reported for ERT, continued musculoskeletal and central nervous system deterioration have been observed. In a study of patients with severe MPS I younger than 5 years of age, follow-up was too short to draw any conclusions on neurocognitive outcome (Wraith et al. 2007). Another potential limitation of ERT is the induction of an immune response to the therapeutic enzyme. Antibodies produced against IDUA may neutralize the effect of enzyme replacement by reducing the efficiency of enzyme uptake and redirecting the enzyme to other target tissues (Dickson et al. 2008). To circumvent the blood–brain barrier, intrathecal ERT is currently being studied; however, the long-term safety and efficacy remain unclear (Tolar et al. 2009). Transplantation of HSCs genetically modified to express supranormal levels of IDUA showed promising results in MPS I mice (Visigalli et al. 2010). Whether this therapy represents an effective and safe therapeutic option for human patients in the future warrants further clinical studies.

In several other lysosomal storage disorders, early treatment with HSC transplantation has resulted in superior neurodevelopmental outcomes. We have also demonstrated benefits for Hurler syndrome, with transplantation before 9 months of age associated with improvements in cognitive function, receptive and expressive language, and adaptive behavior (Poe et al. 2014). Early treatment may have similar effects for some of the somatic manifestations of Hurler syndrome, but whether orthopedic complications can be prevented remains to be elucidated. Our results regarding neurodevelopmental function and growth suggest that newborn screening for Hurler syndrome is needed to optimize the benefits from transplantation. The challenges with newborn screening, however, will be predicting which children who screen positive will develop neurological disease and therefore require transplantation.

Our results are consistent with previous studies reporting that certain disease manifestations continue to progress despite successful transplantation. Insufficient enzyme delivery to poorly vascularized tissues is thought to account for musculoskeletal deterioration after transplantation, similar to that of untreated children (Weisstein et al. 2004; Aldenhoven 2008). Although HSCT may improve ventricular hypertrophy and ventricular function, cardiac valve disease persists (Braunlin et al. 2010), and hearing

impairment, persistent corneal clouding, and noninfectious pulmonary complications (e.g., diffuse alveolar hemorrhage, idiopathic pneumonia syndrome) after transplantation are also common (Souillet et al. 2003; Kharbanda et al. 2006). Therefore, these patients require close follow-up consisting of at least annual assessment by a multidisciplinary team with experience treating children with MPS I (Muenzer et al. 2009).

In our study long-term follow-up of patients with Hurler syndrome who underwent UCBT showed significant improvement of cardiopulmonary function and neurocognitive functioning, prevention of hydrocephalus and severe orthopedic complications, amelioration of vision and hearing impairment, and preservation of cognitive development in the large majority of the patients. These effects accounted for the dramatically increased life expectancy and improved quality of life after transplantation. Further studies are needed to determine whether even earlier transplantation might further improve the prognosis of these patients.

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Synopsis

Unrelated umbilical cord transplantation is associated with improved cognitive development and cardiopulmonary function, attenuated vision and hearing impairment, and prevention of hydrocephalus and severe orthopedic complications.

Compliance with Ethics Guidelines

Conflict of Interest

Hannah Y. Coletti, Mieke Aldenhoven, Karina Yelin, Michele D. Poe, Joanne Kurtzberg, and Maria L. Escolar declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

HYC and KY collected and entered the data and drafted the initial manuscript; MLE was the principal investigator of the study and secured funding; MA and MLE finished the manuscript; MDP performed statistical analysis; MLE interpreted the results; and JK and MLE critically revised the manuscript.

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Psychological Health in Adults with Morquio Syndrome

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Abstract Mucopolysaccharidosis type IV (MPSIV), also known as Morquio syndrome, is a progressive genetic condition which predominantly affects skeletal development. Research thus far has focused on physical manifestations, with little attention to psychological characteristics. As a first step in determining the natural occurrence of psychological symptoms in this population, we administered Achenbach measures of psychological functioning (ASEBA ASR and OASR), quality of life (SF-36), and pain severity (BPI) questionnaires to 20 adults with Morquio syndrome. 11/20 subjects (55%) scored within the symptomatic range on at least one or more ASEBA problem scales. These subjects also had higher pain severity scores ($p = 0.051$) and pain interference scores ($p = 0.03$) on the BPI. However, subjects with psychological symptoms did not differ significantly on QOL measures from those without psychological symptoms. Overall, subjects scored below the US mean only in physical health QOL ($p < 0.001$) on the SF-36, not mental health QOL. Implications of this study include the need for greater attention to psychological health in persons with Morquio syndrome, including regular assessment for psychological symptoms in addition to the quality of life measures typically used, as the latter may miss important information. Greater attention to psychological symptoms may help maximize overall health in adults with Morquio syndrome. Comparison with psychological studies on other lysosomal storage diseases suggests these results may be disease specific, rather than

the result of living with chronic pain or having an LSD in general.

Abbreviations

ASEBA	Achenbach system of empirically based assessment
ASR	Adult self-report
BPI	Brief pain inventory
LSD	Lysosomal storage disease
MPS	Mucopolysaccharidosis
OASR	Older adult self-report
PI	Pain interference
PS	Pain severity
QOL	Quality of life
SAF	Social-adaptive functioning

Introduction

Mucopolysaccharidosis IV (MPSIV), or Morquio syndrome (OMIM 253000 & 253010), is a rare autosomal recessive genetic lysosomal storage disorder (LSD). Although exact disease incidence is unknown, a clinical review by Northover et al. (1996) estimated it to be between 1:40,000 and 1:50,000 live births. A more recent review by Tomatsu et al. (2011) reports epidemiologic data according to country, stating “MPS IV is a rare disorder, and precise epidemiologic data are scarce. . . . At this time, there are no documented reports of incidence for the USA population.”

There are two types of Morquio syndrome, Morquio A and Morquio B. Morquio A is caused by a deficiency of lysosomal enzyme *N*-acetylgalactosamine-6-sulfatase (GALNS; EC 3.1.6.4), while Morquio B is caused by a

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deficiency of enzyme beta-galactosidase. Both enzyme deficiencies result in excessive accumulation of partially degraded glycosaminoglycans (GAGs), specifically keratan sulfate and chondroitin-6-sulfate, causing systemic skeletal dysplasia. Growth is stunted and patients often undergo multiple surgeries and/or are confined to a wheelchair by an early age. The connective tissue of the cornea, airways, and heart valves are also typically affected. Unlike in several other MPS diseases, intellectual abilities are usually spared. Morquio syndrome includes mild, moderate, and severe forms. Although all forms are characterized by skeletal disease, individuals affected by milder cases may live over 70 years, while severe cases do not typically live beyond age 30. Until recently, there was no primary disease treatment for Morquio A or B. However, in 2014, the United States Food and Drug Administration approved the enzyme replacement therapy (ERT) Vimizim for the treatment of Morquio A. There remains no treatment for Morquio B.

The majority of research characterizing Morquio syndrome has focused on physical aspects. Less attention has been paid to psychological symptoms, quality of life, or neurocognition. Available data include two studies. Hendriksz et al. (2014) examined overall quality of life (QOL) in children and adults with Morquio A, using the Health-Related Quality of Life (HRQoL) EuroQoL (EQ)-5D-5L questionnaire. Overall QOL was inversely related to patient mobility, such that high reliance on a wheelchair for mobility significantly reduced QOL. Employed adults also had better QOL than those who were unemployed. In a second study, Davison et al. (2012) suggested there may be subtle neurocognitive and neurological abnormalities previously unrecognized in Morquio syndrome; however, longitudinal assessment of such has not yet been undertaken. There have been no studies to date which have looked at psychological symptoms in this population.

Research on psychological issues in other forms of MPS is also limited and focuses primarily on behavioral problems of aggression and destructiveness displayed in MPS II (Hunter disease) and MPS III (Sanfilippo disease) (Bax and Colville 1995). Kuratsubo et al. (2009) documented psychological health among people with MPS-II in Japan. Results suggested that psychological health worsened as physical disabilities progressed, particularly within the attenuated phenotype, which does not manifest the intellectual disability associated with the severe phenotype. Kuratsubo et al. interpret their results to suggest that as the attenuated phenotype patients understand their disease predicament more fully, their psychological health worsens as a result. It is reasonable to postulate that patients with Morquio syndrome, in whom intelligence is also typically preserved, may similarly display increased psychological difficulties as a result of understanding their disease predicament more fully.

It is critical to pay attention to psychological symptoms associated with LSDs and expand our standard of care to include mental health treatment, if necessary. The present study is a first step toward understanding and treating psychological conditions associated with Morquio syndrome, by determining their natural occurrence in this population.

Methods

Subjects

Subjects with Morquio syndrome were enrolled from July 2012 through October 2013. They were recruited through Emory's clinical population, Annual Morquio Conferences, patient support groups, and word of mouth.

Eligibility criteria included English-speaking men and women with Morquio syndrome ≥ 18 years old, untreated with enzyme replacement therapy.

Measures

All subjects provided informed consent before completing three self-report questionnaires: the Achenbach System of Empirically Based Assessment (ASEBA) Adult Self-Report (ASR) or Older Adult Self-Report (OASR), the Short Form 36-Item Health Questionnaire (SF-36), and the Brief Pain Inventory (BPI).

The ASEBA ASR is a reliable, validated, widely used measure of social-adaptive and psychological functioning in adults aged 18–59 and the OASR in adults aged 60 to 90+ (Achenbach and Rescorla 2003). Norms are representative of the mix of ethnicities, socioeconomic status, urban–rural–suburban residency, and geography within the USA. Raw scores are converted to T-scores for each scale to permit comparisons with the general population. Scale scores are normed by gender and age and then categorized as normal (below the 93rd percentile), borderline–clinical (93rd to 97th percentiles), or clinical (above the 97th percentile). The ASEBA has been used with a wide variety of medical conditions, including Fabry disease, Turner syndrome, Williams syndrome, Angelman syndrome, cystic fibrosis, and Prader-Willi syndrome (Achenbach and Rescorla 2003).

The BPI is used to quantify the degree of pain and interference of pain in a person's daily life (Cleeland and Ryan 1994). It yields a mean pain severity (PS) score and a mean pain interference (PI) score. PI scores provide a measure of how much a subject's pain interferes with seven categories: general activity, mood, walking ability, normal work, relationships, sleep, and enjoyment of life. PI scores can be broken down into two dimensions: physical activity

(walking ability, normal work, sleep, and general activity) and affective (mood, relationships and enjoyment of life). The BPI has demonstrated good reliability and validity (Cleeland 2009) and been used in more than 400 studies worldwide with a variety of medical conditions, including other LSDs (Cleeland 2009; Laney et al. 2010).

The SF-36 is a 36-item survey used to measure quality of life. Scores provide summary measurements of both physical and mental well-being. Raw scores are converted to T-scores and norm-based. The SF-36 is a reliable, validated questionnaire (Maruish and DeRosa 2009; Maruish and Kosinski 2009), used with a variety of medical conditions, including other LSDs (Hoffmann et al. 2005; Laney et al. 2010; Watt et al. 2010; Weinreb et al. 2007; Wilcox et al. 2008).

Data Analysis

ASEBA ASR raw data was entered into assessment data manager (ADM) ASEBA scoring software, which produces detailed profiles on multiple aspects of psychological functioning. Subjects with T-scores in the borderline–clinical and clinical ranges were considered to have psychological symptoms for the purposes of this study. BPI raw data was scored according to instructions in the BPI User Guide (Cleeland 2009). Raw data from the SF-36 was entered into QualityMetric Health Outcomes Scoring Software 4.5, which utilizes T-scores to provide overall Physical Health Summary and Mental Health Summary scores.

Due to the limited number of subjects in subgroups, most results are presented as descriptive statistics. T-tests and Pearson's correlation coefficient were used to compare quantitative scores. We verified the underlying assumptions of these tests by fitting linear regression models and checking the residuals for constant variance, linearity, and absence of outliers. *P* values less than 0.05 were considered to be statistically significant and are reported exactly or as <0.01 or <0.001.

Results

Of the 20 subjects, 19 completed the ASEBA ASR questionnaire and 1 completed the ASEBA OASR, for a 100% response rate. 19 of 20 subjects completed the BPI and SF-36 respectively, for a response rate of 95% on both measures.

