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Contents

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RESEARCH REPORT

4-Hydroxyglutamate Is a Biomarker for Primary Hyperoxaluria Type 3

James J Pitt • Frank Willis • Nicholas Tzanakos • Ruth Belostotsky • Yaacov Frishberg

Received: 22 April 2013 / Revised: 09 November 2013 / Accepted: 25 November 2013 / Published online: 22 February 2014

Abstract Primary hyperoxaluria type 3 (PH3) is a recently identified inborn error of 4-hydroxyproline metabolism causing kidney stone disease. Diagnosis to date has relied on mutation detection. The excretion of 4-hydroxyglutamate (4OHGlu) was investigated in controls and a cohort of nine patients with PH3 and their parents using flow injection tandem mass spectrometry. 4OHGlu was stable in acidified urine samples and was not influenced by diet. Its measurement was readily incorporated into an existing multi-analyte panel for comprehensive screening for inborn errors of metabolism. There was a steady decline with age in 4OHGlu levels, expressed as μmol/mmol of creatinine, in controls. Levels in patients with PH3 ranged from 6.5 to 98 μmol/mmol of creatinine and were all significantly increased when compared to age-matched controls (<4.2). Levels in eight parents (obligatory carriers of the corresponding mutation) were moderately, but significantly increased, ranging from 0.6 to 2.5 (age-matched controls <1.4, $p = 0.03$). Urine 4OHGlu screening was used to prospectively diagnose PH3 in an 18-month-old boy with calcium oxalate kidney stone disease associated with hyperoxaluria. 4OHGlu was also increased in a stored newborn screening dried blood spot sample from this child (37 μmol/L, controls <2.53). 4OHGlu testing provides a robust and high-throughput biochemical screen for PH3.

Communicated by: Rodney Pollitt, PhD

Competing interests: None declared

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Introduction

The two main determinants of kidney stone disease among the paediatric age group are genetic factors and anatomic anomalies. Children, as compared to adults, are more likely to have an underlying metabolic derangement which results in a higher rate of stone recurrence. Urinary metabolic profiling is therefore recommended in all children with nephrolithiasis, as timely treatment may prevent stone formation (Habbig et al. 2011). Primary hyperoxaluria type 3 (PH3, OMIM 613616) is a recently identified childhood oxalate kidney stone disease caused by mutations in the *HOGA1* gene, formerly *DHDPSL* (Belostotsky et al. 2010). A recent study gave an in silico estimate of ~1:165,000 for the incidence of PH3 in the US population (Hopp et al. 2013). *HOGA1* encodes for 4-hydroxy-2-oxoglutarate

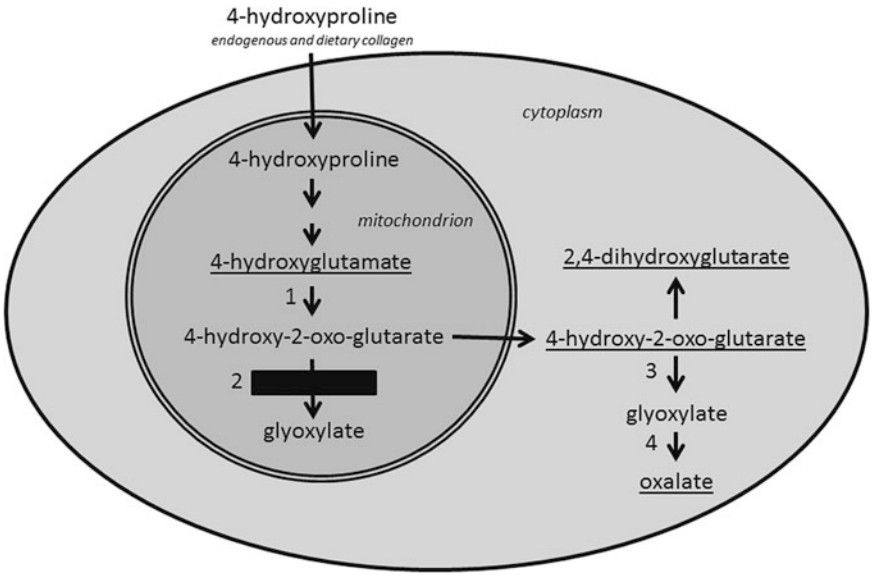

Fig. 1 Metabolic pathway for 4-hydroxyproline showing the enzymatic block in primary hyperoxaluria type 3 and the proposed metabolic re-routing mechanism. Enzymes are as follows: *1* aspartate aminotransferase, *2* 4-hydroxy-2-oxo-glutarate aldolase, *3* unidentified aldolase, *4* lactate dehydrogenase. Principal PH3 urine metabolites that exit the cell are underlined

aldolase (HOGA1), a mitochondrial enzyme in the 4-hydroxyproline (4OHPro) catabolic pathway which converts 4-hydroxy-2-oxoglutarate (HOG) to glyoxylate (Fig. 1). *HOGA1* mutations cause decreased HOG aldolase activity (Riedel et al. 2012), and it was recently shown that patients with PH3 accumulate HOG, 2,4-dihydroxyglutarate (the reduced form of HOG) and 4-hydroxyglutamate (4OHGlu, the metabolic precursor of HOG) in their urine (Belostotsky et al. 2012). It was difficult to envisage how oxalate accumulates when glyoxylate, its immediate precursor, is downstream of the metabolic block. We recently obtained evidence (Belostotsky et al. 2012) of a "re-routing" mechanism in which accumulating HOG exits mitochondria and is converted to glyoxylate in the cytoplasm by a yet-to-be-identified aldolase enzyme, distinct from HOGA1 (Fig. 1).

Until now, PH3 diagnosis has relied on mutation detection by sequencing of the *HOGA1* gene in cases with a high index of suspicion, e.g. persistent hyperoxaluria and exclusion of other genetic (i.e. primary hyperoxaluria types 1 and 2) and acquired causes of hyperoxaluria. The finding of abnormal PH3 metabolites opens the possibility of more comprehensive biochemical screening for this disorder in subjects with a lower index of suspicion. This communication describes the performance of 4OHGlu as a biomarker for PH3, the development of a high-throughput tandem mass spectrometry urine screening method for symptomatic patients and the potential feasibility of newborn screening for PH3.

Materials and Methods

Nine patients with PH3 from four unrelated families of Ashkenazi-Jewish descent were enrolled in this study. The diagnosis was confirmed by mutation screening of *HOGA1*. Three families have been previously described (Belostotsky et al. 2010). The affected individuals from the fourth family are compound heterozygotes for the following mutations: c.107C>T and c.944_946delAGG. Of note, four out of nine patients have never formed a kidney stone despite having persistent hyperoxaluria. Their newborn screening dried blood spots were not available for analysis.

Random urine samples were collected from patients and their parents. Controls were random urine samples submitted for routine screening for inborn errors of metabolism including those causing kidney stone disease. Ages ranged from 4 days to 86 years. The influence of diet was checked by performing an oral loading test of 20 g of gelatine, containing ~14% 4-hydroxyproline (Eastoe 1955), on a healthy male volunteer and collecting random urine samples. Urines were stored at −30°C. Urine creatinine was measured by a kinetic Jaffe reaction using a Cobas Mira analyser. 4OHGlu, 4OHPro and acetyl chloride were purchased from Sigma Aldrich; n-butanol, acetonitrile and methanol were from Merck, while $^{2}H_{3}$-glutamate and $^{2}H_{3}$-4-hydroxyproline were from CDN Isotopes.

Urine 4OHGlu and 4OHPro were measured using a modification of a previously described multi-analyte

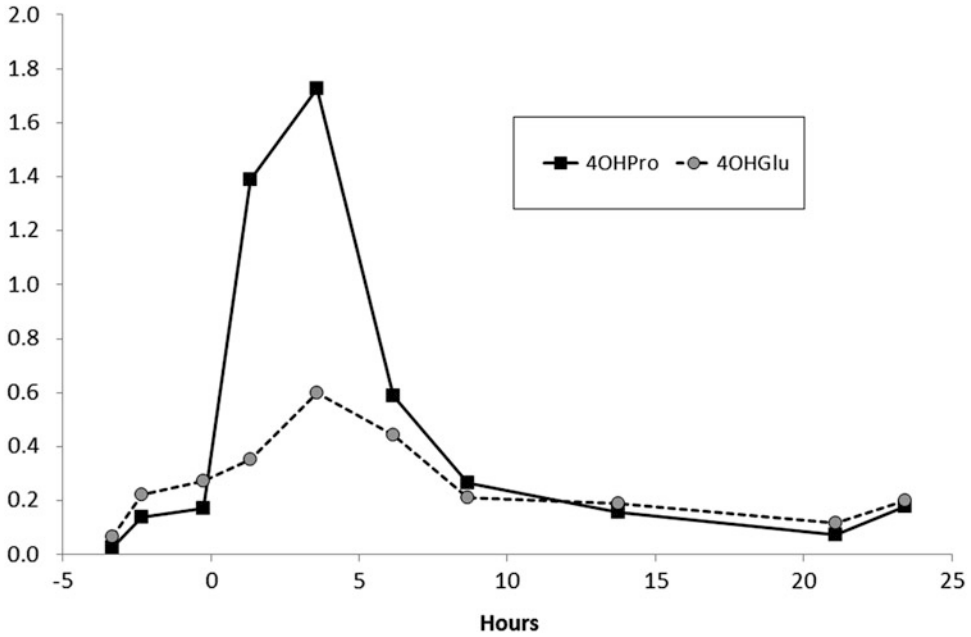

Fig. 2 Response of urinary 4-hydroxyglutamate and 4-hydroxyproline levels (μmol/mmol of creatinine) to gelatine loading (20 g at 0 h) in an adult control

method for comprehensive screening for inborn errors of metabolism using electrospray tandem mass spectrometry (Pitt et al. 2002). Briefly, aliquots of urine containing 20 nmoles of creatinine were mixed with 20 μL of internal standard solution (containing 100 μmol/L of $^{2}H_{3}$-glutamate, $^{2}H_{3}$-4-hydroxyproline and other internal standards). Amino acids and other metabolites were then converted to n-butyl esters and analysed by flow injection analysis electrospray tandem mass spectrometry in positive ion multiple reaction monitoring mode as previously described using a Waters Acquity TQD system. The relevant mass transitions are given in Supplementary Material Table 1 and were added to an existing panel of ~115 metabolites.

The assay was calibrated with three standards (12.5, 25 and 50 μmol/L) and a blank, all prepared in a liquid matrix that approximately matched salt and urea concentrations in urine samples. Urine samples from different control individuals were spiked with 4OHGlu and 4OHPro for determining the assay recovery. To assess storage stability, aliquots of a spiked urine sample were stored at various temperatures with and without acidification to pH 2 using hydrochloric acid. Urine blotters were prepared applying samples to Whatman 903 paper, dried and stored at room temperature.

Neonatal dried blood spots were eluted with methanol containing the internal standards, dried and then derivatised as described above. Mass spectrometry transitions for 4OHGlu and its internal standard (see Supplementary Material Table 1) were added to a standard newborn screening amino acid/acyl-carnitine panel. De-identified dried blood spots from controls were analysed with approval from the Royal Children's Hospital Human Research Ethics Committee.

Results

Assay Performance

The analytical performance for urine 4OHGlu and 4OHPro is summarised in Supplementary Material Fig. 1 and Table 2. Linearities were >0.984, imprecision <11.7% (normal and high levels) and recovery 83–112% for both analytes. A typical calibration curve is shown in Supplementary Fig. 1. As expected, the performance of 4OHPro was superior because a stable isotope internal standard was used.

A spiked urine sample was analysed after 28 days of storage under various conditions and expressed as a percentage of the same sample stored at −80 °C. Samples stored at −30 °C, acidified and stored at room temperature, and on blotters, gave values of 101%, 92% and 99%, respectively.

Intake of 4OHPro, in the form of gelatine, caused an increase in urine 4OHPro, peaking at 1.7 μmol/mmol of creatinine, with a smaller concomitant increase in 4OHGlu levels, peaking at 0.6 μmol/mmol of creatinine (Fig. 2), well within the reference range.

4OHGlu Levels in Controls and PH3 Families

Urine 4OHGlu in μmol/mmol of creatinine for controls, PH3 patients and their parents are shown in Fig. 3. There was a

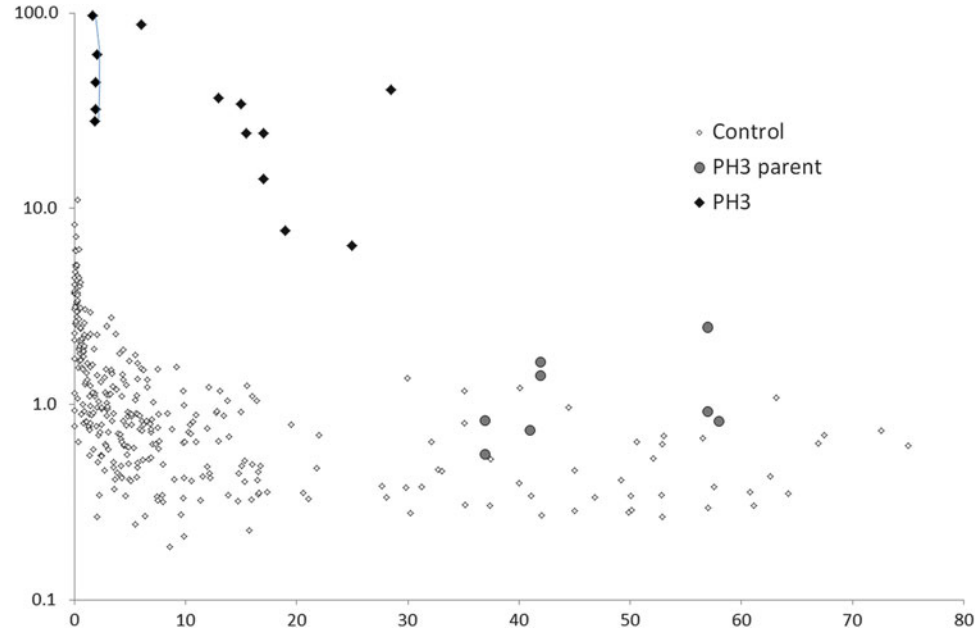

Fig. 3 Urine 4-hydroxyglutamate levels (y-axis, μmol/mmol of creatinine, log scale) vs age (x-axis in years). Samples from patient A are connected by lines

steady decline in 4OHGlu with age in controls (<11.1 for <0.5 years of age; <4.2 for 0.5–1 years; <2.9 for 1–5 years; <1.8 for 5–10 years; <1.4 for >10 years). Levels were grossly increased in PH3 patients (all >0.5 years), ranging from 6.5 to 98 and also showing an apparent decline with age. The levels in eight PH3 obligate heterozygous parents ranged from 0.6 to 2.5, showing a moderate but significant ($p = 0.03$) increase when compared to controls >10 years. There was an approximate correlation between 4OHGlu and 4OHPro levels in both controls and PH3 patients, and consideration of both of these metabolites appeared to give greater discrimination between controls and PH3 (see Supplementary Material Fig. 2).

4OHGlu levels were normal for age in subjects with kidney stone diseases such as cystinuria ($n = 22$), xanthine oxidase deficiency ($n = 2$), hyperoxaluria type 1 ($n = 4$) and hereditary orotic aciduria ($n = 1$). One patient with hyperoxaluria type 2 had a slight increase at 3.5 μmol/mmol of creatinine (controls <1.4).

We subsequently screened a larger population of children with kidney stone disease for PH3. Patient A was diagnosed after paediatric nephrologists throughout Australia and New Zealand were contacted and offered biochemical testing for suspected cases of PH3. He was an 18-month-old boy with multiple calcium oxalate kidney stones from the age of 13 months. Urine oxalate levels ranged from 179 to 379 μmol/mmol of creatinine (reference range: 29–174) with normal glycolate and glycerate. Consistently increased urine 4OHGlu levels were detected by the screening method (Fig. 3), and urine HOG and 2,4-dihydroxyglutarate were also increased (data not shown). The diagnosis of PH3 was confirmed by the finding of a novel deletion (c.803_805delTGC causing an in-frame deletion: p.Lys268del) and a known splice site variant (c.700+5G>T) in the *HOGA1* gene.

The newborn screening dried blood spot card, stored for 2 years, was retrieved and analysed with parental consent. This showed an increase in 4OHGlu (37 μmol/L; storage-matched controls <2.1, $n = 50$, fresh controls <2.5, $n = 50$). Each of the storage-matched and fresh control groups included ten premature babies (30–35 week gestation) to check for possible influences of prematurity. There were no significant differences in 4OHGlu levels between premature and normal gestation babies.

Discussion

Since the initial report of *HOGA1* mutations causing PH3 (Belostotsky et al. 2010), several cohorts of patients with persistent hyperoxaluria of unknown aetiology (also referred to as non-type I/type II PH) have been retrospectively studied, and *HOGA1* mutations have been identified in ~45% of these patients (Beck et al. 2013; Monico et al. 2011; Williams et al. 2012). These findings have significant implications for the way in which patients with hyperoxaluria are investigated and also suggest possible treatments for PH3, now that the gene and enzyme involved have been identified. 4OHPro is almost exclusively derived from collagen (Fig. 1), from either endogenous turnover or

dietary sources, and it may be possible to modulate 4OHPro metabolism in PH3, either through restriction of dietary collagen or by pharmaceutical means, to minimise stone formation. Reliable biomarkers for PH3 would be very useful for further research in these areas as well as for the initial diagnosis of PH3 which has relied on *HOGA1* mutation detection to date.

Our previous study (Belostotsky et al. 2012) found large increases of these metabolites in the urine of patients with PH3, and these findings were later replicated for HOG (Riedel et al. 2012). The analytical methods used in those studies are poorly suited for routine diagnostic work and screening large numbers of samples. 4OHGlu could potentially be measured by standard methods of amino acids analysis which are widely available in biochemical genetics laboratories. Preliminary investigations showed that the peak of 4OHGlu eluted close to normal amino acid urine constituents in both ion exchange (Biochrom 30 system) and reverse phase (Waters Masstrak/Acquity system) chromatography, precluding reliable measurement. We therefore added 4OHGlu to a panel of metabolites measured by electrospray tandem mass spectrometry as part of comprehensive screening for inborn errors of metabolism. A stable isotope version of 4OHGlu was unavailable commercially and we were unable to synthesise one. We chose $^{2}H_{3}$-glutamate as a "surrogate" internal standard for 4OHGlu. Despite this limitation, analytical performance was acceptable for a screening method (see Supplementary Material Table 2) with between batch CVs $<12\%$ and recoveries ranging from 83% to 112%.

4OHGlu was stable in urine with recoveries $>90\%$, when samples were stored frozen or on urine blotters. Acidification with hydrochloric acid also prevented the breakdown of 4OHGlu in liquid urine at room temperature, with a recovery of 92% after 28 days storage. For routine diagnostic work, we recommend acidification of samples (to prevent decomposition of 4OHGlu, oxalate and other metabolites) and transport in the frozen state or at ambient temperatures. In contrast, HOG is unstable at ambient temperatures (Dekker and Maitra 1975) making it less reliable as a diagnostic biomarker. We also found urine 4OHGlu to be relatively unaffected by dietary influences (Fig. 2).

There was a decline in urine 4OHGlu levels in the first few years of life (Fig. 3). This is consistent with increased collagen turnover (Mora et al. 1998) and 4OHPro metabolism in young children. Indeed, 4OHPro excretion steadily declines with age (Shih 2003), and there was a rough correlation between 4OHGlu and 4OHPro (Supplementary Material Fig. 2). 4-OHGlu levels in ten PH3 patients were much higher than age-matched controls and ranged from 6.5 to 98 μmol/mmol of creatinine. A decline in 4OHGlu levels with age was also apparent in the PH3 patients, presumably the result of a similar mechanism to controls. In our previous study, we did not detect a significant difference between controls and obligate carriers (parents) of PH3 patients (Belostotsky et al. 2012). However, this, more extensive, study showed that some carriers do indeed have modest, but significant, increases in 4OHGlu levels compared to age-matched controls. Heterozygous *HOGA1* mutations may be a risk factor for stone formation (Monico et al. 2011), and increased levels of 4OHGlu in carriers may be useful in stratifying this risk.

4OHGlu testing was easily added to a multi-analyte test for comprehensive screening for inborn errors of metabolism (Pitt et al. 2002), and we now test all urines submitted to our laboratory for 4OHGlu. This resulted in rapid biochemical diagnosis of PH3 in patient A. 4OHGlu was specific for PH3 with normal values being obtained for most subjects with other genetic forms of kidney stone diseases. One patient with primary hyperoxaluria type 2 had a slight increase in 4OHGlu levels that did not overlap with the PH3 range which may reflect accumulation of metabolites in the 4-hydroxyproline pathway. When samples are analysed in parallel in negative ion mode, biomarkers for these other kidney stone diseases are readily detected, e.g. hyperoxaluria type 1 (glycolate), hyperoxaluria type 2 (glycerate), xanthine oxidase deficiency (xanthine), hereditary orotic aciduria (orotate) and cystinuria (cystine and arginine).

Retrospective analysis of the stored newborn screening dried blood spot from patient A showed a ~25-fold increase in 4OHGlu relative to controls. This feature may be useful in situations where a rapid diagnosis is requested, e.g. diagnosis of subsequent siblings. PH3 does not meet the traditional criteria for newborn screening test, but 4OHGlu testing would only involve a minor modification of the current newborn tandem mass spectrometry screening test with minimal additional laboratory costs. Newborn screening laboratories may wish to consider the feasibility of newborn screening for PH3 in order to prevent the "diagnostic odyssey" that many PH3 patients experience. Further studies would be required to determine the metrics of this screening, in particular the false positive rate.

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Compliance with Ethics Guidelines

Author Contributions

Ruth Belostotsky: performed mutation analysis, provided samples from the Israeli cohort, revised the manuscript

Frank Willis: clinically managed, organised samples and consent for patient A, revised the manuscript

Nicholas Tzanakos: performed dried blood spot analysis, revised the manuscript

Yaacov Frishberg: advised on study design, managed and provided data for the Israeli cohort, contributed to the manuscript

James Pitt: conceived, designed and supervised the study, wrote the manuscript, acts as guarantor

Funding Disclosure: None relevant to this research

Conflicts of Interest

James Pitt, Ruth Belostotsky, Frank Willis, Nicholas Tzanakos and Yaacov Frishberg declare that they have no conflict of interest.

Ethical Approval

Control neonatal dried blood spots were analysed with the approval of the Royal Children's Hospital Human Research Ethics Committee. This study was also approved by the Helsinki Committee of the Shaare Zedek Medical Center, Jerusalem, Israel.

Consent: Parental consent was obtained for testing the neonatal blood spot from patient A.

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

Animal Rights: Not applicable, no animal experiments were performed

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DATA REPORT

Excellent Response to a Ketogenic Diet in a Patient with Alternating Hemiplegia of Childhood

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Abstract Alternating hemiplegia of childhood (AHC) is a rare disorder caused by heterozygous mutations in *ATP1A3*. AHC is associated with early-onset plegic and tonic/dystonic attacks and permanent neurologic deficits. Attacks tend to persist through life. Flunarizine therapy occasionally reduces the severity, duration and frequency of attacks. A ketogenic diet/modified Atkins diet (KD/MAD) can attenuate paroxysmal movement disorders associated with GLUT1 deficiency syndrome (GLUT1DS), but there are no reports on the effect of KD/MAD in AHC. We describe the case of a young girl with AHC who had tonic/dystonic and plegic attacks, mostly triggered by exercise, together with mild permanent dystonia and mental retardation. Her family had a history of dominant (three affected generations) paroxysmal exercise-induced dystonia. A history of plegic attacks that ceased after childhood was retraced from the medical records of the three affected adults, leading to the diagnosis of familial AHC due to *ATP1A3* p.Asp923Asn mutation (Roubergue et al 2013). KD/MAD was considered for the proband when she was 3½ years old, following initial misdiagnosis of GLUT1DS. MAD, a KD variant, was chosen because it is easier to manage than KD and is similarly effective to KD in most GLUT1DS patients. MAD resulted in complete disappearance of the attacks during 15 months of follow-up. *Conclusions:* A modified Atkins diet had a sustained beneficial effect on attacks associated with AHC. Although preliminary, this observation suggests that a ketogenic diet might be a therapeutic option for paroxysmal disorders in some patients with alternating hemiplegia of childhood.

Communicated by: Michael J Bennett, PhD

Competing interests: None declared

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Introduction

Alternating hemiplegia of childhood (AHC) (OMIM #614820) is a rare disorder characterized by episodes of alternating hemiplegia/quadriplegia and tonic/dystonic attacks associated with permanent neurological deficits (Sweney et al 2009; Panagiotakaki et al 2010). Paroxysmal events typically start before age 18 months and are often precipitated by specific triggers such as physical exertion

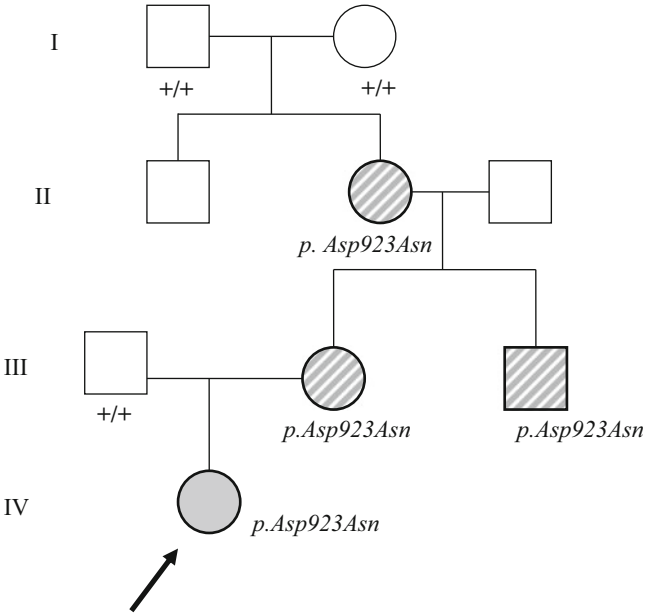

Fig. 1 Family pedigree. *Uniform grey shading:* individual with plegic and tonic/dystonic episodes. *Diagonal shading:* adults with isolated dystonic episodes (mainly exercise-induced), and no plegic episodes after childhood. *Shapes surrounded by a thick line:* individuals with permanent mild dystonia/chorea and mild cognitive deficits. *+/+:* individuals with a normal genotype. *p.Asp923Asn: ATP1A3* heterozygous mutation. The *arrow* indicates the index patient

(Panagiotakaki et al 2010; Sweney et al 2009). Most cases are sporadic. The main genetic cause of AHC was recently identified as heterozygous mutations in *ATP1A3*, the gene encoding the neuron-specific Na+/K+-ATPase α3 subunit (Heinzen et al 2012; Rosewich et al 2012). Mutations in this gene had previously been shown to cause rapid-onset dystonia parkinsonism (RDP) (de Carvalho Aguiar et al 2004).

Current treatments for AHC are disappointing. Flunarizine, a calcium channel blocker, reduces the frequency, duration and severity of attacks in some patients (Panagiotakaki et al 2010; Mikati et al 2000).

The ketogenic diet (KD), or its variant the modified Atkins diet (MAD), is used to treat some metabolic paroxysmal movement disorders such as those encountered in glucose transporter type 1 deficiency syndrome (GLUT1DS) (De Vivo et al 1991; Ito et al 2011). The effect of such diets on the paroxysmal disorders associated with AHC is not known. Here we tested MAD in a child with AHC, in whom paroxysmal attacks were the predominant disease manifestation.

Patient and Methods

The index case and her family have been described briefly elsewhere (Roubergue et al 2013). Their history and neurological and genetic findings are summarized in Fig. 1. The treatment response of their paroxysmal features is reported in Table 1.

The proband, now 4 years 8 months old, was referred to us at age 2½ years for plegic attacks. The attacks had begun at age 2 years with transient upper-limb paresis. Episodes of monoparesis/plegia, hemiplegia and quadriplegia subsequently occurred 2–4 times monthly, lasting from several minutes to 15 days. They were triggered by prolonged crying, nervousness or physical exertion. They disappeared during night-time sleep and, when long lasting, recurred within half an hour after awakening.

Previously, the child had had episodes of paroxysmal dystonia, mostly induced by prolonged physical exertion. At age 8 months, she had wrist dystonia induced by walking on all fours and, from age 20 months, had orofacial-laryngeal dystonia induced mainly by playing with toys. She had an attack of generalized dystonia at age 11 months. The attacks lasted from 30–60 min to 2–3 days and occurred about twice a month.

The family had a history of dominant exercise-induced paroxysmal dystonia (Fig. 1, Table 1). Plegic attacks during childhood, of which the affected adult members of the family were unaware, were retraced from the medical records.

Glycorrhachia was 2.65 mmol/l in the proband. Genetic analysis of DYT1, GLUT1/SLC2A1, PDHE1 alpha, ATP1A2 and CACNA1A, as well as karyotyping and the Illumina CGH array were negative in the proband or in one

Table 1 Treatment responses of attacks in the proband and other affected family members

	Age	Age at onset of Pl/ Dyst attacks	Duration of Pl/ Dyst attacks	Monthly frequency of Pl/ Dyst attacks	Triggers (alleviating factors) of Pl/ Dyst attacks	Current therapy, age at outset [previous therapy, age at outset]	Attack-free periods: age of onset, duration	Age at end of Pl / Dyst attacks
Proband	4 years 8 months	2 years/ 8 months	Mins to 15 days/ mins to 2 days	2 to 4/ 2	Exercise, stress (sleep)/ exercise +++, fever	MAD, 3 years 5 months		3 years 5 months/ 3 years 5 months
Mother	27 years	2 years/ 2 months	NA/ mins to hours	NA/ 4	NA/ exercise +++ (rest, sleep)	PHT, 8 years [CZP, 13 months]; [FNR, 7 years]	Age 8 years, 4 months Age 7 years, 6 months	5 ½ years/ ongoing
Maternal uncle	25 years	2 years/ 8 years	NA/ mins to hours	30 then 4/ 10 to 20	Exercise/ exercise +++	FNR, 2 years 9 months; PHT, 6 years		5 years/ ongoing
Maternal grandmother	52 years	3 years/ NA	5 min to 1 hour/ 30 min to hours	NA/ 4	NA/ exercise +++ (rest)	PB, < 30 years; PHT, 34 years [CZP, < 30 years]; [FNR, 32 years]; [other AEs]		NA/ ongoing

Abbreviations: *Pl* plegic, *Dyst* dystonic, *MAD* Modified Atkins Diet, *AE* antiepileptic drug, *CZP* carbamazepine, *FNR* flunarizine, *PB* Phenobarbital, *PHT* phenytoin, *NA* not available, +++ usual trigger

or several symptomatic family members. Analysis of *ATP1A3* revealed the heterozygous substitution c.2767G>A (p.Asp923Asn) in all four symptomatic family members (Roubergue et al 2013). The mutation had appeared de novo in the maternal grandmother and then segregated over three generations (Fig. 1).

Procedure

The modified Atkins diet, a KD variant, was considered before the final diagnosis of AHC was made, as the patient's clinical phenotype resembled that of mild GLUT1DS, in which KD/MAD was known to be effective.

MAD was introduced at age 3 years 5 months, using the protocol recommended by Kossoff and Dorward (2008), i.e. starting without a fasting period, with no calorie, fluid or protein restriction, and with an initial upper limit of 10 g of carbohydrates per day. Fat intake was encouraged. MAD was initiated with 1,250 cal (88 cal/kg) daily, provided by 93 g of fat, 94 g of protein and 10 g of carbohydrates. The ketogenic ratio (ratio of g of fat to g of protein plus g of carbohydrate) was 0.9:1, compared to 4:1 in the classic KD. Carbohydrate intake was increased to 20 g after 4 months, and then to 30 g daily after a further 6 months. The duration of the diet was 15 months at the last follow-up.

Results

After MAD initiation, the dystonic and plegic attacks disappeared completely during the 15 months of follow-up (Table1). This prompted the family to continue the diet even after the child started school. Interictal neurological status was unchanged, with persistent mild permanent dystonia.

The diet was well tolerated. The child lost 1.5 kg in the early phase but regained it after her caloric intake was increased. Urine ketosis varied from 1+ to 4+. Mild hypercholesterolemia, present before MAD initiation (5.1 mmol/l), increased early after MAD initiation (6.2 mmol/l) and had returned close to the pretreatment level when controlled at 5 months (5.4 mmol/l; $N = 1.8–4.6$ mmol/l). Five months after MAD initiation, she was found to have elevated calciuria (urine calcium/creatinine ratio 2.91, $N = 0.22–0.50$) and low carnitinemia (free carnitine 13.7 µmol/l, $N = 24–63$; total L carnitine 23.7 µmol/l, $N = 35–84$; free to total carnitine ratio 0.58, $N = 0.70–0.90$).

Discussion

We describe the case of a child with a mild form of typical AHC. Initial misdiagnosis of GLUT1DS deficiency led to treatment with a modified Atkins diet, resulting in complete cessation of her dystonic and plegic attacks. This beneficial effect was sustained for more than 15 months. Although based on a single observation, our findings suggest that KD might be a therapeutic option in AHC.

Attacks associated with AHC usually persist throughout life (Bourgeois et al 1993; Panagiotakaki et al 2010; Sweney et al 2009). At best, current drug therapies reduce the severity, duration and frequency of attacks. Flunarizine is the most consistently effective drug, as shown in large cohorts of patients (Casaer 1987; Sweney et al 2009; Panagiotakaki et al 2010).

The cessation of both types of attack in our patient supports the role of the modified Atkins diet in the observed improvement. We found only three other reports of AHC patients whose attacks ceased completely after treatment, which always consisted of flunarizine; they included the first patient to be treated with flunarizine, whose publication ultimately led to current use of this drug to prevent attacks in AHC patients (Casaer and Azou 1984; Silver and Andermann 1993; Mikati et al 2000). Follow-up was reported in only one of these three cases and, as in our patient, lasted more than 1 year.

We cannot rule out the possibility that the link between MAD initiation and the cessation of attacks might have been fortuitous in our patient. In particular, the childhood plegic attacks experienced by her family's three affected adults gradually ceased, leaving only isolated tonic/dystonic attacks. Isolated cessation of either plegic or tonic/dystonic attacks has previously been reported in a small number of AHC patients (Bourgeois et al 1993; Mikati et al 2000; Panagiotakaki et al 2010). In addition, lengthy attack-free periods (up to 2 years) have been reported in very few patients (Bourgeois et al 1993; Salmon and Wilson 1984). Such periods were experienced by our patient's mother after treatment with flunarizine and phenytoin (6 and 4 months, respectively). Yet these attack-free periods were far shorter than that experienced by our patient after MAD initiation (currently 15 months).

We considered the ketogenic diet following initial misdiagnosis of GLUT1DS with dominant paroxysmal exercise-induced dystonia (PEID) and plegic attacks. PEID occurs in some AHC individuals (Sweney et al 2009), but autosomal dominant PEID is not a known phenotype of AHC. By contrast, autosomal dominant PEID is a core feature of GLUT1DS type 2 (OMIM # 612126), the mild form of GLUT1DS, and may be associated with episodes of hemiplegia, quadriplegia, mild mental retardation and permanent neurological symptoms (Suls et al 2008; Weber et al 2008; Rotstein et al 2009; Pons et al 2010). The long duration (up to several days) of some attacks in our patient was not typical of paroxysmal episodes in GLUT1DS (Suls et al 2008; Weber et al 2008). However, the absence of frank hypoglycorrhachia in our patient, and the apparent absence of *SLCA2/GLUT1* mutation, did not rule out

GLUT1DS. Indeed, glycorrhachia in the lower range of normal has been reported in GLUT1DS, and only 70–80% of patients harbour a mutation in the *SLC2A/GLUT1* gene (Klepper 2012, 2013).

KD is the treatment of choice for patients with GLUT1DS (De Vivo et al 1991; Suls et al 2008; Klepper 2012). KD may also be considered for *SLCA2/GLUT1*-negative patients, even in the absence of frank hypoglycorrhachia (as in our proband), and an immediate response to the diet would support the diagnosis of GLUT1DS (Klepper 2012, 2013). We opted for a modified Atkins diet, a KD variant used in GLUT1DS (Ito et al 2011; Leen et al 2013), because it is easier to manage (the proband's mother was mentally impaired), and more palatable than KD.

The mechanism underlying the therapeutic effect of KD is unknown. Ketones provide an alternative brain fuel that may compensate for defective cerebral glucose metabolism (De Vivo et al 1991) as observed in GLUT1DS (Pascual et al 2007; Ito et al 2011). KD might act similarly in AHC. Indeed, cerebral glucose hypometabolism is observed in AHC patients, as well as in AHC model mice and, interestingly, in the only rapid-onset-dystonia-parkinsonism patient studied by means of F-18 fluorodeoxyglucose positron emission tomography, who harboured precisely the same *ATP1A3* mutation (p.Asp923Asn) as the family described here (Sasaki et al 2009; Kirshenbaum et al 2013; Anselm et al 2009). KD might also reduce neuronal excitability (for reviews see Lutas and Yellen 2013; Stafstrom and Rho 2012; Dhamija et al 2013), thereby correcting the altered membrane excitability due to ATP1A3 dysfunction and thus reducing the frequency and/or severity of the paroxysmal features of AHC.

Synopsis

This is the first report on the effect of a ketogenic diet in a patient with alternating hemiplegia of childhood.

Compliance with Ethics and Guidelines

Conflict of Interest

- Anne Roubergue, Bertrand Philibert, Agnès Gautier, Alice Kuster, Karine Markowicz, Thierry Billette de Villemeur, Sandrine Vuillaumier-Barrot, and Diane Doummar declare that they have no conflict of interest.
- Sophie Nicole has received research grants from the French association against alternating hemiplegia (AFHA).
- Emmanuel Roze declares he is the recipient of a grant "poste d'accueil" AP-HP/CNRS. He received research support from INSERM (COSSEC), AP-HP (DRC-PHRC), Fondation pour la Recherche sur le Cerveau (FRC), the Dystonia Coalition (Pilot project), Ipsen, and Merz-Pharma, Novartis, Teva, Lundbeck, Orkyn; served on scientific advisory boards for Orkyn, Ipsen, and Merz-pharma; received speech honorarium from Novartis and Orkyn; received travel funding from Teva, Novartis, the Dystonia Coalition, the Movement Disorders Society, the World Federation of Neurology Association of Parkinsonism and Related Disorders, International Federation of Clinical Neurophysiology

Informed Consent

No ethical approval was required for this work.

The child's parents have given their consent for the publication of this case study.

Animal Rights

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

Details of the Contributions of Individual Authors

Planning: AR, BP, AG, AK, KM, TBV, SV, SN, ER, DD
Conduct: AR, TBV, DD
Reporting of the work described in the article: AR, BP, AG, AK, KM, DD
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RESEARCH REPORT

Thiamine-Responsive and Non-responsive Patients with PDHC-E1 Deficiency: A Retrospective Assessment

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Abstract Pyruvate dehydrogenase complex (PDHC) deficiency is a disorder of energy metabolism that leads to a range of clinical manifestations. We sought to characterise clinical manifestations and biochemical, neuroimaging and molecular findings in thiamine-responsive and nonresponsive PDHC-deficient patients and to identify potential pitfalls in the diagnosis of PDHC deficiency. We retrospectively reviewed all medical records of all PDHC-deficient patients ($n = 19$; all had *PDHA1* gene mutations) and one patient with severe PDHC deficiency secondary to 3-hydroxyisobutyryl-CoA hydrolase deficiency managed at our centre between 1982 and 2012. Responsiveness to thiamine was based on clinical parameters. Seventeen patients received thiamine treatment: eight did not respond, four showed sustained response and the others responded temporarily/questionably. Sustained response was noted at thiamine doses >400 mg/day. Age at presentation was 0–6 and 12–27 months in the nonresponsive ($n = 8$) and responsive ($n = 4$) patients, respectively. Corpus callosum abnormalities were noted in 4/8 nonresponsive patients. Basal ganglia involvement (consistent with Leigh disease) was found in four patients (including 2/4 thiamine-responsive patients). Diagnosis through mutation analysis was more sensitive and specific than through enzymatic analysis. We conclude that patients presenting at age >12 months with relapsing ataxia and possibly Leigh syndrome are more likely to be thiamine responsive than those presenting with neonatal lactic acidosis and corpus callosum abnormalities. However, this distinction is equivocal and treatment with thiamine (>400 mg/day) should be commenced on all patients suspected of having PDHC deficiency. Mutation analysis is the preferable first-line diagnostic test to avoid missing thiamine-responsive patients and misdiagnosing patients with secondary PDHC deficiency.

Short Summary: Thiamine responsiveness is more likely in patients presenting at age >12 months with relapsing ataxia and possibly Leigh syndrome than in those presenting with neonatal lactic acidosis and corpus callosum abnormalities. Thiamine doses >400 mg/day are required for sustained response. Mutation analysis is more sensitive and specific than enzymatic analysis as a first-line diagnostic test.

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Abbreviations

HIBCH	3-Hydroxyisobutyryl-CoA hydrolase
MRI	Magnetic resonance imaging
PDHC	Pyruvate dehydrogenase complex
TPP	Thiamine pyrophosphate

Introduction

The pyruvate dehydrogenase complex (PDHC) is pivotal for energy metabolism in that it catalyses the oxidative decarbo-

xylation of pyruvate into acetyl-CoA, linking glycolysis to the tricarboxylic acid cycle. The PDHC is comprised of several copies of enzymatic subunits, E1 (pyruvate dehydrogenase), E2 (dihydrolipoamide transacetylase) and E3 (dihydrolipoamide dehydrogenase) and an E3 binding protein (E3BP), and its activity is regulated by several isoforms of kinases and phosphatases through reversible phosphorylation (Linn et al. 1969; Pagliarini and Dixon 2006). The E1 enzyme consists of two α- and two β-subunits that share a binding site for thiamine pyrophosphate (TPP), a metabolite of thiamine, which is an essential cofactor for the enzymatic reaction and also helps to maintain the PDHC in an activated state by inhibiting its phosphorylation (Roche and Reed 1972).

PDHC deficiency causes impairment of energy metabolism and leads to a broad range of symptoms. Four main neurological presentations have been reported: neonatal encephalopathy with lactic acidosis, non-progressive infantile encephalopathy, Leigh syndrome and relapsing ataxia (Robinson et al. 1987; Brown et al. 1988, 1989a; Barnerias et al. 2010; Patel et al. 2012). The majority of patients have a mutation located in the *PDHA1* gene encoding the E1α subunit, which is located on the X chromosome (Robinson and Sherwood 1984; McKay et al. 1986; Wicking et al. 1986; Brown et al. 1989b; Lissens et al. 2000). The differences in presentation result from variations in mutations and from the degree of X inactivation in females (Brown et al. 1989b; Dahl et al. 1992).

A high-fat diet, with the amount of calories provided from fat exceeding 50%, has been shown to be effective in many patients with PDHC deficiency. It leads to decreased blood lactate and pyruvate concentrations and provides an alternative source of energy in the form of ketones (Wexler et al. 1997) and has been shown to be helpful in reducing childhood onset epilepsy and paroxysmal dystonia (Barnerias et al. 2010). However, its efficacy has been variable (Weber et al. 2001). The administration of thiamine is an additional treatment that can potentially be effective, given its role as a cofactor (as TPP) of the enzyme-complex activity (Di Rocco et al. 2000; Lee et al. 2006; Barnerias et al. 2010; Giribaldi et al. 2012).

