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JIMD Reports

Volume 16

SSIEM

Springer

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Springer

SSIEM

ISSN 2192-8304 ISSN 2192-8312 (electronic)
ISBN 978-3-662-44586-0 ISBN 978-3-662-44587-7 (eBook)
DOI 10.1007/978-3-662-44587-7
Springer Heidelberg New York Dordrecht London

Contents

JIMD Reports
DOI 10.1007/8904_2014_309

RESEARCH REPORT

Leucine Loading Test is Only Discriminative for 3-Methylglutaconic Aciduria Due to AUH Defect

Saskia B. Wortmann · Leo A.J. Kluijtmans · Silvia Sequeira · Ron A. Wevers · Eva Morava

Received: 04 September 2013 / Revised: 07 March 2014 / Accepted: 11 March 2014 / Published online: 23 April 2014

Abstract Currently, six inborn errors of metabolism with 3-methylglutaconic aciduria as discriminative feature are known. The "Primary 3-methylglutaconic aciduria," 3-methylglutaconyl-CoA hydratase deficiency or AUH defect, is a disorder of leucine catabolism. For all other subtypes, also denoted "Secondary 3-methylglutaconic acidurias" (TAZ defect or Barth syndrome, SERAC1 defect or MEGDEL syndrome, OPA3 defect or Costeff syndrome, DNAJC19 defect or DCMA syndrome, TMEM70 defect, "not otherwise specified (NOS) 3-MGA-uria"), the origin of 3-methylglutaconic aciduria remains enigmatic but is hypothesized to be independent from leucine catabolism. Here we show the results of leucine loading test in 21 patients with different inborn errors of metabolism who present with 3-methylglutaconic aciduria. After leucine loading urinary 3-methylglutaconic acid levels increased only in the patients with an AUH defect. This strongly supports the hypothesis that 3-methylglutaconic aciduria is independent from leucine breakdown in other inborn errors of metabolism with 3-methylglutaconic aciduria and also provides a simple test to discriminate between primary and secondary 3-methylglutaconic aciduria in regular patient care.

Communicated by: Jerry Vockley, M.D., Ph.D.

Competing interests: None declared

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Introduction

Currently, six inborn errors of metabolism with 3-methylglutaconic aciduria (3-MGA-uria) as a discriminative feature are known (3-MGA-uria-IEM; for classification and nomenclature, see (Wortmann et al. 2013a). The "Primary 3-MGA-uria" 3-methylglutaconyl-CoA hydratase (3-MGH) deficiency or AUH defect is a disorder of leucine catabolism. For all other subtypes the origin of 3-MGA-uria has not been discovered but is hypothesized to be independent from leucine catabolism (for review (Wortmann et al. 2012)). These three groups of "Secondary 3-methylglutaconic aciduria" encompass disorders of defective phospholipid remodeling (TAZ defect or Barth syndrome, SERAC1 defect, or MEGDEL syndrome) and mitochondrial membrane associated disorders (OPA3 defect or Costeff syndrome, DNAJC19 defect or DCMA syndrome, TMEM70 defect). The remaining patients with significant and consistent 3-MGA-uria in whom the abovementioned syndromes have been excluded are referred to as "not otherwise specified (NOS) 3-MGA-uria" until elucidation of the underlying pathomechanism enables proper classification.

One can discriminate AUH defect from the other 3-MGA-uria-IEMs based on (1) the level of 3-MGA increase which is higher than in all other subtypes, (2) the elevated urinary 3-hydroxyisovaleric acid (3-HIVA), and (3) the 2:1 ratio for *cis*:*trans* isoforms of 3-MGA (compared to 1:1 in all other types; (Engelke et al. 2006; Wortmann et al. 2009).

Here we test the hypothesis, that, as opposed to the observation in patients with the AUH defect, other types of 3-MGA-uria-IEM are independent of leucine catabolism by

presenting leucine loading test in 21 patients with different types of 3-MGA-uria-IEM.

Material and Methods

Patients

Twenty-one patients with different 3-MGA-uria-IEM (three AUH defect, nine TAZ-defect, one OPA3-defect, one SERAC1-defect, seven NOS-3-MGA-uria) were included in this study. In seven patients leucine loading tests were performed at our center, the other patients were reported in literature (Barth et al. 1999; Christodoulou et al. 1994; Duran et al. 1982; Ensenauer et al. 2000; Haan et al. 1987; Kelley et al. 1991; Ruesch et al. 1996).

Leucine Loading Tests

The tests of the patients P12–P18 were performed as part of the individual diagnostic work-up, not as a scientific study. Oral informed consent was obtained from all patients. In our center 100 mg/kg (max. 6 g) leucine powder dissolved in vanilla pudding or lemonade was given orally to all patients. Prior to the loading test, and 1 h after the leucine gift, a urine portion was collected for urinary organic acid analysis. Besides, a venous blood sample was drawn to determine ammonia, lactate and glucose, blood serum amino acids, and blood gas analysis 1 h after leucine loading. In children who were very scared no venous blood sample was taken, only bedside glucose measurements were performed ("finger prick"). After the loading, also a 24 h urine sample was also collected for another urinary organic acid analysis.

Urinary Organic Acid Analysis

Briefly, a urine sample, in volume equivalent to 4 μmol of creatinine, is acidified to pH 2, after which the organic acids are extracted by ethylacetate twice, derivatized with trimethylsilyl (TMS), and analyzed on an Agilent 7890A gas chromatograph (GC), coupled to a flame ionization detector (FID) and an Agilent 5975C inert XL MSD mass spectrometer. Quantification of organic acids was done by peak area. The concentration of 3-MGA was estimated by calculating the peak area of 3-MGA, which was compared to the calibration curve obtained with a 3-MGA standard. For this purpose the sum of both isoforms (*cis–trans*) was taken.

Leucine Loading Tests in Patients Described in the Literature

These were performed essentially as described above. Fasting hours before the challenge and time points for sampling after leucine loading slightly differed between studies. Loading was performed with 100 mg/kg leucine in nine patients, one patient received 580 mg, one 1,200 mg leucine; two patients received 4 g/kg protein (comparable with 200 mg/kg leucine); one patient received 100 mg/kg leucine followed by 4 g/kg protein. Leucine, isoleucine, or protein was administered orally in 12 and intravenously in three patients (for details see Table 1).

Results

The results are summarized in Table 1. Serum ammonia and lactate levels, blood gas analysis, and glucose levels 1 h after leucine loading in our patients were within normal limits (data not shown). Serum amino acid analysis showed elevated leucine levels proving the intake of leucine powder (see Table 1). The patients felt well during and after the test, no one showed signs or symptoms suggesting hypoglycemia, no one reported side effects. To improve comparability of results between different centers all 3-MGA-uria levels are provided in mmol/mol creatinine (in our laboratory reference <10) and the lowest and highest 3-MGA-uria levels before and after leucine loading per patient are presented, as well as the ratio between these levels.

In AUH defect 3-MGA-uria levels were generally higher than in all other 3-MGA-uria-IEM (range before loading 400–761 mmol/mol creatinine, range after loading 1,600–2,982 mmol/mol creatinine). The mean ratio after/before loading was 4.1 ($N = 3$, range 3.9–4.5).

In TAZ-defect 3-MGA-uria levels ranged between 36 and 202 mmol/mol creatinine. The mean ratio after/before leucine loading was 1.8 ($N = 9$, range 1.1–5.3). In OPA3-defect levels ranged between 26 and 29 mmol/mol creatinine. The ratio after/before leucine loading was 0.9 ($N = 1$). In SERAC1-defect levels ranged between 60 and 75 mmol/mol creatinine. The ratio after/before leucine loading was 1.25 ($N = 1$). In NOS-3-MGA-uria levels ranged between 21 and 410 mmol/mol creatinine. The mean ratio after/before leucine loading was 1.0 ($N = 7$, range 0.5–1.3).

Discussion

The pathomechanism leading to 3-MGA-uria in non-AUH-related defect ("Secondary 3-MGA-uria") remains unclear. Based upon the finding of elevated serum 3-MGA in a subgroup of patients with Smith Lemli Opitz syndrome a link with cholesterol biosynthesis has been hypothesized (Kelley and Kratz 1995). Two possible shuntways are known, the Popjak shunt linking dimethylallyl-PPi in the cholesterol biosynthesis with 3-methylcrotonyl-CoA in the

Table 1 Results of leucine loading tests

	Mutation	Loading	Serum glucose level 1 h after loading (mmol/L, N 4–6 mmol/L)	Serum leucine after loading (μmol/L)	Elevated urinary 3-HIVA	Lowest urinary 3-MGA before loading	Peak urinary 3-MGA after loading	Ratio high/low	Ratio *cis*:*trans* isoforms	Mean ratio (range, *N* patients)
P1 (P1 Duran et al. (1982))	*AUH*	Protein 4 g/kg p.o.	NA	819[a]	+	518	2,328	4,5	NA	4,1 (3,9–4,5; $N = 3$)
P2 (P2 Duran et al. (1982))	*AUH*	Protein 4 g/kg p.o.	NA	875[a]	+	761	2,982	3,9	NA	
P3 Ensenauer et al. (2000)	*AUH*	100 mg/kg leucine followed by 4 g/kg protein	NA	NA	+	400	1,600	4,0	NA	
P4 (P5 aged 6 years Kelley et al. (1991))	*TAZ*	Leucine 100 mg/kg p.o.	NA	Three to fourfold elevated	–	61[b]	75[b]	1,2	NA	1,8 (1,1–5,3; $N = 9$)
P5 (P5 aged 7 years Kelley et al. (1991))	*TAZ*	Leucine 100 mg/kg p.o.	NA	NA	–	36[b]	38[b]	1,1	NA	
P6 (P1 Barth et al. (1999))	*TAZ*	Leucine 100 mg/kg i.v.	NA	NA	NA	69[b]	159[b]	2,3	NA	
P7 (P2 Barth et al. (1999))	*TAZ*	Leucine 100 mg/kg i.v.	NA	NA	NA	38[b]	202[b]	5,3	NA	
P8 (P3 Barth et al. (1999))	*TAZ*	Leucine 100 mg/kg i.v.	NA	NA	NA	106[b]	150[b]	1,4	NA	
P9 (P3 Barth et al. (1999))	*TAZ*	Leucine 100 mg/kg p.o	NA	NA	NA	123[b]	192[b]	1,6	NA	
P10 (JD Christodoulou et al. (1994))	*TAZ*	Leucine 100 mg/kg p.o	NA	211 (N 71–175)	–	100	110	1,1	NA	
P11 (ST Christodoulou et al. (1994))	*TAZ*	Leucine 100 mg/kg p.o	NA	NA	–	130	140	1,1	NA	
P12	*TAZ*	Leucine 100 mg/kg p.o	4.3	NA	–	32	40	1,3	1:1	
P13	*OPA3*	Leucine 100 mg/kg p.o	5.2	NA	–	29	26	0,9	1:1	0,9
P14	*SERAC1*	Leucine 100 mg/kg p.o	4.5	536 (N 22–276)	–	60	75	1,3	1:1	1,3
P15	NOS	Leucine 100 mg/kg p.o	5.8	425 (N 22–276)	–	21	23	1,1	1:1	1,0 (0,5–1,1; $N = 7$)
P16	NOS	Leucine 100 mg/kg p.o	NA	NA	–	33	16	0,5	1:1	
P17	NOS	Leucine 100 mg/kg p.o	4.9	310 (N 22–276)	–	52	53	1,0	1:1	
P18	NOS	Leucine 100 mg/kg p.o	5.3	NA	–	187	234	1,3	1:1	

(continued)

Table 1 (continued)

	Mutation	Loading	Serum glucose level 1 h after loading (mmol/L, N 4–6 mmol/L)	Serum leucine after loading (μmol/L)	Elevated urinary 3-HIVA	Lowest urinary 3-MGA before loading	Peak urinary 3-MGA after loading	Ratio high/low	Ratio *cis*:*trans* isoforms	Mean ratio (range, *N* patients)
P19 (TH Ruesch et al. (1996))	NOS	Leucine 580 mg/kg p.o	NA	NA	–	370	410	1,1	NA	
P20 (TH Ruesch et al. (1996))	NOS	Leucine 1,200 mg/kg p.o	NA	NA	–	360	360	1,0	NA	
P21 Haan et al. (1987)	NOS	Leucine 100 mg/kg p.o	NA	NA	NA	100	100	1,0	NA	

3-methylglutaconic acid (3-MGA) levels are in mmol/mol creatinine

[a] Reference range not provided

[b] Sum of 3-methylglutaric and 3-methylglutaconic acid

leucine catabolism and the HMG Salvage pathway, linking 3-hydroxy-3-methylglutaryl-CoA with 3-methylglutaconyl-CoA. Recently we and others have shown that there is no 3-MGA-uria in a total of 27 (treated and untreated) Smith Lemli Opitz patients as well as in three patients with mevalonate kinase deficiency (Roullet et al. 2012; Wortmann et al. 2013b). This makes significant shunting via the mevalonate shunt as source of 3-MGA-uria unlikely. However, the HMG-salvage pathway has recently been proven to account for the elevated 3-MGA production in a zebrafish model of OPA3 defect. The authors show that simvastatin inhibited mevalonate production from extra-mitochondrial HMG-CoA, which leads to elevated 3-MGA levels (Pei et al. 2010). For the vast majority of patients with 3-MGA-uria mitochondrial dysfunction and in more detail disturbances in the mitochondrial membranes seem to be the common denominator (Wortmann et al. 2013a), but there is no working hypothesis how this membrane imbalance leads to 3-MGA-uria.

Currently six 3-MGA-uria-IEM (Wortmann et al. 2013a) are known. AUH defect is the only "primary 3-MGA-uria," a disorder of leucine catabolism. For all other types (TAZ defect or Barth syndrome, SERAC1 defect or MEGDEL syndrome, OPA3 defect or Costeff syndrome, DNAJC19 defect or DCMA syndrome, TMEM70 defect) the origin of 3-MGA-uria remains enigmatic but is hypothesized to be independent from leucine catabolism (for review, see (Wortmann et al. 2012)).

Here, we report on the results of leucine loading tests in 21 patients with different 3-MGA-uria-IEM (Table 1). Urinary 3-MGA-excretion only showed a significant increase (with a factor 4) in patients with AUH defect, but not in all other types (factor 0.9–1.8). The results for patients with TAZ-defect or Barth syndrome (factor 1.8) should be interpreted with care. The ratio suggests that 3-MGA-uria levels nearly double after leucine loading. But, this is predominantly due to the results of one patient (patient seven; patient two in (Barth et al. 1999)) who has a quite low 3-MGA-level of 38 mmol/mol creatinine before and a high level after leucine loading (202 mmol/mol creatinine; ratio high/low 5.3). This is in contrast to the nine other TAZ-patients described. Leaving patient seven out, the ratio for the remaining nine patients is 1.4 which is compatible with the normal fluctuations of urinary organic acid levels as seen in all patients. The authors discuss this issue in their article and argue that in this patient the daily 3-MGA-uria levels in the morning and in the afternoon only differ up to a factor 1.75 which is still lower than the increase after leucine loading. They speculate that the results could be influenced by different fasting times before leucine loading in this patient (Barth et al. 1999).

Our data prove the hypothesis that 3-MGA-uria is independent from leucine breakdown in all secondary 3-MGA-uria-IEM. Neither the patients reported in the literature nor our own patients encountered any side effects of the leucine powder or other medical problems while undergoing the leucine loading test. This test is a simple, inexpensive test to distinguish between AUH defect and all other 3-MGA-uria-IEM. One should keep in mind that also 3-HIVA excretion and the 2:1 ratio between *cis* and *trans* isoforms of 3-MGA is unique to AUH defect patients and can be used for the same purpose.

In conclusion, this article proves the hypothesis that 3-MGA-uria only stems from leucine metabolism in AUH defect, but not in other types of 3-MGA-uria-IEM.

Disclosures

None declared.

Compliance with Ethics Guidelines

Conflict of Interest

Saskia B. Wortmann, Leo A.J. Kluijtmans, Silvia Sequeira, Ron A. Wevers, and Eva Morava 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). Oral informed consent was obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

Saskia B. Wortmann and Eva Morava planned the study. Saskia B. Wortmann, Silvia Sequeira, and Eva Morava conducted the study in patients. Leo A.J. Kluijtmans and Ron A. Wevers conducted the biochemical measurements. All authors interpreted and discussed the results. Saskia B. Wortmann wrote the manuscript which was read and corrected by all authors.

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JIMD Reports
DOI 10.1007/8904_2014_310

RESEARCH REPORT

Fabry Disease: Multidisciplinary Evaluation After 10 Years of Treatment with Agalsidase Beta

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Received: 31 January 2014 / Revised: 12 March 2014 / Accepted: 13 March 2014 / Published online: 22 May 2014

Abstract Fabry disease is an X linked disorder of metabolism due to deficient α-galactosidase A activity. Enzyme replacement therapy (ERT) with agalsidase Beta was approved by EMA in 2001 and FDA in 2003.

Patients and methods: Six patients were enrolled. Baseline data was measured for renal, cardiac, and cerebrovascular functioning. We compared baseline quality of life scales with the current results. These parameters were assessed during the 10 years of follow-up period.

Results: Before ERT four patients showed normal eGFR, one stage 2 of CKD, and one hyperfiltration stage. All presented microalbuminuria and just two cases showed proteinuria. After 10 years of ERT, no patient showed decrease in renal functioning. One patient decreased from proteinuria to microalbuminuria range. Before treatment one case showed left ventricular (LV) hypertrophy and LV Mass Index was abnormal in two female patients. After 10 years echocardiographic values did not present progression to LVH and one female showed regression to normal values of LV posterior wall and interventricular septum. Brain MRI showed ischemic lesions in one female and vertebrobasilar dolichoectasia in one male. From baseline and during the follow-up period MRI did not progress to new ischemic lesions and there were no clinical signs of cerebrovascular damage. After 10 years quality of life showed improvement in all domains measured.

Conclusion: Early treatment of agalsidase Beta is related to a better outcome regarding stability and regression of signs and symptoms in Fabry disease. Our results in patients with mild organ involvement showed good outcomes and support an early and continuous ERT.

Communicated by: Verena Peters

Competing interests: None declared

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Introduction

Fabry disease (FD) is the result of deficient lysosomal α-galactosidase A (alpha-Gal A) activity, which leads to excessive deposition of glycosphingolipids, predominantly Globotriaosylceramide (Gl_3). The incidence of this X chromosome-linked disorder is estimated at 1/40,000 live births in its classic phenotype and according to recent reports the incidence of the late-onset variant may be as high as 1/3,100 (Germain 2010; Spada et al. 2006). The deposition of Gl_3 occurs in endothelial, perithelial, smooth muscle cells of the blood vessels, neurons, podocytes, cardiomyocytes, etc. The first symptoms appear in hemizygous males during childhood with distal neuropathic pain in the four limbs and hypohidrosis, associated with skin lesions known as angiokeratomas. During adolescence further symptoms include Gl_3 deposition in the cornea (cornea verticillata), autonomic dysfunctions, fatigue, depression, and reduced hearing ability, followed by signs

of kidney and heart failure and cerebrovascular accidents during the third decade (Germain 2010).

Subsequent to the approval of enzyme replacement therapy (ERT) in Europe in 2001 and in the USA in 2003, FD has taken a significant turn, arousing the interest of a large number of specialists (Eng et al. 2001; Schiffmann et al. 2001).

ERT with agalsidase Beta has returned positive results, reducing neuropathic pain (Hilz et al. 2004), stabilizing the renal and auditory functions (Germain et al. 2007; Sergi et al. 2010), preventing the development of cardiac complications (Weidemann et al. 2009), in addition to improving quality of life and reversing angiokeratomas in certain cases (Watt et al. 2010; Furujo et al. 2013). These findings are more evident in relation to early initiation of ERT.

Objective

To describe the results of the multidisciplinary evaluation in six patients suffering from FD after 10 years of treatment with agalsidase Beta.

Materials and Methods

Six adult patients (four males) from Buenos Aires, Argentina were included in this study. All the patients were diagnosed with FD via an enzyme activity test and a molecular test with a mutation search. All the patients had been treated with agalsidase Beta (Fabrazyme®) at 1 mg/kg every 14 days for the last 10 years without interruption. Due to a temporary shortage of agalsidase Beta, three patients were given a dose of 0.3 mg/kg every 14 days for a period of 10 consecutive months in the year 2010. The evaluation of the renal function was conducted by means of the estimated glomerular filtration rate (eGFR) using the MDRD formula, measurement of microalbuminuria (>30 mg/24 h) and proteinuria (>300 mg/24 h); kidney disease was broken down into five stages in accordance with the eGFR: (1) between 120 and 90 mL/min, (2) between 90 and 60 mL/min, (3) between 60 and 30 mL/min, (4) between 30 and 15 mL/min, and (5) <15 mL/min. The evaluation of the cardiac function was conducted by measuring the thickness of the interventricular septum (IVS) and the left ventricle posterior wall (LVPW) and the left ventricular mass index (LVMI) measured by echocardiography. The normal values for IVS and LVPW were 6–11 mm in both genders; the normal LVMI was 43–95 g/m^2 in females and 49–115 g/m^2 in males (Lang et al. 2005). The presence of cerebrovascular compromise was assessed by means of brain magnetic resonance imaging (MRI) using a 1.5T Signa Echo Speed (GE) MRI system with data acquisition techniques standardized for cerebrovascular diseases (conventional FLAIR, T1, T2, diffusion and proton density) and a magnetic resonance angiogram of the intracranial vessels. Quality of life was evaluated using the SF36 scale, which, similar to MRI, describes the findings prior to commencement of ERT and after 10 years of treatment. The SF36 scale is a tool which has been validated in several languages and is used to assess the quality of life of the population as a whole and patients with chronic diseases. It can be conducted via the telephone or by instructing the patient to conduct a self-assessment; the assessment of eight independent items related to the day-to-day life of the individual: physical functioning, limitations due to physical problems (or physical role), bodily pain, general health perceptions, vitality, social functioning, limitations due to emotional problems (or emotional role) and mental health. All the items are given a score of between 0 and 100, whereby the higher the score, the better the quality of life. The reference value was based on the average registered for the general population of the USA (Ware et al. 1993), ranging from 64 to 87 points in males and 58 to 82 points in females for each item. An increase of more than seven points between one assessment and another is regarded as a significant improvement in all the items, with the exception of vitality, where a five-point difference is regarded as a significant improvement. Informed consent was obtained from all patients for being included in the study.

Statistical Analysis

Given the characteristics of this study, no official statistical analysis of the sample in question was conducted. The data is expressed as an average, mean, or percentage, as applicable.

Results

The demographic data is set forth in Table 1. Six patients from two different unrelated families were assessed initially. All the patients registered deficient enzyme activity measured in leukocytes. The mutation study revealed the same mutation in all the patients. A subsequent study of the family tree revealed a common origin in the fourth generation prior to the cases under analysis. The eGFR prior to commencement of ERT was normal in four patients and one patient registered stage 2 baseline CKD. All the patients registered microalbuminuria before beginning of ERT, whereby proteinuria was two cases only. After 10 years of treatment with agalsidase Beta, the state of CKD remained in all the patients (Fig. 1). Microalbuminuria values remained stable throughout the study period,

Table 1 Demographic data

Patient	Gender	Age when symptoms commenced	Age on diagnosis	Age on commencement of ERT	Alpha-Gal A activity in leukocytes	Mutation
1	M	12	17	17	2.2	L415P
2	M	5	18	19	1.1	L415P
3	M	6	18	20	1.9	L415P
4	M	10	29	30	0.7	L415P
5	F	10	49	50	2.6	L415P
6	F	35	45	46	1.4	L415P

Alpha-Gal A activity in leukocytes. Normal values: 30.5–57.7 nmol/h/mg

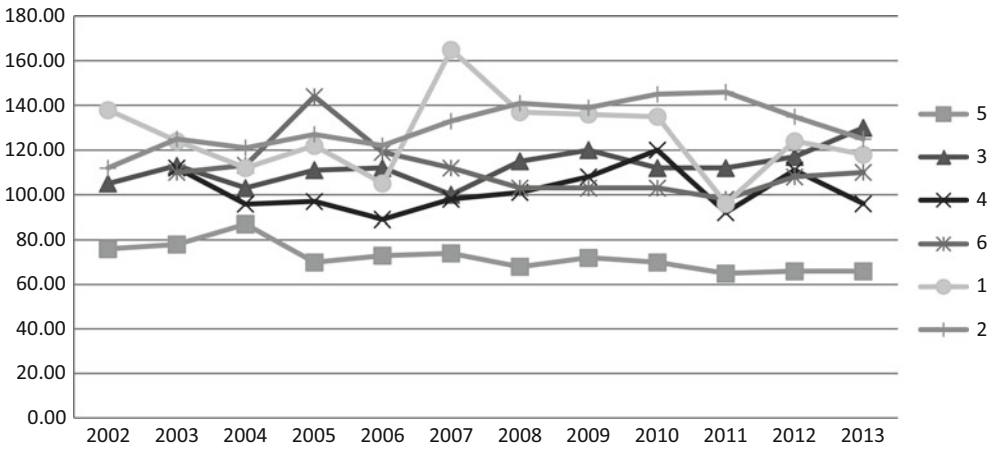

Fig. 1 Evaluation of eGFR after 10 years of treatment

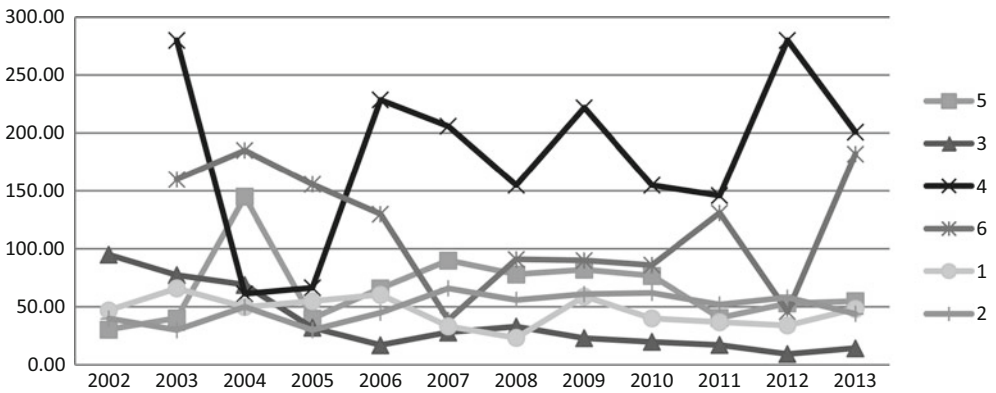

Fig. 2 Evaluation of microalbuminuria after 10 years of treatment

whereby one patient registered a reduction in microalbuminuria at values of less than 30 mg/24 h (Fig. 2). The proteinuria results revealed no increases whatsoever, whereby one of the two patients with baseline values of 300 mg/24 h concluded the study with a value of 224 mg/24 h (Fig. 3).

Cardiac assessment prior to start of ERT demonstrated only one patient with an increased thickness of the IVS and LVPW. There were no cases of progression to left ventricular hypertrophy (LVH) after the study, whereby the only patient with abnormal values returned to the normal range for these parameters (Figs. 4 and 5). The LVMI was normal in four male patients and pathological in the female patients on commencement of the study. No progression to abnormal values was registered in the male patients after 10 years of ERT. One of the heterozygous

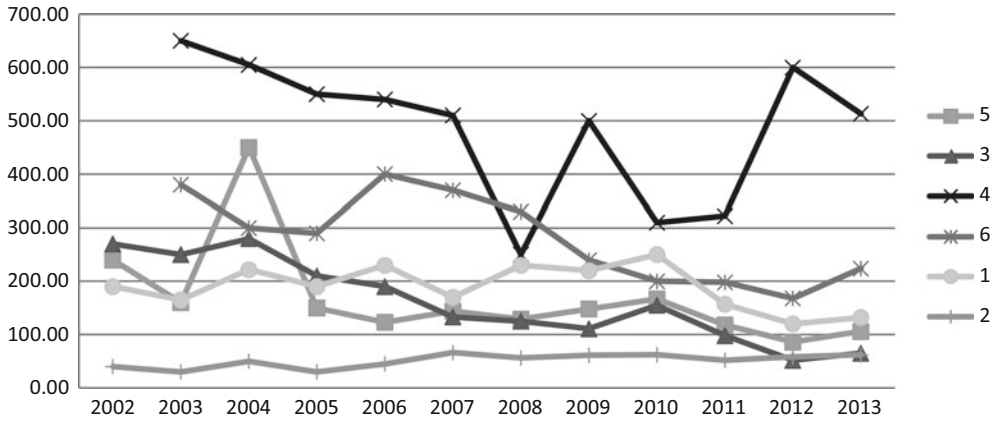

Fig. 3 Evaluation of proteinuria after 10 years of treatment

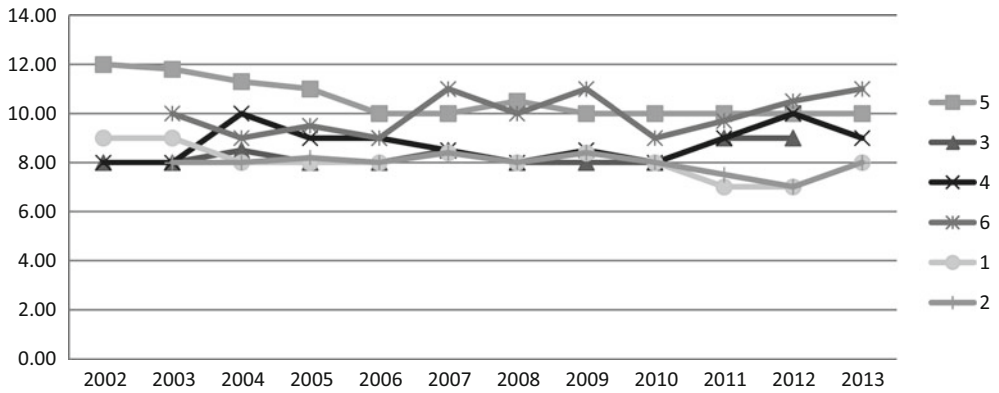

Fig. 4 Evaluation of the interventricular septum after 10 years of treatment

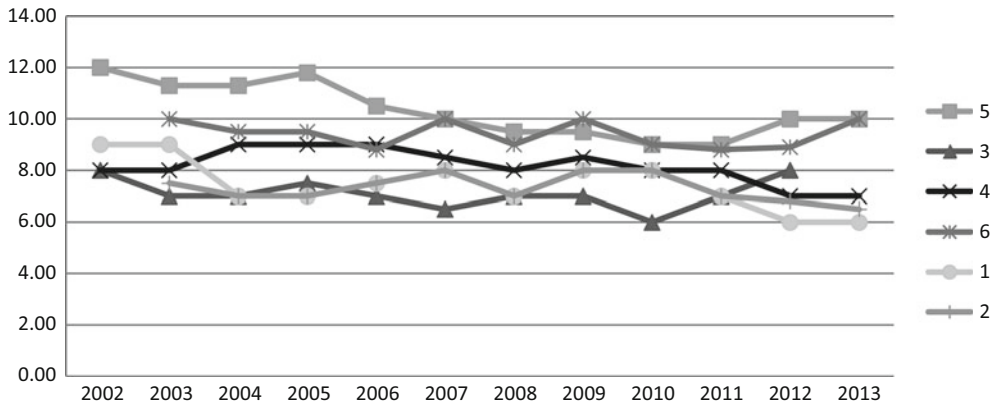

Fig. 5 Evaluation of the left ventricle posterior wall after 10 years of treatment

patients showed no change and the other a clear reduction in LVMI, but without registering normal values (Fig. 6).

The brain MRI prior to beginning of ERT revealed punctate periventricular ischemic lesions in one patient and vertebrobasilar dolichoectasia in another. Monitoring after 10 years of ERT revealed no progression to the lesions described in both cases nor the appearance of new images in the normal baseline assessment cases.

The SF36 scale registered significant improvements in the eight domains analyzed in all the patients (Fig. 7). Compared

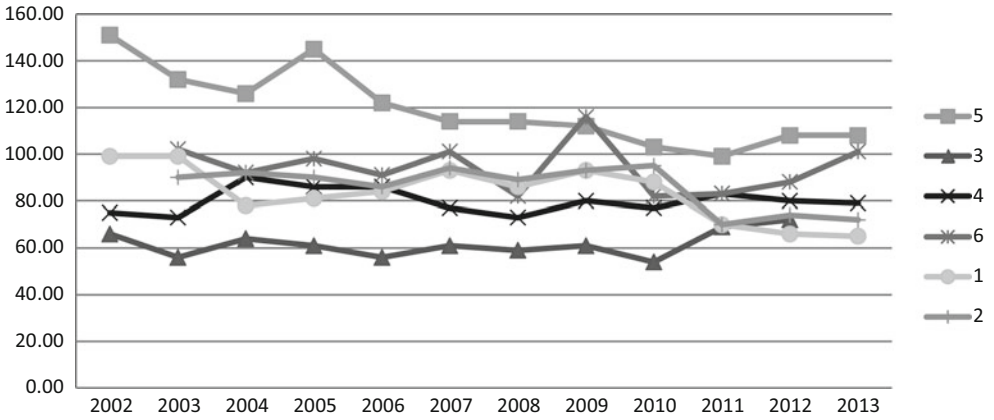

Fig. 6 Evaluation of the left ventricular mass index after 10 years of treatment

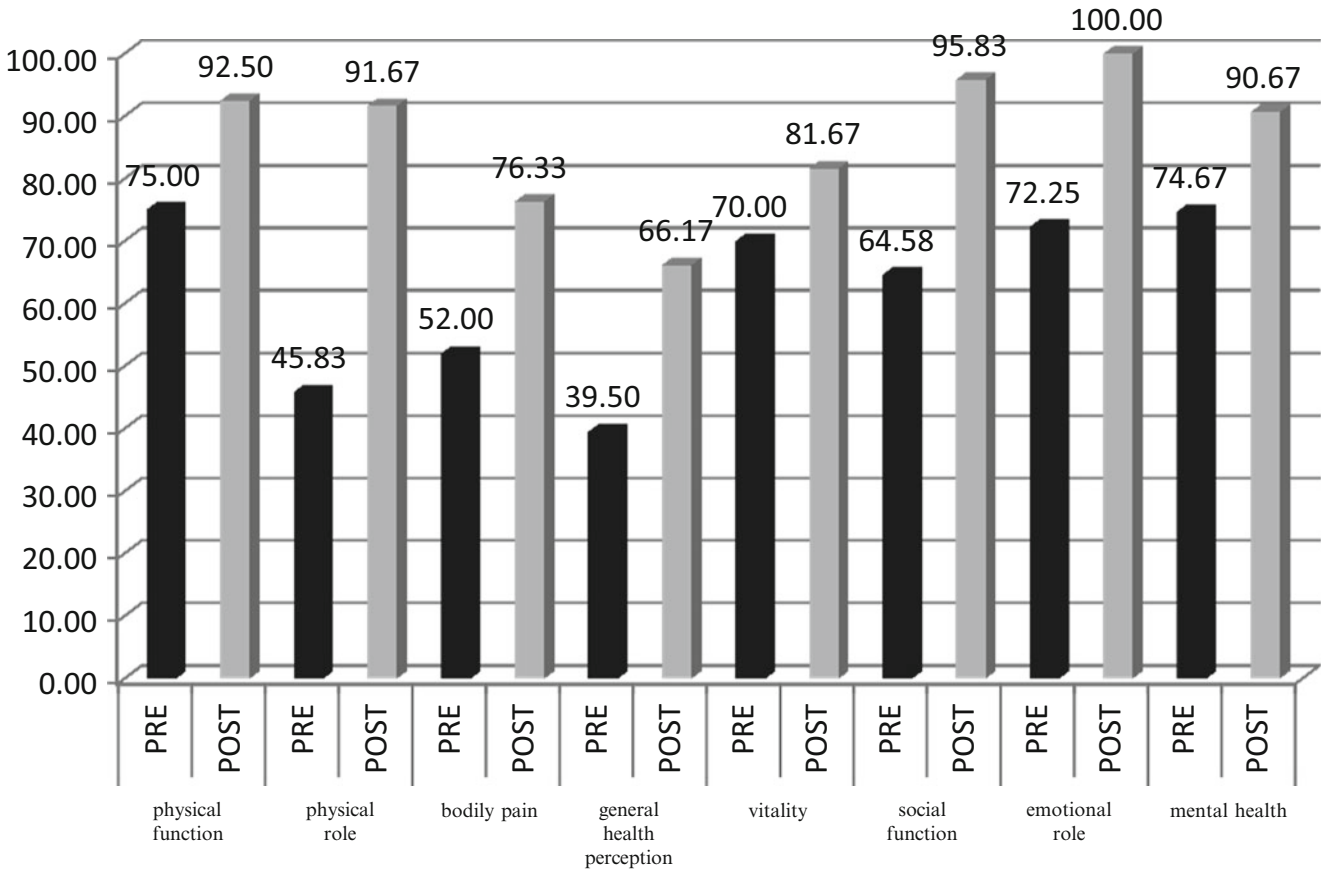

Fig. 7 Evolution of the SF36 scale before (*gray bars*) and after 10 years of treatment (*black bars*)

with the reference values registered for the general population, before ERT four items were below the lower limit in females and three in males, those being: limitations due to physical problems, bodily pain, and general health perceptions in both genders and social functioning in females only. All the scores in relation to 10 years of treatment fell within the normal reference values.

Discussion

ERT with agalsidase Beta has been shown to change the natural history of FD. Histopathological studies conducted prior to commencement and months or years before the use of agalsidase Beta showed a significant reduction of Gl_3 in the renal, cardiac, and dermal capillary endothelium (Eng

et al. 2001). It has recently been reported that a 1 mg/kg dose of agalsidase Beta every 14 days has resulted in the clearance of substrate in the podocytes (Tøndel et al. 2013). A dose of 0.2 mg/kg failed to show any statistically significant results in relation to a reduction of Gl_3 in renal tissue, and recent evidence has revealed no change with regard to podocytes in renal biopsies of young patients on comparing histological results with those in relation to 5 years of treatment with the aforementioned dose (Tøndel et al. 2013).

This patient's cohort is part of the same family as the lineage showed us. The L415P mutation is related to severe classical phenotype and enhancement with chemical chaperones showed no significant response. Recently, one study showed that this mutation overexpressed the mutant alpha-Gal A in the cells, notably above the endogenous level at the expected size of 46 kDa. Thus, the protein is evidently processed, but it is not catalytically active (Lukas et al. 2013). Clinical signs and symptoms were shared in all patients as dysmorphic features, gastrointestinal complaints, and neuropathic pain before 10 years old and presence of proteinuria before third decade. Female patients were under ACEi treatment before ERT and maintained same doses during the study period.

The results of our case series showed a stable renal function measured using eGFR and no increase in proteinuria. We decided to include microalbuminuria as a separate parameter, because it is assumed that the prognostic effect in patients with FD is relatively large and that increased microalbuminuria is also associated with a poor renal prognosis.

One of the factors which may have influenced this response is early initiation of ERT in the hemizygous patients (all of an age $\leq$30). Although the heterozygous patients commenced treatment in the fourth decade of life, the phenotypic expression of the same was less severe than normally registered (Wang et al. 2007). The current evidence of unsatisfactory results in relation to male patients who started ERT at the average age of 40 years (Weidemann et al. 2013a; Rombach et al. 2013) has been one of the main reasons for justifying therapy at an early age. Furthermore, evidence of agents causing cellular hypertrophy in cardiomyocytes and vascular smooth muscle cells, such as markers associated with fibrosis in different tissues, has clearly demonstrated the need for early treatment (Weidemann et al. 2013b). Previously, the Phase IV study showed in patients with reduced GFR treated with agalsidase Beta there was a 61% reduction in renal, cardiac, and cerebrovascular events when corrected for baseline proteinuria when compared to placebo (Banikazemi et al. 2007).

There was no sign of progression to LVH in any of the patients in relation to cardiac assessment after the study period. Only one patient showed echocardiographical signs of LVH, whereby the thickness of the IVS and the LVPW after 10 years of ERT was normal. As is repeated in the bibliography, an increase in the thickness of the IVS, LVPW, and LVMI is related to age, justified by the fact that the only patient with signs of LVH was the oldest in our series (Germain et al. 2013). Agalsidase Beta has proved to be effective in reducing left ventricular mass in patients who commenced ERT between the ages of 18 and 30 according to a recent survey conducted by the Fabry Registry (Germain et al. 2013).

The analysis of the brain MRI revealed microangiopathic lesions in one female patient prior to starting ERT and vertebrobasilar dolichoectasia in one of the male patients. These lesions underwent no change during the study period. The remaining cases showed no change in the images, remaining free of lesions. To date ischemic lesions have been registered in many patients from the third decade of life on, with no major differences between the two genders (Fellgiebel et al. 2005). Moreover, certain series have shown a greater frequency of cerebrovascular compromise in heterozygous patients than in hemizygous patients. Our series of male cases commenced ERT $\leq$ 30 years of age, and this may be the reason for which the males did not develop white matter lesions, although there is still no clear evidence that ERT is capable of preventing the appearance of the same. Vertebrobasilar dolichoectasia seems to be the earliest finding in relation to cerebrovascular damage in patients with FD (personal observation in press). Certain authors have demonstrated that measuring the diameter of the basilar artery may be a useful monitoring tool, as this has been the only parameter which separated general population with a stroke (group control) from patients with FD and stroke (Fellgiebel et al. 2011).

Interference with daily activities due to neuropathic pain, fatigue, and other symptoms registered in patients with FD may be assessed using life quality scales such as SF36. Reports exist of significantly reduced quality of life in men and women with FD (Gold et al. 2002; Miners et al. 2002; Street et al. 2006), this being one of the reasons for the common occurrence of depression (Cole et al. 2007). Of the eight domains assessed using the SF36 scale, four were under the normal limit in the females and three in the males. All the domains showed a significant improvement after 10 years of ERT, including the domains where the score prior to treatment fell within the normal range. Changes in the SF36 scale after the use of agalsidase Beta for 3 years have been registered in a group of 71 men and 59 women belonging to the Fabry Registry (FR) population. In both our series and that conducted by the FR, the domain most affected in both genders was the physical role. Beginning of ERT at an early age may explain why five domains in the male patients registered scores within the normal range.

The FR report described a similar situation, where on dividing the male patients into two groups (>0 < 30 years of age), the older patients registered lower baseline values in all the domains.

Conclusions

The use of agalsidase Beta in our series has shown a significant improvement in quality of life, in addition to the stabilization of renal, cardiac, and cerebrovascular parameters. The determining factor in these results may be the early treatment with ERT, particularly in the hemizygous patients.

Take-Home Message

Early treatment with enzyme replacement therapy in Fabry disease is related to a better outcome.

Compliance with Ethics Guidelines

Conflict of Interest

Politei Juan has received speaker honorarium from Genzyme, Shire HGT and Amicus Therapeutics.

Amartino Hernan, Schenone Andrea Beatriz, Cabrera Gustavo, Michref Antonio, Tanus Eduardo, Dominguez Raul, Larralde Margarita, Blanco Mariana, Gaggioli Daniela, and Szlago Marina 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 of Juan Fernandez Hospital, Buenos Aires, Argentina and with the Helsinki Declaration of 1975, as revised in 2000.

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

The informed consents are available if the journal needed them.

Details of the Contributions of Individual Authors

All authors have contributed in the planning, conduct, and reporting of the work described in the article.

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JIMD Reports
DOI 10.1007/8904_2014_311

RESEARCH REPORT

Chondroitin 6-Sulfate as a Novel Biomarker for Mucopolysaccharidosis IVA and VII

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Received: 05 February 2014 / Revised: 06 March 2014 / Accepted: 12 March 2014 / Published online: 22 May 2014

Abstract Chondroitin 6-sulfate (C6S), a glycosaminoglycan (GAG), is distributed mainly in the growth plates, aorta, and cornea; however, the physiological function of C6S is not fully understood. One of the limitations is that no rapid, accurate quantitative method to measure C6S has been established. Mucopolysaccharidosis IVA and VII (MPS IVA and VII) are caused by the deficiency of *N*-acetylgalactosamine-6-sulfate sulfatase and β-D-glucuronidase, respectively, resulting in accumulation of C6S and other GAG(s). While levels of keratan sulfate (KS), heparan sulfate, and dermatan sulfate in samples from MPS patients are well described, this is the first report of quantitative analysis of C6S levels in samples from MPS IVA and VII patients.

We developed a method to digest polymeric C6S and measure resultant disaccharides using liquid chromatography–tandem mass spectrometry (LC-MS/MS). C6S levels were measured in the blood from control subjects and patients with MPS IVA and VII aged from 0 to 58 years of age. We also assayed KS levels in the same samples for comparison with C6S.