Demographic Characteristics

A total of 20 adults (14 women and 6 men) with Morquio syndrome participated in this study. Sixteen had Morquio A (80%) and four had Morquio B (20%). Due to the small

Table 1 Demographic characteristics: mean (standard deviation) or frequency (percentage)

	Morquio A	Morquio B
Mean Age	27.3 (8.3)	40.8 (17.6)
<i>Gender</i>		
Women	12 (75%)	2 (50%)
Men	4 (25%)	2 (50%)
<i>Race</i>		
Caucasian	11 (69%)	2 (50%)
Hispanic	4 (25%)	1 (25%)
Mixed	1 (6%)	1 (25%)
<i>Education</i>		
In or completed HS	6 (38%)	1 (25%)
In or completed college	8 (50%)	2 (50%)
In or completed graduate school	2 (13%)	1 (25%)
<i>Employment</i>		
Employed	6 (38%)	2 (50%)
Student	5 (31%)	0 (0%)
Retired	0 (0%)	1 (25%)
Disabled	1 (6%)	0 (0%)
None	4 (25%)	1 (25%)
<i>Marital status</i>		
Single	13 (81%)	2 (50%)
Married	2 (13%)	1 (25%)
Separated	1 (6%)	0 (0%)
Divorced	0 (0%)	1 (25%)

number of subjects with Morquio B, it was not possible to conduct statistical comparisons between the two types. Demographic characteristics are presented in Table 1. All subjects were English speaking, with 17 (85%) residing in the USA and 3 (15%) outside the USA. Ages ranged from 18 to 67 years.

Psychological Symptoms

Analysis of ASEBA data determined that 11/20 subjects (55%) scored within the borderline–clinical to clinical range on at least one ASEBA scale (see Table 2), with some subjects scoring in this range on more than one scale. The somatic complaint scale was not included, due to the confounding effect of somatic complaints associated with Morquio syndrome itself.

In comparing the group of 11 subjects who scored within the borderline–clinical to clinical range on at least one or more ASEBA scales with the group of nine subjects whose scores all fell within the normal range, no differences in demographic variables were significant. However, subjects within the borderline–clinical to clinical range had higher PS scores ($p = 0.051$) and PI scores ($p = 0.03$) on the BPI than those

Table 2 Prevalence of social-adaptive function deficits and psychological symptoms

ASEBA scale	Frequency (%) within the borderline–clinical to clinical range
Intrusive	6 (30%)
Attention deficit/ hyperactivity	4 (20%)
Thought problems	4 (20%)
Depressive	3 (15%)
Anxiety	2 (10%)
Withdrawn	2 (10%)
Social-adaptive functioning deficits	2 (10%)
Aggressive	1 (5%)
Avoidant	1 (5%)
Antisocial	1 (5%)
Substance use	1 (5%)

within the normal range. Of note, the higher PI scores were due to differences in interference on physical ($p = 0.02$) rather than mental activities ($p = 0.10$). The two groups did not differ significantly with regard to SF-36 Physical Health ($p = 0.77$) or Mental Health scores ($p = 0.16$).

Two subjects (10%) reported being treated with antidepressant and/or antianxiety medications at the time of evaluation, with one of the subjects being in psychological counseling for anxiety. Of these subjects, one scored within the depressed and anxious ranges on the ASR, while the other did not.

Quality of Life

Subjects' scores ranged from 18.62 to 57.58 (mean = 36.49) on the SF-36 Physical Health component and from 33.46 to 67.76 (mean = 53.26) on the Mental Health component, in comparison to US mean scores of 50. For both components, lower numerical values indicate poorer health. While the difference between subjects' scores on the Physical Health component and the US mean was statistically significant ($t(18) = -6.10$, $p = <0.001$), the difference between scores on the Mental Health component and the US mean was not ($t(18) = 1.19$, $p = 0.25$).

Subjects' SF-36 Physical Health scores were not significantly correlated with SF-36 Mental Health scores (Pearson's $R = -0.25$; $p = 0.31$).

Subjects who were employed (working or full-time students) were not statistically different in QOL from subjects who were unemployed (not working, disabled, or retired), on either the Physical Health ($p = 0.38$) or the Mental Health ($p = 0.41$) dimension.

Pain

Subjects' pain severity scores on the BPI ranged from 0.5 to 7.5 (mean = 4.39). Pain interference scores ranged from 0.014 to 9.43 (mean = 3.84). PS scores were positively correlated with PI scores ($R = 0.75$; $p < 0.001$). This correlation was observed regarding interference on both physical activity ($R = 0.84$, $p < 0.001$) and affective ($R = 0.53$; $p = 0.025$) dimensions.

PS scores were negatively correlated with SF-36 Physical Health component scores ($R = -0.72$; $p < 0.001$), but were not significantly correlated with SF-36 Mental Health component scores ($R = -0.21$; $p = 0.40$).

Discussion

The present study documents for the first time the presence of psychological symptoms in adults with Morquio syndrome (11/20 subjects, 55%) as compared to population norms. These symptoms were associated with higher pain severity and pain interference in physical activities.

Psychological symptoms were separate, however, from self-report on QOL measures. Subjects scored significantly below the US mean in physical health QOL, but not mental health QOL. Similarly, although pain severity was correlated with psychological symptoms, it was not correlated with mental QOL. This difference between psychological symptoms and QOL illustrates the need to assess psychological symptoms in patient care separate from QOL measures, as the latter may miss important health symptoms.

Although Hendriksz et al. (2014) did not examine psychological symptoms, their study found QOL with Morquio syndrome to be inversely related to degree of wheelchair use. As data concerning mobility and independence were not specifically collected in this study, a direct comparison is not possible; however, over 50% of present subjects were observed to be independent of a wheelchair at least some of the time and at least 45% mentioned living independently of their families (or were married). In comparison with Hendriksz et al., the present study examined physical and mental QOL separately and found them to be uncorrelated. Also in comparison, the majority of our subjects were either employed or full-time students. While Hendriksz et al. observed unemployed adults to have worse QOL than employed adults, no such difference was observed in the present study.

While direct comparison with other MPS syndromes and other LSDs in general is complicated by the differing nature of each syndrome, we believe such comparisons are nonetheless beneficial to extending our understanding of the broad umbrella of LSDs. The results of the present

study are consistent with Kuratsubo's (2009) suggestion with MPS II subjects that when cognitive abilities are preserved, subjects may display increased psychological symptoms as a result of understanding their disease burden more fully.

Research in other LSDs, such as Fabry disease (FD) and Gaucher disease (GD), has likewise documented decreased QOL and begun to examine psychological functioning (Crosbie et al. 2009; Gold et al. 2002; Masek et al. 1999; Packman et al. 2006; Watt et al. 2010; Weinreb et al. 2007; Wilcox et al. 2008). Prevalence estimates of depression in FD range from 15 to 62% (Bolsover et al. 2014; Grewal 1993; Wang et al. 2007), with the largest study ($n = 296$) reporting 46% (Cole et al. 2007). The present study found people with Morquio syndrome to likewise report decreased QOL, though only in physical health QOL, while prevalence estimates of depressive symptoms were 15%.

Laney et al. (2010) examined social-adaptive functioning (SAF) in patients with FD. SAF measures how effectively an individual copes with the daily demands of everyday tasks and responsibilities as parents, children, students, caregivers, and employees. Their results showed eight FD patients (26.7%) had mean SAF deficits as compared to population norms. Poorer SAF was associated with greater rates of depression, anxiety, antisocial personality, attention deficit/hyperactivity, and aggressive behavior. In comparison, only 10% of subjects with Morquio syndrome were observed to have mean SAF deficits. While it is unclear why this difference exists, it suggests results may be disease specific rather due to having an LSD or chronic disease in general. Similarly, as FD and Morquio syndrome both involve chronic pain, these results suggest SAF deficits in FD are not exclusively a result of living with chronic pain. It is possible the presence of vascular symptoms in FD may affect SAF in that population, whereas people with Morquio syndrome do not typically have vascular symptoms.

The bidirectional interaction between physical and psychological health is well established. Just as physical health affects us emotionally (e.g., chronic pain can contribute to depression), so can psychological health affect us physically (e.g., anxiety can contribute to feelings of chest pain). Psychological states can also alter immune function and affect adjustment to illness, leading to poor health practices (e.g., noncompliance), interfering with social functioning (e.g., irritability, withdrawal, and isolation behaviors), and resulting in diminished use of the health care system, leading to poorer overall medical outcomes and QOL. For example, extensive evidence suggests that depressive symptoms are associated with noncompliance in common chronic disorders such as diabetes mellitus (Katon et al. 2009) and coronary artery disease (Khawaja et al. 2009). Good adjustment to illness

has been linked to increased attempts to gain control over one's health and better overall health outcomes. It is thus critical to pay attention to psychological symptoms associated with LSDs like Morquio syndrome and expand our standard of care to include mental health treatment, if necessary.

Limitations of this study include small sample size, due to the rarity of Morquio syndrome and limitation to adults over age 18. This is particularly true with regard to Morquio B. Statistical analysis was thus limited primarily to descriptive statistics. It is possible a larger sample would have revealed statistically significant differences not currently detectable. It is also possible those subjects who chose to contact the researcher to participate may have differed from those who chose not to do so. For example, while gender distribution in Morquio syndrome is typically equal, more female subjects chose to participate in the present study than males. Another limitation is that although this study compares subjects' physical QOL to their mental health QOL, it does not include objective measurements of disease severity or clinical symptoms, including height and degree of wheelchair use. Finally, subjects were assessed at one point in time, via self-report. Although all measures possess high test–retest reliability, it is possible subjects may have presented differently at another time or via different measurement.

Implications of this study include the need for greater attention to psychological health in persons with Morquio syndrome, including regular assessment for psychological symptoms in addition to QOL measures. This will enable us to maximize treatment and pursue therapy for any psychological issues which may be contributing to poorer health outcomes among patients with Morquio syndrome.

Recommendations for future research include objective measurements of both disease severity and clinical symptoms (e.g. height, degree of wheelchair use, etc.), for correlational analysis with psychological symptoms. As growth is typically stunted, exploration of body image and its relation to psychological symptoms in subjects with Morquio syndrome may also prove informative. Finally, comparison of treatment-naïve patients to patients on ERT would be beneficial, as well as longitudinal studies to assess the efficacy of such treatment on psychological issues. As ERT does not penetrate the blood–brain barrier, it would be unlikely to improve primary psychological symptoms of Morquio syndrome, should such exist, but might improve secondary psychological symptoms caused by living with this chronic progressive disease.

In conclusion, the present study suggests that some adults with Morquio syndrome exhibit psychological symptoms which may heretofore have been underreported and overlooked. Although 11 out of 20 subjects displayed psychological symptoms, only one subject reported being

in counseling during the study and only two were on psychiatric medication. Greater attention to psychological symptoms may help maximize overall health in adults with Morquio syndrome. When taken together with similar studies in other LSDs, these results allow us to draw further conclusions about the impact of chronic pain and living with an LSD on psychological health and quality of life.

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1-Sentence Take Home Message

Many adults with Morquio syndrome exhibit psychological symptoms which may currently be overlooked during treatment of physical disease manifestations.

Compliance with Ethical Guidelines

Contributions of Individual Authors

Nadia Ali, Ph.D., is responsible for the conception and design of the research, data collection, data preparation and interpretation, and writing the original and final drafts of the manuscript to be submitted for publication. She is the guarantor.

Stephanie Cagle, MS, assisted with some of the data collection and reviewed the article before submission for publication.

Conflict of Interest

Nadia Ali, Ph.D., has received research grants and a speaker honorarium from BioMarin Pharmaceuticals.

Stephanie Cagle, MS, declares that she has no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed

consent was obtained from all patients for being included in the study.

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A Novel Homozygous *YARS2* Mutation in Two Italian Siblings and a Review of Literature

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Abstract *YARS2* encodes the mitochondrial tyrosyl-tRNA synthetase that catalyzes the covalent binding of tyrosine to its cognate mt-tRNA. Mutations in *YARS2* have been identified in patients with myopathy, lactic acidosis, and sideroblastic anemia type 2 (MLASA2). We report here on two siblings with a novel mutation and a review of literature. The older patient presented at 2 months with marked anemia and lactic acidemia. He required periodic blood transfusions until 14 months of age. Cognitive and motor development was normal. His younger sister was diagnosed at birth, presenting with anemia and lactic acidosis at 1 month of age requiring periodical transfusions. She is now 14 months old and doing well. For both our patients, there was no clinical evidence of muscle involvement. We found a new homozygous mutation in *YARS2*, located in the α -anticodon-binding (α ACB) domain, involved in the interaction with the anticodon of the cognate mt-tRNA^{Tyr}.