The diagnostic process usually includes measuring PDHC activity in cultured fibroblasts or skeletal muscle, but there may be great variation in fibroblast activities reported by different laboratories. Thus, it is likely that not all patients are correctly diagnosed and therefore the exact prevalence of PDHC deficiency is not known (Barnerias et al. 2010; Patel et al. 2012). Moreover, thiamine-responsive patients might be missed, because enzyme analysis is usually performed with high TPP concentrations and will not show a decreased PDHC enzyme activity in these patients (Di Rocco et al. 2000; Naito et al. 2002a; Lee et al. 2006). Amongst patients with thiamine-responsive PDHC deficiency, several different mutations have been found; the majority located in the *PDHA1* gene, encoding the E1α subunit, some in the region encoding the TPP-binding site, some outside the TPP-binding site (Naito et al. 1994, 2002a, b; Benelli et al. 2002; Debray et al. 2008). At present, it is not known whether thiamine-responsive patients present differently from those not responsive to thiamine and what doses of the vitamin are required to gain significant therapeutic benefits.

The aim of this study was to review all clinical and laboratory data on our patients with PDHC deficiency in order to elucidate whether a prediction of thiamine responsiveness can be made in patients with PDHC deficiency based on clinical, biochemical or neuroimaging studies and to demonstrate the difficulties in diagnosing PDHC deficiency accurately and reliably by enzymatic analysis. To this end, we included in this study a patient with secondary PDHC deficiency, presumed to have primary PDHC deficiency at the time of presentation.

Methods

We conducted a retrospective review of all medical records of all patients with symptoms and biochemical findings consistent with PDHC deficiency at the Royal Children's Hospital in Melbourne, Australia. Included patients were born in Victoria or Tasmania between 1 January 1982 and 2012. Available clinical, laboratory and neuroimaging characteristics were collected into a database. We defined patients with PDHC deficiency as those with a pathogenic mutation in one of the genes encoding the PDHC subunits or a combination of clinical presentation consistent with PDHC deficiency (e.g. neonatal lactic acidosis, Leigh disease, relapsing ataxia etc.) and diagnostically low PDHC enzyme activity with no other confirmed diagnosis. We also included in this study one patient with severe PDHC deficiency secondary to 3-hydroxyisobutyryl-CoA hydrolase (HIBCH) deficiency determined enzymatically. The study was approved by the Human Research Ethics Committee of the Royal Children's Hospital (HREC # 32087A).

Enzyme analyses were performed usually in cultured skin fibroblasts as previously published (Rahman et al. 1996). Briefly, total (dichloroacetate-activated) PDHC activity was assayed by measuring the rate of $^{14}C–CO_2$ formation from [1-^{14}C]pyruvate. PDHC activity was also measured in lymphocytes and/or skeletal muscle biopsies from some patients. Activities were expressed as percentages of normal control mean relative to protein concentration and relative to citrate synthase. Mutations were identified by sequencing the coding region of the *PDHA1* gene in cDNA prepared from patient fibroblasts and then confirmed in genomic DNA as described previously (Ridout et al. 2008).

Clinical characteristics included pregnancy and perinatal history, age at onset, presenting symptoms and signs, details on thiamine treatment and outcome. Responsiveness to thiamine treatment was defined as sustained clinical improvement (i.e. regaining spontaneous breathing after a period of intubation; decline in seizure activity; developmental gain with progress in communication, intellectual or motor skills; improvement in mobility, tone or coordination; or a decrease in movement disorders (Pastoris et al. 1996; Di Rocco et al. 2000; Naito et al. 2002a, b)) and/or improvement suggested by neuroimaging after the start of thiamine administration. Findings of brain computerised tomography (CT), ultrasonography (US) or magnetic resonance imaging (MRI) before and after the start of thiamine treatment were reviewed, as available. The lowest and highest concentrations of lactate, pyruvate and alanine in blood (arterial, venous and capillary) and cerebrospinal fluid (CSF) before and after start of thiamine treatment were noted but are not reported here. We did not consider biochemical improvement in the definition of responsiveness to thiamine because values of lactate and pyruvate range broadly, are inconsistent and can be influenced by a variety of factors, as has been well documented previously (Chariot et al. 1994). Moreover, lack of correlation between lactate and pyruvate concentrations and clinical outcome has been shown in previous studies on treatment of patients with PDHC deficiency (Barnerias et al. 2010).

Results

We identified 19 patients with primary PDHC deficiency and one patient with secondary PDHC deficiency due to 3-hydroxyisobutyryl-CoA hydrolase (HIBCH) deficiency. Details regarding the presenting symptoms and signs and confirmation of diagnosis are presented in Table 1. In the group of patients with PDHC deficiency feeding difficulties were a common presenting symptom in the neonatal period ($n = 7/9$) as well as lethargy (5/9), hypotonia (4/9) and abnormal respiration (3/9). Seizures were noted in two patients who presented in the neonatal period and two who presented later. Intermittent ataxia was noted in two patients (or possibly three, including patient 15). The patient with HIBCH deficiency also presented with feeding difficulties.

Other neurological and non-neurological symptoms and signs, noted through follow-up reviews, are summarised in Table 2. Ataxia was noted in 8/19 and dystonia in 4/19 patients, combined with chorea in two. Tremor was noted in 3/19 patients. Microcephaly was documented in 4/19 patients, all female. One female patient (patient 19, sister of patient 18) was asymptomatic. Only 4/19 patients had normal development.

Details on treatment and responsiveness to treatment are summarised in Table 3. High-fat diet (50–60% of total calorie intake) was prescribed routinely or as part of a "sick day regime" in 15 patients, alone or in combination with thiamine. Two patients (patients 2 and 5) did not receive thiamine treatment and one was lost to follow-up (patient 14). Eight patients (patients 3, 4, 6, 7, 9, 10, 12, 13) received thiamine at doses ranging from 50 mg to 300 mg/day with no clinical effect. All were on a high-fat diet (including one on expressed breast milk) and five were on anticonvulsants. The age at onset in these patients ranged from the neonatal period (five patients) to 6 months. Seizures occurred in 6/8, dystonia and bulbar dysfunction were noted in 3/8 and chorea in 2/8 patients. Ataxia was reported in only 2/8 patients. Five of these patients have deceased. Patients 1 and 11 (age at presentation: neonatal and 2 months, respectively) seemed to respond favourably but temporarily and deceased later (one at age 1 year; ~25 years ago). Both had seizures and one had chorea.

Four patients (patients 15, 16, 17, 18) were treated with thiamine doses 200–1,200 mg/day and showed sustained response at doses above 500 mg/day. Of these, one was not on a high-fat diet, another was only temporarily on the diet and one was on a "moderately" (40%) high-fat diet. The age at presentation of these patients ranged from 12 to 27 months. Ataxia was reported in 3/4, tremor in 2/4 and bulbar dysfunction and chorea/dystonia in one patient, each. Seizures occurred in only one of these patients. Two of the three patients who regained spontaneous breathing after a period of acute deterioration had only transient response; hence, this feature cannot be ascribed unequivocally to thiamine responsiveness and may reflect the natural history of the disorder. Patient 8, who presented in the neonatal period, is severely disabled, and although she seems to have improved on thiamine treatment, it is hard to confirm improvement objectively. The patient with HIBCH deficiency did not respond to thiamine.

Neuroimaging was done in all 20 patients except for one nonresponsive patient and one asymptomatic PDHC-deficient patient (Table 4). Structural abnormality of the corpus callosum was noted in 6/19 patients (4/9 patients who presented in the neonatal period; two who presented in the second month of life, including one prenatal ultrasonographic diagnosis). Of these patients, 4/6 did not respond to thiamine treatment, 1/6 responded temporarily and 1/6 did not receive thiamine treatment. Cystic lesions were noted in 6/9 patients who presented in the neonatal period. Pre-thiamine treatment imaging revealed basal ganglia involvement (consistent with Leigh disease) in 2/4 thiamine-responsive patients, one who was not treated with thiamine and one patient who had transient response to treatment. The patient with HIBCH deficiency had "abnormalities in brainstem and basal ganglia".

Table 1 Presenting symptoms/signs and results of confirmatory tests

Patient number/sex	Age at onset	Presenting symptoms/signs	PDHC activity (%)	Mutation
1/male	Neonatal	Feeding difficulties Lethargy Irregular breathing Hypertonia	F: 39	PDHA1: c.1133G > A, p.Arg378His
2/female	Neonatal	Feeding difficulties Lethargy Hypotonia	F: 36 (PDH E1: 28) SM: 39	PDHA1: c.419-(17_14)delTGTT
3/female	Neonatal	Feeding difficulties Lethargy Hypotonia Seizures Facial asymmetry	F: 21	PDHA1: c.927_933delAGTAAGA, p.Ser312Valfs11
4/male	Neonatal	Feeding difficulties Lethargy Respiratory distress Hypotonia Seizures	F: 32	PDHA1: c.483C > T, p.Tyr161Tyr
5/male	Neonatal	Feeding difficulties Lethargy Respiratory distress Hypotonia	F: 35	PDHA1: c.604-10C > G
6/female	Neonatal	Feeding difficulties	F: 19	PDHA1: c.511G > A, p.Val171Met
7/female	Neonatal	Feeding difficulties Hyperextension neck	F: 50	PDHA1: c.947C > T, p.Pro316Leu
8/female	Neonatal	Mild lethargy	F: 33 (relative to protein)	PDHA1: c.1139_1142dupCCAA, p.Trp383Serfs5
9/female	Neonatal	Congenital retinal dystrophy	F: 40 (PDH E1: 37)	PDHA1: c.853C > T, p.Gln285*
10/male	M2	Seizures Respiratory distress	F: 43 L: 53	PDHA1: c.628A > G, p.Met210Val
11/male	M2	Episodes of irregular breathing Seizures	F: 11	PDHA1: c.1132C > T, p.Arg378Cys
12/male	M4	Feeding difficulties Hypotonia	F: 43 L: 18 SM: <5	PDHA1: c.1156_7insT, p.Lys387*
13/female	M6	Feeding difficulties Lethargy Hypotonia Developmental delay	L: 39	PDHA1 c.1156_7insT, p.Lys387*

14/female	M12	Developmental delay Progressive microcephaly	F: 64	PDHA1: c.858_861dupTTAC, p.Arg288Leufs9
15/male	M12	Tremor Frequent falling General weakness	F: 37	PDHA1: c.787C > G, p.Arg263Gly
16/male	M14	Intermittent ataxia	F: 75	PDHA1: c.787C > G, p.Arg263Gly
17/female	M24	Developmental delay Right hemiparesis	F: 10	PDHA1: c.1156_7insT, p.Lys387*
18/male	Y2M3	Intermittent ataxia	F: 41	PDHA1: c.261T > G, p.Ile87Met
19[a]/female	(Cascade testing)	No symptoms	ND	PDHA1: c.261T > G, p.Ile87Met
20[b]/female	Neonatal	Feeding difficulties	F: 26	

Y years, *M* months, *Sz* seizures, *F* fibroblasts, *L* lymphocytes, *SM* skeletal muscle
[a] Asymptomatic female patient diagnosed through cascade testing
[b] PDHC deficiency secondary to HIBCH deficiency

Table 2 Follow-up: neurological and non-neurological manifestations

Patient/sex	Seizures (age)	Ataxia (age)	Movement disorder (age)	Tone/tendon reflexes	Other neurological symptoms and signs	Ophthalmological findings	Other
1/male	M1	–	–	Axial hypotonia, distal hypertonia/ hyperreflexia	Aspiration		
2/female	–	–	–	Axial hypertonia, distal hypertonia			Episodes of cyanosis
3/female	D6	–	–	Axial hypotonia, fluctuating distal tone	–		
4/male	D1	–	Dystonia (D13)	Axial hypotonia, distal hypertonia	Autonomic dysfunction		
5/male	M19	–	–	Generalised hypotonia/ hyperreflexia	Bulbar dysfunction	Fluctuating ptosis	
6/female	M4	M14	Chorea/dystonia (Y9M10)	Axial hypotonia, distal hypertonia	Microcephaly	Alternating exotropia (Lt)	Skeletal deformities
					Dysphagia	Pale optic disks	Fatigue
							Peripheral oedema
7/female	–	–	–	Spastic tetraparesis/hyperreflexia	Microcephaly		Skeletal deformities
					Cortical blindness		Irritability hepatomegaly
8/female	–	Y5M2	–	Axial hypotonia, distal hypertonia/ hyperreflexia	Microcephaly Mild pseudobulbar dysfunction Sensorineural hearing loss	Ptosis (Lt) Retinal changes	Skeletal deformities
9/female	Y3	–	–	Axial hypotonia, distal hypertonia/ hyperreflexia	Microcephaly	Divergent squint	Hyperlaxity of small joints
					Dysphagia		Sleep disturbance
					Lethargy		
					Contractures		
10/male	M2	–	Intermittent dystonia (Y10)	Axial hypotonia	Lethargy	Episode of unequal pupils	Feeding difficulties
				Developed spastic tetraparesis/ hyperreflexia	Axonal neuropathy Aspiration Episodes of respiratory distress		Skeletal deformities Growth retardation Anxiety/panic attacks Easy bruising
11/male	M2	–	Chorea (M6)	Axial hypotonia, distal hypertonia	Lethargy	Pale optic disks; nystagmus	Feeding difficulties
					Distal weakness		Skeletal deformities
					Bulbar dysfunction		
					Episodes of irregular breathing		
					Fluctuating consciousness		

12/male	–	–	–	Generalised hypotonia	Episodes lethargy and sweating Quadriplegia Bulbar dysfunction Respiratory decompensation		
13/female	M22	Y3M9	Tremor (Y2M1)	Axial hypotonia, distal hypertonia	Episodes of irregular breathing Contractures	Alternating divergent squint Pale optic disks	Skeletal deformities Osteopenia Patella alta Premature pubarche Prolonged QT interval Fatigue Headaches Mild memory loss Episodic chest pain Sleep disturbance
14/female	–	Y4M11	–	Spastic tetraparesis	–		Growth retardation Autistic features
15/male	–	M12	Tremor (M12)	Generalised hypotonia/areflexia	Lethargy Episodic respiratory decompensation Bulbar dysfunction Fasciculations		
16/male	M20	M14	Tremor (M20)	Generalised hypotonia/areflexia	Dysphagia Respiratory decompensation Dysarthria Sixth nerve palsy	Intermittent ophthalmoplegia Convergent strabismus	Feeding difficulties Fatigue Hypertension Tachycardia Prolonged QT interval orthopnoea Thumb hypermobility Episodes of hyperacusis
17/female	–	Y8M0	–	Distal hypertonia	Intermittent hemiplegia Dysdiadochokinesia later persistent hemiplegia Sensorineural hearing loss		Skeletal deformities Osteopenia Fatigue Headaches Short concentration span

(continued)

Table 2 (continued)

Patient/sex	Seizures (age)	Ataxia (age)	Movement disorder (age)	Tone/tendon reflexes	Other neurological symptoms and signs	Ophthalmological findings	Other
18/male	–	Y2M3	Tics/grunts/chorea	Episodic axial hypotonia	Hyperreflexia Episodic lethargy/ weakness	Astigmatism	Skeletal deformities
			(Y3M1)	Distal hypertonia	Peripheral neuropathy		Growth retardation
			Dystonia		Acute hemiparesis		Headaches
			(Y5M0)		Dysarthria		Daytime urine incontinence
19[a]/female	–	–	–	Normal	–		
20[b]/female	M3	–	–	Distal hypotonia	Episodic respiratory distress	Optic atrophy	
				Fluctuating distal tone	Aspiration		

Y years, *M* months, *D* days, *Sz* seizures

[a] Asymptomatic female patient diagnosed through cascade testing

[b] PDHC deficiency secondary to HIBCH deficiency

Enzyme analysis was done in all but patient 19, who was diagnosed by mutation analysis through cascade screening. Fibroblast PDHC activity, expressed relative to citrate synthase, ranged from 19% to 50% and from 10% to 75% of normal in thiamine-nonresponsive and thiamine-responsive patients, respectively (with no substantial difference between samples from male or female patients in these groups). In two thiamine-responsive patients (patients 14 and 16), enzyme activity was not low enough to be diagnostic for PDHC deficiency, and mutation analysis eventually established the diagnosis. Of particular note is the very low PDHC activity (26% of normal) in cultured fibroblasts of the patient later found to have HIBCH deficiency.

Pathogenic mutations leading to PDHC deficiency were identified in all patients with PDHC deficiency (Table 1). All our patients had mutations in the *PDHA1* gene (Table 1) with at least 7/19 having de novo mutations (three mothers were carriers; information regarding the other mothers is not available). Patients 1, 3, 4, 7, 10, 11, 15 and 16 had mutations that had been described previously. All other patients were found to have novel mutations, splicing, insertions and duplications (some in the same region as other similar mutations; e.g. p.Ile87Met in thiamine-responsive patients 18, 19). The patient with HIBCH deficiency was found to be a compound heterozygote for mutations in the HIBCH gene.

Discussion

We sought to identify clinical, laboratory and imaging findings that may predict clinical responsiveness to thiamine treatment in patients with PDHC deficiency. Despite the limitations of it being a retrospective observational study, with a limited number of patients, our results highlight the pitfalls in the clinical and biochemical diagnosis of PDHC deficiency and the need for high thiamine doses to achieve sustained clinical benefit from this treatment. Prospective studies with larger cohorts of patients are needed to further elucidate the clinical features that may clearly distinguish and enable prediction of responsiveness and the thiamine doses for sustained response.

The spectrum of clinical manifestations in our cohort of patients is in line with previous reports (Barnerias et al. 2010; Giribaldi et al. 2012; Patel et al. 2012). The high prevalence of feeding difficulties and lethargy soon after birth and the high prevalence of skeletal deformities are intriguing, as they have not been previously reported (Barnerias et al. 2010; Patel et al. 2012), possibly because they had not been directly associated with this diagnosis. It is also likely that some of the skeletal deformities were secondary to dystonia. Our observations indicate that patients who present with relapsing ataxia and those with Leigh syndrome are more likely to be responsive to thiamine therapy. Ataxia has been previously described in thiamine-responsive patients (Kinoshita et al. 1997; Di Rocco et al. 2000) and in patients with mutations within the thiamine pyrophosphate domain (Debray et al. 2008). From a neuroimaging perspective, basal ganglia abnormalities were more common in thiamine-responsive patients, in support of the diagnosis of Leigh disease, whereas corpus callosum abnormalities and cystic lesions were found mainly in nonresponsive patients, in support of an early, possibly intrauterine insult to the CNS. However, these characteristics are not absolutely distinctive as they can be present in both responsive and nonresponsive patients. No differences were found in other clinical or biochemical characteristics (plasma and CSF lactate and pyruvate concentrations, PDHC enzyme activity).

Our observations highlight difficulties and potential pitfalls in diagnosing PDHC deficiency. For example, enzyme analysis of patient 16 was not low enough to be diagnostic for PDHC deficiency and a diagnosis could only be made through mutation analysis. Patient 18 was found to have a novel missense mutation adjacent to two previously reported missense mutations associated with thiamine responsiveness. This patient would not have been diagnosed by standard enzymatic assay as the activity in his cultured fibroblasts was borderline low yet within the normal range both with and without TPP in the assay mixture. Similarly, patient 12 had substantial residual PDHC activity in cultured cells but undetectable activity in skeletal muscle, possibly due to different levels of TPP in tissue versus culture media or to other factors affecting PDHC stability in different cell types. Such pitfalls have been previously described (Di Rocco et al. 2000; Lee et al. 2006). Interpretation of results is further complicated in female patients, where enzyme activity reflects the impact of X inactivation (e.g. patient 14). On the other hand, PDHC activity was very low in fibroblasts from the patient with HIBCH deficiency, who presented with a clinical phenotype, blood and CSF biochemistry and neuroimaging findings suggestive of PDHC deficiency, indicating that secondary PDHC deficiency could mistakenly lead to a diagnosis of PDHC deficiency unless molecular analysis is done. It may therefore be concluded that in order to avoid missing thiamine-responsive PDHC deficiency and misdiagnosing secondary PDHC deficiency, mutation analysis would be the preferred diagnostic test. Given that the vast majority of patients with PDHC deficiency have defects in the X-linked *PDHA1* gene, it would seem prudent to sequence that gene first.

Table 3 Treatment and response

Patient number/sex	Start/dose/day	Response	High-fat diet start	Anticonvulsants	Outcome (age)
1/male	D14/400 mg	Regained spontaneous breathing	D16	+	Deceased (Y1)
2/female	None	N/A	None	None	Deceased (M1D3)
3/female	D5/50 mg	No clinical effect	D7	+	Deceased (D27)
4/male	D21/150 mg	No clinical effect	Expressed breast milk	+	Deceased (M1D13)
5/male	None	N/A	None	+	Deceased (Y1M9)
6/female	Y9M10/150–300 mg	No clinical effect	Y10M8	+	Alive (Y13M6)
7/female	M9D19/NI	No clinical effect	Y1 (for 1 year)	None	Deceased (Y27M8)
8/female	M7D21/200–600 mg	Improved development	M9D17	None	Alive (Y7M6)
9/female	Y6M7/300 mg	No clinical effect	Y2M10D12 (stopped by family)	+	Alive (Y17M11)
10/male	M3D5/300 mg	No clinical effect	M3	+	Deceased (Y11M3)
11/male	M6D20/150–600 mg (1,200 mg attempted before death)	Regained spontaneous breathing Improved mobility and development Decline in seizures	M14D20	+	Deceased (Y2M9)
12/male	M6D30/150 mg	No clinical effect	M6	None	Deceased (Y1M1)
13/female	Y14M8/100 mg	No clinical effect	M6	None	Alive (Y18M4)
14/female	Y4M11/300 mg	No information	Y4M11		Lost to follow-up (Y6M6)
15/male	Y1M3/200–600 mg	Regained spontaneous breathing Improved mobility and strength Resumed gait and speech within weeks. Attends regular kinder	None	None	Alive (Y3M10)
16/male	Y1M8/300–1,200 mg	Improved coordination and tone Developmental gains: intellectual, communication and mobility Improved neuropsychological performance at age Y7M3. Attends mainstream school	Y1M8 (for 1 year; then when unwell)	None	Alive (Y8M11)
17/female	Y13M6/100–500 mg	Improved mobility and strength Increased energy level	Y16M3 (40% of calories)	None	Alive (Y31M4)
18/male	Y2M5/300–600 mg	Improved development: intellectual, communication and mobility (limited by contractures) Increased energy level	Y2M5	None	Alive (Y9M8)
19[a]/female	Y3M5/300–600 mg	Remains asymptomatic	None	None	Alive (Y9M8)
20[b]/female	Y5M2/200–300 mg	No clinical effect	None	+	Deceased (Y8M1)

Y years, *M* months, *D* days, *Sz* seizures

[a] Asymptomatic female patient diagnosed through cascade testing

[b] PDHC deficiency secondary to HIBCH deficiency

Table 4 Neuroimaging

Patient number	Neuroimaging	
	Before thiamine Rx	After thiamine Rx
1	US: multiple cystic lesions in anterior aspect choroid plexus. Mild asymmetry and dilatation of ventricular system	CT (8 D after start Rx): no abnormalities US (14 D later): cystic lesions floor lateral ventricles, possible resolving sub-ependymal haemorrhage
2	US: CC agenesis, right choroid cyst. Gross dilatation lateral ventricles. Diminished amount of cerebral substance. Prominent cisterna magna	–
3	US: possible cystic structures floor lateral ventricles. Gross hydrocephalus lateral and third ventricles	–
4	MRI: thin CC, periventricular cysts, mild lateral and third ventriculomegaly. Immature cerebral sulcation. Under opercularisation. Globally abnormal signal white matter, generous extra-axial surface spaces. MRS: No elevated lactate	–
5	MRI: symmetric signal abnormalities cerebellar hemispheres, brain stem, R occipital pole, L hypothalamus (consistent with Leigh syndrome) MRI (15D later): interval evolution of signal abnormalities. Resolution of acute oedema, generalised cerebral atrophy. Possible signal changes ganglio-thalamic regions MRS: elevated choline, lactate and pyruvate	–
6	MRI: microcephaly, partial CC agenesis, posterior fossa arachnoid cyst. Asymmetrical ventriculomegaly. Cerebral atrophy Lt > Rt MRI (9Y10M later): myelination of remaining white matter occurred, no other changes MRS: abnormal lactate peaks over left periventricular white matter	–
7	–	–
8	MRI: asymmetric lateral ventricles, white matter loss Lt > Rt, cerebral atrophy. Extra-axial collection R frontal region	–
9	MRI: partial CC agenesis. Probable large arachnoid cyst posterior fossa. Possible stenosis in aqueduct of Sylvius causing hydrocephalus	–
10	Prenatal US: agenesis of the CC	MRI (1 M after start Rx): CC agenesis, dysmorphic ventricles. MRS: increase lactate MRI (5 M after stop Rx): CC agenesis, colpocephaly, signal change in globus pallidus and deep cerebellar white matter. MRS: lactate peak brainstem and basal ganglia
11	CT[c]: mild prominence lateral and third ventricles MRI: thin CC. Symmetrical signal abnormalities hippocampi, globus pallidi, lateral thalami (consistent with Leigh Syndrome). Enlarged extra-axial CSF spaces	CT(7 M after start Rx): stable prominent ventricles and extra-axial spaces
12	–	–
13	MRI: brachycephaly. Possible white matter loss, but myelination not yet completed	–
14	CT: ventricular dilatation and cerebral atrophy MRI: cerebral atrophy	–
15	MRI: no abnormalities	–

(continued)

Table 4 (continued)

Patient number	Neuroimaging	
	Before thiamine Rx	After thiamine Rx
16	MRI: symmetrical signal changes in globus pallidi, putamen, dentate nuclei, ventral medulla and around fourth ventricle (consistent with Leigh syndrome)	MRI[d]: decrease of signal changes except for increased signal in dentate nucleus. New areas in cerebral peduncles
	MRS: raised lactate	MRI (1 Y9 M after restart Rx): decrease in signal changes, areas partially replaced by more cystic changes
		MRI (5 M later): progression of swelling, patchy signal changes globus pallidi. Slight increase signal change medulla
		MRI (4 Y7 M later): Slight increase hyperintensity dorsal medulla. Decrease in signal changes and resolution of swelling, with residual cyst
17	MRI: decreased signal in L globus pallidus and small area of increased signal in R globus pallidus and signal change L cerebral peduncle	–
18	MRI: no abnormalities	MRI (2 Y6 M after start Rx): extensive gliosis, bilateral cystic and asymmetrical signal changes basal ganglia
		MRI (6 M later): stable appearance
19[a]	–	–
20[b]	MRI: thin CC, prominence of ventricular system, cerebral atrophy. Delayed myelination, abnormalities brain stem and basal ganglia	–

Y years, *M* months, *D* days, *CC* corpus callosum, *Lt* left, *Rt* right

[a] Asymptomatic female patient diagnosed through cascade testing

[b] PDHC deficiency secondary to HIBCH deficiency

[c] 4 days after starting thiamine treatment

[d] 5 days after restarting thiamine treatment, after ceasing treatment for 6 months

It is difficult to establish the exact relationship between mutations in the *PDHA1* gene and "thiamine responsiveness". Before the structure of the E1 enzyme was elucidated, a number of mutations were proposed to involve amino acid residues located within the TPP-binding region. These assignments are not consistent with the known structure of the enzyme, so interpretations of their biochemical consequences are difficult to evaluate. None of the mutations that have been well defined as causing "thiamine-responsive" PDHC deficiency have involved amino acid residues that are directly involved in TPP binding; most are adjacent to the binding site and probably alter the position of the actual side chains that interact with the cofactor, weakening their binding. There are two groups of these: p. Ile87Met (present report), p.Arg88Ser (Marsac et al. 1997), p.Arg88Cys (Fujii et al. 2006) and p.Gly89Ser (Naito et al. 1999) on one side and p.Phe205Leu (Naito et al. 2002a), p.Met210Val (Tripatara et al. 1999), p.Trp214 (Lissens et al. 2000), p.Leu216Phe (Naito et al. 2002a) and p. Pro217Leu (also denoted as Pro188Leu) (Hemalatha et al. 1995) on the other. A number of patients have been reported as thiamine responsive but have mutations involving amino acid residues that are located well away from the TPP-binding site. These include p.Val71Ala and p.Cys101Phe (Naito et al. 2002b), p.Tyr161Cys (Lee et al. 2006), p.Tyr243Ser (Benelli et al. 2002), p.Arg263Gly (Bachmann-Gagescu et al. 2009) and other C-terminal residues (Narisawa et al. 1992). At present, there is no structural explanation as to why these should influence TPP binding.

As the TPP-binding site is in the interface between the E1α and β subunits, it might be expected that defects in either one could be thiamine responsive. However, given that the total number of E1 β patients is very small, it is not surprising that no such patients have been described so far. Patients with defects in thiamine transport or the activation of thiamine to TPP would also be expected to be thiamine responsive.

The proportion of thiamine-responsive patients in our cohort (4–5/19) is high in comparison with previous reports (Barnerias et al. 2010; Patel et al. 2012). This difference could be due to differing definitions of responsiveness, namely clinical, biochemical or other. In this regard, we found that clinical response does not correlate with biochemical response (e.g. lactate, pyruvate; data not shown). Another possible reason for our relatively high number of thiamine-responsive patients is an increasing awareness to the possibility of thiamine-responsive PDHC deficiency, coupled with molecular testing. Indeed, there has been an increase in our diagnosis of thiamine-responsive patients within the last 5 years. A third possibility is the use of high (>400 mg/day) or very high (>1,000 mg/day) thiamine doses in our patients. Previous reports have highlighted the discrepancy between in vitro and in vivo response to thiamine. In some reports, recovery of activity has been demonstrated in vitro when the enzyme was assayed in high concentrations of TPP, but there has been no clear clinical response to vitamin supplementation. Debray et al. reported two siblings who were found to have a mutation in the thiamine-binding domain but did not show any clinical benefit from thiamine treatment, at 150 and 250 mg/daily, respectively (Debray et al. 2008). Likewise, Barnerias et al. reported only 1 of 22 patients who was thiamine responsive, but the daily thiamine dose was low (50 mg) (Barnerias et al. 2010). Patel et al. reviewed data on 371 patients and reported that only 73 received thiamine (from a few milligrams to >1,000 mg daily), but no information was given regarding the correlation between doses and responsiveness (Patel et al. 2012). Thus, there are insufficient reported data on the optimal daily thiamine dose that patients with PDHC deficiency should receive. In our cohort, the lowest effective daily dose for sustained benefit was 400 mg/day, but we noted that higher doses should be used in order to maximise the clinical response.

We conclude that patients with PDHC deficiency who present with relapsing ataxia or Leigh disease are more likely to respond to thiamine treatment than those who present with neonatal lactic acidosis and have structural abnormalities of the corpus callosum on imaging. However, responsiveness to thiamine cannot be reliably predicted based on these clinical and neuroimaging differences since some overlap between the two groups exists. We suggest that high-dose (>400 mg/day) thiamine treatment be initiated as soon as PDHC deficiency is suspected. Early treatment will quickly reverse the manifestations of thiamine deficiency and might prevent irreversible complications (e.g. contractures). Plasma thiamine concentration and mutation analysis of the *PDHA1* gene should be done as first-line investigations (along with the usual metabolites analysis), in order to avoid missing thiamine-responsive patients and misdiagnosing patients with secondary PDHC deficiency. If negative, enzymatic analysis, molecular analysis of other PDHC genes and a search for other diagnoses should be done. New molecular tests for defects in thiamine transport will enable the correct diagnosis in more patients who present with manifestations suggestive of PDHC deficiency.

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Compliance with Ethics Guidelines

Conflict of Interest

All the authors of this chapter declare that there are no conflicts of interest.

Details of the Contributions of Individual Authors

Sanne Van Dongen: Reviewed the literature, reviewed all patients' records, collected data into Excel spreadsheets, wrote first manuscript and corrected and rewrote subsequent versions. Reviewed the final version and approved it.

Ruth Brown: Performed mutation analysis, reviewed mutations results and participated in writing the methods, results and discussion sections. Critically reviewed the manuscript and approved its final version.

Garry Brown: Reviewed mutations results and participated in writing the methods, results and discussion sections. Critically reviewed the manuscript and approved its final version.

David Thorburn: Reviewed enzymology and mutation results and participated in writing the methods, results and discussion sections. Critically reviewed the manuscript and approved its final version.

Avihu Boneh: Initiated the study, supervised and reviewed all clinical and laboratory data collection and tabulation, participated in writing all versions of the manuscript and wrote the final version of the manuscript.

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RESEARCH REPORT

Diagnostic Exome Sequencing and Tailored Bioinformatics of the Parents of a Deceased Child with Cobalamin Deficiency Suggests Digenic Inheritance of the *MTR* and *LMBRD1* Genes

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Abstract Disorders of cobalamin deficiency are a heterogeneous group of disorders with at least 19 autosomal recessive-associated genes. Familial samples of an infant who died due to presumed cobalamin deficiency were referred for clinical exome sequencing. The patient died before obtaining a blood sample or skin biopsy, autopsy was declined, and DNA yielded from the newborn screening blood spot was insufficient for diagnostic testing. Whole-exome sequencing of the mother, father, and unaffected sister and tailored bioinformatics analysis was applied to search for mutations in underlying disorders with recessive inheritance. This approach identified alterations within two genes, each of which was carried by one parent. The mother carried a missense alteration in the *MTR* gene (c.3518C>T; p.P1173L) which was absent in the father and the sister. The father carried a translational frameshift alteration in the *LMBRD1* gene (c.1056delG; p.L352Lfs*18) which was absent in the mother and present in the heterozygous state in the sister. These mutations in the *MTR* (MIM# 156570) and *LMBRD1* (MIM# 612625) genes have been described in patients with disorders of cobalamin metabolism complementation groups cblG and cblF, respectively. The child's clinical presentation and biochemical results demonstrated overlap with both cblG and cblF. Sanger sequencing using DNA from the infant's blood spot confirmed the inheritance of the two alterations in compound heterozygous form. We present the first example of exome sequencing leading to a diagnosis in the absence of the affected patient. Furthermore, the data support the possibility for potential digenic inheritance associated with cobalamin deficiency.

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Introduction

The inborn errors of intracellular cobalamin metabolism are a group of clinically and genetically heterogeneous disorders inherited in an autosomal recessive manner and have been categorized based on complementation analysis and designated *mut* (MIM #251000), *cblA-cblG* (MIM #25110, 277400, 277410, 236270, 277380, 250940) (reviewed in Watkins and Rosenblatt 2011), *CblJ* (MIM #614857) (Coelho et al. 2012), and *CblX* (Yu et al. 2013). Cobalamin deficiency generally leads to an accumulation of methylmalonic acid and homocysteine in the blood and urine stemming from decreased activity of the enzymes methylmalonyl-CoA mutase and methionine synthase (Gulati et al. 1996, 1997; Gailus et al. 2010; Watkins and Rosenblatt 2011). Clinical symptoms generally include hematologic and neurologic defects (Watkins and Rosenblatt 2011). However, even within a single complementation class clinical symptoms

and biochemical profile can be highly variable, which led to the need to distinguish the disorders based on the affected gene (Froese and Gravel 2010). Autosomal recessive mutations within at least 19 genes are associated with disorders of cobalamin metabolism. To our knowledge, there have been no reports of digenic inheritance (Sarafoglou and Hoffman 2009; Froese and Gravel 2010).

The number of patients diagnosed through research and clinical exome sequencing has increased exponentially since the inception of the technology in 2008 and the introduction of the clinical test in 2011 (NIH Health Consensus Conference Statement, 2001; Worthey et al. 2011; Dixon-Salazar et al. 2012; Need et al. 2012; Sailer et al. 2012; Hanchard et al. 2013; Shamseldin et al. 2013). Exome sequencing has been instrumental in discovering the pathogenic etiology in patients in whom traditional molecular methods were uninformative and is uniquely useful in overcoming limitations posed by traditional molecular diagnostic strategies in the identification of oligogenic findings. A recent meta-analysis of digenic inheritance publications points out that despite the capacity of high-throughput sequencing to discover digenic inheritance, strikingly few reports have been published (between 1 and 7 annually), and the rate of discovery has not increased over the last decade (Schaffer 2013). Often complicating the interpretation of genetic variants are factors such as reduced penetrance and variable expressivity, which has been, in part, attributed to a two-locus model (Strauch et al. 2003).

To our knowledge, there are no reports of exome sequencing leading to a diagnosis in a patient using only DNA from the affected individual's family members. Herein, we describe this approach and the subsequent postmortem diagnosis of an affected neonate.

Subjects and Methods

DNA from the mother, father, and sister of a patient with cobalamin deficiency were referred to Ambry Genetics (Aliso Viejo, CA) for clinical diagnostic exome sequencing (DES). The patient had previously died at 3.5 months before DNA could be collected. A newborn screening blood spot was available from the patient, providing sufficient DNA for Sanger sequencing confirmation of findings, but not for whole-exome sequencing due to limited DNA quantity and quality. The family was consented for DES and research. The patient was born to non-consanguineous parents after an unremarkable prenatal course. Though B12 levels were not measured, the mother was on a normal diet including meat during pregnancy and breast-feeding. At 27 4/7 weeks gestation, CBC was normal. The patient was apparently healthy for the first 2.5 months. Newborn screening was normal. Specifically, propionylcarnitine (C3) level at birth was 1.43 μmol/L (normal <7.0 μmol/L), propionylcarnitine/acetylcarnitine (C3/C2) was 0.06 μmol/L (normal <0.20 μmol/L), methylmalonylcarnitine (C4-DC) was 0.10 μmol/L (normal <1.00 μmol/L), and methionine was 0.08 μmol/L (normal <1.25 μmol/L). At 2.5 months, he had recurring apnea episodes and was diagnosed with reflux. He then presented with seizures 10 days later. MRI showed brain atrophy with volume decrease and sulcal enlargement. EEG was consistent with diffuse encephalopathy. He had microcephaly with height and weight at the 50% tile. Ammonia, lactate, and pyruvate were normal. Total homocysteine was elevated at 125 μmol/L (normal 4.0–15.4 μmol/L). Urine organic acids showed mild elevation of MMA (8, normal 0–5) and hydantoin-5-propionate. Urine amino acids revealed homocystinuria (204 μM/gCR) and plasma amino acids showed low methionine (3, normal 10–60). Serum MMA was 0.19 nmol/mL. Vitamin B12 was low at 173 pg/mL (211–946), but mom's level was normal. RBC folate was 580 ng/mL. CSF amino acids showed normal methionine levels, and homocystine was not detected. CSF neurotransmitter studies were normal (5-methylterahydrofolate in CSF was 59 nmol/L). Blood smear was normal without megaloblastic anemia or cytopenia. He was placed on daily hydroxocobalamin IM injections, betaine, B6, folate, and carnitine. His homocysteine fell from 125.2 to 30.5 to 18.6. His methionine increased from 3 to 12 in the same period. RBC folate changed from 508 to 503. The patient was ventilated for nearly 2 weeks and failed extubation due to continued respiratory distress with paradoxical episodes of respiratory pauses. He subsequently died at 3.5 months.

Genomic deoxyribonucleic acid (gDNA) was isolated from whole blood from each parent and the sister. Samples were prepared using the SureSelect Target Enrichment System (Agilent Technologies, Santa Clara, CA) (Gnirke et al. 2009). Briefly, each DNA sample was sheared, blunt-end repaired, and adaptor-ligated using indexed adapters. Using solution-based hybridization with oligonucleotide probes, the coding exons and neighboring intronic sequence of the genome were enriched and the nontargeted sequences were washed away. The enriched exome libraries were then applied to the solid surface flow cell for clonal amplification and sequencing using paired-end, 100 bp cycle chemistry on the Illumina HiSeq 2000 (Illumina, San Diego, CA). Each sample was sequenced sufficiently to yield greater than 90% of the targeted region with at least 10x base-pair coverage and greater than 85% of targeted bases with quality score of Q30 or higher, which translates to an expected base-calling error rate of 1:1,000 or an expected base-calling accuracy of 99.9%.

Initial data processing and base calling, including extraction of cluster intensities, was done using RTA

1.12.4 (HiSeq Control Software 1.4.5). Sequence quality filtering script was executed with the Illumina CASAVA software (ver 1.8.2, Illumina, Hayward, CA). Data yield (Mbases), %PF (pass filter), # of reads, % of raw clusters per lane, and quality scores were examined in Demultiplex_Stats.htm file. The sequence data were aligned to the reference human genome (GRCh37) and variant calls were generated using CASAVA and PINDEL (Ye et al. 2009). Exons plus at least two bases into the 5′ and 3′ ends of all the introns were analyzed. Data analysis focused on nonsense variants, small insertions and deletions, canonical splice site alterations, or non-synonymous missense alterations. The human gene mutation database (HGMD) (Strauch et al. 2003), the single nucleotide polymorphism database (dbSNP) (Sherry et al. 2001), 1,000 Genomes (1000, Genomes Project Consortium, 2010), HapMap data (International HapMap C 2003), and online search engines (e.g., PubMed) were used to search for previously described gene mutations and polymorphisms. The filtering pipeline protects all variants annotated within the HGMD (Stenson et al. 2009) and/or the online Mendelian inheritance in man (OMIM) databases. Stepwise filtering included the removal of common SNPs, intergenic and 3′/5′ UTR variants, non-splice-related intronic variants, and lastly synonymous variants. Variants were then filtered further based on family history and possible inheritance models. Data are annotated with the ambry variant analyzer tool (AVA), including nucleotide and amino acid conservation, biochemical nature of amino acid substitutions, population frequency (ESP and 1,000 genomes), and predicted functional impact (including PolyPhen (Gitiaux et al. 2013) and SIFT (Grohmann et al. 2004) in silico prediction tools). Each candidate mutation was assessed by a molecular geneticist to identify the most likely causative mutation(s). Multiple sequence alignments were viewed using IGV (integrative genomics viewer) software (Robinson et al. 2011).

Nonstandard bioinformatics filtering was performed in order to search for gene alterations which may have been transmitted to the deceased son. Specifically, analysis was performed to search for mutations in recessive genes carried by both the mother and father and not present in the homozygous or compound heterozygous state in the sister, as well as for X-linked recessive genes carried by mother. This analysis included all captured genes, including clinically novel genes. Additionally, a manual review of the known cobalamin deficiency genes was performed including the following genes: *ABCD4*, *AMN*, *CD320*, *CUBN*, *FTCD*, *GIF*, *HCFC1*, *LMBRD1*, *MCEE*, *MMAA*, *MMAB*, *MMACHC*, *MMADHC*, *MTHFR*, *MTR*, *MTRR*, *MUT*, *PCFT*, *TCN1*, and *TCN2*.

Identified candidate alterations were confirmed using automated fluorescence dideoxy sequencing. Co-segregation analysis was performed using each available family member. Amplification primers were designed using PrimerZ (Guenther et al. 2009). PCR primers were tagged with established sequencing primers on the 5′ end. Sequencing was performed on an ABI3730 (Life Technologies, Carlsbad, CA) using standard procedures.