Levels of C6S in the blood decreased with age and were significantly elevated in patients with MPS IVA and VII, compared with age-matched controls. Levels of KS in patients with MPS IVA were also higher than those in age-matched controls, although differences were less pronounced than with C6S. Combining KS and C6S data, discriminated patients with MPS IVA from age-matched control subjects were better than either C6S or KS levels alone.

In conclusion, this first report showing that blood levels of C6S are quantitatively evaluated in patients with MPS IVA and VII indicates that C6S could be a useful biomarker for these metabolic disorders.

Communicated by: Frits Wijburg, MD, PhD

Competing interests: None declared

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors.

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Introduction

Mucopolysaccharidoses (MPS) are a family of inheritable metabolic disorders caused by deficiency of lysosomal enzymes required for degradation of glycosaminoglycans (GAGs). Each known MPS type involves deficiency of a specific lysosomal enzyme required for the stepwise degradation of specific GAG(s) (chondroitin sulfate, CS; dermatan sulfate, DS; heparan sulfate, HS; and keratan sulfate, KS).

Mucopolysaccharidosis IVA (MPS IVA, Morquio A syndrome) is caused by the deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS), resulting in the

accumulation of chondroitin 6-sulfate (C6S) and KS mainly in the cartilage and its extracellular matrix (ECM). MPS IVB is caused by deficiency of β-galactosidase, leading to the accumulation of KS but not C6S. Clinically, a classic (severe) form of MPS IVA is characterized by systemic skeletal dysplasia such as short trunk dwarfism, kyphoscoliosis, coxa valga, odontoid hypoplasia, abnormal gait, joint mobility problems, restriction of chest wall movement, and a life span of 20–30 years. Patients with an attenuated form can have a nearly normal life span, with mild involvement of the skeleton (Dũng et al. 2013; Yasuda et al. 2013; Tomatsu et al. 2011, 2012a, 2013a, b; Northover et al. 1996; Montaño et al. 2007, 2008; Suzuki et al. 2001; Hendriksz et al. 2013; Möllmann et al. 2013; Harmatz et al. 2013). In general, patients with MPS IVB have a milder phenotype of skeletal dysplasia. Patients with MPS VII have accumulation of HS, DS, chondroitin 4-sulfate (C4S), and C6S in various tissues and have coarse facial features, mental retardation, short stature, hepatomegaly, bony deformities, GAG excretion, and striking metachromatic granules in peripheral leukocytes (Sly et al. 1973; Tomatsu et al. 1991).

Elevation of total urinary GAG or KS in the blood and/or urine of MPS IVA patients has been detected by colorimetric analysis using dimethylmethylene blue (Melrose and Ghosh 1988), ELISA (Tomatsu et al. 2004, 2005a), and HPLC methods (Linhardt et al. 1989; Whitham et al. 1999). We developed a rapid, reproducible, sensitive, and specific assay system in which the disaccharides produced from DS, HS, and KS in the blood, urine, and dried blood spot (DBS) samples are analyzed simultaneously by using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Oguma et al. 2007a, b; Tomatsu et al. 2010a, b, 2013a; Hintze et al. 2011). KS levels in the plasma and urine from patients with MPS IVA were associated with age and clinical severity and decreased with enzyme replacement therapy in MPS IVA mouse models (Tomatsu et al. 2008). These findings indicate that KS values in the blood and/or urine could be a suitable biomarker for early diagnosis and screening, assessment of disease severity, and monitoring therapeutic efficacy in MPS IVA (Tomatsu et al. 2008, 2010c, 2013b). It is noteworthy that there is some overlap of KS values from patients with MPS IVA and age-matched controls and that this is more pronounced in children over 10 years old, suggesting that better biomarkers are required to monitor all patients with MPS IVA (Tomatsu et al. 2010c).

CS levels have been determined by HPLC, LC-MS/MS, and capillary electrophoresis, based on the differences in enzymatic digestion using chondroitinase ABC and/or chondroitinase ACII (Imanari et al. 1996; Koshiishi et al. 1998; Oguma et al. 2001; Karamanos and Hjerpe 2001; Lamari et al. 2002). Although capillary electrophoresis studies provided subjective data that CS is elevated in the urine of MPS IVA (Hopwood and Harrison 1982; Hata and Nagai 1972), C4S and C6S were not separated. Thus, no quantitative analyses have yet been reported on specific levels of C6S in patients with MPS IVA and VII. Blood specimens (plasma, serum, or DBS) are appropriate and stable for enzymatic diagnosis, newborn screening, assessment of clinical severity, and monitoring therapeutic efficacy (Tomatsu et al. 2008, 2010b, c, 2013a, b; Wang et al. 2007; Blanchard et al. 2008; de Ru et al. 2013; de Ruijter et al. 2012). We are developing a pilot study in newborn screening for MPS by measuring GAGs (DS, HS, and KS) in blood specimens (Tomatsu et al. 2013a). Therefore, development of methods to measure C6S levels in blood specimens should provide an additional approach to have a significant impact on clinical practice in caring for MPS patients.

In this study, we evaluated C6S levels in the blood of control subjects and patients with MPS IV and VII by LC-MS/MS after digestion with chondroitinase ABC and keratanase II and show the potential of C6S as a biomarker for MPS IVA and VII. In addition, we investigated the correlation between C6S and KS levels in patients with MPS IVA.

Materials and Method

Subjects

Blood (plasma or serum) samples were obtained from 35 patients with MPS IVA (phenotype: severe, 32; undefined, 3), four patients with MPS IVB (attenuated, 4), and three patients with MPS VII. Informed consent was obtained from the patients and/or their guardians through the physicians who were in charge of the patients with MPS at each local institute approved by the Institutional Review Board (IRB). For all samples, the ages of the patients were identified. Blood samples were also obtained from 138 healthy controls. In previous experiments by LC-MS/MS, we confirmed that there is no difference of GAG value in specificity and sensitivity between plasma and serum (Oguma et al. 2007a, b; Tomatsu et al. 2010a, b, c). Diagnosis of MPS IVA, MPS IVB, and MPS VII was made on the basis of enzyme activity (GALNS, β-galactosidase, and β-D-glucuronidase, respectively) reduced to $\leq$5% the normal level in plasma, leukocytes or fibroblasts. We classified clinical severity for patients with MPS IVA based on growth charts, as described previously (Montaño et al. 2008; Tomatsu et al. 2012b). According to the isopleth upon which the patient falls, patients above the 90th centile on the growth chart for each gender are defined as attenuated (Tomatsu et al. 2012b). We classified the severity of the disease in MPS IVB in reference with the

growth charts of MPS IVA since the growth chart of MPS IVB was not available.

Chemicals and Materials

To digest "polymer" C6S and KS to disaccharides, chondroitinase ABC and keratanase II were provided from Seikagaku Co. (Tokyo, Japan). Chondroitinase A produced disaccharides of C4S, while chondroitinase B and chondroitinase C produced disaccharides of DS and C6S, respectively. All of these disaccharides have identical molecular masses. Chondrosine (internal standard, IS), ΔDi-6S (C6S) [2-acetamido-2-deoxy-4-*O*-(4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose], and ΔDi-4S (DS) [2-acetamido-2-deoxy-4-*O*-(4-deoxy-L-threo-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-glucose] were provided from Seikagaku Co. Stock solutions of ΔDi-6S (100 μg/mL), ΔDi-4S (100 μg/mL), "polymer" KS (20 μg/mL), and IS (5 mg/mL) were prepared separately in ddH_2O and stored at −80°C. Standard solutions of ΔDi-6S (10, 20, 100, 200, and 1,000 ng/mL) and KS (0.1, 0.2, 1.0, 2.0, and 10.0 μg/mL) and IS solution (500 ng/mL) were prepared freshly.

Sample Preparation

Blood specimens and standards were prepared as follows. Ten microliters of each serum or plasma sample and 90 μL of 50 mM Tris–hydrochloric acid buffer (pH 7.0) were placed in wells of AcroPrep™ Advance 96-Well Filter Plates that have Ultrafiltration Omega 10 K membrane filters (PALL Corporation, NY, USA). The filter plates were placed on a receiver and centrifuged at 2,000 g for 15 min to remove free disaccharides. The membrane plates were transferred to a fresh receiver plate. Standards were added to unused wells of the filter plate. Twenty microliters of IS solution (500 ng/mL), 60 μL of 50 mM Tris–hydrochloric acid buffer (pH 7), and 10 μL of chondroitinase ABC and keratanase II mixture solution (2 mU each per sample) were added to each filter well. The plate was incubated in a water bath at 37°C for 15 h and centrifuged at 2,000 g for 15 min. The receiver plate containing disaccharides was stored at −20°C until injection to LC-MS/MS.

Apparatus

The chromatographic system consisted of a 1,260 infinity (Agilent Technologies, Palo Alto, CA, USA) and a Hypercarb column (2.0 mm i.d. 50 mm, 5 μm, Thermo Electron, USA). The column temperature was kept at 50°C. The mobile phase was a gradient elution of 5 mM ammonium acetate in acetonitrile −5 mM ammonium acetate buffer (pH 11.0). The gradient condition was programmed as follows. The initial composition of 0% acetonitrile was kept for 0.1 min, linearly modified to 30% over 1.8 min, maintained at 30% for 0.3 min, modified to 0% over 0.01 min, and finally maintained at 0% for 2.5 min. The flow rate was 0.7 mL/min. The 6460 Triple Quad mass spectrometer (Agilent Technologies) was operated in the negative ion detection mode. In the multiple reaction monitoring (MRM) mode, the mass spectrometer detected ions by monitoring the decay of the *m/z* 462 precursor ion to the *m/z* 97 production for Galβ1 → 4GlcNAc(6S) disaccharides derived from KS, the decay of the *m/z* 458.4 precursor ion to the *m/z* 282.1 product ion for ΔDi-6S (C6S), the decay of the *m/z* 458.4 precursor ion to the *m/z* 300.2 product ion for ΔDi-4S (DS), and the decay of the *m/z* 354.29 precursor ion to the *m/z* 193.1 product ion for IS. Peak areas for all components were integrated automatically by using QQQ Quantitative Analysis Software (Agilent Technologies), and peak area ratios (area of analytes/area of IS) were plotted against level by weighted linear regression. Raw LC-MS/MS data were automatically preserved. The levels for each disaccharide were calculated using QQQ Quantitative Analysis Software.

Method Validation

Intraday precision evaluated as coefficient of variation (CV) was determined by replicate analyses ($n = 5$) of three different control serum. Inter-day precision was determined by replicate analyses ($n = 5$) of three different serum controls on three separate days.

The selectivity of the assay was investigated by processing and analyzing five independent samples by the procedure described above without enzymatic digestion. Calibration curves were constructed by plotting the peak area ratio of the analytes to IS against the level of the analytes. Each calibration curve consisted of seven calibration points ($n = 1$).

Statistical Analysis

Analysis was performed using SPSS for Windows (version 17.0, SPSS Inc., Chicago, IL, USA). For age-matched comparisons, patient and control samples were grouped in age ranges <3, 3–4, 5–9, 10–14, 15–35, and 36+. Data are shown as the mean ± SD.

Results

LC-MS/MS Conditions

The peaks of ΔDi-6S (C6S) and ΔDi-4S (DS and C4S) with the same molecular weight were separated by the LC

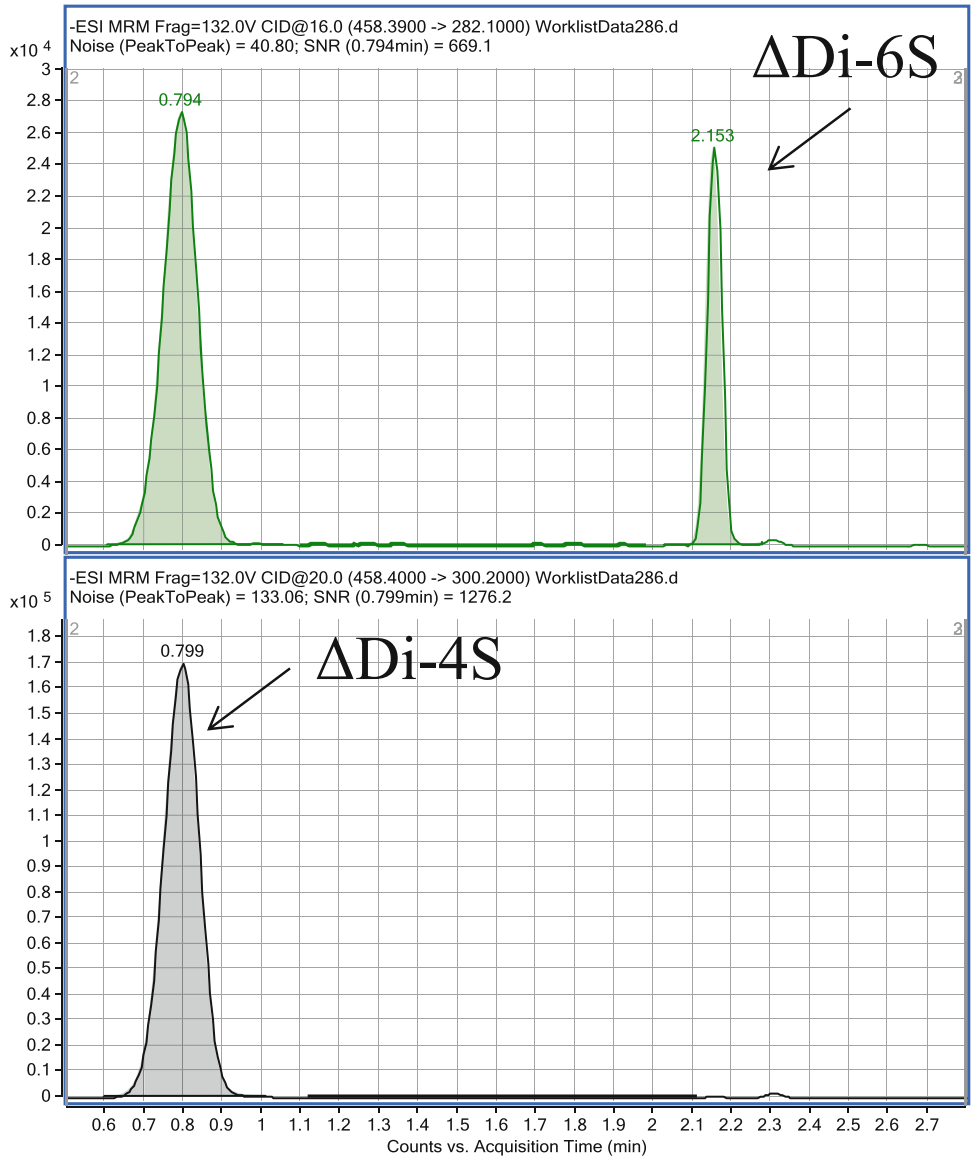

Fig. 1 Representative ΔDi-4S and ΔDi-6S MRM chromatograms of extracts obtained from a patient with MPS IVA

component (Fig. 1). The retention time of ΔDi-6S and ΔDi-4S was 2.15 and 0.83 min, respectively.

Calibration Curves

Calibration curves for ΔDi-6S and Galβ1 → 4GlcNAc(6S) (KS) obtained on five separate days were linear over the level ranges of 10–1,000 ng/mL and 0.1–10 μg/mL, respectively. The correlation coefficients of determination (r) were not less than 0.99.

Precision and Accuracy

Results of intra- and inter-assay precision for ΔDi-6S and Galß1-4GlcNAc(6S) in control specimens are as follows. The intra-assay precision values/coefficient of variation (CV) determined from analysis of ΔDi-6S and Galß1-4GlcNAc(6S) for control serum are less than 11.9 and 6.8%, respectively. The inter-assay precision values/CVs for these disaccharides in control serum are less than 12.2 and 6.5%, respectively. These results demonstrate the reproducibility and accuracy of the method.

Chondroitin 6-Sulfate (C6S) Levels

The C6S values for the blood samples from 35 MPS IVA patients (average age 16.1 years, range 3.4–56 years), 4 MPS IVB patients (average age 15.2 years, range 12.7–17.7 years), 3 MPS VII patients (average age 15 years, range 0–30 years), and 138 control subjects (average age

Table 1 Levels of C6S (ng/ml) in patients with MPS IVA, IVB, and VII

Age (year)	Control	MPS IVA	MPS IVB	MPS VII
<3	156.7 ± 116.9 ($n = 81$)			940.7 ($n = 1$)
3–4	58.7 ± 17.4 ($n = 20$)	166.4 ± 83.8 ($n = 2$)		
5–9	70.5 ± 17.1 ($n = 18$)	175.7 ± 87.2*** ($n = 12$)		
10–14	66.1 ± 25.9 ($n = 13$)	141.7 ± 54.9*** ($n = 8$)	94.9 ± 6.2 ($n = 2$)	
15–35	33.6 ± 19.2 ($n = 6$)	109.2 ± 45.0** ($n = 11$)	33.2 ± 6.8 ($n = 2$)	468.2 ± 533.8 ($n = 2$)
>36		103.7 ± 68.0 ($n = 3$)		

Data represent the mean ± SD values
** and ***; significantly different from the control at $p < 0.005$, and 0.001, respectively

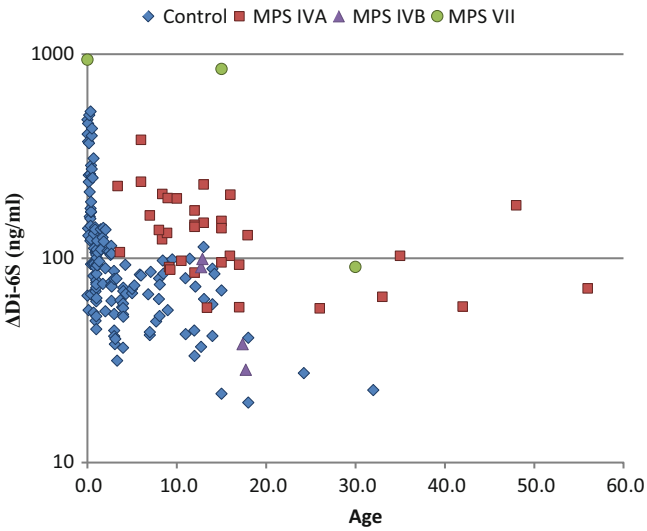

Fig. 2 Level of blood ΔDi-6S of patients with MPS and control subjects. Results of all specimens from patients and control subjects were plotted on a semilogarithmic scale with respect to age (years)

4.1 years, range 0–32 years) are described in Table 1 and Fig. 2. Blood C6S levels in control subjects were found to vary with age. The level was the highest in newborns and rapidly decreased until 5 years of age. After 5 years of age, there was a gradual decline with age.

The C6S levels in samples from patients with MPS IVA in age groups 5–9, 10–14, and 15–35 years were significantly higher than those in age-matched controls ($p < 0.001$, $p < 0.001$, and $p < 0.005$, respectively). Only two MPS IVA patients were younger than 5 years old, so although levels of C6S were high in these patients, significance could not be determined (Table 1, Fig. 2). Although in these group comparisons mean levels of C6S in MPS IVA samples are higher than controls in age ranges 5–35, there is no clear distinction between patients and controls over the age of ten.

The level of C6S was also compared between patients with MPS IVB and VII and the age-matched controls (Table 1, Fig. 2). Four patients with MPS IVB showed that the level of C6S were similar to those of age-matched controls (Fig. 2). Blood C6S levels in all three patients with MPS VII were more than 2 SD above the mean of age-matched controls (Tables 1 and 3).

Keratan Sulfate (KS) Level

KS levels in blood samples from the same patients with MPS IVA, IVB, and VII and the same control subjects in C6S assay are shown in Table 2 and Fig. 3. Blood KS levels in control subjects were also age dependent. Blood KS levels were highest in children up to 10 years of age and then gradually declined in older children and adults. As seen for C6S, KS in the blood of patients with MPS IVA in age groups between 5–9, 10–14, and 15–35 years were significantly higher than those in age-matched control subjects ($p < 0.001$, $p < 0.005$, and $p < 0.001$, respectively) (Table 2, Fig. 3). KS levels in the blood from two MPS IVA patients younger than 5 were indistinguishable from controls, and there was significant overlap of KS values between control subjects and patients with MPS IVA in the entire age range (Fig. 3).

The levels of KS were also compared between patients with MPS IVB and VII and the age-matched controls (Table 2, Fig. 3). All samples in patients with MPS IVB showed that blood KS level was within the level of the age-matched controls (Table 2). Blood KS values for two younger patients with MPS VII were more than 2 SD above the mean of the age-matched controls (Tables 2, 3).

Correlation Between KS and C6S Levels

Correlation between KS and C6S levels was not observed ($r^2 = 0.1136$).

For C6S, 75% (24 out of 32) of the patients with MPS IVA were more than 2 SD above the mean of age-matched controls. For KS this proportion was 71.9% (23 out of 32). For either C6S or KS, 29 out of 32 (90.6%) patients were

Table 2 Levels of KS (μg/ml) in patients with MPS IVA, IVB, and VII

KS (μg/mL)	Control	MPS IVA	MPS IVB	MPS VII
<3	3.2 ± 0.9 (n = 81)			3.4 (n = 1)
3–4	3.2 ± 0.9 (n = 20)	3.0 ± 0.9 (n = 2)		
5–9	2.9 ± 0.4 (n = 18)	4.3 ± 0.9*** (n = 12)		
10–14	2.5 ± 1.1 (n = 13)	4.2 ± 1.2** (n = 8)	3.1 ± 0.1 (n = 2)	
15–35	0.9 ± 0.3 (n = 6)	2.7 ± 0.9*** (n = 11)	1.3 ± 0.2 (n = 2)	2.4 ± 0.5 (n = 2)
>36		1.9 ± 1.0 (n = 3)		

Data represent the mean ± SD values
** and ***; significantly different from the control at $p < 0.005$, and 0.001, respectively

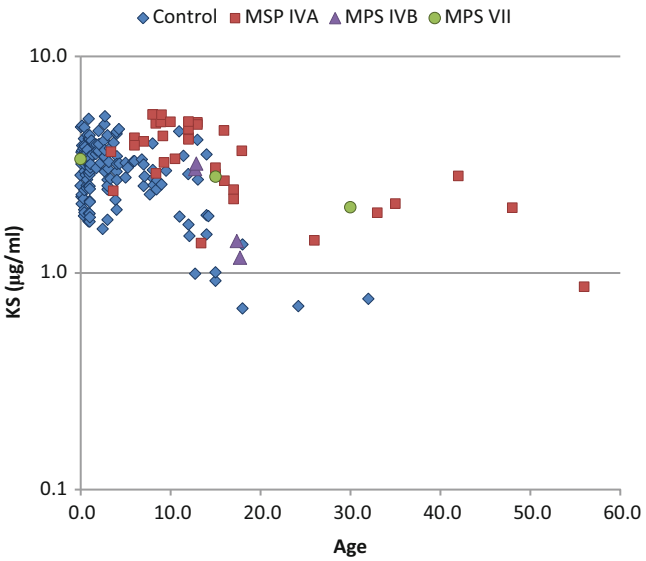

Fig. 3 Level of blood KS of patients with MPS and control subjects. Results of all specimens from patients and control subjects were plotted on a semilogarithmic scale with respect to age (years)

more than 2 SD above the mean for either C6S or KS level in the age-matched control subject (Table 3). The combined data of C6S and KS provided more difference between controls and patients with MPS IVA, compared with C6S or KS by alone.

Discussion

In this study, we have demonstrated (1) that the level of blood C6S in control subjects is age dependent at the peak in newborns and declines with age, (2) that C6S levels in patients with MPS IVA and VII are significantly higher than that in age-matched control subjects, (3) that combination of C6S and KS levels distinguishes patients with MPS IVA and control subjects more clearly compared with either C6S or KS level alone, and (4) that blood C6S and KS levels in patients with MPS IVB are overlapped with those in age-matched control subjects.

CS is involved in specific biological functions including cell adhesion, morphogenesis, neural network formation, and cell division (Sugahara et al. 2003). Historically, CS was divided into three major subtypes: chondroitin A (chondroitin 4-sulfate, C4S), chondroitin B (dermatan sulfate, DS), and chondroitin C (chondroitin 6-sulfate, C6S). Chondroitin B is no longer classified as CS. C6S is distributed in the growth plates (increasing from the proliferative zone to the hypertrophic zone, Ling et al. 1996), aorta (Yasuda et al. 2013), and cornea (Zhang et al. 2005). C6S has been identified in pathological states, including (1) contributing to arterial retention of cholesterol-rich, atherogenic lipoproteins (Mourão et al. 1981), a key event that initiates atherosclerosis (Williams and Tabas 1995), (2) accumulating in the connective tissue stroma of human colon carcinomas (Adany et al. 1990), and (3) excessive secretion of total CS in urinary excretions of MPS IVA and VII patients, although C4S and C6S are not clearly separate (semiquantitative) (Hopwood and Harrison 1982; Hata and Nagai 1972; Haskins et al. 1984). However, physiological and pathological roles and distributions of C6S are not well investigated because of the lack of accurate quantitative methods for measuring of C6S. We show here that the level of C6S in the blood is highest at birth and decreases with age. Total CS has also been shown to decrease with age in human and sheep cartilage (Dziewiatkowski et al. 1989; Elliott and Gardner 1979). Olczyk reported that progressive decrease in C6S in intervertebral discs is observed, most rapidly during the first two decades of life and then more slowly (Olczyk 1993). Age-dependent alterations of C6S level in cartilage most likely account for the age-dependent decrease of C6S levels in the blood in the current study. Levels of C6S in the blood of patients with MPS IVA are significantly higher than in age-matched control subjects, suggesting that C6S is

Table 3 Ratio of 2 SD above the mean of the age-matched controls in MPS IVA, IVB, and VII

	C6S	KS	Either C6S or KS
MPS IVA	75% (24 out of 32)	71.9% (23 out of 32)	90.6% (29 out of 32)
MPS IVB	0% (0 out of 4)	0% (0 out of 4)	0% (0 out of 4)
MPS VII	100% (3 out of 3)	66.7% (2 out of 3)	100% (3 out of 3)

a potential biomarker for MPS IVA, and may be better than KS. In all three patients with MPS VII, blood levels of C6S were markedly elevated compared to age-matched control subjects, showing that C6S level in the blood is likely to be a good biomarker for MPS VII. Two out of the three MPS VII patients also had elevated levels of KS in the blood; this secondary elevation could be related to underlying bone disease, especially of cartilage tissues (Tomatsu et al. 2010c). These MPS VII patients also had elevated levels of HS (data no shown), compatible with our previous report (Tomatsu et al. 2005b). Additional data from more patients with MPS VII will be required to validate these biomarkers.

Blood KS level in control subjects were high during the first 5 years of life and then steadily declined with age before stabilizing in late teenage years, in agreement with prior studies (Tomatsu et al. 2005a; Thonar et al. 1988). The level of blood KS in patients with MPS IVA was significantly higher than age-matched controls in older children and adults. KS in the blood is primarily a result of turnover of cartilage during development (Tomatsu et al. 2005a; Thonar et al. 1988). Elongation of the long bones during growth occurs through a process of endochondral ossification. Chondrocytes degrade the cartilage for replacement by the bone, releasing KS from the cartilage and secretion into the circulation. The decreased level of KS in the blood as healthy teenagers move towards adulthood is consistent with the fact that their growth rate begins to decline during this period. In previous studies we showed that blood KS level in severely affected MPS IVA patients less than 10 years old is markedly elevated and that KS level declines to near-normal or normal levels after 15 year of age, following closing of growth plate (Tomatsu et al. 2005a, 2010c). In the present study, KS levels are less dramatic and may reflect fewer young patients in this study and/or a cohort of patients with a less severe phenotype.

KS levels in the blood appears to be a suitable biomarker for early diagnosis, screening, assessment of disease severity, and monitoring therapeutic efficacy in young MPSIVA patients (Tomatsu et al. 2008, 2010c, 2013a); however, substantial overlaps of KS values between control subjects and MPS IVA patients especially, those older than 15 years of age, questions whether KS alone is a good biomarker for patients with MPS IVA at any age.

We did not see any elevation of KS in the blood of patients with MPS IVB in this study. All four of these patients were over 12 years of age and have the attenuated phenotype. Furthermore, patients with MPS IVB typically have a milder skeletal dysplasia compared with those with MPS IVA. Further study, especially of younger patients and those with a severe type with MPS IVB, is required to determine whether measurement of KS levels in these patients will be of value.

Levels of both C6S and KS in patients with MPS IVA and control overlapped after the age of five. However, over 93% of patients with MPS IVA showed elevation of either C6S or KS level more than 2 SD higher than age-matched control subjects, compared to only 75% for just one of these GAGs. This finding indicates that measurements of both GAGs could be more useful as a biomarker for early diagnosis, screening, assessment of disease severity, and monitoring therapeutic efficacy in patients with MPS IVA.

Our previous LC-MS/MS method determined levels of three GAGs, DS, HS, and KS, simultaneously after digestion with chondroitinase B, heparitinase, and keratanase II, respectively, but could not distinguish easily the peaks of ΔDi-4S (DS) and ΔDiHS-6S (HS) (Tomatsu et al. 2013b), since these products have the same molecular weight. Moreover, the method used chondroitinase B, heparitinase, and keratanase II that do not digest polymer CS to disaccharides, making it impossible to detect ΔDi-4S and ΔDi-6S derived from CS. This study and preliminary data showed that the peaks of ΔDi-4S (DS), ΔDi-6S (C6S), and ΔDiHS-6S (HS) can be separated with the current LC-MS/MS procedure. The peak of ΔDi-4S remains as a mixture of disaccharides derived from C4S and DS (stereoisomers with the same molecular weight). However, if we digest the samples with chondroitinase ABC or chondroitinase B separately and calculate the differences of the peaks for each disaccharide, all major disaccharides derived from KS, HS, CS, and DS can be determined.

By the current method, we could measure C6S level in the urine (human, rat, mouse), DBS (human), shark cartilage, bovine cornea (data not shown), as well as plasma or serum investigated in this study, suggesting that understanding of distribution pattern and physiological role of C6S can be explored more extensively. C6S was also found to be elevated in the urine of patients with MPS IVA

and VII (data not shown). C6S levels in the urine and potential correlation with levels in the blood require further study. Preliminary data indicate that an alternative approach to measure C6S is to use chondroitinase C to digest polymer C6S to disaccharides. Digestion of polymer CS by chondroitinase C provided similar results as digestion of chondroitinase ABC for detection of disaccharides C6S. Thus, both chondroitinases can be used for detection of C6S, although chondroitinase ABC is more cost-effective (personal communication with Seikagaku Co.).

We discussed that measurement of C6S and/or KS is useful for the diagnosis of MPS IVA and VII patients. However, there was a limitation of data interpretation by a small number of patients, especially under 5 years of patients with MPS IV or MPS VII. Therefore, it is still unclear whether this assay could be used for diagnosis in young patients.

In conclusion, we have established the method to measure C6S levels by LC-MS/MS, leading to the finding of age-dependent alteration of C6S in control subjects and patients with MPS IVA. Significant difference in levels of C6S between control subjects and patients with MPS IVA and VII indicates that C6S may be a valuable biomarker for early diagnosis, screening of the disease, assessment of disease severity, and monitoring therapeutic efficacy in patients with MPS IVA and VII.

Acknowledgement This work was supported by grants from the Austrian MPS Society and International Morquio Organization (Carol Ann Foundation). This work was also supported by the Japanese MPS Family Society. R.W.M. and S.T. were supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of NIH under grant number P20GM103464.

S.T. was supported by the National Institutes of Health grant 1R01HD065767-02. The content of the article has not been influenced by the sponsors. Editorial assistance to the manuscript was provided by Michelle Stofa at Nemours/Alfred I. duPont Hospital for Children.

Compliance with Ethics Guidelines

Conflict of Interest

All the authors contributed to this "Original Article" and have no conflict of interest with any other party.

Tsutomu Shimada, Eriko Yasuda, Robert W. Mason, William G. Mackenzie, Yuniko Shibata, Seiji Yamaguchi, Yasuyuki Suzuki, Tadao Orii, Francyne Kubaski, Roberto Giugliani, and Shunji Tomatsu declare that they have no conflict of interests.

Informed Consent

Informed consent was obtained from the patients and/or their guardians through the physicians who were in charge of the patients with MPS at each local institute approved by the Institutional Review Board (IRB).

Animal Rights

Not applicable

Contributions to the Project

Tsutomu Shimada: He has contributed to the concept of project, the planning, performance of experiments (LC-MS/MS), data analysis, and reporting of the work described in the article.

Eriko Yasuda: She has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Robert W. Mason: He has contributed to the planning, performance of LC-MS/MS, data analysis, and reporting of the work described in the article.

William G. Mackenzie: He has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Yuniko Shibata: She has contributed to analysis of standards, data analysis, and reporting of the work described in the article.

Seiji Yamaguchi: He has contributed to collecting samples and reporting of the work described in the article.

Yasuyuki Suzuki: He has contributed to collecting samples and reporting of the work described in the article.

Tadao Orii: He has contributed to collecting samples and reporting of the work described in the article.

Kenji E. Orii: He has contributed to collecting samples and reporting of the work described in the article.

Francyne Kubaski: She has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Roberto Giugliani: He has contributed to collecting samples and reporting of the work described in the article.

Shunji Tomatsu: He is a Principal Investigator and is responsible for the entire project. He has contributed to the concept of the project, planning, analysis of data, and reporting of the work described in the article.

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JIMD Reports
DOI 10.1007/8904_2014_312

RESEARCH REPORT

Carnitine Profile and Effect of Suppletion in Children with Renal Fanconi Syndrome due to Cystinosis

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Received: 24 November 2013 / Revised: 17 March 2014 / Accepted: 19 March 2014 / Published online: 6 May 2014

Abstract *Background*: Cystinosis is an autosomal recessive disorder marked by intralysosomal cystine accumulation. Patients present with generalized proximal tubular dysfunction called renal Fanconi syndrome. Urinary carnitine loss results in plasma and muscle carnitine deficiency, but no clinical signs of carnitine deficiency have been described. Also, the optimal dose of carnitine supplementation is undefined. This study aimed to determine whether currently recommended carnitine doses result in adequate correction of plasma carnitine.

Methods: Five cystinosis patients with renal Fanconi syndrome, aged 2–18 years, were included. L-carnitine was prescribed 50 mg/kg/day since diagnosis: median 36 (range 18–207) months. Total and free plasma and urine carnitine and carnitine profiles were measured at study onset, after stopping L-carnitine for 3 months and 3 months after reintroducing L-carnitine 50 mg/kg/day.

Results: At study onset, plasma free carnitine was normal in all patients, total carnitine (1/5), acetylcarnitine (3/5), and several short- and medium-chain acylcarnitines $\leq$10 carbons (5/5) were increased indicating carnitine over-supplementation. Three months after cessation, carnitine profiles normalized and 3/5 patients showed plasma carnitine deficiency. Three months after reintroduction, plasma free carnitine normalized in all patients, however, carnitine profiles were disturbed in 4/5 patients. Urine free carnitine, acetylcarnitine, and acylcarnitines $\leq$10 carbons were increased in all patients independent of carnitine supplementation.

Conclusion: Administration of recommended doses L-carnitine (50 mg/kg/day) resulted in over-supplementation. Although the drug is considered to be rather safe, long-term effects of over-supplementation remain unknown warranting cautious use of high doses. Plasma carnitine profile might be used as a monitor, to prevent overdosing.

Communicated by: Ertan Mayatepek, MD

Competing interests: None declared

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Background

Cystinosis is a rare, autosomal recessive disorder. It is caused by mutations in the *CTNS* gene (17p13) which encodes for the lysosomal cystine carrier cystinosin. Mutations in the *CTNS* gene lead to the intralysosomal accumulation of cystine (Town et al. 1998, Kalatzis et al. 2001). The most frequent infantile, nephropathic form manifests around the age of 3–6 months with renal Fanconi syndrome. This general proximal tubular damage is characterized by the enhanced excretion of various small molecules, including salts, glucose, amino acids, low-molecular weight proteins and carnitine. If untreated with the cystine-depleting agent cysteamine, cystinosis

leads to end-stage renal failure around the age of 10 years. Besides the specific treatment with cysteamine, cystinosis patients with renal Fanconi syndrome are treated symptomatically by supplementation of solutes lost into urine, such as bicarbonate, potassium, phosphate, citrate, and in some cases L-carnitine (Gahl et al. 2002).

Carnitine is an important transport molecule of long-chain fatty acids (acyl groups) and acyl-Co-enzyme A esters (acyl-CoA) across the mitochondrial inner membrane. The latter are degraded inside the mitochondrium by the process of β-oxidation until acetyl-CoA is left, which is further channelled as a substrate for several enzymes. In most tissues, acetyl-CoA enters the Krebs cycle with subsequent ATP generation; in ketogenic tissues, such as liver, it can also be used for ketone synthesis during carbohydrate starvation. Toxic fatty acyl-CoA esters can bind carnitine to form an acylcarnitine, which can be transported out of the mitochondrion. The cytosolic acetylcarnitine can act as an "acetyl-CoA buffer" and re-enter the mitochondrion when intramitochondrial acetyl-CoA levels have decreased, while acylcarnitines can be exported out of the cell and excreted in the urine. Since acyl and acetyl-groups can be transferred between carnitine and CoA, carnitine does not only affect the intracellular and intramitachondrial concentrations of acyl-CoA and acetyl-CoA, but it also maintains the acyl-CoA/CoA ratio and protects the body from toxic fatty acids (Bartlett and Eaton 2004).

In cystinosis, decreased tubular absorption of free carnitine and acylcarnitines leads to a deficiency of free carnitine in both plasma and muscle (Bernardini et al. 1985, Steinmann et al. 1987). Carnitine has a molecular weight of 162 Da and is freely filtered by the glomerulus. It is subsequently reabsorbed for 99% in the proximal tubule by a sodium-dependent transporter called OCTN2 (Tamai et al. 1998). Recently, a second carnitine transporter was identified in mouse proximal tubular cells, called Oat9, which transports carnitine without the need for a sodium or H^+ gradient (Tsuchida et al. 2010). The latter transporter has, however, not been demonstrated in humans thus far.

It has been suggested to administer oral L-carnitine in a daily amount of 50–100 mg/kg, resulting in normalization of plasma carnitine levels within days (Gahl et al. 1988). Muscle carnitine levels, however, may need years to normalize (Gahl et al. 1993). Since clinical signs of carnitine deficiency such as cardiomyopathy or metabolic encephalopathy have never been reported in cystinosis patients, it remains unclear whether there is an indication for carnitine supplementation in cystinosis patients, and if so, what would be the optimal dose regimen. Also, the effect of carnitine supplementation on the altered carnitine profile in cystinosis patients is unknown. Therefore, we aimed to study whether the currently recommended oral supplementation of 50 mg L-carnitine/kg/day leads to alterations in the carnitine profile in cystinosis patients with renal Fanconi syndrome.

Patients and Methods

Five cystinosis patients with renal Fanconi syndrome, aged 2–18 years old, were included in this study, two of them were male. No patient had a renal graft. Their clinical characteristics are shown in Table 1. Glomerular filtration rate (GFR) was calculated using the bedside Schwartz formula (Schwartz et al. 2009).

All patients were treated with 50 mg/kg/day L-carnitine since they were diagnosed with cystinosis and had received L-carnitine supplements during at least 18 months prior to enrolment in this study. Blood and urine carnitine profiles were measured at the study onset (daily dose 50 mg/kg), after 3 months of cessation of L-carnitine supplements and 3 months after reintroduction of L-carnitine at a daily dose of 50 mg/kg. In patient 1, no urine sample could be obtained after 3 months without carnitine supplements. Free carnitine concentrations and a carnitine profile were measured in blood and urine. Total carnitine concentrations were determined only in plasma, not in urine. All samples were measured in the laboratory of Radboudumc (Nijmegen, The Netherlands). Normal values were provided by the laboratory, different reference ranges were used for different age groups (Mueller et al. 2003).

Concentrations of free carnitine and acylcarnitines were measured by tandem mass spectrometry, essentially as described before (Vreken et al. 1999). Total carnitine concentrations were calculated by summation of free and acylcarnitine concentrations.

Results

The diagnosis of cystinosis was confirmed by a molecular analysis of the *CTNS* gene showing a disease causing mutation on both alleles in all five patients (Table 1). GFR ranged between 21 and 123 mL/min/1.73 m^2, all patients had full-blown renal Fanconi syndrome with pronounced aminoaciduria. None of the patients reported muscle weakness before or during the study.

At the onset of this study, all patients were supplemented with a daily L-carnitine dose of 50 mg/kg. Plasma free carnitine was normal in all patients, plasma total carnitine was elevated in patient 1 and plasma acetylcarnitine was elevated in patients 1, 2, and 4. Several short- and medium-chain acylcarnitines (up to 10 carbons) were increased in all patients on carnitine therapy (Fig. 1). Urine analysis showed increased excretions of free

Table 1 Clinical characteristics

Patient	Sex	Age at diagnosis (months)	*CTNS* gene mutations	Age at onset study (years)	Glomerular filtration rate (mL/min/1.73 m^2)
1	Female	14	57 kb del 57 kb del	11	21
2	Female	9	57 kb del c.926 dup	2	75
3	Male	6	57 kb del 57 kb del	18	52
4	Female	10	57 kb del c.926 dup	4	87
5	Male	11	c.681G>A c.1015G>A	3	123

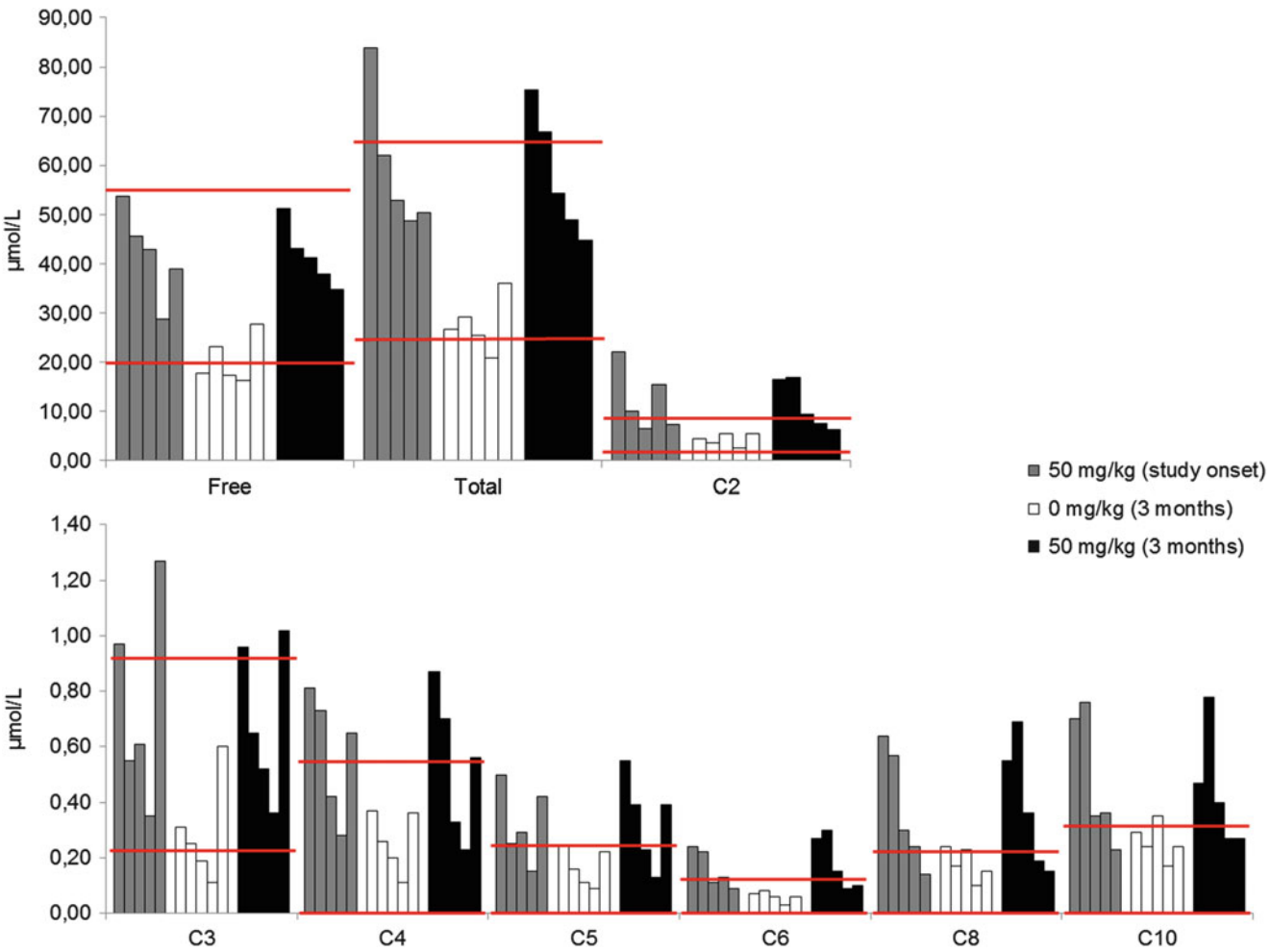

Fig. 1 Carnitine profile in plasma. *Red lines* indicate normal range. Each *bar* represents an individual patient

carnitine, acetylcarnitine, and acylcarnitines with a length of up to 10.