Our study confirms that MLASA must be considered in patients with congenital sideroblastic anemia and underlines the importance of early diagnosis and supportive therapy in order to prevent severe complications. Clinical severity is variable among *YARS2*-reported patients: our review of the literature suggests a possible phenotype-genotype correlation, although this should be confirmed in a larger population.

Introduction

Mitochondrial protein synthesis requires the RNA apparatus encoded by mtDNA genes (2 rRNAs and 22 tRNAs) and a remarkable number of protein factors encoded by nuclear genes, including ribosomal, structural, and assembly proteins; tRNA-modifying enzymes; rRNA-methylating enzymes: initiation, elongation, and termination translation factors; and aminoacyl-tRNA synthetases (aaRSs) that charge the 20 amino acids to the cognate mt-tRNA molecule (Rötig 2011). With the exception of *GARS* and *KARS*, mitochondrial and cytoplasmic aaRSs are encoded by two distinct nuclear genes (Diodato et al. 2014). Mutations in sixteen mt-aaRSs genes have been found to cause heterogeneous mitochondrial disorders, often characterized by a remarkable tissue or organ specificity (Konovalova and Tyynismaa 2013; Diodato et al. 2014; Hallmann et al. 2014; Schwartzentruber et al. 2014).

YARS2 encodes the mitochondrial tyrosyl-tRNA synthetase. Mutations in *YARS2* have been identified in patients with myopathy, lactic acidosis, and sideroblastic anemia (MLASA) phenotype (Riley et al. 2010, 2013; Sasarman et al. 2012; Shahni et al. 2013; Nakajima et al. 2014). MLASA is a rare autosomal recessive condition associated

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with two distinct genetic forms: MLASA1 (MIM #600462) results from mutations in *PUS1* gene (Bykhovskaya et al. 2004; Fernandez-Vizarrá et al. 2007) and MLASA2 (MIM #613561) caused by *YARS2* mutations. *PUS1* encodes pseudouridine synthase, a tRNA-modifying enzyme that stabilizes the tRNA structure and facilitates the peptidyl transfer reaction of rRNAs (Fernandez-Vizarrá et al. 2007). Both *PUS1* and *YARS2* mutations lead to decreased mitochondrial protein synthesis resulting in mitochondrial respiratory chain dysfunction. *PUS1* mutations have been associated with combined defects of complex I (CI) and IV (CIV) and *YARS2* with CI, complex III (CIII), and CIV deficiency (Riley et al. 2010, 2013; Sasarman et al. 2012).

Congenital sideroblastic anemia may be caused by mutations in several nuclear DNA genes involved in mitochondrial pathways: (1) heme biosynthesis, *ALAS2* (erythroid-specific delta-aminolevulinic synthase) and *SLC25A38* (a mitochondrial carrier protein of still unknown function); (2) iron-sulfur cluster biosynthesis, *ABCB7* (adenosine triphosphate binding cassette B7) and *GLRX5* (glutaredoxin 5); and (3) mitochondrial protein synthesis, *PUS1* and *YARS2*. Congenital sideroblastic anemia has also been described in association with sporadic single mtDNA deletions in Pearson's syndrome (Fujiwara and Harigae 2013) and more recently in a patient with an mtDNA point mutation in *ATP6* gene (Burrage et al. 2014).

We report the clinical, biochemical, and molecular findings of two Italian siblings with a new mutation in *YARS2* and a review of literature.

Case Report

The two siblings are children of unrelated healthy parents. Family history is unremarkable.

Patient 1 is a boy born after uncomplicated pregnancy and delivery. At 2 months of age, hematological examination performed because of extreme pallor with no other symptom revealed marked anemia (Hb 5.2 g/dl, Hct 14.8 %, RBC $1.52 \times 10^6/\mu\text{l}$) with mild neutropenia ($1.36 \times 10^3/\mu\text{l}$) but normal total leukocyte count and platelets. Serial controls confirmed anemia and occasionally neutropenia and thrombocytopenia. Parvovirus 19 infection, Fanconi anemia and Diamond-Blackfan anemia were ruled out. Liver and renal function and cardiac and abdominal ultrasound were normal. Bone marrow analysis performed at 3 months of age revealed mild erythroblastopenia and marked vacuolization of erythroid precursors. He was found to have lactic acidemia 4,416 $\mu\text{mol/l}$ (normal range 580–2,100 $\mu\text{mol/l}$). At this age he received the first transfusion, and since then he required transfusions every 3–4 weeks until 14 months when he spontaneously improved with normalization of blood parameters. The patient was examined in our unit at 13

months of age: he presented good general conditions, normal physical development, and mild psychomotor delay without achievement of autonomous gait. Laboratory data showed recovery of anemia (Hb 10.4 g/dl, Hct 31.7 %) and a normal blood lactate (2077 $\mu\text{mol/l}$). Analysis of urinary organic acids showed mild elevation of lactic, 3-OH butyric, and 3-OH isovaleric acids. EEG and ophthalmological examination were normal. T2-weighted MRI showed mild hyperintensity in the pallida and two tiny areas of abnormal signal in the pontine tegmentum. HMR spectroscopy was normal. Muscle biopsy performed at 16 months showed a few hypo-atrophic fibers, normal content of lipids, and glycogen with minimal increase of SDH activity in subsarcolemmal fibers. Activities of CI, CIII, and CIV were markedly reduced in muscle homogenate (CI = 13%, CIII = 18%, and CIV = 15% of the mean control values) but normal in fibroblasts; however, seahorse-based microscale oxygraphy revealed a consistent reduction of maximal respiratory rate in fibroblasts (28% of controls). As these results suggested a mitochondrial respiratory chain disorder, treatment with coenzyme Q₁₀ and riboflavin was started. He had a good clinical evolution with normal psychomotor development and achieved autonomous gait at the age of 18 months. At the last examination, at 6 years of age, neurological examination was normal, body weight and height were at the 50th centile, and no other signs of organ dysfunction appeared.

Patient 2, the younger sister, was born after uncomplicated pregnancy and delivery. The parents refused prenatal diagnosis. At 1 month of age, hematological examination revealed high lactate 9,900 $\mu\text{mol/l}$ (normal 580–2,100) and mild anemia: Hb 10.5 g/dl, Hct 27.7 %, RBC $3 \times 10^6/\mu\text{l}$, normal leukocytes, and platelets. Serial controls confirmed anemia, and she was started on periodical transfusions, the last one at 9 months of age. She is now 14 months old with a normal psychomotor development without any other major problems.

After receiving written informed consent from the parents, in agreement with the Declaration of Helsinki and approved by the Ethical Committees of the Foundation 'Carlo Besta' Neurological Institute, Milan, Italy, we performed Southern blot analysis of mtDNA extracted both from blood and muscle of patient 1 that did not show alteration, as well as the sequence analysis of mtDNA. On the basis of the similarity between the clinical features of our patient and of previously reported MLASA patients, we sequenced *PUS1* and *YARS2*. While the *PUS1* gene was normal, we identified a homozygous missense mutation c.933C>G/p.Asp311Glu in *YARS2*, which affects amino acid residue in the α -anticodon-binding (α ACB) domain that is invariant from humans to yeast. The mutation was heterozygous in the healthy parents and absent in the public single nucleotide polymorphism databases, including dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP>) and

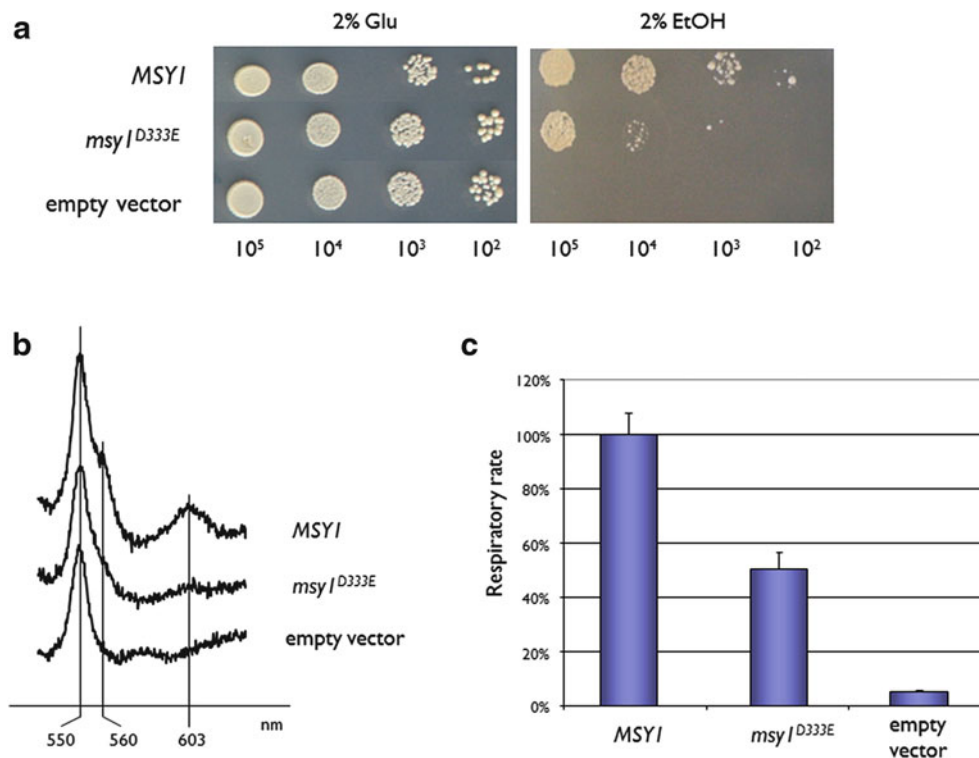


Fig. 2 Study in yeast strain W303-1B *msy1D*. **(a)** Oxidative growth phenotype; **(b)** cytochromes absorption spectra; **(c)** respiratory rate

Discussion and Conclusion

As disease mutations in mt-aaRS hit housekeeping nuclear DNA genes, they should in principle cause impaired mitochondrial translation in all organs. Nevertheless, a tissue-organ specificity of mt-aaRS disease mutations has been observed for each of the fourteen aaRS2 genes associated with disease mutations in humans (Diodato et al. 2014; Hallmann et al. 2014; Schwartzenruber et al. 2014).

To date nine patients with *YARS2* mutations have been described (Table 1). Two different homozygous missense mutations affecting the catalytic domain of mtTyrRS have been reported in seven different subjects of Lebanese origin: c.156C>G/p.Phe52Leu and c.137G>A/p.Gly46Asp. Later, Riley et al. described a French compound heterozygous patient carrying two novel *YARS2* mutations: c.572G>A/p.Gly191Asp and c.1078C>T/p.Arg360*. The p.Gly191Asp mutation results in a 38-fold loss in catalytic efficiency; the p.Arg360* mutation produces an unstable protein (Riley et al. 2013). The most recent *YARS2*-mutated patients reported in the literature (Nakajima et al. 2014) carried a homozygous missense mutation c.1303A>G/p.Ser435Gly. The p.Ser435Gly mutation is located in the S4-like domain of the C-terminal portion of the protein, which is essential to strengthen the interaction between mt-tRNA^{Tyr} and the mtTyrRS (Bonfond et al. 2007). The mutation found in our patients is located in the α ACB domain. Asp311 is at the

beginning of the α -12 helix, which plays a crucial role in the interaction with the anticodon of cognate tRNA^{Tyr} (Bonfond et al. 2007). In the homologous TyrRS from the bacterium *T. thermophilus*, where Asp259 is the equivalent to human Asp311, Yaremchuk et al. demonstrated a specific interaction between the Asp carboxyl group and G34 in the anticodon of tRNA^{Tyr} (Yaremchuk et al. 2002). This essential function is further supported by the complete observation of this Asp residue throughout evolution, while other putative anticodon-binding residues are not conserved (Bonfond et al. 2007).