Several months subsequent to exome sequencing, we were notified of the availability of Guthrie blood spot card from the patient and isolated gDNA for genotyping to confirm the presence of identified mutations.

Results

Exome sequencing of the mother, father, and sister resulted in an average of 16.7 Gb of sequence per sample (Fig. 1a; Table 1). Mean coverage of captured regions was 141x per sample, with 89% covered with at least 10x coverage and an average of 89% base call quality of Q30 or greater. Stepwise filtering for the removal of SNPs, intergenic and 3′/5′ UTR variants, non-splice-related intronic variants, and synonymous variants resulted in ~111,000 variants per sample (Table 2). Nonstandard bioinformatics filtering was performed in order to search for gene alterations which may have been transmitted to the deceased neonate but not to his unaffected sister. Five alterations met these criteria. Four were within two genes with described autosomal recessive inheritance and the fifth by the mother on the X chromosome. Of these three genes none related to the patient's phenotype. Therefore, all of the autosomal recessive genes with alterations carried by the father (58) and mother (51) were analyzed for relation to the patient's phenotype. Among these, two notable genes (two alterations) with potential clinical relevance were identified: (1) a missense alteration in the *MTR* gene (c.3518C>T; p.P1173L) present in the mother and (2) a single nucleotide deletion predicting a translational frameshift alteration in the *LMBRD1* gene (c.1056delG; p.L352Lfs*18) present in the heterozygous state in the father. Automated fluorescence dideoxy sequencing using DNA from the infant's blood spot confirmed the inheritance of each of the two alterations in heterozygous state (Fig. 1b). Furthermore, familial co-segregation analysis revealed that the *MTR* alteration was absent in the father and sister and the *LMBRD1* alteration was absent in the mother and present in the heterozygous state in the sister (Table 3).

Special consideration was given to the known cobalamin metabolism genes which had almost complete base-pair coverage at ≥10x (>99%), with exceptions noted in some exons in *AMN*, *CD320*, *FTDC*, *MMAB*, and *HCFC1* (Supplementary Table 1). No candidate alterations were identified, other than the described *MTR* and *LMBRD1* alterations.

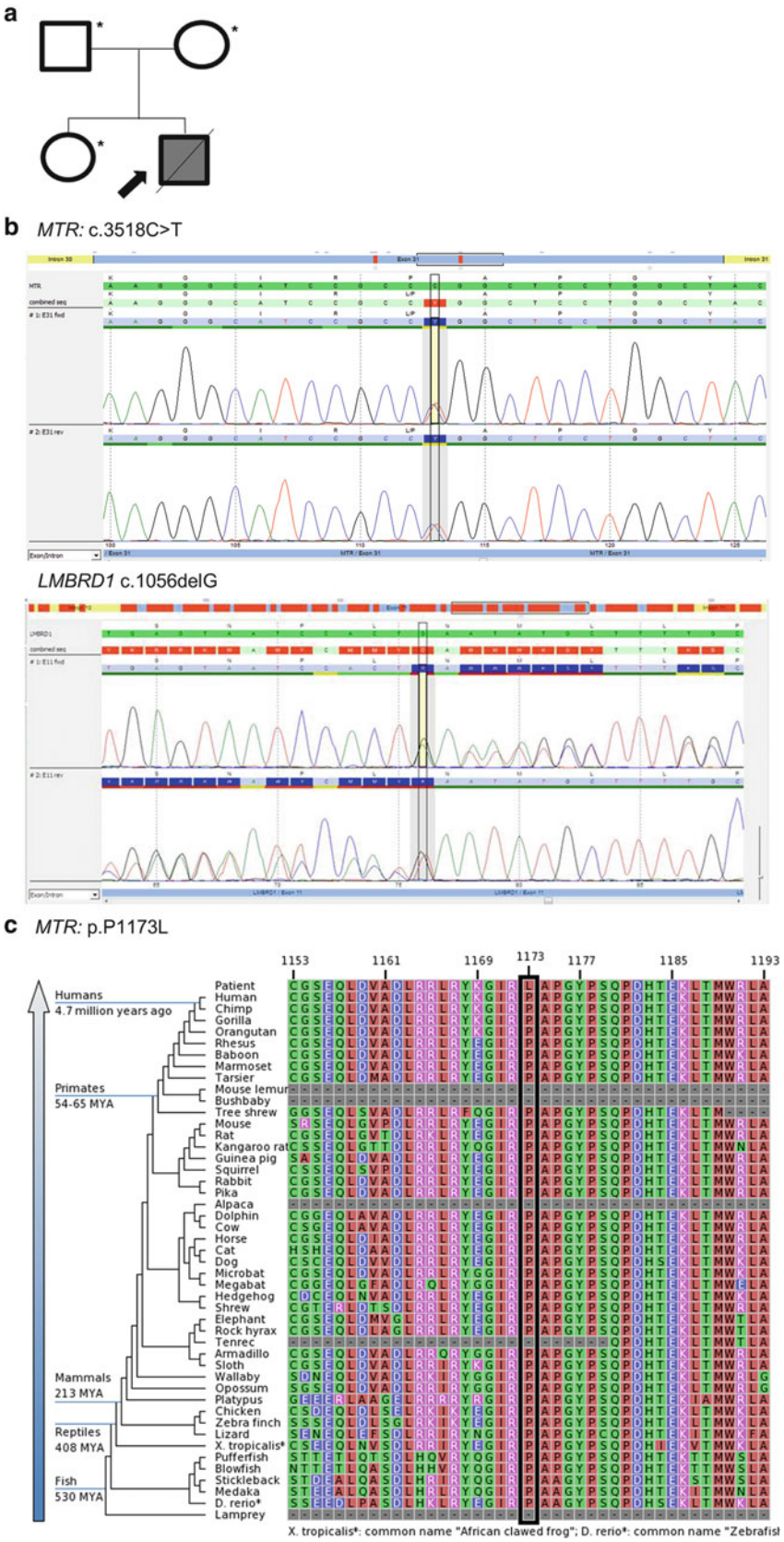
a
b
MTR: c.3518C>T
LMBRD1 c.1056delG
c
MTR: p.P1173L
1153
1161
1169
1173
1177
1185
1193
Humans
4.7 million years ago
Primates
54-65 MYA
Mammals
213 MYA
Reptiles
408 MYA
Fish
530 MYA
Patient
Human
Chimp
Gorilla
Orangutan
Rhesus
Baboon
Marmoset
Tarsier
Mouse lemur
Bushbaby
Tree shrew
Mouse
Rat
Kangaroo rat
Guinea pig
Squirrel
Rabbit
Pika
Alpaca
Dolphin
Cow
Horse
Cat
Dog
Microbat
Megabat
Hedgehog
Shrew
Elephant
Rock hyrax
Tenrec
Armadillo
Sloth
Wallaby
Opossum
Platypus
Chicken
Zebra finch
Lizard
X. tropicalis*
Pufferfish
Blowfish
Stickleback
Medaka
D. rerio*
Lamprey
X. tropicalis*: common name "African clawed frog"; D. rerio*: common name "Zebrafish"

Table 1 HiSeq sequencing run metrics

	Father	Mother	Sister	Average
Yield (Gb)	14.722	17.446	18.036	**16.735**
Quality				
% of >= Q30 bases (PF)	88.715	88.945	88.995	**88.89**
Mean quality score (PF)	35.115	35.195	35.205	**35.17**
Base coverage				
%Base_1x (%)	94.03	94.24	94.05	**94.11**
%Base_4x (%)	91.75	92.21	92.07	**92.01**
%Base_5x (%)	90.72	91.31	91.18	**91.07**
%Base_10x (%)	89.31	90.05	89.96	**89.77**
%Base_20x (%)	85.22	86.44	86.43	**86.03**
%Base_50x (%)	73.65	76.95	77.26	**75.95**
%Base_100x (%)	50.60	58.85	59.74	**56.40**
Mean_coverage (%)	121.46	148.73	153.09	**141.09**

The bold values signify the average of the three individual's (father, mother, sister), values

Table 2 Bioinformatics stepwise variant filtering

Stepwise filtering[a]	Father	Mother	Sister	Average
No. of variants in coding regions[b]	110,636	108,825	112,270	**110,577**
No. post-removal of intergenic and 3′/5′ UTR variants	82,300	81,626	83,470	**82,465**
No. post-removal of non-splice-related intronic[c] variants	21,855	21,963	22,319	**22,046**
No. post-removal of synonymous variants	11,631	11,622	11,874	**11,709**
(A) Autosomal recessive[d]	2 genes (4 alterations)			
No. of genes in (A) related to phenotype	0 genes (0 alterations)			
(B) X-linked recessive[e]	1 gene (1 alteration)			
No. of genes in (B) related to phenotype	0 genes (0 alterations)			
(C) Autosomal recessive carrier variants[f]	58	51	N/A	**N/A**
No. of genes in (C) related to phenotype	2 genes (2 alterations)			

The bold values signify the average of the three individual's (father, mother, sister), values

[a] Stepwise filtering protects variants annotated within the human gene mutation database (HGMD) and/or the online Mendelian inheritance in man (OMIM) databases

[b] Variants refer to single nucleotide alterations, insertions, deletions, and indels with at least 10x base-pair coverage

[c] Intronic refers to >3 bp into the introns

[d] Mother and father carrier, daughter negative or carrier

[e] Mother carrier

[f] Mother and father

Fig. 1 Pedigree and *MTR* c.3518C>T (p.P1173L) and *LMBRD1* c.1056delG (p.L352fsX18) mutations. (**a**) Familial pedigree. *Shaded shapes* indicate affected individuals. *Asterisk* (*) indicates whole-exome sequencing performed. (**b**) An electropherogram of the *MTR* c.3518C>T (p.P1173L) and *LMBRD1* c.1056delG (p.L352fsX18) alterations in the proband. (**c**) Sequence conservation plots at the *MTR* p.P1173L mutated site amino acid position across different species

Table 3 Familial co-segregation analysis results

Gene(s)	Protein	RefSeq id	Alteration	Mother	Father	Sister	Proband
LMBRD1	LMBR1 domain containing 1	NM_018368	c.1056del (p.L352fsX18)	−/−	+/−	+/−	+/−
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	NM_000254	c.3518C>T (p.P1173L)	+/−	−/−	−/−	+/−

The P1173L (c.3518C>T) alteration is located in exon 31 of the *MTR* gene (NM_000254) (OMIM), is predicted to be deleterious by PolyPhen (Adzhubei et al. 2010) and SIFT (Ng and Henikoff 2006) in silico analyses, and is located at highly conserved amino acid position (Fig. 1c). Based on data from the NHLBI Exome Sequencing Project (ESP), the T-allele has an overall frequency of approximately 0.04% (4/10,744) total alleles.

The c.1056delG (p.L352Lfs*18) alteration is located in exon 11 of the *LMBRD1* gene and results from the deletion of 1 nucleotide, causing a translational frameshift with a predicted alternate stop codon.

Discussion

Herein, whole-exome sequencing and nonstandard bioinformatics analysis of the parents and sister of a deceased infant with cobalamin deficiency identified alterations within well-described genes associated with cobalamin deficiency, of which each parent was a carrier. Sanger sequencing using DNA from the infant's blood spot confirmed the presence of both alterations in compound heterozygous form. To our knowledge, these results represent both the first example of the molecular diagnosis in the absence of the affected patient as well as the first example of digenic inheritance associated with cobalamin deficiency.

Mutations in the *MTR* and *LMBRD1* genes have been described in patients with disorders of cobalamin metabolism. Specifically, *MTR* gene alterations are associated with homocystinuria/hyperhomocysteinemia, and *LMBRD1* gene alterations are observed in patients with methylmalonic acidemia/aciduria and hyperhomocysteinemia/homocystinuria (Watkins and Rosenblatt 2011). c.3518C>T (p.P1173L) is the most common alteration observed in the *MTR* gene, with a frequency of about 40% (16/38 chromosomes) of patients with cblG deficiency (Watkins et al. 2002). *LMBRD1* c.1056delG (p.L352Lfs*18) is the most frequently reported alteration, found to be present in 75% (18/24 chromosomes) of one cohort of 12 patients with cblF deficiency (Rutsch et al. 2009, 2011).

The identified alterations are likely founder mutations among individuals of European ancestry, consistent with the family's reported ancestry (Watkins et al. 2002; Rutsch et al. 2011). *MTR* and *LMBRD1* are two of the several proteins essential to cobalamin metabolism (reviewed in Sarafoglou and Hoffman 2009; Watkins and Rosenblatt 2011). *MTR* is a cobalamin-dependent enzyme that catalyzes a methyl transfer reaction from methyltetrahydrofolate to homocysteine to form methionine and tetrahyrofolate (Gulati et al. 1997). Mutations in the *MTR* gene lead to the cblG complementation class of the cobalamin metabolism disorders (Gulati et al. 1996; Watkins et al. 2002). Patients with cblG typically present in the first year of life with elevated homocysteine, decreased methionine, seizures, and cerebral atrophy (Sarafoglou and Hoffman 2009). Other clinical findings in patients with cblG include decreased S-adenosylmethionine, lethargy, feeding difficulties, vomiting, abnormal tonus, mental retardation, failure to thrive, blindness, ataxia, delayed myelination, and megaloblastic anemia (Sarafoglou and Hoffman 2009). *LMBRD1* is a lysosomal membrane protein thought to be involved in lysosomal export of cobalamin in which alteration leads to the cblF complementation class of the cobalamin metabolism disorders (Rutsch et al. 2009, 2011; Gailus et al. 2010). Cells from cblF patients accumulate large amounts of free cobalamin within the lysosomes, but there is a deficiency of both cobalamin coenzyme derivatives and decreased activity of MMA mutase and methionine sythetase (reviewed in Watkins and Rosenblatt, 2011). Immortalized fibroblasts from patients with cblF show restored cobalamin coenzyme synthesis and function upon transfection with an *LMBRD1* wild-type complementary DNA (cDNA) construct (Rutsch et al. 2011). Typical clinical findings in patients with cblF are evident by the first year of life and include increased homocystine and MMA, encephalopathy, poor head growth, decreased cobalamin levels, mental retardation, ataxia, and megaloblastic anemia and/or pancytopenia (Sarafoglou and Hoffman 2009).

The proband's clinical phenotype did not fit any of the well-characterized cobalamin metabolism disorders but had overlapping features. His elevated homocysteine and low methionine are seen in both cblF and cblG, while the elevated MMA and low cobalamin levels are seen with cblF but not typically with cblG. He did not have the megaloblastic anemia that is observed in both cblF and

cblG. However, the phenotype produced from digenic inheritance is often distinct from that observed in either of the contributing genes (e.g., Helwig et al. 1995; Van Goethem et al. 2003; Yang et al. 2009). In fact, a recent review on digenic inheritance cautions medical geneticists to consider digenic inheritance as a possible mechanism for patients with novel phenotypes (Schaffer 2013). Further, the clinical features observed among patients with *LMBRD1* are widely variable (Froese and Gravel 2010; Watkins and Rosenblatt 2011), and specifically patients with the c.1056delG alteration in the compound heterozygous or homozygous state show considerable phenotypic variability ranging from death in infancy to asymptomatic long-term survival (Rutsch et al. 2009).

The unaffected mother, unaffected father, and unaffected sister do not carry both the *MTR* and *LMBRD1* alterations which would have ruled out the possibility for a digenic affect. The alterations were present in the compound heterozygous state in the proband, reducing, but not eliminating, the likelihood for copy-number variation (CNV) on the corresponding allele of each gene. Gross deletions and duplications have not been reported in *MTR*, but a single report of a 6,785 bp deletion of exon 2 has been reported in *LMBRD1* (Miousse et al. 2011). A gross deletion in *LMBRD1* would either have occurred *de novo* in the proband or be inherited from a parent (most likely the mother). The mother and father's next-gen sequencing data show comparable coverage within this gene compared with other genes, reducing the likelihood for the presence of deletion within the gene (~120X vs. mean = 134X ± 44.4). Due to insufficient DNA isolated from the blood spot of the deceased proband we are unable to entirely exclude this possibility. Whole- or partial-gene deletions including exon 11 of *LMBRD1* or exon 31 or *MTR* are excluded on the basis of heterozygosity of the mutation call.

Although a digenic inheritance pattern has not been previously reported in a patient with cobalamin deficiency, it is well described in a growing number of inherited conditions including nonsyndromic hearing loss, Bardet–Biedl syndrome, retinitis pigmentosa, polycystic kidney disease, and facioscapulohumeral muscular dystrophy type 2 (Vockley 2011; Schaffer 2013). Common to syndromes in which digenic inheritance has been described are variable expressivity, reduced penetrance, pleiotrpy, and genetic heterogeneity (reviewed in Vockley 2011), all of which are observed in cobalamin deficiency disorders. The data herein suggest that digenic inheritance may represent a novel molecular mechanism for disorders of cobalamin deficiency. It should be noted, however, that we could not completely rule out an alteration occurring in a dominant *de novo* manner, a novel gene alteration, or alterations not detectable by exome sequencing (including copy number variation). Biochemical analysis and *in vitro* transfection studies are needed to further define the impact of digenic haploinsufficiency on this metabolic pathway. This unique case highlights the power of whole-exome sequencing as a diagnostic tool even in the absence of a sample from the affected individual and suggests a novel mechanism and inheritance pattern for disorders of cobalamin metabolism.

Acknowledgments We are grateful to the family of the patient for their participation.

Compliance with Ethics Guidelines

Synopsis

In this report, we simultaneously demonstrate the power of new molecular technologies to diagnose a deceased patient while uncovering the first potential example of digenic inheritance associated with cobalamin deficiency.

Conflict of Interest

Kelly Gonzalez, Xiang Li, Hsiao-Mei Lu, Hong Lu, Elizabeth Chao, and Wenqi Zeng are employed and receive a salary from Ambry Genetics. Exome sequencing is among the commercially available tests. Joan Pellegrino and Ryan Miller declare that they have no conflict of interest.

Informed Consent

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

Animal Rights

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

Details of the Contributions of Individual Authors

Kelly Gonzalez was involved in the study's conception and design, analysis and interpretation of data, and drafting the article and is the guarantor for the manuscript. Xiang Li, Hsiao-Mei Lu, and Hong Lu were involved in analysis and interpretation of data and critical review and revision of drafts for important intellectual content. Joan Pellegrino and Ryan Miller were involved in drafting the article, analysis

and interpretation of data, and critical review and revision of drafts for important intellectual content. Elizabeth Chao and Wenqi Zeng were involved in the study's conception and design, analysis and interpretation of data, and critical review and revision of drafts for important intellectual content.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

ESP [Internet]: Exome variant server, NHLBI GO exome sequencing project (ESP), Seattle, WA. http://evs.gs.washington.edu/EVS/. Accessed Feb 2013.

OMIM [Internet]: Online Mendelian inheritance in man, OMIM®. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD. World Wide Web. http://omim.org/. Accessed 2 June 2013.

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CASE REPORT

Report of Two Never Treated Adult Sisters with Aromatic L-Amino Acid Decarboxylase Deficiency: A Portrait of the Natural History of the Disease or an Expanding Phenotype?

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Abstract Two sisters were diagnosed in their adulthood with aromatic L-amino acid decarboxylase (AADC) deficiency (OMIM#608643). They experienced early myasthenia-like manifestations, myoclonic jerks, oculogyric crises, tremors, and developmental delay during childhood; clinical stabilization afterwards; and spontaneous improvement during adolescence and young adulthood. Two novel pathogenic mutations on *DDC* gene [p.Tyr37Thrfs*5 (c.105delC) and p. F237S (c.710 T>C)] were associated with undetectable enzyme activity in plasma and only a mild reduction of biogenic amines in cerebrospinal fluid (CSF). The increase of both 3-*O*-methyldopa and 5-hydroxytryptophan on CSF was the most relevant biochemical alteration denoting AADC defect in these subjects. Transdermal rotigotine remarkably improved their gross motor functions and the asthenic status they complained. The present cases broaden the phenotypic spectrum of AADC deficiency and suggest that (1) AADC defect is not a progressive neurological disease and behaves rather as a neurodevelopmental disorder that improves during the second decade of life; (2) treatment-naïve adults can still respond well to neurotransmitter therapy; and (3) the possibility of a mild presentation of AADC deficiency should be considered when examining young adults with asthenic and parkinsonian symptoms.

Introduction

Aromatic L-amino acid decarboxylase (AADC) deficiency (OMIM#608643) is a rare and severe disorder of biogenic amine synthesis (Hyland et al. 1992; Brun et al.

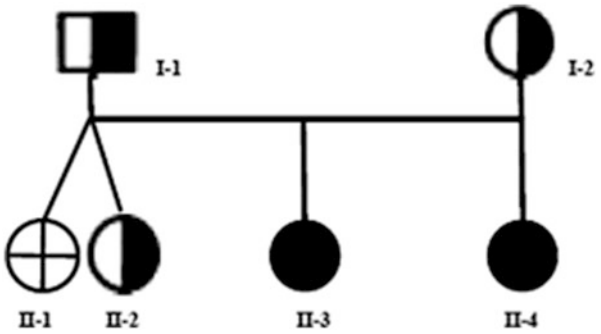

Fig. 1 Pedigree of the family

2010). Unlike other congenital defects of biogenic amine metabolism, no exhaustive data are available on the natural history of the disease and the efficacy of treatments during adulthood (Segawa 2011). Reported here is a novel family, whose follow-up lasted over than 30 years, demonstrating that treatment-naïve adults with AADC deficiency can respond remarkably well to therapy.

Patients

Figure 1 shows the pedigree, of the family, which includes two unrelated healthy Italian parents (Fig. 1: I-1 and I-2), two sisters with AADC deficiency [Fig. 1: II-3 (Patient 1) and II-4 (Patient 2)], and a heterozygote eldest sister (Fig. 1: II-2) (see below for genetic analysis). This last subject, a 35-year-old woman, was born after a complicated twin pregnancy (her sister was stillborn – Fig. 1: II-1; clinical details are not available) and soon after birth suffered from epileptic seizures and non-epileptic oculogyric crises. Later in childhood, she was diagnosed as affected by severe cerebral palsy and intellectual disability. Upon her most recent examination, at the age of 34, she showed microcephaly, profound mental disability, and severe spastic-dystonic tetraparesis with dystonic scoliosis.

Patient 1 (Fig. 1; II-3)

This 33-year-old female was born from an emergency cesarean section after a pregnancy with reduced fetal movements. At birth, she was floppy and weak. Multifocal myoclonic jerks, oculogyric crises, occasional palpebral ptosis, and tremors of upper limbs emerged over the following months. She sat unaided after the age of 3, stood up at 5, and walked unaided at 7. She had never been able to run. A diurnal fluctuation of weakness and fatigability was constantly recorded since early childhood. Mental and language development were impaired, and she attained a secondary school degree with educational support. By the age of 15, oculogyric crises disappeared. Because of her persisting marked weakness and easy fatigability, at the age of 17, she underwent single-fiber electromyography (EMG), which suggested a defect of neuromuscular transmission of congenital myasthenia type. Even though anti-acetylcholine receptor (AChR) antibodies and mutations of a panel of AChR genes subunits yielded negative results, she went under 3,4 diaminopyridine (DAP) (10 mg/4 times a day) for 4 years with transient improvements of weakness, motor performances, and speech. At the age of 20, she experienced irregular menstrual cycles: specific investigations revealed hyperprolactinemia with normal brain MRI. She was rehospitalized at the age of 32 because of increasing focal jerks. Upon examination, she appeared as a friendly and talkative woman with borderline cognitive impairment (WAIS-R total IQ = 74; verbal IQ = 78; performance IQ = 72), fluctuating palpebral ptosis and muscular weakness, clumsy gait and mild derangement of postural reactions, nasal speech, spontaneous multifocal myoclonic jerks mainly at the upper limbs, hypo- and bradykinesia, brady-palilalia, and occasional dystonic postures of the hands.

Patient 2 (Fig. 1; II-4)

This 23-year-old female presented with a severe motor developmental delay, muscular weakness, hypotonia, and recurrent non-epileptic focal jerks since birth. Bilateral fluctuating palpebral ptosis became evident at the age of 5 months. She was not able to walk unsupported until the age of 5. She experienced frequent oculogyric crises, often precipitated by tiredness and a myasthenia-like phenotype milder than patient 1. Notwithstanding some learning difficulties, she reached a secondary school educational level with special teaching support. She suffered from headache and orthostatic hypotension since adolescence. Hyperprolactinemia was detected at the age of 17. Upon examination at the age of 22, she showed mild dysarthria, bradylalia, fluctuating palpebral ptosis, multifocal myoclonic jerks, orthostatic hypotension, moderate muscular weakness with fatigability, unsupported gait, and balance

Table 1 Biochemical and molecular alterations in members of the reported family

Sample	Exam[a]	Reference range	Subject/age (years) (Fig. 1)				
			I-I/57	I-II/57	II-2/35	II-3/33	II-4/23
CSF	HVA	98–450 nmol/L	NP	NP	NP	124.3	169.3
	5HIAA	45–135 nmol/L	NP	NP	NP	30.3	50.4
	MHPG	28–60 nmol/L	NP	NP	NP	18.6	12.9
	3-OMD	<50 nmol/L	NP	NP	NP	521.8	328.4
	5HTP	<10 nmol/L	NP	NP	NP	65.6	64.3
Blood	VMA	26–63 nmol/L	NP	NP	28	6.2	10.2
	HVA	41–119 nmol/L	NP	NP	23	25.8	24.8
	5 HIAA	44–79 nmol/L	NP	NP	8.1	8.1	7,2
	MHPG	8–22 nmol/L	NP	NP	12.8	3.5	2,3
	Prolactin	4.80–23.30 ng/mL	NP	NP	9,9	159	145
Urine	Dopamine	65–400 μg/24 h	NP	NP	NP	1494	619
Plasma	AADC activity	18–43 nmol/L/min	6.2	7.2	3.7	Undetectable	Undetectable
DNA	*DDC* genotype		p.Tyr37Thrfs*5 (c.105delC)	p.F237S (c.710 T>C)	p.Tyr37Thrfs*5 (c.105delC)	p.Tyr37Thrfs*5 (c.105delC) p. F237S (c.710 T>C)	p.Tyr37Thrfs*5 (c.105delC) p. F237S (c.710 T>C)

[a] *AADC* aromatic L-aminoacid decarboxylase, *HVA* homovanillic acid, *5HIAA* 5-hydroxyindoleacetic acid, *3OMD* 3-*O*-methyldopa, *MHPG* 3-methoxy-4-hydroxyphenylglycol, *5HTP* 5-hydroxytryptophan, *VMA* vanillylmandelic acid, *DDC* dopa decarboxylase gene, *NP* not performed

impairment with frequent falls especially in the evening. WAIS-R IQ scored 72 (verbal IQ = 77; performance IQ = 65). Language and socialization skills were relatively preserved.

Biochemical Phenotype and Genotype

Table 1 shows the dosage of biogenic amines in blood, urine, and cerebrospinal fluid (CSF).

CSF of patients 1 and 2 were collected after an overnight fast when they were 33 and 23 years old, respectively. 5-Hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 3-*O*-methyldopa (3OMD), and 3-methoxy-4-hydroxyphenylglycol (MHPG) were assessed in CSF by high-performance liquid chromatography (HPLC) with electrochemical detection.

The HPLC analysis of catecholamines in urine was carried out using Chromsystems 6000 Catecholamines Reagent kit (Chromsystems Instruments & Chemicals GmbH). Compounds were identified and quantified by an electrochemical detector at a potential of 400–500 mV.

For the evaluation of blood biogenic amines, plasma was quickly separated after blood drawing, stored at −80°C and subsequently shipped on dry ice to the lab. Their measurement was performed according to Hartleb et al. (2003).

AADC activity in plasma was measured essentially according to Hyland and Clayton (1992).

DDC gene was analyzed by direct sequencing of exons and intron-exon boundaries by using BDT v1.1, and the samples were run on 3130XL (Applied Biosystems, Foster City, CA).

Two novel mutations, segregating with the disease, were identified (Table 1). The first [p.Tyr37Thrfs*5 (c.105delC)] was a deleterious frame shift mutation, the second a missense mutation. To assess the pathogenic role of p.F237S (c.710T>C) transition, a bioinformatics analysis was performed (Table 2).

Figure 2 shows the possible effects of the mutation on AADC protein structure.

Treatments

Patients 1 and 2 were treated with rotigotine (6 mg/day), escitalopram (200 mg/day), and pyridoxine (300 mg/day) when 32 and 22 years old, respectively. Rotigotine treatment resulted in a significant improvement in gross motor functions in both sisters (reduction in fluctuating weakness, increase in muscular strength, and improvement in balance and gait). Motor items of Movement Disorder Society Unified Parkinson's Disease Rating scale (UPDRS-part III) assessed a prominent improvement in gait and postural

Table 2 Bioinformatics evaluation of pathogenicity of p.Phe237Ser

Software	Output	Interpretation
SIFT: http://sift.jcvi.org/	Score: 0	Amino acids with probabilities <0.05 are predicted to be deleterious
POLYPHEN2: http://genetics.bwh.harvard.edu/pph2/	Score: 1	Probably damaging with a score of 1
MUPRO: http://mupro.proteomics.ics.uci.edu/	Confidence Score: −0.5427525	Score near −1 means high confidence in decreased stability
MUTATION TASTER: http://www.mutationtaster.org/	Prob: 0.99999985746648	Prediction – disease causing: the probability value is the probability of the prediction, i.e., a value close to 1 indicates a high "security" of the prediction

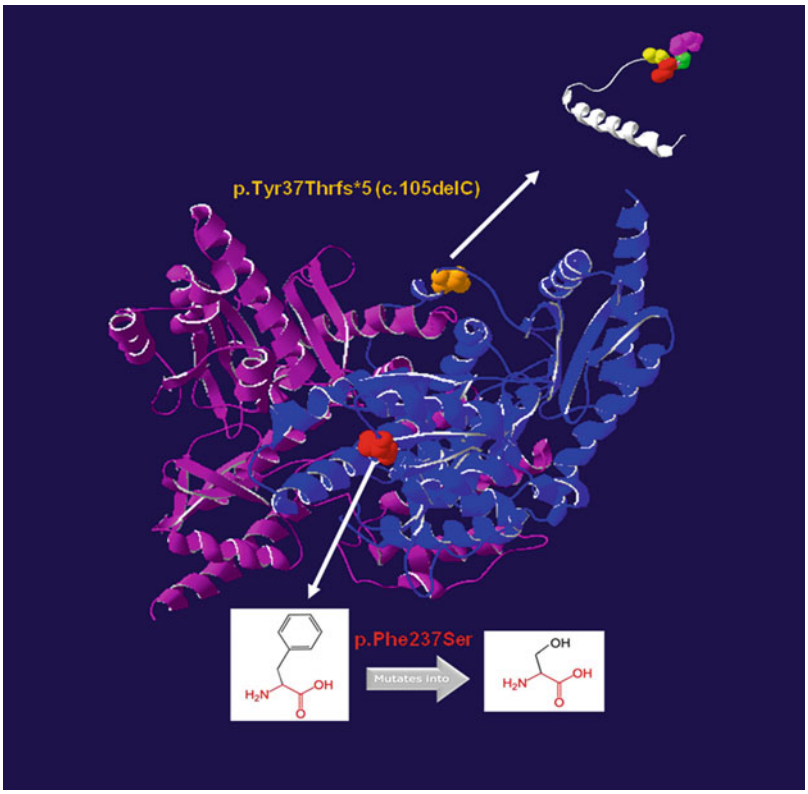

Fig. 2 The figure shows the consequences of the two mutations on the protein encoded by the gene *DDC*, as assessed by the software SwissPdbViewer version 4.1.0. The mutation c.105delC generates a premature stop codon after the change of 4 amino acids (Tyr>Thr in *yellow*, Leu>Cys in *red*, Arg>Gly in *green*, and Pro>Arg in *pink*): p.Tyr37Thrfs*5. Nevertheless, it is very likely that the stop mutation causes an early degradation of RNA by NMD (Nonsense Mediated Decay). Project HOPE (retrieved from http://www.cmbi.ru.nl/hope/home) (Venselaar et al. 2010) analysis of the mutation p.F237S suggested that : i) while the wild-type residue secondary structure, according to Uniprot, is a β-strand, the mutant residue forms a different secondary structure, which potentially destabilizes the local conformation; ii) the residue is hidden in the core of a domain that may be altered by the mutant residue; iii) the mutant residue is smaller than the wild-type residue; therefore, the mutation causes an empty space in the core of the protein; iv) the mutation would cause loss of hydrophobic interactions in the protein core

stability in patient 1 and in hand movement in patient 2. Patient 1 also became able to run slowly without falling.

Patient 1 experienced a transient recurrence of oculogyric crises. Patient 2 presented, with rotigotine at the dosage of 6 mg/day, an increase in multifocal myoclonias and in the reappearance of oculogyric crises, which required a temporary dosage reduction. A return to 6 mg/day dose, three months later, was well tolerated. In both patients, rotigotine normalized the blood prolactin levels within a few days [prolactinemia decreased from 159 to 14.8 ng/mL in patient 1 and from 145 to 12.4 ng/mL in patient 2 (reference ranges: 4.80–23.30 ng/mL)]. In both sisters, no effects were observed on orthostatic hypotension.

The heterozygote sister received rotigotine and pyridoxine with no obvious improvement.

Discussion

Even though presenting with the typical early symptoms denoting the severe forms of AADC deficiency, our patients experienced a relevant clinical stabilization and spontaneous improvement of their neurological status during adolescence and young adulthood (Pons et al. 2004; Brun et al. 2010). This clinical pattern broadens the phenotypic spectrum of AADC deficiency (Pons et al. 2004; Manegold et al. 2009; Lee et al. 2009; Brun et al. 2010).

In a larger series of AADC-deficient patients published so far, the overall mortality associated with the disease was about 10% and the majority of the remaining subjects showed a poor response to the treatment, experiencing a several neurological impairment (Brun et al. 2010). Furthermore, among the few cases with milder presentation and protracted clinical observation, the spontaneous improvements we observed in our cases as they grew older have not been reported (Pons et al. 2004; Chang et al. 2004; Tay et al. 2007; Manegold et al. 2009; Brun et al. 2010). Despite the severe clinical presentation and the undetectable plasma AADC activity (Brun et al. 2010), in our patients CSF homovanillic acid (HVA) was surprisingly within the normal range, and 5-hydroxyindoleacetic acid (5HIAA) was only marginally reduced. Therefore, the increase of 3-*O*-methyldopa and 5-hydroxytryptophan was the most relevant biochemical alteration denoting AADC defect (Table 1). We are not aware of serial assessments of biogenic amines in CSF of AADC-deficient patients. In a single case examined at the ages of 3 and 18 (Claudia Carducci, personal communication), the concentrations of HVA and 5HIAA remained relatively stable (69 and 66 nmol/L and 18 and 36 nmol/L, respectively). However, taking into account the brain requirement of biogenic amines and their age-related reference values in CSF, the gap between pathological and normal levels of HVA and 5HIAA declines with the age. Accordingly the biphasic clinical course in our patients might arise from the combined effect of a high residual enzyme activity in the brain and the decreased demand of mature brain for biogenic amines. As the blood AADC activity is concerned, it has been already reported that the enzyme activity in blood does not predict the level of biogenic amines in CSF (Hyland et al. 1992; Fiumara et al. 2002; Pons et al. 2004; Wassenberg et al. 2012). An adjunctive compensatory mechanism has been suggested by the murine model of AADC deficiency where a progressive autoregulation of dopaminergic (but not of serotoninergic) network, resulting in an increase of brain dopamine levels, developed in adulthood, as a consequence of pre- and postsynaptic adaptive mechanisms (Lee et al. 2013).

Transdermal rotigotine was extremely effective in improving gross motor functions as well as in normalizing blood prolactin in AADC defect both in these two sisters and in a previously reported 12-year-old boy (Mastrangelo et al. 2013). The transient signs of dopaminergic dysregulation, observed in the present patients, but not in the previously reported pediatric patient, could reflect the abovementioned age-related dopaminergic change leading to a possible postsynaptic hyper-recruitment of dopamine receptors (Lee et al. 2013; Mastrangelo et al. 2013). It is unclear whether sexual differences in morphology and functions of the monoaminergic system, which have been demonstrated in murine models, could also play a role (Reisert and Pilgrim 1991)[14]. Previous clinical observations suggested that women are relatively unresponsive to therapy (Pons et al. 2004). While, in general term, our experience does not support this hypothesis, the lack of affected males carrying the mutation we found makes impossible for us to contribute to this topic.

Eyelid ptosis and fluctuating muscle power in AADC-deficient patients were sometimes misinterpreted as myasthenic symptoms (Tay et al. 2007; Brun et al. 2010). The presence of the electromyographic myasthenic-like alteration in patient 1 is difficult to interpret. Presently, systematic EMG studies in patients with AADC deficiency are lacking. The well-known effects of DAP on dopamine and norepinephrine release can explain the partial improvement observed in patient 1 (Scheer and Lavoie 1991).

A mean AADC enzyme activity of 35–40% of the normal reference range has been reported in asymptomatic heterozygotes (Verbeek et al. 2007). The concurrence of a low residual AADC activity and unusual severity of cerebral palsy in the sister of the two affected patients is intriguing. It is a matter of conjecture if this was a change association or, alternatively, if AADC defect may have contributed in increasing the brain vulnerability in this patient.

Conflicting opinions exist as to the importance of early treatment for the outcome of the disease (Brun et al. 2010). The present adult diagnosed sisters were responsive to a combined pharmacologic treatment as previously reported in early diagnosed and treated patients (Hyland et al. 1992, Pons et al. 2004; Manegold et al. 2009; Brun et al. 2010). In conclusion, present cases suggest that (1) AADC defect is not a progressive neurological disease and behaves rather as a neurodevelopmental disorder that improves during the second decade of life; (2) treatment-naïve adults can still respond well to neurotransmitter therapy; and (3) the possibility of a mild presentation of AADC deficiency should be considered when examining young adults with asthenic and parkinsonian symptoms.

Acknowledgements We thank Professor Kerry R. Mills (University of Oxford, UK) for EMG analysis in patients 3 and 4 and the late Professor John Newsom-Davis for having examined both patients in the late 1990s.

Compliance with Ethics Guidelines

The work described in the manuscript has been realized in accordance with Italian law and with international ethics guidelines.

Synopsis

AADC deficiency behaves as a neurodevelopmental disorder that improves and responds to neurotransmitter therapy during the second decade of life.

Conflict of Interest

All the authors of this chapter declare that there are no conflicts of interest.

Ethics Approval

Not applicable, not required.

Patient's 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

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

Authors' Contribution

Vincenzo Leuzzi planned the manuscript and realized the first draft and the final revision. He directed the diagnostic and therapeutic management of the patients since the moment of the diagnosis. He serves as guarantor for the article, he accepts full responsibility for the work and/or the conduct of the study, and he has access to all the related data and controlled the decision to publish.

Mario Mastrangelo took part in the planning of the work described in the manuscript, in its conduct and reporting. He collaborated in the diagnostic and therapeutic management of the patients since the moment of the diagnosis.

Agata Polizzi took part in the reporting of the work described in the manuscript, and she was responsible for the follow-up of the patients since the early infancy.

Cristiana Artiola realized the molecular genetic analysis of DDC gene in all the members of the reported family and the related bioinformatic evaluation that is reported in Fig. 1. She also took part in the conduct and in the reporting of the work described in the manuscript.

André B.P. van Kuilenburg participated in the conduct and in the reporting of the work described in the manuscript and realized AADC enzyme activity and plasma biogenic amine measurement in all the members of the reported family.

Carla Carducci realized the molecular genetic analysis of DDC gene in all the members of the reported family and revised the bioinformatic evaluation that is reported in Fig. 1. She also took part in the conduct and in the reporting of the work described in the manuscript.

Martino Ruggieri took part in the conduct and in the reporting of the work described in the manuscript, and he collaborated in the follow-up of the patients since the early infancy.

Rita Barone took part in the conduct and in the reporting of the work described in the manuscript, and he collaborated in the follow-up of the patients since the early infancy.

Barbara Tavazzi took part in the conduct and in the reporting of the work described in the manuscript and realized biogenic amine urinary measurements in the members of the reported family.

Nico G.G.M. Abeling participated in the conduct and in the reporting of the work described in the manuscript and realized AADC enzyme activity and plasma biogenic amine measurement in all the members of the reported family.

Lida Zoetekouw participated in the conduct and in the reporting of the work described in the manuscript and realized AADC enzyme activity and plasma biogenic amine measurement in all the members of the reported family.

Vito Sofia participated in the conduct and in the reporting of the work described in the manuscript, and he collaborated in the follow-up of the patients in adult age.

Mario Zappia took part in the drafting of the manuscript, and he collaborated in the follow-up of the patients in adult age.

Claudia Carducci participated in the conduct and in the reporting of the work described in the manuscript and realized the cerebrospinal fluid measurements of biogenic amine in the two reported patients.

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RESEARCH REPORT

Lysine-Restricted Diet as Adjunct Therapy for Pyridoxine-Dependent Epilepsy: The PDE Consortium Consensus Recommendations

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Abstract *Background*: Seventy-five percent of patients with pyridoxine-dependent epilepsy (PDE) due to Antiquitin (ATQ) deficiency suffer from developmental delay and/or intellectual disability (IQ < 70) despite seizure control. An observational study showed that adjunct treatment with a lysine-restricted diet is safe, results in partial normalization of lysine intermediates in body fluids,

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and may have beneficial effects on seizure control and psychomotor development.

Methods: In analogy to the NICE guideline process, the international PDE Consortium, an open platform uniting scientists and clinicians working in the field of this metabolic epilepsy, during four workshops (2010–2013) developed a recommendation for a lysine-restricted diet in PDE, with the aim of standardizing its implementation and monitoring of patients. Additionally, a proposal for a further observational study is suggested.

Results: (1) All patients with confirmed ATQ deficiency are eligible for adjunct treatment with lysine-restricted diet, unless treatment with pyridoxine alone has resulted in complete symptom resolution, including normal behavior and development. (2) Lysine restriction should be started as early as possible; the optimal duration remains undetermined. (3) The diet should be implemented and the patient be monitored according to these recommendations in order to assure best possible quality of care and safety.

Discussion: The implementation of this recommendation will provide a unique and a much needed opportunity to gather data with which to refine the recommendation as well as improve our understanding of outcomes of individuals affected by this rare disease. We therefore propose an international observational study that would utilize freely accessible, online data sharing technologies to generate more evidence.

Introduction

Pyridoxine-dependent epilepsy (PDE, MIM #266100) is an autosomal recessive epileptic encephalopathy characterized by resistance to conventional anti-epileptic drugs but responsiveness to pharmacological dosages of pyridoxine (Mills et al. 2010). In 2006, the underlying genetic defect was identified as deficiency of α-aminoadipic semialdehyde dehydrogenase (antiquitin), which is involved in cerebral lysine catabolism (MIM #107323) (Mills et al. 2006). Antiquitin (ATQ) deficiency results in the accumulation of intermediates arising from lysine degradation proximal to the deficient enzyme activity including α-aminoadipic semialdehyde (AASA), Δ-1-piperideine-6-carboxylate (P6C), and pipecolic acid (see Fig. 1). Inactivation of pyridoxal 5′phosphate (PLP) via chemical reaction with P6C is the pathophysiological mechanism of pyridoxine dependency. While treatment with pyridoxine compensates for PLP deficit, intermediates from lysine degradation remain elevated. These compounds may contribute to the observation that despite adequate seizure control, 75–80% of patients suffer from developmental delay or intellectual disability (IQ < 70) despite pyridoxine treatment (Basura et al. 2009; Bok et al. 2012). Approximately 20% of patients need additional anti-epileptic drugs for seizure control. Further, there is an increase in the white matter abnormalities over time (Gospe 2012). Recently, a magnetic resonance spectroscopy study suggested ongoing neuronal damage with reduction in the *n*-acetylasparatate (NAA)/choline ratio in a single patient with PDE between the ages of 9 and 18 years, despite pyridoxine treatment (Dogan et al. 2012).