After stopping L-carnitine supplementation during 3 months, blood and urine carnitine-ester profiles normalised in all patients. However, free carnitine was decreased in three patients (patients 1, 3, and 4) and total carnitine was decreased in one patient (patient 4).

Three months after reintroduction of L-carnitine at a daily dose of 50 mg/kg, free plasma carnitine normalised in plasma while it remained above normal values in urine in

all patients; plasma total carnitine was above normal values in patients 1 and 2. Plasma acetylcarnitine was above normal values in patients 1, 2, and 3, while it was above normal values in urine in all patients. Several short-chain acylcarnitines with a length of up to 10 carbons were above normal values in 4/5 patients in both blood and urine, patient 4 was the only one who had a normal plasma carnitine profile.

Discussion

In this study, we analyzed plasma and urine carnitine levels in children with Fanconi syndrome due to cystinosis, both on and off L-carnitine supplementation.

Since children with renal Fanconi syndrome may have a high urinary loss of both free carnitine and carnitine-esters (Bernardini et al. 1985, Steinmann et al. 1987), it is recommended to treat them with L-carnitine. In the USA a daily dose of 100 mg/kg L-carnitine is advised (Gahl et al. 1988), while in Europe a daily dose of 50 mg/kg L-carnitine is mostly prescribed. We found alterations in plasma and urine carnitine profiles in all five cystinosis patients with renal Fanconi syndrome supplemented with 50 mg/kg L-carnitine during at least 18 months and 3 months after reintroducing L-carnitine 50 mg/kg, after 3 months cessation of L-carnitine supplementation. These alterations include increased levels of total carnitine, acetylcarnitine, and several short- and medium-chain acylcarnitine with a length up to 10 carbons in plasma and urine. On the other hand, plasma free carnitine (patients 1, 3, and 4) and plasma total carnitine (patient 4) declined below normal values when L-carnitine supplementation was stopped during 3 months.

The increased urinary (acyl)carnitine excretion in these patients can be explained by the fact that they all suffered from renal Fanconi syndrome (Bernardini et al. 1985, Steinmann et al. 1987), since 99% of filtered carnitine is reabsorbed in the proximal tubule by OCTN2 transporter (Tamai et al. 1998). Strikingly, the patient with the lowest GFR had the highest plasma total, free, and acetylcarnitine levels. One could hypothesize that a lower GFR is accompanied by lower urine carnitine losses. However, it is impossible to draw this conclusion in only one patient. Also, in previous studies in cystinosis patients, no correlation was found between serum creatinine (as a measure of GFR) and plasma carnitine levels (Bernardini et al. 1985, Gahl et al. 1988).

The increased (acyl)carnitine levels in plasma are more peculiar. Since these patients excrete high amounts of (acyl) carnitines in their urine, it could be expected to find corresponding decreased (acyl)carnitine profiles in patients' plasma. However, the plasma carnitine profile in patients under supplementation in our study mimics plasma carnitine profiles in healthy subjects who receive L-carnitine supplements. In a study conducted by Hongu et al. 6 healthy women received 680 mg L-carnitine l-tartrate per day and were compared to 7 healthy woman receiving placebo and 6 healthy woman receiving 940 mg choline bitartrate per day. After 7 days they increased plasma and urine levels of total carnitine, free carnitine, acid-soluble acylcarnitine, and acetylcarnitine were found in the group receiving L-carnitine supplementation, while acid-insoluble acylcarnitine was normal in both plasma and urine (Hongu and Sachan 2003). Thus, increased levels of plasma total carnitine, acetylcarnitine, and short- and medium-chain acylcarnitines in our patients most likely reflect a state of over-supplementation.

It remains unclear whether over-supplementation with L-carnitine can be harmful. L-carnitine is considered to be a rather safe drug, with the only reported side effects being a fishy body odor, diarrhea, and nausea. Doses up to 2,000 mg/day are considered safe, the available data regarding doses above 2,000 mg/day are not sufficient for a confident conclusion on long-term safety (Hathcock and Shao 2006). However, in very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD), controversy exists regarding the L-carnitine supplementation. It does not prevent decreased tissue carnitine levels and the accumulation of certain acylcarnitines can have toxic effects (Spiekerkoetter and Wood 2010).

Hypothetically, L-carnitine could be carcinogenic since it can be metabolized into trimethylamine and trimethylamine-*N*-oxide, both of which can be further metabolized into the potential carcinogenic compound *N*-nitrosodimethylamine (Bain et al. 2005). However, at this moment there is no evidence that L-carnitine might indeed be carcinogenic in vivo in doses used in this study. In addition, dietary L-carnitine can be metabolized into trimethylamine-N-oxide by gut bacteria, which is proatherogenic, and chronic dietary L-carnitine supplementation in mice can cause atherosclerosis (Koeth et al. 2013). Most of the cystinosis patients develop ESRD requiring renal replacement therapy associated with increased risk of cardiovascular disease and malignancy due to the use of immunosuppressive treatment. However, most cystinosis patients with renal graft are not receiving carnitine treatment, since renal Fanconi syndrome becomes much less pronounced after renal transplantation. Whether carnitine overdose in pre-transplant cystinosis patients can enhance the risk of malignancy and/or cardiovascular disease in the post-transplant period is impossible to estimate.

The rationale to prescribe high doses of L-carnitine of up to 100 mg/kg/day originates from the fact that muscle carnitine levels take years to normalize (Gahl et al. 1993). The clinical consequences of low muscle carnitine levels in cystinosis patients, however, remain unknown. Clinical

symptoms of carnitine deficiency are highly variable and may include metabolic encephalopathy and cardiomyopathy, while other patients remain asymptomatic (Breningstall 1990). Myopathy, which can be another symptom of carnitine deficiency (Breningstall 1990), is frequently reported in cystinosis patients. However, myopathy in cystinosis patients generally develops after 10 years of age and progresses after renal transplantation (Gahl et al. 2002), when secondary carnitine deficiency is no longer present (Gahl et al. 2007). Therefore, the myopathy observed in older cystinosis patients is most likely a trait of the disease and not linked to excess urinary carnitine excretion. Noteworthy, rat studies have shown that the induction of carnitine deficiency does not alter exercise capacity despite decreased muscle carnitine levels (Simi et al. 1990).

As for the clinical relevance of carnitine supplementation in cystinosis patients, controversy exists regarding the recommended dose for L-carnitine administration. Plasma carnitine profiling could be a useful tool to determine the optimum L-carnitine dose in individual patients. The goal of L-carnitine supplementation should be to normalize total carnitine, free carnitine, and short-chain acylcarnitines. If plasma carnitine profiling is not easily accessible, we suggest using plasma free carnitine, total carnitine, and acetylcarnitine levels to monitor L-carnitine supplementation.

In conclusion, we demonstrate that the current dosing schemes of carnitine result in over-supplementation. Plasma carnitine profiling can be a useful tool to monitor carnitine doses in cystinosis patients under carnitine therapy.

Synopsis

The indications, optimal dose regimen, and efficacy of L-carnitine supplementation in cystinosis are unknown; however, current dosing schemes result in over-supplementation.

Compliance with Ethics Guidelines

Conflict of Interest

All the authors of this chapter declare that there are no conflicts 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 2005. Informed consent was obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

Martine Besouw planned the study, gathered data, and wrote the manuscript.

David Cassiman interpreted data and corrected the manuscript.

Elisabeth Cornelissen conducted the study and corrected the manuscript.

Leo Kluijtmans interpreted data and corrected the manuscript.

Lambertus van den Heuvel interpreted data and corrected the manuscript.

Elena Levtchenko supervised the study, planned the study, and corrected the manuscript.

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JIMD Reports
DOI 10.1007/8904_2014_315

RESEARCH REPORT

Cognitive and Antipsychotic Medication Use in Monoallelic *GBA*-Related Parkinson Disease

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Received: 09 January 2014 / Revised: 15 March 2014 / Accepted: 18 April 2014 / Published online: 22 May 2014

Abstract Mutations in glucosidase, beta, acid (*GBA*) are associated with cognitive impairment in Parkinson disease (PD) as well as dementia with Lewy bodies. For both of these diseases, dementia and hallucinations are typically treated with cholinesterase inhibitors and antipsychotics. However, in some lysosomal storage disorders certain antipsychotic medications are poorly tolerated. This study examined cholinesterase inhibitor and antipsychotic use in monoallelic *GBA*-related PD to explore potential pharmacogenetic relationships. Monoallelic *GBA* mutation carriers with PD (*GBA*-PD) with at least two clinic visits ($n = 34$) were matched for age-of-onset and gender to *GBA* and leucine-rich repeat kinase 2 (*LRRK2*) mutation negative idiopathic PD subjects (IPD) ($n = 60$). Information regarding cholinesterase inhibitor and antipsychotic use as well as impaired cognition (UPDRS Mentation >1) and hallucinations (UPDRS Thought Disorder >1) were obtained. *GBA*-PD more frequently reported hallucinations (HR = 5.0; $p = 0.01$) and they were more likely to have cognitive impairment but this was not statistically significant (HR 2.2, $p = 0.07$). Antipsychotic use was not significantly different between *GBA*-PD and IPD (HR = 1.9; $p = 0.28$), but *GBA*-PD were more likely to have sustained cholinesterase inhibitor use (HR = 3.1; $p = 0.008$), even after adjustment for cognition and hallucinations. Consistent with reports of worse cognition, *GBA*-PD patients are more likely to use cholinesterase inhibitors compared to IPD. While there was no difference in antipsychotic use between IPD and *GBA*-PD, persistent use of quetiapine in *GBA*-PD suggests that it is tolerated and that a significant interaction is unlikely. Further prospective study in larger samples with more extensive cognitive assessment is warranted to better understand pharmacogenetic relationships in *GBA*-PD.

Communicated by: Peter Burgard, PhD

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Introduction

Gaucher disease (GD) is a rare lysosomal storage disease caused by homozygous or compound heterozygous glucosidase, beta, acid (*GBA*) mutations. In addition to GD, both biallelic and monoallelic *GBA* mutations have been linked to Parkinson disease (PD) and dementia with Lewy bodies (DLB): individuals with GD type 1 have an increased risk of PD (Bultron et al. 2010), and heterozygous *GBA* mutations have emerged as the most important genetic contributor to PD and DLB risk (Sidransky et al. 2009; Nalls et al. 2013). Estimates of the prevalence of heterozygous *GBA* mutations in PD vary from 3 to 20% depending on the population assessed (Sidransky et al. 2009).

Although heterozygous *GBA* mutations have been considered a risk factor rather than causal for PD, a reported penetrance as high as 30% by age 80 (Anheim et al. 2012; Kumar et al. 2012) suggests autosomal dominant inheritance with reduced penetrance.

While overall clinically similar to idiopathic PD (IPD), PD patients with monoallelic *GBA* mutations (*GBA*-PD) as a group have greater cognitive impairment (Sidransky et al. 2009; Neumann et al. 2009; Seto-Salvia et al. 2012; Brockmann et al. 2011; Alcalay et al. 2012). In a multicenter analysis of 5,691 PD subjects, cognitive changes were more prevalent in *GBA*-PD (Sidransky et al. 2009), and in pathologically confirmed PD with *GBA* mutations, dementia or cognitive decline was a common finding (Neumann et al. 2009). Another study found that *GBA*-PD subjects have a 6-fold greater risk of dementia compared to IPD after adjustment for age of onset and duration of disease (Seto-Salvia et al. 2012). Prospective studies with quantitative assessments demonstrate greater cognitive impairment in *GBA*-PD compared to IPD: *GBA*-PD subjects had lower Montreal Cognitive Assessment (MoCA) scores (Brockmann et al. 2011) and lower Mini-Mental State Exam scores (Alcalay et al. 2012). As cognitive impairment is associated with psychosis in PD and *GBA* mutations are associated with DLB (Nalls et al. 2013; Fenelon et al. 2000), it is not surprising that there is a high prevalence of hallucinations in *GBA*-PD (Neumann et al. 2009). Cognitive impairment is also a prominent feature of PD in patients with biallelic *GBA* mutations (Saunders-Pullman et al. 2010), but this represents a much smaller group of patients compared to those with monoallelic *GBA* mutations.

As monoallelic *GBA* mutations are the most important genetic risk factor for PD and DLB, it is important to understand the pharmacogenetic implications of *GBA* mutations in these two conditions. In biallelic lysosomal storage disorders, cationic amphiphilic drugs, which include quetiapine and clozapine, may worsen lysosomal function (Anderson and Borlak 2006). As a first step to assess pharmacogenetic implications of *GBA*-PD, we performed a retrospective study to determine whether *GBA*-PD is associated with differential medication usage of cholinesterase inhibitors and antipsychotic medications.

Methods

Participants. At the Movement Disorder Center at Beth Israel Medical Center, patients with two or more Ashkenazi Jewish grandparents who met diagnostic criteria for PD were invited to participate in a genetic study of PD (Pankratz et al. 2002). Consent was obtained, blood or saliva samples were collected, and DNA was isolated and screened for the eight common Ashkenazi Jewish *GBA* mutations (N370S, L444P, 84GG, IVS2+1G→A, V394L, del55bp, D409H, and R496H) (Saunders-Pullman et al. 2010) and the leucine-rich repeat kinase 2 (*LRRK2*) G2019S mutation (Ozelius et al. 2006). The institutional review board at Beth Israel Medical Center approved the study procedures, and all subjects provided informed consent.

After excluding subjects with symptom onset prior to January 1990, only one clinical visit, carriers of the LRRK2 G2019S mutation, and *GBA* mutation homozygotes or compound heterozygotes, 34 PD subjects with heterozygous *GBA* mutations were identified. Each of these was gender-matched and age-of-onset-matched (±2 years) with at least one PD subject without *GBA* mutations from the same patient population. Controls met the same criteria as cases. Potential subjects with symptom onset prior to 1990 were excluded because quetiapine and donepezil were approved in the USA in 1997 and 1996, respectively. The gene status of subjects was unknown to treating clinicians during the study period; therefore, clinical care and treatment were provided irrespective of genotype.

Data Collection

A retrospective chart review blinded to gene status was performed to assess demographics and UPDRS scores and to determine: (1) the date after which subjects were rated as having cognitive impairment for two or more consecutive visits based on patient and/or caregiver report; (2) whether a cholinesterase inhibitor was prescribed and compliance reported for two or more consecutive visits; (3) the date after which subjects reported hallucinations for two or more consecutive visits; and (4) whether an antipsychotic medication was prescribed and compliance reported for two or more consecutive visits. All clinic visits prior to April 1, 2012 were considered. In order to reduce the likelihood of detecting a single aberrant finding or acute but limited cognitive worsening, cognitive impairment was defined as a United Parkinson Disease Rating Scale (UPDRS) Mentation score (Item I.1) >1 for at least 2 visits encompassing greater than 6 months. Similarly, hallucinations were defined as a UPDRS Thought Disorder (Item I.2) score >1 for greater than 6 months. Cholinesterase inhibitor and antipsychotic medication usage were only recorded if the subject reported medication use for more than 6 months. For each date a subject met an outcome and for the last recorded clinic date, medications and UPDRS scores were recorded. Duration of disease was calculated by determining the time between the date of symptom onset and visit date. A subset of patients also had formal testing with the MoCA. A MoCA score of <26, which was previously identified as the optimal screening cutoff for

Table 1 Demographics, clinical features, and medication use in *GBA*-PD and IPD

	GBA-PD ($n = 34$)	IPD ($n = 60$)	p value
Women, *n* (%)	17 (50.0)	28 (46.7)	0.76
Age of onset, years	57.5 (48, 65)	59 (51, 66)	0.44
Time to first evaluation, years	2.4 (1.0, 5.7)	2.0 (1.4, 5.0)	0.90
Duration of disease, years	9.3 (5.9, 15.1)	8.4 (7.0, 12.5)	0.99
Duration of clinical follow-up, years	5.1 (3.1, 9.2)	5.4 (3.0, 8.5)	0.83
Education, *n* (%)			0.28
High School	4 (11.8)	2 (1.8)	
Undergraduate	13 (38.2)	27 (48.2)	
Graduate	17 (50.0)	27 (48.2) ($n = 56$)	
UPDRS III at last visit	20.5 (15, 30)	16 (13, 26.5) ($n = 52$)	0.26
Levodopa equivalents at last visit, mg	715.8 (400, 1,000)	598.5 (432.5, 887.5)	0.22
Dopamine agonist at last visit, *n* (%)	12 (35.3)	26 (43.3)	0.29
Mentation consistently >1, *n* (%)	9 (26.5)	7 (11.7)	0.07
Cholinesterase inhibitor >6 months, *n* (%)	14 (41.2)	9 (15.0)	0.005
Hallucinations >1, *n* (%)	17(50.0)	18 (30.0)	0.054
Hallucinations >1 for 6 months, *n* (%)	9 (26.5)	4 (6.7)	0.01
Antipsychotic >6 months, *n* (%)	6 (17.7)	7 (11.7)	0.54

Continuous variables are reported as medians with interquartile ranges. *GBA-PD* Parkinson disease with heterozygous *GBA* mutations, *IPD* idiopathic Parkinson disease, *UPDRS* Unified Parkinson's Disease Rating Scale

mild cognitive impairment in PD (Dalrymple-Alford et al. 2010), was used to differentiate mild cognitive impairment and dementia from normal cognition in PD. Subjects with any missing UPDRS items were not included in analyses utilizing motor UPDRS.

Statistical Analysis

Mann–Whitney and Chi-squared or Fisher's exact tests were used for bivariate comparisons of continuous and categorical variables, respectively. Cox proportional hazard models were applied for the outcomes of: reported cognitive impairment, sustained psychotic symptoms, and usage of cholinesterase inhibitor as well as atypical antipsychotics. The predictor of interest was heterozygous *GBA* mutation status. Significance of the Cox proportional hazard models was determined using the Wald statistic with a p value set at 0.05. All models met the proportional hazards assumption based on Schoenfeld residuals. Analyses were performed using Stata 11.1 (Statacorp, College Station, TX, USA). Post-hoc assessment was also performed in the subgroup with available MoCA scores.

Results

Demographic characteristics of *GBA*-PD and age-of-onset and gender-matched IPD subjects are presented in Table 1. As expected, there were no differences in gender distribution ($p = 0.76$), median age of onset ($p = 0.44$), disease duration (0.99), and duration of clinical follow-up ($p = 0.83$). Levodopa equivalent dose at last visit between the *GBA*-PD and IPD groups also did not differ ($p = 0.22$). There were also no significant differences between the *GBA*-PD and IPD groups in the UPDRS III score ($p = 0.26$) at the last visit.

Of the 34 *GBA*-PD subjects, 27 had a mild mutation (22 with N370S and 5 with R496H) and 7 had a severe mutation (4 with 84GG, 2 with L444P, and 1 with V394L) (Beutler et al. 2005). Demographic characteristics of the *GBA*-PD groups with mild and severe mutations are presented in Table 2. While the duration of clinical follow-up time was longer in the mild mutation group compared to the severe mutation group (9.5 vs. 4.6 years, $p = 0.02$), there were no significant differences in gender, age of onset, disease duration, UPDRS III, or levodopa equivalent dose at the last visit between the two groups.

GBA-PD subjects had a significantly higher risk of reporting hallucinations (HR = 5.0; 95%CI: 1.5, 16.9) compared to IPD subjects. *GBA*-PD subjects also were more likely to report cognitive impairment, but this was not statistically significant (HR = 2.2; 95%CI: 0.8, 5.8). *GBA*-PD subjects were maintained on cholinesterase inhibitor more often than IPD subjects (HR = 3.1; 95%CI: 1.3, 7.2) (see Fig. 1). Of these, 19 received donepezil (12 *GBA*-PD) and 4 received rivastigmine (2 *GBA*-PD). Neither adjustment for the UPDRS mentation score nor presence of

Table 2 Demographics, clinical features, and medication use in *GBA*-PD with severe mutations versus *GBA*-PD with mild mutations

	GBA-PD with mild mutations ($n = 27$)	*GBA*-PD with severe mutations ($n = 7$)	p value
Women, *n* (%)	11 (40.7)	6 (85.7%)	0.09
Age of onset, years	57 (48, 67)	58 (45, 62)	0.61
Time to first evaluation, years	2.5 (1.0, 6.6)	1.9 (1.1, 3.4)	0.80
Duration of disease, years	7.8 (5.0, 12.8)	11.6 (9.0, 16.9)	0.06
Duration of clinical follow-up, years	4.6 (2.3, 7.2)	9.5 (5.2, 15.0)	0.02
UPDRS III at last visit	20 (15, 32)	26 (8, 30)	0.65
Levodopa equivalents at last visit, mg	675 (400, 1,000)	733 (575, 1,330.5)	0.69
Mentation consistently >1, *n* (%)	8 (29.6)	1 (14.3)	0.64
AcheI >6mo, *n* (%)	9 (33.3)	5 (71.4)	0.10
Hallucinations >1, *n* (%)	11 (40.7)	6 (85.7)	0.09
Hallucinations >1 for 6 months, *n* (%)	7 (25.9)	2 (28.6)	1.0
Antipsychotic >6 months, *n* (%)	5 (18.5)	1 (14.3)	1.0

Continuous variables are reported as medians with interquartile ranges. *GBA-PD* Parkinson disease with heterozygous *GBA* mutations, *UPDRS* Unified Parkinson's Disease Rating Scale

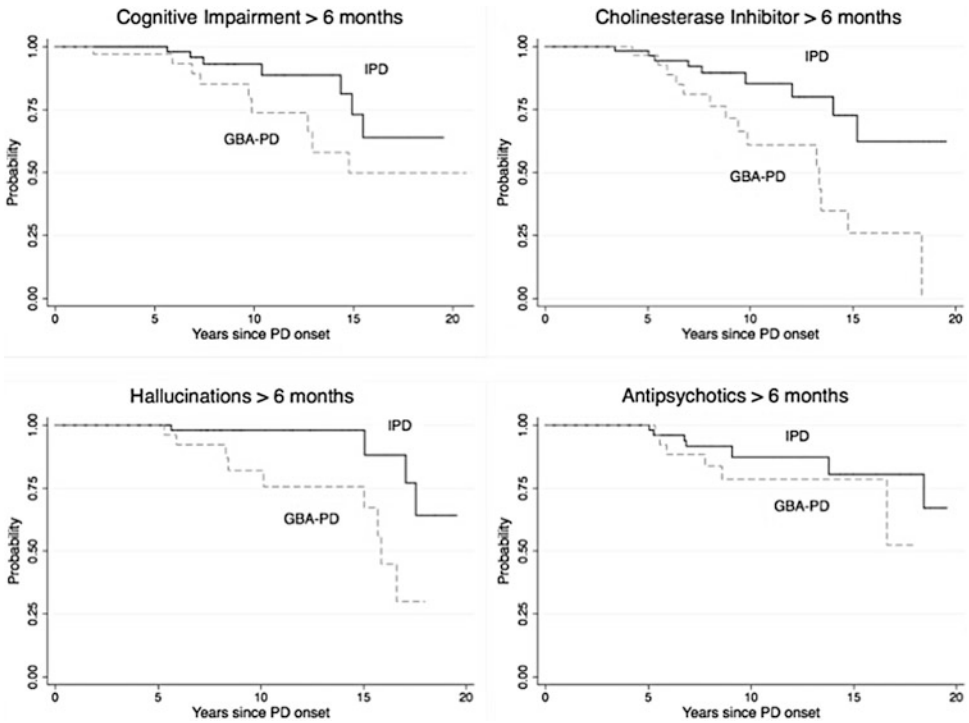

Fig. 1 Kaplan–Meier survival curves for cognitive outcomes in GBA-PD vs. IPD

hallucinations at the time subjects met this outcome or at the last visit date altered the finding that *GBA*-PD subjects were more likely to use cholinesterase inhibitors (HR = 3.2; 95%CI: 1.3, 7.9).

GBA-PD were not significantly more likely be prescribed an antipsychotic for greater than 6 months (HR = 1.9; 95%CI: 0.6, 5.8). Of those treated, 12 received quetiapine (6 *GBA*-PD) and 1 received clozapine (IPD). While a greater proportion of *GBA*-PD subjects with severe mutations reported hallucinations and used cholinesterase inhibitors compared to *GBA*-PD subjects with mild mutations, the differences were not significant (see Fig. 2). As anticipated, for the subset with available MoCA scores, UPDRS mentation scores >1 were more frequently

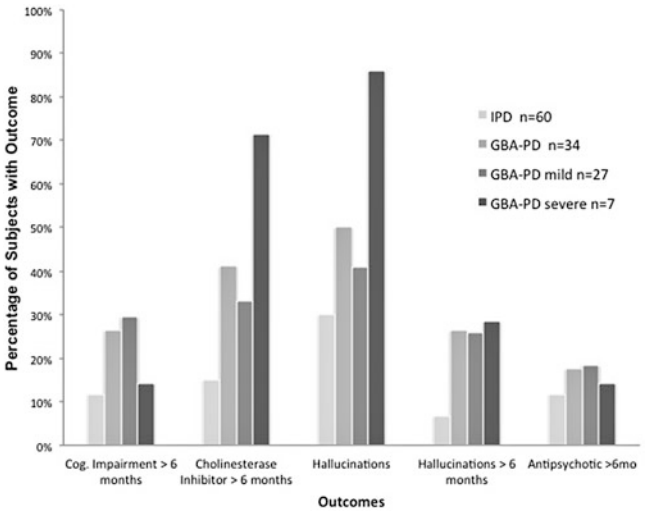

Fig. 2 Percentage of IPD and *GBA*-PD subjects meeting cognitive outcomes. *Bar graph* representing percentage of subjects in each group that met outcomes of UPDRS mentation score >1 for >6 months, usage of cholinesterase inhibitor >6 months, ever having hallucinations, UPDRS thought disorder score >1 for > 6 months, and usage of antipsychotic medication >6 months

observed in the group with MoCA scores <26 compared to those with scores ≥ 26 (45.5% vs 8%, $p = 0.04$).

Discussion

Our study found no difference in sustained use of quetiapine and clozapine between *GBA*-PD and IPD, despite the fact that *GBA*-PD was more likely to have sustained hallucinations. The greater use of cholinesterase inhibitors in *GBA*-PD suggests that PD genotype could be associated with differential medication use. The greater cholinesterase inhibitor use and sustained hallucinations in *GBA*-PD are consistent with previous reports of greater cognitive impairment and hallucinations in this condition (Sidransky et al. 2009; Neumann et al. 2009; Seto-Salvia et al. 2012; Brockmann et al. 2011; Alcalay et al. 2012). While greater cognitive impairment in *GBA*-PD is likely related to higher cholinesterase use, the association with cholinesterase use was maintained even when adjusted for cognitive score, raising the possibility that additional factors, such as better response, may be present.

In some autosomal recessive metabolic diseases, medications may worsen symptoms. For example, in Tay–Sachs disease, treatment with risperidone, haloperidol, and chlorpromazine may precipitate neurological worsening and should be avoided (Shapiro et al. 2006). This effect is postulated to result from reduction in residual lysosomal function. Because monoallelic *GBA* mutations may disturb lysosomal dysfunction (Westbroek et al. 2011), there is a parallel concern for *GBA*-PD. As cationic amphiphilic drugs, both quetiapine and clozapine tend to accumulate in lysosomes and can interfere with lysosomal function and autophagy (Anderson and Borlak 2006). While this study found no difference in quetiapine and clozapine use between *GBA*-PD and IPD, suggesting tolerability of this medication in *GBA*-PD, we lack the data to assess whether the response to quetiapine, both its benefit and side effect profile, was the same in both groups.

While our study is limited by its retrospective nature and the increased frequency of cholinesterase use is not a proxy for efficacy, more and persistent use of cholinesterase inhibitors in *GBA*-PD is consistent with the expected response based on the prominence of Lewy body pathology in *GBA*-related parkinsonism. Lewy bodies have been reported in both monoallelic *GBA*-related PD and DLB (Goker-Alpan et al. 2006; Clark et al. 2009; Poulopoulos et al. 2012; Tsuang et al. 2012), as well as in GD1 patients with parkinsonism, who had Lewy bodies in the cortex and hippocampal pyramidal cell layers CA2–CA4 (Wong et al. 2004). The presence of Lewy bodies has been associated with greater reduction in midfrontal choline acetyltransferase compared to Alzheimer's disease (AD) pathology (Tiraboschi et al. 2000), a rationale for better cholinesterase inhibitor efficacy in dementia associated with PD (PDD) and DLB compared to AD. Increased hallucinations in *GBA*-PD may also be explained by the predominance of Lewy body pathology. Evidence linking Lewy body pathology to visual hallucinations as well as a greater cortical cholinergic deficit helps to explain the finding that PDD subjects with visual hallucinations showed greater benefit from rivastigmine compared to those without visual hallucinations (Williams and Lees 2005; Harding et al. 2002; Burn et al. 2006).

Consistent with prior studies that demonstrated greater PD risk and earlier PD onset with severe *GBA* mutations

(Gan-Or et al. 2009; Gan-Or et al. 2008; Barrett et al. 2013), among the *GBA*-PD group, 86% of those with severe mutations reported hallucinations during clinical follow-up compared to 41% of those with mild mutations, suggesting a more malignant phenotype. Comparatively, a pathological series found that the lifetime prevalence of hallucinations in PD was 50% (Williams and Lees 2005).

While this study is retrospective and exploratory, it has the advantages of including a large number of *GBA*-PD subjects and extended follow-up time. One limitation is that by targeted sequencing of common Ashkenazi Jewish *GBA* mutations, it is possible, albeit unlikely, that there was misclassification of IPD subjects. It is also possible that genetic mutations linked to PD, besides those in *LRRK2* and *GBA*, were present in the *GBA*-PD or IPD groups. As this study relied on retrospective clinical information, we did not capture short medication trials, especially those outside routine clinic visits, nor did we formally measure medication compliance. Therefore, we cannot assess whether *GBA*-PD subjects were less likely to stop cholinesterase inhibitors once they were started. Additionally, as Part I of the UPDRS is determined with input from the patient and caregiver, and the source of information was not systematically recorded, reporting bias may have affected the mentation score. Lastly, clinician practice was not standardized and may have evolved over time; however, this would not be expected to differentially affect our comparison groups as they were both sampled from the same time period. To address the need for standardization in the assessment of neuropsychiatric symptoms, a prospective study evaluating cognitive outcomes and medication effects, including detailed pre- and post-medication assessment of the quality and frequency of psychotic symptoms, cognition with neuropsychological testing, and adverse events, is warranted. Further studies will also be necessary to determine if cholinesterase inhibitor use is increased in *GBA*-PD secondary to increased dementia, better response to treatment, or a combination of both.

Acknowledgments The research presented in this manuscript was supported by the Empire State Clinical Research Training Program, the Marcled Foundation, and NIH-NINDS NS073836.

Sources of Support

Empire State Clinical Research Training Program, the Marcled Foundation, and NIH-NINDS NS073836.

Synopsis Sentence

Monoallelic *GBA* mutation carriers with PD have the same rate of antipsychotic medication use as PD patients without *GBA* mutations and are more likely to use cholinesterase inhibitors.

Compliance with Ethics Guidelines

Conflict of Interest

Neither Matthew Barrett, Vicki Shanker, Lawrence Severt, Debbie Raymond, Susan Gross, Nicole Schreiber-Agus, Ruth Kornreich, Laurie Ozelius, Susan Bressman, nor Rachel Saunders-Pullman have any disclosures, financial or otherwise, that would represent a conflict of interest with the research presented in this manuscript.

Other Disclosures

Dr. Barrett reports no disclosures. Dr. Shanker reports no disclosures.

Dr. Severt served as a speaker and/or on advisory boards for Teva, Allergan, Merz, Impax, and UCB. Ms. Raymond reports no disclosures. Dr. Gross serves as PI on a research grant sponsored by PerkinElmer Inc. for development of new genetic platforms and as co-PI on an NIH research grant for preterm labour (primary PI Dr. S. Gennaro at Boston College). She received no personal compensation of any kind from either grant. All research funds are to the hospital system. Dr. Schreiber-Agus served as an investigator on a research grant sponsored by PerkinElmer Inc. for development of new genetic platforms. She received no personal compensation of any kind. All research funds were to the hospital system. Dr. Kornreich reports no disclosures. Dr. Ozelius receives salary support from NIH [NS058949, NS037409, NS075881, DC011805] and has received grant support from the Bachmann-Strauss Dystonia and Parkinson Foundation, the Benign Essential Blepharospasm Research Foundation, and The Dystonia Medical Research Foundation. She is a current member of the scientific advisory boards of the National Spasmodic Dysphonia Association, the Benign Essential Blepharospasm Research Foundation, and Tourette Syndrome Association, Inc. Dr. Ozelius receives royalty payments from Athena Diagnostics related to patents. Dr. Bressman serves on the advisory boards of the Michael J. Fox Foundation, the Dystonia Medical Research Foundation, the Bachmann Strauss Dystonia and Parkinson's Foundation, and the Board of We Move. She has consulted for Bristol Meyer Squibb. She has received research support from the Michael J. Fox Foundation, National Institutes of Health (NIH), and Dystonia Medical Research Foundation. Dr. Bressman received royalty payments from Athena Diagnostics related

to patents. Dr. Saunders-Pullman serves on the Scientific Advisory Board of the Dystonia Medical Research Foundation. She receives research support from the NIH (K02 NS073836), the Michael J Fox Foundation for Parkinson's Research, the Bachmann-Strauss Dystonia and Parkinson's Foundation, the Marcled Foundation, and the Empire State Clinical Research Training Program.

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.

Animal Rights

No animals were involved in the conduct of this study.

Individual Author Contributions

Study conception and design: Dr. Barrett, Dr. Saunders-Pullman; Conduct of research: Dr. Barrett, Dr. Shanker, Dr. Severt, Ms. Raymond, Dr. Gross, Dr. Schreiber-Agus, Dr. Kornreich, Dr. Ozelius, Dr. Bressman, Dr. Saunders-Pullman. Analysis and interpretation of data: Dr. Barrett, Dr. Bressman, Dr. Saunders-Pullman; Drafting the manuscript or revising it critically for important intellectual content: Dr. Barrett, Dr. Shanker, Dr. Severt, Ms. Raymond, Dr. Gross, Dr. Schreiber-Agus, Dr. Kornreich, Dr. Ozelius, Dr. Bressman, Dr. Saunders-Pullman.

Guarantor

Dr. Barrett

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JIMD Reports
DOI 10.1007/8904_2014_316

CASE REPORT

Laronidase Replacement Therapy and Left Ventricular Function in Mucopolysaccharidosis I

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Received: 01 February 2014 / Revised: 07 April 2014 / Accepted: 17 April 2014 / Published online: 22 May 2014

Abstract We assessed the effects of long-term laronidase replacement therapy (LRT) on the left ventricular (LV) function of a 52-year-old adult woman with mucopolysaccharidosis I (MPS I). The urinary uronic acid concentration significantly decreased by 78.7% (from 75 to 16 mg/g creatinine) after LRT; thereafter, estimated LV weight as assessed by two-dimensional (2D) echocardiography significantly decreased by 33.3% (from 189 to 126 g). Although systolic LV function of the ejection fraction as assessed by 2D echocardiogram did not change after LRT, the diastolic LV function parameters of the deceleration time (DcT) and the ratio of early (E) to late (A) ventricular filling velocities (E/A ratio) significantly improved. The DcT significantly decreased from 355 to 300 ms and the E/A ratio significantly decreased from 1.8 to 0.98. These diastolic parameters were normalized. In the contraction synchrony assessed by 2D speckle tracking imaging, the maximum time delay of contraction decreased from 148 to 14 ms. In addition, the LV weight significantly correlated with the E/A ratio ($p < 0.001$, $r = 0.63$), DcT ($p < 0.05$, $r = 0.48$), and contraction synchrony ($p < 0.001$, $r = 0.61$), respectively. This is the first study to report that LRT significantly improves diastolic LV function and contraction synchrony in a patient with MPS I.

Communicated by: Carla E. Hollak, M.D.

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Introduction

Mucopolysaccharidosis I (MPS I) is an autosomal recessive inherited disorder caused by mutations in the gene encoding α-L-iduronidase, located on chromosome 4p16.3 (Scott et al. 1992). The prevalence is approximately one in 100,000 live births; however, as with all MPS disorders international variation exists (Brown and Trivette 1998). α-L-iduronidase is a lysosomal enzyme that contributes to the degradation of glycosaminoglycans (GAG) (Neufeld and Muenzer 2001). The accumulation of GAG in cells and tissues results in a variety of organic dysfunctions, including joint disorders, hepatosplenomegaly, clouding of the cornea, degradation of the retina, cardiovascular disorders, mental retardation, umbilical herniation, and respiratory disorders (McKusick et al. 1965). The cardiovascular system is often affected; its prevalence is reported at 60–100% of MPS patients. Valvular disease, such as mitral valve or aortic valve regurgitation, is most commonly found in MPS I patients with valve and endocardium thickening caused by GAG accumulation, and valve replacement therapy is necessary for overcoming heart failure. Coronary artery disease, conduction abnormalities, and pulmonary hypertension are also often present. In left ventricular (LV) function, systolic LV function is preserved; however, diastolic LV dysfunction and LV hypertrophy are frequently found in patients with MPS (Braunlin et al. 2011).

Enzyme replacement therapy with recombinant human α-L-iduronidase (laronidase) provides an etiology-specific treatment by delivering enough α-L-iduronidase to prevent GAG accumulation in patients with MPS I (Wraith 2001). Laronidase replacement therapy (LRT) has been shown to be safe and effective in animal and human studies (Kakkis et al. 2001; Shull et al. 1994) and has been shown to improve respiratory function and physical capacity by reducing GAG

retention in patients with MPS-I (Wraith et al. 2004). We previously reported that in an adult patient with MPS-I, short-term LRT was effective in improving myocardial function, including myocardial strain and torsion, as assessed by the two-dimensional (2D) speckle tracking imaging method (Harada et al. 2011). However, little is known about the effects of LRT on LV systolic and diastolic functioning long-term, as adult patients are rare and thus there is little opportunity for cardiologists to observe their outcomes. Therefore, we investigated whether long-term LRT improves LV function in an adult patient with MPS I.

Case Report

The patient is 52-year-old female with MPS I (Scheie) who was diagnosed at 35 years of age with deficient activity of the enzyme α-L-iduronidase. At 11 years of age, she started showing her first clinical symptoms, which included stiffness, joint deformity, hepatosplenomegaly, and umbilical herniation. At the age of 20, she developed clouding of the bilateral corneas. At 33, degradations in her retinal pigmentosa emerged, leading to amblyopia. She also had congestive heart failure (CHF) due to aortic and mitral valvular regurgitation, and underwent a double valvular replacement. After her procedure, a small amount of paravalvular leakage was found at the site of the aortic prosthetic valve, resulting in chronic heart failure. She was then placed on anti-heart failure medications, including candesartan, carvedilol, and amlodipine, as well as an antiarrhythmogenic agent (cibenzoline) and an anticoagulant (warfarin) for paroxysmal atrial fibrillation (PAF).

In 2006, LRT (Aldurazyme®, Genzyme Corporation, Japan) was approved for use in Japan by the Ministry of Health, Labor and Welfare. The patient in this study was placed on LRT in November 2007; her dosage was 0.58 mg/kg administered once per week via a three-hour intravenous infusion, based on the manufacturer's recommendations.

Methods

Clinical assessment of the patient after LRT was performed over a 42-month follow-up period. This included a physical examination, measuring urinary uronic acid, estimating liver and spleen volumes via abdominal computed tomography, and monitoring LV function and weight through 2D echocardiography.

Conventional, Pulse Doppler, and Speckle Tracking Echocardiograms

The 2D echocardiography was performed using a Vivid 7 Dimension ultrasound machine with an M4S probe (GE Medical Systems, Milwaukee, WI, USA). The LV ejection fraction and weight were measured using M-mode and area-length methods, respectively, and LV inflow velocity for early (E) wave deceleration time (DcT) and the ratio of early (E) to late (A) ventricular filling velocities (E/A ratio) by pulse Doppler. For speckle tracking echocardiography (D'hooge et al. 2000; Hurlburt et al. 2007; Notomi et al. 2005; Voigt and Flachskampf 2004), an offline speckle tracking analysis was performed on all digitally stored grayscale imaging using customized software (EchoPAC PC Dimension: GE Medical Systems, Milwaukee, WI, USA). The LV myocardium of the short or long axis in B-mode was divided into six segments. Ten to twelve speckles, which are a high echoic region of interest, are automatically determined in each segment of the myocardium and monitored during contraction.

For the assessment of contraction synchrony, the maximum time delay was measured. For the short axis view, the time for reaching the end-systolic phase was different in each of the six segments. The maximum time delay was obtained by measuring the difference between the maximum time and minimum time in each of the six segments. The maximum time delay became shorter in a well-synchronized contraction but was longer in a dyssynchronized contraction. The high reproducibility of speckle tracking echocardiography has previously been reported by us and others (Beaver et al. 2011; Harada et al. 2011).

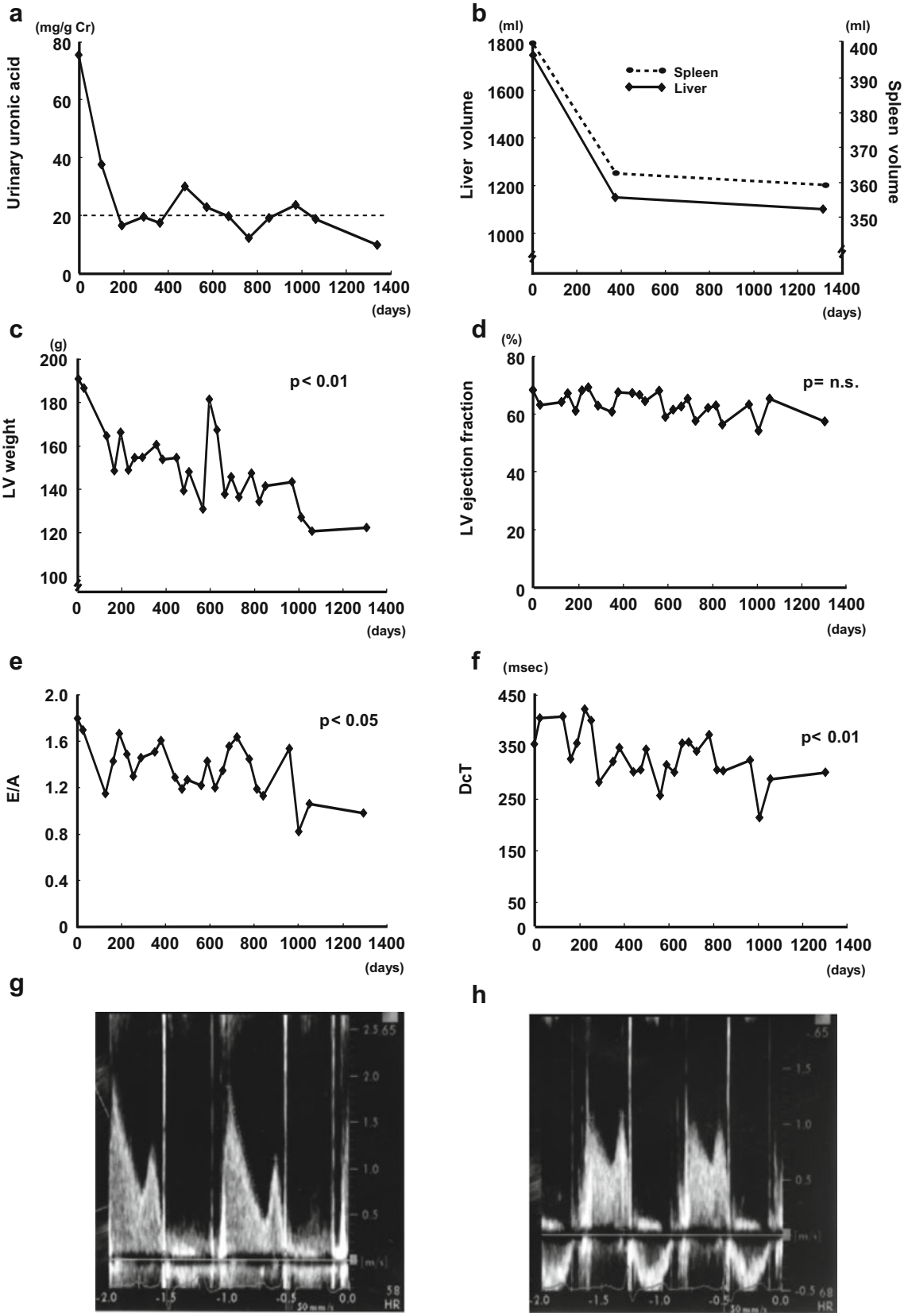
Statistical Analysis

The time course values of the echocardiographic parameters were analyzed using one-way ANOVA. The correlations between LV weight and the echocardiographic parameters were analyzed through a single regression analysis using SPSS software (SPSS Inc. Chicago, IL, USA).