The *MSY1*-defective yeast strain (*msy1 Δ*) displays an OXPHOS phenotype characterized by failure to grow in media containing ethanol, an obligatory respiratory compound as the only carbon source. The expression in the *msy1 Δ* strain of *msy1^{D333E}* variant, equivalent to *YARS2* D311E, gave a clearly defective OXPHOS phenotype, but less drastic than that of the *msy1 Δ* null strain, indicating the preservation of some functional competency. Likewise, the decrease of two components of mtDNA-related respiratory chain complexes, cytochrome aa3, part of CIV, and cytochrome b, part of CIII, was marked but milder than in the *msy1 Δ* strain (Fig. 2). In no case cytochrome c was decreased, as expected in mtDNA-specific translation defects.

The patient described by Nakajima et al. showed the most severe clinical phenotype among those previously reported in association with *YARS2* mutations, characterized

Table 1 Clinical features of reported patients with *YARS2* mutations

	Pt 1 ^a	Pt 2 ^a	Pt 3 ^a	Pt 4 ^b	Pt 5 ^c	Pt 6 ^d	Pt 7 ^d	Pt 8 ^d	Pt 9 ^e	Pt 10 ^f	Pt 11 ^f
Anemia	2 months	First months	7 years	31 years	1 years	2 months	23 years	<1 years	1–2 months	2 months	1 month
Transfusion	Yes (2 months-n.a.)	Yes (onset-n.a.)	No (parents' refusal)	No	Yes (11.5 years)	Yes (2 months-n.a.)	No	Yes (onset-1 year)	Yes (2 months)	Yes (2–12 months)	Yes (1 month-currently)
Myopathy	Yes (7 years)	Yes	Yes (n.a.)	Yes	Yes	n.a.	Yes (mild 23 years)	Yes (6 years)	? (axial hypotonia)	No	No
Lactic acidosis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Hypertrophic cardiomyopathy	Yes (3 months)	n.a.	n.a.	n.a.	Yes (11.5 years)	Yes (2 months)	No	n.a.	Yes	No	No
Outcome	Deceased (18 years, respiratory failure)	Last observation 16 years	Last observation 24 years	Last observation 34 years	Last observation 16 years	Deceased (3 months, cardiomyopathy)	Last observation 28 years	Last observation 6 years	Deceased (3 months, cardiopulmonary arrest)	Last observation 6 years, good clinical evolution	Last observation 13 months, periodic transfusion
Muscle RC	Reduced I–IV	Reduced I–III–IV	Reduced I–IV	Reduced I–III–IV	n.a.	n.a.	n.a.	n.a.	n.a.	Reduced I–III–IV	n.a.
Fibroblast RC	Normal	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Reduced I–III–IV–V	n.a.	Normal	n.a.
<i>YARS2</i> mutations	c.156C>G p.Phe52Leu	c.156C>G p.Phe52Leu	c.156C>G p.Phe52Leu	c.137G>A p.Gly46Asp	c.156C>G p.Phe52Leu	c.156C>G p.Phe52Leu	c.156C>G p.Phe52Leu	c.572G>A p.Gly191Asp c.1078C>T p.Arg360*	c.1303A>G p.Ser435Gly	c.933C>G p.Asp311Glu	c.933C>G p.Asp311Glu
Mutations location	Catalytic domain	Catalytic domain	Catalytic domain	Catalytic domain	Catalytic domain	Catalytic domain	Catalytic domain	Catalytic domain	S4-like domain	α ACB domain	α ACB domain

^a Riley et al. (2010), ^b Sasarman et al. (2012), ^c Shahmi et al. (2013), ^d Riley et al. (2013), ^e Nakajima et al. (2014), ^f this report; n.a. not achieved

by a rapidly progressive anemia and recurrent metabolic decompensation leading to fatal outcome at 3 months of age. Disease onset was in the first months in our patients, who became transfusion-dependent, similar to the six previously reported patients whose disease onset was during the first year. An additional patient was characterized by later onset, at 7 years of age, and her parents refused transfusion. She was treated with multivitamin cocktails and her hemoglobin maintained between 8.0 and 11.0 g/dl (Riley et al. 2010). Finally, two patients presented an adult onset and did not require transfusion. These observations suggest that early onset of anemia is a prognostic factor for transfusion dependence.

In contrast with most of the patients previously reported in the literature, our children had no clinical evidence of muscle involvement, although the muscle biopsy in patient 1 showed mild myopathic features. Measurement of the respiratory chain complexes confirmed a combined defect of CI-III-IV in muscle of patient 1 but not in his fibroblasts. The absence of clinical involvement of the CNS of MLASA patients is confirmed also in our cases: the neurological examination has been normal and the brain MRI findings in patient 1 can be considered as unspecific. Likewise, a brain MRI in a previously reported child showed a thin corpus callosum which was not associated with any clinical sign of neurological impairment (Nakajima et al. 2014).

Lactic acidosis is confirmed in our patients, and it is a constant feature in MLASA2. Interestingly, in our patient 1, the recovery of anemia coincided with normalization of lactic acidemia.

Hypertrophic cardiomyopathy was observed in four patients reported in the literature but was absent in our siblings.

In summary, we report a new mutation in *YARS2* gene and confirm that MLASA must be considered in patients with congenital sideroblastic anemia: early diagnosis and supportive therapy may be important to prevent severe complications.

Anemia and lactic acidosis are constant features, CNS is spared, and myopathy is a variable finding probably appearing later in life. The severity of the clinical course is quite variable, considering the small number of patients and the prevalence of the same mutations in the original Lebanese patients (Riley et al. 2010, 2013; Sasarman et al. 2012; Shahni et al. 2013). The prognosis is relatively good in most of the cases but somehow unpredictable; two of them had a fatal course in the first year of life (Riley et al. 2013; Nakajima et al. 2014), and one with a severe form of myopathy and cardiomyopathy died suddenly because of respiratory failure at the age of 18 years (Riley et al. 2010).

The only patient with a mutation in the S4-like domain in the anticodon-binding region (Nakajima et al. 2014) had a very poor clinical outcome, suggesting a possible

correlation between the severity of the phenotype and specific mutations, since all the other previously described patients carried mutations in the catalytic domain. Our siblings also harbor a mutation involving the anticodon-binding region, but in the α ACB domain, their clinical phenotype is mild. Thus, definite conclusions about genotype-phenotype correlation are difficult to draw due to the exiguity of the cohort of patients so far reported in the literature. Increased awareness of the existence of this rare disorder will hopefully lead to the identification of additional cases, which will help to achieve a better definition of both clinical spectrum and prognostic clues.

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Take-Home Message

MLASA must be considered in patients with congenital sideroblastic anemia: early diagnosis and supportive therapy may be important to prevent severe complications.

Compliance with Ethic Guidelines

Conflict of Interest statements

Anna Ardissonne, Eleonora Lamantea, Jade Quartararo, Cristina Dallabona, Franco Carrara, Isabella Moroni, Claudia Donnini, Barbara Garavaglia, Massimo Zeviani, and Graziella Uziel declare that they have no conflict of interest.

Informed Consent

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

Animal Rights

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

Contributions of Individual Authors

AA, IM, and GU evaluated the patients and wrote the case report. FC performed biochemical and molecular analyses.

EL monitored biochemical and genetic analyses. JQ and CDa performed experiments in yeast model. CDo coordinated the yeast studies. AA and EL wrote the manuscript; MZ, BG, and GU critically revised the manuscript for important intellectual content. All authors read and approved the manuscript.

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Carnitine Levels in Skeletal Muscle, Blood, and Urine in Patients with Primary Carnitine Deficiency During Intermission of L-Carnitine Supplementation

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Abstract *Background:* Primary carnitine deficiency (PCD) is a disorder of fatty acid oxidation with a high prevalence in the Faroe Islands. Only patients homozygous for the c.95A>G (p.N32S) mutation have displayed severe symptoms in the Faroese patient cohort. In this study, we investigated carnitine levels in skeletal muscle, plasma, and urine as well as renal elimination kinetics before and after intermission with L-carnitine in patients homozygous for c.95A>G.

Methods: Five male patients homozygous for c.95A>G were included. Regular L-carnitine supplementation was stopped and the patients were observed during five days.

Blood and urine were collected throughout the study. Skeletal muscle biopsies were obtained at 0, 48, and 96 h.

Results: Mean skeletal muscle free carnitine before discontinuation of L-carnitine was low, 158 nmol/g (SD 47.4) or 5.4% of normal. Mean free carnitine in plasma (fC0) dropped from 38.7 (SD 20.4) to 6.3 (SD 1.7) $\mu\text{mol/L}$ within 96 h ($p < 0.05$). Mean $T_{1/2}$ following oral supplementation was approximately 9 h. Renal reabsorption of filtered carnitine following oral supplementation was 23%. The level of mean free carnitine excreted in urine correlated ($R^2 = 0.78$, $p < 0.01$) with fC0 in plasma.

Conclusion: Patients homozygous for the c.95A>G mutation demonstrated limited skeletal muscle carnitine stores despite long-term high-dosage L-carnitine supplementation. Exacerbated renal excretion resulted in a short $T_{1/2}$ in plasma carnitine following the last oral dose of L-carnitine. Thus a treatment strategy of minimum three daily separate doses of L-carnitine is recommended, while intermission with L-carnitine treatment might prove detrimental.

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Introduction

Primary carnitine deficiency (PCD, OMIM #212140) is an autosomal recessive disorder of fatty acid beta-oxidation caused by a dysfunctional OCTN2 carnitine transporter, coded by the *SLC22A5* gene on chromosome five (Shoji et al. 1998; Nezu et al. 1999; Longo et al. 2006). Patients continually lose large amounts of carnitine in urine leading to low blood and tissue levels of carnitine (Scaglia et al. 1998; Tein 2003; Longo et al. 2006). PCD has a relatively high prevalence in the Faroe Islands (1:300) and has been associated with several sudden deaths among young Faroese individuals due to cardiac arrhythmia (Rasmussen

et al. 2013, 2014b). Cardiomyopathy has especially been reported in children and patients may develop metabolic disturbances and fatigue – though many remain asymptomatic (Tein 2003; Stanley 2004; Cano et al. 2008; Magoulas and El-Hattab 2012; Rasmussen et al. 2014a). The principal role of carnitine is to transport long-chain fatty acids into the mitochondria for beta-oxidation and to preserve the intracellular CoA homeostasis (Engel et al. 1981; Rebouche 2004). Patients suffering from PCD are treated with daily oral L-carnitine supplements (Lund et al. 2007; Rasmussen et al. 2014a). Four different PCD-related mutations and a risk haplotype have been uncovered in the Faroese population, with the c.95A>G mutation being the most prevalent and severe (Rasmussen et al. 2014b, c). Patients homozygous for the c.95A>G mutation have the lowest mean plasma carnitine levels and mean residual OCTN2 transport activity among the Faroese patients (Rasmussen et al. 2014c). Furthermore all patients found previously to suffer from severe complications and sudden death were homozygous for the c.95A>G mutation indicating a phenotype–genotype correlation (Rasmussen et al. 2013, 2014c). Although it is documented that continued daily L-carnitine supplementation increases blood carnitine levels in PCD patients, the effect on carnitine levels in skeletal muscle tissue, which is the main store of carnitine in the human body, is currently unknown (Rasmussen et al. 2014a). Additionally the kinetics of renal carnitine elimination in PCD patients following interruption of regular L-carnitine supplementation is to our knowledge not fully described.

The objectives of the present study were to investigate and monitor the effects of stopping L-carnitine supplementation in a small cohort of patients homozygous for the c.95A>G mutation during a 5-day period with regard to levels of carnitine in skeletal muscle, blood, and urine prior to and following the intervention.

Materials and Methods

Five male patients with a mean age of 32.6 years (range 19–73) known to be homozygous for the c.95A>G mutation gave informed consent to participate (Table 1). They had all taken L-carnitine supplements continually for at least 3 years. All patients were requested to ingest the same amount of L-carnitine relative to their weight (75 mg/kg/day) in three daily doses a week prior to the study. The patients had no symptoms of PCD and had previously only had symptoms of fatigue, which had been treated effectively by L-carnitine supplementation. One patient was treated with warfarin because of chronic atrial fibrillation; apart from that, the patients did not receive any medication other than L-carnitine.