Standard treatment for inborn errors of metabolism (IEM) affecting catabolic pathways of essential amino acids includes reduction in the substrate of the deficient enzyme through dietary modification. For ATQ deficiency, a similar strategy was pursued to explore whether dietary lysine restriction could reduce the accumulation of lysine-derived intermediates and help improve cerebral function (neurodevelopment, cognition, and behavior).

The first dietary intervention for PDE was reported in an open-label, observational study that evaluated the effectiveness and safety of concomitant dietary lysine restriction and pyridoxine therapy using chemical biomarkers, seizure control, and developmental/cognitive outcomes in seven children with ATQ deficiency (van Karnebeek et al. 2012). The results show that dietary lysine restriction (evidence level IV) in these children: (1) is tolerated without short-term adverse effects; (2) leads to decrease of potentially neurotoxic biomarkers in different body compartments; and (3) has potential benefit for seizure control and neurodevelopmental outcome.

However, evidence regarding the benefits of a lysine-restricted diet remains limited. Additionally, lysine restriction poses a burden on patients and families, and can conflict with social and cultural traditions (Stockler et al. 2012). Further, it requires monitoring by a specialist and (metabolic) dietitian with regular clinical follow-up, dietary protocols, and laboratory testing.

During the past three decades, important experience has been gained with the implementation and optimization of a lysine-restricted diet to prevent brain injury in Glutaric Aciduria Type I (GA-I). This rare organic aciduria is caused by an inherited deficiency of glutaryl-CoA dehydrogenase, which is involved in the catabolic pathways of L-lysine, L-hydroxylysine, and L-tryptophan (Kölker et al. 2011). Despite distinct differences between PDE and GA-I, the former being associated with chronic toxicity and the latter with a risk of acute metabolic stroke (striatal necrosis) during catabolism requiring acute intervention in illness (especially before the age of 6 years), and a need for L-tryptophan restriction, the experience with a lysine-restricted diet in GA-I provides a critical framework for the following recommendations in PDE. Furthermore, in IEMs, as in other diseases, standardization of treatments and monitoring improves outcomes (Heringer et al. 2010).

Motivated by the positive outcomes of our first study on the one hand and the limited evidence level on the other,

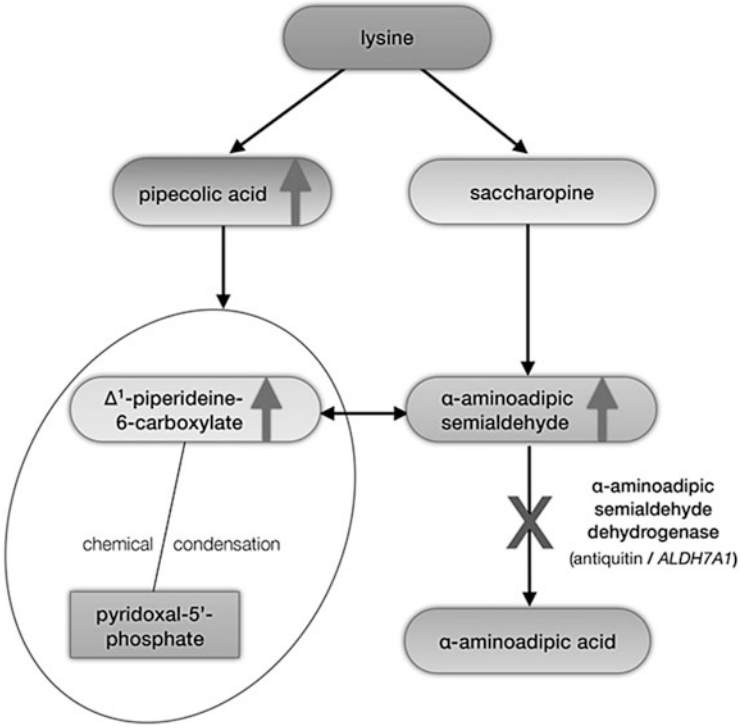

Fig. 1 Schematic overview of the metabolism of L-lysine via the saccharopine and pipecolic acid (PA) pathways and biochemical pathophysiology of ATQ deficiency. The two pathways converge where L-Δ1-piperideine 6-carboxylate (P6C), produced via the pipecolic acid pathway, and α-aminoadipic semialdehyde (α-AASA), produced via the saccharopine pathway, are in equilibrium. α-AASA is then converted to α-aminoadipic acid (α-AAA) by ATQ. In ATQ deficiency, P6C and α-AASA accumulate due to a block in α-aminoadipic semialdehyde dehydrogenase (Antiquitin, *ALDH7A1*). P6C undergoes chemical condensation with pyridoxal phosphate (PLP) resulting in PLP deficiency. PA accumulates due to back-pressure from the enzymatic block

the PDE Consortium has developed the following consensus recommendation for implementation, monitoring, and follow-up of a lysine-restricted diet in patients with PDE due to ATQ deficiency, and proposes an observational study to generate further evidence for this adjunctive therapy and the pathophysiology of PDE in general.

Methods

In 2010, an initiative was launched to bring together clinicians and scientists with experience in PDE due to ATQ deficiency to identify research questions, which could be answered via collaborative efforts and data sharing, analogous to the NICE guidelines (http://www.nice.org.uk). Scientists publishing in the field or attending conferences on the topic were invited to join. The PDE Consortium was established, and by 2013 comprised over 50 scientists including: pediatric and adult neurologists; metabolic diseases specialists; laboratory biochemical geneticists; metabolic dieticians; methodologists; and basic and clinical scientists in neurology, genetics and biochemistry. As an initial step, the Consortium published recommendations for diagnosis and *standard* treatment of PDE based on a review of existing literature and experts' experience (Stockler et al. 2011).

In analogy to other disorders of amino acid degradation (specifically GA-I), adjuvant treatment of ATQ deficiency with a lysine-restricted diet had been offered to some patients showing neurodevelopmental deficits in Vancouver (Canada), Hannover (Germany), and Denver (Colorado, US), as part of an observational study, with promising results (van Karnebeek et al. 2012). Consequently, the Consortium undertook a systematic review of a lysine-restricted diet in PDE as a basis for recommendations on its application and a proposal for future studies. The specific review question was: "What is the evidence for benefit and costs of an adjunct lysine-restricted diet in PDE?" It was also agreed that the review and recommendation should not cover other issues related to PDE, specifically the optimal dosage of pharmacological treatment with pyridoxine.

The "International PDE Consortium" convened for the 2nd, 3rd, and 4th International PDE workshops in Geneva, CH (2012), Birmingham, UK (2012), and Barçelona, Spain (2013) respectively, to draft and consolidate the dietary protocol. Milupa/Nutricia sponsored the room rental for the Barcelona meeting; a company employee was in attendance but did not contribute to the content or design

of the recommendations. During these workshops it was agreed that, based on first and only evidence (van Karnebeek et al. 2012), neonates and children with PDE due to ATQ deficiency may be offered a lysine-restricted diet as part of "improved clinical care." The importance of systematic outcome monitoring was also acknowledged and options for further studies to generate more evidence were discussed. Randomized-controlled trials designed to generate the highest level of evidence were not deemed possible due to; the inability to blind the patient and physician to the intervention (i.e., use a placebo for a diet restriction); the small number of patients scattered throughout the world; and the lack of funding despite multiple applications. Furthermore, the UBC/BCCHW REBs advised that the 2012 study presented sufficient evidence in favor of the diet, making an RCT unjustified (even in the form of an "n-of-1-trial").

Results

Literature Search

The literature search (PubMed, 1966–2011), using combinations of the keywords "pyridoxine dependent epilepsy" and "lysine", "antiquitin" and "lysine", "antiquitin" and "pipecolic acid", and "pyridoxine" and "pipecolic acid" yielded 20 results (seven case series, four case reports, three biochemical methodological studies, one animal study, one comment, and four review articles). Except for one review (Stöckler et al. 2011), no article discussed lysine restriction as possible adjunct treatment in ATQ deficiency. A second search using "pyridoxine dependent epilepsy" combined with "treatment" yielded an additional 12 publications (seven case series, two case reports, one biochemical methodological study, two reviews), but no controlled trials. To date, the only evidence (level IV) for this diet in PDE is that of Van Karnebeek et al. (2012). This evidence, together with the updated guidelines for management of GA-1 and clinical expertise, provided the basis for the following recommendations.

Pharmacotherapy: Recommendation

Lifelong treatment with pyridoxine-HCl is the standard treatment for ATQ deficiency. Reported dosages vary considerably, however, and current evidence for an optimum is limited (Haenggeli et al. 1991; Baxter 2001; Nabbout et al. 1999; Basura et al. 2009). Specific recommendations on treatment with pyridoxine-HCl are beyond the scope of this paper. To avoid grossly diverging dosages, which may impede evaluation of the lysine-restricted diet, the Consortium recommends that pharmacotherapy in patients on a lysine-restricted diet should be administered according to the 2011 Consortium publication (Stockler et al.). Furthermore, in view of the uncertainty regarding safety of long-term treatment with high dosages of pyridoxine (Bender 1999), peripheral neuropathy should be screened for via regular clinical neurological examinations and, if in doubt, nerve conduction studies should be performed (Footitt et al. 2013).

Add-on Dietary Treatment: General Recommendations and Rationale

All patients with confirmed ATQ deficiency are eligible for the diet. Diagnosis must be confirmed by: (1) elevated AASA in plasma or urine (or CSF); and (2) at least one disease-causing mutation (or deletion/duplication) in the *ALDH7A1* gene. Measurement of pipecolic acid in plasma is less specific and sensitive. Therefore it is only acceptable as an initial suggestive but not diagnostic test (Mercimek-Mahmutoglu et al. 2013). Confirmation via molecular analysis is important as AASA elevations are not specific for ATQ deficiency, but rather are observed in molybdenum cofactor deficiency and sulphite oxidase deficiency (Mills et al. 2012). In addition to newly diagnosed patients, adjunct dietary lysine restriction may be considered in all patients with a pre-existing diagnosis of ATQ deficiency, except in those who are seizure-free with normal IQs and behaviors on pyridoxine mono-therapy.

Recommendations (Fig. 2)

1. *Dietary lysine restriction is an adjunct therapy*, not a substitute for pharmacotherapy.
2. *All confirmed ATQ-deficient patients are eligible* (unless pyridoxine mono-therapy has resulted in complete symptom resolution with cessation of seizures, and the establishment of normal behavior and development)—regardless of age and gender.
3. *Initiation and duration of treatment*: lysine restriction should be started as early as possible, ideally in early infancy (see below). If tolerated without adverse effects, the patient should continue to follow the lysine-restricted diet. The optimal duration of the diet remains to be determined.
4. *Diet and monitoring*: see below.
5. *Discontinuation*: In case of the unavailability of, or intolerance to, a lysine-free amino acid formula, or severe adverse effects (nutritional, neurological or other), the diet should be terminated. In the former case, a natural protein-restricted diet may be considered.
6. *Quality assurance*: A multi-disciplinary team consisting of a medical specialist knowledgeable in PDE treatment (a neurologist and/or a specialist in IEMs), a metabolic dietitian with experience in amino acid/protein restriction, and a nurse should implement dietary treatment and monitoring. Parents or caregivers and the patients

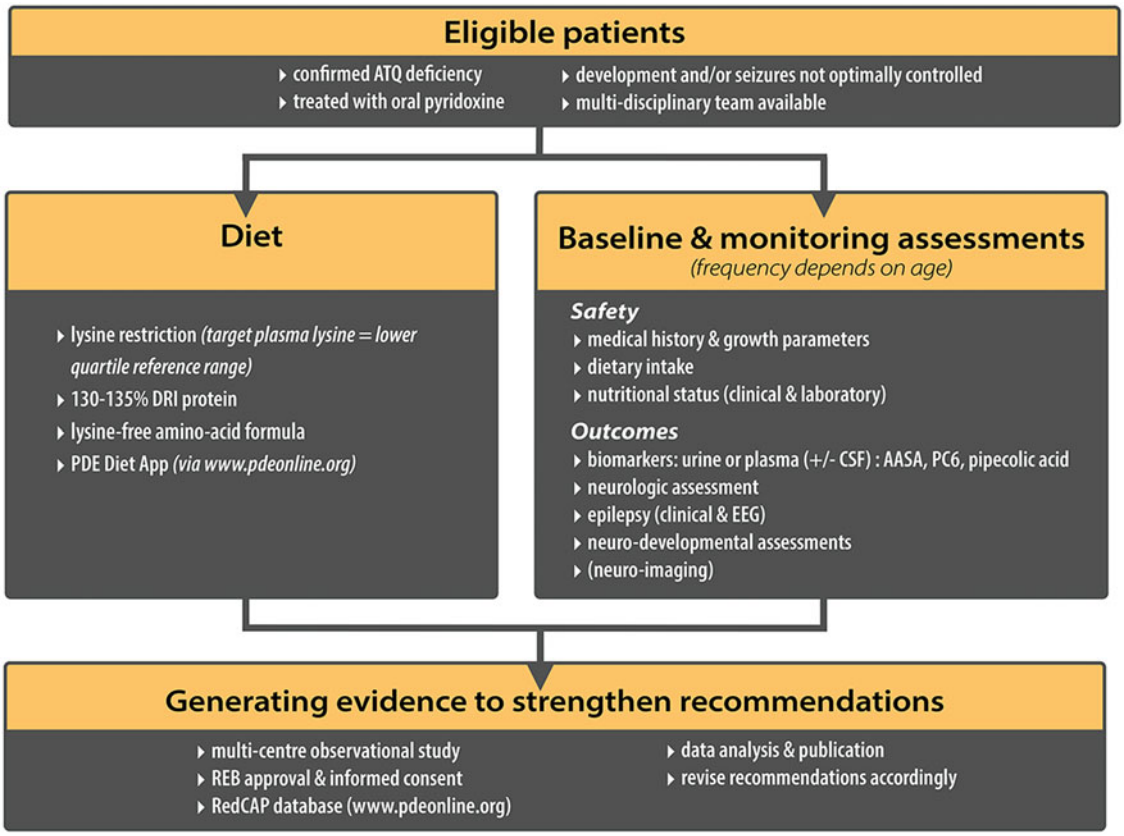

Fig. 2 Consortium recommendations for the lysine-restricted diet in PDE patients with confirmed ATQ deficiency

should be trained and monitored regularly for dietary compliance.

Rationale

Based on our current understanding of the pathophysiology and derived from experience in conditions with comparable pathogenic backgrounds (accumulation of potentially toxic substrates in the brain), such as phenylketonuria, GA-I, and guanidine-acetate methyltransferase deficiency, the brain may be considered a separate pathogenic compartment which can be influenced by extra-cerebral/systemic metabolic manipulation. The goal of dietary treatment is to restrict lysine as a precursor of potentially neurotoxic intermediates generated by disturbed lysine degradation, whilst avoiding lysine deficiency and maintaining sufficient intake of essential nutrients and energy substrates. Although a decline of pipecolic acid following the supplementation of pharmacological doses of pyridoxine has been observed, the reactive compounds AASA and P6C remain elevated, even during long-term treatment. The potential neurotoxicity of permanently raised AASA or other metabolites could have a substantial impact on neurodevelopmental outcomes. In a cohort of 32 PDE patients, no clear genotype-phenotype correlation could be established (Mills et al. 2010). Lysine restriction should, therefore, be initiated as early as possible in newly diagnosed PDE patients to optimize developmental outcomes, and be maintained over an extended period of time.

Most patients with PDE are diagnosed and followed by pediatric neurologists, often with limited access to dietitians experienced in treating patients with IEMs, which raises inherent challenges for the management of this metabolic epilepsy. These standardized dietary recommendations are intended to support the managing team in this process.

Already routinely used in GA-I, this diet has demonstrated efficacy and safety, if properly monitored. The degree of protein restriction is more liberal than in many IEMs, such as phenylketonuria or maple syrup urine disease, and therefore compliance is easier. This is supported by data from our observational study, which showed dietary adherence in six out seven patients who varied in age from infancy to early teens (van Karnebeek et al. 2012).

Diet Prescriptions (See Online Supplement for More Details)

We recommend the use of a diet based on the restricted lysine intake combined with a lysine-free amino acid

Table 1 Age-dependent lysine restriction according to the PDE Consortium[a]

Age (year)	Lysine[b,c] (mg/kg)	Protein[d,e] (g/kg)	Energy
0.0 < 0.5	70–100	2.75–3.50	125–145 kcal/kg
1.0 < 1.0	55–70	2.50–3.25	140–115 kcal/kg
1 < 4	50–80	1.80–2.60	900–1,800 kcal/day
4 < 7	40–70	1.60–2.00	1,300–2,300 kcal/day
7 < 11	35–65	1.55–1.85	1,600–2,800 kcal/day
Female			
11 < 15	35–40	1.50–1.80	1,500–2,800 kcal/day
15 < 19	33–40	1.45–1.75	1,200–2,800 kcal/day
>19	30–40	1.45–1.75	1,400–2,400 kcal/day
Male			
11 < 15	35–40	1.45–1.75	2,000–3,200 kcal/day
15 < 19	33–45	1.45–1.75	2,100–3,200 kcal/day
>19	30–40	1.45–1.75	2,000–3,000 kcal/day

[a] These recommendations are based on the guidelines published for GA-I by Kölker et al. (2011) and Yanicelli (2010), and adapted for PDE by the Consortium based on experience

[b] The lysine/protein ratio varies considerably in natural food: thus natural protein intake in children on a low lysine diet is dependent on the source

[c] The continued chronic damage model in PDE requires as low as reasonably possible-chronic lysine levels. This contrasts with GA1, where currently the focus is on preventing damage during acute episodes. In some cases, this translates into lower intakes in PDE than currently recommended in GA1

[d] Tryptophan restriction is not needed in the management of PDE, in contrast to GA-I. Lysine-free amino acid formulas developed for managing GA-I are often low in tryptophan. The individual's diet should be assessed for tryptophan adequacy and, if inadequate, should be supplemented

[e] Lysine-free amino acid mixtures should be supplemented with minerals and micronutrients necessary to maintain normal levels. Adequate intake of essential amino acids is provided by natural protein and lysine-free amino acid supplements. The amount of amino acid supplementation is adjusted to meet 130% of the patient's age-appropriate DRI (Table C)

supplement to maintain adequate daily total protein and micronutrient intake. This diet has been associated with favorable neurological outcomes and normal growth in several GA-I studies (Heringer et al. 2010; Kölker et al. 2006). The natural protein allowed in the individual's diet should be based on the daily lysine prescription.

The Consortium has adapted the guidelines for GA-I by Kölker et al. (2011), using both the WHO guidelines (FAO/WHO/UNU 1985) and a paper by Yannicelli et al. (2010) as additional references, and presents these recommendations for age-dependent lysine restriction in Table 1. This can be used as a starting point; thereafter, the lysine prescription must be individually tailored (also beyond the age of 6 years) based on the results of plasma lysine levels, adequate nutrition, and growth. Lysine-free amino acid formulas must be used to provide additional protein intake to meet total protein needs and provide micronutrients.

Nutritional Aims

1. The aim is to achieve 130–135% of the daily-recommended intake (DRI) for total protein. The rationale for this recommendation is the rapid digestion and absorption of free amino acids provided in the supplement and the subsequent decreased nitrogen retention.
2. Daily lysine intake should be prescribed at an amount that maintains the plasma lysine level within the lower normal age-dependent reference range, preferably in the lower quartile. Lysine intake should be kept at the lowest possible levels that allow for adequate growth and nutrition. In contrast to GA-I, tryptophan restriction is *not* indicated in the management of PDE. As some lysine-free amino acid formulas are also low in tryptophan, this amino acid must be supplemented to meet the daily DRI (FAO/WHO/UNU 1985).
3. Lysine-free amino acid formulas are often supplemented with vitamins and minerals to provide adequate or significant intakes of these nutrients: an adequate supply of iron, minerals, and vitamins must be confirmed by regular laboratory testing and nutritional evaluation. Possible interactions between the pharmacological treatment with pyridoxine-HCl and altered nutritional B6 vitamers due to the diet, specifically lower PLP intake due to reduced animal protein consumption, are not understood and require further research. If, in an adolescent or adult patient, lysine-free amino acid

formulas are abandoned, and a protein-restricted diet is continued, vitamin and mineral supplements become mandatory. Because the consumption of meat, fish, and dairy products will be limited, the use of fats and oils, carbohydrates, and special low protein foods is often necessary to provide adequate caloric intake. Fats and oils also provide essential dietary fatty acids. Low protein foods provide variety to the diet in addition to needed calories (Refer: Online Supplement).

Diet Management in (Breastfed) Infants

Mothers of neonates and infants with PDE should be encouraged to continue breastfeeding. Since the average lysine content of breast milk after the neonatal period (68–86 mg/100 mL) (sources: www.bls.nvs2.de; http://www.ars.usda.gov/nutrientdata) is considerably lower than that of formula milk (120–160 mg/100 mL) a greater amount of breast milk may be consumed.

In order to guarantee normal growth, use of a lysine-free amino acid supplement is also advised for breastfed children. As in organic aciduria (Francis and Smith 1981), this is best achieved by feeding a defined amount of lysine-free formula before breastfeeding ad libitum, or alternatively, by feeding expressed breast milk which can be mixed with the amino acid supplement in the bottle-feedings prepared once daily, and kept refrigerated for a maximum of 24 h. Expressed breast milk may be required when the adequacy of breastfeeding is doubtful, or inconsistent. If breastfeeding is not possible, a combination of an appropriate infant formula and lysine-free amino acid supplement is recommended.

As in healthy infants, measured amounts of solid foods should be introduced from an age of 4 to 6 months onwards with the lysine content included in the daily dietary calculations.

Assessment of Lysine Content

The lysine content varies considerably in different proteins. Cereals, fruit, and vegetables tend to have a low lysine content, e.g., 2–4% (lysine/protein) in cereals, whereas meat and animal protein is rich in lysine, e.g., 8% in beef, chicken or cow milk, and 9% in fish. Therefore, actual daily lysine intake may be less well controlled by calculation of total protein intake rather than lysine intake. Lysine restriction is preferred to protein restriction, especially during the first 2 years of life, as there is ongoing myelination of the brain, and a reported potential for white matter damage in ATQ-deficient patients (Niermeijer et al. 2012).

Switching to protein counting may be considered only where lysine counting is not feasible, and should be undertaken only after the individual/family has followed a lysine-restricted diet for 1 year (if possible), to allow the patient and caregivers to become familiar with the lysine content of different foods, the taste of the formula, and the rhythm of the diet.

Older patients who refuse the lysine-free formula may still benefit from a protein-restricted diet, which can substantially reduce levels of AASA and P6C.

Lysine-Free Amino Acid Formulas

The use of commercially available formulas with amino acid supplements is recommended, as this ensures a well-balanced diet. It must be noted that some lysine-restricted formulas exist specifically for management of GA-1 patients and so are also tryptophan-restricted. In the Online Supplement (under 10) useful resources are listed that provide information on metabolic diets, nutritional content of foods, and medical formulas.

Monitoring

Clinical Monitoring

1. Prior to initiating the diet, a detailed medical and nutritional history should be obtained from each patient, including symptoms; date of seizure onset; history of epilepsy; current medical developmental and nutritional status; and documentation of weight, length, and head circumference.
2. Patient monitoring must include both clinic visits and follow-up by telephone or email. Interim history during the follow-up visits should include questions regarding seizure recurrence, developmental progress, growth parameters, and any change in health status or concomitant medications, to monitor for safety and adverse events.
3. A nutritional intake history should be reviewed via a 3-day diet record or, if unavailable, a 24-hour dietary recall, for age-appropriate daily nutritional adequacy of energy, macro- and micronutrients, and lysine-restricted diet adherence.
4. Follow-up clinic visits include, at a minimum, the initial visit, 1-month post dietary initiation, then every 3 months during the first year, and every 6 months thereafter.
5. Assessments during clinic visits should include vital signs, a physical exam, and anthropometric measures (height, weight, and head circumference), and nutritional assessment and counseling. Weights may be recorded with greater frequency, as guidance for

Table 2 summarizes the recommended follow-up for biochemical and routine laboratory monitoring

Parameter	Rationale	Following initiation of diet	Thereafter, frequency at age	
			0–3 years	>3 years
AASA, P6C, and pipecolic acid (urine and plasma)	Efficacy in reducing metabolites of lysine degradation	1 month, 3 months	3 months	6 months
Lysine and amino acids in plasma	Medical adherence, safety	1 month, 3 months	1 months (age 0–1 years), 3 months (age 1–3 years)	6 months
Albumin, pre-albumin, iron parameters, calcium, phosphate, 25-OH-vitamin D_3, zinc, selenium, complete blood count, folic acid, and vitamin B_{12} in serum or plasma	Safety panel	1 month, 3 months	6 months	6 months

clinical care, i.e., weekly weight will be taken in patients less than 1 year of age, every 4 weeks in patients from ages 1 to 6 years, and every 3 months for patients >6 years of age, to adjust the total dose of pyridoxine and lysine restriction (both calculated per weight).

Biochemical and Routine Laboratory Monitoring

Table 2 provides an overview of the frequency for various laboratory tests. AASA, P6C, and pipecolic acid are the biochemical markers for PDE that will be measured in plasma or urine.

1. For infants <1 year of age, a plasma sample for the biochemical markers should be taken at least 3 h after a meal; for children >1 year of age, samples should be taken at least 4 h after a meal.
2. Since AASA and P6C are unstable, plasma and urine samples need to be stored at −80°C, and, if required, transport to the laboratory should be done on dry ice.
3. If a lumbar puncture is performed for clinical purposes, it is recommended that additional samples be collected to monitor the level of AASA, P6C, and pipecolic acid in the CSF. Levels of PLP, neurotransmitter metabolites, and amino acids can also be analyzed as they may be abnormal. CSF should be frozen immediately, stored at −80°C, and shipped on dry ice. The rationale for the different CSF analyses is provided in Table 3. CSF is the body fluid most closely reflecting cerebral lysine metabolism and possible neurotoxic status in the brain, and provides the most relevant biochemical outcome measure.

These CSF results may also be of future clinical relevance, specifically, to guide pyridoxine dosing, based on the levels of PLP-dependent neurotransmitters in CSF, in addition to documenting a response to dietary treatment. Moreover, the assessment of lysine in CSF will demonstrate whether lysine restriction decreases CSF lysine levels, and will give insight into whether P6C, AASA, and pipecolic acid are derived from cerebral lysine catabolism or are imported from the circulation. It is important to indicate the interval from the last pyridoxine intake to time of sampling for appropriate interpretation of results.

Nutritional Markers

To ensure adequate nutritional status, regular testing of a number of laboratory parameters should be done, including pre-albumin, albumin, plasma amino acids; complete blood count; comprehensive metabolic panel; and micronutrients (for details see Table 2).

Neurological Monitoring

1. A pediatric neurological exam should be performed during every clinic visit and be tailored to the age of the individual with a focus on the achievement of developmental milestones; muscle tone; strength; deep tendon reflexes; and coordination. Sensitivity to light touch and vibration should be assessed in children >4 years. If deep reflexes of the Achilles tendon are absent, peripheral neuropathy should be considered and nerve conduction velocity studies performed.
2. Electroencephalogram (EEG) should be performed every 6 months, or more frequently if clinically indicated, to evaluate the development of age-appropriate background activity and to assess the presence of epileptiform discharges.
3. Patients who continue to have seizures should maintain a seizure logbook for review during clinic visits.

Table 3 Rationale for CSF analysis

CSF Metabolite	Rationale/expected insights
AASA and P6C and pipecolic acid	Metabolites reflecting lysine metabolism in the brain/surrogate parameters of toxicity
	We expect reduction in these metabolites upon lysine-restricted diet
Pyridoxal-5-phosphate (PLP)	Metabolite reflecting the biochemical interaction of accumulating AASA/P6C with PLP
	We expect increase of this metabolite upon lysine-restricted diet
Monoaminergic neurotransmitter metabolites (HVA, 5-HIAA)	Synthesis of Dopamin and Serotonin requires pyridoxal phosphate as cofactor
	We expect that these neurotransmitter metabolite levels increase with increasing availability of pyridoxal phosphate upon the lysine-restricted diet
Amino acids	We are primarily interested in CSF lysine, which has been observed in the low normal range in patients on the lysine-restricted diet.[7] Other amino acids of interest are (but not restricted to) tryptophan (due to inadequate tryptophan content of most lysine-free amino acid formulas), glycine, serine, and branched chain amino acids (given that many reactions the metabolism of these amino acids requires PLP as a cofactor)
Cells count, protein, and glucose	CSF specimen that is bloody, or has an increased protein level have to be excluded from analysis)
1 mL	To store for possible additional research studies such as metabolomics. AASA as a semialdehyde is a reactive substance and its accumulation might result in changes in numerous other metabolites. Metabolomic metabolite patterns might reveal chemical compounds that might contribute to the pathophysiology of ATQ deficiency prior and during treatment with a lysine-restricted diet

Neurodevelopmental Monitoring

Table 4 lists the recommended psychometric measures (to be performed by a psychologist) to evaluate the effect of treatment prior to starting the diet and at annual follow-up.

Neuro-Radiological Monitoring

Magnetic resonance imaging of the brain should be performed using sedation prior to initiating the diet and considered after 3 years of age, or more frequently, if clinically indicated, to detect structural abnormalities and to monitor for myelination and brain development.

Emergency Treatment

In contrast to IEMs of the intoxication type, such as MSUD or GA-I (Kölker et al. 2011), *acute* neurological injury resulting in permanent brain damage is not a typical feature of PDE. However, degenerative cerebral MRI findings over time have been reported (Gospe 2012; Niermeijer et al. 2012). Based on this and unpublished individual experience, the Consortium reached the following consensus.

During an acute illness (with inherent increase of catabolism, and potential increase of PLP-inactivating P6C) routine treatment may not sufficiently protect against seizures and neurologic dysfunction, and the following precautions are recommended:

1. Doubling the dosage of pyridoxine up to a maximum of 40 mg/kg/day (or 500 mg in older children and adults) for up to 3 days.

Table 4 Summarized the standard psychological scales to monitor neurodevelopmental outcome

Measurement tool	Age group
Bayley Scale for infant and toddler development	0–3 years
Vineland Adaptive Behavior Scales	0–90 years
Wechsler Preschool and Primary Scale of Intelligence	2 years 6 months to 7 years
Wechsler Intelligence Scale for Children	6–16 years 11 months

2. Maintenance of caloric intake to prevent catabolism of endogenous proteins.
3. Intravenous fluid, caloric, and pyridoxine supplementation should be administered only if justified by the severity of the acute disease and inability to tolerate medications or fluids orally or enterally.

Discussion

It is the Consortium's impression that the potential benefits of lysine restriction in PDE could outweigh its risks and burdens, if recommendations such as these are followed closely by a clinical team, including an expert physician (neurologist or IEM specialist), a (metabolic) dietitian, and a nurse or counselor. The greatest risk associated with the diet is lysine over-restriction, which can be avoided by close monitoring and adjustment.

The recommended standardization of treatment and outcome evaluation, together with the creation of an online database with a standard research ethics board (REB) protocol and consent form, paves the way for data collection as part of a multicenter observational study to increase evidence regarding the benefit and optimal duration of the diet in PD. Therefore, the Consortium proposes that an observational study (level III) be done which would include PDE patients of all ages, and compare those on- (active) and off-diet (controls). To this end, we have designed an Internet-accessible RedCAP database for entry of patient data and we will post a standardized REB protocol on our website (www.pdeonline.org), which can be adapted for local submission and approval. With appropriate institutional ethics board approvals, de-identified data on phenotype and genotype will also be collected.

We invite physicians from across the globe to participate. Data entered will be analyzed at periodic intervals to further consolidate evidence and be published with all participants as co-authors. The current recommendations will be adapted according to new insights and evidence.

In support of patients following the lysine-restricted diet and to enhance compliancy, a digital PDE diet-App has been developed for use on handheld devices and desktops. The App will be freely available from the beginning of 2014. It provides the following functions: password coded access; option to enter weight and customized diet plan; lysine-arginine-protein-caloric content of foods; digital calculator for advised and realistic lysine and protein intake; recipes for low protein/lysine-restricted foods; storage and export of daily food records; and tracking of clinical and laboratory values (with charts and graphs).

Not only is this kind of initiative essential for collecting evidence for treatment of rare disorders where it is not practically possible to conduct randomized-controlled studies, it will also provide a framework to generate evidence regarding the evaluation of any new strategies, such as arginine fortification, which might be added in the future (Strauss et al. 2011; Kölker et al. 2012).

The Consortium recognizes that, aside from potential neurotoxic damage, the ID seen in PDE could be explained by the role of ATQ in neuronal migration (Jansen et al. 2013), as well as the increased rate of fetal distress leading to premature birth and/or associated pathology (Mills et al. 2010). Additionally, an association between delay in diagnosis and poor development has been reported (Bok et al. 2012). The latter issue can be addressed by improving the awareness of PDE among neonatologists and pediatric epileptologists, or, in the future, perhaps newborn screening (Jung et al. 2013).

Conclusions

In summary, we realize that for PDE, many challenges remain. These include the limited insights into the natural history and pathophysiology, and the potential impact of long-term hyper-physiologic, high-dose pyridoxine therapy. Through collaboration, standardization of diagnosis and treatment, and the establishment of registries and databases for data collection on natural history as well as interventional outcomes, it will be possible to address these issues and improve the clinical management of individuals affected by PDE.

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Synopsis

The first recommendation for the implementation and monitoring of the lysine-restricted diet as adjunct treatment for PDE generated as consensus statement by the international PDE Consortium.

Conflict of Interest

Clara van Karnebeek and Hans Hartmann declare that the room rental for the 4th PDE Consortium meeting in Barcelona, Spain was sponsored by Milupa/Nutricia; a company employee was in attendance but did not contribute to the content or design of the recommendations. Clara van Karnebeek, Sylvia Stockler, Sravan Jaggumantri, Birgit Assmann, Peter Baxter, Daniela Buhas, Levinus A. Bok, Barbara Cheng, Curtis R. Coughlin II, Anibh M. Das, Alette Giezen, Wahla Al-Hertani, Gloria Ho, Uta Meyer, Philippa Mills, Barbara Plecko, Eduard Struys, Keiko Ueda, Monique Albersen, Nanda Verhoeven, Sidney M. Gospe Jr., Renata C. Gallagher, Johan Van Hove, Hans Hartmann declare that they have no (other) conflict interest to declare.

Informed Consent

Not applicable

Animal Rights

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

Details of Contributions of Individual Authors' Contributions

Clara van Karnebeek and Hans Hartmann performed the literature review, led the consensus meetings, and drafted the recommendations, revising it according to the author group's input. Sylvia Stockler, Sravan Jaggumantri, Barbara Plecko, Sidney Gospe, Renata Gallagher, and Johan van Hove provided critical input for the content and format of the recommendations at each stage, and drafted individual parts of the manuscript. Peter Baxter, Daniela Buhas, Levinus A. Bok, Barbara Cheng, Curtis R. Coughlin II, Anibh M. Das, Alette Giezen, Wahla Al-Hertani, Gloria Ho, Uta Meyer, Philippa Mills, Barbara Plecko, Eduard Struys, Keiko Ueda, Monique Albersen, Nanda Verhoeven provided input for the recommendations specific to their field (pediatric neurology, metabolic diseases, laboratory biochemical genetics, dietetics, and nutrition), both at a clinical and scientific level. All authors are active members of the PDE Consortium and reviewed/edited the manuscript.

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RESEARCH REPORT

Mortality in Patients with Morquio Syndrome A

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Abstract *Background*: Morquio syndrome A (mucopolysaccharidosis type IVA) is an autosomal recessive, life-limiting lysosomal storage disease characterized by deficient activity of the enzyme galactosamine-6-sulfatase. The disease affects multiple body systems, and patients require multidisciplinary care from an early age.

Methods: To better understand the natural progression of the disease, life expectancy and common causes of death, death certificates were evaluated for 27 patients (15 male, 12 female) with Morquio syndrome A in the UK, covering the years 1975–2010.

Results: Mean age at death (±standard deviation) was 25.30 ± 17.43 years, with female patients living longer than male patients (26.55 ± 12.28 years versus 22.95 ± 17.63 years, respectively). Respiratory failure was the primary cause of death in nearly two-thirds of patients (63%). Other causes of death were cardiac failure (11%), post-traumatic organ failure (11%), complications of surgery (11%) and myocardial infarction (4%). Life expectancy increased gradually over time ($R^2 = 0.0963$), and mean age at death due to respiratory failure improved from 17.42 ± 9.54 years in the 1980s to 30.74 ± 10.84 years in the 2000s.

Conclusions: The current data suggest that survival of patients with Morquio syndrome A in the UK has improved in recent decades. It is possible that improvements in multidisciplinary care and referral of patients to specialist centres underlie this trend. It is hoped that novel disease-specific treatments such as enzyme replacement therapy and haematopoietic stem cell therapy will help to extend the lifespan of patients with Morquio syndrome further still.

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Introduction

Morquio syndrome A (mucopolysaccharidosis type IVA, MPS IVA; OMIM 253000) is an autosomal recessive lysosomal storage disease characterized by deficient activity of the enzyme galactosamine-6-sulfatase (GALNS). The absence of GALNS activity results in impaired catabolism of two glycosaminoglycans (GAGs), keratin sulphate and chondroitin-6-sulphate (Dorfman et al. 1976; Glössl and Kresse 1982). The progressive accumulation of GAGs in various tissues means that the disease affects multiple body systems. Short stature and skeletal dysplasia are observed in most patients (Wraith 1995), with bone deformity as the most common initial symptom (Montaño et al. 2007). The digestive, cardiovascular and respiratory systems, and visual and auditory function may also be affected (Northover et al. 1996).

The incidence of Morquio syndrome A is estimated to be between 1 in 76,000 and 1 in 450,000 in Europe, and 1 in 200,000 in the USA and Canada (Nelson 1997; Poorthuis et al. 1999; Applegarth et al. 2000). Clinical presentation of the disease ranges from a severe, rapidly progressing form (which represents the classical description of this disorder) to a phenotype that evolves more slowly. Onset of disease symptoms commonly occurs before the age of 1 year in

patients with a severe phenotype or as late as the second decade of life in patients with the slowly progressing form of the disease (Montaño et al. 2007).

Cardiac valve disease and respiratory complications leading to limitations in endurance are common in patients with Morquio syndrome A from late childhood (John et al. 1990; Hendriksz et al. 2013), and both are associated with disease involvement in multiple body systems (Hendriksz et al. 2013). GAG accumulation in the upper airways and tonsils predisposes the patient to the development of obstructive sleep apnoea and upper airway obstruction (Walker et al. 2003; Montaño et al. 2007). Respiratory function is further compromised by chest wall deformities and displacement of the diaphragm due to short stature coupled with hepatosplenomegaly (Hendriksz et al. 2013). Atlantoaxial instability and spinal cord compression may also result in respiratory muscle weakness (Tomatsu et al. 2011; Hendriksz et al. 2013). Owing to these changes, patients with Morquio syndrome A may experience recurrent infections, progressive loss of pulmonary function and, ultimately, respiratory failure (Montaño et al. 2007; Pelley et al. 2007; Tomatsu et al. 2011; Hendriksz et al. 2013). Although the central nervous system is not impacted directly by GAG accumulation (Wraith 1995), patients with Morquio syndrome A have a high risk of developing neurological complications owing to skeletal abnormalities (Nelson and Thomas 1988).

Patients with Morquio syndrome A require multidisciplinary care from primary care physicians, orthopaedic surgeons, pulmonologists, cardiologists and anaesthesiologists (Algahim and Almassi 2013). Multiple interventions are required to maintain optimal respiratory function, and ongoing management of skeletal manifestations and the associated neurological complications is critically important (Hendriksz et al. 2013). At present, there is no disease-specific treatment for Morquio syndrome A, although enzyme replacement therapy (ERT) is in development (Algahim and Almassi 2013). Owing to a limited effect on skeletal manifestations of other MPS diseases, haematopoietic stem cell therapy (HSCT) is not recommended for patients with Morquio syndrome A (Peters and Steward 2003).

To be able to optimize current management and evaluate the effectiveness of novel treatments for Morquio syndrome A, it is necessary to understand the natural progression of the disease, life expectancy and common causes of death. Here, we analyse survival and causes of death in patients with Morquio syndrome A, and how these have changed in recent decades, using data collected by the Society for Mucopolysaccharide Diseases (UK). These data will be of interest to clinicians, healthcare authorities and commissioning bodies, as well as to patients, their families and patient societies.

Methods

The Society for Mucopolysaccharide Diseases made available death certificates of all deceased patients with Morquio syndrome A held in its database. The death certificates provided information on date of birth, gender, date of death and primary cause of death. The society has aimed to collect data on every patient with Morquio syndrome A in the UK. Although the number of patients missing from the database is unknown, it is estimated by the collators to be very few because most, if not all, individuals with Morquio syndrome A are treated at a small number of designated centres.

Results

Patient Characteristics

Death certificates were available for 27 patients (15 male, 12 female) with Morquio syndrome A, covering the years 1975–2010 (Table 1).

Mean Age at Death and Primary Cause of Death

Mean age at death (± standard deviation [SD]) was 25.03 ± 17.43 years. In general, female patients tended to live slightly longer than male patients (mean age at death [±SD], 26.55 ± 12.28 years versus 22.95 ± 17.63 years, respectively), but the difference was not significant. Respiratory failure was the primary cause of death in nearly two-thirds of patients (Fig. 1a). Other reported causes of death were cardiac failure, post-traumatic organ failure, complications of surgery and myocardial infarction. Although respiratory failure was proportionally a more common cause of death in male patients than in female patients (67% versus 59%, respectively), this difference was not considered significant, nor were there other numerical differences in cause of death between the genders (Fig. 1b, c).