Results

Clinical Findings After LRT

The clinical findings in the patient on joint movements, vision, and distance by 6-minute walk test were not improved after LRT during the follow-up period. However, the concentration of urinary uronic acid and liver and spleen volumes were improved (Fig. 1). As we previously described in a six-month follow-up study of this patient (Harada et al. 2011), the concentration of urinary uronic acid decreased by 78.7% (from 75 to 16 mg/g creatinine after LRT). Thereafter, urinary uronic acid concentration was maintained during the follow-up period (Fig. 1a).


a
(mg/g Cr)
Urinary uronic acid
80
60
40
20
0
0 200 400 600 800 1000 1200 1400
(days)
b
(ml)
(ml)
Liver volume
Spleen volume
Spleen
Liver
1800
1600
1400
1200
1000
400
390
380
370
360
350
0 200 400 600 800 1000 1200 1400
(days)
c
(g)
LV weight
p< 0.01
200
180
160
140
120
100
0 200 400 600 800 1000 1200 1400
(days)
d
(%)
LV ejection fraction
p= n.s.
80
60
40
20
0
0 200 400 600 800 1000 1200 1400
(days)
e
E/A
p< 0.05
2.0
1.6
1.2
0.8
0.4
0
0 200 400 600 800 1000 1200 1400
(days)
f
(msec)
DcT
p< 0.01
450
350
250
150
50
0
0 200 400 600 800 1000 1200 1400
(days)
g
h

Hepatosplenomegaly also was improved after LRT. Estimated liver and spleen volumes decreased by 34.1% (from 1,746 to 1,150 mL) and 9.5% (from 400 to 362 mL), respectively (Fig. 1b). Most importantly, the disease state of the patient's CHF and PAF was stabilized following LRT.

Echocardiographic Findings After LRT

The cardiothoracic ratio as shown through chest X-ray imaging and LV ejection fraction (Fig. 1d) were unchanged after LRT. However, estimated LV weight decreased by 33.3% (from 189 to 126 g) during the follow-up period (Fig. 1c). LV diastolic function significantly improved after LRT (Fig. 1e–h). The E/A ratio significantly decreased from 1.8 to 0.98, with a normal range from 0.75 to 1.5 (Fig. 1e, g, h). The improved E/A ratio is attributable to decreased E wave velocity but not to an alteration in A wave velocity. The DcT significantly decreased from 355 to 300 ms, with a normal range from 150 to 250 ms (Fig. 1f–h). In the contraction synchrony, the maximum time delay decreased from 148 to 14 ms, with a normal range from 36 to 49 ms (Fig. 2a–c).

Relation of LV Weight to Urinary Uronic Acid Concentration, Diastolic Function, and Contraction Synchrony

LV weight correlated significantly with urinary uronic acid concentration ($n = 13$, $r = 0.73$, $p < 0.01$) (Fig. 3a). In the relationship between LV weight and diastolic function parameters, LV weight correlated significantly with E/A ratio ($n = 26$, $p < 0.001$, $r = 0.63$) and DcT ($n = 26$, $p < 0.05$, $r = 0.48$), respectively (Fig. 3b, c). Furthermore, LV weight correlated significantly with contraction synchrony ($n = 26$, $p < 0.001$, $r = 0.61$) (Fig. 3d).

Discussion

We previously reported on the improvement of LV myocardial longitudinal strain (shortening ratio), LV myocardial radial strain (thickening ratio), and LV myocardial torsion using speckle tracking echocardiography for a six-month observation period (Harada et al. 2011). However, we could not recognize changes in systolic and diastolic function because of a short-term observation period. The effects of LRT on LV function during a long-term follow-up period remain unknown in patients with MPS I. This is the first report that long-term LRT has a beneficial effect on LV diastolic function but not on systolic function in an adult patient with MPS I.

In this report, the therapeutic effectiveness of LRT was confirmed by our clinical findings, which included a reduction of urinary uronic acid, LV weight, and hepatosplenomegaly. In fact, a good correlation was observed between LV weight and urinary uronic acid concentration. These findings are similar to the previous results of our studies and others (Arora et al. 2007; Harada et al. 2011; Kakkis et al. 2001). These findings suggest that decreased LV weight was due to GAG degradation in cardiac tissue, resembling volume reduction of the liver or spleen.

In MPS I, GAG accumulation in the heart affects the LV tissue and cytoplasmic structural changes. In an electromicroscopic examination, the interstitial fibroblast as a "clear cell" infiltrates the LV wall and the membranous cytoplasmic body is found in the interstitial fibroblast cytoplasm. However the sarcomeric structure of the cardiomyocyte is normally conserved (Braunlin et al. 2011; Dekaban et al. 1976; Nagashima et al. 1976; Shimamura et al. 1976). In the optical microscopic examination, GAG accumulation was predominantly found in the valves and endocardium and these tissues thickened (Braunlin et al. 2011; Dekaban et al. 1976; Nagashima et al. 1976; Shimamura et al. 1976).

These histological findings suggest that diastolic function is impaired by these thickened tissues. In the mouse MPS I model in which α-L-iduronidase was deleted, the structure of sarcomeres appeared normal; however, numerous empty vacuoles were observed in the cardiomyocyte, and endothelial cells of the mitral valve were also vacuolated on electromicroscopy. These empty vacuoles in the cardiomyocyte disappeared significantly and the endothelial cells in the mitral valve appeared normal, without vacuoles, when the MPS I mice were treated with gene

Fig. 1 Time courses of urinary uronic acid concentration, estimated liver and spleen volumes, LV weight, LV systolic and diastolic function after LRT. (**a**) Urinary uronic acid concentration. *Dotted line*: Normal range of urinary uronic acid is less than 20 mg/g creatinine. (**b**) Estimated liver and spleen volumes. (**c**) Estimated left ventricular weight. Significant decrease in LV weight was observed by LRT. LV weight in healthy women is about 66–110 g. (**d**) Ejection fraction did not change with LRT. (**e**) E/A ratio decreased significantly after LRT ($p < 0.05$). Normal range: 0.75–1.5, abnormal relaxation pattern:<0.75, restriction (pseudo-normalization) pattern:≧1.5. (**f**) DcT decreased significantly after LRT ($p < 0.01$). Normal range:160–240 ms, abnormal relaxation pattern:>240 ms, restriction (pseudo-normalization) pattern:<160 ms. (**g**) LV in-flow velocity at 25 days after LRT. DcT was 406 ms and E/A was 1.7. The 1.87 m/s of E was in hyper in-flow velocity. The LV diastolic function status was within the range of impaired relaxation to pseudo-normalization. (**h**) LV in-flow velocity at 1,206 days after LRT. DcT was 299 ms and E/A was 0.98. The 1.06 m/s of E was at normal velocity. These findings suggest that LV diastolic function was within the normal range

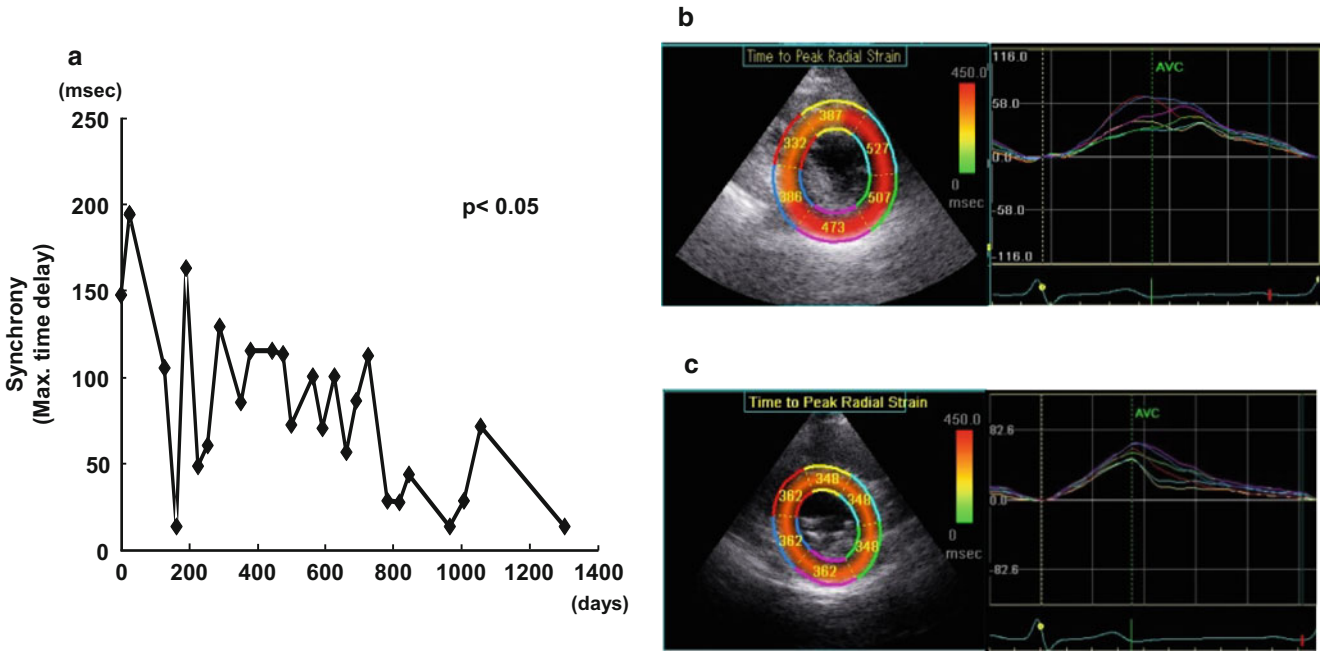


Fig. 2 Time course of LV contraction synchrony. (**a**) Maximum time delay was measured with 2D speckle tracking echocardiogram imaging. Contraction synchrony decreased significantly after LRT ($p < 0.05$). The normal maximum time delay was from 36 to 49 ms in five healthy women. (**b**) Maximum time delay of LV contraction was 195 ms at 25 days after LRT. (**c**) Maximum time delay of LV contraction was 14 ms at 1,206 days after LRT. Synchrony improved significantly

therapy (Jordanb et al. 2005). In a patient with MPS I, we believe that LRT thins the thickened valve and endocardium and decreases both infiltrated interstitial fibroblasts in the LV wall and the empty vacuoles in the cardiomyocyte by GAG degradation.

In the mechanism of heart failure, both the contractile and diastolic functions of the LV are very important. With hypertensive heart disease, hypertrophic cardiomyopathy, restrictive cardiomyopathy, early-stage cardiac amyloidosis, etc., all manifest heart failure with diastolic dysfunction but show almost normal LV contraction. In the patient in this study, LV contraction was normally conserved and not significantly changed by long-term LRT as reported previously (Braunlin et al. 2006; Sifuentes et al. 2007). As mentioned above, since the sarcomeric structure of the cardiomyocyte in MSP I is well conserved, this is why LV systolic function perhaps is conserved.

In a previous report, diastolic function was impaired in the attenuated forms of MPS I (Soliman et al. 2007). To the best of our knowledge, there was no report of the correlation between LRT and diastolic function. In this report, we are the first to clarify the improvement of LV diastolic function in the E/A ratio and DcT by LRT. Before LRT, the diastolic function status showed moderate diastolic dysfunction (pseudo-normalization) (Leite-Moreira 2006; Redfield et al. 2003; Sohn et al. 1997). After long-term LRT, the indices of E/A and DcT were improved. The E wave alteration was much more apparent than that of the A wave, suggesting that the improvement of LV function by LRT was more effective than that of the atrium (data not shown). Furthermore, we found a significant correlation between LV weight and diastolic parameters, and LV contraction synchrony. Accordingly, it is possible that an LRT-induced decrease in LV weight results in the improvement of LV diastolic function, leading to the improvement of LV contraction synchrony.

In conclusion, based on our survey of the literature, our findings are the first to report that long-term LRT improves LV diastolic function and LV contraction synchrony in a patient with MSP I. If LRT had been started when the patient was younger, her clinical status might have been improved. This case report suggests the importance of starting LRT in the initial stages of the disease to prevent clinical and organic complications.

Limitations

In this study, DcT and E/A ratio improved; however, E/e' as another index of diastolic function did not change. This discrepancy might be attributed to perivalvular muscle deformity after valve replacement. Further investigation may be needed.

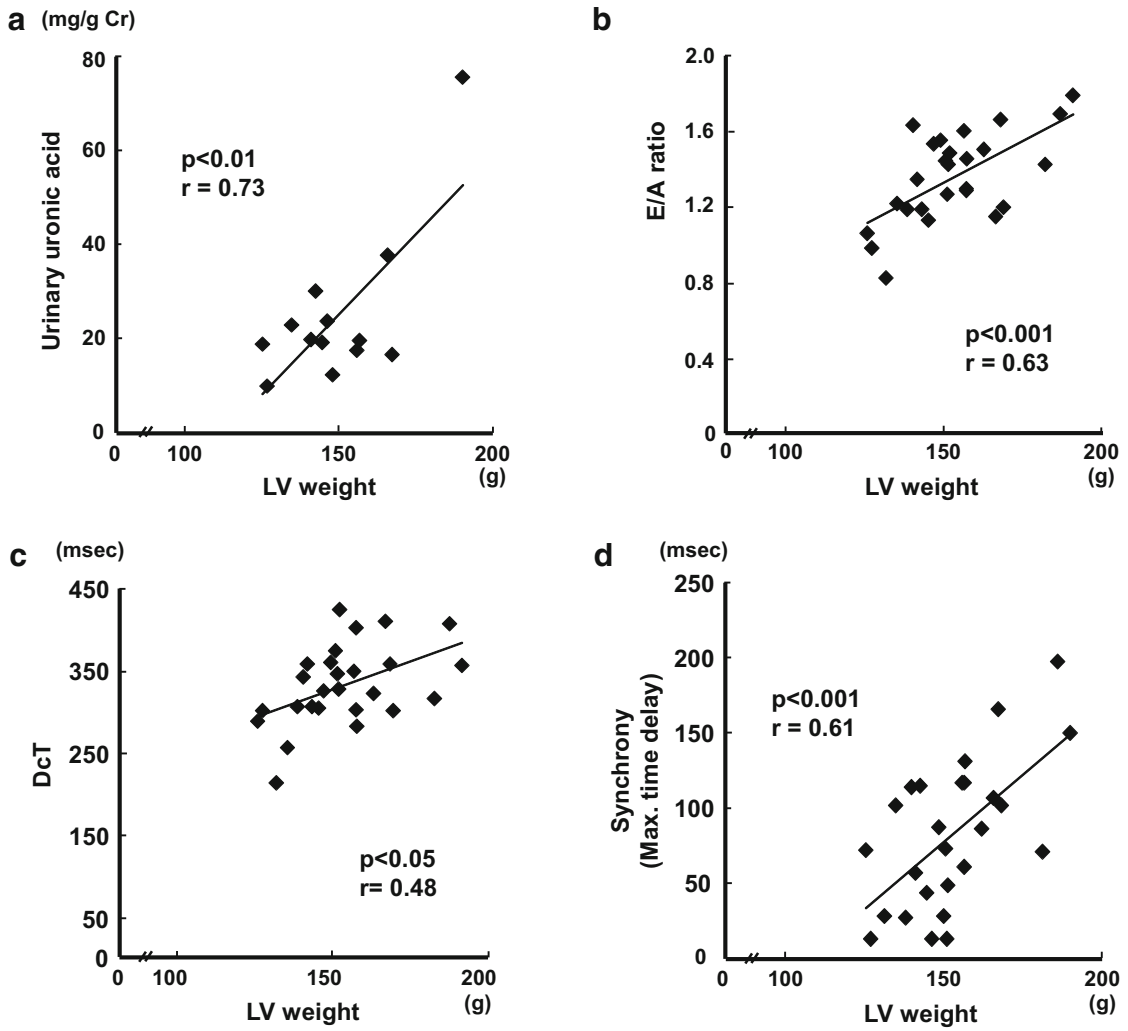


Fig. 3 (**a**) Relationship between estimated left ventricular weight and urinary uronic acid concentration. A significant correlation ($n = 13$, $r = 0.73$, $p < 0.01$) was observed between urinary uronic acid concentration and LV weight. (**b**, **c**, and **d**) Relationship between estimated LV weight and cardiac function. Significant correlations were observed between estimated LV weight and E/A ratio (**b**, $n = 26$, $r = 0.63$, $p < 0.001$), DcT (c, $n = 26$, $r = 0.48$, $p < 0.05$), and synchrony (**d**, $n = 26$, $r = 0.61$, $p < 0.001$), respectively

Acknowledgments We would like to thank Hiroyoshi Ryu and Masaji Honda for their technical assistance in echocardiography and Emiko Shiotani for her excellent secretarial assistance.

Funding

We had no funding source for this study.

Disclosure

All authors declare there is no conflict of interest associated with this study.

Synopsis

Long-term laronidase replacement therapy leads to improvements in left ventricular diastolic function and contraction synchrony in an adult patient with mucopolysaccharidosis I.

Compliance with Ethics Guidelines

Conflict of Interest

Haruhito Harada, Hiroshi Niiyama, Atsushi Katoh, and Hisao Ikeda declare that they have no conflict of interest.

Informed Consent

All procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation in Kurume University and with the Helsinki Declaration of 1975, as revised in 2005. The signed

informed consent form was obtained from the patient for their inclusion in the study.

Animal Rights

This article does not refer to any animal studies performed by the authors.

Author Contributions

Haruhito Harada contributed to data collection, data analysis, and preparation of the manuscript and figures.

Hiroshi Niiyama contributed in terms of clinical therapy.

Atsushi Katoh contributed in terms of clinical therapy and data interpretation.

Hisao Ikeda contributed to data interpretation, literature search, and preparation of the manuscript and figures.

Competing Interests

There are no competing interests.

Funding

There is no funding source for this study.

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JIMD Reports
DOI 10.1007/8904_2014_318

CASE REPORT

Fibrolamellar Hepatocellular Carcinoma Mimicking Ornithine Transcarbamylase Deficiency

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Received: 14 March 2014 / Revised: 23 April 2014 / Accepted: 28 April 2014 / Published online: 5 July 2014

Abstract We report an unusual case of recurrent non-hepatic hyperammonaemic encephalopathy in an adult patient. She had a previous history of treated fibrolamellar hepatocellular carcinoma (FLC). This posed a diagnostic challenge, as she had normal liver function tests and normal looking liver on imaging but with extra hepatic metastases. This case highlights the importance of measuring plasma ammonia levels in all patients presenting with unexplained acute confusion. Clinical awareness of non-hepatic hyperammonaemic encephalopathy can contribute to early diagnosis and timely initiation of life-saving treatment. Delay in treatment results in irreversible brain damage, deep coma and death. Treatment of hyperammonaemia must begin prior to confirmation of aetiology, for a favourable outcome. This case also highlights the need for further research to understand the exact mechanism of hyperammonaemia in hepatocellular carcinoma.

Introduction

Non-hepatic hyperammonaemic encephalopathy in an adult is a diagnostic challenge for the clinician. Its aetiology includes both inherited and acquired causes. Late onset ornithine transcarbamylase (OTC) deficiency is one of the most common inherited urea cycle defects presenting in adult life as hyperammonaemia. Our patient presented with biochemical features of OTC deficiency though this was not confirmed on genetic testing. Rapidly growing hepatic tumours are known to cause hyperammonaemia, however, the exact mechanism remains to be elucidated. Physicians should be aware of this rare complication in order to provide early diagnosis and treatment.

Case Report

A 20-year-old woman was admitted to hospital with an acute onset of confusion and agitation following repeated vomiting. She had an underlying history of fibrolamellar hepatocellular carcinoma(FLC), diagnosed at the age of 14, and initially treated by left lateral hepatectomy. At age 17 she had developed extra hepatic disease recurrence (abdominal lymphadenopathy) but with no recurrent tumour in the liver. She was treated by chemotherapy, which was well tolerated and she remained in partial remission for the succeeding three years. She last received chemotherapy ten months before this admission. Blood tests on admission including liver function, renal function, serum urate, full blood count and coagulation screen were normal. Computed tomography (CT) of head, including CT venogram, and magnetic resonance imaging (MRI) of brain were also normal. Lumbar puncture revealed normal cerebrospinal fluid. No bacterial infection was detected. Toxicology, viral and autoimmune disease screen were negative. A working diagnosis of hepatic encephalopathy was made and she was initially treated with oral lactulose and phosphate enema with resolution of her symptoms. However, she was re-admitted within few weeks of discharge, with similar symptoms. She then had electroencephalography which suggested metabolic encephalopathy with diffuse slowing and high incidence of triphasic waves

Communicated by: Pascale de Lonlay

Competing interests: None declared

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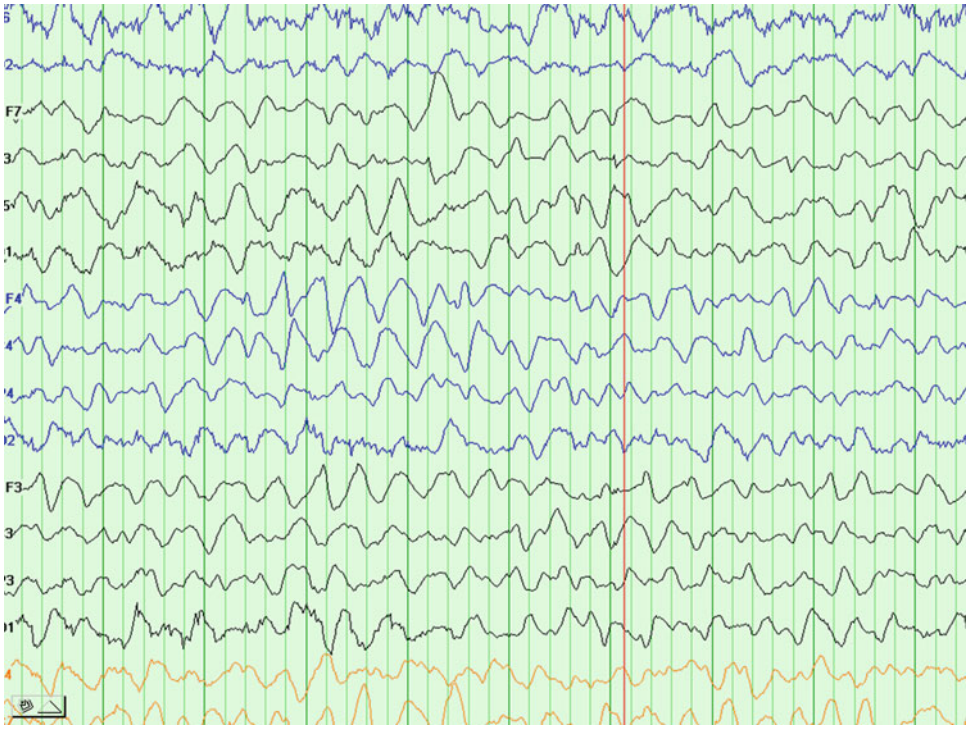

Fig. 1 Electroencephalography showing a diffuse slowing and high incidence of triphasic waves

(Fig. 1). A subsequent plasma ammonia level was 281 μmol/L (<50 μmol/L) with peak ammonia level of 410 μmol/L. CT scan of abdomen (Fig. 2) showed widespread intra-abdominal lymphadenopathy, consistent with progression of her recurrent cancer, encasing blood vessels, but there were no focal hepatic lesions and no portosystemic shunting was evident. Further metabolic investigations revealed high plasma glutamine levels 825 μmol/L (413–690 μmol/L), low ornithine 11 μmol/L (29–125 μmol/L), low citrulline18 μmol/L, arginine113 μmol/L and grossly elevated urinary orotic acid levels 420.3 μmol/mmol creatinine (<3 μmol/mmol creatinine), suggesting an inherited urea cycle disorder namely, ornithine transcarbamylase (OTC) deficiency. Genetic testing, however, did not reveal a pathogenic sequence, variant, or exon deletions/duplication in OTC gene. In addition, there was no relevant family history. She was treated with intravenous sodium phenyl butyrate, sodium benzoate and arginine with good response.

Over the next few months, she had multiple admissions with hyperammonaemic encephalopathy, each time responding well to intravenous sodium benzoate and phenyl butyrate infusion. She was then commenced on regular oral sodium benzoate, phenyl butyrate and arginine. During follow-up, a repeat plasma amino acid levels showed high glutamine 716 μmol/L, low citrulline 23 μmol/L and low arginine 16 μmol/L while urine orotic acid level was very high 687 μmol/mmol creatinine. However later, with improved compliance to ammonia scavenging medication she had no further episode of acute encephalopathy. She was also commenced on palliative chemotherapy with Sorafenib. She remained metabolically stable though her general condition gradually deteriorated due to her underlying malignancy and she passed away in 6 months.

Discussion

Hyperammonaemic encephalopathy is a well-known complication of advanced liver disease. Other causes include rare inherited metabolic disorders such as urea cycle defects and organic acidaemias. It has also been reported in association with drugs like Valproate, porto-systemic shunts, malignancies including multiple myeloma, hepatocellular carcinoma (HCC), following chemotherapy for haematological malignancies, bone marrow transplantation and reconstructive surgeries like bariatric surgery and ureterosigmoidoscopy. Fibrolamellar hepatocellular carcinoma (FLC) is a rare variant of hepatocellular carcinoma of unknown aetiology, arising almost exclusively in a non-cirrhotic liver of young adults and in the absence of chronic viral hepatitis. This patient received conventional treatment comprising liver

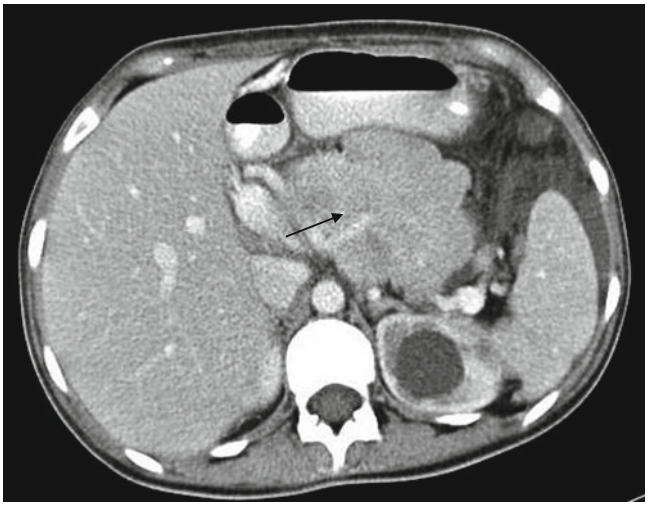

Fig. 2 CT of the abdomen showing epigastric neoplastic mass with encasement of major coeliac axis branches (*arrow*)

resection and upon disease recurrence cytotoxic chemotherapy neither of which precipitated encephalopathy, and she remained well for several years. It was unexpected of her to present with hyperammonaemic encephalopathy with normal liver function and no imaging evidence of hepatic lesion or porto-systemic shunt. Although initial metabolic investigations suggested inherited OTC deficiency, this was not confirmed by DNA analysis. Similar cases have been reported in the literature of patients with FLC (Jeffers et al. 1988; Sethi et al. 2009) presenting with hyperammonaemic encephalopathy, in some cases related to chemotherapy (Chan et al. 2008; Winter et al. 1997). A previous study on rats has shown an inverse relationship between ornithine carbamyl transferase activity and the rate of growth of hepatoma (Weber et al. 1972). It is possible that FLC may release a chemical inhibitor of OTC enzyme or there is increased activity of ornithine decarboxylase. There may be genetic alteration associated with under expression of OTC gene during the course of development of FLC leading to hyperammonaemia.

In view of her deteriorating condition, liver biopsy was not performed to assess OTC enzyme activity.

This case illustrates the diagnostic challenge posed by unexplained encephalopathy with normal routine biochemical, haematological and microbiological studies as well as brain imaging. Such patients should be evaluated thoroughly to identify any treatable cause, including measurement of blood ammonia levels and referral for specialist metabolic assessment. Although the precise mechanism of the development of hyperammonaemia in FLC remains unclear, treatment with ammonia scavenging medication resolves encephalopathy. Further studies are required both at a molecular and biochemical level to determine the cause for the lack of urea cycle expression in hepatic tumour cells causing significant hyperammonaemia.

Acknowledgement We are grateful for the contribution of Dr Rachel Cooney, Adam Gerrard, and Prof. D H Palmer in the management of this patient.

Synopsis

Patients with unexplained encephalopathy should also be investigated for hyperammonaemia for early initiation of potentially life-saving treatment.

Details of the Contributions of Individual Authors

Dr R A Sulaiman did the planning, literature search, and wrote the manuscript of this case report.
Dr T G Hiwot reviewed and edited the manuscript.
Guarantor of this article: Dr T G Hiwot

Compliance with Ethics Guidelines

Conflict of Interest

R A Sulaiman and T G Hiwot declare that they have no conflict of interest.

Informed Consent

Patient had signed an informed consent form, giving permission for the publication of this case report. This will be available on request.

Animal Rights

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

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JIMD Reports
DOI 10.1007/8904_2014_319

CASE REPORT

Reversal of Respiratory Failure in Both Neonatal and Late Onset Isolated Remethylation Disorders

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Received: 31 December 2013 / Revised: 04 May 2014 / Accepted: 08 May 2014 / Published online: 6 July 2014

Abstract Respiratory failure is a well-documented potential presentation of inherited isolated remethylation disorders (IRDs). It appears to be a combination of both central and peripheral neuropathy and has previously often been considered to herald an irreversible neurological decline. We present three patients, one with methionine synthase (cblG) and two with methyltetrahydrofolate reductase deficiency (MTHFR). One patient with MTHFR presented in infancy, and other patients in later childhood. All three patients required intubation for respiratory failure but in all three, this was totally reversed by the initiation of medical therapy. This consisted of betaine and folinic acid supplementation in all three, methionine in two and cobalamin supplementation in two. The rate of respiratory improvement was variable, though two of the cases were successful extubated within a week of commencement of medical therapy. We document their subsequent clinical, biochemical and electrophysiological progress and review the potential pathological mechanisms underlying respiratory failure in these disorders.

Communicated by: Garry Brown

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Introduction

Isolated remethylation disorders (IRDs) are a rare, inherited group of enzymatic deficiencies, which result in impaired conversion of homocysteine to methionine without accumulation of methylmalonic acid. They include the rare Cobalamin D1 variant (cblD-HC) and deficient activity in the enzymes, Methyltetrahydrofolate reductase (MTHFR), methionine synthase (cblG) and methionine synthase reductase (cblE). While the exact pathological mechanisms are still be fully elucidated, the neurological impairment seen in these disorders appears to be chiefly derived from the disturbance in the cellular methylation index (Weir and Scott 1995) and the toxic effects of homocysteine. Clinically IRDs can present with, or develop respiratory failure (de Ogier et al. 1998; Rosenblatt and Fenton 2001); with this often being heralded by an acute irreversible decline in overall clinical state (Narisawa et al. 1977; Nishimura et al. 1985; Al Tawari et al. 2002; Tsuji et al. 2011). Up to now, there have been extremely limited examples of successful therapeutic intervention (Surtees et al. 1991; Schiff et al. 2011). We document the resolution of the respiratory failure of three previously unreported patients with isolated remethylation defects (one methionine synthase deficiency and two MTHFR deficient patients). One patient with MTHFR deficiency presented neonatally, while the two other patients presented in later childhood. The respiratory failure in each case seems to have resulted from a combination of central and peripheral neurological impairment. We report on their presentation and response to therapy.

Case Reports

Patient 1

Patient 1, diagnosed with methionine synthase deficiency both on complementation and functional fibroblast studies (MeCbl synthesis in fibroblast 16%, total reference range 58 ± 18%), is the only child of an Asian father and Caucasian mother. After an uneventful antenatal course she was born at term weighing 2.44 kg. There were no initial perinatal concerns, but by 8 months of age she underwent genetic investigation for global developmental delay and generalised hypotonia. At 9½ years of age, when reviewed for two isolated seizures, she had ten distinct words, could run and scribble on paper but was still urinary incontinent. Reinvestigation at that time demonstrated a raised homocysteine and reduced methionine (see Table 1), a raised mean corpuscular volume (MCV) but normal plasma B12 and folate and urinary methylmalonic acid levels (MMA). A putative diagnosis of a remethylation disorder was made, however successful introduction of folinic acid and betaine were hampered by the patient's refusal to take oral medication.

While initiation of therapy was being attempted, her parents noted a decrease in her walking capacity and increasing lethargy. This worsened rapidly over the course of several weeks, culminating in her becoming unrousable. On admission to hospital, she had a partially compensated respiratory failure (pH 7.28, PCO_2 = 13 kPa, bicarbonate = 35 mmol/L), requiring ventilation. Extensive viral and bacterial investigations, including lumbar puncture, showed no evidence of infection. Her subsequent medication and biochemical responses to these are documented in Table 1. On admission to intensive care she had bilateral flaccid paralysis and absent deep tendon reflexes (DTRs) in her legs and limited antigravity movements with decreased reflexes in her arms. Diaphragmatic ultrasound indicated complete bilateral diaphragmatic palsy, while nerve conduction and electromyography (EMG) showed absent phrenic nerve responses and neurogenic changes bilaterally in both the intercostal muscles and the diaphragm. Further electrophysiological investigations showed undetectable sensory responses and only minimally recordable motor responses in her legs, while the upper limb and bulbar investigations also showed patchy neurogenic involvement. Her cranial MRI on admission showed no acute changes but a generalised lack of cerebral volume with some non-specific periventricular white matter changes. The magnetic resonance imaging (MRI) of her spine was normal.

Within 24 h of initiation of therapy to improve remethylation she was able to interact with her parents. She was extubated on day 5, though required 1 L of oxygen and demonstrated marked tachypnoea and respiratory distress. Antigravity movements in her legs were noted on day 10. Polysomnography on day 14 showed a mean nocturnal transcutaneous CO_2 of 58 mmHg. After a month on therapy, she only required nocturnal oxygen supplementation and by 3 months post admission full polysomnographic examination was normal. Six months post admission she had regained all her previously acquired skills and had a completely normal physical examination.

Patient 2

Patient 2, a patient who has MTHFR confirmed on fibroblast studies (MTHFR activity 0.23 nmol/h/mg, control 25.3 ± 8.9 nmol/h/mg), was born at term, to non-consanguineous Asian parents, with no antenatal complications and weighed 2.98 kg. Again initial investigations for global developmental delay at a year were uninformative. At the age of 6½ years old, just prior to her clinical decompensation, she was fully fed orally, able to stand in a standing frame and to say her own name. Over the course of a 6-week period, she developed increasing sleepiness and poor feeding, culminating in respiratory failure, necessitating her transfer to the regional intensive care unit for ventilation. A cranial MRI on admission revealed a generalised global diffuse atrophy but no acute lesions. She was started on betaine, mefolinate and cyanocobalamin (see Table 1) on day 7 of her admission, when her raised homocysteine, low methionine and absence of urinary MMA or macrocytosis suggested MTHFR deficiency. After 48 h, she was alert and her previous apnoeic spells had improved markedly. Nerve conduction studies at onset of remethylation therapy demonstrated suppressed amplitude in both posterior tibial, median and ulnar nerves and her EMG findings were also consistent with decreased innervation. This corresponded to the clinical findings of profound hypotonia, areflexia and an MRC power assessment score of grade 1 in all four limbs. Her ventilatory support was initially weaned rapidly and extubation was first attempted at 48 h after betaine was introduced. However it was prevented by recurrent left lower lobe collapse, which occurred at low pressures. Thoracic CT and bronchoscopy excluded extrinsic compression and intrinsic obstruction, while diaphragmatic ultrasound indicated unilateral diaphragmatic palsy.

Twenty-one days post initiation of therapy the power in the patient's upper limbs had improved to an MRC score of 4 but the left diaphragmatic palsy continued. A tracheostomy allowed gradual weaning, with the patient coming off all ventilatory support at 127 days post initiation of therapy. Two years post her acute decompensation, she has normal respiratory function, reflexes and upper limb strength, though her lower limbs still show some residual weakness.

Table 1 Therapy and the biochemical responses

Time	Plasma THCy (μmol/L) [<15]	Plasma Met (μmol/L) [10–60]	CSF Met (μmol/L) [1–4]	CSF MTHF (nmol/L) [52–178]	Betaine (g/day)	B12 (mg/day IM)	Calcium folinate (mg/day)	B6 (mg/day)	Met supplement (g/day)
Patient 1: Methionine synthase									
Pre Rx	140	14							
PICU day 0	78	14	4		8	1	15	200	
PICU Day 3	88	285			8	1	15	200	2
PICU Day 4	80	345			8	1	15		1
+8 months	61	54			8	1	15		1
	Plasma THCy (μmol/L) [5–15]					B12 (mg once per week)	Mefolinate (mg/day)		
Patient 2: MTHFR									
Pre	269	7		0					
1 month	88	10			6	10	15		
2 month	80	6			6	10	15		
+1 year	123	18		18	6	10	15		
+2 years	113	24			6	10	15		
+3 years	111	21			6	10	15		
	Plasma THCy (μmol/L) [<15]					B12 (mg/day)	Calcium folinate (mg/day)	Riboflavin (mg/day)	
Patient 3: MTHFR									
Pre Rx	166	16	1						
2 weeks	212	6						50	
3 weeks	115	24			4	1 IM	15		200
8 months	112	34			4		15		200
+2 years	123	19	1	10	4		15		200

The normal ranges for each of the metabolite are indicated in the square brackets. NB all medication given orally unless indicated

Patient 3

Patient 3 is homozygous for a novel splice site mutation c.1530G>A in the 3′ splice site of exon 9 of MTHFR. This is predicted to be pathogenic by splice site prediction software MaxEntscan, NNSplice and Genesplicer. She is the second child of consanguineous Asian parents, whose only previous child had died of respiratory failure at 2 months of age, shortly after placement of a VP shunt for a Dandy–Walker malformation. After delivery at term, weighting 2.97 kg, she was initially well but developed increasing feeding difficulties from 2 weeks. At referral, for investigation of recurrent cyanotic spells at 4½ months of age, she had marked failure to thrive, global developmental delay and acquired macrocephaly. On examination she had gross hypotonia with head lag, and minimal lower limb movement (MRC power score 3/5) but her DTR could be elicited. Given her hypotonia and respiratory weakness, she was initially started on riboflavin for possible Brown–Vialetto–Van Laere Syndrome. Subsequent biochemical investigations indicated an isolated remethylation disorder and her subsequent medication and biochemistry are noted (see Table 1).

Subsequent to a cranial MRI, which suggested obstructive hydrocephalus, she failed 2 extubations due to poor respiratory drive and a chest X-ray indicated a raised right hemidiaphragm, consistent with right phrenic nerve palsy. Nerve conduction studies showed prominent denervation with re-innervation in both upper and lower limbs and some bulbar impairment. She remained intubated for 7 days post

initiation of therapy and required CPAP for a further 1½ weeks. In total she required 3½ weeks of respiratory support. Her cyanotic episodes proved to be seizure related, this initially settled without specific seizure medication. A sleep study at 1 year of age was normal and gastronomy was successfully inserted without complications. At 2 years of age she is starting to eat more and stand unsupported, however she now requires sodium valproate for seizure control.

Discussion

All three patients described here had remethylation defects and had varying degrees of respiratory compromise on presentation. Respiratory failure is a well-documented complication of isolated remethylation disorders (Rosenblatt and Fenton 2001), being particularly prominent in children presenting between 3 months to 10 years though it can occur at any age (de Ogier et al. 1998). The pathophysiological mechanisms underlying this respiratory failure are poorly defined but on the evidence of the patients described here, appear to be multifactorial in nature, encompassing both central and peripheral neurological impairment with the combination of both ultimately leading to inadequate ventilation.

Peripheral neuropathy is a well-described late onset complication in IRDs, (Pasquier et al. 1994; Haworth et al. 1993; Tonetti et al. 2003; Chaabene-Masmoudi et al. 2009) but is also increasingly recognised in the infantile onset age group (Tsuji et al. 2011; Al Tawari et al. 2002). In this case series there was direct electrophysiological evidence of phrenic nerve dysfunction in patient 1, while patients 2 and 3 had radiological evidence strongly suggestive of diaphragmatic, i.e. phrenic nerve dysfunction. Also all three patients had other electrophysiological evidence of generalised peripheral nerve involvement. As was seen in patients 1 and 2 here, worsening lower limb neuropathy can herald potential respiratory decompensation (Nishimura et al. 1985; Kishi et al. 1994). The response of this peripheral neuropathy is often at best limited (Chaabene-Masmoudi et al. 2009; Schiff et al. 2011); however, in this case series complete electrophysiological resolution was documented in the infantile onset MTHFR case accompanied by normalisation of clinical symptomology, while apparent clinical resolution was seen in patients 1 and 2.

Given the limited numbers and the retrospective nature of these cases, it is difficult to be certain as to why there seems to be such a greater improvement in peripheral nervous function between these cases and those previously described in literature. It is possible that the early introduction of methionine may have played a role, with two of the patients described here, having methionine started as part of the initial attempt to normalise their biochemical milieu. Patient 2 who was not supplemented took the longest to resolve and indeed continued initially to have low levels of plasma methionine despite betaine supplementation, though low methionine has been documented in other patients even with methionine supplementation (Clayton et al. 1986; Chaabene-Masmoudi et al. 2009). It has previously been seen that methionine supplementation has resulted in a rapid improvement in a neonate with MFHTR already on betaine and folate (Abeling et al. 1999); with the suggestion that the betaine:homocysteine methyltransferase was inadequate to fully maintain remethylation. It was thought to also be benefical in three patients in a recent case series by Schiff et al. (2011), however it is still not standard therapy for MTHFR and has not been frequently used historically. It is also possible that betaine therapy has in these patients been started early enough (Diekman et al. 2014) to prevent the decrease in neuronal numbers that has been previously noted (Nishimura et al. 1985) and attributed to the reduction of myelin (Surtees et al. 1991). Certainly adequate concentrations of methionine are required to produce *S*-adenosylmethionine (AdoMet) (Surtees et al. 1991), the major methyl donors (Katz et al. 2003) with low levels of AdoMet being seen in a range of remethylation disorders (Surtees 1998). In turn methylation is crucial in myelin formation as it determines both the tertiary structure of myelin basic protein (Amur et al. 1986) and is vital for the production of phosphatidylcholine, a key constituent of the myelin sheath (Weir and Scott 1995).

It has also been suggested that that *S*-adenosylhomocysteine (AdoHcy), a potent inhibitor of methyltransferases (Schatz et al. 1981), shown to accumulate with high levels of homocysteine (Dayal et al. 2001; de la Haba and Cantoni 1959) may prevent methylation and inhibit myelination (Weir and Scott 1995). However these cases seem to suggest that this may not be as important a factor, given patient 3's improvement despite her persistent homocysteine levels >110 μmol/L.

The major presynaptic input for the phrenic nerve is derived from the dorsal and ventolateral respiratory centres in the medulla. Thus central nervous system depression leading to presynaptic inhibition may also cause phrenic nerve inhibition. Central causes of respiratory failure seem to have been a major cause of in the early onset case of MTHFR reported here, with recurrent apnoeas occurring. This lack of central drive may have resulted from pressure effects since patient 3 did show ventriculomegaly suggestive of hydrocephalus. This has been described in neonatal MTHFR patients who presented with apnoea and required shunting (Tsuji et al. 2011; Baethmann et al. 2000). However the very

rapid improvement in this patient is hard to explain purely in terms of pressure effects. Other potential mechanisms for the improved respiratory drive would include the improvement of the low levels of brain choline seen in patients with remethylation disorders (Debray et al. 2008) or improved cerebral glucose uptake related to increase AdoMet levels (Al-Essa et al. 1999).