The patients were admitted to the National Hospital of the Faroe Islands during the study period under close observation, including continuous heart monitoring. Informed written consent was obtained from all participants and the Faroese Ethics Committee approved the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Oral L-carnitine supplements were discontinued when admitted and the patients were without L-carnitine supplementation for initially 97 h (\approx 4 days). L-carnitine was then infused with a rate of 3.75 mg/kg/h for 4 h and then stopped. The patients were monitored for further 24 h before starting their regular oral supplements again. Blood, drawn with venipuncture from an antecubital vein into regular blood sample tubes containing EDTA, and urine samples were collected with predetermined intervals during the study period. The blood was centrifuged and the resulting plasma collected and frozen at -40°C until analysis in Copenhagen. Some blood was though analyzed in the local hospital laboratory to obtain routine blood parameters, e.g., glucose, electrolytes, creatinine, hemoglobin, etc. Urine was stored at -70°C until analysis. Skeletal muscle biopsies were obtained from the medial part of m. vastus lateralis using the Bergstrom technique before discontinuation of L-carnitine ($t = 0$ h), midway through the study ($t = 48$ h), and just before infusion of L-carnitine ($t = 96$ h) (Bergstrom 1975). Muscle tissue was immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Standard echocardiography was performed before the discontinuation and restart of L-carnitine supplementation using a GE Medical Vivid s6 ultrasound system. Measurements were obtained according to accepted standards and techniques (Lang et al. 2005). All reported measures were the average of two separate measurements.

Plasma, urine, and muscle tissue were analyzed at the Centre for Inherited Metabolic Disease in Rigshospitalet, Copenhagen.

Analyses of acylcarnitines and carnitine in plasma, muscle, and urine were performed using stable-isotope dilution combined with ultra-performance liquid chromatography–tandem mass spectrometry, using a Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, Massachusetts).

d_3 -Carnitine, d_3 -acetylcarnitine, d_3 -propionylcarnitine, d_3 -butyrylcarnitine, d_9 -isovalerylcarnitine, d_3 -octanoylcarnitine, d_3 -tetradecanoylcarnitine, and d_3 -hexadecanoylcarnitine (Herman ten Brink, Vrije Universiteit, Amsterdam, The Netherlands) were added to samples before extraction/homogenization. Carnitine and acylcarnitines in all three matrices were quantified using external spiked plasma calibration curves.

Table 1 Baseline values

Patient # ^a	Age (years)	Weight (kg)	Height (cm)	BMI (kg/m ²)	BSA (m ²)	LV mass index (g/m ²)	LVEF (%)	B-hemoglobin (mmol/L)	P-glucose (mmol/L)	P-potassium (mmol/L)	P-sodium (mmol/L)	P-ALAT (U/L)	P-creatinine (μmol/L)	Creatinine clearance (mL/min) ^b
1	23	90	183	26.9	2.12	104	55	9.1	4.9	3.6	138	31	83	167
2	73	120	193	32.2	2.49	90	56	9.1	6.7	3.9	139	43	106 ^c	60 ^c
3	19	70	180	21.6	1.89	84	61	8.0	4.6	3.6	137	24	64	212
4	28	83	185	24.3	2.07	105	64	8.0	5.0	3.8	143	40	75	78
5	20	85	187	24.3	2.11	134 ^c	60	8.5	4.5	4.0	140	16	75	107

BMI body mass index, *BSA* body surface area, *LV* left ventricle, *LVEF* left ventricular ejection fraction

^aAll homozygous for the c.95A>G mutation

^bCorrected for body surface area

^cOut of range

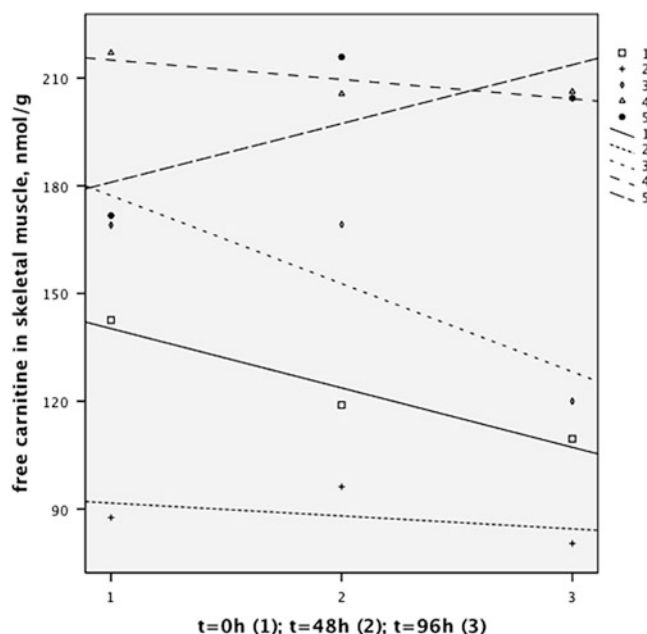


Fig. 1 Free carnitine in muscle measured three times during the study

Pharmacokinetic Methods

Pharmacokinetic parameters were determined using Phoenix WinNonlin 6.2 (Pharsight Certara) and Microsoft Excel. For each urine collection, interval (the amount of excreted carnitine (Ae_{ti})) was determined (ti represents a particular time interval). The area under the plasma concentration curves (AUC_{ti}) for carnitine was determined by non-compartmental analysis in WinNonlin using the same time intervals as the urine collection intervals. Renal clearance (CL_R) was estimated as amount excreted divided by the area under the plasma concentration curve Ae_{ti}/AUC_{ti} . Fraction reabsorbed (F_{reabs}) was calculated as $1-(CL_R/CL_{Crea})$. Half-lives were calculated as $\ln(2)/\lambda_z$, where λ_z (the terminal rate constant) was determined by linear regression of the semilogarithmic plot of the plasma concentration–time profile.

Statistical Analysis

Data analysis was performed using IBM® SPSS® Statistics Version 19 (SPSS Inc., Chicago, IL, USA). All continuous variables were expressed as mean (standard deviation). Paired student's *T*-test was used to test for a significant difference in carnitine levels. Level of significance was set at $p < 0.05$ (two-tailed test).

Results

The mean level of free carnitine in skeletal muscle was severely depressed before discontinuation of L-carnitine,

158 nmol/g (SD 47.4, range 87.6–217) compared to a mean of 2,914 nmol/g (SD 249) found in healthy individuals (Madsen et al. 2013). There was a tendency for the mean level of free carnitine in muscle to decrease slightly (13.5 nmol/g) during the L-carnitine intermission ($p = 0.35$) (Fig. 1).

Mean free carnitine in plasma (fC0) was 38.7 (SD 20.4) $\mu\text{mol/L}$ measured 1 h after the last dose of L-carnitine. The mean $T_{1/2}$ following oral supplementation was approximately 9 h (Table 2). fC0 decreased most rapidly during the first 24 h before leveling off and dropping further to a mean 6.3 (SD 1.7) $\mu\text{mol/L}$ before infusion of L-carnitine at 96 h ($p = 0.02$) (Fig. 2). During the 4-h infusion of L-carnitine, the mean level of fC0 rose quickly to 174 (SD 51) $\mu\text{mol/L}$ but then fell rapidly to 12.3 (SD 6.8) $\mu\text{mol/L}$ within 26 h (Fig. 2). There was practically no difference in mean renal reabsorption of filtered carnitine following oral supplementation (23%) and following infusion with L-carnitine (25%), combined mean 24% (Table 2), when measured in the lower plasma free carnitine concentration range (5.9–54.3 $\mu\text{mol/L}$) where reabsorption would be expected to be more than 90% in healthy subjects.

The level of mean free carnitine excreted in urine correlated ($R^2 = 0.78$, $p < 0.01$) with fC0 in plasma, with a decreased excretion with decreasing plasma levels of fC0 and vice versa (Fig. 3).

The level of mean plasma acetylcarnitine fell significantly within 7 h ($p = 0.037$) from 23.0 (SD 9.2) $\mu\text{mol/L}$ to 10.7 (SD 5.0) $\mu\text{mol/L}$ and then dropped to 0.7 (SD 0.6) $\mu\text{mol/L}$ ($p < 0.001$) before infusion of L-carnitine. Following the start of L-carnitine infusion, we measured a

Table 2 Pharmacokinetics

Patient #	Means			Oral versus infusion					
	CL _R (mL/min)	CL _{crea} (mL/min)	F _{reabsorption}	T _{1/2-oral} (h)	T _{1/2-inf} (h)	F _{reabsorption-oral}	F _{reabsorption-inf}	CL _{R-oral} (mL/min)	CL _{R-inf} (mL/min)
1	121.1	166.8	0.27	9.2	21.2	0.23	0.31	127.6	114.6
2	54.2	60.0	0.10	16.1	11.1	0.28	-0.08	43.4	65.1
3	103.1	211.9	0.51	5.6	14.2	0.45	0.58	117.0	89.3
4	77.2	78.2	0.01	9.5	38.6	0.00	0.02	78.1	76.4
5	77.0	107.3	0.28	5.7	17.8	0.17	0.40	89.1	64.9
Mean (CI 95%)	86.5 (54.3–118.7)	124.9 (46.2–203.4)	0.24 (0.08–0.39)	9.22 (3.9–14.5)	20.6 (7.2–33.9)	0.23 (0.0–0.47)	0.25 (-0.09–0.58)	91.0 (49.6–132.5)	82.1 (56.3–107.8)

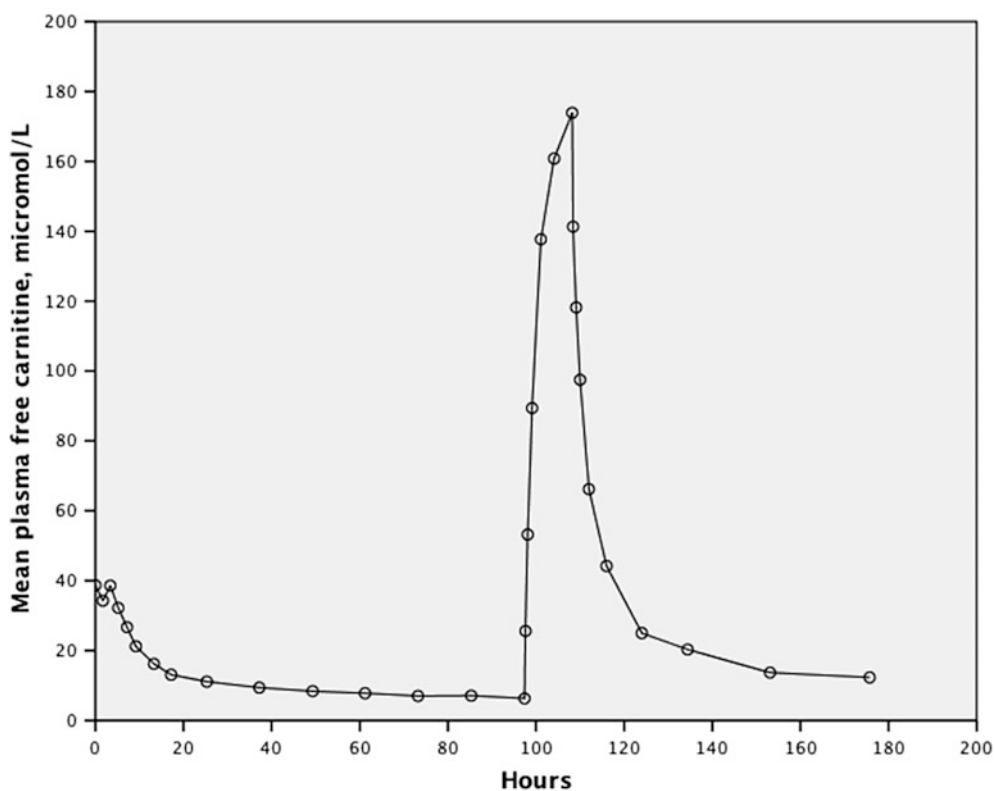


Fig. 2 Mean free carnitine in plasma following discontinuation of oral L-carnitine and then during and after L-carnitine infusion