Differences by Age Group

There was a gradual increase in the proportion of deaths caused by respiratory failure with each decade of life (Fig. 2). A high proportion of fatalities in patients younger than 10 years were attributed to post-traumatic organ failure, suggesting that accidental deaths were more common in children than in older individuals. When death owing to post-traumatic organ failure was excluded from the analysis, mean age at death (±SD) increased to 27.73 ± 16.95 years, with females living longer than male patients (mean age at death [±SD], 32.54 ± 12.68 years

Table 1 Patient characteristics

Patient (gender)	Date of death	Age at death (years)	Primary cause of death	Additional information
1 (M)	07/02/1975	4.00	Post-traumatic organ failure, pneumonia and lung collapse	Fell from swing, head injury
2 (F)	21/05/1986	26.96	Respiratory failure – bronchopneumonia	
3 (M)	28/05/1987	7.88	Respiratory failure	Tracheomalacia
4 (M)	27/02/1989	3.08	Complication of surgery – anoxic brain damage post-surgery	Cervical surgery
5 (F)	26/10/1989	15.16	Complication of surgery – cerebral haemorrhage	Mitral valve replacement
6 (F)	18/10/1990	10.24	Post-traumatic organ failure – cervical stenosis	Post-mortem brain stem compression, atlantoaxial subluxation
7 (M)	28/03/1992	75.32	Respiratory failure – bronchopneumonia	
8 (M)	13/04/1992	22.56	Respiratory failure – bronchial asthma	
9 (M)	21/08/1993	6.24	Respiratory failure	Cervical stenosis
10 (M)	22/10/1993	10.08	Respiratory failure	
11 (F)	27/09/1995	22.48	Respiratory failure	
12 (F)	01/04/1996	46.24	Respiratory failure	
13 (F)	23/03/1996	48.56	Respiratory failure – bronchopneumonia	
14 (M)	20/05/1997	14.16	Respiratory failure	Bronchopneumonia, obstructive airway disease
15 (M)	12/03/1998	20.80	Cardiac failure	Aortic valve disease, hypoplastic lung, trachea abnormality seen at post-mortem
16 (M)	13/01/1999	33.64	Respiratory failure	
17 (M)	17/02/1999	18.56	Cardiac failure	
18 (F)	17/06/2000	52.48	Cardiac failure – acute left heart failure	
19 (M)	22/04/2001	42.80	Respiratory failure	
20 (F)	27/05/2002	36.88	Myocardial infarction	Chest infection
21 (F)	02/06/2003	3.16	Post-traumatic organ failure – cervical compression after trauma	Fell at school after a seizure
22 (F)	28/01/2005	28.16	Respiratory failure – chest infection (bronchopneumonia)	
23 (M)	08/03/2005	20.08	Complication of surgery – aspiration pneumonia	Gastrointestinal haemorrhage, oesophageal tear, post-mortem outcome was misadventure
24 (F)	01/04/2005	15.40	Respiratory failure	
25 (M)	03/12/2005	20.16	Respiratory failure	
26 (M)	24/07/2008	44.88	Respiratory failure – bronchopneumonia, sepsis	Community-acquired pneumonia
27 (F)	03/10/2010	33.06	Respiratory failure – bronchopneumonia	

versus 24.30 ± 18.69 years, respectively). When patients younger than 10 years were excluded from the overall analysis, the mean age at death (±SD) changed to 29.94 ± 16.00 years, with female patients living slightly longer than male patients (mean age at death [±SD], 30.51 ± 13.69 years versus 29.37 ± 17.99 years, respectively).

Changes in Longevity Over Time

An analysis of longevity showed a weak trend over time towards gradual improvement in life expectancy in patients with Morquio syndrome A ($R^2 = 0.0963$; Fig. 3a, b). Importantly, recent deaths due to respiratory failure appear to have occurred later in life than deaths in earlier times,

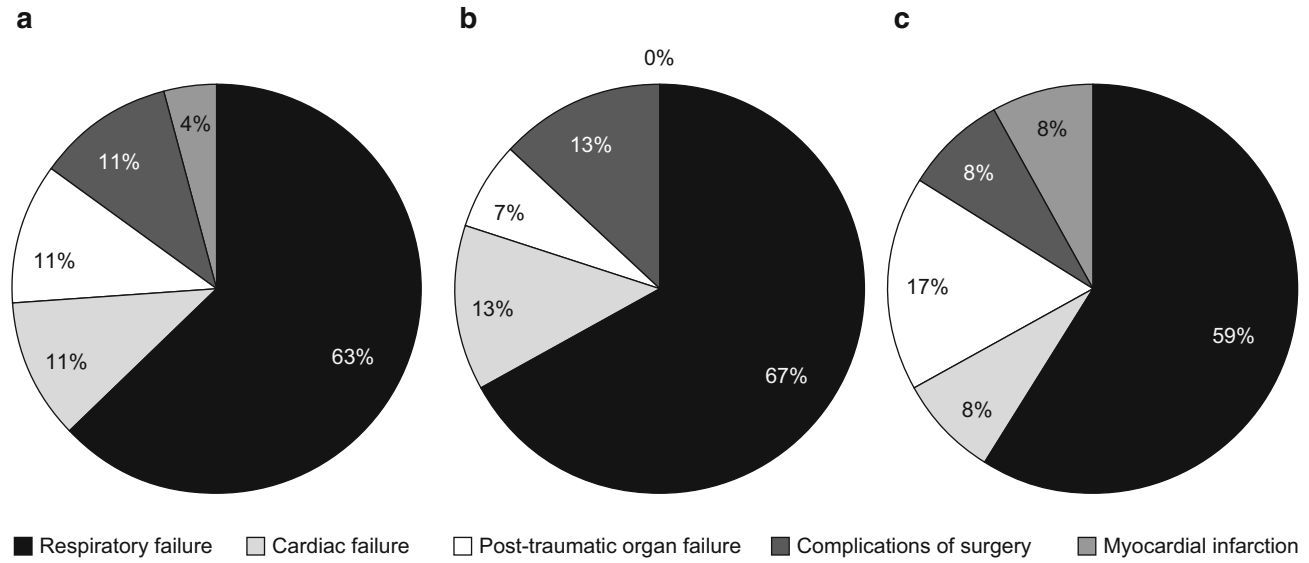

Fig. 1 Primary cause of death in patients with Morquio syndrome A. (**a**) All patients ($n = 27$); (**b**) male patients only ($n = 15$); (**c**) female patients only ($n = 12$)

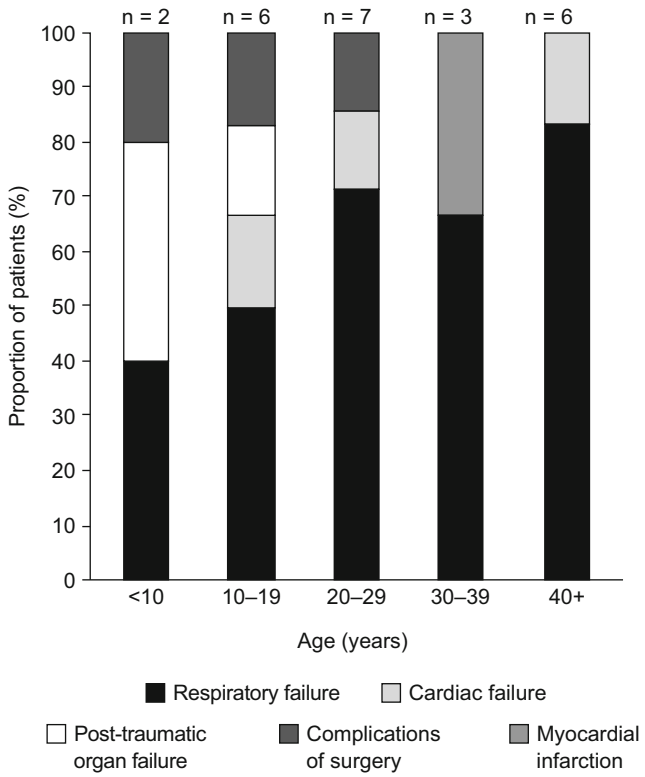

Fig. 2 Primary cause of death in patients with Morquio syndrome A, stratified by age group

with mean age at death improving from 17.42 ± 9.54 years in the 1980s to 30.74 ± 10.84 years in the 2000s (Fig. 3c).

One of the greatest advances in the management of patients with Morquio syndrome A has been the implementation of routine prophylactic management of the spinal stenosis and instability in 1990. To evaluate the impact of this development on patient survival, an analysis of longevity and cause of death before 1990 and from 1990 onwards was carried out. Age at death was found to be greater in patients who died from 1990 onwards than in those who died before 1990 (Fig. 4a), although this difference was observed primarily in males. Respiratory

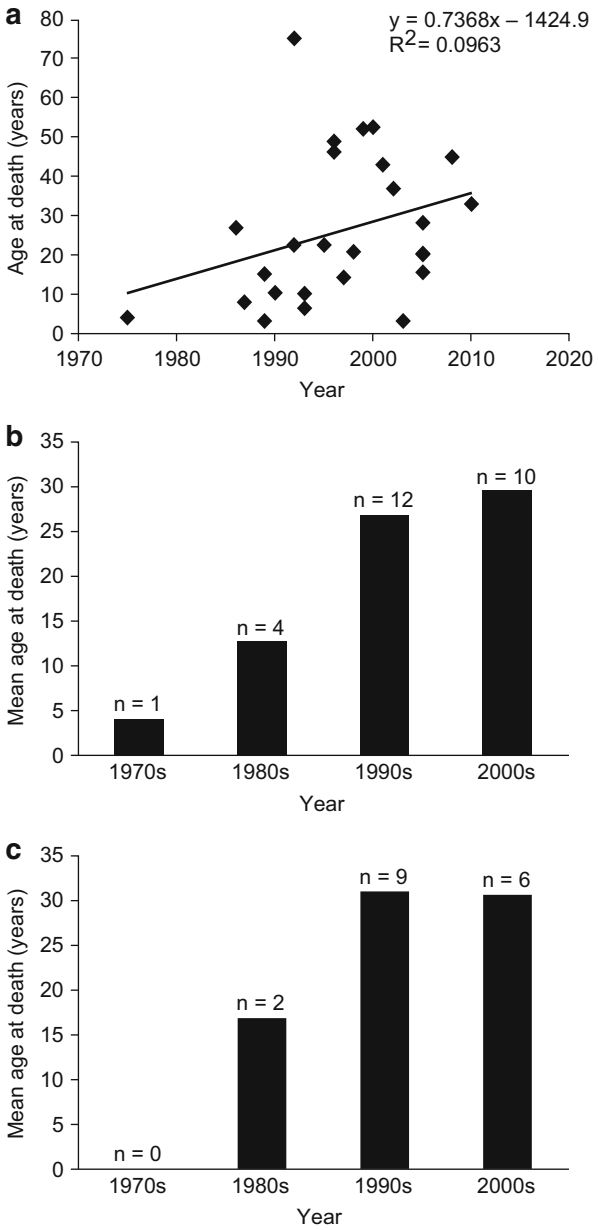

Fig. 3 Longevity in patients with Morquio syndrome A between 1975 and 2010. (**a**) Age at death by individual patient; (**b**) mean age at death over time; (**c**) mean age at death due to respiratory failure

failure was found to be a major cause of death both before 1990 and from 1990 onwards (Fig. 4b). Although death due to complications of surgery appears to have been more common before 1990, the dataset is too small to allow any strong association.

Discussion

This mortality analysis has found that survival in patients with Morquio syndrome A appears to have improved gradually over the past three decades. Although the dataset is too small to allow any strong assertions, it is possible that efforts to improve disease management and multidisciplinary care, and to refer patients to specialist centres, are being reflected in extended lifespans for individuals with this life-limiting condition.

Respiratory failure remains the primary cause of death in patients with Morquio syndrome A; however, the current data suggest that this occurs later in recent years than previously. One reason for this may be advances in pulmonary care for these patients, including regular immunizations, prompt and aggressive treatment of respiratory tract infections, prevention of weight gain and

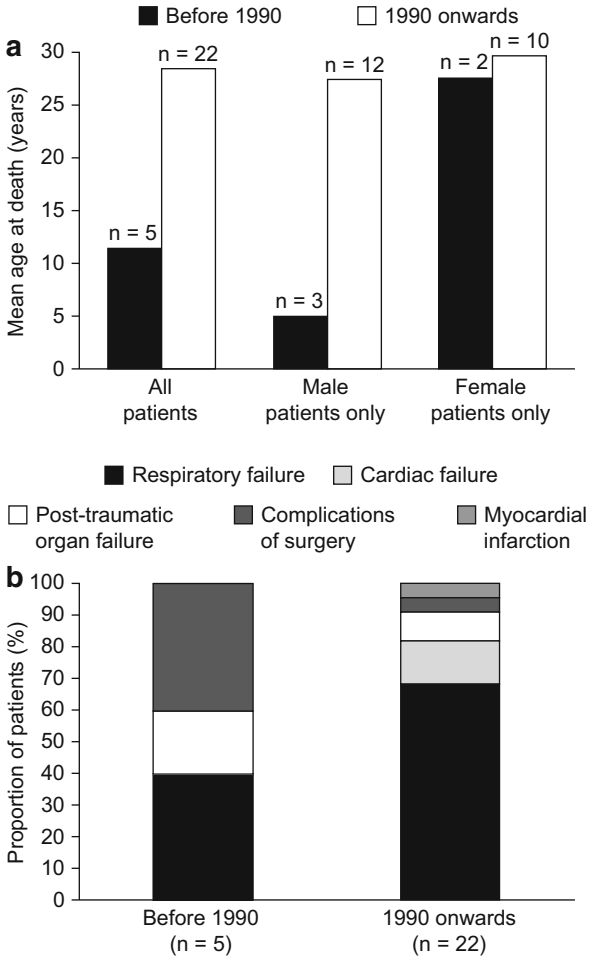

Fig. 4 (**a**) Longevity and (**b**) primary cause of death in patients with Morquio syndrome A before 1990 and from 1990 onwards

availability of respiratory support (Hendriksz et al. 2013). In addition, improvements in prophylactic neck management in the last two decades may have helped to prevent respiratory compromise due to atlantoaxial instability and spinal cord compression. The introduction of C1/C2 cervical fusion to improve neck stability in the mid-1980s is particularly notable. This was driven by the patient organization investing in expertise and inviting world leaders to collaborate and proactively manage this known risk for the cohort. Once diagnosed, patients now undergo regular assessment to evaluate spinal involvement and monitor its progression. It is recommended that patients undergo a neurological examination at least every 6 months, with annual magnetic resonance imaging and radiological examination every 2–3 years (Solanki et al. 2013). Pathological changes observed during any of these evaluations may indicate a need for surgical intervention (Solanki et al. 2013).

Complications of surgery were reported as a cause of death in three patients, one in each of the first three decades of life. It is reassuring that the incidence of fatal complications of surgery is low, although the fact that two of the deaths were probably related to intubation or extubation (patients 4 and 22) highlights the risks associated with general anaesthesia in patients with Morquio syndrome A. Most anaesthetists are not familiar with the issues associated with surgery in individuals with Morquio syndrome A, so it is recommended that these patients should be referred to specialist centres with an experienced paediatric anaesthetist supported by a multidisciplinary team practised in the perioperative management of mucopolysaccharide (MPS) diseases (Walker et al. 2013). In general, the key to successful anaesthesia in patients with MPS diseases is planning, with a thorough preoperative evaluation of anaesthetic risk factors in consultation with a multidisciplinary team (Walker et al. 2013). Assessment of existing respiratory and cardiac manifestations, as well as cervical and tracheolaryngeal anatomy, may help the anaesthetist to anticipate potential problems that may arise during the procedure, such as difficult intubation and ventilation, and cardiac and cervical spine issues. Consideration should also be given to monitoring and postoperative care.

It is notable that the accidental deaths associated with trauma all occurred during the first two decades of life. This is perhaps to be expected, because children and adolescents tend to be more susceptible to falls and accidents than adults. The fact that only one death in this age group was attributed to cardiac failure may also indicate that the cardiovascular complications associated with Morquio syndrome A have yet to become life-threatening in this age group, particularly as cardiac failure is likely to develop secondary to respiratory impairment and loss of mobility due to skeletal and respiratory complications.

There is an opportunity for novel treatment options such as ERT to slow or halt the development of skeletal, respiratory and cardiac complications by preventing the accumulation of GAGs in bodily tissue. It is too early to determine what effect ERT will have on the somatic manifestations of Morquio syndrome A, but functional endpoints (such as the 6-minute walk test and forced vital capacity) will be useful in making these assessments, especially if they could be linked to patient-reported outcomes or quality of life measures.

The current data are based entirely on death certificates from patients with Morquio syndrome A, supplemented with limited information from their families. Although death certificates provide a useful source of information regarding mortality rates and causes of death in patients with rare diseases, a limitation of this study is the absence of clinical notes for each of the patients. Anecdotal reports indicate that all except two patients in the current dataset (patients 2 and 7) had short stature and experienced severe signs and symptoms of Morquio syndrome A. However, there is no information available about each patient's functional ability and the presence or absence of comorbidities, which may have provided useful context for the trends observed here. For example, it is unclear why the mean age of mortality in boys with Morquio syndrome A is so dramatically different before and after 1990. It can be speculated that this may simply be an anomaly owing to small patient numbers, but without further clinical information, no firm conclusion can be made.

In conclusion, the findings of this study suggest that survival in patients with Morquio syndrome A in the UK has improved in recent decades. It would be of interest to assess whether a similar trend has occurred in other countries. In addition, it would be of interest to evaluate whether changes in multidisciplinary care have led to improvements in patient and carer quality of life. Although quality of life will inevitably decline as the disease progresses, steps can be taken to keep patients active, pain-free and independent for as long as possible. It is hoped that as experience of caring of individuals with Morquio syndrome A increases, this will be reflected in improvements in outcomes for patients and their carers.

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Take-Home message

Evaluation of death certificates from patients with Morquio syndrome A in the UK has shown that survival in this population has improved in recent decades.

Compliance with Ethics Guidelines

Conflicts of Interest

C. Lavery serves on advisory boards for BioMarin Pharmaceutical Inc. and has received travel grants and lecture fees from BioMarin Pharmaceutical Inc.

C. Hendriksz is a consultant and chair of advisory boards for BioMarin Pharmaceutical Inc. and has received travel grants and lecture fees from BioMarin Pharmaceutical Inc.

Informed Consent

Informed consent was not required for this study. This article does not report on any studies with human or animal subjects performed by any of the authors.

Author Contributions

C. Lavery conceived the idea for the study, collected death certificates and contributed to the development of the manuscript.

C. Hendriksz analysed the study data and contributed to the development of the manuscript.

Both authors have seen and approved the final manuscript.

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CASE REPORT

Neurogenic Bladder Dysfunction Presenting as Urinary Retention in Neuronopathic Gaucher Disease

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Abstract Neuronopathic Gaucher disease can present as a continuum of clinical findings, including somatic symptoms of anemia, thrombocytopenia, hepatosplenomegaly, and bone disease as well as neurologic sequelae. There is a spectrum of neurologic symptoms ranging from oculomotor apraxia to severe convulsions. The heterozygosity of phenotypes makes it difficult to predict the disease course. We describe an 8-year-old male with neuronopathic type III Gaucher disease who developed bladder dysfunction and was unable to completely void. He also presented with hypertension and acute renal insufficiency, most likely secondary to urinary retention. A complete evaluation was done for causes of urinary retention and bladder dysfunction. A renal bladder ultrasound demonstrated marked hydroureteronephrosis. There was no clinical evidence of infection and cystoscopy revealed no anatomic obstruction. In addition, MRI showed no spinal abnormalities. His bladder dysfunction was managed operatively by creating a catheterizable stoma, using his appendix, to empty his bladder, and surgical findings were consistent with neurogenic bladder. He continues to be managed for his Gaucher disease and neurogenic bladder by genetics, nephrology and urology. This is the first clinical report of neurogenic bladder dysfunction in neuronopathic Gaucher disease.

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Introduction

Gaucher disease is an autosomal recessive lysosomal storage disease caused by a deficiency of the enzyme glucocerebrosidase. The accumulation of glucosylceramide within the macrophage-monocyte system causes the signs and symptoms of this disease (Grabowski 2008). Gaucher disease presents as a continuum of clinical findings; patients with no primary central nervous system disease are classified as type I/non-neuronopathic, and patients with both somatic symptoms and primary neurologic disease are classified as types II and type III, neuronopathic, with type II being more severe.

The neuronopathic subtypes occur in less than 1/100,000 births and are characterized by both somatic symptoms of anemia, thrombocytopenia, fatigue, hepatosplenomegaly, and bone disease and neurologic sequelae. Neuronopathic symptoms may be limited to oculomotor apraxia but can also manifest with progressive myoclonic epilepsy, cognitive impairment, bulbar signs, and pyramidal signs (Pastores and Hughes 2000). While there have been reports of urinary retention and neurogenic bladder in other lysosomal storage disorders, there have been no such reports to date in non-neuronopathic or neuronopathic Gaucher disease (Koyama et al. 1994; Gografe et al. 2009).

Herein we present a case of a young boy with neuronopathic Gaucher disease who developed chronic urinary retention requiring surgical intervention.

Case Presentation

An 8-year-old African-American male with Gaucher disease presented to the pediatric urology clinic with three-week history of dysuria, urinary urgency, frequency, dribbling, and incontinence. He had several emergency room (ER) visits with normal urinalyses, renal/bladder sonogram, and voiding cystourethrogram. He was thought to be in urinary retention and had been catheterized in the ER for 250 cc of urine (expected bladder capacity for age = 300 cc) and was discharged home with terazosin, an alpha-blocker to potentially relax the internal urinary sphincter, and phenazopyridine, a urinary analgesic. He was able to void in clinic, but sonogram revealed a post-void residual of at least 185 cc.

He had been diagnosed with neuronopathic Gaucher disease at 26 months of age. At 27 months he began enzyme replacement therapy (ERT) with IV Cerezyme (imiglucerase injection) at a dose of 60 units/kg every 2 weeks. Given both his clinical presentation and genotype (L444P/L444P), he was classified as having neuronopathic disease. As suggested by preliminary data outlined in a 2001 European consensus statement for management of neuronopathic disease indicating that "high-dose" ERT could perhaps reverse, stabilize, or slow neurologic involvement, his dose was increased to 120 units/kg every 2 weeks before the age of 33 months (Vellodi et al. 2001). The family has been highly compliant with every other week infusions since his diagnosis. His infusion dose has been adjusted for growth over time and has remained above 60 units/kg/infusion as is advised by the revised management guidelines for neuronopathic disease published in 2009 (Vellodi et al. 2009).

Despite ongoing treatment of his Gaucher disease, before the age of 8 years, he demonstrated multiple clinical symptoms of neuronopathic disease. These symptoms include:

- History of swallowing difficulties, abnormal swallowing studies, and aspiration
- Abnormal EEG consistent with a nonspecific diffuse brain dysfunction/encephalopathy
- Abnormal BAER consistent with severe diffuse brainstem conduction delay and mild conductive hearing loss
- Two abnormal sleep studies demonstrating obstructive events with accompanying O2 desaturations
- Significant esotropia
- Developmental delay

After the initial urologic evaluation, the dose of terazosin was increased from 1 to 2 mg. Over the next 4 weeks, he was noted to have worsening constipation, which was treated, and the urinary symptoms were mildly improved without further episodes of retention.

Two weeks later, he returned to the clinic with increasing abdominal pain and was found to have a markedly distended bladder and BP of 138/118 mmHg. The patient had no other somatic complaints at this time. A renal bladder ultrasound showed bilateral moderate hydroureteronephrosis as well as a distended bladder with thickened wall consistent with functional or anatomic bladder outlet obstruction (Fig. 1). Serum chemistry was normal except for an elevated creatinine of 1.5 mg/dL. He was catheterized for a volume of 500 cc of urine; however, the hydronephrosis and hypertension did not resolve. He was, therefore, admitted for management of hypertension, acute renal insufficiency, and urinary retention. Serum creatinine returned to normal baseline value with continuous bladder drainage, and blood pressure normalized with IV and then oral medication. Attempts were made to initiate clean intermittent catheterization, but he was unable to tolerate catheterization due to normal urethral sensation. He was taken to surgery for further evaluation. Cystourethroscopy did not show posterior urethral valves or any other obstructive lesions. The bladder was noted to be normal except for signs of chronic distention. A suprapubic catheter was placed to allow regular and complete bladder emptying without the discomfort of a urethral catheter. A total spine MRI was obtained and revealed no abnormalities.

The patient returned to the ER 2 weeks after discharge with the suprapubic catheter dislodged and unable to void. Due to the persistence of urinary retention, the family elected to proceed with a more permanent intervention. Urodynamics were not performed at this time because he would not tolerate urethral catheterization and results were unlikely to affect management. An appendicovesicostomy (Mitrofanoff procedure) was performed to create a continent catheterizable channel from the umbilicus to the bladder using the appendix. The bladder was noted to be markedly thickened, consistent with a neurogenic bladder. The patient did well after surgery and was discharged home with normal renal function.

Follow-up renal bladder ultrasound 6 months postoperatively demonstrated complete resolution of hydroureteronephrosis. Urodynamic evaluation at 8 months postoperatively demonstrated normal bladder compliance and capacity (300 cc) with no detrusor overactivity. Bladder sensation was noted only at capacity. He was unable to void during the study.

Currently now 22 months after surgery, he remains on low amlodipine for hypertension. He has no proteinuria. His constipation is managed with polyethylene glycol 3350

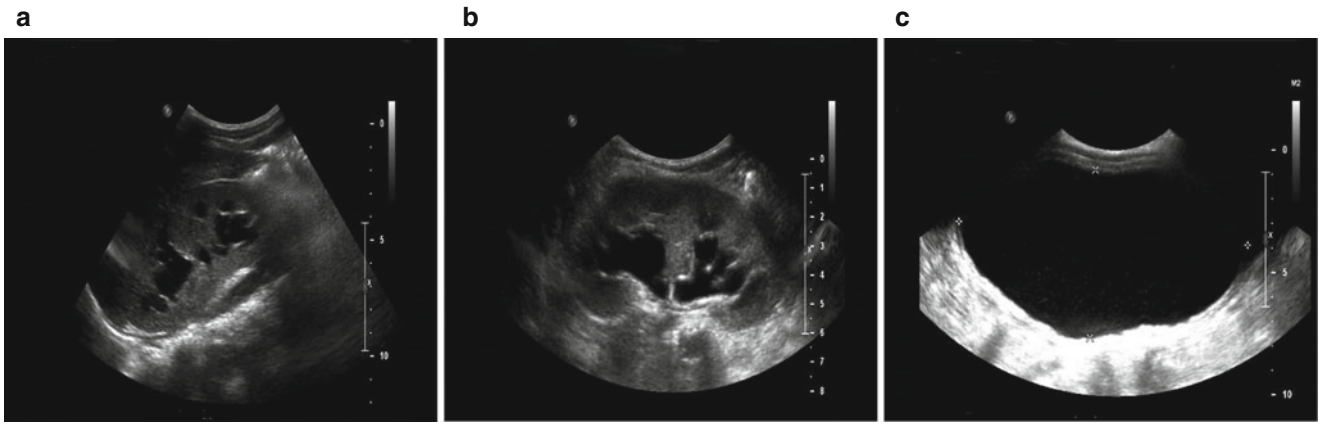

Fig. 1 (**a**) Right Kidney, long. (**b**) Left Kidney, prone. (**c**) Bladder, long

as needed. He has had only one documented urinary tract infection. He continues to catheterize himself independently with a 12 French catheter without supervision and is completely dry during the day with only rare nocturnal enuresis. He can void spontaneously, but his elevated post-void residual urine volume mandates ongoing intermittent bladder catheterization twice daily.

While there continues to be some concern regarding neuronopathic symptoms, recent evaluation demonstrated moderate oropharyngeal dysphagia; his somatic disease appears to be well controlled. On his most recent physical examination with the primary specialist following his diagnosis of Gaucher disease, his spleen was not palpable and his liver was just palpable below the right costal margin. The family has denied any bone fractures, pain, or crisis, and his laboratory parameters demonstrate normal hemoglobin and platelet levels at all evaluated time points since his surgery. Biomarkers used for monitoring disease progression and response to therapy (tartrate-resistant acid phosphatase, angiotensin-converting enzyme, and chitotriosidase) have been stable over this time period excepting one increase chitotriosidase level at a semi-annual evaluation. After dose adjustment of ERT for growth/weight gain initiated at that visit, a recheck one month later showed decease in chitotriosidase trending in the direction of his previous baseline.

Discussion

This is the first case report of chronic urinary retention and neurogenic bladder in a patient with neuronopathic Gaucher disease. The bladder dysfunction led to acute renal insufficiency that would have been progressive without definitive urologic management. There was no evidence for spinal cord abnormalities on the MRI in our patient; however, this patient experienced severe bladder and bowel dysfunction that led to acute renal insufficiency and hypertension, requiring a permanent surgical intervention to address the bladder issues. While constipation is often linked to acute urinary retention, the urinary retention remained an issue after treatment of constipation, and the intraoperative findings of a thickened bladder wall were consistent with neurogenic bladder.

There are no reports of urinary sequelae of Gaucher disease, specifically the neuronopathic type. A February 2011 query to the International Collaborative Gaucher Group (ICGC) Gaucher registry identified no other reported cases of patients with neuronopathic Gaucher disease who also had symptoms of urinary retention, incomplete bladder emptying, and/or neurogenic bladder. At the time of the query, 488 of 5,943 patients enrolled in the Registry were identified as type II or III Gaucher disease.

There is scant literature on lysosomal storage disease and urinary sequelae in general. One preclinical study has shown mononuclear cell infiltrate in the lower urinary tract, specifically the bladder, of a mouse mucopolysaccharidosis type IIIB model that led to urinary retention, although this has not been demonstrated in humans (Gografe et al. 2009). There is one case report of neurogenic bladder in a patient with Hunter syndrome, but this was secondary to cervical myelopathy (Koyama et al. 1994).

This patient has had good somatic response to his ongoing enzyme replacement therapy with resolution of his anemia and thrombocytopenia within 8 months of treatment initiation. However, he continues to have ongoing neuronopathic symptomatology despite high compliance with enzyme replacement therapy. We hypothesize that this is bladder autonomic dysfunction secondary to neurologic sequelae of type III Gaucher Disease. Bladder autonomic dysfunction can be seen in other neurologic diseases and can manifest as urinary retention. Gaucher disease may

affect the nerves that promote contraction of the detrusor muscle and/or relaxation of the internal (involuntary) and/or external (voluntary) sphincter(s)—simultaneous functions that are required for efficient bladder emptying—causing his neurogenic bladder.

Synopsis

A case report of neurogenic bladder that had to be managed surgically, presenting as urinary retention in an 8-year-old boy with neuronopathic Gaucher disease.

Compliance with Ethics Guidelines

Funding Source

No external funding was secured for this study.

Conflict of Interest

Dr. McNamara, Jennifer Sullivan, and Dr. Nagaraj do not have any conflict of interest, financial or otherwise, to disclose.

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John S. Wiener served as a consultant for Eli Lilly & Co. in 2013.

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

Contribution

Erin R. McNamara—responsible for analysis and interpretation of data and drafting the article or revising it critically for important intellectual content

Jennifer Sullivan—responsible for revising the article critically for important intellectual content

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Drs. John S. Wiener, Priya S. Kishnani—responsible for conception and design as well as revising the article critically for important intellectual content. Provided expertise in their respective fields of pediatrics

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RESEARCH REPORT

Common and Novel *TMEM70* Mutations in a Cohort of Italian Patients with Mitochondrial Encephalocardiomyopathy

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Abstract ATP synthase or complex V (cV) of the oxidative phosphorylation system is responsible for the production of ATP, dissipating the electrochemical gradient generated by the mitochondrial respiratory chain. In addition to maternally transmitted cV dysfunction caused by mutations in mtDNA genes (*MT-ATP6* or *MT-ATP8*), encoding cV subunits, recessive mutations in the nuclear *TMEM70* are the most frequent cause of ATP synthase deficiency.

We report on a cohort of ten Italian patients presenting with neonatal lactic acidosis, respiratory distress, hypotonia, cardiomyopathy and psychomotor delay and harbouring mutations in *TMEM70*, including the common splice mutation and four novel variants. TMEM70 protein was virtually absent in all tested *TMEM70* patients' specimens.

The exact function of TMEM70 is not known, but it is considered to impact on cV assembly since *TMEM70* mutations have been associated with isolated cV activity reduction. We detected a clear cV biochemical defect in *TMEM70* patients' fibroblasts, whereas the assay was not reliable in frozen muscle. Nevertheless, the evaluation of the amount of holocomplexes in patients with *TMEM70* mutations showed a nearly absent cV in muscles

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and a strong decrease of cV with accumulation of sub-assembly species in fibroblasts. In our cohort we found not only cV deficiencies but also impairment of other OXPHOS complexes. By ultrastructural analysis of muscle tissue from one patient with isolated cV deficiency, we found a severely impaired mitochondrial morphology with loss of the cristae. These findings indicate that cV impairment could indirectly alter other respiratory chain complex activities by disrupting the mitochondrial cristae structure.

Introduction

The mitochondrial oxidative phosphorylation (OXPHOS) system consists of five multi-subunit complexes, acting in concert to generate energy in the form of ATP molecules. Genetic OXPHOS disorders result from mutations in either mitochondrial DNA (mtDNA) or nuclear genes encoding structural subunits of the OXPHOS complexes or factors involved in their synthesis, assembly and function. Complex V or mitochondrial ATP synthase consists of 16 different polypeptides, two of which, ATPase 6 and ATPase 8, being encoded by mtDNA (Holt et al. 1990; Rahman et al. 1996). Most isolated cases of ATP synthase deficiency are caused by mutations in the mitochondrial genes *MT-ATP6* (MIM516060) and *MT-ATP8* (MIM516070) and are associated with different clinical phenotypes, including maternally inherited Leigh syndrome (MILS) (Rahman et al. 1996), adult-onset NARP (neuropathy, ataxia and retinitis pigmentosa), Leber hereditary optic neuropathy and hypertrophic cardiomyopathy. Disease-causing mutations in patients with isolated cV deficiency have been identified in only four nuclear genes, two encoding assembly factors: *ATPAF2* and *TMEM70*, and two structural subunits—*ATP5E* encoding the epsilon subunit of the F1 domain (Mayr et al. 2010) and *ATP5A1* encoding the alpha subunit of the F1 complex (Jonckheere et al. 2013). Whilst mutations in *ATPAF2* (MIM604273), *ATP5A1* (MIM615228) and *ATP5E* (MIM614053) have been detected in single patients, *TMEM70* (MIM614052) mutations are a frequent cause of autosomal recessive ATP synthase deficiency, usually associated with a distinctive phenotype consisting of neonatal-onset hypertrophic cardiomyopathy, facial dysmorphisms, severe lactic acidosis and 3-methylglutaconic aciduria (3-MGA) (CízkovÄ et al. 2008). Several patients share a common Roma descent, being homozygous for a founder c.317-2A>G mutation which causes aberrant splicing with marked loss of *TMEM70* transcript (Honzík et al. 2010). Few other mutations have been described, mainly in Arab-Muslim families (Spiegel et al. 2011).

Herein, we report the clinical, biochemical and genetic characteristics of ten patients presenting with neonatal severe hypertrophic cardiomyopathy and carrying mutations in *TMEM70*, including four novel changes.

Patients and Methods

Patients

Written informed consent was obtained from patients' parents, in agreement with the Declaration of Helsinki upon approval of the Ethical Committees of the Foundation IRCCS Istituto Neurologico "C.Besta", Milan, Italy.

A total of ten patients (five boys and five girls) from eight apparently unrelated families were examined. The clinical characteristics and biochemical investigations carried out in the patients are summarized in Table 1. All our patients showed hypertrophic cardiomyopathy as the main clinical feature, associated with high lactate levels, psychomotor delay, respiratory distress and muscular hypotonia. All showed intrauterine growth restriction (IUGR) and most of them had an emergency delivery because of oligohydramnios, indicating antenatal disease onset. Dysmorphic features were observed in all subjects of our cohort; none of the boys presented hypospadias, often reported in *TMEM70*-mutant patients (Spiegel et al. 2011; Torraco et al. 2012). One patient (P3) showed subacute intestinal obstruction, as reported elsewhere (Spiegel et al. 2011). Although there was no consistent brain MRI pattern, lesions included bulbar and cerebellar atrophy (P1, P2), pseudocysts in frontal (P3) or occipital lobe (P1), signs of incomplete brain development (P5, P6 and P7) and in one case a severe haemorrhagic lesion in the parieto-occipital lobe (P8). Six patients died during the first year of life because of respiratory distress consequent to cardiomyopathy. Four cases are still alive, with the oldest patient, P1, being 9 years old. Biomarkers in body fluids reported in *TMEM70*-mutant cases, such as 3-MGA, organic acids in urine and hyperammonaemia (Wortmann et al. 2009; Honzik et al. 2012; Wortmann et al. 2013), were detected in some, but not all, of our patients (Table 1).

Biochemistry and Morphology

Muscle and skin biopsies were performed after receiving written informed consent. Muscle morphology and histochemistry were performed as previously described (Dubowitz 1985) in all patients but P2, P8 and P9. Electron microscopy of muscle tissue from P3 was performed as described (Jonckheere et al. 2011).

Fibroblasts were grown in 35 mm imaging dishes (iBIDI, Thistle Scientific) to 50–70% confluence. Cells

Table 1 Clinical, genetic and biochemical features of individuals with *TMEM70* mutations

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Origin	Italian	Italian	Italian	Roma	Macedonian	Roma	Italian	Italian	Italian	Italian
Consanguinity	No	No	No	Yes	No	Yes	No	No	No	No
Sex	F	F	F	F	M	M	M	M	F	M
Onset	2 years	1 year	8 months	Neonatal	Neonatal	Neonatal	Neonatal	Birth	Prenatal	Prenatal
Age/age of death	Alive (9 years)	Died (1 year)	Alive (3 years)	Died (2 years)	Alive (4 years)	Died (4 years)	Alive (1 years)	Died (1 months)	Died (48 h)	Died (3 months)
Delivery weeks	37	40	33	35 + 6	39	36	36 + 6	33	37	34
Birth weight	Reduced	2,460 g	Reduced	1,680 g	2,620 g	2,000 g	2,140 g	1,975 g	2,650 g	1,855 g
Oligohydramnios	Yes	Yes	Yes	No	Yes	N.a.	No	Yes	Yes	Yes
IUGR	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Apnoea/distress	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Hypotonia	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	N.a.	Yes
Hypertrophic cardiomyopathy	Yes	Yes	Yes	Yes (NCC)	Yes	Yes (NCC)	Yes	Yes	Yes	Yes
Psychomotor delay	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.a.	N.a.	Yes
Dysmorphic features	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Lactic acidosis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.a.	Yes
Hyperammonaemia	Yes	Yes	No	No	No	Yes	Yes	No	No	No
3-MGA mmol/Mol creatinine	Normal	High	Normal	High	High	High	High	Normal	N.d.	Normal
Brain imaging	Occipital cysti, cerebellar hypoplasia	Bulbar hypoplasia	Frontal pseudocyst, vermis hypotrophy, WM alterations	Cerebellar hypoplasia, thin corpus callosum, lactate peak	Incomplete hippocampal inversion, WM alterations	Incomplete hippocampal inversion, WM alterations	Abnormal cortical development lactate peak, WM alterations	Haemorrhagic lesion in the parietal occipital lobe	N.d.	N.d.
Mutations	c.317-2A > G; c.349_352del	c.317-2A > G; c.317-2A > G	c.317-2A > G; c.783A > G	c.317-2A > G; c.317-2A > G	c.701A > C; c.701A > C	c.317-2A > G; c.317-2A > G	c.238C > T; c.238C > T	c.317-2A > G; c.317-2A > G	c.317-2A > G; c.317-2A > G	c.317-2A > G; c.317-2A > G
	Transcript loss[a] p.Ile117Alafs*36	Transcript loss[a]	Transcript loss[a] p.*261Trpext*17	Transcript loss[a]	p.His234Pro	Transcript loss[a]	p.Arg80*	Transcript loss[a]	Transcript loss[a]	Transcript loss*
MRC deficiency (muscle)	I (48 %); V (53 %)	N.d.	Normal	V (23 %); I (67 %); II (48 %); III (55 %)	Normal	Normal	Normal	N.d.	N.d.	I (15 %)
MRC deficiency (fibroblasts)	V (24 %)	V (28 %)	V (47 %)	V (35 %)	V (37 %)	V (17 %)	V (25 %)	N.d.	N.d.	Normal

N.d. not done/not available, *N.a.* not applicable, *IUGR* intrauterine growth restriction, 3-*MGA* 3-methylglutaconic aciduria, *WM* white matter, *MRC* mitochondrial respiratory chain, *NCC* non-compaction cardiomyopathy

[a] The intronic variant c.317-2A > G leads to aberrant splicing and loss of *TMEM70* transcript

were then incubated with complete DMEM media (Gibco) supplemented with 100 nM MitoTracker Red (Invitrogen) for 25 min at 37° C. After washing with PBS, cells were visualized with a live-imaging system (CARV-II, Crisel) and analysed using the MetaMorph Imaging System (version 7.1.2, Molecular Devices Corporation).

Respiratory chain activities of complexes I to V were measured by spectrophotometric methods (Bugiani et al. 2004) in supernatants of 800 x g muscle homogenates or digitonin-treated cultured skin fibroblasts. The ATPase activity of cV was assessed although this assay in frozen tissues is considered to be poorly reliable (Jonckheere et al. 2012) and a high oligomycin-resistant activity may interfere with the dosage in cultured cells (Barrientos et al. 2009).

Genetic Analyses

DNA was extracted from blood samples obtained from affected patients and available parents. Sequence analyses of the three *TMEM70* exons and their flanking splice junction consensus sequences were performed using the BigDye Terminator Kit (Applied Biosystems, Foster City, California, USA) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). For P10, whole-exome next-generation sequencing (WES) and variant filtering were performed as described (Haack et al. 2012). The complete sequencing of mtDNA was performed on DNA extracted from muscle homogenate, as previously described (Greaves et al. 2006).

Immunoblot Analyses

Protein gel electrophoresis (GE) and blotting analyses were performed in P1, P3 and P8 skeletal muscle, in P8 cardiac autopsy muscle and in P1, P2, P3, P4, P5 and P7 fibroblasts.

For SDS-GE, samples containing 50 μg protein were separated by 12% SDS–polyacrylamide gels, transferred to nitrocellulose membranes (Tiranti et al. 1999) and incubated with antibodies against TMEM70 (Proteintech) and GAPDH (Millipore).

The analysis of the assembled respiratory complexes was performed by using Blue Native Gel Electrophoresis (BNGE). Samples obtained from 25 mg of skeletal/cardiac muscle biopsy or from 2×10^6 fibroblasts (Nijtmans et al. 2002) were loaded and run into a 5–13 % gradient non-denaturing 1-dimensional gel. After protein blotting, immunodetection was carried out using antibodies against alpha subunit of ATP synthase, NDUFA9 (for complex I), MTCOI (for complex IV), SDHA (for complex II) from MitoSciences, Invitrogen, and against HSP60 (Abcam). Densitometric analysis was performed using Quantity One software (BioRad, Hercules, CA, USA).

Results

Muscle morphology, including histochemical reactions, was normal in all studied patients, with no RRF or COX-deficient fibres being present. Electron microscopy, performed only in P3 muscle, showed several swollen and irregularly shaped mitochondria with disruption of the central part and complete loss of the cristae. Some mitochondria were small with simplified cristae (Fig. 1a, b). MitoTracker-based visualization in P1 and P4 showed increased fragmentation of the mitochondrial network, suggesting altered fission/fusion dynamics (Fig. 1c–e)

In muscle homogenate we found isolated cI deficiency in P10 (15 %) and multiple defects in P1 (cI 48 %–cV 53 %) and P4 (cI 67 %–cII 48 %–cIII 55 %–cV 23 %), whereas in the other available samples, MRC activities were normal (Table 1). In autopsy skeletal and cardiac muscles from P8, all the RC activities were markedly reduced, probably because of post-mortem autolysis. In fibroblasts, we measured low cV activity in all but one analysed patients (P1 to P7), whereas all the other OXPHOS activities were in the control range. Normal cV activity was found in fibroblasts from P10, who displayed an isolated complex I deficiency in muscle.