While it is likely that improved muscle function resulted from improved neurological function, all the patients had a period of inadequate nutrition prior to their admission to PICU. However the timing of the improvements in respiratory function all coincided with the introduction of medication targeted at improving remethylation, not with the institution of general supportive measures. It also is to be noted, that although the clinical manifestations of remethylation disorders are very similar (de Ogier et al. 1998), the levels of tetrahydrofolate/available cellular folate pool is reduced to a greater degree in MTHFR than CblG which may result in differing pathological cascades in the different remethylation disorders.

Summary

Respiratory failure in isolated remethylation disorders is likely to be multifactorial in nature with these cases suggesting that the prompt institution of appropriate therapy can result in its complete resolution. While the timeframe to complete resolution varied some definitive improvements were seen within the first couple of weeks of therapy in all patients.

Acknowledgements The authors would like to acknowledge the contribution of Professor B Fowler and the University Children's hospital Zurich for the fibroblast work on patients 1 and 2 and Dr Rodenburg and Prof Van Den Heuvel at the Stichting klinisc-Genetisch Centrum Nijmegen for their work on patient 3.

Synopsis

Acute respiratory failure seen in remethylation disorders is multifactorial but with aggressive medical management can be reversed.

Compliance with Ethics Guidelines

Alexander Broomfield, Lara Abulhoul, Matthew Pitt, Elisabeth Jameson and Maureen Cleary declare that they have no conflict of interest.

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

Contributions of Each Author

Dr Broomfield conception and design of study, draft article. Dr Abulhoul revision of article and collection of data. Dr Jameson design of article and revision. Dr Pitt analysis and interpretation of data. Dr Cleary revision of article and provision of data.

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JIMD Reports
DOI 10.1007/8904_2014_320

RESEARCH REPORT

Seven Novel Mutations in Bulgarian Patients with Acute Hepatic Porphyrias (AHP)

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Received: 23 February 2014 / Revised: 07 May 2014 / Accepted: 09 May 2014 / Published online: 6 July 2014

Abstract Acute intermittent porphyria (AIP), variegate porphyria (VP), and hereditary coproporphyria (HCP) are caused by mutations in the hydroxymethylbilane synthase (HMBS), protoporphyrinogen oxidase (PPOX), and coproporphyrinogen oxidase (CPOX) genes, respectively. This study aimed to identify mutations in seven Bulgarian families with AIP, six with VP, and one with HCP. A total of 33 subjects, both symptomatic ($n = 21$) and asymptomatic ($n = 12$), were included in this study. The identification of mutations was performed by direct sequencing of all the coding exons of the corresponding enzymes in the probands. The available relatives were screened for the possible mutations. A total of six different mutations in HMBS were detected in all seven families with AIP, three of which were previously described: c.76C>T [p.R26C] in exon 3, c.287C>T [p.S96F] in exon 7, and c.445C>T [p.R149X] in exon 9. The following three novel HMBS mutations were found: c.345-2A>C in intron 7–8, c.279-280insAT in exon 7, and c.887delC in exon 15. A total of three different novel mutations were identified in the PPOX gene in the VP families: c.441-442delCA in exon 5, c.917T>C [p.L306P] in exon 9, and c.1252T>C [p.C418R] in exon 12. A novel nonsense mutation, c.364G>T [p.E122X], in exon 1 of the CPOX gene was identified in the HCP family. This study, which identified mutations in Bulgarian families with AHP for the first time, established seven novel mutation sites. Seven latent carriers were also diagnosed and, therefore, were able to receive crucial counseling to prevent attacks.

Abbreviations

AHP	Acute hepatic porphyrias
AIP	Acute intermittent porphyria
ALA	δ-Aminolevulinic acid
CPOX	Coproporphyrinogen oxidase
HCP	Hereditary coproporphyria
HMBS	Hydroxymethylbilane synthase
PBG	Porphobilinogen
PPOX	Protoporphyrinogen oxidase
VP	Variegate porphyria

Introduction

Acute intermittent porphyria (AIP) (OMIM 176000), variegate porphyria (VP) (OMIM 176200), and hereditary coproporphyria (HCP) (OMIM 121300) are autosomal dominant, low-penetrant inborn errors of the heme biosynthesis pathway that result in the decreased activity of hydroxymethylbilane synthase (HMBS) (EC 4.3.1.8), protoporphyrinogen oxidase (PPOX) (EC 1.3.3.4), and coproporphyrinogen oxidase (CPOX) (EC 1.3.3.3), respectively.

AIP, VP, and HCP present with clinically identical recurrent neurovisceral attacks. Additionally, erosive bullous cutaneous lesions and hyperpigmentation on sun-exposed areas are more common in VP than in HCP (Sassa 2006). The acute attacks include three major classes of symptoms: gastrointestinal, neurological, and psychiatric. These symptoms are represented by severe abdominal pains, motor neuropathy, depression, and psychosis. The most common factor trigger-

Communicated by: Eva Morava, MD PhD

Competing interests: None declared

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ing these attacks is the use of numerous porphyrinogenic drugs. Infections, alcohol, a low-calorie diet, and natural sex hormones fluctuations in women, related to menstrual cycle and pregnancy can also provoke attacks (Kappas et al. 1995).

The diagnosis of acute porphyric attack is based on both clinical manifestations and typical biochemical abnormalities. The main laboratory finding is the dramatic increase of the porphyrin precursors porphobilinogen (PBG) and δ-aminolevulinic acid (ALA) in urine. The exact distinction between the three different diseases requires measuring the urinary and fecal porphyrin excretion patterns, which are characteristic for each enzymatic defect. A fluorescence scan of native plasma is also an important diagnostic criterion, presenting (or not) a characteristic peak for each entity (Sassa 2006; Hift et al. 2004). Decreased levels of HMBS activity in AIP, PPOX activity in PV, and CPOX activity in HCP clarify the diagnosis in the proband and establish the enzymatic defect in the latent carriers (Meyer et al. 1972; Deybach et al. 1981). Unfortunately, when measuring enzymatic activities, results similar to the reference values may bring in uncertainty in the precise diagnosis of the latent carriers (Mustajoki 1981). Thus, the optimal strategy for the detection of these individuals includes the implementation of molecular genetic methods (Whatley et al. 2009).

At least 600 different mutations in the HMBS, PPOX, and CPOX genes have been identified so far (www.hgmd.cf.ac.uk). Most of these mutations are specific to one or a few families, although a founder effect has been clearly demonstrated for both AIP and VP (Thunnel et al. 2006; Meissner et al. 1996). Most AIP, VP, and HCP carriers are heterozygotes. Mutations are heterogeneous and are comprised of single nucleotide substitutions, small insertions and deletions. Recently, large insertions/deletions have been described in the HMBS, PPOX, and CPOX genes (Whatley et al. 2009; Barbaro et al. 2013). In Bulgaria, during a 50-year period as the sole porphyria service at University Hospital "Saint Ivan Rislki" Sofia, 35 families with AIP, 20 with VP, and 2 with HCP have been diagnosed, treated, and followed up. However, the molecular analysis of the patients with AIP, VP, and HCP has not yet been performed. The aim of this study was to identify mutations in the HMBS, PPOX, and CPOX genes in Bulgarian families with acute hepatic porphyrias.

Materials and Methods

Patients

Seven independent index cases with AIP were included. The diagnosis was based on clinical symptoms and increased urinary PBG and ALA values. Decreased levels of HMBS activity in erythrocytes and the absence of a plasma fluorescence peak at 624–627 nm confirmed the diagnosis of AIP. Six independent index cases with VP were also studied. The diagnosis of these patients included the evaluation of the clinical symptoms, a typical plasma fluorescence peak at 624–627 nm, elevated urinary PBG and ALA levels during acute attacks and increased stool porphyrins, with a predominance of protoporphyrin over coproporphyrin in the cases with cutaneous symptoms. One patient with HCP was included. The diagnosis was based on the symptoms that occurred during acute attack, increased urinary PBG and ALA levels, markedly increased total porphyrins in the urine, and a plasma fluorescence peak at 618 nm. Molecular analysis of the HMBS, PPOX, and CPOX genes confirmed the precise diagnosis. These 14 probands were diagnosed, treated, and followed up in the Porphyria Unit of "Saint Ivan Rilski" University Hospital Sofia. The precise places of birth and the pedigree trees of the patients were determined, with no apparent signs of consanguinity. Once the diagnosis of AIP was confirmed, the HMBS activity was evaluated in the available asymptomatic family members. A total of 33 individuals, including the probands and asymptomatic relatives, from 15 families with AIP, VP, and HCP gave their written consent to participate in this study, which was approved by the Ethics Committee.

Methods

Biochemical Measurements

PBG and ALA levels in the urine were measured according to the method described by Mauzarell and Granick (1956). Urinary and fecal porphyrins were assessed according to the method of Rimington (1971). Total fecal total porphyrin levels were measured according to the method of Lockwood et al. (1985). Total porphyrin levels in urine were evaluated according to our modification and optimization of the method described by Deacon and Elder (2001). HMBS activity in the erythrocytes was determined according to the method described by Adjarov et al. (1994). Plasma fluorescence scanning was performed on a Perkin–Elmer fluorescence spectrophotometer MPF 43, with an excitation wavelength of 398 nm and an emission spectrum from 580 to 700 nm.

Identification of Mutations

Genomic DNA was isolated from peripheral whole blood samples using the innuPREP Blood DNA Midi kit (Analytik Jena Life Science, Germany) according to the manufacturer's protocol. PCR amplification of exons 1 to

Table 1 Clinical and biochemical data and mutations detected in the HMBS gene in families with acute intermittent porphyria

Fam	Pts	Sex	Age/age at first symptoms	Nucleotide change	Amino acid change	Symptoms	HMBS	No. of acute attacks	Urine		TF	TT
									PBG	ALA		
I	P1	F	40/19	c.76C>T	p.R26C	+	18.7	1	715	95	MC	Glu
	Fa	M	65/44	c.76C>T	p.R26C	+	20.1	–	129	122	Inf + M	–
II	P2	F	47/20	c.279-280insAT[a]	Frameshift	+	14.1	2	153	42.7	–	C + Glu
	Sc1	F	45/26	c.279-280insAT[a]	Frameshift	+	10	2	189	–	P	Glu
	Sc2	F	37/30	c.279-280insAT[a]	Frameshift	+	14.3	1	304	–	Inf + M	Glu
	Mo	F	69	c.279-280insAT[a]	Frameshift	–	14	–	–	–	–	–
III	P3	F	44/24	c.887delC[a]	Frameshift	+	20.6	1	320	19.4	MC	Hem
	So	M	20	Neg		–	34.3	–	–	–	–	–
IV	P4	F	27/22	c.445C>T	p.R149X	+	ND	2	955	100	MC	Glu
	Mo	F	50	c.445C>T	p.R149X	+	ND	–	350	78	MC	–
	Si	F	28	Neg	–	–	ND	–	–	–	–	–
V	P5	F	59/39	c.345-2A>C[a]	Splice acceptor site	+	18.7	1	341	214	Inf + M	C + Glu
VI	P6	F	43/25	c.287C>T	p.S96F	+	25.1	1	421	45	MC	Glu
	D	F	21	c.287C>T	p.S96F	–	ND	–	–	–	–	–
VII	P7	F	42/28	c.287C>T	p.S96F	+	ND	1	384	130	MC	Glu
	D	F	15	c.287C>T	p.S96F	–	ND	–	18.2	–	–	–

Fam family, *Pts* patients, *P* proband number, *Fa* father, *Sc* second cousin, *Mo* mother, *So* son, *Si* sister, *D* daughter, *F* female, *M* male, *HMBS* hydroxymethylbilane synthase activity in the erythrocytes, normal range: 25–45 pkat/gHb, *PBG* porphobilinogen, normal value: <15 μmol/24 h, *ALA* δ-aminolevulinic acid, normal range: 11.4–57.2 μmol/24 h, *TF* triggering factor, *Inf* infection, *M* medication, *MC* menstrual cycle, *P* pregnancy, *TT* treatment, Glu, 10% glucose i.v. infusion, *C* cimetidine, *Hem* Hem-arginate (Normosang), *ND* not determined
[a] Novel mutations identified

15 for HMBS, exons 1 to 13 for PPOX, and exons 1 to 7 for CPOX, with corresponding flanking intron–exon boundaries, was performed; the primers and PCR conditions are available upon request. The PCR products were automatically sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, USA).

To prove the rarity of the identified novel missense mutations, the corresponding exons of the HMBS and PPOX genes were screened in 96 control DNA samples. In silico prediction of the pathogenicity of these mutations was determined by the HumVar score using the PolyPhen-2 tool (http://genetics.bwh.harvard.edu/pph2/).

Results and Discussion

Detailed clinical, biochemical, and genetic data are presented in Table 1 for the AIP families and in Table 2 for the VP and HCP families. In three of our female AIP patients, the attacks were related to the patient's menstrual cycle. Infections and/or medications played a triggering role in three AIP and four VP cases, as well as in the HCP patient. Nine subjects with AIP manifested with one or two acute attacks. Some patients (family I-F, family II-P2 and II-Sc1, family IV-P4 and family V-P5) suffered chronic symptoms, including fatigue, lower back pain, paresthesia in the lower limbs, and depression. The proband P2 had suffered from two unrecognized acute attacks and had residual paresis at the time of presentation in our clinic. She also had chronic neurological symptoms when Cimetidine treatment was applied. During the 6 months course of Cimetidine administration, a reduction in porphyrin precursors levels and clinical improvement was achieved. Family V-P5 suffered from one acute attack following infection and antibiotic treatment after surgery. She also had chronic neurological symptoms. Cimetidine was administered after the acute onset, but over the first weeks the pains worsened and no biochemical improvement was noticed. Both acute and cutaneous symptoms were present in four symptomatic VP patients, and only acute symptoms were present in one patient and only cutaneous symptoms in four patients. All VP patients manifested with a single acute attack, and only family III-So exhibited chronic symptoms similar to those observed in AIP. The majority of our VP patients presented with severe photodermatosis alone or accompanying acute

Table 2 Clinical, biochemical and genetic characteristics of families with variegate porphyria and hereditary coproporphyria

Fam	Pts	Sex	Age/age at first symptom	Gene	Nucleotide change	Amino acid change	Symptoms		Plasma scan	Urine					Feces					
							Acute	Skin		PBG	ALA	TPu	Uro	Cop	TPf	Uro	Cop	Proto	TF	TT
I-VP	P1	F	28/21	PPOX	c.441-442delCA	Frame-shift	+	–	626	37	40	4,675	2,340	2,335	–	–	–	–	Inf + M	Glu
	D	F	11	PPOX	c.441-442delCA	Frame-shift	–	–	ND	–	–		–	–	–	–	–	–	–	–
II-VP	P2	F	30/24	PPOX	c.917T>C	p.L306P	+	+	626	383		7,076	–	–	2,422[a]	–	–	–	Inf + M	Glu
	So	M	3	PPOX	c.917T>C	p.L306P	–	–	Neg	2	13	0	–	–	0.6	–	–	–	–	–
III-VP	P3	M	69/39	PPOX	c.917T>C	p.L306P	–	+	626	3	19	1,326	143	1,183	671	84	241	346		Ph
	So	M	43/30	PPOX	c.917T>C	p.L306P	+	+	627	75	223	930	–	–	1,753	–	–	–	–	Glu
	G	M	10	PPOX	Neg	–	–	–	Neg	3	15	120	–	–	138	–	–	–	–	–
IV-VP	P4	F	80/45	PPOX	c.917T>C	p.L306P	–	+	626	2.2	16.8	1,340	79	1,261	3,182	890	662	1,630	–	Ph
	D	F	58	PPOX	c.917T>C	p.L306P	–	+	626	3.7	30	199[a]	–	–	1,202[a]	–	–	–	–	–
	So	M	48	PPOX	c.917T>C	p.L306P	–	–	Neg	5	32	0	–	–	142	–	–	–	–	–
V-VP	P5	F	31/25	PPOX	c.1252T>C	p.C418R	+	+	626	505	309	14,326	–	–	–	–	–	–	Inf + M	
	Si	F	38/25	PPOX	c.1252T>C	p.C418R	+	+	626	11[a]	33[a]	217[a]	–	–	–	–	–	–	Inf	
	So	M	19	PPOX	Neg	–	–	–	Neg	7	29	68	–	–	–	–	–	–	–	
	Ni	F	16	PPOX	c.1252T>C	p.C418R	–	–	ND	–	–		–	–	–	–	–	–	–	
	Nf	M	17	PPOX	Neg	–	–	–	ND	–	–		–	–	–	–	–	–	–	
VI-VP	P6	M	63/35	PPOX	c.1252T>C	p.C418R	–	+	626	8	66	626	74	552	268	3	88	177	–	
I-HCP	P	F	22/22	CPOX	c.364G>T	p.E122X	+	–	618	53	63	5,100	1,425	4,100	4,960	–	–	–	Inf + M	Glu

Fam, family, *Pts* patients, *P* proband number, *Si* sister, *D* daughter, *Ni* niece, *Nf* nephew, *G* grandson, *F* female, *M* male, *PBG* porphobilinogen, normal value: <15 μmol/24 h, *ALA* δ-aminolevulinic acid, normal range: 11.4–57.2 μmol/24 h, *TPu* total porphyrins in urine, normal values <200 nmol/24 h, *Plasma scan* plasma fluorescence emission peak in nm, *TPf* total porphyrins in feces, normal values <150 nmol/g dry weight, *Uro* uroporphyrin, normal values: <36 nmol/24 h in urine, <1 nmol/g dry weight in feces, *Cop* coproporphyrins, normal values: <122 nmol/24 h in urine, <26 nmol/g dry weight in feces, *Proto*, protoporphyrins, normal value: <80 nmol/g dry weight in feces, *TF* triggering factor, *Inf* infection, *M* medication, *TT* treatment, *Glu* 10% glucose i.v. infusion, *Ph* photoprotection, *ND* not determined, *Neg* no emission peak on plasma scan

[a] Mark, values measured after resolution of the acute attack

onset, with a frequency similar to that reported in South Africa and Western Europe (Whatley et al. 1999). The patient with HCP manifested with acute symptoms only. During onset, all of the patients had markedly increased levels of PBG and ALA. In all of the investigated AIP probands, except for one (family VI-P6), the HMBS activity in the erythrocytes was decreased. In P6, the HMBS activity was measured during acute onset, when transitory normal levels could be expected. Heme-arginate is not available in Bulgaria; therefore, all patients with acute onset, except for one, were treated with i.v. glucose infusions (from 200 to 500 g/day according to the severity of the attack) or Cimetidine until the clinical and biochemical parameters improved.

A total of six different mutations in HMBS were detected in all seven families with AIP, three of which were previously described as single nucleotide substitutions: c.76C > T [p.R26C] in exon 3, c.287C > T [p.S96F] in exon 7, and c.445C > T [p.R149X] in exon 9; the other three mutations were newly detected. The novel ones included a single nucleotide change, a small insertion and a single nucleotide deletion: c.345-2A > C in intron 7–8, c.279-280insAT in exon 7, and c.887delC in exon 15. Overall, there were two missense, one nonsense, one splice-site, and two frameshift mutations. The alterations identified in the Bulgarian patients were heterogeneous, as previously reported in many other populations (Whatley et al. 2009; Puy et al. 1997).

Three of the substitutions identified in the Bulgarian AIP patients have been reported in various ethnic populations. The mutations identified in families I-P1 (p.R26C) and IV-P4 (p.R149X) were initially identified in Finish patients (Kauppinen et al. 1995). p.R26C has been subsequently reported in Slavic (Hrdinka et al. 2006), Spanish (To-Figueras et al. 2006), French (Puy et al. 1997), Chinese (Yang et al. 2008), and Venezuelan populations (Paradisi and Arias 2010). The p.R149X mutation was found to be one of the relatively prevalent mutations (approximately 5%) in a large cohort of 109 mutation-positive AIP families (Puy et al. 1997). It has been shown that the R26 and R149 residues are located in the substrate-binding site and are crucial for enzymatic activity (Llewellyn et al. 1998; Gill et al. 2009). AIP family IV is of particular interest due to its gypsy origin. The gypsy people represent the largest minority in Bulgaria, and the search for the p.R149X substitution in this population deserves further attention.

In families VI-P6 and VII-P7, an identical p.S96F missense mutation was found in the HMBS gene; this mutation was first described by Kaupinnen et al. (2002). There was no significant difference in the clinical phenotype of the two probands; both patients presented with a single acute attack that was triggered by hormonal changes during their twenties. At present, both patients are symptom-free.

Novel substitutions in the HMBS gene that resulted in frameshift and splice-site alterations were identified in families II, III, and V. A small insertion (c.279-280insAT in exon 7) was identified in the II-P2 proband, resulting in a frameshift mutation that led to the formation of premature stop codon after the incorporation of 24 different amino acid residues, compared to the reference transcript. A small out-of-frame deletion (c.887delC) was identified in the III-P3 family, and this mutation led to a premature stop codon after 3 amino acids, compared to the reference sequence. The nucleotide change revealed in family V-P5 (c.345-2A>C in intron 7) affected the invariant AG acceptor splice site and possibly interfered with mRNA processing. At the same position, a different nucleotide change (c.345-2 A>G) has been reported in Swedish patients, listed in a table (Floderus et al. 2002). These three alterations have not been reported in reference databases (such as dbSNP, HGMD, 1KG, and ESP). To exclude the possibility of the novel mutations being SNPs, 96 control DNA samples were screened, and no samples revealed the presence of the novel mutations of the HMBS gene (c.345-2A>C in intron 7, c.279-280insAT in exon 7, and c.887delC in exon 15). The above finding suggests that these mutations are pathogenic. RNA analysis is needed to prove the pathogenicity of the c.345-2A > C change.

After detecting specific mutations in the families, the available family members ($n = 4$ symptomatic and $n = 5$ asymptomatic) were screened for the presence of the identified mutations (Table 1). All symptomatic relatives harbored the corresponding family-specific nucleotide changes. Among the five asymptomatic subjects three were mutation positive, including family II-M, VI-D, and VII-D subjects. Subject II-M was a postmenopausal female with a low level of HMBS activity in the erythrocytes and concomitant diabetes mellitus type II. Even if the diabetes has been treated, a slightly elevated serum glucose levels could exert a protective effect against overt disease (Andersson and Lithner 2001). Subjects VI-D and VII-D, females presently aged 21 and 15 years, respectively, whose HMBS activity levels in the erythrocytes were unavailable, shared identical causative mutations, which facilitated genetic counseling to prevent acute attacks. The HMBS levels in the four mutation negative subjects were within the normal range in two cases and were unavailable in the other two cases.

A total of three different novel substitutions in the PPOX gene were found in all six families with VP: c.441-442delCA in exon 5, c.917T>C [p.L306P] in exon 9, and c.1252T>C [p.C418R] in exon 12. In total, two missense mutations and one small deletion were observed in the VP families. All of the probands from families I, II, III, IV, V, and VI had positive plasma scan at 626 nm, increased levels of urinary porphyrins and their precursors PBG and

ALA. Probands from families II, III, IV, and VI had increased fecal porphyrins levels as well. A small out-of-frame deletion (c.441-442delCA) was found in family I-P1; this mutation led to a premature stop codon after the introduction of nine different amino acids. Families II-P2, III-P3, and IV-P4 shared an identical mutation (c.917T>C) that resulted in a leucine to proline substitution at position 306 (p.L306P). The V-P5 and VI-P6 probands harbored an identical change (c.1252T>C) that led to the replacement of a cysteine with proline in codon 418 (p.C418R). These three novel alterations have not been reported in reference databases (such as dbSNP, HGMD, 1KG, and ESP) and were not detected in the 96 control DNA samples. Residues L306 and C418 are located in the highly conserved FAD-binding domain (Qin et al. 2011), and these alterations most likely disrupt this interaction. The HumVar scores of 1.00 for p.L306P and 0.921 for p.C418R predicted that these mutations are most likely damaging. A small deletion (c.916_917delCT) in codon 306 has been described by Wiman et al. (2003), while no mutations have been reported in codon 418 so far. The available family members ($n = 3$ symptomatic and $n = 7$ asymptomatic) were screened for the presence of the corresponding nucleotide changes, and the details of these individuals are shown in Table 2. The symptomatic subjects III-So, IV-D, and V-Si harbored the family-specific nucleotide changes. Family III-So had an acute attack followed by chronic neurological symptoms and skin involvement and increased levels of PBG, ALA, total porphyrins in urine and feces. Family IV-D suffered from cutaneous lesions only. She had a plasma emission peak at 626 nm, normal PBG and ALA levels due to the lack of acute symptoms, and increased levels of urinary and fecal porphyrins. Family V-Si suffered from acute onset and cutaneous legions, unfortunately PBG and ALA levels shown in Table 2 were measured after the acute onset and were within normal range. However, plasma emission peak at 626 nm and increased total porphyrins in urine were observed even after the resolution of the attack. Among the seven asymptomatic subjects, four harbored the specific alterations, family I-D, II-So, IV-So, and V-Ni. Unfortunately, no biochemical data was available for the adolescent individuals I-D and V-Ni. Family II-So and IV-So had normal total porphyrins levels in urine and feces and urinary PBG and ALA levels. Plasma scan was also negative in both subjects. These results could be expected considering the fact that the significance of plasma emission peak at 626 nm is partly limited in the elderly asymptomatic carriers (IV-So). It is usually absent in asymptomatic children (II-So) (Hift et al. 2004). Unfortunately, PPOX activity could not be measured to check the cosegregation of the mutation and low enzymatic activity levels. Further analysis is needed to prove the pathogenicity of these alterations.

The majority of Bulgarian VP patients can be associated with 2 endemic regions. One of these regions is populated with ethnic Bulgarians, who are Muslim. These individuals reside in remote villages around the mountain town of Velingrad in the Rhodope mountains. Only 2 families, V and VI, from this region agreed to take part in this study, although more patients were invited. Most likely, the majority of VP patients share the identical p.C418R mutation due to consanguinity. The ancestors of families II, III, and IV originated from the small remote village of Buynovtsi, which is situated in the central Balkan mountains. Thus, a possible founder effect can also be anticipated for the p.L306P change. The endemic aggregation of families with VP and the distribution of the corresponding novel missense mutation also emphasize the pathogenic effect of these novel alterations.

A novel nonsense mutation (c.364G>T [p.E122X]) was identified in the patient with HCP shortly after her first acute attack in 2013; the details of this patient can be found in Table 2. She had increased total porphyrins levels in urine and feces and urinary PBG and ALA. Plasma scan emission peak was at 618 nm. Cutaneous involvement was absent. The p.E122X mutation is located in exon 1 and would lead to an unstable and inactive CPOX protein, which is likely removed by proteolytic degradation. The CPOX activity measurements could not be performed to prove the cosegregation of the novel alteration and low enzymatic activity.

This is the first report to describe mutations in Bulgarian patients with AIP, VP, and HCP. We identified a total of seven novel mutations in these families. Seven latent gene carriers were also detected. The identification of the latent gene carriers can result in the prevention of acute attacks by avoiding the well-known exogenic triggering factors.

Acknowledgements We would like to thank all of the families that participated in this study. We would also like to gratefully acknowledge Prof. Dimcho Adjarov for the fruitful discussions and suggestions, as well as for supplying the clinical and biochemical data used in this study. Mrs. Doroteya Leonkeva, Mrs. Kumi Kato, and Ms. Yoko Tateda are also gratefully acknowledged for their skillful technical assistance. We acknowledge the support from Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT)

Take-Home Message

This is the first report to describe mutations in Bulgarian patients with acute hepatic porphyrias, and a total of seven novel mutations were identified in this study.

References to Electronic Databases

MIM 176000; OMIM 176200; OMIM 121300; EC 4.3.1.8; EC 1.3.3.4; EC 1.3.3.3; HMBS; PPOX; CPOX; HMBS Ensembl Accession number ENST00000442944; PPOX Ensembl Accession number ENST00000367999; CPOX Ensembl Accession number ENST00000264193; www.hgmd.cf.ac.uk

Compliance with Ethics Guidelines

Conflict of Interest

Sonya Dragneva, Monika Szyszka-Niagolov, Aneta Ivanova, Lyudmila Mateva, Rumiko Izumi, Yoko Aoki, and Yoichi Matsubara declare that they have no conflicts 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.

Details of the Contribution of Individual Authors

Sonya Dragneva: involved in design, analysis, and interpretation of data, drafted the initial version of the manuscript; Monika Niagolov: involved in conception and design and critical revision of the manuscript; Aneta Ivanova: involved in conception, design, and critical revision of the manuscript; Lyudmila Mateva: involved in conception, design, and critical revision the manuscript; Rumiko Izumi: involved in analysis and interpretation of data and critical revision of the manuscript; Yoko Aoki: involved analysis and interpretation of data and critical revision of the manuscript; Yoichi Matsubara (Guarantor): involved in analysis and interpretation of data and critical revision of the manuscript

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JIMD Reports
DOI 10.1007/8904_2014_321

RESEARCH REPORT

Analysis of Methylcitrate in Dried Blood Spots by Liquid Chromatography-Tandem Mass Spectrometry

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Received: 10 March 2014 / Revised: 01 May 2014 / Accepted: 13 May 2014 / Published online: 6 July 2014

Abstract Accumulation of propionylcarnitine (C3) in neonatal dried blood spots (DBS) is indicative of inborn errors of propionate metabolism including propionic acidemia (PA), methylmalonic aciduria (MMA), and cobalamin (Cbl) metabolic defects. Concentrations of C3 in affected newborns overlap with healthy individuals rendering this marker neither specific nor sensitive. While a conservative C3 cutoff together with relevant acylcarnitines ratios improve screening sensitivity, existing mass spectrometric methods in newborn screening laboratories are inadequate at improving testing specificity. Therefore, using the original screening DBS, we sought to measure 2-methylcitric acid (MCA), a pathognomonic hallmark of C3 disorders to decrease the false positive rate and improve the positive predictive value of C3 disorders. MCA was derivatized with 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE). No separate extraction step was required and derivatization was performed directly using a 3.2-mm disc of DBS as a sample (65°C for 45 min). The reaction mixture was analyzed by liquid chromatography tandem mass spectrometry. MCA was well separated and eluted at 2.3 min with a total run time of 7 min. The median and (range) of MCA of 0.06 μmol/L (0–0.63) were in excellent agreement with the literature. The method was applied retrospectively on DBS samples from established patients with PA, MMA, Cbl C, Cbl F, maternal vitamin B12 deficiency ($n = 20$) and controls ($n = 337$). Comparison with results obtained by another method was satisfactory ($n = 252$). This method will be applied as a second tier test for samples which trigger positive PA or MMA results by the primary newborn screening method.

Communicated by: Rodney Pollitt, PhD

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Introduction

Accumulation of propionate metabolites in body fluids can be caused by defects in propionyl coenzyme A or cobalamin (Cbl) metabolism (Fenton et al. 2001). These metabolites are chiefly derived from the catabolism of certain amino acids and odd chain fatty acids. Propionate accumulation results in increased propionylcarnitine (C3) concentration in biological samples. C3 is a marker of a number of inborn errors of metabolism including methylmalonyl CoA mutase deficiency (OMIM ID: 251000) and propionic acidemia (PA, OMIM ID: 606054) (Zytkovicz et al. 2001; Rashed et al. 1997). Elevated C3 can also be associated with defects in several other genes involved in Cbl metabolism (Fowler et al. 2008; Watkins and Rosenblatt 2011). A subset of C3 related metabolic disorders have been recommended as screening targets and are widely adopted by newborn screening programs around the world

(Watson et al. 2006). Clinically, these are often accompanied by episodic metabolic acidosis, ketosis, hyperammonemia, and may result in severe sequelae including neurological symptoms or death in infancy. Early diagnosis is key to effective management and favorable outcome is expected should treatment start before the appearance of symptoms (Hori et al. 2005; Schulze et al. 2009).

In neonatal dried blood spot (DBS) specimens, concentration of C3 in affected newborns overlaps with healthy individuals rendering screening for relevant disorders using this metabolic intermediate as a sole marker neither specific nor sensitive. In Ontario, screening for PA and MMA started in 2006 based on C3 and one baby with a false negative screen (methylmalonyl-CoA mutase deficiency) was identified among 507,428 infants screened in the first 4 years. By adjusting C3 cutoff and introducing the ratio of C3 to acetylcarnitine (C3/C2), the sensitivity and specificity can be improved (Wilcken et al. 2003; Chace et al. 2003; Lindner et al. 2008), however, the positive predictive value remains generally poor (La Marca et al. 2007).

In our program, screen positive newborns are recalled for further diagnostic workup. Simultaneous testing of mothers to eliminate a maternal condition is also not uncommon and most of these babies turn out to be unaffected. The high false positive rate negatively impacts the cost-benefit ratio of newborn screening, leads to parental anxiety, and increases risk for parent–child dysfunction (Gurian et al. 2006). Unfortunately, routine MS/MS-based newborn screening methodology is inadequate to reduce the false positive rate and new analytical strategies are needed to improve the screening performance.

Second tier testing whereby the initial screening sample is tested for a different marker or using alternative technology is efficient in improving specificity (Minutti et al. 2004; Janzen et al. 2007). 2-Methylcitric acid (MCA) is generally described as a pathognomonic hallmark for disorders involving propionyl CoA metabolism. MCA can be detected in DBS and has been shown to reduce the false positive rate and improve the positive predictive value for PA and MMA (Turgeon et al. 2010).

In this paper, we describe a novel method to analyze MCA in DBS as a derivative of 4-[2-(*N*,*N*-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE). Similar to other carboxylic acids, the poor ionization and fragmentation of MCA in electrospray ionization (ESI)-MS/MS can be improved by this approach (Al-Dirbashi et al. 2007, 2008). The superior sensitivity attained allows for MCA detection using a single 3.2 mm disc. In a newborn screening laboratory setting, this method can be used for quantifying MCA in the original screening DBS to provide additional analytical information and reduce the number of false positive results.

Materials and Methods

Chemicals and Standard Solutions

MCA and d3-MCA used as internal standard (IS) were obtained from CDN Isotopes (Pointe-Claire, QC, Canada). DAABD-AE was synthesized according to the published method (Tsukamoto et al. 2005) but can also be purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 4-(dimethylamino)pyridine (DMAP), and perfluorooctanoic acid (PFOA) were obtained from Sigma-Aldrich. HPLC grade acetonitrile was from Burdick's and Jackson (Muskegon, MI, USA). Water used throughout this study was generated by a Milli-QUV Plus Ultra pure water system (Millipore SA, Molsheim, France).

Stock solutions of MCA and IS were prepared by dissolving the proper amounts in 50% methanol to give a concentration of 10 mmol/L. These solutions were stable for a minimum of 6 months when stored at −20°C. A working IS solution at 2.5 μmol/L was prepared in 50% acetonitrile.

Controls and Patients Samples

Our Institutional Research Ethics Board granted approval to this study. DBS specimens received at Newborn Screening Ontario laboratory and producing normal profiles for all screened conditions were used to determine MCA reference range ($n = 337$). In general, samples were collected at 24–72 h on Whatman 903™ Specimen Collection Paper. Archived DBS samples from confirmed patients with PA ($n = 2$), MMA ($n = 8$), Cbl C ($n = 1$), Cbl F deficiency ($n = 1$), and maternal vitamin B12 deficiency ($n = 8$) were also analyzed. These samples were stored at −80°C from the time of the initial positive screening result.

Sample Preparation

A single 3.2 mm disc was punched from the DBS into a 2.0 mL polypropylene tube. After adding 20 μL of IS working solution, the following were added successively: 25 μL of EDC (25 mmol/L in water), 25 μL of DMAP (25 mmol/L in acetonitrile), and 50 μL of DAABD-AE (2 mmol/L in acetonitrile). The tubes were tightly capped and heated at 65°C for 45 min. The reaction was stopped by adding 120 μL of 10% methanol containing PFOA at a concentration of 0.5 g/L. The sample tubes were centrifuged at 13,000 rpm for 1 min and a 10 μL portion of the resultant supernatant was injected onto the LC-MS/MS. The derivative stability was evaluated by analyzing the reaction mixture stored at 8°C in a tightly sealed vial at 0, 2, 4, 8, 24, and 48 h after reaction.

LC-MS/MS system

The LC-MS/MS consisted of a Waters ACQUITY Ultra Performance LC system (Waters, Milford, MA, USA) for solvent delivery and sample introduction interfaced with a Xevo XE tandem mass spectrometer (Micromass, Manchester, UK). The ESI source was operated in the positive ion mode at a capillary voltage of 3.5 kV. Cone voltage and collision energy were 35 V and 22 eV, respectively, using argon as collision gas. The ion source and desolvation temperatures were maintained at 120 and 350°C, respectively. MCA and IS were detected by selected reaction monitoring (SRM) using transitions of mass to charge (*m*/*z*) of 499 to 151 and 502 to 151, respectively, with a dwell time of 0.07 s. Separation was performed on an ACQUITY UPLC BEH C_8 column (2.1 × 50 mm, 1.7 µm, Waters). Mobile phase A was 10% methanol and mobile phase B was 90% methanol, and both contained 0.5 g/L PFOA. The following gradient program was used: 0–1.3 min 98% of A, 1.3–2.6 min from 98% to 50% of A, and 2.6-2.7 min 50% A at a flow rate of 0.4 mL/min. The column was re-equilibrated with 98% of mobile phase A for 4.3 min at a flow rate of 0.65 mL/min. The injection-to-injection time was 7 min.

Method Validation

To determine the linear range, DBS calibrators were prepared by adding MCA stock solution to whole blood from healthy volunteers to yield 0.5, 1, 2, 4, 8, and 16 µmol/L. Non-enriched blood was used as a blank. Quality control (QC) samples at 2.5 and 10 µmol/L were also prepared. Calibrators and QCs were applied manually onto Whatman 903™ Specimen Collection Paper and allowed to dry at ambient temperature overnight. DBS calibrators were stored at −20°C in sealed plastic bags with a desiccant.

The limit of quantification of MCA was calculated using DBS samples prepared from spiked blood that was serially diluted to give a final concentration of 0.1 µmol/L.

Within-day ($n = 19$) and between-day ($n = 14$) variations were evaluated by repeatedly analyzing QC samples. Coefficient of variation (CV%) was calculated according to the following equation [CV% = 100 × standard deviation/mean]. Analytical recovery was calculated using data obtained from QC samples as follows [Recovery % = 100 × (concentration measured − concentration in non-enriched sample)/concentration added].

Stability of MCA in DBS was assessed by storing samples spiked at 1.5 and 3.5 µmol/L at various temperatures (ambient, −20°C and 32°C). Analysis was carried out as described over a period of 3 weeks.

For method comparison, samples with normal and abnormal MCA levels ($n = 252$) were analyzed in parallel by the current methods and a published method after minor modification (Turgeon et al. 2010). The modification involved extending the derivatization reaction time from 15 min to 4 h.

Results

Sample Preparation

Extraction and derivatization of MCA was accomplished in a single step. IS and reagents required for derivatization were added directly onto a 3.2 mm DBS disc and incubated at 65°C. The derivatization yield which represents the extraction of MCA from DBS and the formation of DAABD-MCA derivative reached its maximum at 45 min or more. All the following experiments hence were performed at 65°C for 45 min. DAABD-MCA derivatives were stable for at least 48 h when stored in a tightly sealed vial at 8°C.

MS/MS and LC-MS/MS Experiments

The reaction mixture was infused into the first quadrupole of the MS/MS. Scanning in the positive ion ESI-MS revealed ions at *m*/*z* of 517 and 520 corresponding to $[MH]^+$ of DAABD-AE derivatives of MCA and d3-MCA, respectively. We also observed another set of more intense ions at *m*/*z* of 499 and 502. These were attributed to intramolecular condensation of MCA and d3-MCA and loss of water. The transmission of these ions into the collision cell and subsequent scanning by the second resolving quadrupole for fragments revealed a simple fragmentation pattern with an intense fragment at *m*/*z* of 151 common to all studied ions. This was assigned to the *N*,*N*-dimethylaminoethylaminosulfonyl moiety originating from DAABD-AE. Figure 1 shows the MS (Fig. 1a) and MS/MS (Fig. 1b) spectra of DAABD-MCA. It is noteworthy to mention that water loss may vary between different ion source designs, therefore, the use of appropriate stable isotope labeled IS is essential to compensate for this potential cause of variation.

Various chromatographic columns were evaluated aiming at the retention of DAABD-AE derivatives while allowing other compounds which may cause ion suppression including excess reagents to elute. This was achieved using a reversed phase C8 column in combination with a gradient program that increases methanol concentration from 2% to 50% (*v*/*v*) over the course of the run. The use of PFOA as a mobile phase additive improved the ionization process and enhanced the peak shape. Figure 2 shows

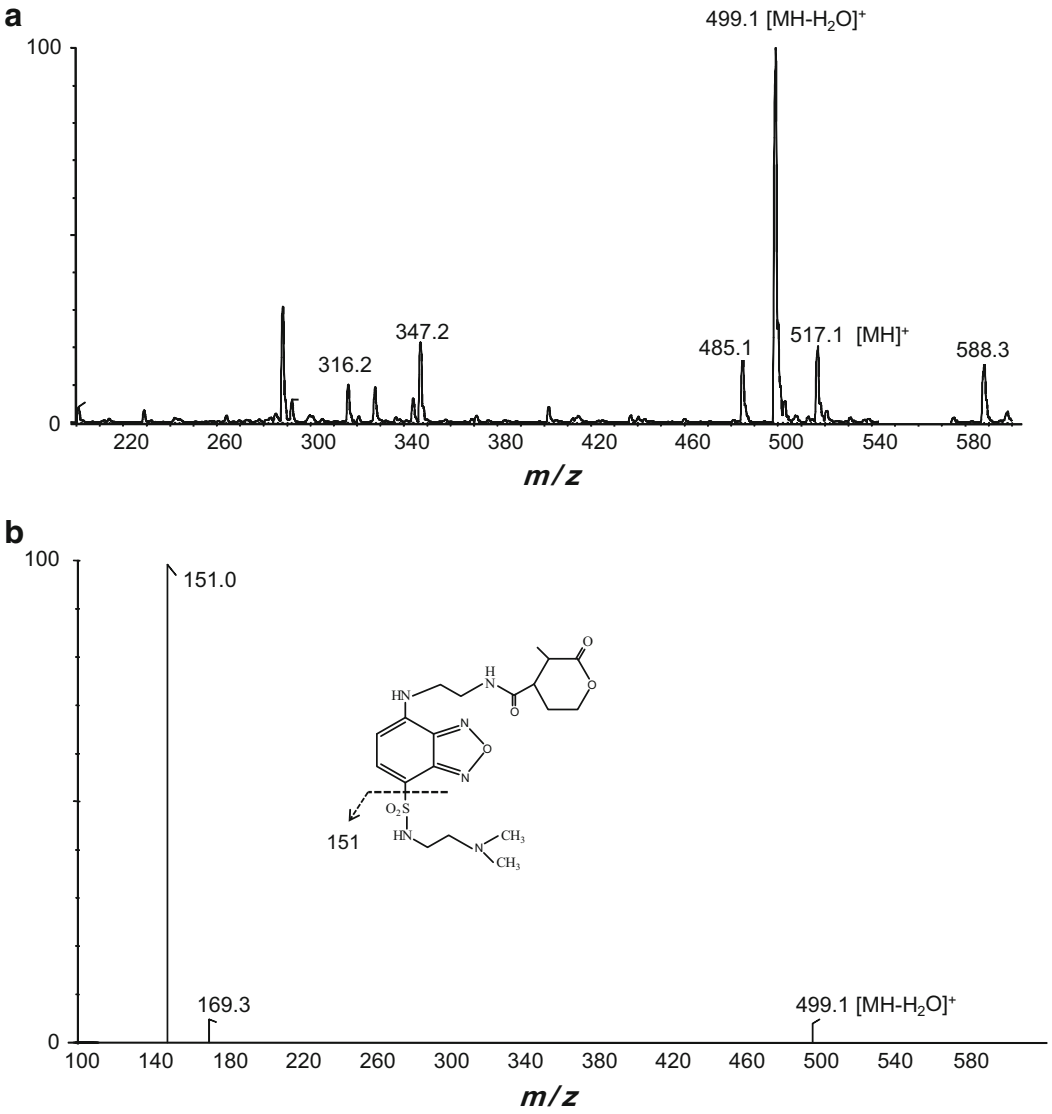

Fig. 1 ESI-MS spectrum of DAABD-MCA derivative (**a**) and ESI-MS/MS spectrum of *m/z* 499 (**b**). Experimental details are as described in the text

extracted mass chromatograms obtained with a 3.2 mm DBS disc from a healthy control (Fig. 2a) and that from a patient with an MMA (Fig. 2b). As shown, MCA was well separated and eluted at 2.3 min. After each injection, the column was reconditioned for 4.3 min to remove ion suppression effects of late eluting compounds, therefore the analytical time between successive injections was 7 min. It is worth mentioning that the automatic switching valve was programmed to divert column effluent to waste for the first 1.8 min and the last 4 min of each run to avoid loading the mass spectrometer with contaminating material from samples or reagents.