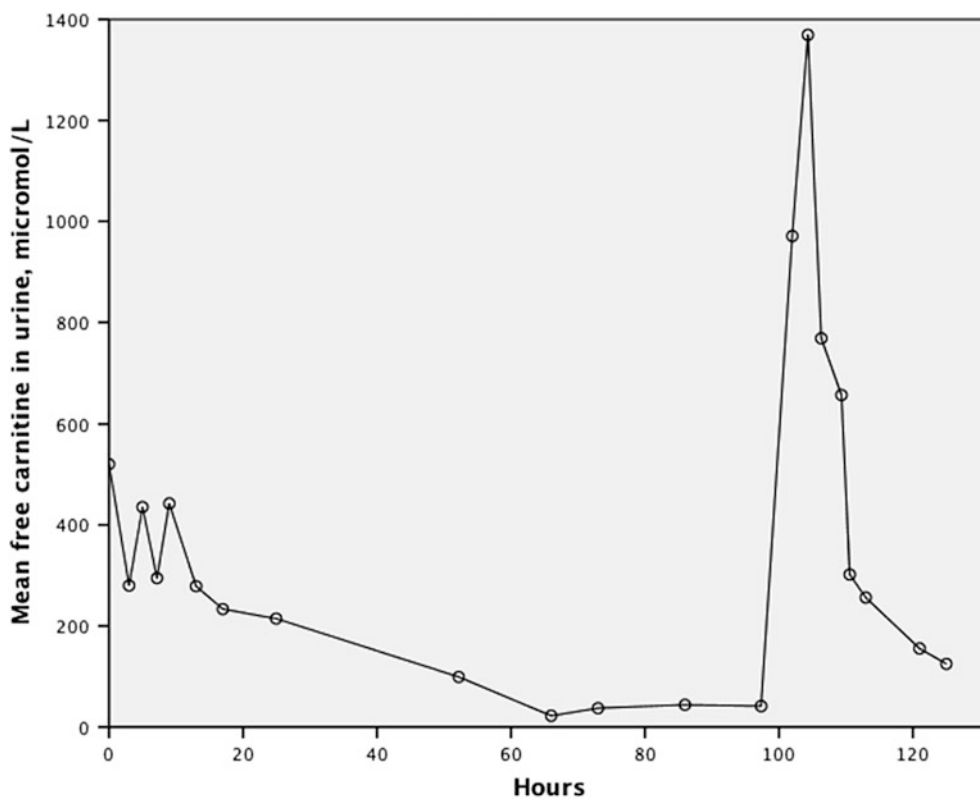


Fig. 3 Mean free carnitine in urine following discontinuation of oral L-carnitine and then during and after L-carnitine infusion

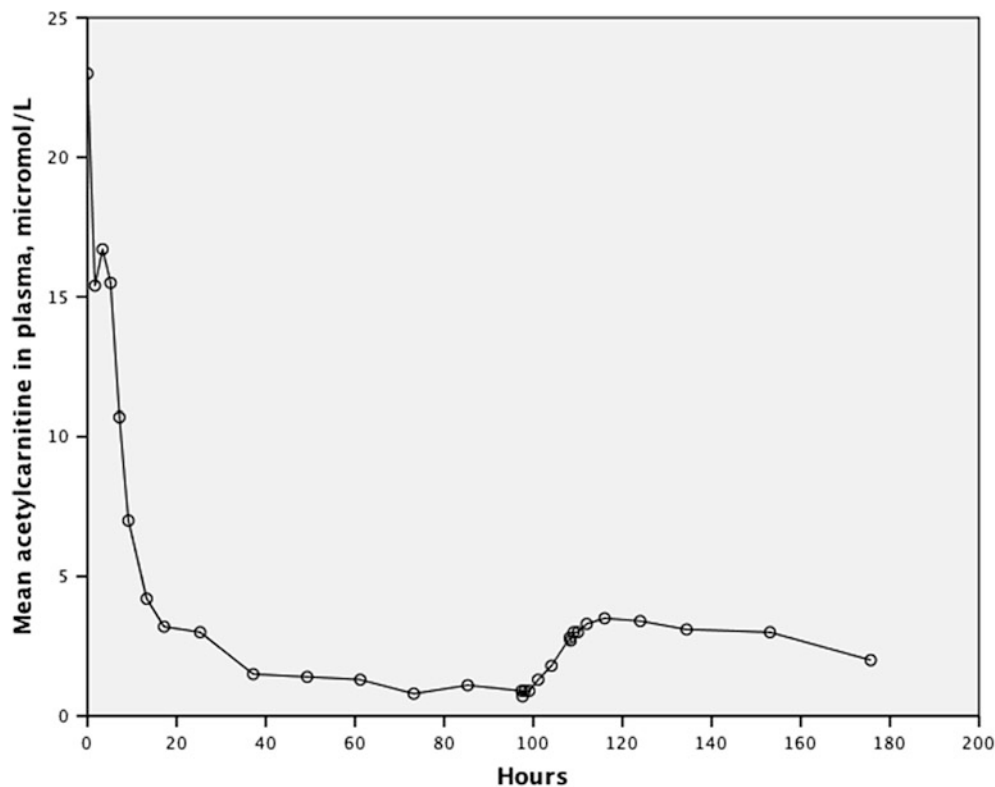


Fig. 4 Mean acetylcarnitine in plasma following discontinuation of oral L-carnitine and then during and after infusion of L-carnitine

nonsignificant increase in the level of mean plasma acetylcarnitine to 3.5 (2.9) $\mu\text{mol/L}$ ($p = 0.13$), before decreasing again following the infusion. As with fC0, the acetylcarnitine in plasma was rapidly excreted in urine following the intermission of supplementation and then following the L-carnitine infusion (Fig. 4). The elderly patient (#2 in Table 1) who had a decreased renal function exhibited a slower decrease in plasma fC0 than the other younger patients and reached a higher level of plasma acetylcarnitine (from 0.4 to 8.6 $\mu\text{mol/L}$) following L-carnitine infusion.

Baseline values including routine safety blood samples and echocardiography were normal – apart from a mild renal insufficiency in the elderly patient (Table 1). There was no change in echocardiographic parameters following 4 days without L-carnitine supplementation. Except for the changes in carnitine levels, there were no changes in other measured blood parameters, including hemoglobin, glucose, electrolytes, ALAT, and creatinine.

Discussion

Our primary findings were that L-carnitine supplemented adult PCD patients homozygous for the c.95A>G mutation had very limited skeletal muscle carnitine stores and that the level of mean free carnitine in plasma dropped rapidly

with a mean $T_{1/2}$ of 9 h following intermission with oral L-carnitine.

The mean concentration of free carnitine in skeletal muscle was only 158 nmol/g in the participants corresponding to approximately 5.4% of the reported normal mean level of 2,914 nmol/g (Madsen et al. 2013). Previous data from children with PCD, showing only a slight increase in skeletal muscle carnitine following supplementation, supports our findings (Stanley et al. 1991). In normal healthy individuals, skeletal muscle carnitine stores account for 97% of all carnitine in the body with a slow estimated turnover of 105 h (Reuter and Evans 2012). There was a tendency toward a decrease in mean muscle carnitine following intermission with L-carnitine supplementation, which was not significant, possibly due to the low number of subjects. It has been hypothesized that if the intramuscular concentration of carnitine is above 4% of normal then fatty acid oxidation in muscle is not compromised (Stanley et al. 1991). Four of our patients reported prior to the study improved physical ability and endurance following long-term L-carnitine supplementation, compared to before being diagnosed with PCD when they did not receive L-carnitine, indicating that even though the obtained skeletal muscle stores were only just above 5% of normal during supplementation, the patients may have benefited with improved physical fitness.

Mean free carnitine in the participants following long-term L-carnitine supplementation was in the lower normal range (Reuter et al. 2008). Carnitine in plasma accounts for only 0.1% of total body carnitine in normal subjects (Reuter and Evans 2012). Plasma carnitine levels are normally maintained by renal tubular reabsorption, a rapidly equilibrating compartment (liver and kidneys) and a slowly equilibrating compartment (muscle) (Evans et al. 2000). It is likely that a compromised renal reabsorption, and a lack of sufficient carnitine stores to maintain plasma levels in the PCD patients, led to rapidly decreasing carnitine levels toward pretreatment plasma levels and an $t_{1/2}$ half-life of only 9 h following the last oral dose of L-carnitine (Fig. 2).

The patients experienced continued excessive loss of carnitine in urine because the mean renal tubular reabsorption of filtered L-carnitine was only 24% compared to more than 90% in normal subjects under normal conditions (Engel et al. 1981; Rebouche et al. 1993; Rebouche 2004; Steiber et al. 2004). We have previously shown that the mean residual OCTN2 transport activity measured in fibroblasts from patients homozygous for the c.95A>G mutation was only 4% compared to normal – the dysfunctional transporter is thus not capable of adequately reabsorbing the filtered carnitine, which is lost in urine instead (Rasmussen et al. 2014c). When L-carnitine was infused, the plasma levels rose quickly, but the infused L-carnitine was excreted in urine within a short period of time in all participants. One exception was the elderly individual with a decreased glomerular filtration, who may not have been able to clear the L-carnitine as effectively as his younger counterparts. Acetylcarnitine is formed intracellularly during normal metabolic activity when L-carnitine is present (Flanagan et al. 2010). The level of plasma acetylcarnitine in the participants fell when they stopped oral supplementation, which may also indicate decreasing intracellular levels of L-carnitine as plasma levels were decreasing (Fig. 4). Plasma acetylcarnitine levels only increased marginally in the young participants following L-carnitine infusion, which might indicate that the amount of infused L-carnitine reaching the intracellular compartment was negligible (Fig. 4). The rise in the level of plasma acetylcarnitine in the elderly man was more pronounced – which might indicate that a greater fraction of infused L-carnitine reached the intracellular space due to reduced glomerular filtration and a slower excretion of the infused carnitine. This may support a strategy of maintaining plasma carnitine levels at a reasonable level with several daily doses of L-carnitine in order to ensure sufficient intracellular carnitine levels. This was partially achieved clinically as shown by a mean plasma acetylcarnitine level of 23 $\mu\text{mol/L}$ after long-term L-carnitine supplementation. The mean plasma half-life of 9 h in the

patients following oral intermission supports a daily regime of at least three daily dosages of L-carnitine, which is also recommended in all Faroese PCD patients.

This study demonstrated that adherence to continued oral L-carnitine supplementation is necessary to ensure and maintain plasma carnitine levels at close to normal levels. It should not be expected that the patients develop sufficient carnitine stores during continued supplementation to compensate for an intermission in their daily supplementation regime. Even though a short-term pause in oral supplementation might not be harmful, our study shows that the plasma levels fall rapidly, which could lead to decreased intracellular carnitine levels, which again might affect cellular metabolism and prove detrimental.

Conclusion

Patients homozygous for the severe c.95A>G mutation had only limited skeletal muscle carnitine stores following long-term high-dosage L-carnitine supplementation. Mean renal reabsorption of L-carnitine was only 24% and the mean plasma half-life of free carnitine following the last oral dose of L-carnitine was 9 h. A treatment strategy of at least three daily separate doses of L-carnitine is recommended from a pharmacokinetic perspective, and while the biochemical consequences of intermission with supplementation in PCD patients are clear, the associated clinical risk is unknown from this study.

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Compliance with Ethics Guidelines

Conflict of Interest

Jan Rasmussen, Jákup A. Thomsen, Jess H. Olesen, Trine M. Lund, Magni Mohr, Jón Clementsen, Olav W. Nielsen, and Allan M. Lund have no conflicts of interest to report.

Informed Consent

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

Contributions of Individual Authors

Jan Rasmussen: First author and involved in all aspects of the work including conception, design, recruiting patients, analysis, and drafting of the manuscript. Guarantor of the article.

Jákup A. Thomsen: Involved in recruiting patients, collecting data, and critical revision of the manuscript.

Jess H. Olesen: Analyzed blood, skeletal muscle, and urine for carnitine and was also involved in critically revising the manuscript.

Trine M. Lund: Involved in design, pharmacokinetic analyses, and critical revision of the manuscript.

Magni Mohr: Involved in collecting data, performed the muscle biopsies, and revised the manuscript critically.

Jón Clementsen: Involved in collecting data and biomaterial and critically revising the manuscript.