In all patients, sequencing of mtDNA did not reveal any pathogenic variants. Because of the clinical and biochemical presentation, we sequenced *TMEM70* (NM_017866.5) in patients 1 to 7. The DNA sample of P10, who presented an isolated cI defect in muscle, underwent whole-exome sequencing (Haack et al. 2012): the founder intronic mutation c.317-2A > G in *TMEM70* was confirmed by Sanger sequence in both P10 and his affected siblings P8 and P9. The c.317-2A > G change was found in homozygosity in three additional patients (P2, P4, P6) and as a compound heterozygous mutation in P1 and P3, associated with a c.349_352del and c.783A > G mutations, respectively. The c.349_352del microdeletion causes a frameshift predicted to result in a truncated protein (p.Ile117Alafs*36); the c.783A > G change affects the termination codon and predicts the synthesis of a protein with an aberrant 17aa-long extra C-terminus (p.*261Trpext*17). We identified a novel homozygous missense mutation in P5 (c.701A > C, p.His234Pro) and a novel homozygous nonsense mutation in P7 (c.238C > T, p.Arg80*); these variants are not reported in public SNP databases, including dbSNP and the Exome Variant Server.

SDS-GE and immunodetection using an anti-TMEM70 in total lysates from P1, P2, P3, P4, P5 and P7 fibroblasts and from P8 cardiac muscle revealed virtual absence of TMEM70 protein in all tested patients' samples, suggesting a deleterious effect of all identified *TMEM70* mutations, including novel variants (Fig. 2a)

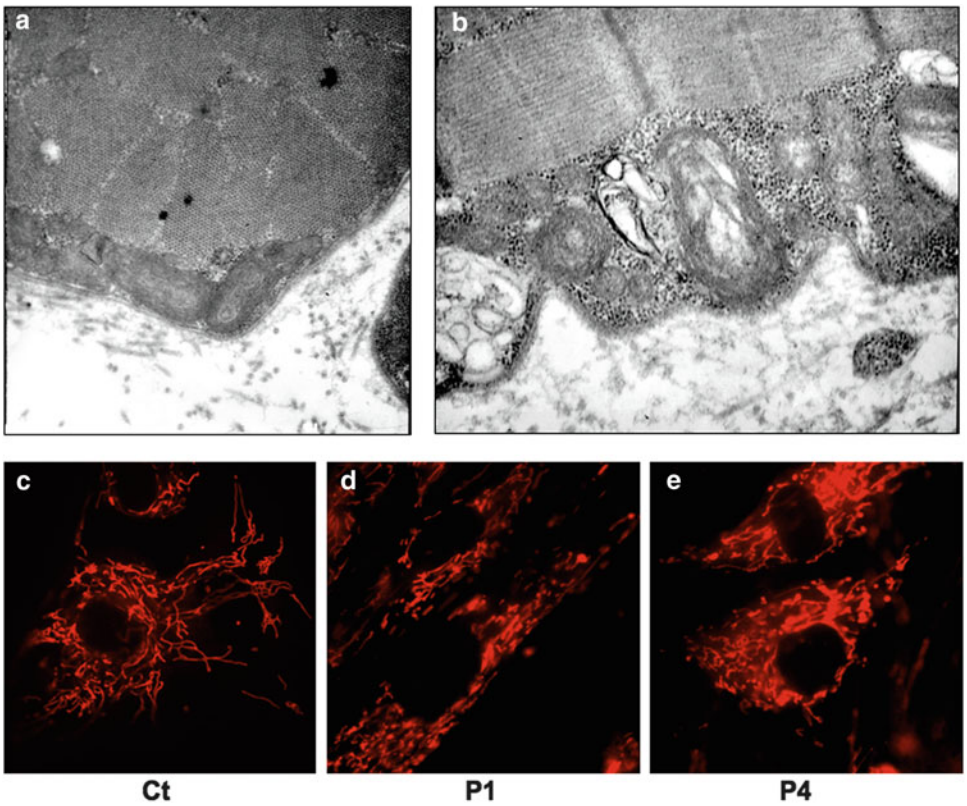

Fig. 1 Morphological studies. (**a**, **b)** Electron microscopy in P3 muscle showing irregularly shaped mitochondria with disruption of the central part and loss of the cristae. (**c**–**e)** Representative images of mitochondrial morphology, showing the filamentous (**c**) or the more fragmented (**d**, **e**) mitochondrial network of fibroblasts from a control subject (**c**), P1 (**d**) and P4 (**e**)

1D BNGE showed undetectable cV in all tested skeletal muscle samples but displayed a moderate to severe reduction of cI in P4 (64 %) and P1 (30 %) compared to a control muscle (see Fig. 2b). In both P8 skeletal and cardiac autopsy muscles we confirmed the absence of cV, associated with accumulation of complex I sub-assembly species. However, since P8 samples were from an autopsy biopsy, we cannot exclude that these results are due to partial sample degradation (Fig. 2b). BNGE analysis in P1 and P4 fibroblasts showed a partial cV reduction, with accumulation of subassembly intermediates (F1 subcomplexes), as already reported in *TMEM70* mutant cells (CízkovÄ et al. 2008; Torraco et al. 2012) (Fig. 2c). Unfortunately no fibroblast samples were available for further investigation on P10, the only subject who showed normal cV activity in fibroblasts.

Discussion

Over 40 patients carrying mutations in *TMEM70* have been thus far described (CízkovÄ et al. 2008; Honzík et al. 2010; Spiegel et al. 2011; Honzik et al. 2012; Cameron et al. 2011), and though they originated from different European countries, most carried a homozygous splice site mutation and shared an identical Roma-gypsy origin that explains genetic homogeneity. Few additional mutations have been reported and often result in absent or prematurely truncated protein.

Our study presents a cohort of ten patients harbouring *TMEM70* mutations. All subjects were Italians; however, P5 parents were from Macedonia (Table 1). Although most of our patients did not have a Roma origin, six patients were homozygous and 2 compound heterozygous for the common Roma mutation, suggesting spreading of an ancestral mutant allele. In the compound heterozygous subjects, the second mutation is predicted to result in a truncated (P1) or in an aberrant (P3) protein, with an extended C-terminus that can impair the function or triggers accelerated degradation. P7 is homozygous for a novel nonsense mutation, predicted to produce a truncated protein (p.Arg80*). On the other hand, P5 is the second patient carrying a homozygous missense mutation. Two missense changes have been described, both in compound

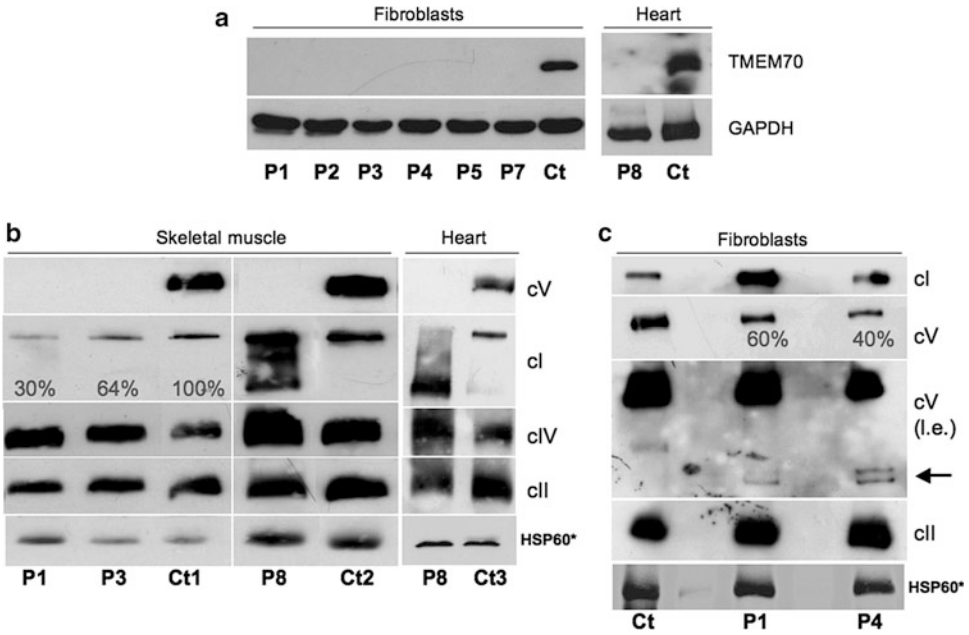

Fig. 2 Protein characterization in muscle and fibroblasts from individuals with TMEM70 mutations. (**a**) SDS-gel electrophoresis of fibroblasts from patients 1,2,3,4,5,7 (P1, P2, P3, P4, P5, P7) and of autopsy cardiac muscle from patient 8 (P8), with corresponding controls (Ct). We used antibodies against TMEM70 and GAPDH (as loading control). (**b**) One-dimensional Blue Native Gel Electrophoresis (1D BNGE) of skeletal muscle homogenates from patients 1, 3 and 8 (P1, P3, P8) and controls (Ct1 and Ct2) and of cardiac muscle homogenates from patient 8 (P8) and a control (Ct3). Autopsy samples were used for P8, Ct2 and Ct3. We used an antibody against the subunit α of ATP synthase to detect complex V (cV), an antibody against SDH 70 kDa for complex II (cII), an antibody against NDUFA9 for complex I (cI), an antibody against subunit COX4 for complex IV (cIV) and an antibody against HSP60, used as loading control. The reported percentages correspond to the values of cI/cII signals obtained by densitometric analysis. (**c**) 1D BNGE of mitochondrial-enriched fibroblasts from patients 1 and 4 (P1, P4) and a control (Ct). We used the same antibodies reported above. For complex V a long exposure (l.e.) allowed the immunovisualization of subassembly species in samples from the patients (*arrow*). *Asterisk* for HSP60 immunodetection, the same samples used for 1D-BNGE were separated by SDS-GE and incubated with an anti-HSP60 antibody

heterozygosity with the "common" variant: the p.Gly165Asp mutation was described in a patient with a milder phenotype characterized by normal growth parameters and cognitive development, no dysmorphic features, improvement of symptoms with age and persistence of hypertrophic cardiomyopathy (Shchelochkov et al. 2010). The p.Thr210Pro was instead identified in two siblings presenting with hypertrophic cardiomyopathy that reversed spontaneously at around 1 year of age. Recently, a homozygous c.535C > T (p.Tyr179His) mutation has been described in a Turkish patient presenting with neonatal lactic acidaemia, severe hypotonia and hypertrophic cardiomyopathy (Atay et al. 2013). At the latest examination at age 1 year, the child showed stable cardiac hypertrophy and had not suffered of metabolic crises requiring hospital admissions. Our P5 was born at term, with a weight >3rd percentile, and he is still alive at the age of 4 years. Taken together, these observations may suggest that infants with missense, rather than nonsense variants in *TMEM70*, have a milder clinical course. However, the virtual absence of TMEM70 protein in P5 as observed in all the other patients carrying different loss-of-function mutations does not agree with this conclusion. Moreover, genotype to phenotype correlation is inconsistent, especially for the common splice mutation, which in our as well as in other studies (Honzik et al. 2012) is associated with variable degree of severity, ranging from fatal outcome at two days of age (P9 in this study) to survival at the age of 17 years (CízkovÄ et al. 2008). The same variability has been reported for other *TMEM70* mutations: the oldest patient reported to date (a 24-year-old man) harboured the same homozygous c.578_579delCA mutation detected in his brother, deceased at the age of 3.5 years (Spiegel et al. 2011).

The biochemical presentation was an interesting feature in our cohort of patients. Mutations in *TMEM70* have been reported to cause isolated cV deficiency (CízkovÄ et al. 2008). Often the spectrophotometric analysis for cV (ATP hydrolysis) is not performed (Spinazzi et al. 2012) because of its insufficient reliability in frozen muscle (Jonckheere et al. 2012) and the presence of oligomycin-insensitive ATPase activity in cultured cells (Barrientos et al. 2009). In our investigation, the measurement of cV activity in total

homogenate from frozen muscles was unreliable, since normal values were obtained in samples with nearly absent cV holocomplex analysed by 1D BNGE Western blot. On the contrary, the ATPase activity in digitonin-treated fibroblasts was reliable, being reduced in all but one patient (P10).

In muscle, the spectrum of defective OXPHOS activities was broader than isolated cV deficiency, including low cI (in P1) or multiple OXPHOS defects (in P4). Likewise, a profound, apparently isolated reduction of cI was found in muscle from P10, which prompted us to carry out WES analysis and, unexpectedly, identify the common splicing mutation in *TMEM70*. Interestingly, a compound heterozygous patient harbouring the common splice site mutation and a missense variant has recently been reported to have no biochemical OXPHOS defects in muscle and fibroblasts (Shchelochkov et al. 2010). Therefore, the screening of *TMEM70* should be performed in patients presenting a mitochondrial disorder characterized by early-onset cardiomyopathy, irrespective of the biochemical profile. In male patients, the analysis of *TMEM70* should also be considered in the differential diagnosis with Barth syndrome (MIM#302060), an X-linked disease characterized by dilated or hypertrophic cardiomyopathy, skeletal myopathy, growth retardation and organic aciduria, including 3-MGA.

The exact molecular function of TMEM70 is still not completely clear and its specific role in ATP synthase assembly is still unknown, since no direct interaction has been demonstrated between this protein and the holocomplex V. In addition to synthesis of ATP from ADP and inorganic phosphate as a consequence of energy derived from proton translocation, cV has an important role for the correct structure of mitochondria. It constitutes 17–30 % of the inner membrane-bound proteins and is important for the formation and invaginations that form the cristae (Ozawa and Asai 1973). Dimeric and higher oligomeric forms of ATP synthase (Paumard et al. 2002) seem critical to maintain the shape of mitochondria by promoting the formation of the inner membrane cristae. Studies in yeast (Paumard et al. 2002; Lefebvre-Legendre et al. 2005) indicate that the cV structure rather than its enzymatic activity dictates cristae morphology. We found ultrastructural alterations in mitochondrial shape of the single TMEM70 patient where electron microscopy could be performed (P3), supporting the link between ATP synthase and mitochondrial morphology.

In conclusion, clinical features, including cardiomyopathy, respiratory distress at birth, lactic acidosis and muscular hypotonia often associated with dysmorphic features, sometimes supported by suggestive biochemical data (cV deficiency and 3-MGA), are still extremely important to point to the diagnosis.

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The authors declare that they have no conflict of interest.

Sentence Take-Home Message

TMEM70 patients present with a mitochondrial cardiomyopathy with early-onset hypotonia, respiratory distress and psychomotor delay, irrespective of the biochemical defect.

Compliance with Ethics Guidelines

Daria Diodato, Federica Invernizzi, Eleonora Lamantea, Gigliola Fagiolari, Rossella Parini, Francesca Menni, Giancarlo Parenti, Lina Bollani, Maria A.Donati, Denise Cassandrini, Elisabetta Pasquini, Filippo M. Santorelli, Tobias B. Haack, Holger Prokisch, Daniele Ghezzi, Costanza Lamperti and Massimo Zeviani declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

DD, DG, CL and MZ designed the study; DD, FI, EL, GF, EP, FMS, TBH, HP, DG, CL and MZ performed experiments, collected and analysed data. RP, FM, GP, LB, MAD, DC, FMS and CL evaluated the patients and wrote case reports. DD, DG, CL and MZ wrote the manuscript. FI, EL, GF, RP, FM, GP, LB, MAD, DC, EP, FMS, TBH and HP critically revised the manuscript for important intellectual content.

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RESEARCH REPORT

Newborn Screening for Galactosemia in the United States: Looking Back, Looking Around, and Looking Ahead

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Abstract It has been 50 years since the first newborn screening (NBS) test for galactosemia was conducted in Oregon, and almost 10 years since the last US state added galactosemia to their NBS panel. During that time an estimated >2,500 babies with classic galactosemia have been identified by NBS. Most of these infants were spared the trauma of acute disease by early diagnosis and intervention, and many are alive today because of NBS. Newborn screening for galactosemia is a success story, but not yet a story with a completely happy ending. NBS, follow-up testing, and intervention for galactosemia continue to present challenges that highlight gaps in our knowledge. Here we compare galactosemia screening and follow-up data from 39 NBS programs gathered from the states directly or from public sources. On some matters the programs agreed: for example, those providing relevant data all identify classic galactosemia in close to 1/50,000 newborns and recommend immediate and lifelong dietary restriction of galactose for those infants. On other matters the programs disagree. For example, Duarte galactosemia (DG) detection rates vary dramatically among states, largely reflecting differences in screening approach. For infants diagnosed with DG, >80% of the programs surveyed recommend complete or partial dietary galactose restriction for the first year of life, or give mixed recommendations; <20% recommend no intervention. This disparity presents an ongoing dilemma for families and healthcare providers that could and should be resolved.

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Introduction

Classic galactosemia is a potentially life-threatening autosomal recessive inborn error of metabolism that affects between 1/30,000-1/60,000 live births in the USA and worldwide (reviewed in (Fridovich-Keil and Walter 2008)). Most affected infants are born looking healthy but experience a rapid and devastating decline following exposure to breast milk or milk formula, which contain large quantities of galactose. Acute symptoms can progress in a matter of days from jaundice, vomiting, and diarrhea to failure to thrive, hepatomegaly, and *E. coli* sepsis. Without treatment, affected infants often die in the neonatal period (reviewed in (Fridovich-Keil and Walter 2008)).

With the advent of population newborn screening (NBS) for galactosemia in the early 1960s it became possible to identify affected newborns before they became critically ill, sometimes even before they were symptomatic. Unlike most newborn screens that quantify small molecules, NBS for galactosemia is based on a coupled assay quantifying the activity of an enzyme: galactose-1P uridylyltransferase (GALT) (Beutler and Baluda 1966). Many NBS labs also quantify "total galactose" (galactose + galactose-1P), which can be elevated in affected infants, especially if they have consumed milk.

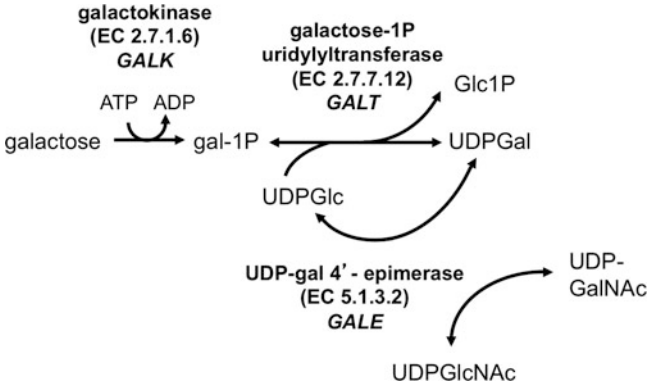

Fig. 1 The Leloir Pathway of galactose metabolism. Classic galactosemia results from profound loss of galactose-1P uridylyltransferase (GALT); Duarte galactosemia results from partial loss of GALT. Profound loss of GALK leads to galactokinase deficiency galactosemia, and partial loss of GALE results in generalized, intermediate, or peripheral epimerase deficiency galactosemia depending on the degree of impairment and the tissues impacted

Once identified by a positive NBS result, infants suspected of having galactosemia may be shifted from milk to a low-galactose soy or elemental formula until follow-up testing can be completed. For those infants ultimately diagnosed with classic galactosemia, the recommended intervention is continued and lifelong dietary restriction of galactose. For those infants who receive a normal follow-up testing result, the galactose restriction is lifted. However, interpreting NBS and follow-up testing results in the case of some infants can be difficult, because galactosemia is not a single or discrete condition; it is a family of disorders resulting from partial to profound loss of activity of any of the three enzymes of the Leloir pathway (Fig. 1). The biochemical NBS presentation and follow-up testing results of these different types of galactosemia can overlap, complicating diagnosis. Additionally, the clinical implications of forms other than classic galactosemia are often poorly understood, resulting in a lack of evidence-based standards of care once a diagnosis is reached. Identification of these individuals and recommendations for follow-up care vary by state and by practice of the individual infant's healthcare provider.

Classic and Duarte Galactosemia

Newborn screening for galactosemia was originally designed to detect *classic* galactosemia, the rare and potentially lethal condition resulting from profound to complete loss of activity of galactose-1P uridylyltransferase (GALT), the middle enzyme in the Leloir pathway (Fig. 1). Patients with classic galactosemia carry functionally severe (G) mutations in each of their *GALT* alleles, so that classic galactosemia is sometimes referred to as "GG" galactosemia. These mutations may be the same (molecular homozygote) or different (compound heterozygote). Blood spots from affected newborns show very low to absent GALT activity (Beutler and Baluda 1966) and elevated total galactose (Table 1), especially if the infant consumed milk before the NBS blood spot was collected. However, not all mutations in *GALT* are functional nulls; many are "hypomorphs," mutations that leave some residual activity intact (e.g., (Riehman et al. 2001)). Indeed, one extremely mild variant called the Duarte (D or D2) allele is associated with about half of the normal level of GALT activity (Carney et al. 2009; Elsas et al. 2001; Greber et al. 1995; Levy et al. 1978; Mellman et al. 1968; Podskarbi et al. 1996; Tighe et al. 2004; Trbusek et al. 2001; Tyfield 2000). Worldwide, D2 alleles are found at an allele frequency of >11% in Europeans, less than 3% in Asians, and almost zero in Africans (http://hapmap.ncbi.nlm.nih.gov/).

Duarte galactosemia (DG) results from compound heterozygosity for one functionally severe (G) *GALT* mutation together with a D2 allele. As explained previously (Ficicioglu et al. 2008), while classic galactosemia occurs with a prevalence of 1/40,000 to 1/60,000 infants, DG is identified in approximately 1/4,000 Caucasian infants, making DG approximately 10 times as common as classic galactosemia among US newborns. Fernhoff (2010) also confirmed this ratio, noting that of 405,000 newborns screened in a 3-year period in the state of Georgia, eight were identified with classic galactosemia and 83 were identified with DG.

The NBS results for DG infants can overlap those of classic galactosemics (Table 1). Follow-up testing results for most infants with Duarte galactosemia demonstrate close to 25% residual GALT activity, which is well above the level typically seen in classic galactosemia. However, this residual activity may be lost from the sample by exposure of the dried blood spot to heat or humidity during storage or transport before testing (Dobrowolski et al. 2003).

Table 1 Newborn screening results anticipated for different diagnostic categories of galactosemia

Condition (gene)	Estimated prevalence (US)	NBS Results	Follow-up biochemical test results
Classic galactosemia (*GALT*)	~1/50,000 live births (Suzuki et al. 2001)	GALT activity low to absent gal+gal-1P elevated	Hemolysate GALT activity very low to absent Hemolysate gal-1P elevated (diet-dependent) Urinary galactitol may be elevated if the infant is consuming milk
Duarte variant galactosemia (*GALT*)	~1/4,000 live births (Ficicioglu et al. 2008)	GALT activity low gal+gal-1P elevated	Hemolysate GALT activity low Hemolysate gal-1P elevated (diet-dependent) Urinary galactitol may be elevated if the infant is consuming milk
Epimerase-deficiency galactosemia (*GALE*)	~1/7,000 Afr-Amer ~1/70,000 Cauc (Alano et al. 1997)	GALT activity normal gal+gal-1P elevated[a]	Hemolysate GALE activity low in RBC Hemolysate GALT activity normal Hemolysate gal-1P elevated (diet-dependent) Urinary galactitol may be elevated if the infant is consuming milk
Kinase-deficiency galactosemia (*GALK*)	<1/100,000 live births in the US (reviewed in (Fridovich-Keil and Walter 2008))	GALT activity normal gal+gal-1P elevated[a]	Hemolysate GALK activity low to absent in RBC Hemolysate GALT activity normal Hemolysate galactose elevated (diet-dependent) but gal-1P NOT elevated Urinary galactitol may be elevated if the infant is consuming milk

[a] Other conditions, independent of galactosemia, which compromise liver function or circulation can also lead to this NBS result

Epimerase- and Kinase-Deficiency Galactosemia

Abnormal NBS results can also occur in infants with epimerase-deficiency galactosemia (Fridovich-Keil et al. 2011), which results from partial impairment of UDP-galactose 4′ epimerase (GALE), or kinase-deficiency galactosemia, which results from profound loss of galactokinase (GALK) (Fig. 1, Table 1). GALE deficiency presents as a continuum disorder, both clinically and biochemically (Openo et al. 2006). The vast majority of patients identified with GALE deficiency in the USA are clinically mild to asymptomatic and demonstrate peripheral or intermediate GALE deficiency, with profound enzyme impairment restricted to the circulating red and white blood cells (reviewed in (Fridovich-Keil and Walter 2008)). Peripheral and intermediate GALE deficiencies together have been estimated to impact approximately 1/7,000 African-American and 1/70,000 Caucasian US newborns (Alano et al. 1997). Cases of generalized GALE deficiency, with profound enzyme impairment evident in all tissues tested, are extremely rare and present clinically with acute symptoms similar to those seen in classic galactosemia (Walter et al. 1999).

GALK-deficiency is considered clinically mild with the exception of neonatal cataracts that can be prevented by immediate dietary restriction of galactose. GALK deficiency is diagnosed in (<1/100,000) US newborns but may be more common in some populations (e.g., the Romani) (Hennermann et al. 2011; Janzen et al. 2011; Kalaydjieva et al. 1999; Sangiuolo et al. 2004).

Strengths and Challenges of NBS for Galactosemia

As a screening tool for classic galactosemia, current NBS protocols are highly effective. Principal challenges stem from high false positive rates, defined here as the number of infants identified by NBS each year who require follow-up testing but ultimately are determined not to have classic or Duarte galactosemia. A small number of these infants may turn out to have variant forms of galactosemia (e.g., kinase or epimerase-deficiency) and some are carriers (GN or DN) or Duarte homozygotes (DD); others may have no recognized cause for GALT deficiency. In the majority of cases, high false positive rates necessitate clinic visits for what can be large numbers of ostensibly healthy infants each year, and for many of these infants the positive galactosemia NBS result imposes what can be an extended interruption of breastfeeding while the family awaits the follow-up test result.

Challenges associated with NBS and follow-up testing for galactosemia also stem from differences of opinion

among healthcare professionals and policymakers regarding which forms of galactosemia newborn screening should be tailored to identify; most notably with regard to DG. Of course, the answer to this question also feeds back to the false positive rate, because testing schemes designed to identify infants with 25% residual GALT activity may have a higher likelihood of also picking up galactosemia carriers (GN), who represent almost 1% of the US population. False positives due to other causes may also increase when the NBS GALT cutoff level is raised.

Here we describe the results of a comparative review of state NBS programs in the US queried with regard to their historical and current approaches to galactosemia newborn screening and follow-up testing. The findings of this study document the successes and lessons learned from NBS for galactosemia in the past 50 years, but also highlight areas where there is room for improvement (Wilcken 2013) in the years to come.

Methods

Though most of the data collected for this study were either publicly available or did not involve any Protected Health Information because they concerned populations and not individuals, those aspects of the study that required a HIPAA waiver were conducted with approval of the Emory Institutional Review Board (Emory IRB# 00024933) and also the Georgia Department of Public Health Institutional Review Board (Georgia DPH IRB#130306), PI: JL Fridovich-Keil.

Publicly Available Data

Information concerning when each state initiated NBS testing for galactosemia and the total numbers of newborns screened per state were collected from publicly available sources (e.g., the Centers for Disease Control and Prevention's National Vital Statistics Reports (http://www.cdc.gov/nchs/products/nvsr.htm) and the National Newborn Screening Information System (http://nnsis.uthscsa.edu).

Data from Newborn Screening Programs and Follow-up Centers

We attempted to reach all 51 NBS programs in the USA (all 50 states plus Washington DC) by email and/or telephone using names and contact information listed at the National Newborn Screening Information System (http://nnsis.uthscsa.edu). We asked each program for information concerning their approach to galactosemia screening and follow-up testing for as many years back as the data were available. We also asked for information concerning total numbers of newborns screened, total positive NBS test results for galactosemia reported each year, and how those total numbers translated into cases of classic or Duarte galactosemia, or false positives. Where possible, we also asked for information concerning detection rates by gender and racial group.

Some programs responded and generously shared their aggregate data with us, though not every respondent program was able to share data relevant to every question posed. Some programs responded, but explained that they were short-staffed and simply could not spare the time to assemble the requested information. Finally, some programs never responded despite repeated contact attempts. The data presented here concerning detection rates for different diagnostic categories of galactosemia were derived from those programs that provided data to us, in some cases supplemented by data we found published online (e.g., for Arizona, Arkansas, Indiana, New Jersey). Whenever possible, detection rates were calculated using the actual number of newborns screened in a given state and year, but when those numbers were not available, we substituted available data giving the total number of births, rather than the total number of screens. In these instances, the detection rates we calculated should be considered slight underestimates assuming there were at least some newborns in those states who were not screened. Other inaccuracies in our estimates may stem from instances where an infant was born in one state but for some reason transferred to and screened in another state. The 39 states from which we either received NBS data or for which we were able to find publicly available NBS data are listed in Supplementary Table 1.

GALT Activity, gal-1P Values, and *GALT* Genotypes Determined on Follow-up Testing by the Emory Genetics Lab

Samples from infants less than 1 month old that were sent to the Emory Genetics Lab (EGL, http://genetics.emory.edu/egl/) between 2008 and 2012 for galactosemia testing were accessed from the EGL MEDGIS (Medical Genetics Information System) via a HIPAA waiver granted by the Emory University Institutional Review Board (Emory IRB# 00024933). Data used for the preparation of Figs. 3, 4, and 5, and Supplementary Table 5 were limited to samples from infants <1 month old who had GALT activity measured, and in most cases also gal-1P level, and *GALT* genotyping performed. Of note, these samples derived from many states, not just Georgia. *GALT* genotyping at EGL during the time period 2008–2012 was conducted by testing DNA samples for the presence or absence of a panel of specific known mutations or variations, including: S135L (c.404C>T), T138M (c.413C>T), Q188R (c.563A>G),

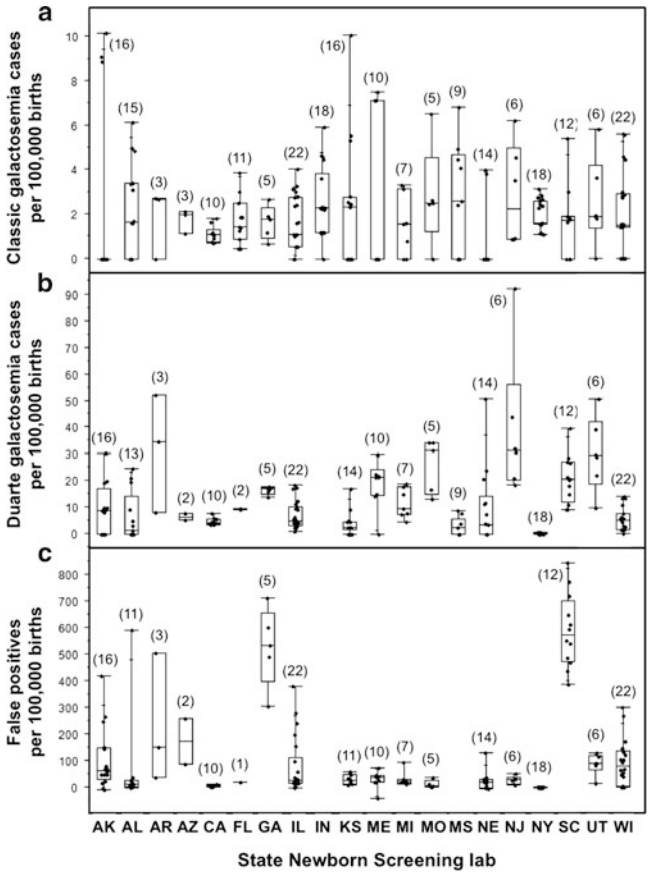

Fig. 2 Classic, Duarte, and false positive rates from galactosemia newborn screening in respondent states. (**a**) Detection rates for classic galactosemia per 100,000 newborns screened. (**b**) Detection rates for Duarte galactosemia per 100,000 newborns screened. We were not able to calculate a DG detection rate for Indiana. (**c**) False positive rates defined as the total number of positive galactosemia screening results minus the numbers of cases diagnosed with classic, variant, or Duarte galactosemia. We were not able to calculate false positive rates for Indiana or Mississippi. The number of years of data included in the analysis for each box and whisker plot is indicated in parentheses above the box. For each plot the lower, middle, and upper boundaries of the box represent the 25th, 50th, and 75th percentiles for the data set, respectively, and the whiskers indicate the full range of the data. The 25th, 50th, and 75th percentile limits for each population plotted in Fig. 2 are also presented in Supplementary Table 2

L195P (c.584T>C), Y209C (c.626A>G), L218L (c.652C>T), K285N (c.855G>T), N314D (c.940A>G), c.-119_-116del, IVS2-2A>G, and the 5.5 kb deletion (c.[-1041_751del;820+50_*790delinsGAATAGACCCCA]) (Coffee et al. 2006). Samples from patients with biochemical findings consistent with the presence of a G allele that did not test positive for a recognized deleterious GALT mutation from this panel were said to carry an "unknown" G mutation.

Data Analysis and Presentation

In some tables actual numbers of cases identified with classic or Duarte galactosemia are presented (Table 3 and Supplementary Table 3), while in other tables (Supplementary Table 4) detection rates per 100,000 newborns screened are presented.

The data graphed in Supplementary Fig. 1a were calculated for each year between 1963 and 2004 by multiplying the average number of annual births for each state screening for galactosemia at that time and calculating what percentage of the total US births were in that group. These values are estimates as we did not have state newborn population numbers for all states reaching back to 1963 and so calculated proportions using birth numbers from the available years.

The data illustrated in Fig. 2 are presented as "box and whisker" plots in which the top and bottom of each box indicate the 75th and 25th percentiles, respectively, the midline in each box presents the median (50th percentile), and the whiskers indicate the full range of the data points. The number of years of data included in the analysis for each box and whisker plot is indicated in parentheses above the box. Box and whisker plots are also presented in Figs. 4 and 5, with added hatch marks on the top and bottom whiskers indicating the 90th and 10th percentiles, respectively.

Table 2 Newborn screening testing strategies of respondent state labs

State	Testing method	GALT cutoff (trigger for further action)	Total galactose cutoff (trigger for further action)
Alabama (AL)	Perkin Elmer	<2.7 U/dL	Not tested
Colorado (CO)	Perkin Elmer	<2.5 U/dL	Not tested
Delaware (DE)	Perkin Elmer	<2.4 U/gHb	>10 mg/dL
Florida (FL)	Perkin Elmer	<3.5 U/dL	Not tested
Georgia (GA)	Astoria Pacific	<60 μM NADPH[a]	>11 mg/dL; only tested if GALT low
Illinois (IL)	Not specified	Partial/no activity	>6.5 mg/dL
Kansas (KS)	Qualitative	Partial/no activity	Not specified
Louisiana (LA)	Perkin Elmer	<4.3 U/dL	Not tested
Michigan (MI)	Perkin Elmer	≤3.1 U/gHb (changed to ≤3.6 U/gHb in 2012)	Only tested if GALT low; not used to define a sample as abnormal
Mississippi (MS)	Astoria Pacific	<40 μM NADPH[a]	>15.0 mg/dL
Missouri (MO)	Perkin Elmer	<3.0 U/gHb	Not tested
Nebraska (NE)	Astoria Pacific	<40 μM NADPH[a]	>15 mg/dL
New York (NY)	Qualitative	Partial/no activity	Not specified; tested only in samples with reduced or absent GALT
Ohio (OH)	Astoria Pacific	≤1.7 U/gHb (confirmatory testing if ≤2 U/gHb)	≥10 mg/dL; Only tested if GALT ≤2 U/gHb)
South Carolina (SC)	Astoria Pacific	<60 μM NADPH[a]	All samples tested; cutoff not specified
Tennessee (TN)	Astoria Pacific	<40 μM NADPH[a]	>15 mg/dL
Utah (UT)	Perkin Elmer	<3.0 U/gHb	Not tested
West Virginia (WV)	Astoria Pacific	<50 μM NADPH[a]	>10 mg/dL
Wisconsin (WI)	Perkin Elmer	<3.0 U/gHb	> 6.0 mg/dL

Note: Testing strategies used by specific labs may change over time; these are the responses received at the time requested (2011–2012)

[a] GALT activity was measured using a coupled assay that produced NADPH so that μM NADPH produced in a given period of time corresponded to a given level of GALT activity in the sample (Freer et al. 2010))

Results

Toward Universal Newborn Screening for Galactosemia in the USA

To determine what fraction of newborns in the USA were screened for galactosemia in the early years of NBS, we gathered and analyzed historical data from the National Newborn Screening Information System (http://nnsis.uthscsa.edu). From 1963, when the first NBS for galactosemia was conducted, until 2004, when the last state added galactosemia to their NBS panel, there was a slow, steady rise in both percentage of US newborns screened (Supplementary Fig. 1a) and the number of programs doing the screening (Supplementary Fig. 1b). By 2004, essentially all infants born in the USA were screened for galactosemia through the combined efforts of 51 NBS programs. Thanks to these programs and their associated follow-up testing centers, each year an estimated >80 newborns affected with classic galactosemia were identified and diagnosed, and for most of these infants the potentially lethal sequelae of their disease were prevented by early intervention.

Detection Methods and Rates

To compare the detection methods used for galactosemia newborn screening, we compiled responses from 19 state programs (Table 2). We also compared NBS detection rates for classic and Duarte galactosemia and false positive rates reported for these and other programs for which the data were available (Fig. 2). Of note, while not all data were available from every program, enough programs provided information about both their detection methods and detection rates to reveal patterns.

The screening approaches for all respondent programs included an assay for GALT activity (Table 2) though the cutoffs applied to distinguish normal from abnormal results varied. Some states also measured total galactose (galactose + galactose-1-phosphate); however, some measured total galactose in all samples (e.g., SC) while others measured total galactose only in samples already flagged by a borderline, low, or absent GALT activity level (e.g., GA, MI, NY, OH). Further, not every state that measured total galactose used that information in the

decision of whether or not to flag a sample as abnormal for galactosemia, and some states also had a special approach to NBS if the baby had been transfused prior to sample collection.

Prevalence and Detection of Classic Galactosemia

When comparing detection rates among respondent NBS programs for classic galactosemia, it was clear that all identified affected infants at close to the anticipated 2/100,000 births (Fig. 2a, Supplementary Table 2) (Suzuki et al. 2001). The variance and scatter evident among states, and within states over time, presumably reflect statistics of small numbers, especially for the less populous states (e.g., AK, KS, NE). Differences in screening approach, the definition of each diagnostic category, and ancestry of the populations screened might also have contributed to the differences observed.

Comparing Table 2 with Fig. 2a, it is worth noting that programs that used both GALT activity and total galactose level in their definition of an abnormal NBS result (GA, IL, KS, MS, NE, NY, SC, and WI) showed the same median detection rate for classic galactosemia (1.67 per 100,000 newborns screened) as states that based their definition solely on GALT activity (e.g., AL, FL, MI, MO, and UT). Further, states that had a higher GALT cutoff level (e.g., FL) did not show a higher detection rate for classic galactosemia than states with a lower cutoff level (e.g. AL). These results suggest that there are very few, if any, infants with classic galactosemia who slip through the US newborn screening net undetected regardless of where in the country they are born.

Prevalence and Detection of Duarte Galactosemia

In contrast to classic galactosemia, median detection rates for Duarte galactosemia (DG) varied from essentially zero (e.g., NY) to more than 1/3,500 (30/100,000) newborns screened (e.g., AR and NJ, Fig. 2b, Supplementary Table 2) suggesting that many DG infants go undetected by NBS in some states. For example, Georgia detected close to 20 DG cases per 100,000 newborns screened, while Mississippi detected a median of fewer than 5. This difference likely reflects, at least in part, the differing GALT cutoff levels used by the two states: in Georgia a sample showing <60 μM (NADPH) (Freer et al. 2010) GALT activity was defined as abnormal, whereas in Mississippi a sample needed to show <40 μM (NADPH) GALT activity to be declared abnormal (Table 2).

Comparing Table 2 with Fig. 2b, it is interesting to note that programs that used both GALT activity and total galactose level in their definition of an abnormal NBS result (GA, IL, KS, MS, NE, NY, SC, and WI) collectively showed a lower median detection rate for Duarte galactosemia (4.37 per 100,000 newborns screened) than states that used only GALT activity (9.7 per 100,000 newborns screened; AL, FL, MI, MO, and UT). Whether or not this difference is meaningful, including total galactose in the definition of an NBS positive result clearly did not increase the detection rate for DG, and may have decreased it.

False Positives

The number of false positives, defined here as the total number of NBS galactosemia positives minus the numbers of infants diagnosed with classic or Duarte galactosemia, also differed strikingly from state to state, and in some states from year to year (Fig. 2c, Supplementary Table 2). Some of these "false positive" infants likely were carriers (GN or DN) or Duarte homozygotes (DD) or had a variant form of galactosemia (e.g., kinase or epimerase-deficiency); however, in many cases these infants had no recognized cause for GALT deficiency.

Notably, false positive rates did not always go hand-in-hand with DG detection rates. For example, New Jersey reported a high DG detection rate but a moderate to low false positive rate (Fig. 2b, c), while both Georgia and South Carolina reported moderate DG detection rates but strikingly high false positive rates. The way states defined a positive result may also have contributed to these differences.

Comparing Table 2 with Fig. 2c, it is interesting to note that programs that used both GALT activity and total galactose level in their definition of an abnormal galactosemia NBS result (GA, IL, KS, NE, NY, SC, and WI) collectively showed a higher median false positive rate for galactosemia NBS (30.26 per 100,000 newborns screened) than did states that used only GALT activity (20.47 per 100,000 newborns screened; AL, FL, MI, MO, and UT). This difference might reflect the prevalence of infants with any of the multitude of causes of elevated total galactose in a newborn other than classic or Duarte galactosemia (Ono et al. 1999; Ono et al. 2000; Raffel et al. 1993). It is also important to note that for the purposes of this study we classified states according to their current NBS approach at the time we collected the data. At least some of the states that currently do versus do not test total galactose as part of their galactosemia NBS protocol may have changed their approach during the time frame of the data shared with us.