Assay Validation

Linearity was established by using DBS enriched with MCA at 0.5, 1, 2, 4, 8, and 16 μmol/L. Non-enriched DBS was also analyzed to correct for endogenous MCA. Regression analysis over the studied range revealed a linear relationship ($y = 0.041x - 0.002$, $r = 0.9999$), with y as the peak area ratio (MCA/IS) and x as the concentrations in DBS (μmol/L). The limit of quantification defined as MCA concentration that gives a signal to noise ratio (S/N) of ≥ 10 was found to be 0.1 μmol/L whereas the limit of detection ($S/N = 3$) was calculated to be 0.03 μmol/L.

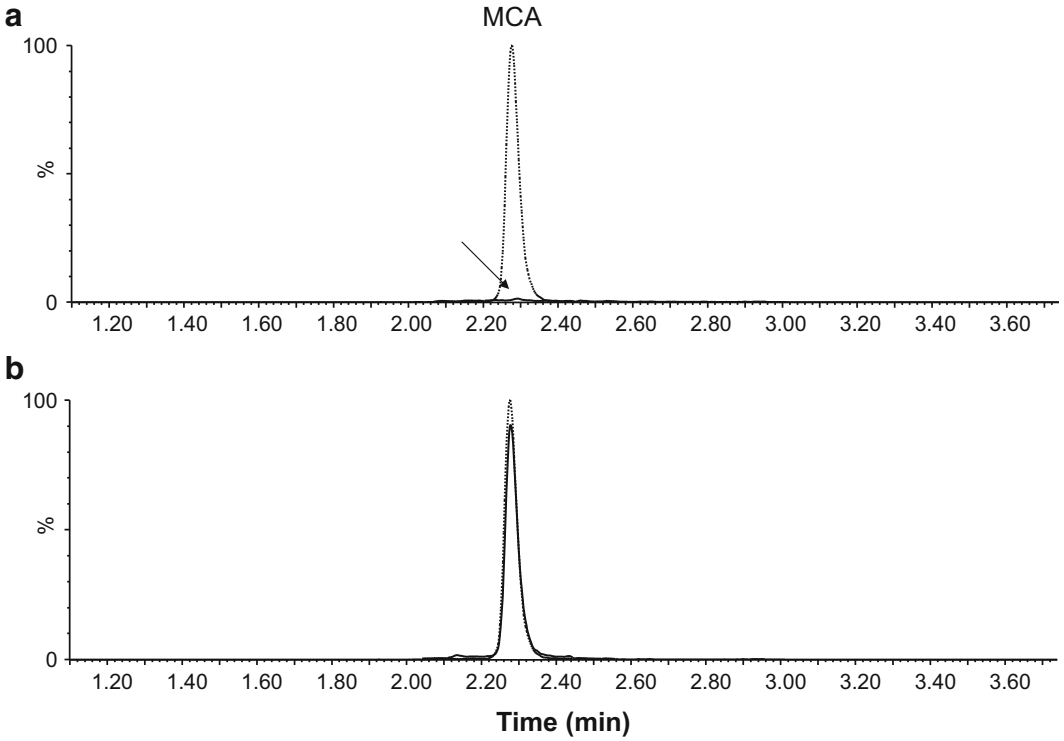

Fig. 2 Extracted mass chromatograms obtained with a DBS from a healthy individual (**a**) and from an MMA patient (**b**). Solid lines represent MCA and dotted lines represent the IS. The *arrow points* at MCA in the healthy individual's sample

Analysis of DBS specimens containing MCA at 1.5 and 3.5 μmol/L stored for a period of 3 weeks at −20°C, 23°C (ambient) and 32°C revealed that this compound is reasonably stable at the conditions described (Fig. 3).

Within-day ($n = 19$) and between-day ($n = 14$) imprecision were evaluated by repeated analysis of QC samples at 2.5 and 10 μmol/L. Table 1 summarizes the imprecision expressed as coefficient of variation (%) and analytical recovery.

Figure 4 illustrates the correlation between concentrations of MCA in DBS samples ($n = 252$) measured by the current method and in parallel by a published method (Turgeon et al. 2010).

Analysis of Controls and Patients Samples

MCA concentrations obtained by the current method using DBS samples from healthy individuals ($n = 337$) and from patients ($n = 20$) with confirmed PA, MMA, Cbl C, Cbl F, and maternal vitamin B12 deficiency are summarized in Table 2 which also shows the corresponding C3 and C3/C2 levels obtained by the primary newborn screening method.

Discussion

As proposed by the American College of Medical Genetics, PA, MMA, Cbl A, and Cbl B defects are included in the newborn screening core panel whereas Cbl C and Cbl D are considered secondary targets (Watson et al. 2006). These disorders are screened for by MS/MS using C3 as a primary marker together with appropriate ratios. Due to the overlap in C3 concentration between affected and unaffected newborns these disorders present a significant challenge to newborn screening laboratories. In affected patients, a gradient of C3 concentration is observed including subtle elevations that may be overlooked. To maximize screening sensitivity, laboratories tend to apply conservative cutoffs but this decreases test specificity resulting in a high rate of false screen positives, and potentially reveals incidental findings of non-targeted disorders. To mitigate this, second tier tests were devised to improve the screening process for this group of disorders aiming at measuring MMA, 3-hydroxypropionic acid, and MCA in DBS samples (Turgeon et al. 2010; Matern et al. 2007; La Marca et al. 2007). Among these, MCA, which is traditionally detected by gas chromatography mass spectrometry as part of the

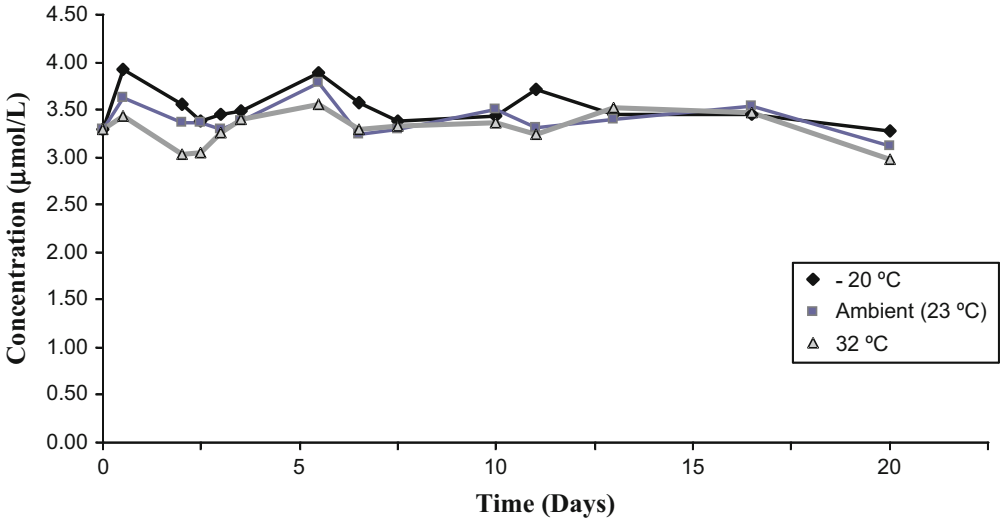

Fig. 3 Stability of MCA in DBS samples stored at different temperatures

Table 1 Recovery, intra- and inter-day reproducibility of MCA analysis

	Intra-day (n = 19)				Inter-day (n = 14)			
Concentration added (µmol/L)	Mean (µmol/L)	SD[a]	CV[b] (%)	Recovery[c] (%)	Mean (µmol/L)	SD	CV (%)	Recovery (%)
2.5	2.2	0.1	4.5	88.0	2.3	0.2	8.7	92.0
10.0	11.4	0.7	6.1	114.0	11.5	0.7	6.1	115.0

[a] SD = standard deviation
[b] CV = coefficient of variation
[c] Recovery (%) = 100 × found concentration /added concentration

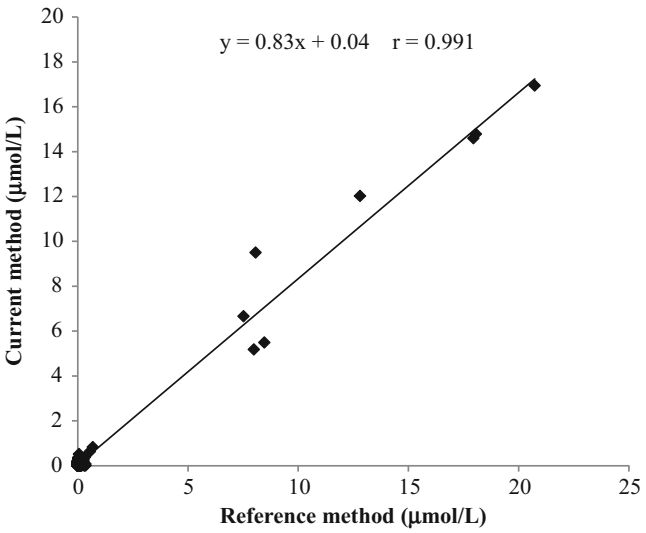

Fig. 4 Comparison of MCA concentrations in DBS (n = 252) obtained by the current method versus a reference method after modification (Turgeon et al. 2010). Experimental details are as described in the text

organic acid profile (Rinaldo 2008) is known to accumulate in patients with defects in propionate metabolism. The MCA method is appealing because it is also capable of measuring MMA and homocysteine simultaneously. We attempted to adopt the MCA method of Turgeon et al. (2010) as described, but we encountered inadequate sensitivity and large fluctuations in reproducibility. We decided to extend the butylation reaction time beyond 15 min and monitor the formation of the tributyl MCA derivative and found a 10-fold increase in peak intensity at 4 h of incubation or more. Under the modified conditions, the reproducibility was also improved, but this modification together with a lengthy chromatographic run of 15.6 min did not meet our screening objective which necessitates that samples not be batched and that reflexive testing is completed within the same working day as the initial screen positive result.

We sought to develop a more efficient method for MCA in DBS with sufficient sensitivity, still allowing for early referral of screen positive results. MCA, a tricarboxylic acid is a hydrophilic compound with disappointing chromatographic

Table 2 Median and range of MCA, C3, and C3/C2 levels in healthy individuals and confirmed patients studied in this work

Sample type	MCA (μmol/L) Median (range)	C3 (μmol/L) Median (range)	C3/C2 Median (range)
Control ($n = 337$)	0.06 (0–0.63)	1.87 (0.46–7.15)	0.08 (0.03–0.21)
Propionic acidemia ($n = 2$)	10.4 (7.3–13.4)	20.8 (16.5–25.1)	2.3 (1.97–2.55)
Methylmalonyl CoA mutase deficiency ($n = 8$)	13.3 (5.2–19.4)	17.6 (8.1–49.0)	0.79 (0.38–2.06)
Cbl C deficiency ($n = 1$)	6.7	18.5	0.77
Cbl F deficiency ($n = 1$)	0.83	5.73	0.27
Maternal vitamin B12 deficiency ($n = 8$)	0.28 (0–2.76)	8.4 (6.4–14.7)	0.26 (0.14–0.40)

and mass spectrometric behavior when analyzed by LC-MS/MS. To overcome this, we used chemical derivatization with DAABD-AE to generate a highly ionizable hydrophobic derivative. DAABD-AE forms stable amides upon reaction with carboxylic acids and introduces a chargeable moiety suitable for detection by ESI-MS/MS in the positive ion mode (Al-Dirbashi et al. 2007; Al-Dirbashi et al. 2008). Using reversed-phase chromatography, the hydrophobic DAABD-MCA derivative can be well separated from the early eluting ion-suppressing compounds. The high organic content coinciding with DAABD-MCA peak elution enhanced the ionization process and contributed to the increased overall sensitivity. DAABD-AE offers outstanding reactivity and allows for derivatization using DBS specimens without the need for a dedicated extraction step. The simple sample preparation which consisted of a single 45-min step for both extraction and derivatization resulted in excellent recovery and reproducibility. With a 7-min chromatographic time, we are able to meet our required turn around time and easily integrate this second tier method into our routine screening process. Compared with the published MCA method (Turgeon et al. 2010), our sample preparation does not require extraction and subsequent evaporation and our injection-to-injection time is more than halved conserving more than 50% of the instrument time.

MCA is present in DBS from healthy individuals at low, yet detectable levels (<0.7 μmol/L) (Turgeon et al. 2010). On the contrary, this marker is detected at significantly higher concentrations in PA or MMA patients. To obtain the maximum diagnostic value, we designed our calibrators to cover a wide concentration range encompassing physiological and pathological MCA levels (0.5–16 μmol/L). The use of stable isotope IS to generate the calibration curve enhanced the quality of quantitative data obtained.

MCA levels in DBS achieved in this work were compared to those obtained by a modified version of a published LC-MS/MS method (Turgeon et al. 2010). The two methods performed adequately and showed satisfactory agreement as demonstrated by linear regression analysis shown in Fig. 4.

The potential usefulness of the proposed method as a second tier test was assessed by a retrospective study using archived DBS samples from known patients ($n = 12$) with MMA, PA, Cbl C, and Cbl F defects randomized with DBS samples from babies of maternal vitamin B12 deficiency ($n = 8$) and healthy newborns ($n = 337$). Median MCA concentration was 0.06 μmol/L (range 0–0.63 μmol/L) in healthy newborns. As shown in Table 2, elevated MCA was detected in all known patients regardless of the underlying genetic defect. In DBS samples from babies born to mothers with confirmed vitamin B12 deficiency ($n = 8$), MCA was elevated in only two out of the eight samples in contrast to C3 which was elevated in all samples. With the implementation of MCA analysis as second tier test, we anticipate reduction of the false positive rate while maintaining excellent sensitivity. As previously reported (Turgeon et al. 2010), some patients with certain Cbl metabolic defects may not be detected by MCA analysis, hence, complementary analysis of other relevant markers such as MMA may be considered.

Many enzymes and small molecule markers are known to be stable in blood collected on filter paper as the dry nature of this matrix provides a favorable environment that decreases degradation. In this work, we found MCA in DBS to be invariably stable for at least 3 weeks at temperatures ranging between −20°C and 32°C as shown in Fig. 3. This finding is significant as stability during transport of DBS samples is essential to guarantee sample integrity and result validity.

In this work we developed and validated a novel, simple, and robust method to determine MCA in DBS using a single 3.2 mm disc. The excellent reactivity of the commercially available DAABD-AE reagent allowed for derivatization within 45 min, completely eliminating the extraction step. Injection-to-injection time was 7 min. The short sample preparation and chromatography time permits integration of this lean assay as a second tier method into routine newborn screening work flow without prolonging the turn around time. Reference intervals obtained are in agreement with the literature, and the method was able to

detect confirmed cases of MMA, PA, Cbl C, and Cbl F defects with 100% sensitivity. Prospective application of this method as a second tier test to improve screening for C3 disorders is in progress.

Take-Home Massage

Measuring methylcitric acid in dried blood spots improves newborn screening for disorders in which propionate metabolism is impaired.

Compliance with Ethics Guidelines

Conflict of Interest

Osama Y. Al-Dirbashi, Nathan McIntosh, Christine McRoberts, Larry Fisher, Mohamed S. Rashed, Nawal Makhseed, Michael T. Geraghty, Tomofumi Santa, and Pranesh Chakraborty declare that they have no conflict of interest.

Informed Consent

Not applicable.

Details of the Contribution of Individual Authors

OYA and PC designed and coordinated the study. NM, CM, and LF carried out the analysis of samples. TS synthesized the derivatization reagent. OYA, MSR, NM, MTG, and PC provided subspecialist consultation services, clinical examination, and diagnostic testing. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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JIMD Reports
DOI 10.1007/8904_2014_322

CASE REPORT

The Expanding MEGDEL Phenotype: Optic Nerve Atrophy, Microcephaly, and Myoclonic Epilepsy in a Child with *SERAC1* Mutations

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Received: 27 December 2013 / Revised: 03 April 2014 / Accepted: 20 May 2014 / Published online: 6 July 2014

Abstract The inborn errors of metabolism associated with 3-methylglutaconic aciduria are a diverse group of disorders characterized by the excretion of 3-methylglutaconic and 3-methylglutaric acids in the urine. Mutations in several genes have been identified in association with 3-methylglutaconic aciduria. We describe a patient of Saudi Arabian descent with 3-methylglutaconic aciduria, sensorineural hearing loss, encephalopathy, and Leigh-like pattern on MRI (MEGDEL syndrome), as well as developmental delay and developmental regression, bilateral optic nerve atrophy, microcephaly, and myoclonic epilepsy. The patient had an earlier age of onset of optic atrophy than previously described in other MEGDEL syndrome patients. Whole exome sequencing revealed two loss-of-function mutations in *SERAC1* in trans: c.438delC (p.T147Rfs*22) and c.442C>T (p.R148X), confirmed by Sanger sequencing. One of these mutations is novel (c.438delC). This case contributes to refining the MEGDEL phenotype.

Communicated by: Marc Patterson

Competing interests: None declared

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Introduction

Inborn errors of metabolism (IEM) with 3-methylglutaconic aciduria (3-MGA-uria) are a diverse group disorders associated with the excretion of 3-methylglutaconic and 3-methylglutaric acids in the urine. The IEMs with 3-MGA-uria were recently reclassified as primary 3-MGA-uria caused by defective leucine degradation or secondary 3-MGA-uria caused by defective phospholipid remodeling, mitochondrial membrane associated repair, or an unknown origin (NOS 3-MGA-uria) (Wortmann et al. 2013). Primary 3-MGA-uria is due to a deficiency of 3-methylglutaconic-CoA hydratase and due to mutations in the *AUH* gene (MIM #250950) (Ijlst et al. 2002; Ly et al. 2003; Wortmann et al. 2010). Mutations in other genes have been associated with the secondary 3-MGA-urias. Mutations in *TAZ* (MIM #300394) cause Barth syndrome, an X-linked disorder characterized by skeletal and cardiac myopathy, short stature, and neutropenia (Barth et al. 1983; Bione et al. 1996). *OPA3* (MIM #606580) mutations cause Costeff syndrome, characterized by early onset optic atrophy and neurological abnormalities including spasticity, extrapyramidal dysfunction, and cognitive deficits (Anikster et al. 2001; Sheffer et al. 1992). *DNAJC19* (MIM #608977) mutations cause DCMA syndrome, characterized by dilated cardiomyopathy and ataxia (Davey et al. 2006; Ojala et al. 2012). Defects in *TMEM70* (MIM #612418), involved in complex V of the electron transport chain, have been associated with cataracts, gastrointestinal dysfunction, and hypertonia (Jonckheere et al. 2012). Mutations in all of these genes have been associated with secondary 3-MGA-uria, but the mechanism responsible for the 3-MGA-uria has not been elucidated (Wortmann et al. 2013).

MEGDEL syndrome (MIM #614739) is another autosomal recessive form of secondary 3-MGA-uria that is caused

by defective phospholipid remodeling and was formerly classified as a form of type IV 3-MGA-uria. It is characterized by the features of defects of oxidative phosphorylation, sensorineural deafness, encephalopathy, dystonia, and Leigh-like syndrome. Wortmann et al. (2012) identified 14 different loss-of-function mutations in *SERAC1* in 15 patients with MEGDEL syndrome. A single patient with identified optic atrophy and microcephaly was found to have a homozygous nonsense mutation in *SERAC1* (Tort et al. 2013), and four patients with features of MEGDEL syndrome and infantile mitochondrial hepatopathy were found to have novel homozygous nonsense mutations in *SERAC1*, further expanding the phenotype of *SERAC1* (Sarig et al. 2013).

SERAC1 is a member of the PGAP-like protein domain family (PFAM PF07819) and is localized to the interface between the endoplasmic reticulum and the mitochondria that is necessary for phospholipid exchange. *SERAC1* mutations are associated with an abnormally increased ratio of phosphatidylglycerol-34:1 to phosphatidylglycerol-36:1. It is hypothesized that this leads to lower bis(monoacylglycerol)phosphate levels and accumulation of cholesterol in the perinuclear region. SERAC1 is hypothesized to be involved in phosphatidylglycerol remodeling and is essential for mitochondrial function and intracellular cholesterol trafficking (Wortmann et al. 2012). The abnormal cardiolipin profile that results from *SERAC1* mutations may in part explain the defects in oxidative phosphorylation in these patients (Wortmann et al. 2012).

Here we describe a patient with remote consanguinity who has compound heterozygous mutations in *SERAC1* detected by exome sequencing with symptoms of MEGDEL syndrome, with an earlier age of onset of optic atrophy than previously described in other MEGDEL syndrome patients.

Case Description

The proband is a 5-year-old boy with developmental delay and developmental regression, bilateral optic nerve atrophy, microcephaly, sensorineural hearing loss, myoclonic epilepsy, and 3-methylglutaconic aciduria. The child was born at 37 weeks gestation via spontaneous vaginal delivery following an uncomplicated pregnancy. The parents are of Saudi Arabian descent with a remote history of consanguinity as fourth cousins. His birth weight was 2,767 g (10th percentile) and birth length was 43.9 cm (<3rd percentile) with Apgar scores at 1 and 5 min of 9 and 10, respectively. At 24 h after birth, he was noted to have respiratory distress and an anion gap metabolic acidosis. He was treated with restricted protein intake and bicarbonate and improved within 24 h. Laboratory tests at the time revealed mildly elevated liver function tests, with ALT of 59 U/L (normal 7–41 U/L), GGT of 349 U/L (normal 9–58 U/L), and total serum bilirubin of 9.8 mg/dL (normal 0.3-1.3 mg/dL). Tyrosine was elevated at 519 μmol/L (normal <200 μmol/L) on amino acid profile, and 4-hydroxyphenylpyruvic acid and 4-hydroxyphenylacetic acid were elevated in the urine organic acid profile. No succinylacetone was detected in the urine. Diet was liberalized to an unrestricted diet.

Developmental milestones have all been severely delayed. He smiled at 3–4 months, rolled over at 6 months, sat independently at 12 months, walked unassisted at 26 months, and spoke his first words at 24 months. Bilateral hearing loss was first noted at 8 months of age, and he received cochlear implants at 3 years.

Metabolic testing at 2 years of age revealed slightly elevated lactic acid of 2.4 mmol/L (normal 0.5–2.0 mmol/L), mildly elevated pyruvic acid of 166 mmol/L (normal 30–90 mmol/L), and elevated ALT of 59 U/L (normal 7–41 U/L), and mildly increased AFP of 31.6 ng/mL (normal 0–11 ng/mL). Urine organic acids demonstrated 3-methylglutaconic acid of 82 mmol/mol creatinine (normal 1–9.2 mmol/mol creatinine) and 3-methylglutaric acid of 19 mmol/mol creatinine (normal 0.1–3.5 mmol/mol creatinine). Repeat urine organic acids at 29 months demonstrated 3-methylglutaconic acid of 119 mmol/mol creatinine and 3-methylglutaric acid of 38 mmol/mol creatinine. Plasma amino acids, ammonia, and CPK were normal.

An EEG recorded during sleep under chloral hydrate sedation, performed at 22 months, showed a reduction in background amplitude and theta slowing during sleep as well as a few sharp transients arising from the left frontotemporal region. The epileptiform discharges were considered suggestive of partial epilepsy. MRI of the brain at 2 years showed a Leigh-like pattern, with hyperintensity at the anterior aspect of the putamen and caudate, and to a lesser extent involving the globus pallidus. MR spectroscopy showed increased lactate in these areas. MRI findings were thought to be suggestive of a mitochondrial disorder. Follow-up MRIs demonstrated progressive basal ganglia and cerebellar volume loss.

Developmental regression was noted at 3 years of age by which time he could no longer walk, sit, or pick up objects, and his communication was limited to crying. He was hypertonic with dystonia and fisting in his upper extremities and limited movement in his lower extremities. Pharyngeal dysphagia and difficulty with oral motor coordination resulted in feeding difficulty. He demonstrated failure to thrive, with height consistently at the 10th

percentile and weight below the 3rd percentile. He has acquired microcephaly with head circumference measurements of 47 cm at 35 months (5–10th percentile), 47 cm at 43 months (<3rd percentile), and 47.5 cm at 83 months (<3rd percentile). At 4 years of age, he was diagnosed with myoclonic seizures and bilateral optic atrophy.

A right quadriceps biopsy was performed at 3 years of age due to suspicion of a mitochondrial disorder. The biopsy showed nonspecific changes, including slight myofiber atrophy and size variability. There were no ragged red fibers or fibers devoid of cytochrome oxidase staining.

The mitochondrial genome was sequenced from blood and analyzed for deletions due to suspicion of a mitochondrial disorder, and no mutations were detected. No mutations were detected in *AUH*, *TAZ*, *OPA3*, or *TMEM*. Whole exome sequencing and data analysis were subsequently conducted on a clinical basis as previously described (Yang et al. 2013). After multiple steps of variant filtering, 569 rare variants were retained including 124 variants in genes associated with Mendelian disorders and 445 variants in genes with no known association with Mendelian disorders at the time. Six missense variants of unknown clinical significance in five Mendelian genes including *DDX11*, *OTOF*, *PPT1*, *TECTA*, *TRIOBP* were identified as possible candidates. However, the pathogenicity of these changes was unknown and none of the findings could fully explain the patient's clinical phenotype. In addition to the missense changes, deleterious mutations in nine non-disease genes including a heterozygous single nucleotide deletion, c.438delC (p.T147Rfs*22), and nonsense mutation, c.442C>T (p.R148X), in the *SERAC1* gene were also detected and were consistent with 3-methylglutaconic aciduria with deafness, encephalopathy, and Leigh-like syndrome (MEGDEL) syndrome (Wortmann et al. 2012). Sanger sequencing of the proband and his father confirmed that the father was heterozygous for c.438delC and did not carry c.442C>T, establishing that the two mutations in *SERAC1* are in trans. One of these mutations (c.442C>T) has been previously identified in three patients with MEGDEL syndrome (Wortmann et al. 2012). The second mutation is novel (c.438delC) and not present in 1,000 genomes or EVS database. Both mutations are predicted to cause loss of function, with nonsense mediated decay with c.442C>T (p.R148X).

Discussion

We describe a patient with compound heterozygous mutations in *SERAC1* with 3-methylglutaconic aciduria, progressive neurologic deterioration, Leigh-like pattern on MRI, sensorineural hearing loss, acquired microcephaly, myoclonic epilepsy, bilateral optic nerve atrophy, and infantile hepatopathy.

Twenty other patients have been described with *SERAC1* mutations associated with 3-methylglutaconic aciduria, and clinical features including progressive spasticity, dystonia, encephalopathy, Leigh-like MRI, deafness, bilateral optic atrophy, microcephaly, and infantile hepatopathy (Sarig et al. 2013; Tort et al. 2013; Wortmann et al. 2012). Our patient shares similar clinical features to the other 20 patients with *SERAC1* mutations, although our patient had optic atrophy by the age of 4, much earlier than the only other patient diagnosed with optic atrophy at the age of 16 years (Table 1) (Tort et al. 2013).

Eighty percent of previously described patients with *SERAC1* mutations demonstrated developmental regression. What is notable in our patient is the rate of developmental regression, which is not completely attributable to the progressive dystonia and spasticity, given his cognitive decline in addition to the loss of his motor skills. Evidence of dystonia and delayed milestones was apparent by 6 months of age in our patient, with delayed sitting and walking. The patient's developmental regression began at 3 years of age, at which point he demonstrated a rapid decline. Though he had been able to walk at 26 months, by 39 months he was unable to walk, sit, or pick up objects, and he was no longer able to recognize or respond to his parents.

The SERAC1 protein has a serine lipase domain that is hypothesized to play a key role in its function (Wortmann et al. 2012). The majority of the mutations that have been identified in *SERAC1* have either been frameshift, nonsense, or missense mutations within or upstream of the lipase domain (Sarig et al. 2013; Tort et al. 2013; Wortmann et al. 2012). Both of our patient's mutations are upstream of the lipase domain. The frameshift mutation c.438delC (p.T147Rfs*22) and the stop gain mutation c.442C>T (p.R148X) are both disruptive mutations, accounting for the severity of his phenotype.

This case contributes to our understanding of the expanding phenotype of MEGDEL syndrome and the spectrum of *SERAC1* mutations.

Acknowledgments We thank the family for their generous contribution. The work was supported in part by NIH Grant 5 T35 DK 93430-2.

Synopsis

This case further characterizes the phenotype associated with *SERAC1* mutations and expands upon the previously described features of MEGDEL syndrome.

Table 1 Comparison of the clinical features of MEGDEL syndrome in previously reported patients with mutations in *SERAC1* and the patient described here (Sarig et al. 2013; Tort et al. 2013; Wortmann et al. 2012)

		Previously reported patients ($n = 20$)		
	Patient (age of onset)	Yes *N* (%)	No *N* (%)	Not reported *N* (%)
Consanguinity	+	11 (55)	9 (45)	0 (0)
Urinary 3-MGA	+	20 (100)	0 (0)	0 (0)
Sensorineural deafness	+	18 (90)	1 (5)	1 (5)
Leigh-like MRI	+	17 (85)	0 (0)	3 (15)
Dystonia	+	19 (95)	0 (0)	1 (5)
Psychomotor delay	+	19 (95)	0 (0)	1 (5)
Developmental regression	+ (3 years)	16[a] (80)	1 (5)	3 (15)
Microcephaly	+	1 (5)	19 (95)	0 (0)
Epilepsy	+ (4 years)	4[b] (20)	16 (80)	0 (0)
Infantile hepatopathy	+	4 (20)	16 (80)	0 (0)
Optic atrophy (bilateral)	+ (4 years)	1[c] (5)	19 (95)	0 (0)
Lactic acidemia	+	19 (95)	1 (5)	0 (0)
OXPHOS defects – muscle	NA	9 (45)	2 (10)	4 (20)

[a] Mean age of onset = 1.5 years
[b] Mean age of onset = 4 years
[c] Age of onset = 16 years
NA not available

Compliance with Ethics Guidelines

Conflict of Interest

Heidi Lumish, Ashley Wilson, and Wendy Chung declare that they have no conflict of interest.

Yaping Yang works for Baylor College of Medicine in a laboratory that generates revenue from performing clinical genetic tests.

Fan Xia works for Baylor College of Medicine in a laboratory that generates revenue from performing clinical genetic tests.

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

Details of the Contributions of Individual Authors

Heidi Lumish was responsible for drafting the article.

Yaping Yang performed genomic sequencing and data interpretation and critical review of the manuscript.

Fan Xia performed genomic sequencing and data interpretation and critical review of the manuscript.

Ashley Wilson critically reviewed and revised the manuscript.

Wendy Chung was responsible for drafting the article and revising it for intellectual content. Guarantor.

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JIMD Reports
DOI 10.1007/8904_2014_323

CASE REPORT

Isolated Mild Intellectual Disability Expands the Aminoacylase 1 Phenotype Spectrum

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Received: 04 April 2014 / Revised: 30 April 2014 / Accepted: 19 May 2014 / Published online: 6 July 2014

Abstract Aminoacylase 1 (ACY1) deficiency is a rare inborn error of metabolism presenting with heterogeneous neurological symptoms such as psychomotor delay, seizures, intellectual disability and it is characterized by increased urinary excretion of N-acetylated amino acids. We report on a new patient who presented ACY1 deficiency in association with isolated mild intellectual disability, but neither neurological symptoms nor autistic features. The child showed a compound heterozygous mutation (p.Glu233Asp) and a novel p.Ser192Arg fs*64, predicting an unstable transcript and resulting in very low protein levels.

This new ACY1 deficient child was identified through regular screening for inborn error of metabolism adopted in our department in all cases of intellectual disability. This report supports a recommendation to perform metabolic investigations in patients with isolated mild intellectual disability.

Communicated by: Francois Feillet, MD, PhD

Competing interests: None declared

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Introduction

Aminoacylase 1 (ACY1, EC 3.5.1.14) deficiency (MIM 609924) is a rare inborn error of metabolism (IEM) characterized by increased urinary excretion of N-acetylated amino acids with the exception of *N*-acetylaspartic acid (NAA). Since the first report by Van Coster et al. (2005) less than 20 additional patients have been described, most of them presenting with heterogeneous neurological symptoms such as psychomotor delay, seizures, intellectual disability (Sass et al. 2007; Ferri et al. 2013). ACY1 deficiency has also been reported associated with autistic feature in one patient (Tylki-Szymanska et al. 2010). The diagnosis of ACY1 deficiency is based on urinary analysis of organic acid by gas chromatography-mass spectrometry (GC/MS) (Gerlo et al. 2006) or NMR spectroscopy (Engelke et al. 2008), and confirmatory molecular genetic and enzyme analyses (Sass et al. 2006).

Aminoacylase 1 is a soluble homodimeric zinc binding enzyme that catalyzes the formation of free aliphatic amino acids and acetic acid from N-acetylated precursors. Compared to other tissues, the kidney contains the highest levels of ACY1 mRNA, protein and enzyme activity, with a localization throughout the renal tubular epithelium (Lindner et al. 2008). This seems to be in line with a possible functional role of ACY 1 in recycling of N-acetylated amino acids in the kidney and can explain the high concentrations of N-acetylated amino acids in the urine of patients with the biochemical defect (Sass et al. 2006). *ACY1* is also highly expressed in brain and liver, and to a lesser extent in almost all the other tissues (Cook et al. 1993; Sass et al. 2006), suggesting a pivotal role of the enzyme in amino acid metabolism of these organs.

ACY1 is located on the short arm of chromosome 3 (3p21.1) (Naylor et al. 1979, 1982; Miller et al. 1990; Cook

et al. 1993), and the protein is highly conserved, supporting an evolutionary role in physiology. Until now ten different mutations have been reported (Van Coster et al. 2005; Sass et al. 2006, 2007; Tylki-Szymanska et al. 2010; Ferri et al. 2013), with no clear-cut phenotype–genotype correlation.

We report a new deficient patient presenting with mild intellectual disability harboring compound heterozygous mutations in *ACY1*.

Patient

A 10-year-old girl was admitted in our department to evaluate her language and learning disabilities. She was born at term after an uneventful pregnancy as the first child of unrelated healthy parents. At birth she presented a mild respiratory distress (APGAR score after 1 and 5 min was 7–10); birth weight was 3,500 g, length 52 cm, OFC 34.5 cm.

With the exception of multiple abortions reported by the mother there was no family history of epilepsy, other neurologic or psychiatric disorders. The child presented a normal motor development with language delay and mild difficulties in relationship with her peers. The parents also reported several episodes of asthmatic bronchitis since the first year of life.

The phenotypic features of the child were normal; height and weight were in the 75th percentile and head circumference between the 50th and 75th percentile.

At the neurological examination the girl showed only a mild clumsiness; her behavior was characterized by impulsivity, attention deficit, and provocative attitude.

Diagnostic Work-Up

Blood and urine samples from the proband and her parents were obtained after informed consent for metabolic and genetic analyses.

Organic acids determination was performed as part of the metabolic work-up, including amino acids, creatine, thyroid hormones evaluation, and karyotype analysis (550 band level), as recommended by the diagnostic flowchart in our department in all cases of non-syndromic intellectual disability.

Moreover brain Magnetic Resonance Imaging (MRI) with proton spectroscopy, awake and sleep EEG, and fundus oculi evaluation were performed.

For organic acids analysis urine was extracted with ethylacetate (Tanaka et al. 1980) and trimethylsilylated (TMS) by TMCS + BSTFA at 60°C for 1 h prior to the analysis with an Agilent 6890 N GC equipped with an HP5MS capillary column (0.25 mm × 30 m, film thickness 0.25 μm) and an Agilent mass spectrometer 5973 N. Reagents were purchased from Sigma-Aldrich (Italy).

Mutation Analysis

Genomic DNA was purified from peripheral blood using standard procedures. PCR primers were designed by *Primer 3* software (http://frodo.wi.mit.edu/primer3) to cover the 14 exons and part of the flanking intronic regions of the *ACY1* gene (primer sequences are available upon request). Fragments were directly sequenced using the BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA) on an ABI3130xl Genetic Analyzer (Applied Biosystems).

A punch skin biopsy from the anterior area of the left forearm of the patient was obtained for fibroblast culture. Cells were grown in regular DMEM supplemented with 10% fetal bovine serum following standard procedures.

Total RNA was isolated from cultured skin fibroblasts using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Germany) according to the manufacturer's protocol. Both integrity and concentration were assessed on 1% agarose gel. Identical amounts of RNA were retrotranscribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and the cDNA was PCR-amplified using primers 5′-3′ACY1-F CTAAGTCCAGGCCACAGTCA and 5′-3′ACY1-M13R CAGGAAACAGCTATGACCGGGGGAAGAG-GAGGTCCTT.

About 40 μg of cell proteins were separated by 15% denaturating SDS-PAGE as previously described (Nogueira et al. 2013) and subjected to western blotting using a monoclonal anti-mouse antibody specific for the human Aminoacylase 1 protein (dilution 1:100; Novus Biologicals, USA). Quantitative blot analysis was performed using ImageJ software (http://rsbweb.nih.gov/ij/). The same ACY1 antibody was used for immunocytochemical analysis, following procedures already described elsewhere (Fiorillo et al. 2012).

Enzyme Assay

Enzymatic activity of ACY1 was measured in the supernatant of homogenized fibroblasts, according to Sass et al. (2006) with minor modifications. Methionine concentrations produced after incubation of the cells with *N*-acetylmethionine (NAM) were determined by a JLC-500/V amino acids analyzer (Jeol, Japan). Proteins were quantitated by the Lowry method (Lowry et al. 1951). ACY1 activity in the patient and in seven controls cells was analyzed in duplicate.

Neuropsychological Phenotype

The assessment of neuropsychological profile was performed according to the protocols of our department: the WISC IV (Wechsler 2003) for the cognitive level, a short

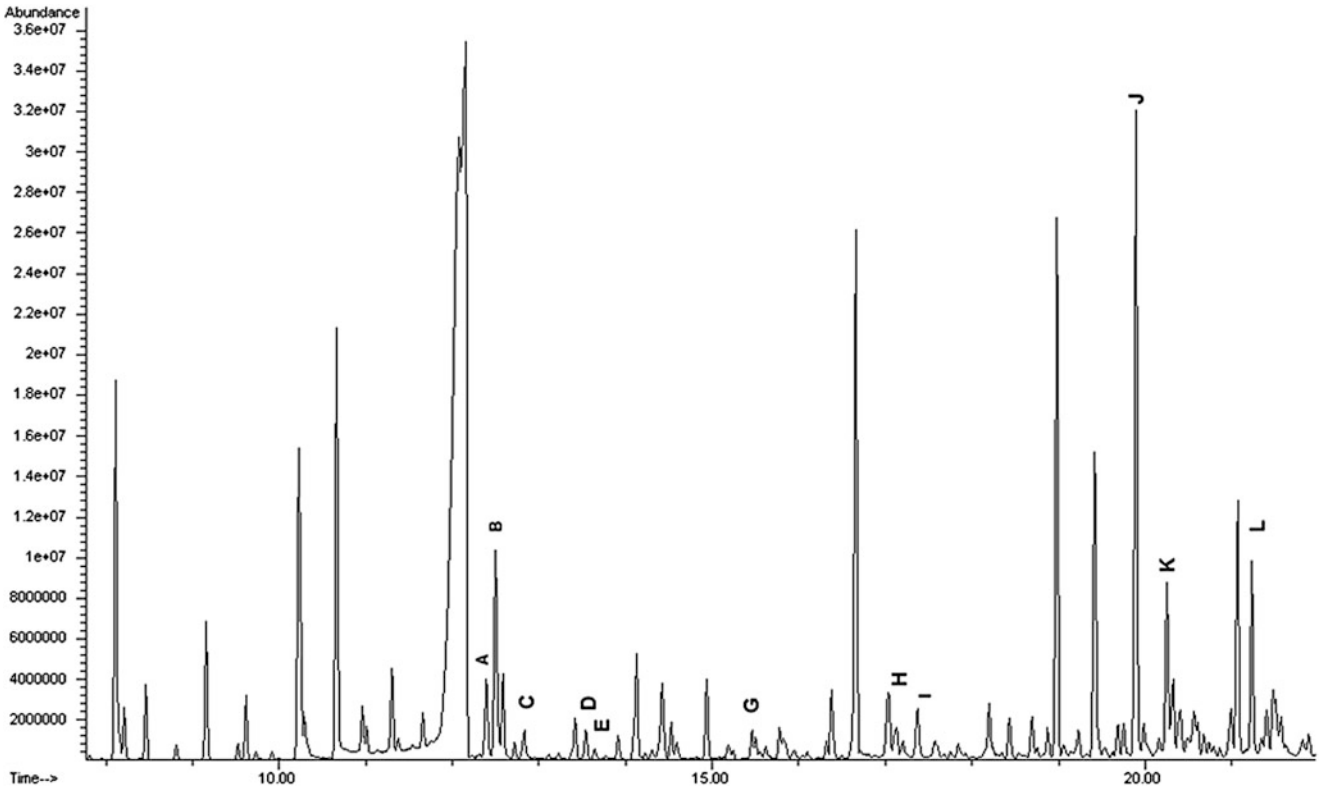

Fig. 1 Section of the total-ion chromatogram of organic acid from urine of the patient after extraction with ethylacetate and derivatization with BSTFA + TMCS (1% v/v) at 60°C for 60 min. In the figure only the TMS derivatives of the N-acetylated amino acids are highlighted with *capital letters*. The suffixes 1 or 2 indicate the number of trimethylsilyl groups, according to Gerlo et al (2006): (**a**) *N*-acetylalanine-1; (**b**) *N*-acetyl-alanine-2; (**c**) *N*-acetylglycine-1; (**d**) *N*-acetylglycine-2; (**e**) *N*-acetylvaline-1; (**f**) *N*-acetylvaline-2; (**g**) *N*-acetylleucine-1; (**h**) *N*-acetylserine-2; (**i**) *N*-acetylthreonine-2; (**j**) *N*-acetylmethionine-2; (**k**) *N*-acetylmethionine-1; (**l**) *N*-acetylglutamate-3

version of a pictured multiple-choice comprehension grammar test for children (TCGB) (Chilosi and Cipriani 1996) for the sentence comprehension, the Peabody Picture Vocabulary Test (PPVT) (Dunn and Dunn 1997; Italian standardization, Stella et al. 2000) for the receptive vocabulary, and a naming test (Gugliotta et al. 2009) for the productive vocabulary.

Results

Diagnostic Work-Up

Amino acids and creatine levels in plasma and urine of the patient were normal, as well as karyotype and standard blood and urine examinations. Brain MRI with proton spectroscopy, EEG awake and sleep, and fundus oculi evaluation were also normal.

Organic acids analysis in three different urine samples disclosed the characteristic pattern of ACY1 deficiency, with the presence of numerous peaks corresponding to N-acetylated amino acids. The most abundant were methionine and glutamate (mono- and di-) TMS derivatives, but alanine, glycine, valine, leucine, serine, and threonine were easily identified (Fig. 1).

All the usual organic acids, including *N*-acetylaspartic acid, both in urine and blood, were within the normal range. Organic acids in the urine of the parents of the patient were normal.

Molecular and Structural Findings

Sequencing of the coding region of *ACY1* revealed that the patient was compound heterozygous for the missense mutation c. 699A>C (p.Glu233Asp) in exon 10 and the c.574_575insG insertion in exon 8. The latter mutation predicts a frameshift and the synthesis of a prematurely truncated protein (p.Ser192Arg fs*64) with a carboxy-tail containing 64 new amino acids. Segregation was confirmed in the parents (Fig. 2).

The expression of Acy-1 protein, evaluated by Western blotting and immunocytochemistry in patient cultured skin fibroblasts, was nearly absent if compared to normal controls (Fig. 3).