Olav W. Nielsen: Involved in design, analysis, and critical revision of the manuscript.

Allan M. Lund: Involved in conception, design, analysis of data, and critically revising the manuscript.

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Newborn Screening for Homocystinuria Revealed a High Frequency of MAT I/III Deficiency in Iberian Peninsula

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Abstract Homocystinuria due to cystathionine β -synthase deficiency or “classical homocystinuria” is a rare autosomal recessive condition resulting in altered sulfur metabolism with elevated methionine and homocysteine in plasma and homocystine in urine. This condition is characterized by a high clinical heterogeneity, which contributes to late clinical diagnosis, usually only made after irreversible damage has occurred. Treatment is effective if started before clinical symptoms. The analysis of methionine levels by tandem mass spectrometry (MS/MS) allows the newborn screening for homocystinuria, but false-positive results can be frequently obtained and lead to the unwanted identification of methionine adenosyl transferase (MAT I/III) deficiency. This latter condition is biochemically characterized by isolated persistent hypermethioninemia, accompanied in some individuals with slightly elevated levels of homocysteine in plasma. A dominant form of MAT I/III deficiency, associated with mutation p.R264H, seems to be very frequent in the Iberian Peninsula and usually has a clinically benign course. Both these metabolic disorders are screened in Galicia and Portugal since the introduction of the MS/MS technology, in 2000 and 2004,

respectively, resulting in the identification of three patients with classical homocystinuria and 44 patients with MAT I/III deficiency. All but one heterozygous parent of MAT I/III patients, identified with the p.R264H mutation, are healthy adults around the age of 30/40. The implementation of a second-tier test for homocysteine in dried blood spots would considerably reduce the number of MAT I/III-deficient patients identified and improve the specificity and positive predictive value for classical homocystinuria screening.

Introduction

Homocystinuria due to cystathionine beta-synthase deficiency (CBS, OMIM #236200) or “classical homocystinuria” is a rare autosomal recessive condition caused by a deficiency in the enzyme cystathionine beta-synthase (CBS, EC 4.2.1.22), the first enzyme in the transsulfuration pathway. Incidence in Europe is estimated to be 1 in 100,000 live births, with an estimated worldwide frequency of 1:344,000 (Mudd et al. 2001). CBS catalyzes the conversion of serine and homocysteine to cystathionine and water and contains three functional domains; the C-terminal domain is responsible for allosteric activation of the enzyme by S-adenosylmethionine (AdoMet); the middle domain contains the catalytic core, which is responsible for the pyridoxal 5'-phosphate-catalyzed reaction; and the N-terminal domain, which contains heme, regulates the enzyme in response to redox conditions (Banerjee and Zou 2005). CBS is mainly expressed in the adult liver and pancreas and is active as a homotetramer (Meier et al. 2001); its deficiency results in altered sulfur metabolism with elevated methionine and homocysteine in plasma and homocystine in urine. The clinical features of untreated

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homocystinuria due to CBS deficiency result in defects in a variety of different organs and systems and include myopia, ectopia lentis, mental retardation, skeletal anomalies resembling Marfan syndrome, and thromboembolic events (Mudd et al. 2001). There is a high clinical heterogeneity, which contributes to late clinical diagnosis, usually only made after irreversible damage has occurred. Treatment is effective if started before clinical symptoms and is based on dietary restriction of methionine and supplementation with betaine, vitamin B12, and folic acid. Without treatment, 50% of patients die by the age of 30 years, usually as a result of arterial thromboembolism (Testai and Gorelick 2010).

This condition is due to mutations in *CBS* gene, with more than 170 known mutations, the majority of which being sporadic mutations (<http://cbs.lfl.cuni.cz>). A genotype-phenotype correlation is reported, with mutations associated with residual enzyme activity leading to a milder clinical phenotype (Mudd et al. 2001).

Patients with classical homocystinuria can be divided into two important biochemical phenotypes: pyridoxine responsive and pyridoxine nonresponsive. The pyridoxine-responsive patients represent approximately 50% of patients; they usually have milder symptoms and slower disease progression, when on oral pyridoxine (vitamin B6) supplementation (Mudd et al. 2001).

The most common *CBS* allele is c.833T>C (p.I278T), which is associated with pyridoxine-responsive homocystinuria; this seems to be rare in Iberian Peninsula (20%; Moat et al. 2004). On the other hand, mutation p.T191M, a pyridoxine nonresponsive mutation, seems to be prevalent in this region (Urreizti et al. 2003; Urreizti et al. 2006). Mutation p.G307S is also a frequent pyridoxine nonresponsive mutation in northern Europe (Gallagher et al. 1995) and is probably of Celtic origin.

The analysis of methionine levels by tandem mass spectrometry (MS/MS) allows newborn screening (NBS) for homocystinuria, but this method raises two important issues: milder pyridoxine-responsive patients may be missed due to slightly elevated levels of methionine at the screening time, and false-positive results can be obtained due to secondary methionine elevations, resulting from other conditions like liver disease, parenteral nutrition, or methionine adenosyl transferase deficiency (MAT I/III deficiency, MIM#250850). This latter condition is biochemically characterized by isolated persistent hypermethioninemia accompanied, in some individuals, with slightly elevated levels of homocysteine in plasma (Mudd et al. 2001). Methionine adenosyl transferase (MAT, EC 2.5.1.6) is the enzyme responsible for the biosynthesis of S-adenosylmethionine from methionine and ATP and has two hepatic forms (MAT I and MAT III) encoded by *MAT1A* gene. Although usually inherited as an autosomal recessive

trait, a dominant form has been reported associated with mutation p.R264H (Chamberlin et al. 1997, 2000). This mutation has been reported in several populations (Chamberlin et al. 1997; Nagao et al. 2013; Chien et al. 2005; Chadwick et al. 2014) and seems to be very frequent in the Iberian Peninsula (Couce et al. 2008, 2013; Martins et al. 2012). The clinical significance of this condition is not clearly elucidated, raising some issues regarding its inclusion in newborn screening programs. Both these metabolic disorders, classical homocystinuria and MAT I/III deficiency, are screened in Galicia (Spanish region) and Portugal since the introduction of the MS/MS technology, in 2000 and 2004, respectively, resulting in the identification of 47 patients, presenting only three of them classical homocystinuria.

Here, we report a high frequency of MAT I/III deficiency in Iberian Peninsula detected through neonatal screening for homocystinuria.

Patients and Methods

During the last 10 years, approximately 850,000 newborns were screened in the single Portuguese Newborn Screening Laboratory by MS/MS technology, through the analysis of amino acids and acylcarnitines as butyl esters (Rashed et al. 1995), in dried blood samples. Most samples were collected between the third and sixth days of life. The positive screening criterion for classical homocystinuria and MAT I/III deficiency was a methionine concentration above 50 $\mu\text{mol/L}$.

Galicia started NBS for both these conditions in 2000, and approximately 374,000 newborns were screened, using the same MS/MS method. If the measured concentration exceeded the 99th centile for the studied population, a second sample was requested. For methionine, this value ranged between 48 and 56 $\mu\text{mol/L}$ over the study period. In Galicia, most of the NBS samples were collected between the second and sixth days of life, and a urine sample was also included in the screening. Homocystine measurement was performed as second-tier test, by MS/MS analysis of urine spots in filter paper (Rebollido-Fernandez et al. 2012).

In both centers, newborns with a persistent, isolated high methionine level in a second sample were sent to a treatment center for further clinical and biochemical evaluation, which included hepatic function evaluation, plasma and urinary amino acid chromatography, plasma total homocysteine, and urinary organic acid analysis.

During this time, 47 patients were identified (Tables 1 and 2); all of them were asymptomatic term newborns. Diagnosis in all patients was confirmed by *MAT1A* or *CBS* gene analysis. Genomic DNA was isolated from blood

Table 1 Characterization of patients with MAT I/III deficiency

Patient data				Neonatal screening time (blood spot)		Confirmation time (plasma)			Molecular analysis	
Patient	Origin	Gender	Current age	Age	Met (μM) ^a	Age	Met (μM) ^b	tHcy (μM) ^c	Gene <i>MAT1A</i>	References
1	Portugal	F	9 years	6 days	85	12 days	80	13	p.R264H/WT	Chamberlin et al. (1997)
2	Portugal	F	8 years	5 days	123	18 days	176	17	p.R264H/WT	Chamberlin et al. (1997)
3	Portugal	M	8 years	4 days	77	19 days	121	16	p.R264H/WT	Chamberlin et al. (1997)
4	Portugal	M	8 years	4 days	52	19 days	168	16	p.R264H/WT	Chamberlin et al. (1997)
5	Portugal	F	7 years	4 days	103	16 days	85	13	p.R264H/WT	Chamberlin et al. (1997)
6	Portugal	M	7 years	3 days	58	29 days	247	18	p.R264H/WT	Chamberlin et al. (1997)
7	Portugal	F	6 years	4 days	80	30 days	195	14	p.R264H/WT	Chamberlin et al. (1997)
8	Portugal	F	6 years	4 days	85	24 days	254	20	p.R264H/WT	Chamberlin et al. (1997)
9	Portugal	M	6 years	6 days	309	10 days	194	n.a.	p.R264H/WT	Chamberlin et al. (1997)
10	Portugal	M	5 years	6 days	73	23 days	145	14	p.R264H/WT	Chamberlin et al. (1997)
11	Portugal	F	5 years	6 days	77	63 days	157	12	p.R264H/WT	Chamberlin et al. (1997)
12	Portugal	F	5 years	5 days	117	18 days	182	21	p.R264H/WT	Chamberlin et al. (1997)
13	Portugal	F	5 years	5 days	124	24 days	155	15	p.R264H/WT	Chamberlin et al. (1997)
14	Portugal	F	5 years	7 days	67	30 days	85	5	p.L137V/p.S247N	This study
15	Portugal	F	5 years	5 days	92	30 days	79	11	p.R264H/WT	Chamberlin et al. (1997)
16	Portugal	F	5 years	4 days	79	20 days	111	64	p.R264H/WT	Chamberlin et al. (1997)
17	Portugal	F	4 years	3 days	72	19 days	465	n.a.	p.L355R/p.L355R	Couce et al. (2013)
18	Portugal	F	4 years	6 days	80	22 days	147	23	p.R264H/WT	Chamberlin et al. (1997)
19	Portugal	F	4 years	5 days	60	16 days	136	10	p.R264H/WT	Chamberlin et al. (1997)
20	Portugal	M	4 years	4 days	100	13 days	105	n.a.	p.R264H/WT	Chamberlin et al. (1997)
21	Portugal	F	3 years	4 days	63	30 days	117	12	p.R264H/WT	Chamberlin et al. (1997)
22	Portugal	F	3 years	5 days	72	19 days	85	11	p.R264H/WT	Chamberlin et al. (1997)
23	Portugal	F	3 years	3 days	71	16 days	110	15	p.R264H/WT	Chamberlin et al. (1997)

(continued)

Table 1 (continued)