Impact of the GALT Cutoff Level

Perhaps the clearest illustration of the relationship among NBS GALT cutoff level, DG detection rate, and false positive rate can be seen from the longitudinal records of a single NBS program compared across a time period when

Table 3 Lowering the NBS GALT cutoff level in Utah lowered the false positive rate and lowered the number of infants detected with Duarte galactosemia, but not the number of infants detected with classic galactosemia

Year	Total resident births	Cases of classic galactosemia detected	Cases of Duarte galactosemia detected	False positives[a] detected	NBS GALT cutoff (U/gHb)
2011	51,223	1	1	4	3.0
2010	52,164	1	5	7	3.0
2009	53,849	1	21	44	3.5
2008	55,605	1	16	72	3.5
2007	55,063	2	12	49	3.5
2006	53,448	0	16	48	3.5
2005	51,517	3	26	58	3.5

Note: These are raw numbers and not scaled per 100,000 births

[a] False positives were defined as total NBS cases reported as abnormal for galactosemia minus the numbers of cases diagnosed with classic or Duarte galactosemia. False positives may include carriers and other genotypes that have lowered GALT activity but are not considered clinically affected

the cutoff level was changed. The Utah NBS program underwent such a change in 2010 and was kind enough to share their detection rate data with us. As presented in Table 3, the number of infants with classic galactosemia detected each year in Utah remained essentially unchanged, between 0 and 3, regardless of whether the NBS GALT cutoff level was 3.5 U/gHb (2005–2009) or 3.0 U/gHb (2010–2011). However, the number of infants detected with Duarte galactosemia changed dramatically, from 12 to 26 per year when the cutoff was 3.5 U/gHb down to 1–5 per year when the cutoff was 3.0 U/gHb. The number of false positives identified annually during this time period changed even more dramatically, from 44 to 72 when the GALT cutoff was 3.5 U/gHb down to 4–7 when the GALT cutoff was 3.0 U/gHb. This was the desired result, since Utah does not recommend dietary intervention for infants with Duarte galactosemia. Streamlining the NBS approach to prevent their detection, and therefore lower the false positive rate, made sense in Utah. A similar experience was reported for newborn screening in Sweden (Ohlsson et al. 2012).

Roles of Gender and Race in Galactosemia NBS Detection

Classic and Duarte galactosemia both result from mutations in the *GALT* gene encoded on chromosome 9 (Leslie et al. 1992) explaining why these conditions are inherited as autosomal recessive traits. Nonetheless, even autosomal or complex traits can show gender bias in expressivity or prevalence in a population (Gethins 2012; Mohamad and Apffelstaedt 2008).

To test whether either classic or Duarte galactosemia showed any gender bias in detection by NBS we assessed available historical data from eight programs: AL, IL, MI, MO, MS, NY, SC, and WI (Supplementary Table 3). The detection rates from some programs showed an apparent bias; for example, Alabama detected twice the number of DG girls (34) as boys (17) from 2006 to 2011. However, when the totals from all eight states were combined it was clear there was no overall gender bias. Of note, while these numbers are informative for gender ratio in the patient population, they should not be used to estimate the relative proportion of newborns diagnosed with classic vs. Duarte galactosemia because they include data from states (e.g., NY) that do not screen for DG.

In contrast to gender, assessing prevalence by race did show some clear differences. For example, while the eight state programs listed in Supplementary Table 4 detected classic galactosemia in an average of 1.7 ± 0.67 per 100,000 "White" or "Caucasian" newborns screened and 1.99 ± 1.55 per 100,000 "Black" or "African-American" newborns screened, the average detection rate among infants identified as "Asian/Pacific Islander" was only 0.18 ± 0.36 per 100,000. Although most respondent states providing data with regard to race did not list "Hispanic" among their categories, New York did, revealing a detection rate of 2.4 per 100,000 newborns screened for this group.

In most states, detection rates for Duarte galactosemia exceeded detection rates for classic galactosemia, and again, there was some apparent influence of race. For example, Duarte galactosemia was detected at an average rate of 14.5 ± 10.65 per 100,000 white newborns screened (excluding NY, which detected almost no DG infants), but the average detection rate was only 3.84 ± 4.82 per 100,000 African-American newborns screened (again excluding NY). The scatter in these numbers was substantial, but in every state the prevalence of Duarte galactosemia detected in white infants exceeded that in

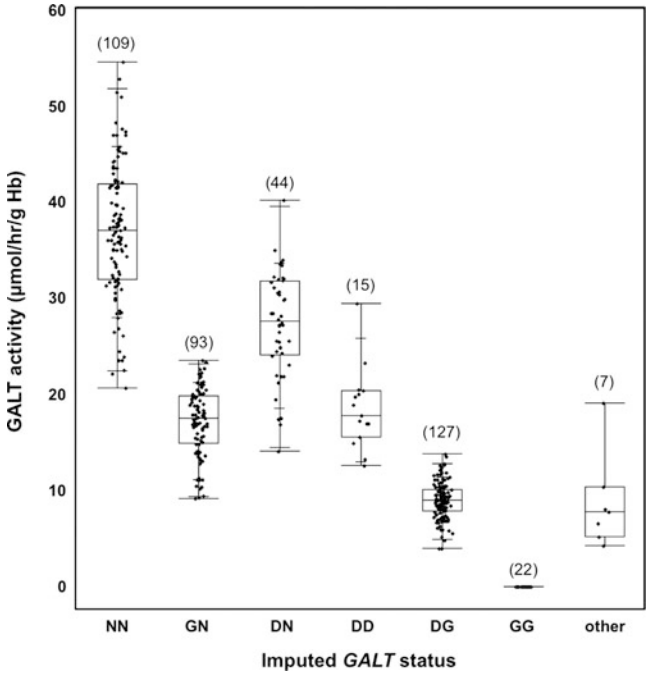

Fig. 3 GALT activity levels detected in hemolysates from infants in different diagnostic categories for galactosemia. GALT activity levels determined as part of follow-up testing at the Emory Genetics Lab (EGL) were ascertained via a HIPAA waiver and presented as box-and-whisker plots by diagnostic group. The upper, middle, and lower boundaries of each box indicate the 25th, 50th, and 75th percentiles of the data set, respectively; the whiskers indicate the full range of the data, and the cross hatches in the upper and lower whiskers indicate the 90th and 10th percentiles of the data set, respectively. The number of points in each data set is indicated in parentheses above the box

African-American infants. This pattern is notably different from the indistinguishable detection rates for classic galactosemia observed for these groups and likely reflects the lower D2 allele frequency among populations of African ancestry (http://hapmap.ncbi.nlm.nih.gov/). As with classic galactosemia, Duarte galactosemia was also strikingly rare among infants categorized as Asian/Pacific Islander in all but three states. For reasons that remain unclear, DG was detected at a high rate among Asian/Pacific Islanders in Missouri, and to a lesser extent in Illinois and Michigan.

Biochemical Complexity

The level of GALT enzyme activity detected in hemolysate is an essential component of follow-up testing for classic or Duarte galactosemia in all states. To explore the range of GALT activity levels detected in hemolysate samples from infants in different diagnostic categories, we reviewed GALT activity data from 2008 to 2012 reported by the Emory Genetics Lab (EGL), which performs follow-up testing for Georgia newborn screening as well as samples from many other sources. In a collection of 417 GALT activity test results identified via a HIPAA waiver, the 22 designated as classic galactosemic (GG) demonstrated no GALT activity (Fig. 3, median 0 μmol/h/g Hb), and all others demonstrated at least some GALT activity. The samples designated normal (NN; two normal *GALT* alleles) had the highest median activity (37 μmol/h/g Hb), followed by DN (one Duarte allele and one normal allele, 27.6 μmol/h/g Hb), then DD (two Duarte alleles, 17.8 μmol/h/g Hb), GN (one classic allele and one normal, 17.5 μmol/h/g Hb), then DG (one Duarte allele and one classic, 9 μmol/h/g Hb), and finally "other" (two unknown alleles; 7.8 μmol/h/g Hb). Notably, there was substantial overlap in the GALT activity levels observed for individual samples in many of the diagnostic categories (Fig. 3), illustrating the difficulty of making a definitive diagnosis based on GALT activity alone.

Because of this complexity, many programs in the USA also measure hemolysate galactose-1-phosphate (gal-1P) as a secondary indicator of diagnostic status. Figure 4 presents the gal-1P values reported by EGL for 413 samples in the indicated diagnostic categories. As illustrated, while there was some overlap of gal-1P values in almost all categories, the majority of samples in all groups, except GG, had low to undetectable gal-1P, while the majority of samples in the GG category had elevated gal-1P. Many samples from infants with DG also had elevated gal-1P, but the median DG gal-1P level (0.4 mg/100 mL) was substantially lower than the median GG gal-1P level (47.3 mg/100 mL).

To further explore the possible relationship between GALT activity and gal-1P values in hemolysate samples

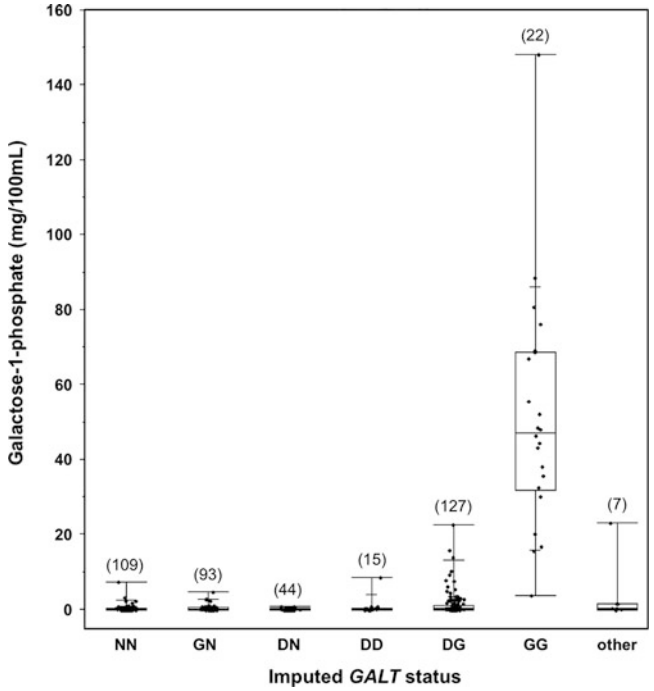

Fig. 4 Gal-1P levels detected in hemolysates from infants in different diagnostic categories for galactosemia. Gal-1P levels determined as part of follow-up testing at the Emory Genetics Lab (EGL) were ascertained via a HIPAA waiver and presented as box-and-whisker plots by diagnostic group. The lower, middle, and upper boundaries of each box indicate the 25th, 50th, and 75th percentiles of the data set, respectively; the whiskers indicate the full range of the data, and the cross hatches in the upper and lower whiskers indicate the 90th and 10th percentiles of the data set, respectively. The number of points in each data set is indicated in parentheses above the box

from infants with classic and Duarte galactosemia we plotted one as a function of the other. As illustrated (Fig. 5), while higher gal-1P values were found in samples with undetectable GALT activity and lower gal-1P values were found in samples with high GALT activity, there was also scatter along both axes.

GALT Genotyping Helps to Resolve Some But Not All Diagnostic Mysteries

To explore the impact of genotyping on NBS follow-up testing, we tabulated the results of *GALT* genotyping conducted at the Emory Genetics Lab from 2008 to 2012 on samples from infants who were less than 1 month old and diagnosed as having classic (GG, 22 infants) or Duarte galactosemia (DG, 127 infants), or who were determined to be galactosemia carriers (GN, 93 infants). Of note, these samples were not all derived from infants born in the same state so they cannot be used to assess the relative prevalence of GG, DG, or GN in a population. As explained in Methods, each sample was tested for the presence or absence of a panel of recognized causal mutations and other variants. Except for the addition of direct testing for the c.-119_-116del non-coding change associated with D2, the panel of mutations screened remained constant during this time frame. Patient *GALT* alleles that did not carry any of the recognized mutations in the panel but that could be inferred from biochemical results to be functionally impaired were designated "unknown."

Genotyping results for 22 neonates with classic galactosemia (Supplementary Table 5, upper section) revealed two recognized *GALT* mutations in 16, effectively confirming the diagnosis for these infants. Three other infants each had one *GALT* allele inferred as "unknown," and another three had both *GALT* alleles inferred as "unknown." For these six infants, genotyping did not confirm the diagnosis of classic galactosemia. In terms of specific *GALT* alleles in this group, 26/44 (59%) identified were Q188R, 5/44 (11%) were S135L, and 8/44 (18%) were unknown. A handful of other known mutations accounted for the remaining 12%.

Genotyping results for 127 neonates with Duarte galactosemia (Supplementary Table 5, middle section) showed that the D2 allele was present in all, and individual recognized G mutations were also identified in 102, confirming the diagnosis in 80% of the infants. Of note, in one DG infant the presumed G mutation presented on a D2 background; the variants characteristic of D2 were homozygous in that sample. This observation stands as a clear reminder that the presence of D2 markers on a *GALT* allele does not indicate absence of a G mutation. In terms of specific G alleles

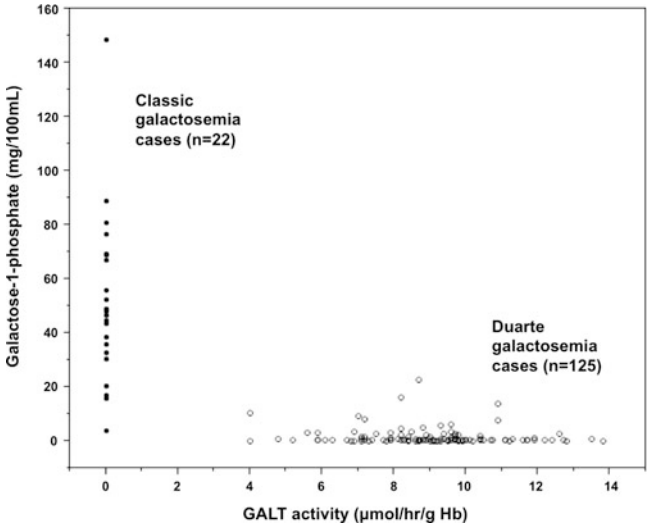

Fig. 5 Relationship between Gal-1P and GALT activity levels detected on follow-up testing of hemolysates from infants with classic or Duarte galactosemia. GALT activity and Gal-1P levels determined as part of follow-up testing at the Emory Genetics Lab (EGL) were ascertained via a HIPAA waiver and presented as "scatter plots." The highest gal-1P levels were all found in infants with undetectable GALT activity, but there was also considerable scatter in both diagnostic groups

identified in the DG infants, 83/127 (65%) were Q188R, 5/127 (4%) were S135L, and 25/127 (20%) were unknown. A handful of other known mutations accounted for the remaining 11%. It is interesting to note that while Q188R was as well represented in this group as among the classic galactosemics, S135L was not. This presumably reflects the African origins of the S135L mutation (Suzuki et al. 2001). Because D2 is particularly rare in African populations (http://hapmap.ncbi.nlm.nih.gov/) ascertaining infants with Duarte galactosemia presumably skewed the G allele frequencies away from S135L.

Finally, *GALT* genotyping results for 93 neonates determined to be GN carriers (Supplementary Table 5, lower section) uncovered single recognized G mutations in 79, confirming those diagnoses but leaving 14 (15%) as "unknown." Absent molecular confirmation it might be difficult to distinguish a "high activity" GN from a "low activity" NN (see Fig. 3). In terms of specific G mutations identified among carriers, 47/93 were Q188R (51%) and 19 were S135L (20%), reminiscent of the allele frequencies seen among GGs.

Recommendations for Intervention

All NBS program and follow-up care providers consulted for this study agreed that immediate and lifelong dietary restriction of galactose is required for patients with classic galactosemia, but the recommendations were mixed for Duarte galactosemia. Of the 28 states that responded to our inquiries on this point, five (18%) said they do not recommend any intervention for infants with Duarte galactosemia. Six (21%) said they do not intervene if the infant is to be breastfed, but if the infant is to drink formula they recommend soy. The rationale for this approach is the belief that the benefits of breastfeeding outweigh the potential risks of milk, but the potential benefits of milk-based formula over soy formula do not. Ten states (36%) said different specialists in their state give different recommendations for intervention in DG, and seven (25%) said they recommend at least partial milk restriction for the first year of life for all DG infants in their state (Supplementary Table 6). Combined, 82% of states said that at least some of the DG infants diagnosed in their state experience at least partial galactose restriction in the first year of life.

Discussion

We initiated this study with two goals in mind: (1) to compare ongoing practices across the USA with regard to newborn screening and follow-up testing for galactosemia to see how uniform or disparate those practices were, and if possible to see if some practices were more effective or more efficient than others and (2) to characterize the landscape of opinion and practice about screening, diagnosis, and intervention for Duarte galactosemia.

From Screen to Diagnosis

Our study revealed a broad range of practices with regard to newborn screening and follow-up testing for galactosemia that, despite their differences, were all clearly effective at

identifying newborns with classic galactosemia (Table 2 and Fig. 2a). The programs differed in their response to finding a result outside their normal range, some erring on the side of certainty, and others erring on the side of speed. Most programs sought to balance both goals by having "panic" thresholds: GALT or total galactose values that would trigger an immediate call to the referring pediatrician or metabolic clinic, and "borderline" cutoffs that would trigger either a re-screen or a follow-up test, but not necessarily an immediate call to a clinician (Berry 2012). The median detection rate for classic galactosemia among states that did *not* use total galactose in their definition of an abnormal NBS result was indistinguishable from the median detection rate in states that *did* use total galactose (1.674 vs. 1.666 per 100,000 newborns screened, respectively). If the singular goal of galactosemia NBS is detecting classic galactosemia, these data imply that including total galactose in addition to GALT activity to flag a sample as abnormal does not add value. Of course, if the goal of NBS includes detecting epimerase (GALE) or kinase (GALK)-deficiency galactosemia (reviewed in (Fridovich-Keil and Walter 2008)), testing for elevated total galactose in all samples is essential.

Follow-up testing for infants who received a positive NBS result for galactosemia involved a quantitative red blood cell GALT enzyme assay in all respondent programs in our study, and for many also a quantitative measurement of red blood cell gal-1P. It is important to note that while there was a relationship apparent among GALT activity, gal-1P, and diagnostic category assigned for infants referred for follow-up testing for galactosemia, in the data available to us there was still substantial overlap among diagnostic categories (Figs. 3, 4, and 5). Some follow-up testing programs also included *GALT* genotyping as part of their diagnostic panel for galactosemia, which clarified the diagnosis in some but not all cases.

The broad range of GALT activities observed upon follow-up testing within all diagnostic categories, except classic galactosemia, likely reflects the combined effects of biological variation and testing differences. This includes the reality that not all samples were tested immediately, and conditions during transport and sample storage may have varied widely. As a testament to these complicating factors, even repeat GALT activity tests from the same child assayed in the same program showed considerable variability (Table 4).

Hemolysate galactose-1P levels measured on follow-up testing (Fig. 4) were low in infants from all diagnostic categories except Duarte and classic galactosemia, where they ranged from low to quite high, especially in classic galactosemia. As has been well documented previously (reviewed in (Fridovich-Keil and Walter 2008)), dietary galactose exposure is a major determinant of gal-1P level for this group, likely explaining a substantial portion of the spread observed (Figs. 4 and 5) and also illustrating the limited utility of gal-1P measurement as a diagnostic tool when reliable dietary information may not be available.

Table 4 Replicate GALT activity assays performed at the Emory Genetics Laboratory between 2005 and 2011

Patient	GALT activity (μmol/h/g Hb)		Years elapsed between 1st and 2nd measurement
	1st measurement	2nd measurement	
1	17.9	11.0	1.4
2	9.2	7.9	1.0
3	8.0	8.8	0.7
4	5.8	6.2	0.4
5	7.3	5.8	0.3
6	4.4	7.1	0.1
7	10.1	13.8	0.1
8	8.7	5.7	0.1
9	7.3	4.9	0.0
10	8.4	8.4	0.0

What to Do About Duarte galactosemia?

One of the most difficult and contentious issues currently facing galactosemia newborn screening is what to do about Duarte galactosemia (Fernhoff 2010). Prior reports state that Duarte galactosemia is identified at least 10 times as frequently as classic galactosemia in some US populations (Ficicioglu et al. 2008), and the data reported here corroborate that prevalence (Fig. 2 and Supplementary Table 2). However, there is still no consensus on whether children with Duarte galactosemia are at increased risk for long-term developmental disabilities (Fernhoff 2010).

A study by Ficicioglu and colleagues testing developmental outcomes in 28 children with DG, including both children who had and children who had not consumed lactose in early childhood, found no evidence of developmental delay in either group (Ficicioglu et al. 2008), but the mean age in both groups was less than 4 years. In contrast, Powell and colleagues noted that children with DG were significantly overrepresented in a cohort of students receiving special educational resources in elementary school (Powell et al. 2009). Whether these apparently contradictory results reflect artifacts or statistics of small numbers in one or both studies, or whether they indicate that DG children are at increased risk of some developmental difficulties in mid- but not early childhood remains unclear. There is also no consensus on whether dietary restriction of galactose in the first year of life, the intervention recommended by some care providers, offers any long-term outcome benefit for children with DG.

As a result of this uncertainty some states seek to identify infants with Duarte galactosemia by NBS while others do not (van Calcar and Bernstein 2011) (Fig. 2b). Considering the prevalence of DG (estimated at close to 1/4,000 Caucasian newborns screened (Ficicioglu et al. 2008)), this uncertainty affects a large number of infants and families, and diagnosis and follow-up care for these infants adds considerably to the combined cost of NBS. Interrupting breastfeeding of infants who may not be at increased risk of negative consequences from milk exposure is also problematic and raises an ethical dilemma for both caregivers and families. Designing the NBS screen to detect infants with DG also raises the false positive rate, at least in some states (Table 3), which again adds to both the financial burden and the human cost.

A large case–control study comparing diet and long-term developmental outcomes between school age DG children with and without dietary galactose restriction in the first year of life could help resolve this uncertainty. Such a study would address whether children with DG as a group are more likely than controls to experience developmental disabilities and could also reveal whether dietary restriction of galactose in the first year of life results in more favorable long-term outcomes. Resolving this issue would empower NBS programs, healthcare providers, and the families they serve to make evidence-based decisions that would maximize public health interests and also ensure DG children the best possible long-term outcomes.

The Challenge of False Positives

False positives are a reality of any large screening protocol, and considering the potentially lethal consequence of a false negative in classic galactosemia, there is no question that NBS screening protocols should err on the side of false positives. However, false positives for galactosemia carry a significant human cost beyond the worry they cause new parents, because these infants are advised to switch from breastfeeding to soy or elemental formula until follow-up testing can be completed, which may take two to three weeks or more (Gurian et al. 2006). While studies have yet to report the success rates of mothers who attempt to resume breastfeeding following an NBS false-positive-imposed breastfeeding interruption, it is reasonable to assume these women may face a substantial challenge. As presented in Fig. 2c and Supplementary Table 2, median false positive rates among states that shared their NBS data with us varied from 1 to 2 to >500 per 100,000 newborns screened.

Tailoring NBS protocols for galactosemia to maximize identification of infants who will benefit from diagnosis and early intervention and minimize identification of infants who would be better left alone is therefore an issue of substantial urgency, especially in the current climate of limited healthcare dollars. Recognizing the shared successes of galactosemia NBS across the USA is clearly warranted, and learning from the combined experiences of these programs offers a first step toward raising the bar even higher.

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Compliance with Ethical 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)."

No data from specific individuals are included in this study and so no one signed an informed consent form to participate.

Please note that this study was approved by both the Emory University Institutional Review Board (IRB# 00024933, PI: Fridovich-Keil) and the Georgia Department

of Public Health Institutional Review Board (GA PDH IRB# 130306, PI: Fridovich-Keil). Data accessed from the Emory Genetics Lab MEDGIS database were ascertained through a HIPAA waiver granted by the Emory IRB (under IRB# 00024933).

Animal Rights

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

Contributions of Each Author

Brook Pyhtila conducted most of the data gathering, performed some of the data analysis, and participated in writing and editing the manuscript.

Kelly Shaw performed some of the data gathering, performed some of the data analysis, and participated in editing the manuscript.

Samantha Neumann performed some of the data gathering and participated in editing the manuscript.

Judith Fridovich-Keil originated the project, oversaw the data gathering and analysis, wrote and edited most of the manuscript, and coordinated the contributions of the other authors.

1-Sentence Synopsis

Newborn screening for galactosemia in the USA is a success story, but significant challenges remain.

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RESEARCH REPORT

Phenotypic Variability in Patients with Fanconi–Bickel Syndrome with Identical Mutations

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Abstract *Objective*: To describe the phenotypic features of an ethnically homogenous group of patients with Fanconi–Bickel syndrome harboring the p.R310X mutation.

Methods: The study group consisted of eight patients from a single Bedouin family with clinically and molecularly diagnosed Fanconi–Bickel syndrome who had been followed at the same tertiary medical center for 8 years or more. All were homozygous for the p.R310X mutation. The medical files were reviewed for presenting signs and symptoms, laboratory and imaging findings, treatment regimens, and disease severity over time.

Results: Seven patients were diagnosed at our center before age 1 year, and one transferred from another center at age 16 years. Most patients presented with failure to thrive and/or hepatomegaly. All had short stature and doll-like facies. Most had biochemical abnormalities. Evaluation of the long-term findings revealed a wide spectrum of disease severity according to the following parameters: growth patterns, maximal electrolyte replacement therapy, skeletal and renal complications, frequency of follow-up visits, and hospitalizations for disease exacerbations. There was no apparent association of the clinical picture at presentation and later disease severity.

Conclusion: Fanconi–Bickel syndrome has a broad phenotypic variability in patients harboring the same homozygous p.R301X mutation. This finding might be explained by genetic elements such as modifier genes and epigenetic factors, as well as the effects of still-undetermined environmental and nutritional factors.

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Introduction

Fanconi–Bickel syndrome (FBS, OMIM 227810) is a rare autosomal recessive disease caused by a deficiency of glucose transporter protein 2 (GLUT2) (Santer et al. 1998), a member of the facilitative glucose transporter family. GLUT passively mediates the bidirectional transport of monosaccharides, mainly D-glucose and D-galactose, in hepatocytes, pancreatic β cells, renal tubular cells, and enterocytes (Santer et al. 2002a). The gene encoding GLUT2 (*SLC2A2*) is located on chromosome 3q26.1–q26.3 (OMIM 138160). Of the 59 or more known mutations that are responsible for FBS, p.R301X, a nonsense mutation at nucleotide c.1213 of exon 7, is the most common (http://www.biobase-international.com).

Signs and symptoms of FBS begin in infancy and include failure to thrive (FTT), hepatomegaly, hypophosphatemic rickets, and short stature (Santer et al. 2002b). Biochemically, the disease is characterized by a general renal proximal tubular defect (glycosuria, bicarbonate wasting, aminoaciduria, renal tubular acidosis, hyperphosphaturia) and carbohydrate abnormalities that include postprandial hyperglycemia, fasting hypoglycemia, and hypergalactosemia (Odièvre et al. 2002). The diagnosis is suspected on the basis of the clinical and laboratory findings and confirmed by molecular genetic testing. Management consists of nutritional supplement with

uncooked cornstarch and fluid and electrolyte replacement therapy (Lee et al. 1995).

FBS is generally considered a well-defined clinical condition (Odièvre et al. 2002), although there are reports of atypical clinical signs or an unusually mild disease course (Aperia et al. 1981; Berry et al. 1995; Grünert et al. 2012). Long-term follow-up studies have focused mainly on specific disease characteristics, such as renal (Manz et al. 1987) and hepatic complications (Saltik-Temizel et al. 2005). In a study of the genotype–phenotype correlation in FBS, Santer et al. (2002b) investigated the various possible presentations of FBS due to null versus missense mutations.

The aim of the present study was to describe the presenting and long-term features of FBS and to assess phenotypic variability in an ethnically homogenous group of patients all harboring the p.R301X mutation.

Patients and Methods

The Bedouin are an ethnic Arabian, traditionally nomadic, group divided into tribes and clans with a complex consanguinity. The cohort for the present study consisted of seven patients (four male) of a single family (Fig. 1) and a male patient born to third-degree consanguineous parents from the same Bedouin clan, all homozygous for the p.R301X mutation. The patients had been followed at the Day Hospitalization Unit of Schneider Children's Medical Center of Israel for more than 8 years. For the present study, the medical files were reviewed for data on presenting symptoms and signs, laboratory and imaging findings, and treatment regimens. Disease severity was evaluated by the following parameters: growth patterns, mean electrolyte replacement therapy, skeletal and renal complications, frequency of follow-up visits, and hospitalizations for disease exacerbation. In addition the parents of the minors and the adult patient were assessed for the severity of the disease on a scale from 1 (mild) to 3 (severe).

Growth was evaluated in the pre-pubertal stage (age 5–7 years) by calculating the standard deviation score (*Z*-score) for mean height for age and sex, as defined by the guidelines of the World Health Organization (WHO) Global Database on Child Growth and Malnutrition (de Onis and Blössner 2003). Hyperglycemia and hypoglycemia were defined as glucose >170 mg/dL and <50 mg/dL, respectively. Biochemical urinary parameters were taken by urine spots; amino acids excretion was measured qualitatively and phosphaturia at presentation/diagnosis was estimated by calculating the tubular reabsorption of phosphate (TRP). The presence of rickets or osteopenia/osteoporosis was evaluated after diagnosis by plain bone X-rays of the distal femur, proximal tibia, distal radius, and distal ulna. The presence of nephropathy and nephrocalcinosis was evaluated by repeated urine analyses and sonography.

Results

Patients

Seven patients were referred to our medical center before age 1 year; 3 were 1 month old or younger. The remaining patient (no. 6) was diagnosed at age 3 years at another hospital, where he was managed until age 16 years. In all cases, the diagnostic investigation was prompted by patient symptoms and signs or by incidental physical findings on routine general checkup. None of the patients was diagnosed prenatally. All were managed by corn starch supplement and fluid and electrolyte replacement therapy.

Detailed Patient Descriptions

Patient 1 presented at age 11 months with FTT, doll-like facies, hepatomegaly, developmental delay, rachitic rosary, and bowed legs. Laboratory work-up at presentation revealed remarkable fluctuations in blood glucose levels, metabolic acidosis, hypocalcemia, hypophosphatemia, hypouricemia, and hypokalemia in addition to massive glucosuria, hyperphosphaturia, and generalized aminoaciduria compatible with proximal tubulopathy. Over the course of follow-up, early radiologic abnormalities of rickets and osteopenia improved considerably. At no time did the patient require hospitalization. Relatively low amounts of phosphorus and potassium supplementation were needed.

Patient 2 presented at age 7 months with FTT, doll-like facies, hepatomegaly, rachitic rosary, and enlarged wrists and ankles. Laboratory work-up at presentation revealed significant fluctuations in blood glucose levels, hypoglycemia, postprandial hyperglycemia, mild metabolic acidosis, hypophosphatemia, hypouricemia, massive glucosuria, excessive phosphate losses, and generalized aminoaciduria. Skeletal evaluation during follow-up revealed severely stunted growth, bilateral genu varum, rickets, and osteopenia; nephrocalcinosis developed at age 5 years. High amounts of electrolyte supplementation were necessary. During follow-up, the patient was hospitalized nine times for metabolic acidosis, hypokalemia, hypophosphatemia, and three events of hypocalcemic tetany.

Patient 3 presented at age 6 months with FTT, doll-like facies, and hepatomegaly. Laboratory work-up at presentation revealed remarkable fluctuations in blood glucose levels, hypokalemia, hypouricemia, massive glucosuria, hyperphosphaturia, and generalized aminoaciduria. Skeletal evaluation over the course of follow-up showed short stature, bilateral genu varum, and osteopenia. Low amounts of phosphorus

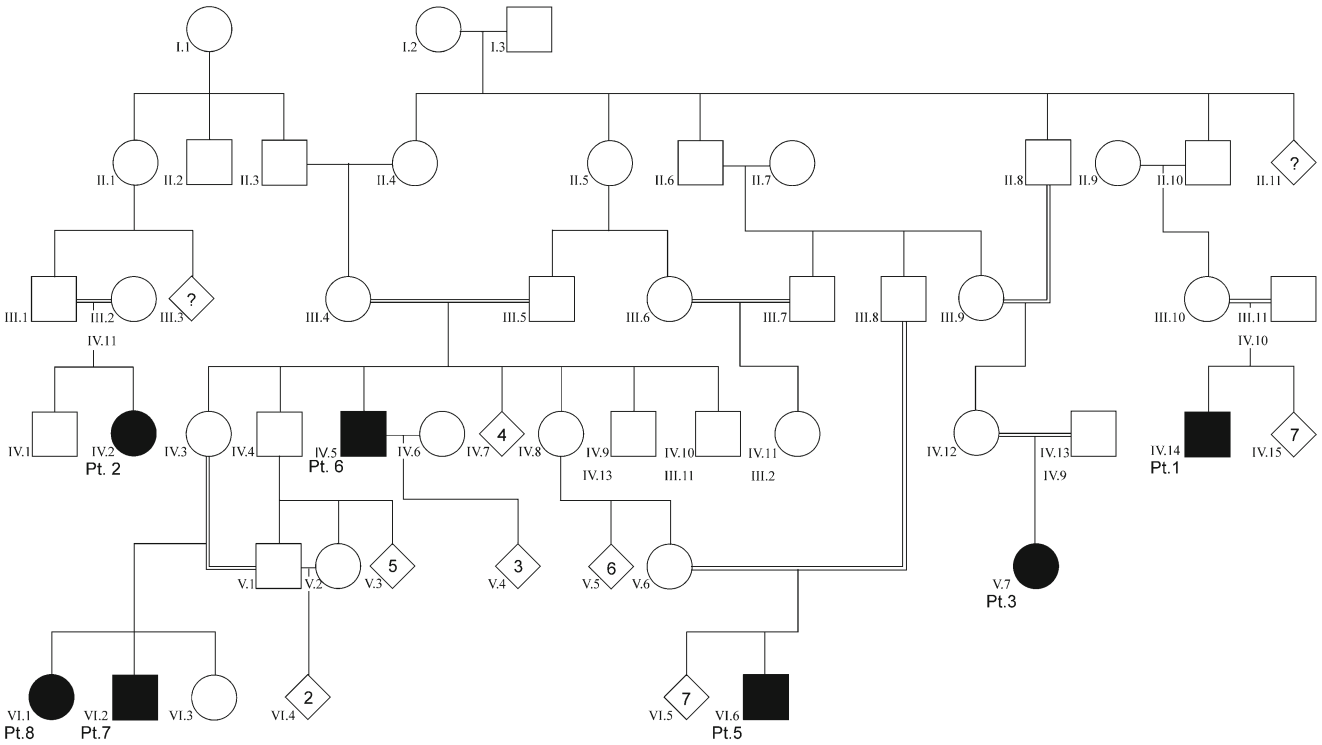

Fig. 1 The pedigree of the Bedouin sibship Note: III.2 and IV.11, III.11 and IV 10, IV. 9, and IV.13 are the same individuals. The intrafamilial relation of patient 4 could not be tracked

replacement therapy were needed. During follow-up, the patient was hospitalized once for metabolic acidosis during acute gastroenteritis.

Patient 4 presented at age 1 month with symptomatic hypoglycemia, prolonged diarrhea, FTT, hepatomegaly, hypotonia, and rickets. Laboratory work-up at presentation showed significant fluctuations in blood glucose levels, hypoglycemia, postprandial hyperglycemia, hypocalcemia, hypophosphatemia, hypouricemia, hypokalemia, and hyponatremia. His urine studies revealed massive glucosuria, hyperphosphaturia, and generalized aminoaciduria compatible with proximal tubulopathy. X-rays showed signs of rickets. Skeletal evaluation during follow-up revealed severely stunted growth and rickets. The patient had recurrent nocturnal symptomatic hypoglycemic episodes that necessitated gastrostomy for continuous night feedings until age 3 years. He had hypercalciuria and developed nephrocalcinosis at age 6 years. He required high amounts of electrolyte replacement therapy. During follow-up, he was hospitalized 8 times for electrolyte imbalance and once, in the pediatric intensive care unit (PICU), for hyponatremia. This patient was initially misdiagnosed with insulin-dependent diabetes mellitus; FBS was diagnosed at age 5 months.

Patient 5 presented at age 1 month with recurrent vomiting, FTT, hypotonia, and hepatomegaly. Laboratory work-up at presentation revealed significant fluctuations in blood glucose levels, postprandial hyperglycemia, hypocalcemia, hypophosphatemia, and hypouricemia; urine studies showed massive glucosuria, excessive phosphate, and generalized aminoaciduria compatible with proximal tubulopathy. Skeletal evaluation during follow-up revealed severely stunted growth, rickets, and osteopenia. Over the course of follow-up he had hypercalciuria, and developed nephrocalcinosis at age 4.5 years. High amounts of phosphorus replacement therapy were needed. During follow-up, he was hospitalized nine times for diarrhea, prerenal azotemia, recurrent pneumonia, and symptomatic hypocalcemia, and twice, in the PICU, for hypocalcemia.

Patient 6 presented at age one month with FTT, doll-like facies, hepatomegaly, and hypotonia. Laboratory work-up at presentation revealed significant fluctuations in blood glucose levels including hypoglycemia and postprandial hyperglycemia, hypocalcemia, hypophosphatemia, hypokalemia, hypouricemia, massive glucosuria, hyperphosphaturia, and generalized aminoaciduria; X-rays revealed signs of rickets. He was diagnosed at another hospital when he was 3 years old. The patient was hospitalized only once, for pathological fracture. Final height was 154 cm, with mild osteopenia. Since he was under our follow-up he did not require electrolyte supplementation to age 29 years, when he was lost to follow-up. The patient is married and has three healthy children.

Patient 7 presented at age 1 year with hepatomegaly. Laboratory work-up at presentation revealed significant

Table 1 Main demographic, clinical, laboratory, and radiological findings at first presentation in eight patients with Fanconi–Bickel syndrome with the p.R310X mutation

Pt. no./year of birth	Sex	Age at presentation/ symptoms and signs	Main laboratory findings at diagnosis		Skeletal findings	
			Blood	Urine	Clinical	Radiologic
1/2001	M	11 months/ FTT Wt 15, length 3 Hepatomegaly (+5) Developmental delay	Ca 8.7 pH 7.29 HCO_3 17.4 K 3.0 Na 132 Phos 2.5 Uric acid 1.5 Glucose 63–157	Glucose >1,000 mg/dL General aminoaciduria TRP 83%	Bowed legs Rachitic rosary	Rickets Osteopenia
2/2002	F	7 months/ FTT Wt <2, length <2 Hepatomegaly (+6)	Ca 8.4 pH 7.36 HCO_3 12.5 K 2.5 Na 135 Phos 1.8 Uric acid 0.8 Glucose 46–202	Glucose >1,000 mg/dL General aminoaciduria TRP 68%	Rachitic rosary Enlarged wrists and ankles	Rickets Osteopenia
3/2001	F	6 months/ FTT Wt 25, length 3 Hepatomegaly (+3)	Ca 10.3 pH 7.33 HCO_3 17 K 2.0 Na 133 Phos 4.4 Uric acid 0.8 Glucose 61–160	Glucose >1,000 mg/dL General aminoaciduria TRP 80%	None	Osteopenia
4/1998	M	1 month/ Symptomatic hypoglycemia Prolonged diarrhea Hepatomegaly(+3)	Ca 7.8 pH 7.30 HCO_3 16.0 K 2.4 Na 127 Phos 2.4 Uric acid 0.7 Glucose 35–271	Glucose >1,000 mg/dL General aminoaciduria TRP 57%	Enlarged wrists and ankles	Rickets
5/2002	M	1 month/ FTT Wt <3, length <3 Recurrent vomiting Hypotonia Hepatomegaly (+2)	Ca 8.7 pH 7.32 HCO_3 18.0 K 4.1 Na 140 Phos 2.4 Uric acid 0.8 Glucose 50–190	Glucose >1,000 mg/dL General aminoaciduria TRP 64%	None	None
6/1980	M	1 month/ FTT Wt 5, length 3 Hepatomegaly (+5) Hypotonia	Ca 7.8 pH 7.36 HCO_3 24.6 K 3.3 Na 138 Phos 2.3 Uric acid 1.1 Glucose 46–202	Glucose >1,000 mg/dL General aminoaciduria TRP 79%	None	Rickets

(continued)

Table 1 (continued)

Pt. no./year of birth	Sex	Age at presentation/ symptoms and signs	Main laboratory findings at diagnosis		Skeletal findings	
			Blood	Urine	Clinical	Radiologic
7/1997	M	1 year/ Hepatomegaly (+4)	Ca 10.8 pH 7.33 HCO_3 19.8 K 3.2 Na 135 Phos 3.5 Uric acid 1.8 Glucose 61–210	Glucose >1,000 mg/dL General aminoaciduria TRP 81%	None	None
8/1999	F	1 year/ FTT Wt 5, length <3 Hepatomegaly (+4)	Ca 7.1 HCO_3 18 pH 7.28 K 3.8 Na 136 Phos 2.0 Uric acid 1.3 Glucose 53–190	Glucose >1,000 mg/dL General aminoaciduria TRP 80%	Bowed legs Enlarged wrists and ankles	Rickets

Note: Patients 4 and 6 were diagnosed at ages 5 months and 3 years, respectively

Normal biochemical values in blood: Hypoglycemia <50 mg/dL, hyperglycemia >170 mg/dL, Ca 9.0–11.0 mg/dL, HCO_3 22–26 mmol/L, pH 7.35–7.45, K 3.5–5.0 mEq/L, Na 135–145 mEq/L, uric acid 2.0–7.0 mg/dL, phosphorus 3.8–6.5 mg/dL. Normal biochemical values in urine: TRP (Total reabsorption phosphate): >85%. Calcium/creatinine ratios by age: 0–6 months. < 0.8, 7–12 months. <0.6, >2 years <0.21

FTT—failure to thrive (growth below the 5rd percentile or a change in growth that has crossed two major growth percentiles in a short time); numbers of weight and length percentiles are presented

Hepatomegaly (centimeters below midline costal margin)

fluctuations in blood glucose levels with postprandial hyperglycemia, mild metabolic acidosis, hypokalemia, hypouricemia, massive glucosuria, hyperphosphaturia, and generalized aminoaciduria. Skeletal evaluation during follow-up showed short stature and radiologic signs of rickets. High amounts of electrolyte replacement therapy were needed. During follow-up, the patient was hospitalized 14 times for bronchopneumonia, acute gastroenteritis, and hypokalemia, and twice, in the PICU, for hypokalemia and metabolic acidosis.

Patient 8 presented at age 1 year with FTT, hepatomegaly, bowed legs, and enlarged wrists and ankles. Laboratory work-up at presentation revealed significant fluctuations in blood glucose levels with postprandial hyperglycemia, metabolic acidosis, hypocalcemia, hypophosphatemia, hypouricemia, massive glucosuria, hyperphosphaturia, and generalized aminoaciduria compatible with proximal tubulopathy; X-rays showed signs of rickets. Skeletal evaluation during follow-up showed severely stunted growth and rickets. Very high amounts of phosphorus supplementation were needed. Nephrocalcinosis developed at age 9 years. During follow-up, the patient was hospitalized six times for bone pain that necessitated intravenous therapy with opiates.

Summary of Findings

Presentation

The main demographic, clinical, laboratory, and radiological findings at presentation and diagnosis are summarized in Table 1. All patients had short stature and doll-like facies. All had prominent fluctuations in blood glucose levels and hypouricemia; most patients had also metabolic acidosis, hypophosphatemia hypocalcemia, and hypokalemia. In addition, all had massive glucosuria, excessive phosphate losses with low TRP and generalized aminoaciduria compatible with proximal tubulopathy; none had microalbuminuria at presentation. Five patients had clinical and/or radiological signs of rickets at presentation.