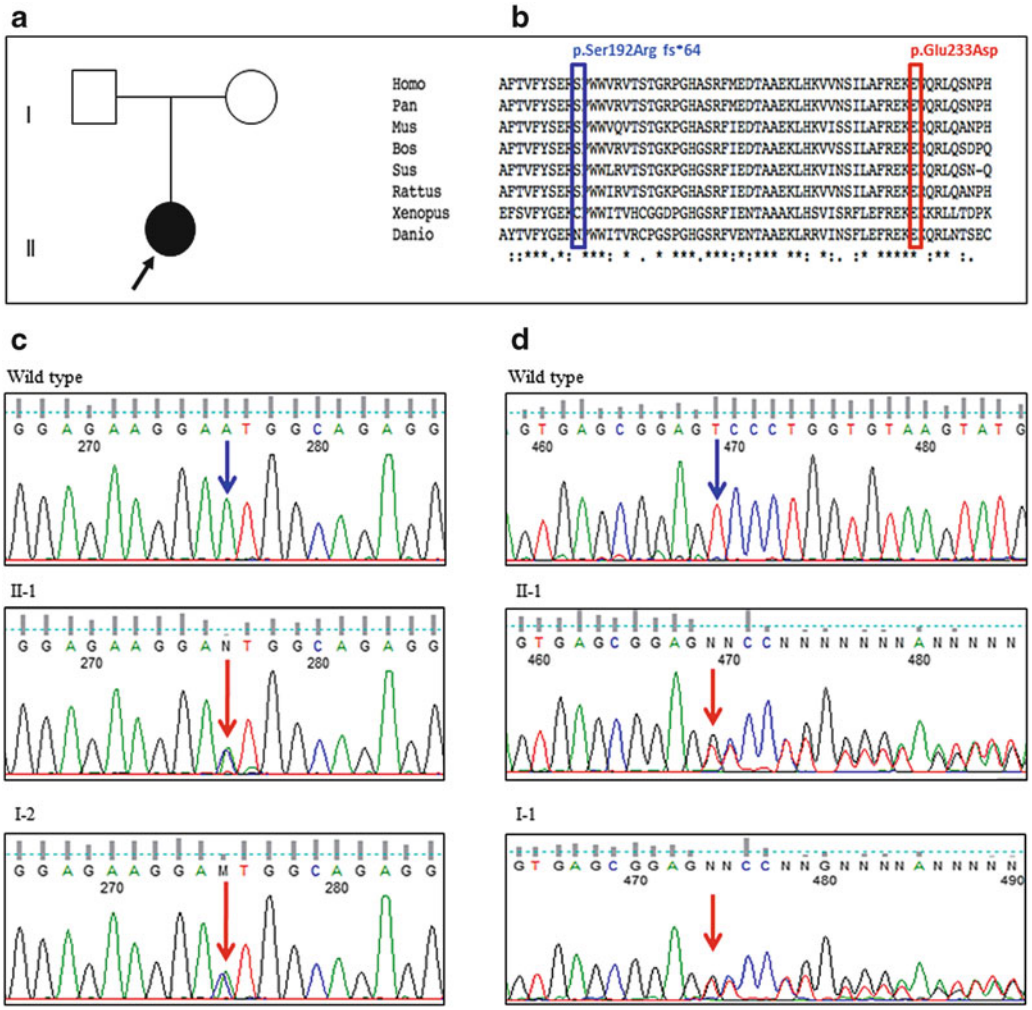

Fig. 2 (**a**) Family tree; the proband (II-1; *filled symbol*) is indicated by the *arrow*. (**b**) Sequence alignment of the human *ACY1* and corresponding orthologs from various species. The amino acids affected by the missense mutation Glu233Asp and the frameshift mutation Ser192Arg fs*64 are highlighted. (**c**) Sequence chromatograph from a wild-type individual, the affected patient (II-1) and her mother (I-2) showing the heterozygous missense mutation c.699A>C/p.Glu233Asp. (**d**) Electropherogram from a wild-type individual, the affected patient (II-1) and her father (I-1) carrying the c.574_575insG/p.Ser192Arg fs*64 change, predicting a frameshift and a prematurely truncated protein

Enzyme Assay

In the fibroblasts of the patient no increase of methionine was detectable after two hours of incubation with the substrate *N*-acetylmethionine; in the controls cells ($n = 7$) methionine production increased over the time with a mean activity of 0.91 ± 0.30 nmol/mg protein/min (mean $\pm$ SD).

Neuropsychological Phenotype

The cognitive psychometric test revealed mild intellectual disability (QIT:59); grammatical comprehension (TCGB) was under 1 SD for a mean score of 5.6 years, productive vocabulary was normal as receptive vocabulary (PVVT) with a raw score of 91. Learning difficulties were in agreement with the cognitive level. Moreover the child presented many difficulties of selective and sustained attention.

Discussion

The principal clinical trait of the patient here presented was an isolated mild intellectual disability, together with absence of neurological symptoms or autistic features, or brain MRI alterations that could suggest an inherited metabolic disease. Our proband, as well as the autistic patient described earlier by Tylki-Szymanska et al. (2010), has been identified during a thorough diagnostic assessment

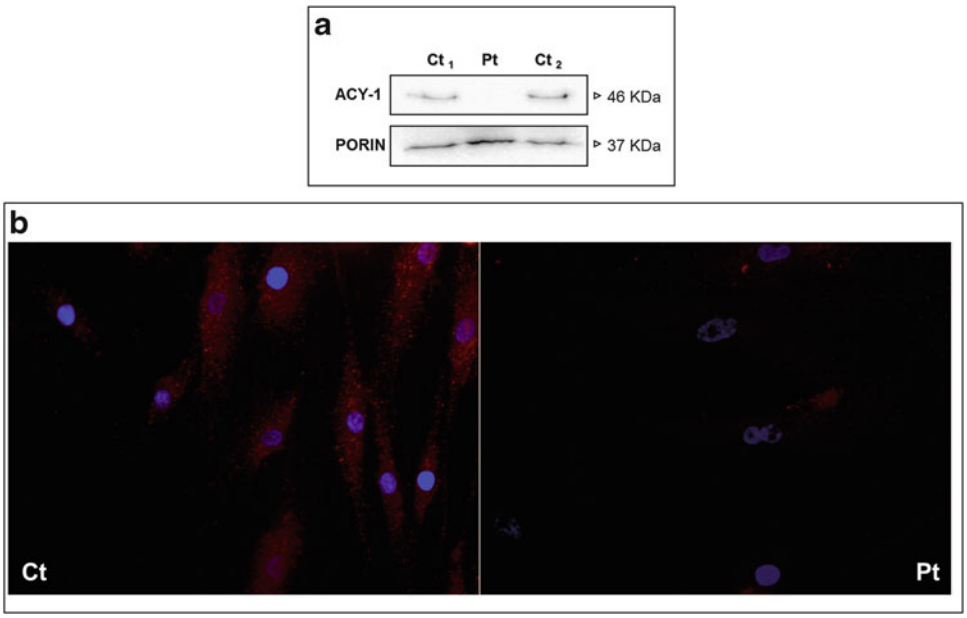

Fig. 3 ACY1 protein analyses on cultured skin fibroblasts, showing a significant reduction of the expression in the patient compared to controls both by Western blot (**a**) and by immunofluorescence (**b**). For immunoblot quantification, ACY1 content was normalized using a monoclonal anti-porin antibody (Mitosciences, USA); DAPI was used for nuclei counterstaining in immunocytochemistry. Representative experiments are shown. *Ct* controls, *Pt* patient

adopted in our department for intellectual disability, whereas a complete metabolic screening is commonly performed only in patients in whom an IEM is suspected.

The mild developmental impairment observed in the child suggests that ACY1 deficiency could be often overlooked for various reasons, such as the clinical phenotype or some technical problems in the diagnostic laboratories. The identification of N-acetylated amino acids, the biochemical hallmark for the ACY1 deficiency, is fairly easy for many laboratories through the organic acids analysis. Unfortunately the extraction and derivatization of these metabolites present a great variability depending on the conditions adopted for the analysis. As reported by Gerlo et al. (2006) the major problems in identifying the N-acetylated amino acids are the lack of reference spectra in most commercial mass spectral libraries, the presence of coeluting compounds and multiple derivatives in the chromatograms. On this respect, the results of qualitative organic acids scheme of the European Research Network for evaluation and improvement of screening for Diagnosis and treatment of Inherited Disorders of Metabolism (ERNDIM) in 2012, where a sample of urine from a patient with ACY1 deficiency was included, showed that only 73% of the participants ($n = 86$) correctly identified the disease.

To date, ten mutations in *ACY1* have been described, the majority in isolated cases, including small deletions, insertions, and missense mutations. The clinical aspects of the patients previously reported are extremely heterogeneous and comprise symptoms as psychomotor retardation, brain and spinal malformations, hypotonia, developmental retardation, seizures (Van Coster et al. 2005; Sass et al. 2006, 2007; Engelke et al. 2008; Tylki-Szymanska et al. 2010).

Mutation analysis of *ACY1* in the proband revealed the presence of two heterozygous mutations, one of which (p.Glu233Asp) has been already reported by Sass et al. (2006) in a heteroallelic child who was evaluated because of a slight psychomotor delay. The second mutation (c.574_575insG/p.Ser192Arg fs*64) is novel and resulted in very low levels of ACY1 protein, as shown by immunoblot and immunocytochemistry. This finding is supported by expression studies on transfected cells with Glu233Asp mutation, in which the enzymatic activity was completely loss (Sommer et al. 2011). Both mutations identified in our patient involve evolutionary conserved amino acids and are localized in the protein dimerization domain. In particular, they are localized towards the second monomer and may contribute to the stabilization of the homodimeric structure (Sommer et al. 2011). Because no phenotypic association has been found so far for different types of mutations, it is likely that additional factors modify the ACY1 phenotype in affected patients. The question of the pathogenicity of ACY1 deficiency requires the identification of additional subjects with this condition; similarly the role of the N-acetylated amino acids in the central nervous system should be better understood. Decreased expression of ACY1 has been found in murine models of Huntington's Disease and Fragile-X syndrome (Zabel et al.

2006), suggesting a role of this enzyme in regulating apoptosis and complex metabolic pathways through protein–protein interactions (Zabel et al. 2006; Maceyka et al. 2004). Among the N-acetylated amino acids, ACY1 preferentially hydrolyzes *N*-acetylleucine, NAM, *N*-acetylcysteine, and to a lesser extent *N*-acetylglutamate (NAG) (Lindner et al. 2008). NAG is the principal excitatory neurotransmitter in the brain, involved in many brain functions including development, cognition, memory and learning, but also in acute or chronic neurodegenerative processes; chronic alterations of its levels can affect these functions. The presence of NAM in the human brain has prompted Smith et al. (2011) to hypothesize that it could be a key player to understanding why individuals with the ACY1 deficiency have neurological or psychiatric defects. They proposed NAM as a secondary source of acetate, similarly to the *N*-acetylaspartate which provides acetate for the myelin lipids synthesis. In addition, NAM could serve as a storage pool of methionine, which has a prominent role in the brain; in this respect it is interesting to note that NAM is the major N-acetylated amino acid excreted in urine of patients harboring pathogenic mutations in *ACY1*. Moreover methionine can be converted to cysteine, the rate-limiting factor for the synthesis of glutathione that is the most important cellular antioxidant; accordingly, the depletion of methionine and cysteine, due to the impairment of ACY1 activity, could lead to an increased susceptibility to oxidative stress, which affects metabolism, signaling, and functioning of the cells. Finally it must be noted the role of methionine as precursor of *S*-adenosylmethionine, which in turn provides methyl groups for transmethylation reactions or can be decarboxylated for polyamine synthesis; these metabolites are involved in various physiological processes and have a critical role on the central nervous system function.

In conclusion, the patient here reported expands the phenotypic spectrum of the ACY1 deficiency and the number of mutations in the responsible gene. Our findings underscore the recommendation to perform a metabolic work-up even in patients with isolated intellectual disability of unknown origin.

Acknowledgments The authors thank Dr. Filippo M. Santorelli for his important suggestions and Mrs. Rita Magni for excellent technical assistance.

Concise 1 Sentence Take-Home Message

The isolated mild intellectual disability in a child harboring two mutations in ACY1 recommends thorough metabolic investigations in patients affected by intellectual disability of unknown origin.

Compliance with Ethics Guidelines

Conflict of Interest

Maria G Alessandrì, Manuela Casarano, Ilaria Pezzini, Stefano Doccini, Claudia Nesti, Giovanni Cioni, and Roberta Battini declare that they have no conflict of interest.

Informed Consent

All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with Helsinki Declaration of 1975, as revised in 2000. Parents of the proband gave their informed consent to participate in the study.

Contributions of Individual Authors

Maria G Alessandrì: biochemical analyses, concept and drafting of manuscript. Manuela Casarano, Roberta Battini: clinical data and drafting of manuscript. Ilaria Pezzini: performed mutation analysis. Stefano Doccini: performed protein and cellular studies. Claudia Nesti: directed molecular genetic studies. Giovanni Cioni: design and revision of manuscript.

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JIMD Reports
DOI 10.1007/8904_2014_324

RESEARCH REPORT

Birth Prevalence of Fatty Acid β-Oxidation Disorders in Iberia

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Received: 16 April 2014 / Revised: 13 May 2014 / Accepted: 19 May 2014 / Published online: 11 July 2014

Abstract Mitochondrial fatty acid β-oxidation disorders (FAOD) are main targets for newborn screening (NBS) programs, which are excellent data sources for accurate estimations of disease birth prevalence. Epidemiological data is of key importance for the understanding of the natural history of the disorders as well as to define more effective public health strategies. In order to estimate FAOD birth prevalence in Iberia, the authors collected data from six NBS programs from Portugal and Spain, encompassing the screening of more than 1.6 million newborns by tandem mass spectrometry (MS/MS), and compared it with available data from other populations. The participating NBS programs are responsible for the screening of about 46% of all Iberian newborns. Data reveals that Iberia has one of the highest FAOD prevalence in Europe (1:7,914) and that Portugal has the highest birth prevalence of FAOD reported so far (1:6,351), strongly influenced by the high prevalence of medium-chain acyl-CoA dehydrogenase deficiency (MCADD; 1:8,380), one of the highest ever reported. This is justified by the fact that more than 90% of Portuguese MCADD patients are of Gypsy origin, a community characterized by a high degree of consanguinity. From the comparative analysis of various populations with comparable data other differences emerge, which points to the existence of significant variations in FAOD prevalences among different populations, but without any clear European variation pattern. Considering that FAOD are one of the justifications for MS/MS NBS, the now estimated birth prevalences stress the need to screen all Iberian newborns for this group of inherited metabolic disorders.

Communicated by: Jerry Vockley, M.D., Ph.D.

Competing interests: None declared

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Introduction

Mitochondrial fatty acid β-oxidation is a key metabolic pathway to the provision of energy for the organism, particularly during periods of fasting and metabolic stress (Bartlett and Eaton 2004; Houten and Wanders 2010). To carry out the process, at least 25 enzymes and specific transport proteins are involved and defects in many of them are associated with human diseases (Kompare and Rizzo 2008; Houten and Wanders 2010).

Fatty acid oxidation defects (FAOD) are a group of inherited metabolic disorders that present heterogeneous clinical phenotypes mainly affecting heart, liver and skeletal

muscles (Kompare and Rizzo 2008). Some patients present the full spectrum and multisystemic disease, while others may stay asymptomatic and only exhibit hypoketotic hypoglycemia during illness or periods of rhabdomyolysis due to vigorous exercise (Wilcken 2010). Presumed pathophysiology of FAOD is supposed to rely mainly on inadequate energy supply, on the toxicity of accumulated metabolites or mutated proteins, and in some cases carnitine depletion. Pathophysiological threshold of some of these factors can be triggered by external factors like insufficient caloric intake, diet changes or infections (Olpin 2013).

Treatment options include avoiding fasting, which is in some cases complemented with carnitine, riboflavin or CoQ10 supplementation (Spiekerkoetter et al. 2010) and leads in most cases to favourable prognosis following diagnosis, being FAOD associated with high mortality/morbidity rates for those diagnosed later in a symptomatic phase (Baruteau et al. 2013). The effectiveness of available treatments for most of FAOD patients, the availability of high throughput adapted tests (acylcarnitine analysis on dried blood spots) and the advantages of an early intervention made this group of disorders main targets for Newborn Screening (NBS) programs worldwide (Lindner et al. 2010; Bennett et al. 2012; Baruteau et al. 2013).

NBS programs, besides being public health programs that allow on an early and positive intervention on affected newborns, are valuable data sources on the screened disorders. Since the introduction of FAOD in the screening panels of NBS programs, generated data has pointed to a significant increase in the disease incidence (Wilcken et al. 2007) that is now believed to be about 1:9,000, although some significant differences can be observed between different populations (Zytkovicz et al. 2001; Wilcken et al. 2003; Frazier et al. 2006; Kasper et al. 2010; Lindner et al. 2011; Lund et al. 2012). In the pre-NBS era, diagnosis was achieved mainly through organic acid analysis in the urine of symptomatic patients that resulted in a low detection rate, which together with the detection of potentially asymptomatic patients through NBS, justifies the observed difference (Sturm et al. 2012).

NBS programs have facilitated the expansion of epidemiological knowledge of screened disorders, with clear advantages over prevalence estimations calculated based on diagnosis of symptomatic cases. The present work incorporates population data derived from the use of tandem mass spectrometry by Iberian NBS programs and has the aim to obtain up-to-date estimates of birth prevalence of FAOD in Iberia and compare it with those of other reported population-based studies.

The increased knowledge on epidemiology is useful for a better understanding of the natural history of the disorders, so that we can define better treatments and public health strategies.

Material and Methods

Study Design

The present study includes data from the metabolic screening by tandem mass spectrometry of 1,672,286 Iberian newborns (812,902 Portuguese and 859,384 Spanish) (Table 1). Data was collected from six NBS programs, the Portuguese one and five from Spain (Galicia, Murcia, Western Andalucia, Eastern Andalucia and Aragón/La Rioja) during several years. All participating programs are well-implemented public health programs that virtually screen all newborns from their regions. All together, the regions covered by participating NBS programs represent about 46.2% of all annual births in Iberia.

Patient Detection

FAO detection criteria applied in the participating NBS programs are identical (all according to the best-accepted practices) and present similar levels of ascertainment. The panel of FAOD screened in the different programs is similar with exception of the Portuguese that doesn't screen for short-chain acyl-CoA dehydrogenase deficiency. After the initial suspicion of FAOD, based on the acylcarnitine profile, all diagnoses were confirmed by molecular and/or enzymatic approaches.

Statistical Analysis

FAOD birth prevalence in Iberia was determined by dividing the number of diagnosed patients by the total number of newborns screened. The calculation of the confidence interval of the prevalence was calculated using Wilson's score method.

Results and Discussion

Accurate assessment of the birth prevalence of screened disorders requires analysis of test results from a birth cohort representative of a geographic population. In this work, data from well-defined Iberian populations was used to assess the birth prevalence of FAOD in this European region.

Analysing the variation of FAOD prevalence within Iberian populations, what clearly emerges is that the prevalence of FAOD in Portugal (1/6,351) is the double of that observed in Spain (1/12,104). If we compare it excluding SCADD (not screened in Portugal), the difference is even bigger (1/6,351 versus 1/14,817). Undoubtedly, MCADD is the major contributor for this difference; nevertheless, all other FAOD present in Portugal higher

Table 1 Number of FAOD detected in the participating Iberian NBS programs and estimated birth prevalences, as well as in other NBS programs with available data reported

Region	Screened newborns	Fatty acid β-oxidation disorders															
Iberia		SCADD		MCADD		VLCADD		LCHADD		MADD		CPT1		CPT2		CUD		Overall
Portugal	812,902	Not screened		97	1/8,380	8	1/101,613	7	1/116,129	3	1/270,967	2	1/406,451	3	1/270,967	8	1/101,613	1/6,351
Galicia	278,371	5	1/55,674	14	1/19,884	0	0	3	1/92,790	0	0	0	0	0	0	0	0	1/12,653
Múrcia	124,942	1	1/124,942	3	1/41,647	0	0	2	1/62,471	1	1/124,942	0	0	0	0	1	1/124,942	1/15,618
Western Andalucia	272,462	3	1/90,981	15	1/18,196	0	0	0	0	1	1/272,462	1	1/272,462	0	0	1	1/272,462	1/12,997
Aragón/La Rioja	54,901	1	1/54,901	3	1/18,300	0	0	0	0	0	0	0	0	0	0	0	0	1/13,725
Eastern Andalucia	128,228	3	1/42,743	8	1/16,029	0	0	0	0	0	0	0	0	3	1/42,743	2	1/60,114	1/8,014
Overall	1,672,286	13	1/66,106	140	1/11,945	8	1/209,036	12	1/139,357	5	1/334,457	3	1/557,429	6	1/278,714	11	1/139,357	1/7.914
		(95% CI: 1/113, 111–1/ 38,634)		(95% CI: 1/14,093–1/ 10,122)		(95% CI: 1/ 415,522–1/ 105,923)		(95% CI: 1/ 243,603–1/ 79,720)		(95% CI: 1/ 783, 014–1/ 142,860)		(95% CI: 1/1,639, 060–1/ 189,576)		(95% CI: 1/608, 135–1/ 127,737)		(95% CI: 1/272, 250–1/ 84,891)		(95% CI: 1/9, 054–1/6,916)
Austria (Kasper et al. 2010)	622,489	1/155,622		1/24,900		1/88,927		1/69,165		1/311,245		0		0		1/311,245		1/12,704
Germany (Lindner et al. 2011)	583,555[a]/ 1,084,195[b]	1/64,839[a]		1/14,0808[b]		1/180,699[b]		1/216,839[b]		1/194,517[a]		1/1,084,195[b]		1/1,084,195[b]		1/194,518[a]		1/9,198
Denmark (Lund et al. 2012)	190,287[a]/ 504,049[b]/ 363,538[c]	1/190,287[a]		1/9,164[b]		1/168,016[b]		1/168,016[b]		0[c]		1/363,538[c]		0[c]		1/100,810[b]		1/7,691
Italy[d]	640,707	1/27,857		1/22,882		1/45,765		1/640,707		0		1/640,707		1/640,707		1/128,141		1/8,777
Greece (Loukas et al. 2010)	45,000	0		1/45,000		0		0		0		0		0		0		1/45,000
Switzerland (Rhead 2006)	57,000			1/11,500														
UK (Oerton et al. 2011)	1,500,000			1/10,204														
Belgium(Bodamer and Pollitt 2005)	120,000			1/15,000														
Netherlands (Derks et al. 2008)	182,850			1/9,624														
USA																		
New England (Zytkovicz et al. 2001)	164,000	1/32,800		1/16,400		1/164,000		0		0		0		1/164,000		0		1/9,647
North Carolina (Frazier et al. 2006)	944,078	1/118,010		1/12,933		1/78,673		1/314,693		0		0		1/472,039		0		1/9,633
California (Feuchtbaum et al. 2006; Gallant et al. 2012)	353,894[a]/ 2,632,058[b]	1/34,632[b]		1/27,223[a]		1/353,894[a]		1/353,894[a]		1/176,947[a]		0		0		0		1/13,002
Michigan[e]	708,257	1/16,097		1/13,620		1/88,532		1/708,257		1/354,129		0		1/708,257		1/354,129		1/6,439
Western USA (Merritt et al. 2014)	2,802,504					1/53,894												
New York (Arnold et al. 2010)	±1,501,022			1/24,210														
Japan (Bodamer and Pollitt 2005)	102,000			1/51,000														
Saudi Arabia (Al-Hassnan et al. 2010)	237,812			1/18,293														
Australia (Wilcken et al. 2003)	362,000	1/72,400		1/21,294		1/120,667		0		0		0		0		1/120,667		1/12,929

[a-c] refer to the number of screened newborns in a given population

[d] Data from Italy was extracted from national NBS reports from 2006 to 2012 (available on http://www.simmesn.it/it/documents/rt_screening/index.html; Accessed on December 2013)

[e] Data from the Michigan NBS program was extracted from program reports from 2006 to 2011 (available on www-michigan.gov/mdch/0,1607,7-132-2942_2950-233593-,00.html, accessed on September 2013). *CI* confidence interval, calculated using Wilson's score method

birth prevalence than in Spain. In Portugal, the prevalence of MCADD (1:8,380: 95% CI; 1:10,221 to 1:6,869) is higher than in any Spanish region and than in any other country with available data reported so far (Table 1). This high prevalence of MCADD can be justified by the fact that the great majority of the patients are of Gypsy origin (over 90%, and all homozygous for the most common mutation c.985 A>G), a community characterised by high inbreeding and where the high occurrence of genetic diseases, namely MCADD is known (Martinez et al. 1998; Kalaydjieva et al. 2001). The same is observed in Spain but to a less extent. This bias in the ethnical distribution of MCADD patients represents a significant difference in MCADD epidemiology in Iberia, namely Portugal, in comparison with other European countries where patients are almost exclusively of non-Gypsy origin (Khalid et al. 2008). This particular characteristic of Portuguese Gypsy community is the main justification for the observed birth prevalence of FAOD in Portugal, the highest among countries with available comparable data. These results are in disagreement with a pre-NBS assumption that there was in Europe a north-to-south gradient in the incident of MCADD (Tanaka et al. 1997).

SCADD prevalence in Iberia reflects its birth prevalence only in Spain, since this FAOD is not screened in Portugal. It has a prevalence of 1:66,106 in line with other available data for Europe, with exception of Italy where this FAOD seems to be significantly more prevalent.

VLCADD presented a birth prevalence of 1:209,036 in our cohort. Its prevalence in Iberia doesn't present significant differences from what is reported to other European populations, with exception of Italy where it presents a prevalence of 1:45,765. Indeed in this country, and in comparison with the other southern countries, Portugal and Spain, and besides an overall similar birth prevalence of FAOD, there are some differences in individual FAOD. Besides the differences in SCADD and VLCADD it is also clear that there is a lower birth prevalence of MCADD in Italy comparing to Portugal and Spain.

For LCHADD Iberia has a prevalence of 1:139,357, similar to that of Germany and Denmark, but less than in Austria. Concerning CUD, Iberia presents a birth prevalence of 1/139,357 in line with available data from other European populations. Comparing available data from Europe, the USA and Australia, it seems that CUD is less prevalent in the USA.

The analysis of the birth prevalence of MADD, CPT1 and CPT2 encompasses some difficulties due to their low frequency, requiring higher numbers of screened newborns in order to get more accurate prevalence estimations.

In Iberia, and influenced by Portuguese population data, MCADD has a birth prevalence of 1: 11,945 one of the highest reported in European countries, alongside with UK, Denmark and the Netherlands. This is higher than those observed, for example, in Austria (1/24,900) or Germany (1/14,080) as well as in the USA or Australia (Table 1).

Iberia presents birth prevalence for FAOD of 1:7,914 one of the highest in Europe, only comparable with Denmark and Michigan-USA. MCADD is the most prevalent FAOD in our cohort (66.3%), being followed by SCADD (12.0%), CUD and LCHADD (5.7%), VLCADD (3.8%), CPT 2 (2.8%), MADD (2.4%) and finally CPT 1 (1.4%) (values corrected for the number of screened newborns). Comparing our data with those from the world collaborative NBS project – Region4genetics (McHugh et al. 2011), what emerges is a higher proportion of MCADD (66.3% versus 56.3%) and lower contribution of SCADD (12.0% versus 15.7%) and VLCADD (3.8% versus 12.2%), for the total number of FAOD patients.

In conclusion, Portugal has the highest birth prevalence of FAOD reported, estimated based on NBS data. Iberian as a whole presents a birth prevalence of FAOD in agreement with available data from other Caucasian populations, but exhibiting one of the highest values reported. As FAOD are one of the main justifications for MS/MS screening in Caucasian populations, the birth prevalence now estimated stresses the need to screen all Iberian newborns for this group of disorders.

Synopsis

Data on the birth prevalence of fatty acid β-oxidation disorders in Iberia is reported for the first time, highlighting the high birth prevalence of this group of metabolic disorders in this European region. From the comparative analysis of various populations with comparable data differences emerge, which points to the existence of significant variations in FAOD prevalences among different populations.

Compliance with Ethics Guidelines

Conflict of Interest

Hugo Rocha, Daisy Castiñeiras, Carmen Delgado, José Egea, Raquel Yahyaoui, Yolanda González, Manuel Conde, Inmaculada González, Inmaculada Rueda, Luis Rello, Laura Vilarinho and José Cocho declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

Hugo Rocha: Conception, design and drafting of the article. Daisy Castiñeiras: Analysis and interpretation of data from Galicia. Carmen Delgado: Analysis and interpretation of data from Western Andalucia. José Egea: Analysis and interpretation of data from Murcia. Raquel Yahyaoui: Analysis and interpretation of data from Eastern Andalucia. Yolanda González: Analysis and interpretation of data from Aragón/La Rioja. Manuel Conde: Analysis and interpretation of data from Western Andalucia. Inmaculada González: Analysis and interpretation of data from Murcia. Inmaculada Rueda: Analysis and interpretation of data from Eastern Andalucia. Luis Rello: Analysis and interpretation of data from Aragón/La Rioja. Laura Vilarinho: Analysis and interpretation of data from Portugal and critical review of the article for important intellectual content. José Cocho: Conception, design and critical review of the article.

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JIMD Reports
DOI 10.1007/8904_2014_325

CASE REPORT

Perioperative Medullary Complications in Spinal and Extra-Spinal Surgery in Mucopolysaccharidosis: A Case Series of Three Patients

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Received: 15 February 2014 / Revised: 03 May 2014 / Accepted: 25 May 2014 / Published online: 20 July 2014

Abstract The mucopolysaccharidoses (MPS) are genetic lysosomal storage diseases. Peripheral bone dysplasia and spinal involvement are the predominant orthopedic damage. The risk of spinal cord compression due to stenosis of the craniocervical junction is well known in these patients, whereas the thoracolumbar kyphosis is often well tolerated over a long period of time. Thus, signs of spinal cord compression at this level occur later and more insidiously. The authors describe three cases of patients with thoracolumbar kyphosis who suffered from postoperative spinal cord compression in the absence of direct surgical trauma. Analysis of these cases and review of the literature helped identify causal factors resulting in spinal cord ischemia. The risk of perioperative spinal cord complications related to a thoracolumbar kyphosis must be discussed with patients with MPS and their families prior to any surgery, including extra-spinal procedures.

Communicated by: Maurizio Scarpa, M.D, Ph.D

Competing interests: None declared

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Introduction

Mucopolysaccharidoses (MPS) are genetic lysosomal storage diseases related to the accumulation of glycosaminoglycans resulting from congenital enzymatic deficiency. Dysostosis multiplex, along with spinal cord damage, is predominant and forms part of a picture of progressive multi-organ impairment appearing as of age one (Kleigman and Muenzer 2004; Feillet et al. 2006). Stenosis of the craniocervical junction and thoracolumbar kyphosis are the characteristic spinal deformities and are responsible for spinal cord compression at both levels (White and Harmatzc 2010). More recently, myelopathy due to cervicothoracic stenosis has been described in patients with Morquio syndrome (Baratela et al. 2014). We report on three children afflicted with MPS and thoracolumbar kyphosis showing no neurological impairment (two Hurler syndrome, or MPS type I, and one Morquio syndrome, or MPS type IV) who suffered perioperative spinal cord damage in the absence of direct surgical trauma.

Case Reports

Patient 1 was a 12-year-old child suffering from Morquio syndrome with recurrent bilateral genu valgum despite repeated treatment by temporary epiphysiodesis. At age 5, the child had been treated for craniocervical stenosis. At age 12, preoperative orthopedic assessment also revealed a T12 apical thoracolumbar kyphosis of 40° in prone position, reduced to 22° when in supine position. A 45° T2 apical craniothoracic kyphosis was present in prone position which remained unchanged when in supine position (Fig. 1). Preoperative neurological assessment was normal. Distal femoral and proximal tibial varisation

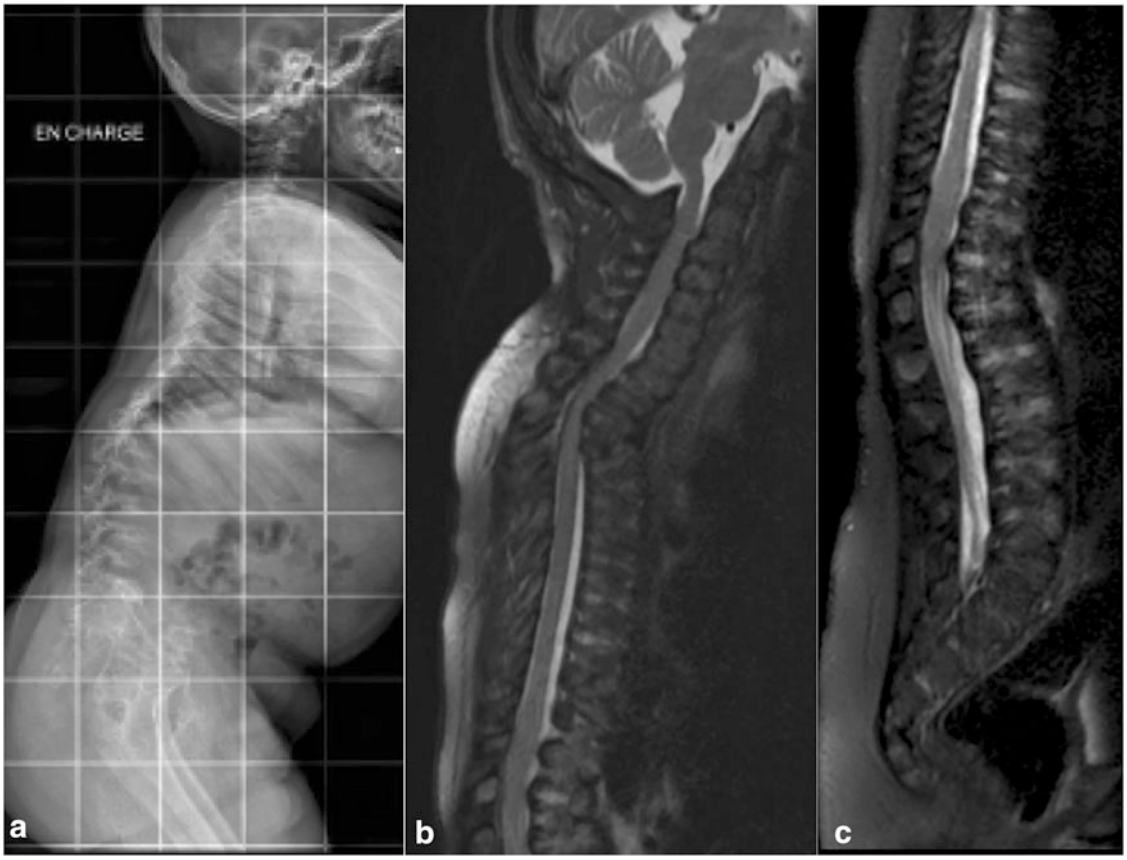

Fig. 1 Preopertive radiograph (**a**) and MRI (**b** and **c**) of patient 1. T12 apical thoracolumbar kyphosis of 40° in prone position, (**a**) reduced to 22° when in supine position. 45° T2 craniocervical kyphosis unchanged when in supine position (**a** and **b**)

osteotomies were carried out under general anesthesia, with no observable perioperative adverse event. Since it was an extra-spinal surgery, evoked potentials have not been monitored before and during surgery. On day 2 postoperatively, L1-L2 motor paraplegia appeared which slowly progressed over three days. No sensory disturbance was observed; damage appeared to be an anterior spinal artery syndrome. Emergency MRI showed no worsening of the two kyphotic zones. The diameter of the medullary canal remained unchanged with persistence of perimedullar fluid spaces next to both kyphotic zones. An extended intramedullary hyperintensity, however, was observed from T1 to T3 which did not correspond to the clinical topography. It was compatible, on the other hand, with ischemia or temporary spinal cord compression. There was no further neurosurgical indication, and the patient had partially recovered six months postoperatively, albeit persistence of sphincter disorders.

Patient 2 was a 4-year-old child afflicted with Hurler syndrome with advanced apical T12, greater than 90° (Fig. 2). No neurological complications existed, and preoperative evoked potentials were normal. Two-stage surgical correction was planned, with initial posterior instrumentation of T12-L2, followed by anterior graft. The first surgery was carried out monitoring somatosensory and motor evoked potentials. A slight modification of motor evoked potentials was observed during the procedure, but which returned to normal with repositioning of the spinal electrode. On recovery, the child developed an asymmetric paraplegia with partial sensory loss, more pronounced on the left side, requiring emergency surgery to remove the hardware. A spine cast was ordered to maintain reduction of this unstable thoracolumbar kyphosis after hardware removal, despite any MRI indication of spinal cord compression (Fig. 3). There was no improvement of neurological deficit with definitive installation of a complete L2 paraplegia with a complete sensory loss.

Patient 3 was a 13-year-old child also suffering from Hurler syndrome who had previously undergone surgery for thoracolumbar kyphosic instability treated by T10-L2 arthrodesis with instrumentation. He consulted for right hip dysplasia. The treatment approach associated a Dega pelvic osteotomy with a proximal femoral varisation and derotation osteotomy under spinal anesthesia (the epidural catheter was removed just after anesthesia). Respiratory distress occurred in the early hours postoperatively,

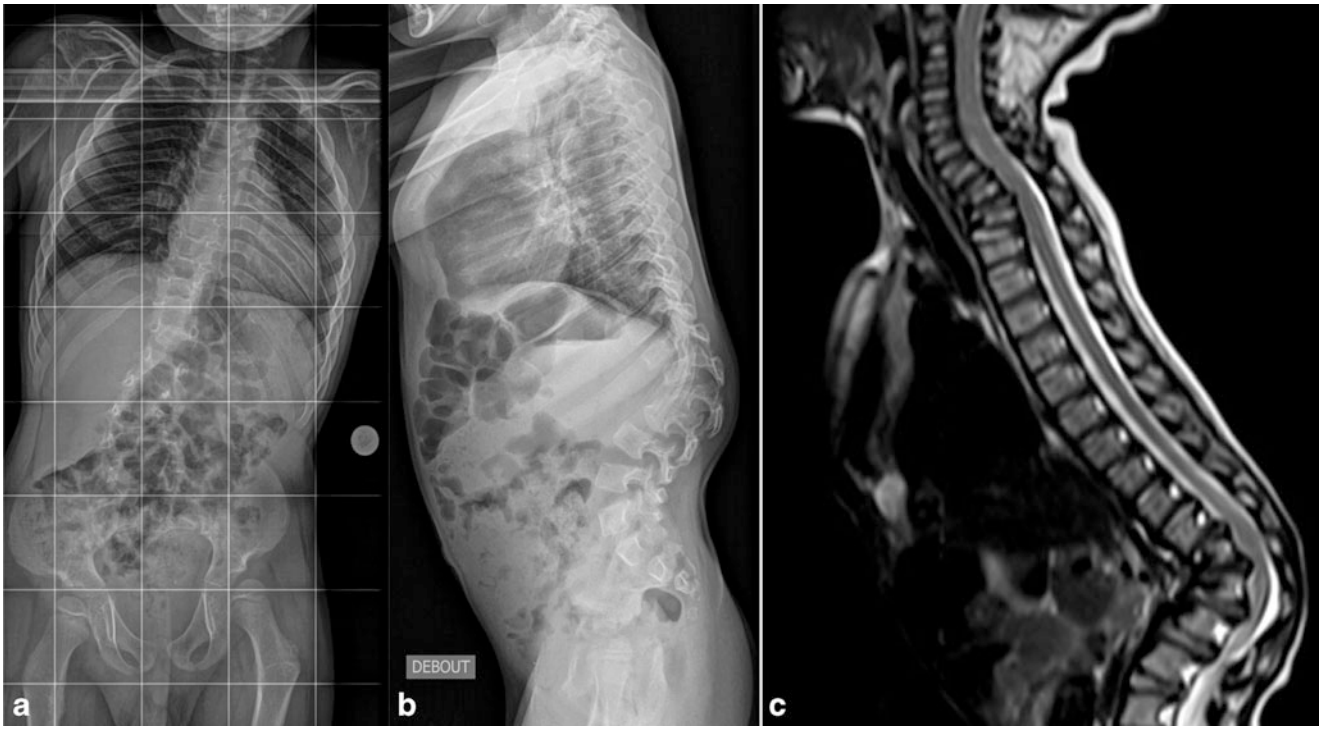

Fig. 2 Preoperative radiographies (**a** and **b**) and MRI (**c**) of patient 2 (Hurler syndrome). Kyphosis greater than 90° with retrolisthesis of the apical T12 vertebra and infiltrated, bulging appearance of intervertebral discs in the spinal cord

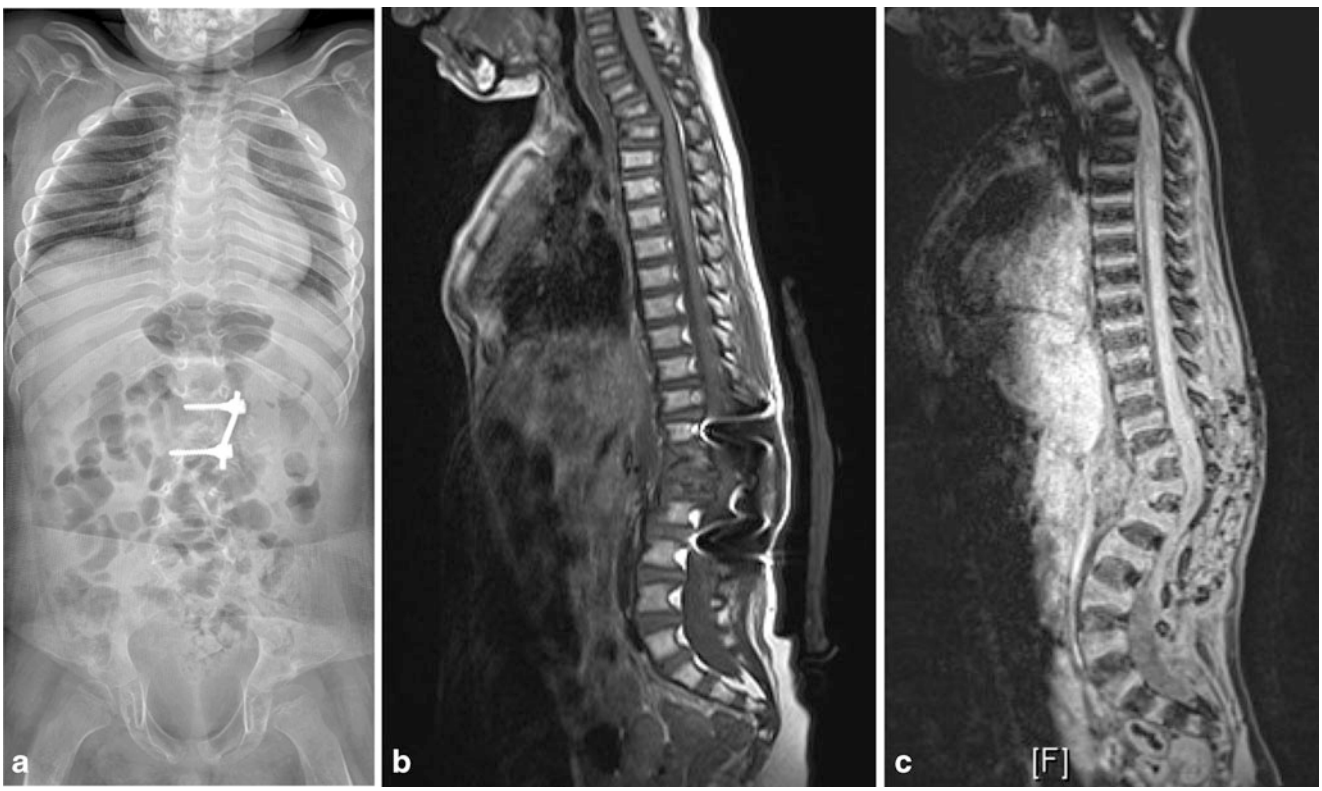

Fig. 3 Immediate postoperative condition of patient 2. (**a**) Anteroposterior radiograph of instrumentation T12-L2. (**b**) MRI before removal of hardware, absence of indication of spinal cord compression (artefacts related to the hardware). (**c**) MRI after removal of hardware showing no sign of spinal cord compression

attributed to pulmonary atelectasis, followed by progressive tetraparesis. Emergency CT-scan revealed a compressive posterior epidural hematoma extending from T1 to T6 but no pathological process at the cervical level. Decompression was carried out, evacuating the hematoma with a T1 to T3 laminotomy. Surgical verification was also carried out in the zone of spinal anesthesia (lower lumbar), revealing no indication of hemorrhagic stigmata. A laminectomy was nevertheless performed. No recovery was observed and the patient died from tetraparesis complications.

For these three patients, a complete retrospective study of anesthesia records failed to find an etiology, including hemodynamic.

Discussion

These three cases illustrate the spinal cord fragility of patients suffering from MPS. This fragility is most likely multifactorial, as few common elements were seen in the cases observed. Medullary complications can occur from spinal surgery as well as in extra-spinal surgical correction.