Patient data				Neonatal screening time (blood spot)		Confirmation time (plasma)			Molecular analysis	
Patient	Origin	Gender	Current age	Age	Met (μM) ^a	Age	Met (μM) ^b	tHcy (μM) ^c	Gene <i>MAT1A</i>	References
24	Portugal	M	2 years	4 days	92	28 days	108	12	p.R264H/WT	Chamberlin et al. (1997)
25	Portugal	M	2 years	5 days	101	16 days	94	8	p.R264H/WT	Chamberlin et al. (1997)
26	Portugal	F	2 years	6 days	110	23 days	148	12	p.R264H/WT	Chamberlin et al. (1997)
27	Portugal	F	1 year, 4 months	5 days	79	21 days	156	12	p.R264H/WT	Chamberlin et al. (1997)
28	Portugal	M	1 year	6 days	86	17 days	126	23	p.R264H/WT	Chamberlin et al. (1997)
29	Portugal	F	9 months	5 days	71	15 days	90	n.a.	p.R264H/WT	Chamberlin et al. (1997)
30	Portugal	M	5 months	6 days	71	21 days	113	10	p.R264H/WT	Chamberlin et al. (1997)
31	Portugal	M	3 months	4 days	74	16 days	93	26	p.R264H/WT	Chamberlin et al. (1997)
32	Galicia	F	8 years	6 days	50	35 days	573	23	p.R264H/WT	Chamberlin et al. (1997)
33	Galicia	M	8 years	3 days	100	1 month, 15 days	189	9	p.R264H/WT	Chamberlin et al. (1997)
34	Galicia	F	8 years	5 days	88	2 months, 21 days	341	10	p.R264H/WT	Chamberlin et al. (1997)
35	Galicia	M	8 years	3 days	147	2 months, 13 days	331	12	p.R264H/WT	Chamberlin et al. (1997)
36	Galicia	M	7 years	5 days	100	25 days	164	11	p.R264H/WT	Chamberlin et al. (1997)
37	Galicia	F	6 years	3 days	80	1 month, 15 days	292	n.a.	p.R264H/WT	Chamberlin et al. (1997)
38	Galicia	F	5 years	4 days	115	1 month, 10 days	283	8	p.R264H/WT	Chamberlin et al. (1997)
39	Galicia	F	4 years	2 days	82	1 month, 28 days	106	15	p.R264H/WT	Chamberlin et al. (1997)
40	Galicia	F	2 years	3 days	82	1 month, 20 days	392	n.a.	p.R264H/WT	Chamberlin et al. (1997)
41	Galicia	M	2 years	5 days	76	4 months, 8 days	157	8	p.R264H/WT	Chamberlin et al. (1997)
42	Galicia	M	1 year, 2 months	6 days	45	3 months	255	12	p.R264H/WT	Chamberlin et al. (1997)
43	Galicia	M	2 years	3 days	67	22 days	91	n.a.	p.R264H/WT	Chamberlin et al. (1997)
44	Galicia	M	5 months	3 days	70	2 months, 22 days	144	11	p.R264H/WT	Chamberlin et al. (1997)

n.a. not available, *Met* methionine, *tHcy* total homocysteine

^a Cutoff value: 50 μM (Portugal) and 56 μM (Galicia)

^b Normal range: 4–44 μM

^c Normal range: 2–14 μM ; patients 3 and 4 are twin brothers

Table 2 Characterization of patients with classical homocystinuria

Patient data			Neonatal screening (blood spot)			Confirmation time (plasma)			Molecular study	
Patient	Origin	Gender	Current age	Age	Met (μM) ^a	Age	Met (μM) ^b	tHcy (μM) ^c	Gene <i>CBS</i>	References
45	Portugal	F	9 years	5 days	130	22 days	744	130	p.V178GfsX23/p.K523SfsX18	Cozar et al. (2011), Castro et al. (2001)
46	Portugal	M	2 years	6 days	130	14 days	293	149	p.L338P/p.L338P	Urreizti et al. (2003)
47	Galicia	F	9 years	3 days	59	29 days	1,086	148	p.T257M/p.T257M	Sebastio et al. (1995)

Met methionine, *tHcy* total homocysteine

^a Cutoff value: 50 μM (Portugal) and 56 μM (Galicia)

^b Normal range: 4–44 μM

^c Normal range: 2–14 μM

samples and sequenced by standard procedures, based on direct sequencing of PCR-amplified fragments containing all the coding regions, the corresponding intron-exon junctions, and the 5'- and 3'-untranslated regions (see Couce et al. 2008 and Martins et al. 2012 for details; oligonucleotide primer sequences and PCR conditions are available upon request).

Results

More than one million newborns have been tested for CBS and MAT I/III deficiencies, in Galicia and Portugal, since 2000, three patients being identified with classical homocystinuria and 44 patients with MAT I/III deficiency. All patients were confirmed by biochemical and molecular analysis (Table 1), and we had a 0.013% recall rate due to elevated methionine in the screening sample; approximately 50% of these cases were premature neonates with parenteral nutrition.

Thirty-one cases of MAT I/III deficiency (approximate frequency 1:27,400) and two cases of classical homocystinuria (approximate frequency 1: 425,000) were identified in Portugal (Tables 1 and 2). Some of these patients were already reported (Martins et al. 2012; Cozar et al. 2011).

In Galicia, 13 cases of MAT I/III deficiency (approximate frequency 1:28,736) and one classical homocystinuria (approximate frequency 1:374,000) were detected (Tables 1 and 2). These patients were already referred to in previous publications (Urreizti et al. 2006; Couce et al. 2008, 2013).

All MAT I/III-deficient patients had moderately increased levels of methionine, both in screening and confirmation samples, with usually high levels in the confirmation sample. Most patients with p.R264H mutation had slightly elevated levels of total homocysteine (Table 1). In regular annual follow-up, these patients maintain the

moderate methionine levels (data not shown). After diagnosis, all patients except patients 35 and 38 received free diets appropriate for their ages with a protein intake within normal limits (FAO/WHO).

The molecular study of MAT I/III-deficient patients revealed that 42 of the 44 patients are heterozygous for p.R264H mutation, with no other mutation identified in *MAT1A* gene. Family studies of these patients lead to the identification of eight new cases and, as expected, one parent (mother or father) of each patient was also confirmed to be heterozygote for p.R264H mutation. All these individuals were clinically well, except the father of patient 15, who died at a young age with a myocardial infarction (Martins et al. 2012). In general, the identified adult patients also had mildly elevated levels of homocysteine and lower levels of methionine, compared with newborns. Two mothers (of twin brother patients 2 and 3 and patient 28) and one great grandmother (of patient 13) even presented completely normal levels of methionine (32, 33, and 49 μM , respectively).

Patient 14 is a heterozygous compound presenting two novel missense mutations: p.L137V (c.409T>G) and p.S247N (c.740G>A). This patient has no Iberian ancestry. Both mutations affect highly conserved amino acid residues, were not found in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) or exome variant database (evs.gs.washington.edu/EVS/), and are predicted to be pathogenic through bioinformatics tools analysis, namely, Condel (bg.upf.edu/condel/), SIFT (sift.jcvi.org/), MutationTaster (www.mutationtaster.org/), and PolyPhen 2 (genetics.bwh.harvard.edu/pph2/index.shtml). In the S247 amino acid residue, another mutation p.S247R (c.739A>C) was recently reported (Nagao et al. 2013).

Patient 17 is homozygous for mutation p.L355R, a recently reported mutation found in a related patient living in Spain (Couce et al. 2013).

All three patients with classical homocystinuria had moderately elevated levels of methionine at screening but extremely high levels of plasma methionine and total homocysteine at confirmation time (Table 2). They have been treated since diagnosis in the neonatal period with dietary restriction of methionine and supplementation with betaine, vitamin B12, and folic acid. In spite of being pyridoxine nonresponsive, they remain asymptomatic to date.

Discussion

Newborn screening has been advocated for classical homocystinuria due to considerable evidence that early detection and treatment can prevent the severe clinical consequences of this condition (Mudd et al. 2001). Nevertheless, newborn screening for classical homocystinuria has been mostly performed by identification of increased methionine concentrations in dried blood spot samples through MS/MS, which is a low-specificity method if a low cutoff value is used for methionine and a low-sensitivity method if, instead, a high cutoff value is used (Gan-Schreier et al. 2010); a compromise value should then be used, but this is never an optimal strategy for screening. In Portugal and Galicia, methionine cutoff values of 50 and 56 $\mu\text{mol/L}$, respectively, have been used, and, as far as we know, there are no missing cases of homocystinuria. Moreover, we identified a considerable number of patients with a possible benign form of MAT I/III deficiency, a condition not known in the Iberian Peninsula before MS/MS-based newborn screening. All MAT I/III-deficient patients are under biochemical and clinical follow-up in different treatment centers, and all have normal growth, development, and neurological examination. Only patient 7 was found to have myelination abnormalities of unknown clinical significance, and in two families (patients 1 and 5), severe vascular disease histories, without other risk factors, were observed. Careful regular clinical monitoring is being performed for all newborn patients and p.R264H carrier relatives, despite their current healthy state (Martins et al. 2012). This is in accordance with the results of Nagao et al. (2013), who reported 14 clinically well patients with p.R264H mutation, mostly detected through newborn screening. Parents and siblings identified in subsequent family studies were also clinically well, and there was also a tendency to a decrease of methionine values with age.

Only three patients were identified with classical homocystinuria, but they are all free of symptoms proving the usefulness of this screening.

A second-tier approach for NBS of homocystinuria, by measuring the total homocysteine on the initial dried blood spot, has been developed (Gan-Schreier et al. 2010;

Tortorelli et al. 2010). This allows the use of an initial low cutoff value for methionine, with the positive cases being then selected for total homocysteine measurement. With this approach, a higher sensitivity would be achieved, and the identification of newborns with elevated methionine levels due to parenteral nutrition or secondary to other liver disease would be avoided, thus reducing the number of parents affected by the parental anxiety associated with a false-positive result. Some of the newborns with MAT I/III deficiency could also be deliberately ignored, although this could be controversial due to the incomplete knowledge of the natural history of the disease in all patients; the complete lack of MAT I/III activity may represent a risk for development of brain demyelination (Mudd 2011). Some MAT I/III-deficient patients have been reported with myelination disorders (Chamberlin et al. 1996; Furujo et al. 2012; Nagao et al. 2013). Forty-two of the forty-four patients identified in Portugal and Galicia are heterozygous for p.R264H mutation, a dominant form of the disease, associated with a high residual enzyme activity and moderate elevated methionine levels. According to previous reports, some residual activity could be sufficient to prevent clinical symptoms in these patients (Chamberlin et al. 1996). If this is the case, there is no advantage for them to be identified; they will never need treatment and their identification will only lead to unnecessary anxiety for the families. Nevertheless, myelination alterations do not seem to be directly related to methionine levels, and a genotype/phenotype correlation regarding methionine levels and CNS involvement has not yet been established (Chamberlin et al. 1996; Mudd et al. 2001). At least one p.R264H heterozygous 3-year-old girl was reported with myelination abnormalities (Martins et al. 2012), which suggests care must be taken with the assumption that none of these individuals will develop symptoms.

In spite of the improving of the specificity and positive predictive value for classical homocystinuria screening, this second-tier approach may not always allow for the unequivocal differentiation between MAT I/III deficiency and classical homocystinuria since there is the possibility of low levels of total blood homocysteine in the first days of life in patients with classical homocystinuria B6-responsive and mildly elevated values of total homocysteine sometimes observed in MAT I/III-deficient patients. Some patients are initially misdiagnosed as having CBS deficiency due to these mildly elevated values (Nagao et al. 2013). In these cases, the molecular study of *MAT1A* and *CBS* genes or the measurement of other metabolites of the methionine cycle could be important for the correct diagnosis (Stabler et al. 2002).

In conclusion, this report tried to address two main questions: should we maintain NBS for classical homocystinuria through elevated methionine levels, in spite of

the low frequency of this disease in Iberian Peninsula, and the identification of a large number of patients with a possible benign form of MAT I/III deficiency or, on the other hand, should we implement a second-tier test for total homocysteine in dried blood spots? Do patients and families of MAT I/III-deficient patients have any advantage from their identification or, on the contrary, is this diagnosis only a burden to the families?

Considering our present knowledge and results, we decided to implement the second-tier test for total homocysteine. For the MAT I/III-deficient cases which are still to be identified, careful full information regarding the most probable benign clinical course of the disease should be provided to the families, together with the recommendation for regular clinical and biochemical follow-up.

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Synopsis

MS/MS-based NBS in Galicia and Portugal, through the analysis of methionine, revealed a very low birth prevalence for classical homocystinuria (approximately 1:400,000) and a high birth prevalence for MAT I/III deficiency (approximately : 1:28,000).

Compliance with Ethics Guidelines

Conflict of Interest

Ana Marcão, María L. Couce, Célia Nogueira, Helena Fonseca, Filipa Ferreira, José M. Fraga, M. Dolores Bóveda, and Laura Vilarinho declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

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

Details of the Contributions of Individual Authors

Ana Marcão – Planned, conducted, and reported the work described in the article.

María L. Couce – Planned and reported the work described in the article.

Célia Nogueira – Planned, conducted, and reported the work described in the article.

Helena Fonseca – Conducted the work described in the article.

Filipa Ferreira – Conducted the work described in the article.

M. Dolores Bóveda – Planned the work described in the article.

José M. Fraga – Planned the work described in the article.

Laura Vilarinho – Planned and reported the work described in the article.

All authors have read and approved the final manuscript.

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