Thoracolumbar kyphosis is a characteristic deformity of MPS, occurring most frequently in type I, type VI (Maroteaux–Lamy syndrome), and type IV. It develops from poor bone growth in the anterior–superior aspect of the cranial lumbar vertebrae. This process leads to anterior wedging with retrolisthesis of the apical vertebra, as well as to posteriorly herniated infiltrated intervertebral discs bulging at the thoracolumbar junction (White and Harmatzc 2010). This kyphosis, moreover, is characterized by its hypermobility (Dalvie et al. 2001a) resulting from the weakening of the intrinsic ligamentary properties due to glycosaminoglycan infiltration. A lesser degree of kyphosis, moreover, is confirmed by diverse radiographic assessment in supine position (Dalvie et al. 2001a). This narrowing of the canal in a particularly mobile spine has already been identified in the literature as a major risk factor in spinal cord compression (White and Harmatzc 2010; Dalvie et al. 2001a, b; Ebara et al. 2003).

Analysis of these three observations leads us to believe that narrowing associated with spinal hypermobility plays a key role in the origin of this medullary damage. But this interrelation is not the sole factor, as evidenced by the two cases of extra-spinal surgery, notably where kyphosis had previously been stabilized. Other factors, particularly vascular and hemostatic, may also impact: these patients could present a risk of compressive epidural hematoma, as in our third case, potentially due to vascular walls infiltration that could make them more fragile and alter endothelial function (Kleigman and Muenzer 2004; Aydin et al. 2006; Kelly et al. 2013), with risk of spontaneous compressive hematoma. This is, however, still an hypothesis which has not been clearly stated. The role of spinal anesthesia in the development of epidural hematoma in our third case has not been established, but taking into consideration the global picture of vascular factors and anomalies of blood crasis, it seems preferable to avoid this type of anesthesia in these patients.

Moreover, a thickening of perispinal tissue, through infiltration of epidural fat and the dura mater, is regularly observed in spinal surgery (White and Harmatzc 2010). The infiltrative hypertrophy of the dura mater results in compressive spinal sheathing, without systematic bone canal narrowing. Hypertrophy of the ligamentum flavum has already been reported twice in the literature as being responsible for spinal compression in Type VI MPS (Sheridan et al. 1992; Mut et al. 2005). Furthermore, spinal sheathing is a factor in the onset of spinal ischemia from compression of the spinal arteries.

This theory of medullary ischemia is most likely involved in the genesis of medullary complications. These patients, for the most part, suffer from cardiovascular damage and thoracic deformity which lead to reduced cardiac ejection fraction. Prone position decreases cardiac index and further contributes to reducing the flow (Brown et al. 2013). Spinal sheathing and narrowing of the medullary canal thus promote medullary ischemia, already generated by the abovementioned factors. The key motor symptomatology is most likely related to the predominance of anteromedullar compression (corticospinal tract). As Tong et al. underscore in their description of perioperative paraplegia in atlanto-axoidian stabilization in a patient with Morquio syndrome, spinal cord infarction remote from maximal compression argues for the role of ischemia in the onset of neurological symptoms (Tong et al. 2012).

Currently accepted practice is to surgically stabilize kyphosis, resecting bulging discs combining an anterior and a posterior approach, in one or two surgeries, before the appearance of neurological complications. The decision to operate must take into account the patient's age, potential for growth and the evolution of the kyphosis.

These three cases raise the question of preventive spinal cord stabilization, prior to lower limb surgery, in order to decrease the risk of compression related to hypermobility, keeping in mind the nonnegligeble risk of spinal cord damage. Close collaboration between anesthesiologists and surgeons is important in maintaining sufficient medullary perfusion pressure and proscribing low cardiac output.

Perioperative monitoring of motor and sensory evoked potentials is an essential procedure in these risky surgeries (Mut et al. 2005; Tong et al. 2012). Nevertheless, our second case shows that it is not infallible, which makes us to hypothesize that the thickening of perimedullary tissue could alter data from the potentials.

Conclusion

The presence of craniocervical stenosis and thoracolumbar kyphosis in patients suffering from MPS must be seen as raising the risk of perioperative medullary complications, even in extra-spinal surgery. These complications are tied, on the one hand, to factors of compression (hypermobile spine, narowwing of the medullary canal accentuated by bulging discs; medullary sheathing from infiltrative hypertrophy of surrounding tissue) and, on the other hand, to ischemia (lowered perfusion pressure due to compression of spinal arteries and altered cardiovascular function). Informing patients and their families is essential prior to any surgical treatment, and neurological monitoring precautions should systematically be incorporated in case management. These precautions include prolonged extra-spinal surgery where the risk of hemorrhage might impact medullary perfusion pressure.

One Sentence Message

Thoracolumbar kyphosis in patients suffering from mucopolysaccharidoses is raising the risk of perioperative medullary complications, even in extra-spinal surgery.

Compliance with Ethics Guidelines

Conflict of Interest

All the authors of this chapter declare that there are no conflicts 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 and their legal representative when necessary for being included in the study.

Details of the Contributions of Individual Authors

Nicolas Pauchard has reviewed all the patients.

Jean-Luc Jouve, Christophe Garin, and Pierre Journeau performed the surgical procedure for the patients and followed them.

Nicolas Pauchard, Pierre Journeau and Pierre Lascombes have written and reviewed the article.

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JIMD Reports
DOI 10.1007/8904_2014_326

RESEARCH REPORT

NMR-Based Screening for Inborn Errors of Metabolism: Initial Results from a Study on Turkish Neonates

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Received: 15 November 2013 / Revised: 19 May 2014 / Accepted: 28 May 2014 / Published online: 11 July 2014

Abstract Approximately 1 in 400 neonates in Turkey is affected by inherited metabolic diseases. This high prevalence is at least in part due to consanguineous marriages. Standard screening in Turkey now covers only three metabolic diseases (phenylketonuria, congenital hypothyroidism, and biotinidase deficiency). Once symptoms have developed, tandem-MS can be used, although this currently covers only up to 40 metabolites. NMR potentially offers a rapid and versatile alternative.

We conducted a multi-center clinical study in 14 clinical centers in Turkey. Urine samples from 989 neonates were collected and investigated by using NMR spectroscopy in two different laboratories. The primary objective of the present study was to explore the range of variation of concentration and chemical shifts of specific metabolites without clinically relevant findings that can be detected in the urine of Turkish neonates. The secondary objective was the integration of the results from a healthy reference population of neonates into an NMR database, for routine and completely automatic screening of congenital metabolic diseases.

Both targeted and untargeted analyses were performed on the data. Targeted analysis was aimed at 65 metabolites. Limits of detection and quantitation were determined by generating urine spectra, in which known concentrations of the analytes were added electronically as well as by real spiking. Untargeted analysis involved analysis of the whole spectrum for abnormal features, using statistical procedures, including principal component analysis. Outliers were eliminated by model building. Untargeted analysis was used to detect known and unknown compounds and jaundice, proteinuria, and acidemia. The results will be used to establish a database to detect pathological concentration ranges and for routine screening.

Communicated by: Ron A Wevers

Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2014_326) contains supplementary material, which is available to authorized users.

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Introduction

Approximately 1:13,00 neonates is affected by congenital metabolic diseases in central Europe and 1:400 in Turkey (Aygen et al. 2011; Saudubray et al. 2006; Leonard and Morris 2006; Hasanoglu et al. 2000). This high prevalence is at least in part due to consanguineous marriages. Undetected and untreated, these diseases can lead to irreversible organ failure, invalidity, or death. With early diagnosis, therapy, and special medication, these developmental disorders may be treated successfully. Therefore, all neonates should be tested for congenital metabolic diseases. The standard screening in Turkey now covers only a few diseases, including phenylketonuria, congenital hypothyroidism, and biotinidase deficiency. Numerous additional diseases are just tested by tandem-MS once symptoms have developed; however, this technique currently uses a maximum of 40 metabolites, as only 40 reference substances are available for quantification (Lehotay et al. 2011; Chace and Kalas 2005).

^{1}H-NMR spectroscopy of body fluids constitutes a complementary technique in the diagnosis of numerous congenital metabolic diseases (Engelke et al. 2008; Moolenaar et al. 2003; Wevers et al. 1994, 1999; Holmes et al. 1997). It shows the majority of proton-containing compounds and therefore provides an overall view of metabolism, giving a characteristic 'fingerprint' of almost all hydrogen nuclei in a metabolite. In the NMR spectrum of urine, more than 1,000 metabolites can be detected simultaneously. As the intensity of the observed resonance is proportional to the number of hydrogen nuclei in the sample, the concentration of each metabolite can be determined as well. Moreover, all metabolites in urine can be analyzed in a single measurement that only takes minutes, allowing fast and cost-effective diagnostic screening. Besides buffering, no pre-processing of the urine samples is necessary. Because of its noninvasive nature, the lack of derivatization, the speed of measurement and its high reproducibility ^{1}H-NMR spectroscopic analysis is a very promising approach.

Some neonates with congenital metabolic diseases have already been investigated qualitatively by NMR spectroscopy (Engelke et al. 2008; Moolenaar et al. 2003). Nevertheless, there is no systematic, automated, and standardized NMR-based analytical technique. Therefore, fully automated NMR spectroscopy of body fluids may be considered as an alternative analytical approach for the early and clear diagnosis of known, and even unknown, inborn errors of metabolism.

In this context, a multi-center clinical trial with 989 neonates in 14 clinical centers was performed. The main objective of this project is the qualitative and quantitative determination of pathological metabolites by high resolution NMR spectroscopy in an automated and standardized procedure. To achieve this, it is necessary to define a statistical model of normality based on healthy neonates, against which each new urine spectrum can be tested.

An additional problem is that healthy neonates can have high concentrations of different pathological metabolites after birth. On subsequent monitoring, it may be found that metabolite levels have returned to normal and that the child is clinically normal. The case can then be eliminated as a false positive result. The transition state in the first days of life can actually be used to build models for the first days after birth and such can give information on the development state of the newborn.

This approach is intended to facilitate the early detection of inherent metabolic diseases, leading to appropriate treatment – which can greatly increase the quality of life of both the neonate and its parents.

Materials and Methods

Study Objectives

The primary objective of the study was to explore the range of variation (concentration and chemical shifts) of specific metabolites without clinically relevant findings. This is necessary for the identification of pathological thresholds of specific metabolites in comparison with healthy neonates (for development of a normal model).

The secondary objective was the integration of the results from a healthy population of neonates into an NMR database, in order to perform routine and completely automatic screening for congenital metabolic diseases as well as the direct quantification of diagnostic metabolites indicating inborn errors of metabolism.

Study Design

In an open, one-arm, non-interventional study, data were collected from healthy Turkish neonates, aged 24 to 168 h after birth. Data included the date of birth, the day of life, gestational age, gender, weight, length, head circumference, and type of birth. Premature infants with gestation period of below 37 weeks (+6 days) as well as neonates, who have clinical symptoms of disease, were excluded. Data were collected in 14 centers in Turkey over a period of 18 months.

Selected Cohort

The study covered 14 birth centers from all over Turkey. In all, 989 neonates were included in the study. 953 of these

samples were measured in INFAI laboratory and 890 in the BRUKER laboratory. Demographic data was collected for the neonate and for the parents. The study was approved by local ethic committees and all parents gave written informed consent.

Sample Collection and Preparation

Urine samples were collected between day 1 and day 7 of life, using a standard pediatric urine bag. The urine samples were deep frozen and then transferred to the NMR laboratories, where they were thawed and homogenized. Five-hundred and forty micro liter of urine was then mixed with 60 µL of 1.5 M phosphate buffer, to give a stable pH of 7.0 ± 0.5. The buffer was prepared in D_2O containing 0.1% sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-1-propionate (TSP) for reference, together with 2 mM sodium azide. Chemicals were obtained from Sigma Aldrich. A pH around 7 was chosen, since at this pH most metabolites show very small variation in chemical shift due to pH changes as can be found from the Bruker Reference Compound Spectral Base. In most cases, chemical shifts between 6 and 8 ppm do not change significantly in more than 650 compounds investigated.

Study Devices

The urine samples were investigated by using fully automated NMR spectrometers in two different laboratories (INFAI and BRUKER). At *INFAI*: 500 MHz AVANCE III, UltraShield Magnet with BOSS III shimming system, BACS-60, TopSpin 2.1, 5 mm SEI 1H/D-13C with *z*-gradient, automated tuning and matching (ATM). The BTO 2000 of the probe combined with a BCU-05 for cooling ensured temperature stability to within 0.01 K. At *BRUKER*: 600 MHz AVANCE III, UltraShield Plus Magnet with BOSS II shimming system, BACS-60, TopSpin 2.1, 5 mm BBI 1H/D-BB with *z*-gradient, automated tuning and matching (ATM). The BTO 2000 of the probe combined with a BCU-05 for cooling ensured temperature stability of 0.01 K.

NMR Experiments

Before measurement, samples were kept for 5 min inside the NMR probe head, to achieve temperature equilibration (300 K). *Experiment 1 – noesygppr1d*: For each sample, a one-dimensional ^1H NMR spectrum was acquired using a standard pulse sequence with 25 Hz CW-based water peak suppression during a relaxation delay of 4 s and a mixing time of 10 ms. Thirty-two free induction decays (FIDs) were accumulated into 64 k data points at a spectral width of 20.5425 ppm. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz before Fourier transformation and fully automated phasing and baseline correction. *Experiment 2 – jresgpprqf*: In addition to the one-dimensional experiment, a two-dimensional fast J-resolved measurement was performed on each sample using a standard pulse sequence with 25 Hz CW-based water peak suppression during a relaxation delay of 1 s. After 16 dummy scans, 2 free induction decays (FIDs) were accumulated into 8 k data points at a spectral width of 16 ppm. 40 such increments were collected covering the second spectral dimension (F1) at a width of 78.125 Hz.

Data Analysis

Data was visualized with AMIX 3.9 (Bruker BioSpin). Metabolite signal identification used the reference spectral database BBIOREFCODE-2.0. (Bruker BioSpin). Automated quantification and multivariate statistics were based on in-house developed algorithms using MATLAB (MathWorks Inc).

Absolute Quantification (mmol/L)

TSP is added to all NMR samples at given concentration. From the TSP signal in the urine spectra, a signal-intensity-per-proton-ratio can be calculated which after correction for T1-relaxation effects can be used to translate any signal of the spectrum into a concentration value.

Relative Quantification (mmol/mol Creatinine)

The ratio between a signal intensity of a metabolite and the signal intensity of, e.g., the CH_3-signal of creatinine, corrected for signal specific proton numbers and relaxation time effects, multiplied by 1,000 directly gives the concentration of the metabolite in mmol/mol creatinine.

Targeted analysis

Targeted analysis aims at the quantitation of a predefined list of metabolites. Concentration is calculated both, absolute or relative. Quantification is done via signal fitting using a simplex algorithm under consideration of signal and metabolite specific constraints and quantification parameters. This includes molecular mass, number of protons, relaxation time, chemical shift, signal multiplicity and coupling constants, line width, Gauss–Lorentz ratio and search ranges for signal detection and quantification. The quantification targets include 20 normal metabolites that are always present in normal urine (see Table 1).

Table 1 Concentrations of 19 urinary metabolites given in mmol/mol creatinine

Metabolite	Mean	Q2.5	Q25	Median	Q75	Q97.5	Max
Acetic acid	120	–/–	–/–	–/–	40	1,200	7,300
Alanine	120	–/–	60	90	160	330	1,900
Betaine	430	–/–	220	390	600	1,100	4,300
Citric acid	440	–/–	160	360	600	1,300	5,100
Creatine	140	–/–	–/–	40	190	700	1,800
Dimethylamine	100	30	70	110	130	160	210
Formic acid	160	–/–	30	80	200	740	1,600
Fumaric acid	12	–/–	–/–	10	20	50	100
Glycine	650	–/–	230	490	960	1,900	6,900
Lactic acid	90	–/–	–/–	40	80	540	5,000
D-Lactose	230	–/–	–/–	190	270	670	19,000
α-D-Galactose	110	–/–	–/–	50	150	500	1,200
β-D-Glucose	250	–/–	60	100	130	380	83,000
1-Methylnicotinamide	60	–/–	–/–	50	70	150	370
Myo-inositol	490	–/–	170	360	640	1,600	6,700
N,*N*-Dimethylglycine	80	–/–	30	60	90	270	950
Succinic acid	110	–/–	40	70	130	440	1,700
Trimethylamine	50	–/–	20	40	60	170	590
Taurine	410	–/–	150	310	540	1,200	12,000

The parameters Mean and Median assign the concentration mean and median values of the metabolites. Q2.5, Q25, Q75, and Q97.5 represent the respective quantiles (2.5%, 25%, 75%, 97.5%) of the concentration distributions. Because of detection thresholds, Q2.5 and Q25 could not always be determined. Related values are marked as '–/–'. Another consequence was that mean values could only be approximated roughly for orientation

In addition, 45 pathological metabolites were analyzed automatically which are generally not found in healthy neonatal urine, except occasionally at low concentrations for short periods: 2-oxoisovaleric acid, 2-hydroxyisovaleric acid, 2-hydroxyphenylacetic acid, 2-oxoisocaproic acid, 2-hydroxyisocaproic acid, 2-phenyllactic acid, 2-hydroxybutyric acid, 3-hydroxyglutaric acid, 3-hydroxyisovaleric acid, 3-hydroxypropionic acid, 3-hydroxyvaleric acid, 3-methyl-2-oxovaleric acid, 3-methylcrotonylglycine, 3-phenyllactic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenyllactic acid, 5-aminolevulinic acid, acetoacetic acid, acetone, allo-isoleucine, citrulline, D-galactonic acid, D-sorbitol, E-glutaconic acid, ethylmalonic acid, galactitol, glutaric acid, isovalerylglycine, L-leucine, L-isoleucine, L-pyroglutamic acid, methylmalonic acid, *N*-acetylaspartic acid, neopterin, orotic acid, phenylacetic acid, L-phenylalanine, phenylpyruvic acid, propionic acid, propionylglycine, uracil, uridine, valine and xanthine.

Limits of detection (LODs) and limits of quantification (LOQs) were determined by real spiking experiments and electronically calculating spectra in which different concentrations of the analyte were added to all urine samples from Turkey. Averaging over the total cohort finally gave a rate for true and false detection at given concentration.

Untargeted analysis

Untargeted analysis is used to identify untypical spectroscopic patterns and peaks in the NMR spectrum, be they known or unknown. For this purpose, extensive statistical analysis is necessary. Moreover, a reliable reference data set must be established to represent the normal condition. The initial step is data reduction and preprocessing by the use of the so-called bucketing. Normalized spectra were segmented into $n = 900$ consecutive integrated spectral regions (buckets) of fixed width, covering the range from 0.5 to 9.5 ppm. This then gives a table in which each line represents a sample and each column represents the intensities within one spectral region (bucket). Each line of the bucket table and hence each sample can be represented by a single point in N-dimensional space.

Statistical Analysis

Principal Component Analysis (PCA) was used to visualize the data and to reduce the number of dimensions for further multivariate statistical analyses. A supervised extension of PCA was used to establish a statistical model of normality in a healthy population of Turkish neonates (Mohammadi

et al. 2011; Aygen et al. 2011; Pomerantsev 2008; Vanderginste and Massart 1998). In this approach hierarchical multi-model/soft independent modeling for class analogy (HMM/SIMCA) was used. At the first level, a SIMCA model is built from the complete bucket table. At the second level, the bucket table is divided into two equally large parts and a SIMCA model is derived for each of these. At the third level, the bucket table is divided into four parts. The process is extended until the differences are no longer significant. This process has the advantage to identify both (a) atypical samples and (b) respective atypical deviations in the spectrum.

Additionally, principal component analysis/canonical analysis/k-nearest neighbor (PCA/CA/k-NN) was used if a sample needs to be classified with respect to multiple co-existing classes.

To avoid overfitting the data, extensive validation is needed (Hastie et al. 2009). For example, test set validation divides a bucket table into two parts, one serving as model set and the other as an independent test set. Models are built from the model set only and the test set is classified on this basis. Alternative approaches involve cross-validation and Monte-Carlo embedded cross-validation (MCCV).

Identification of Outliers

In order to establish a reference set for metabolic normality, it is essential to have a procedure for removing atypical samples by outlier detection. In the first step, univariate outliers are detected with respect to the bucket table, indicating buckets with a deviation from normality. In the second step, MCCV is performed for HMM/SIMCA modeling. The modeling is then repeated after the outlier has been removed. The normal model contains 20% outliers, which were used to avoid false-positive results in the newborn screening. Outliers were then tested for an additional multitude of relevant metabolites.

Results

Completeness of Data Collection and Deviations from Study Protocol

With the exception of head circumference, complete demographic data were collected for 727 neonates (75.9%). Deviations from the study protocol were found in 71 of neonates (7.2% of the initial cohort of 989 neonates). These deviations were mainly in gestational age or birth weight.

Center Effects

Center effects were examined with a confusion matrix (Fig. 1) and have to be accepted as a given property of the cohort. As outlined in the methods part, 14 centers distributed over whole Turkey, some of them more than 1,500 km apart, contributed to this study. It has to be anticipated that environmental, ethnical, and even economic factors (compare the city Van in the far east of Turkey with Istanbul) may result in characteristic effects on the metabolic fingerprints resolved by NMR.

Comparison of Spectra in Two Different NMR Laboratories

Nearly all samples were measured twice independently in different laboratories (Bruker and INFAI). The results of this analysis were compared in Fig. 2.

Reference Ranges

Reference ranges were established for the 20 metabolites that are almost always present in neonatal urine (Table 1). These metabolites are expressed relative to creatinine (except creatinine itself) and these curves can be used to derive percentiles.

LODs and LOQs

The LODs and LOQs are extremely diverse, due to the nature of the metabolites' NMR signals, the number of protons generating the signal and the spectral regions they are located in. Values for the 95% true detection rate range from 8 for 2-hydroxyisovaleric acid to $>>$1,000 for citrulline (in mmol/mol creatinine). Due to the so-called confusion potential of heavily overlapped signal regions, the LOD and LOQ determination had to be done individually for every metabolite under investigation.

Outliers: Detection of atypical Samples

A substantial number of the urine samples shows atypical features in their NMR spectra due to, e.g., atypical metabolites, atypical concentrations or atypical concentration ratios and overall signal patterns. Two-hundred and two and 200 samples were identified as outliers in the sample set measured at BRUKER (890 samples: 22.7% outliers) and in the sample set measured at INFAI (953 samples: 21.0% outliers), respectively. They are summarized in Table 2.

The most frequent reasons for classification as an outlier were medication, ketosis, proteinuria, or jaundice, and to a

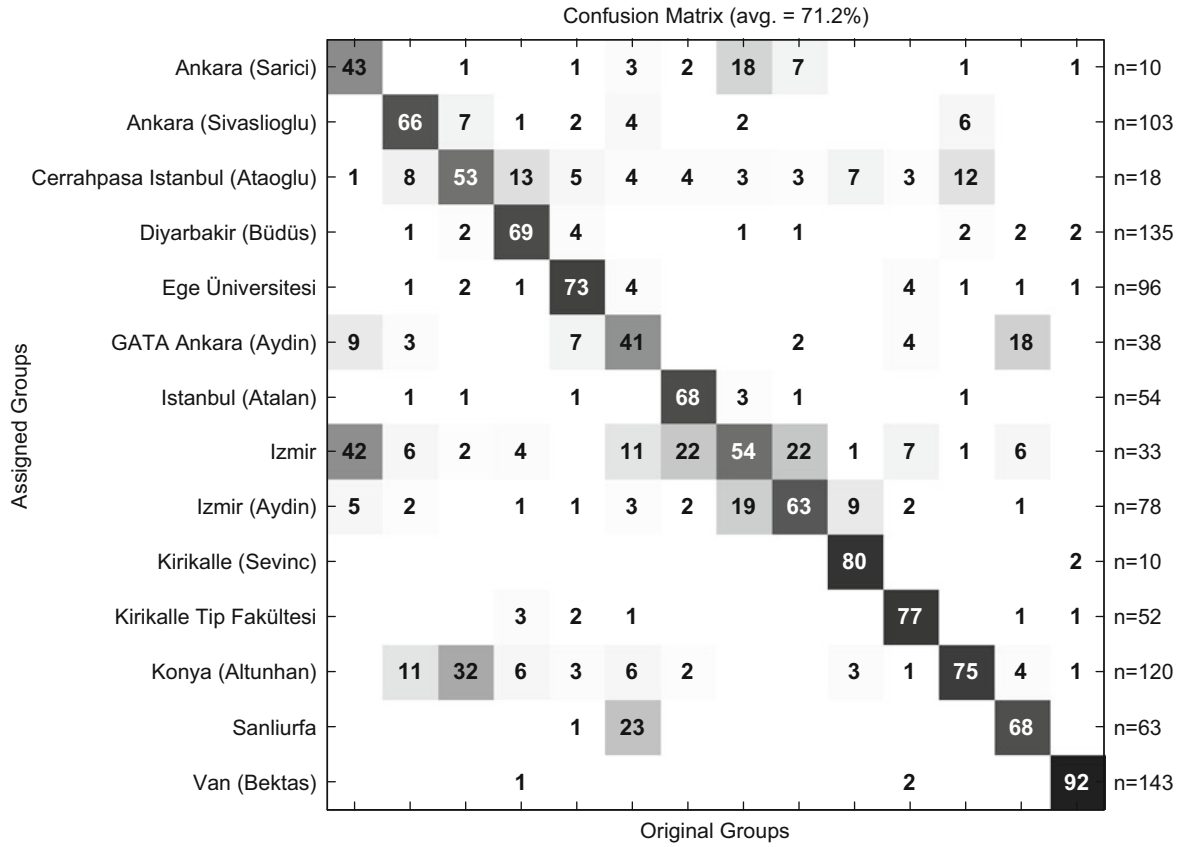

Fig. 1 Center prediction from urinary NMR data using 953 INFAI samples as input. The results are summarized in a confusion matrix giving the rates of correct prediction of the respective centers on the diagonal and the rates of false prediction (cross center prediction) in the off diagonal elements of the matrix

	Total Number of NMR Samples	NMR Samples with clinical metadata
INFAI	953	953
BRUKER	892	890
Both labs	885	885
Only at INFAI	68	68
Only at Bruker	7	5

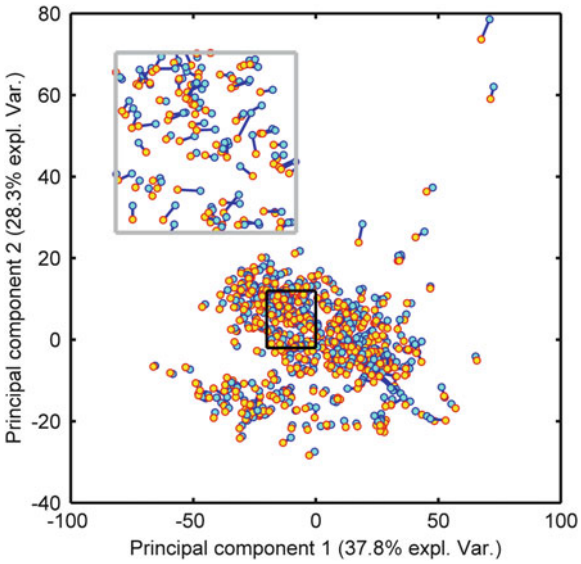

Fig. 2 The *right figure* shows PCA analysis of NMR spectra measured and analyzed at Bruker (600 MHz) and INFAI (500 MHz); each pair of samples is represented by a pair of markers connected by a *blue line*. The variance explained by principal component 1 and principal component 2 is 37.8% and 28.3%, respectively. The *black box* is magnified and replotted in the *inset* at the *upper left corner*

Table 2 List of different classes of outliers and their related atypical deviations

Type of class		Outlier class	Mmol/mol Crea	Number of outliers	Ppm region
Ketosis		2	>1,000	7	2.25–2.45 and 1.18–1.23
Medication		3	\	15	7.39–7.59
Panthenol gel		4	>100	1	0.85–0.96
High concentration of known metabolite	Succinic acid	5	>800	4	2.4–2.42
	Acetic acid	6	>4,000	3	1.91–1.94
	Lactic acid	7	>1,300	3	1.31–1.355
	4-Hydroxyphenyllactic acid	8	>900	4	6.84–6.88 and 7.16–7.20
	4-Hydroxyphenylacetic acid	9	>150	2	7.15–7.18
	Acetoacetic acid	10	>400	1	2.27–2.29
	Myo-inositol	11	>5,000	9	3.27–3.3
	N,*N*-Dimethylglycine	12	>850	1	2.92–2.94
	D-Lactose	13	>2,000	5	4.45–4.48
	D-Glucose	14	>4,000	6	4.63–4.67
	Sucrose	15	>950	1	4.2–4.3
	Tiglylglycine	16	>800	1	6.45–6.55 and 1.75–1.85
	N-Phenylacetylglycine	17	>50	1	7.8–7.85
	Salicylurate	18	>250	1	7–7.05
	Threonate	19	>1,200	1	4–4.2
	Gluconate	20	>4,500	1	3.7–4.1
	Propylene glycol	21	>700	3	1.1–1.18 and 3.4–3.6 and 3.85–3.9
	Ethanol	22	>11,000	1	1.18–1.21
	Mannitol	23	>1,000	1	3.6–4
	Proline	24	>2,000	1	1.95–2.1
	Proteinuria	25	–	8	0.5–2.5
	Jaundice	26	–	4	7.5–7.7
Unknown signal	Single signal	28		19	
	Multiple signals	29		69	
	Unknown sugars	30		4	3–4.5

The number of outliers refers to the INFAI data set. In about half of the outliers related a-typical signals are not yet identified

small extent the presence of unknown signals or known signals in unusually high concentrations. Seven ketosis (see Fig. 3), eight proteinuria, four jaundice, and four 4-hydroxyphenyllactic acid cases were confirmed through clinical data.

Metabolic Fingerprint of Gender

Differences in the metabolic fingerprint of male and female have been observed and documented in several studies in animals and human studies. To study the gender influence on the metabolic fingerprint in the neonate samples, PCA/CA/k-NN modeling with MCCV validation was done on the spectral subset where gender information is available. It was found that gender modulates the NMR metabolic profile in a characteristic way even in neonates. The rate of correct gender prediction could be estimated to about 77% for male and 63% for female, respectively.

Metabolic Fingerprint of Birth Mode

It can be anticipated that the mode of birth, i.e. spontaneous versus intersection, exposes a strong impact on neonate metabolism during the first days of life. In the subcohort of neonates where birth mode information is available, 49% experienced a spontaneous birth and 51% were delivered by section. PCA/CA/k-NN + MCCV could predict the birth mode from the NMR data at a rate of correct prediction of 66% far above random chance.

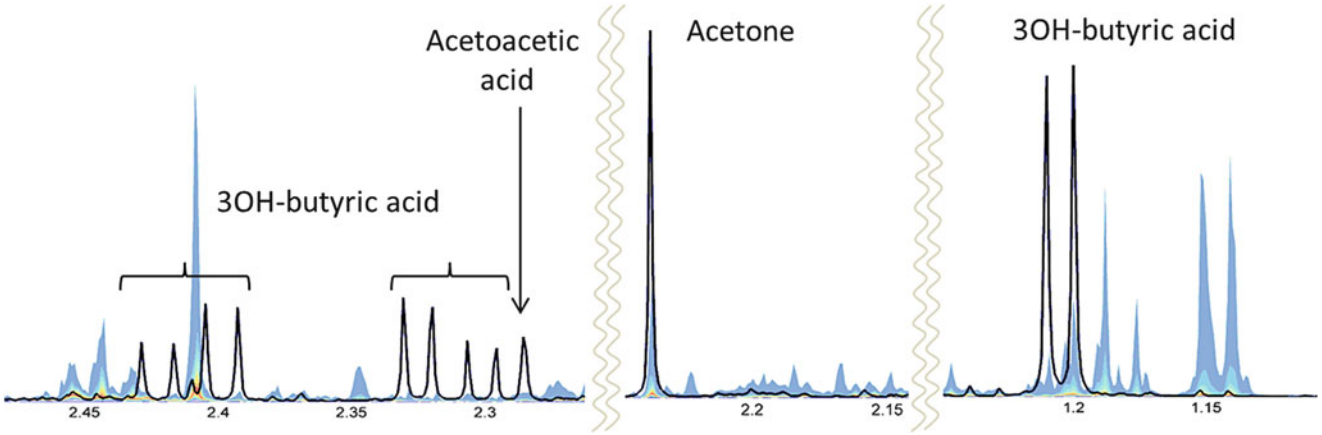

Fig 3 NMR spectrum of a urine sample from a newborn presenting with ketosis (*black line*). The three markers 3OH-butyric acid, acetoacetic acid and acetone are clearly enhanced as can be seen by comparing to the quantile plot (*colored band*) representing the normal ranges of spectral intensities in the respective spectral regions

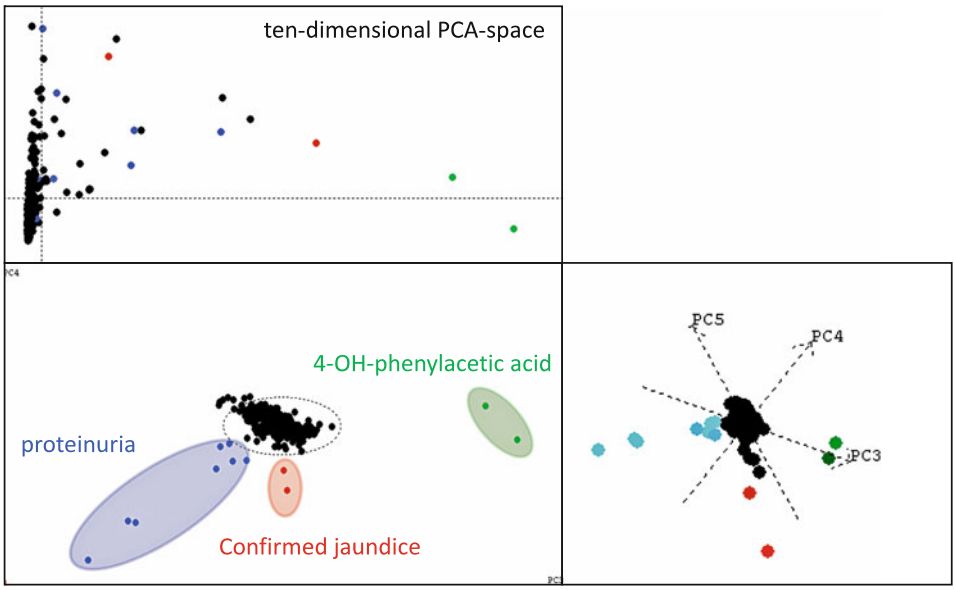

Fig. 4 Use of principal component analysis to detect outliers, e.g. proteinuria and confirmed jaundice, as well as 4-OH-phenylacetic acid. The *upper left subplot* represents the influence plot derived from a 10-dimensional subspace. The *lower left plot* is the scores plot in principal components 1 and 2. The *right plot* is the scores plot in PC 3, 4, and 5. *Color coding* shows the relations of the outlying samples in PC 1, 2 and PC 3, 4, 5. That means, the outlying samples color coded in the figure are outlier in at least a five-dimensional PCA subspace. Proteinuria (*blue color*) and jaundice (*red color*) were confirmed in the clinical records of the respective newborns. The figure illustrates, how some of the atypical samples can be easily detected even with a most simple straight forward PCA

Metabolic Fingerprint of Day of Life

The subset of neonates with day of life information was segmented into three classes: class 1 day 0-1, 193 samples; class 2, days 2–4, 317 samples; class 3, days 4–7, 317 samples. PCA/DA/k-NN modeling with MCCV validation of the NMR gave correct prediction for class 1 of 84%, of class 2 59% and of class 3 79% (Assfalg et al. 2008; Bermini et al. 2009).

Untargeted Analysis and Detection of Atypical Samples

PCA was used as a starting point for untargeted analysis in order to get a first impression on the dispersion of the metabolic phenotypes within the cohort (compare Fig. 4).

Even with this simple approach, some atypical and extremely deviating metabolic profiles could be identified in a straightforward manner, i.e. pathological profiles resulting from enhanced 4-hydroxyphenylacetic acid, or

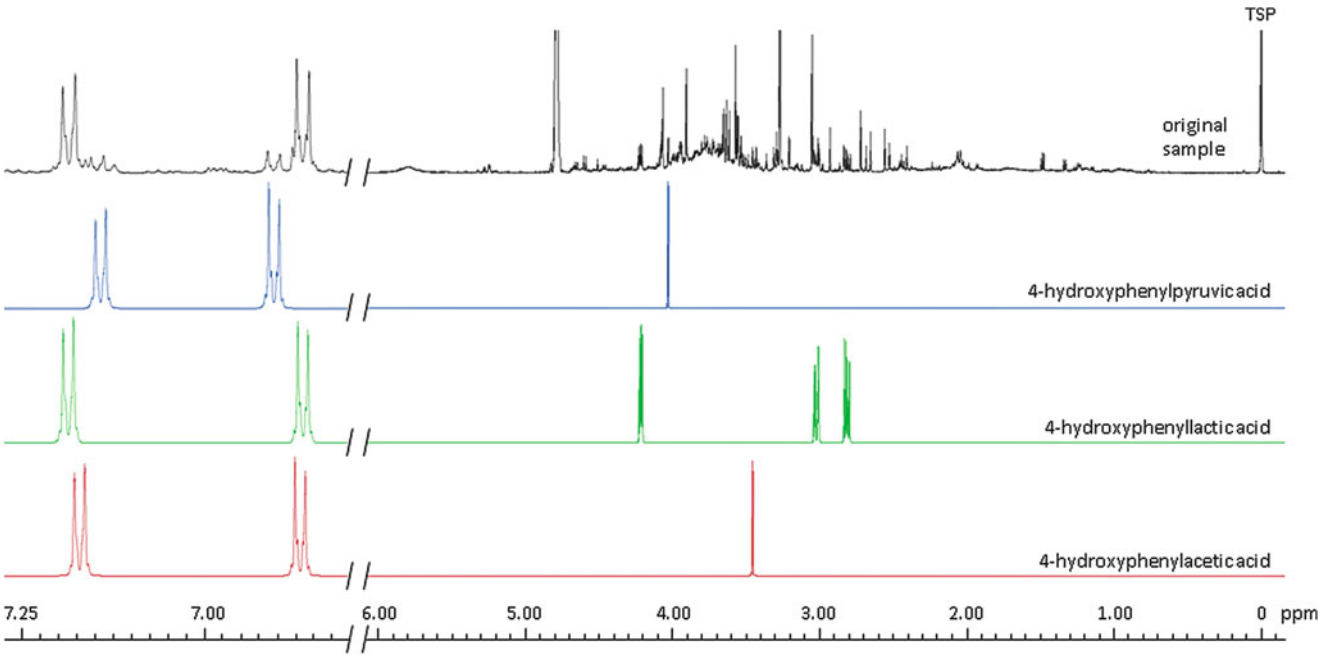

Fig. 5 Matching of urine with database spectra of diagnostic metabolites. Urine from a Turkish child with possible type II or type III tyrosinemia, in comparison with 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid

jaundice or proteinuria, respectively. However, for detection of the majority of atypical profiles, PCA turned out to be not appropriate. Instead, MCCV on HMM/SIMCA was needed. As a result, a substantial number of the urine samples shows atypical features in their NMR spectra due to, e.g., atypical metabolites, atypical concentrations or atypical concentration ratios and overall signal patterns. Two-hundred and two and 200 samples were identified as outliers in the sample set measured at BRUKER (890 samples: 22.7% outliers) and in the sample set measured at INFAI (953 samples: 21.0% outliers), respectively. They are summarized in Table 2.

Clinical Discussion of Outliers

Problem of false positive and false negative results should be avoided by classifying outlier groups. Unknown metabolites need additional investigations and different techniques.

In type II and type III tyrosinemia, 3 metabolites: 4-hydroxyphenylacetic acid, 4-hydroxyphenyllactic acid and 4-hydroxyphenylpyruvic acid (Tomoeda et al. 2000) were found in urine, and the amino acid tyrosine must be detectable in plasma (Blau et al. 2003). Figure 5 shows the results from a neonate with possible type II or type III tyrosinemia.

In type I tyrosinemia additionally to the abovementioned metabolites also 5-aminolevulinic acid should be present, but was not observed. In hawkinsinuria, the first three abovementioned metabolites also appear in urine, but additionally 5-oxoproline and 4-hydroxycyclohexylacetate must be present. However, in this case these metabolites were also not found. Therefore, this neonate may suffer from type II or type III tyrosinemia. Unfortunately, a conclusive diagnosis would only have been possible with a plasma sample, which was not available. Moreover, we have no information on the neonate's subsequent clinical course.

One of the four neonates with jaundice had to be treated in intensive care in the first week of life. Later, its condition normalized and the neonate could leave the hospital. In eight proteinuria cases, we observed high concentrations of proteins in urine. It is known that some neonates may have a temporary kidney insufficiency after birth. One of the seven cases with ketosis (see Fig. 3) had to be treated.

Discussion

Inborn metabolic diseases can pose a major threat to neonates and to the quality of life of their parents and families. Improved methods of diagnosis and screening may improve diagnosis and detection. Even if the disease is not treatable, the parents are spared the necessity of visiting a long series of physicians in their uncertainty and more rapidly receive reliable advice as to whether they should have further children. Moreover, prenatal diagnosis may then be possible in subsequent pregnancies (Lehotay et al. 2011).

Screening for metabolic disorders may be based on tandem MS (Lehotay et al. 2011; Chace and Kalas 2005) or NMR (Engelke et al. 2008; Wevers et al. 1999). Although tandem-MS is more frequently used, NMR possesses several advantages. In particular, NMR is more reproducible and rapid, one reason being that virtually no sample preparation is required. Tandem-MS is destructive and generally requires prior separation of the metabolites, either by chromatography or capillary electrophoresis (Nicholson et al. 2012). In addition, calibration is easier with NMR, as this can be based on the cumulative data from all measurements. Moreover, limits of quantitation and detection can be derived automatically, without having to use a series of separate samples. Detailed statistical analysis of complete spectra can lead to the identification of novel peaks. An additional advantage is that NMR can be used to monitor treatment success (Dietz et al. 1996).

The aim of the present study was to examine the NMR spectra of 989 Turkish neonates, in order to develop a statistical model for this group. The objectives were to characterize the spectra of healthy neonates, to identify statistical outliers, and to identify the urine spectra of diseased neonates.

The resulting spectra were subjected to both targeted and untargeted analyses. While the untargeted analysis started with no preconceptions about changes in spectra, the targeted analysis concentrated on 20 endogenous metabolites that are always present, together with 45 pathological metabolites that are usually absent in normal samples. The targeted analysis was used to establish distributions for metabolites and to identify inborn errors.

Non-targeted analysis employed the techniques of principal component analysis and HMM/SIMCA modeling. Outliers were tested against reference metabolites. A suspect for type II or type III tyrosinemia was detected in this way (Fig. 5).

Agreement between the two NMR laboratories was good (Fig. 2). Clear center effects were found (Fig. 1). Using the PCA/CA/k-NN approach we demonstrated predictivity with respect to gender, day of life, and birth mode. Although predictivity was above random chance and hence significant, it did not jeopardize the validity of confidence regions derived from the newborn cohort. Non-targeted analysis can be used to detect physiological abnormalities, such as jaundice, ketosis, proteinuria, or raised levels of abnormal metabolites (Fig. 4).

The present report describes the initial characterization and validation of the database resulting from the NMR spectra of 953 urine samples from healthy neonates. Therefore, we developed a statistical normal model of healthy Turkish neonates to allow the identification of statistical outliers and to identify the urine spectra or diseased neonates. Further studies will concentrate on identification of unknown pathological peaks, correlation with clinical parameters, and follow-up measurements, to investigate the evolution of metabolic phenotypes over time.

Acknowledgements Support from ZIM (Zentrales Innovationsprogramm Mittelstand) is gratefully acknowledged. We also thank our collaborating partners in Turkey, who helped us to execute this study. Altunhan H, Selcuk University, Konya, Turkey; Aydin A, Dokuz Eylül University, Izmir, Turkey; Bektaş MS, Van University, Van, Turkey; Coşkun T, Hacettepe University, Ankara, Turkey; Pehlivanoglu E, International Children Clinic, Istanbul, Turkey; Sanlı C, Diyarbakır Hospital Kadım Doğum, Diyarbakır, Turkey; Sivaslıoğlu S, Etlik Zübeyde Hanım Hospital Kadın Doğum, Ankara, Turkey.

Compliance with Ethics Guidelines

Sitke Aygen, Ulrich Dürr, Peter Hegele and Johannes Kunig, Manfred Spraul, Hartmut Schäfer, David Krings, Claire Cannet, Fang Fang, Birk Schütz, Selda F.H. Bülbül, H. Ibrahim Aydin, S. Ümit Sarıcı, Mehmet Yalaz, Rahmi Örs, Resit Atalan and Oguz Tuncer 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 the parents or guardians of all patients included in the study.

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