Follow-up

The duration of follow-up ranged from 8.7 to 14 years (average 11.4 years). The following biochemical abnormalities were found in all patients during follow-up: postprandial hyperglycemic events, asymptomatic fasting ketotic hypoglycemia, hypouricemia, hyponatremia, hypokalemia; most had hypocalcemia and metabolic acidosis. All had

Table 2 Clinical findings in eight patients with Fanconi–Bickel syndrome with the p.R310X mutation during follow-up

Pt. no.	F-U duration (year)	Annual visits (average)	No. hospitalizations/diagnoses	No. ICU admissions/ diagnoses	Ht. SDS[a]
1	11.7	5.6	None	None	−2.78
2	9.3	9.6	9/Hypocalcemia with/without tetany Metabolic acidosis Hypophosphatemia	1/Hypocalcemic tetany	−4.33
3	10.5	4.3	1/Metabolic acidosis during acute gastroenteritis	None	−2.70
4	14	12.5	8/Hyponatremia Hypokalemia Hypophosphatemia	1/Hyponatremia	−3.89
5	10	7.5	9/Diarrhea Acute prerenal azotemia Recurrent pneumonia Hypocalcemia	2/Hypocalcemia	−5.32
6	13	2.6	1/Pathological fracture, lt tibia	None	
7	14	6.1	14/Bronchopneumonia Acute gastroenteritis Hypokalemia	2/Hypokalemia Metabolic acidosis	−3.18
8	8.7	6.6	6/Bone pain Hypophosphatemia	None	−3.77

[a] Height standard deviation score (*Z*-score) for mean height for age and gender

massive glucosuria, generalized aminoaciduria, hypercalciuria, and hyperphosphaturia. Tables 2 and 3 summarize the main clinical, laboratory, and radiological findings during the course of the disease; subjective disease-severity assessment is presented in Table 3; Figs. 2a, b show the males and females' height curves, respectively. Longitudinal follow-up revealed variability in disease severity. Two patients (nos.1 and 3) needed small amounts of replacement therapy, and patient 6 (referred to our center at age 16 years) needed no replacement therapy when he was under our follow-up. Two patients (nos. 2 and 4) had exceptionally severe biochemical abnormalities: patient 2 had three hypocalcemic tetanic events, and patient 4 had recurrent nocturnal symptomatic hypoglycemia that necessitated gastrostomy for continuous night feedings until age 3 years.

Clinical and/or radiological bone pathologies (rickets, osteopenia, osteoporosis) occurred in all patients during follow-up. Patient 8 had a severe bilateral genu varum deformity associated with incapacitating pain that warranted intravenous analgesics and surgical orthopedic correction. Four patients (nos. 2, 4, 5, and 8) acquired nephrocalcinosis at ages 4.5–9 years. Microalbuminuria developed in all patients within 3–26 years following diagnosis.

Discussion

The present study describes the early and long-term characteristics of eight patients with FBS from a single Bedouin sibship, all of whom were homozygous for the p.R301X *GLUT2* mutation. The findings highlight the typical clinical features at presentation and point to a phenotypic variability over time despite the presence of the identical mutation.

FTT and/or hepatomegaly, prominent fluctuations in blood glucose levels, and proximal tubulopathy were found at presentation or during diagnosis or both in all our patients, similar to other series of FBS (Santer et al. 1998, 2002b). Although there were only mild differences in age at diagnosis, skeletal involvement ranged from none to radiological and/or clinical signs of rickets and osteopenia.

Evaluation of the follow-up findings revealed a spectrum of disease severity as reflected by growth parameters, frequency of follow-up visits, hospitalizations for disease exacerbations, mean amount of electrolyte replacement therapy, and skeletal and renal complications (Fig. 2a, b, Tables 2 and 3). Of note, the variability in disease severity correlated with the subjective disease-severity scores assessment of the parents and the adult patient.

A broad phenotypic expression in FBS has been reported previously, with specific mutations causing atypical clinical manifestations such as intestinal malabsorption without hepatomegaly (Aperia et al. 1981) and glomerular hyperfiltration (Berry et al. 1995). Others described an unusually mild disease course with normal growth and absence of hepatomegaly, renomegaly, or rickets (Grünert et al. 2012).

Few patients with FBS due to homozygosity for the p.R301X mutation have been described. Santer et al. (1998) followed the first patient reported with what is now

Table 3 Laboratory and radiological findings of 8 patients with Fanconi–Bickel syndrome with the p.R310X mutation during follow-up

Pt. no.	Main laboratory findings		Mean electrolyte replacement (kg/day)	Skeletal findings		Nephrocalcinosis (age at detection)	Disease severity score
	Blood	Urine		Clinical	Radiologic		
1	Ca 8.7–10.2 HCO_3 17.0–23.5 K 3.0–4.6 Na 132–137 Phos 2.5–4.6 Uric acid 1.3–2.0 Alk phos (8–10): 333–336. (11–13): 330–482 PTH 30.7–155.0	Ca/creat 0.26–0.95 Glucosuria >1,000 mg/dL General aminoaciduria Microalbumin (9 years) 460	Phos 70 mg K 0.8 meq	Valgus knee	Mild osteopenia	No	1
2	Ca 8.4–10.1 HCO_3 12.5–23.7 K 2.5–5.2 Na 130–139 Phos 1.8–7.6 Uric acid 0.8 -1.0 Alk phos (3–5): 317–554 (8–10): 504–697 PTH 10.6-68.8	Ca/creat 0.13–0.6 Glucosuria >1,000 mg/dL General aminoaciduria Microalbumin (5 years) 1,133	Phos 110 mg K 6.6 meq	Bilateral genu varum	Rickets Osteopenia	Yes (5 years)	2
3	Ca 8.7–11.0 HCO_3 19.1–27.1 K 2.0–4.7 Na 133–143 Phos 3.3–9.5 Uric acid 0.6–1.8 Alk phos (3–5): 280–522 (8–10): 376–4,400 PTH 14.4–85	Ca/creat 0.14–4.5 Glucosuria >1,000 mg/dL General aminoaciduria Microalbumin (6 years) 563	Phos 25 mg	Bilateral genu varum	Osteopenia	No	1
4	Ca 7.8–9.7 HCO_3 14.0–25.3 K 2.4–4.4 Na 127–139 Phos 1.9–6.2 Uric acid 0.7–2.0 Alk phos (3–5): 600–1,400 (8–10): 388–1,280 (11–13): 505–986 PTH 12.7–224.3	Ca/creat 0.22–2.38 Glucosuria >1,000 mg/dL General amino aciduria Microalbumin (6 years) 302	Phos 98 mg K 4.4 meq	Bilateral genu varum	Rickets Osteopenia	Yes (6 years)	3
5	Ca 6.5–10.7 HCO_3 13.4–28.6 K 2.6–5.9 Na 131–145 Phos 2.2–11.6 Uric acid 0.6–1.5 Alk phos (3–5): 342–677.0 (8–10): 373–1,159 PTH 60.9–191.0	Ca/creat 0.15–5.0 Glucosuria >1,000 mg/dL General aminoaciduria Microalbumin (4 years) 124	HCO_3 0.3 meq Phos 120 mg K 2.1 meq		Rickets Osteopenia	Yes (4.5 years)	2
6	Ca 7.8–11.0 HCO_3 24.6 K 3.3–4.8 Na 132–144 Phos 2.3–3.6 Uric acid 1.0–1.5 Alk phos (20–30): 150–255 PTH 30.0–40.5	Ca/creat 0.34–2.9 Glucosuria >1,000 mg/dL General aminoaciduria Microalbumin (24 years) 60			Mild osteopenia	No	1

(continued)

Table 3 (continued)

Pt. no.	Main laboratory findings		Mean electrolyte replacement (kg/day)	Skeletal findings		Nephrocalcinosis (age at detection)	Disease severity score
	Blood	Urine		Clinical	Radiologic		
7	Ca 9.87–11.0 HCO_3 17.0–22.0 K 3.2–4.7 Na 134–143 Phos 4.3–5.4 Uric acid 1.5–1.8 Alk phos (3–5): 397–4,100 (8–10): 368–440 (11–13): 437–747 PTH 15.3–67.3	Ca/creat 0.22–0.23 Glucosuria >1,000 mg/dL General aminoaciduria Microalbumin (11 years) 293	HCO_3 0.5 meq Phosphor 85 mg K 1.7 meq		Rickets	No	2
8	Ca 6.8–10.6 HCO_3 11.0–21.4 K 2.6–4.9 Na 132–139 Phos 1.4–5.5 Uric acid 0.5–1.4 Alk phos (8–10): 327–1,210 (11–13): 380–740 PTH 68.7–425.0	Ca/creat 0.16–1.0 Glucosuria >1,000 mg/dL General aminoaciduria Microalbumin (9 years) 1,207	HCO_3 3.2 meq Phos 180 mg K 2.8 meq/kg	Bone pains Bilat. genu varum	Rickets Osteoporosis	Yes (9 years)	3

Note: Normal biochemical values in blood: Ca 9.0–11.0 mg/dL, HCO_3 22–26 mMol/L, K 3.5–5.0 mEq/L, Na 135–145 mEq/L
Phosphorus values by age in years: 1–3: 3.8–6.5 mg/dL, 4–11: 3.7–5.6 mg/dL, 12–15: 2.9–5.5 mg/dL, 16–19: 2.7–4.7 mg/dL, Uric acid 2.0–7.0 mg/dL, Hypoglycemia <50 mg/dL, hyperglycemia >170 mg/dL
PTH (parathyroid hormone) 15.0–65 ng/L
For alkaline phosphatase, because norms change with growth, measurements are presented by age (indicated in parentheses). Normal values: 3-5/8–10 years 37–448 U/L, 11–13 years 74–390 U/L, 20–30 years 37–270 U/L
Normal biochemical values in urine: microalbuminuria (age at diagnosis in years) 0–17 mcg/mgCr, calcium/creatinine ratios by age: 0–6 months, <0.8, 7–12 months, <0.6, >2 years <0.21
Disease severity scores as assessed subjectively by the parents of the minors and the adult patient on a scale from 1 (mild) to 3 (severe)

recognized as typical features of FBS. Like our patient 6, he had not been receiving continuous medications since infancy, and like our patients 1, 3, and 6 he had neither rickets nor nephrocalcinosis on follow-up. Riva et al. (2004) described a neonate who presented with fever, vomiting, mild hepatomegaly, and proximal tubulopathy who was managed with galactose-free diet, frequent small meals, and phosphate and vitamin D supplements. At 1 year, growth was normal, and there were no clinical abnormalities. Of our three patients who presented in the neonatal period, one (patient 5) had also recurrent vomiting and hepatomegaly at presentation but he had a severe disease course. The other two presented with prolonged diarrhea with symptomatic hypoglycemia and rickets (patient 4) and hepatomegaly with hypotonia (patient 6). The former had a severe disease course while patient 6 had a mild disease. Another reported patient with the p.R301X mutation presented with hypoglycemic seizures, FTT, hepatomegaly, tubulopathy, and rickets, in addition to facial dysmorphism, global developmental delay, hyperinsulinism, and refractory hypoglycemia (Hoffman et al. 2007). Interestingly, he inherited the disease via maternal isodisomy of chromosome 3. Patient 4 had recurrent symptomatic hypoglycemic episodes but they were not refractory and they resolved in late infancy. In contrast to the patient of Hoffman et al. (2007) none of our patients had facial dysmorphism and none exhibited severe developmental delay.

It is noteworthy that although earlier studies found that disease severity is somewhat ameliorated during the second decade of life (Santer et al. 2002b), we noted no remarkable change in our patients during follow-up from infancy to age 10–14 years.

The bone complications, mainly rickets, in five patients (nos 2, 4, 5, 7, 8) and the development of nephrocalcinosis in most of them may have been due to difficulty in balancing the high tubular phosphorus loss, as reflected by their need for high doses of phosphorus replacement

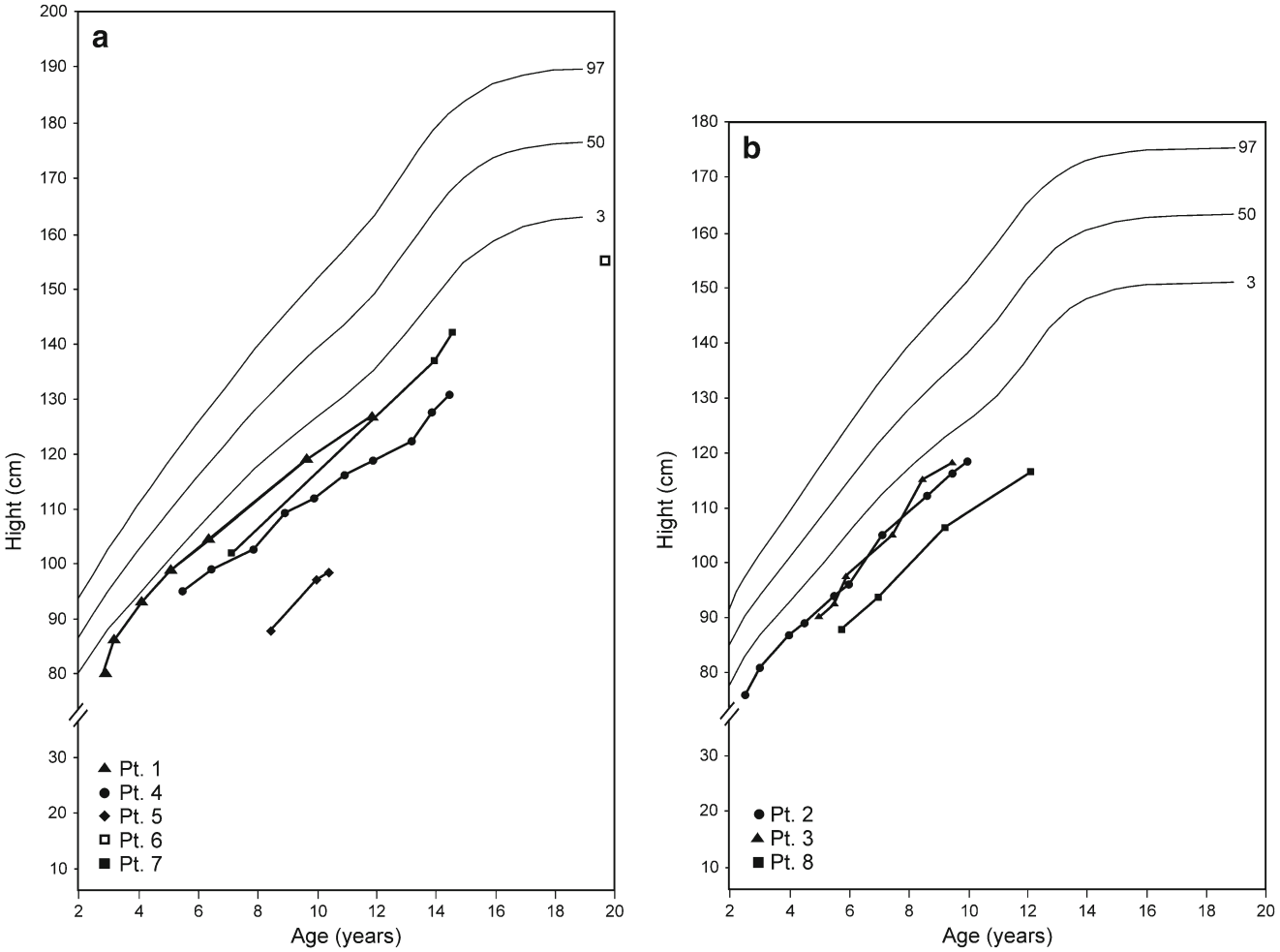

Fig. 2 Patient height curves. (**a**) Males; (**b**) Females

therapy and frequent follow-up visits to monitor electrolyte levels. This led to secondary hyperparathyroidism in most cases as well as to renal calcium–phosphate deposits, with consequent bone mineralization abnormalities and nephrocalcinosis, respectively.

Glomerulopathy is not a common complication of FBS (Santer et al. 1998). It was previously reported in one patient with microalbuminuria, glomerular hyperfiltration, and glomerular mesangial expansion (Berry et al. 2005), and in four patients with reduced glomerular filtration (Manz et al. 1987). It is unclear if microalbuminuria is a manifestation of a glomerular insult. It is possible that in our patients, the microalbuminuria was attributable to excessive fluid intake for chronic dehydration secondary to the osmotic diuresis induced by impaired glucose reabsorption (Clark et al. 2008; Viberti et al. 1982).

Patient compliance with electrolyte replacement therapy and corn starch supplementation is crucial to avoid complications of FBS. Good parental care during follow-up was found for all our patients with severe disease, as reflected by their strict attendance to scheduled visits, frequent telephone consults, and unscheduled visits for minor health problems. At the same time, our sole adult patient (no. 6), who frequently failed to appear for follow-up, had a mild FBS phenotype without significant disease complications or need for replacement therapy. This patient is one of the few reported adults with FBS (Santer et al. 1997; Pena and Charrow 2011), and the third reported fertile male (Von Schnakenburg and Santer 2011).

We speculate that the broad spectrum of disease severity among our patients might be explained by modifier genes, as suggested for other monogenic diseases (Weatherall 2001), or unknown epigenetic factors (Jansen et al. 2010). At the same time, despite the similar lifestyle and place of residence, the disease course in the individual patients could have been modified by external causes, such as insufficient corn starch management or undetermined environmental factors.

In conclusion, the present follow-up study points to the variability in the clinical spectrum of FBS among patients homozygous for the p.R301X mutation. The initial clinical

manifestations are not predictive of the long-term severity of the disease.

Take-Home Message

There is a broad spectrum of clinical severity of Fanconi–Bickel syndrome in patients harboring a homozygous p.R301X mutation.

Author Contributions

Elena Fridman, MD, MSc: Guarantor, data analysis and interpretation, drafting of the manuscript.

Avraham Zeharia, MD: Conception and design of the study, intellectual input, revision of the manuscript.

Tal Markus-Eidlitz, MD: Revision of the content.

Yishai Haimi Cohen, MD: Design, analysis, and revision of the manuscript.

All coauthors have seen the final version of the manuscript, confirm that the work has not been published/submitted elsewhere, and agree with the submission.

Details of funding

No funding was received for the study.

Conflict of Interest

Elena Fridman, Avraham Zeharia, Tal Markus-Eidlitz, and Yishai Haimi Cohen declare that they have no conflict of interest.

Ethics

This retrospective analysis of prospectively collected data was approved by the Rabin Medical Center Research Review Board. Any identifying information about patients was omitted after initial data collection; hence, the need for individual-patient informed consent was waived.

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CASE REPORT

Successful Management of Enzyme Replacement Therapy in Related Fabry Disease Patients with Severe Adverse Events by Switching from Agalsidase Beta (Fabrazyme®) to Agalsidase Alfa (Replagal®)

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Abstract *Background*: Enzyme replacement therapy (ERT) is the only approved therapy for Fabry disease. In June 2009, there was a worldwide shortage of agalsidase beta, necessitating dose reductions or switching to agalsidase alfa in some patients.

Case presentation: We present two cases of Fabry disease (a parent and a child) who received agalsidase beta for 27 months at the licensed dose and 10 months at a reduced dose, followed by a switch to agalsidase alfa for 28 months.

Case 1, a 26-year-old male had severe coughing and fatigue during ERT with agalsidase beta requiring antitussive and asthmatic drug therapy. After switching to agalsidase alfa, the coughing gradually resolved completely.

Case 2, a 62-year-old female had advanced cardiac manifestations at the time of diagnosis. Despite receiving ERT with the approved dose of agalsidase beta, she experienced aggravation of congestive heart failure and was hospitalized. After switching to agalsidase alfa with standard care in heart disease, BNP level, echocardiographic parameters, eGFR rate and lyso-Gb3 levels were improved or stabilized.

Conclusions: We report on two Fabry disease patients who experienced severe adverse events while on approved and/or reduced doses of agalsidase beta. Switching to agalsidase alfa associated with standard care in heart disease led to resolution or improvement in the cardiorespiratory status. And reduction in dose associated with standard care in respiratory disease was useful for decrease in cough and fatigue. Plasma BNP level was useful for monitoring heart failure and the effects of ERT.

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Background

Fabry disease (OMIM 301500) is an X-linked, inherited lysosomal storage disorder caused by a deficiency of the enzyme α-galactosidase A (Nagueh 2003). α-Galactosidase deficiency results in the accumulation of globotriaosylceramide (Gb3 or ceramide trihexoside) and other neutral glycolipids in many tissues and cell types throughout the body, ultimately leading to cellular abnormalities and triggering inflammation and fibrosis (Zarate and Hopkin 2008). The consequences of these biochemical changes can be wide-ranging, and include acroparesthesias, hypohidrosis, angiokeratomas, corneal opacities, cerebrovascular lesions, cardiac disorders and renal dysfunction (Nagueh 2003; Fabry 2002). Organ dysfunction and pain are usually the first clinical manifestations of Fabry disease and, at this stage, some degree of irreversible damage may already have occurred. The onset of symptoms generally starts during childhood, and by middle-age life-threatening complications often develop in untreated patients (Zarate and Hopkin 2008).

Enzyme replacement therapy (ERT) is currently the only approved therapy for Fabry disease (Lidove et al. 2007).

Two preparations of the enzyme α-galactosidase A are now available in Japan: agalsidase alfa (Replagal®; Dainippon Sumitomo Pharma) and agalsidase beta (Fabrazyme®; Genzyme). Since June 2009, there had been a worldwide shortage of agalsidase beta as a result of viral contamination in the manufacturer's production facility. This shortage led to a series of recommendations including dosage reductions (i.e., spreading the usual dose over a longer time period). These guidelines were reviewed by a consensus group of treating physicians, with the outcomes reported by the European Medicines Agency in October 2010 (European Medicines Agency 2011; Sirrs 2011; Linthorst et al. 2011). At this meeting it was noted that there was a steady increase in the number of adverse events reported, presumably because of clinical deterioration in patients receiving the reduced dose of agalsidase beta. Currently, the general consensus is that patients should receive only the licensed dose of agalsidase beta (1 mg/kg once every 2 weeks), and if this is not possible, then switching to treatment with agalsidase alfa (0.2 mg/kg once every 2 weeks) is an option (European Medicines Agency 2011). However, only limited clinical data are available regarding patients in whom treatment has been switched between the different forms of α-galactosidase A enzyme (European Medicines Agency 2011; Sirrs 2011; Linthorst et al. 2011).

Recently, an observational study involving 11 patients with Fabry disease who were switched from agalsidase beta to agalsidase alfa showed that indices of renal function, cardiac mass, pain and quality of life were maintained after switching (Tsuboi and Yamamoto 2012). Another study found that a reduced dose of agalsidase beta was not associated with an increase in the incidence of clinical events compared with the full dose, and neither was a switch to agalsidase alfa (Smid et al. 2011).

In this report, we present two cases with Fabry disease, a parent and child, who received 27 months of treatment with agalsidase beta at the registered dose and 10 months of treatment at the reduced dose, followed by a switch to 28 months of treatment with agalsidase alfa. We analyzed cardiac involvement using echocardiography and plasma brain natriuretic peptide (BNP) levels, renal function (estimated glomerular filtration rate; eGFR), and plasma Gb3 and lyso-Gb3 levels.

Case Presentation

Case 1 (Male, 26 Years Old)

This patient was diagnosed with Fabry disease in 2005 at the age of 19. He was initially suspected of having the disease because of a history of peripheral pain and sweating abnormality since the age of approximately 5 years, and

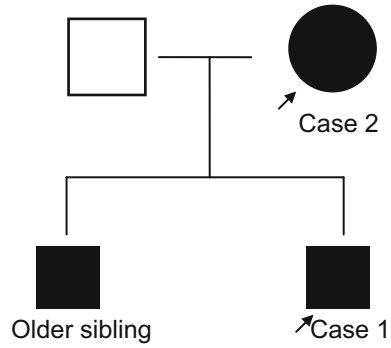

Fig. 1 Familial tree for cases 1 and 2

because of a definitive diagnosis of Fabry disease in his older sibling (Fig. 1). The level of α-galactosidase A activity in a dried blood spot analysis was 2.4 AGal U, which is below the male cut-off level (17.0 AGal U). A genetic analysis showed a missense mutation of the α-galactosidase A gene (D299V in exon 6). Consequently, ERT with the registered dose of agalsidase beta (1 mg/kg every other week) was initiated in February 2006. The patient had coughing from the time of initiation of ERT, and IgG anti-agalsidase beta antibody was detected, with a titre of 1/3,200 to 1/6,400.

In August 2007, Case 1 was transferred to Nagoya Central Hospital voluntarily. An extensive work-up revealed neuropathic pain, dermatological symptoms (angiokeratoma and sweating abnormality), gastrointestinal symptoms (diarrhoea and abdominal pain) and ocular manifestations (cornea verticillata and vessel tortuosity). ERT with agalsidase beta was continued. He had coughing and fatigue at night, and a steroid (betamethasone 1 mg/day) and an antihistamine (chlorpheniramine maleate 8 mg/day) were administered to suppress these symptoms. As this was not successful, an anti-leukotriene agent (montelukast 10 mg/day) and a beta-agonist (tulobuterol 2 mg/day) were co-administered for approximately 1 month from September 2009, followed by a switch to inhalation of fluticasone propionate 0.2 mg twice/day from October 2009 (Fig. 2).

During the supply shortage of agalsidase beta, Case 1 preferred to remain on agalsidase beta at the reduced dose of 0.7 mg/kg every other week from November 2009 to July 2010. Despite the reduction in dose of agalsidase beta, his coughing did not improve and inhaled fluticasone remained necessary.

Because the supply shortage of agalsidase beta continued and his mother (Case 2) was switched from agalsidase beta onto agalsidase alfa, Case 1 also switched onto agalsidase alfa at 0.2 mg/kg every other week. His coughing gradually resolved completely and administration of fluticasone was stopped in August 2010. Administration of betamethasone and chlorpheniramine were also stopped in December 2011. IgG anti-agalsidase alfa antibody was not detected at any point of determination following the switch.

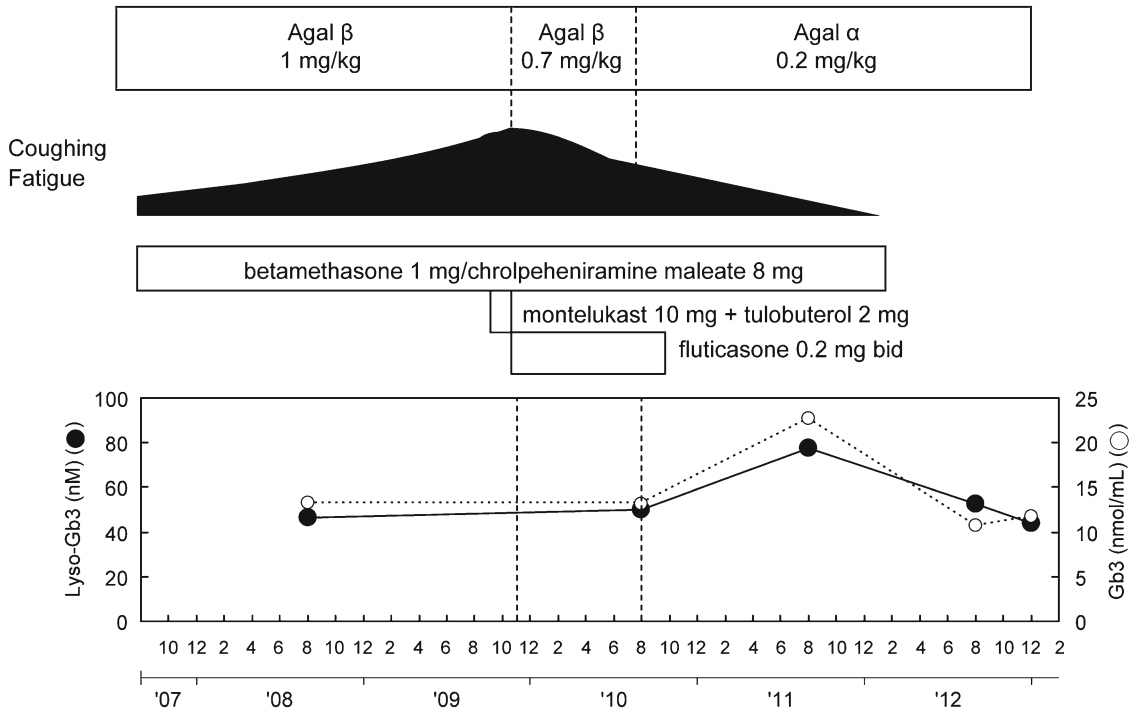

Fig. 2 Case 1: clinical course (Agal β = agalsidase beta; Agal α = agalsidase alfa)

Cardiac structure, function and renal function were maintained during ERT with both agalsidase beta and alfa (data not shown). Changes in plasma lyso-Gb3 and Gb3 levels are shown in Fig. 2; both increased at 12 months after switching to agalsidase alfa, but decreased to their original levels by 24 months.

Case 2 (Female, 62 Years Old)

This patient was diagnosed with Fabry disease in 2005 at the age of 55 years. The diagnosis was suspected because of the presence of left ventricle hypertrophy and the definitive diagnosis of her older son. The level of α-galactosidase A activity in a dried blood spot test was 12.6 AGal U, below the female cut-off level (20.0 AGal U). Genetic analysis confirmed the diagnosis of Fabry disease (D299V in exon 6 of the α-galactosidase A gene). Consequently, ERT with agalsidase beta at 1 mg/kg every other week was initiated in April 2006.

In August 2007, Case 2 was transferred to Nagoya Central Hospital voluntarily. An extensive work-up revealed advanced organ manifestations including heart, kidney and eyes (cornea verticillata). Electrocardiography showed ST segment depression with a negative T wave in leads I, II, aVL, aVF and V_4–V_6. Left ventricular mass (LVM) index was 54 g/m$^{2.7}$. eGFR was 65.7 mL/min/1.73 m^2 (indicating chronic kidney disease stage 2) and proteinuria was present. ERT with agalsidase beta was continued. In contrast to Case 1, Case 2 generally tolerated treatment with agalsidase beta well, and no infusion-related reaction was observed.

Plasma BNP level was monitored during ERT with the registered dose of agalsidase beta (Fig. 3a). Plasma BNP level increased gradually from approximately 200 pg/mL in November 2007 to 500 pg/mL in September 2009. Case 2 was hospitalized for congestive heart failure twice between September 2009 and December 2009. Plasma BNP level increased rapidly to >700 pg/mL in September 2009 and ejection fraction decreased to 42.5% in November 2009. Case 2 was treated for congestive heart failure with a loop diuretic (furosemide 10–30 mg/day), beta-blocker (carvedilol 2.5 mg/day), amiodarone 100 mg/day and an angiotensin-converting enzyme (ACE) inhibitor (perindopril 2 mg/day). Plasma BNP level decreased and ejection fraction improved with this treatment (Fig. 3a, b).

During the supply shortage of agalsidase beta, this patient preferred to remain on agalsidase beta at a reduced dose of 0.7 mg/kg every other week from September 2009 to July 2010. During the initial 27-month period of ERT with the registered dose of agalsidase beta 1 mg/kg, LVM index was maintained at around 50–56 g/m$^{2.7}$; but after the dose reduction, LVM index increased to 62 g/m$^{2.7}$ by June 2010 (Fig. 3b). Interventricular septum thickness and left ventricular posterior wall thickness showed similar changes (data not shown). During ERT with agalsidase beta, IgG anti-agalsidase beta antibody was not detected at any point.

The supply shortage of agalsidase beta continued and Case 2 decided to switch to agalsidase alfa 0.2 mg/kg every other

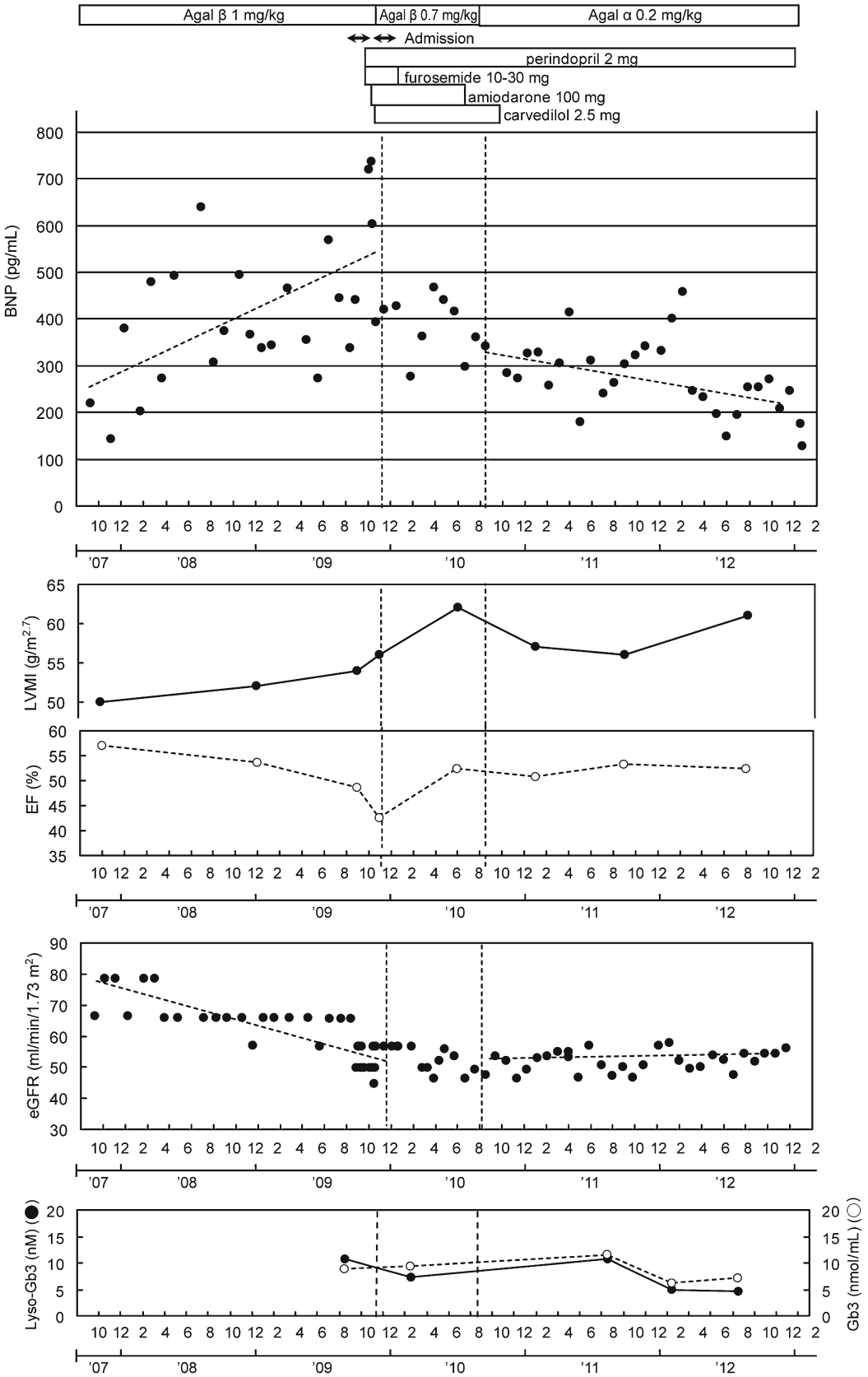

Fig. 3 Case 2: clinical course (Agal β = agalsidase beta; Agal α = agalsidase alfa)

week in August 2010. Administration of an ACE inhibitor (perindopril) continued. Plasma BNP level decreased gradually from >300 pg/mL in August 2010 (before switching) to <200 pg/mL by December 2012 after 28 months' treatment with agalsidase alfa (Fig. 3a). LVM index and ejection fraction were maintained after 6 months' treatment.

Changes in eGFR are shown in Fig. 3c. During 36 months' ERT with agalsidase beta, eGFR decreased from around 78.6 mL/min/1.73 m^2 in November 2007 to 44.5 mL/min/1.73 m^2 by June 2010. Two years after switching to agalsidase alfa, eGFR remained at around 50 mL/min/1.73 m^2.

During ERT with agalsidase alfa, IgG anti-agalsidase alfa antibody was not detected.

Changes in plasma lyso-Gb3 and Gb3 levels are shown in Fig. 3d. Both increased slightly 12 months after switching to agalsidase alfa, but then decreased to a level below that seen while the patient was on agalsidase beta.

Discussion

In this report, we present two cases with Fabry disease (a parent and a child) who received 27 months of treatment with agalsidase beta at the registered dose and 9 months of treatment at a reduced dose, followed by a switch to 28 months of treatment with agalsidase alfa. For both patients the switch was performed because of severe adverse events while receiving the registered dose of agalsidase beta and also because of a worldwide shortage of agalsidase beta.

Case 1 was thought to have an allergic tendency when he was transferred to Nagoya Central Hospital. He had severe coughing and fatigue, and therefore antitussive and antiasthmatic drugs were prescribed for about 4.5 years. After switching to agalsidase alfa because of the shortage of agalsidase beta, the coughing gradually resolved and anti-allergic drugs were no longer needed. These results suggest that Case 1 had severe coughing and fatigue due to an infusion-related reaction to agalsidase beta.

Case 1 developed a high titre of IgG anti-agalsidase beta antibody, but no IgG anti-agalsidase alfa antibody during ERT with the alternative drug. IgE anti-agalsidase beta antibody was not determined. Although the mechanism underlying coughing as an infusion-related reaction to agalsidase beta has not been elucidated, immunological reactions are thought to be involved.

There are significant differences in the frequencies of immunoglobulin responses and infusion-related reactions between agalsidase alfa and beta (Lidove et al. 2007, 2010; Deegan 2012). According to data from Shire HGT and Genzyme Corp held on file with the European Medicines Agency, 24% of men treated with agalsidase alfa develop IgG antibodies and 89% of patients treated with agalsidase beta develop IgG antibodies (European Medicines Agency 2006a, b). An IgE antibody response to ERT has only been reported for patients treated with agalsidase beta (Banikazemi et al. 2007; Bodensteiner et al. 2008), not in those receiving agalsidase alfa (Pastores et al. 2007; Schiffmann et al. 2006; Tesmoingt et al. 2009). The frequency of infusion-related reactions is also higher with agalsidase beta than agalsidase alfa, at about 67% and 13.7%, respectively (European Medicines Agency 2006a, b). Agalsidase alfa seems to be less immunogenic than agalsidase beta, although it should be noted that these frequencies came from different trials using different methods for detecting antibodies in plasma. Tanaka et al. reported that IgE antibodies against agalsidase beta which developed in a male patient with Fabry disease showed no cross-reactivity to agalsidase alfa (Tanaka et al. 2010). This suggests that IgE anti-agalsidase beta antibody could be determined in order to clarify differences between adverse events.

Our case studies showed that decrease in cough and fatigue started at lower dose of agalsidase beta with standard and effective care in respiratory disease, and switching to agalsidase alfa can be helpful for resolving severe infusion-related reactions while maintaining various indices of cardiac and renal function. However, the licensed dose of agalsidase beta is only 1 mg/kg once every 2 weeks and its reduced dose should be used carefully because it has not clarified to be effective in various symptoms of Fabry disease.

Plasma lyso-Gb3 elevation is a hallmark of Fabry disease (Aerts et al. 2008) and is associated with clinical manifestations (Rombach et al. 2010). Recently, Smid et al. reported that an increase in lyso-Gb3 was found both in male patients who switched to agalsidase alfa for approximately 1 year and in patients who received a reduced agalsidase beta dose for approximately 1 year, although no correlation between the incidence of clinical events and an increase in lyso-Gb3 was found (Smid et al. 2011). In our study of Case 1, lyso-Gb3 also increased 12 months after switching to agalsidase alfa, but by 24 months after switching it had decreased to a level similar to that seen prior to switching. The mechanism of such a transient increase of lyso-Gb3 remains to be determined.

Case 2 had advanced cardiac manifestations at the time of her definitive diagnosis. Despite receiving ERT at the approved dose of agalsidase beta, this patient was hospitalized because of aggravation of congestive heart failure, which was treated appropriately. After that, she received a reduced dose of agalsidase beta for 9 months during the supply shortage, which led to an increase in LVM index. After ERT was subsequently switched to agalsidase alfa, all measured indices such as BNP level, echocardiographic parameters, eGFR and lyso-Gb3 level improved or stabilized. The registered dose of agalsidase beta stabilized heart

structure, but was not effective at stabilizing cardiac function. In contrast, switching to agalsidase alfa after receiving conventional treatment for congestive heart failure led to stabilization of both heart structure and cardiac function. In this patient, IgG anti-agalsidase beta antibody did not develop during ERT with agalsidase beta, and an immunological response is not thought to be involved in the events experience by this patient.

BNP is a cardiac neurohormone specifically secreted from the ventricles (Daniels and Maisel 2007) by stimuli such as pressure overload or ventricular wall distension. A recent study showed that NT-proBNP was potentially useful as a biomarker in Fabry disease and correlated with changes in Mainz Severity Score Index (Torralba-Cabeza et al. 2011). BNP gene expression is stimulated by both mechanical strain (Liang et al. 1997; Liang and Gardner 1998) and autocrine/paracrine processes via angiotensin II (Liang and Gardner 1998). Renin–angiotensin system blockade reduces plasma NT-proBNP level and improves prognosis, which is most likely a reflection of improved cardiac function due to the treatment (Anand et al. 2003). Based on these reports, combination therapy with an ACE inhibitor and agalsidase alfa might be useful in achieving a good effect on plasma BNP levels and cardiac function.

Therapy with ACE inhibitors and/or angiotensin II receptor blockers is recommended in patients with renal disease, to treat proteinuria and prevent progression toward renal failure (Chobanian et al. 2003). The role of ACE inhibitors and angiotensin II receptor blockers in patients with Fabry disease is not solely antihypertensive; in combination with ERT, it also helps stabilize kidney function by slowing the progressive decline of eGFR and reducing urinary protein excretion to <500 mg/day (Eng et al. 2006; Warnock and West 2006). In Case 2, combination therapy with an ACE inhibitor and agalsidase alfa is likely to have been responsible for the beneficial effects recorded for renal function.

Plasma BNP level was useful for monitoring several clinical aspects, such as heart failure during Fabry disease, and for evaluating the effect of ERT and/or combination therapy. Our results showed that switching to agalsidase alfa was helpful for maintaining various indices reflecting both cardiac and renal function.

Conclusions

In conclusion, we report two clinical cases that experienced severe adverse events during therapy with the approved and/or reduced doses of agalsidase beta. Switching to agalsidase alfa associated with standard care in heart disease led to resolution or improvement in the cardiorespiratory status. And reduction in dose associated with standard and effective care in respiratory disease was useful for decrease in cough and fatigue. Plasma BNP level was found to be useful for monitoring various clinical aspects, such as heart failure and the effect of ERT and/or combination therapy in patients with Fabry disease.

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Editorial assistance was provided by Content Ed Net.

Synopsis

Switching to agalsidase alfa can ameliorate adverse events experienced during treatment with agalsidase beta, while maintaining cardiac and renal function.

Conflict of Interest

Kazuya Tsuboi has received a speaker honorarium from Genzyme, Shire HGT and Dainippon Sumitomo Pharma.

Hiroshi Yamamoto has received a speaker honorarium from Genzyme.

Fuji Somura and Hiromi Goto declare that they have no conflict of interest.

Informed Consent

The study was approved by the institutional review boards and was conducted in accordance with the ethical provisions set out in the Helsinki Declaration of 1975 as revised in 2000, and all applicable local laws and regulations. Informed consent was obtained from all patients for being included in the study.

Animal Rights

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

Kazuya Tsuboi contributed the planning, conduct and reporting of the work described in the article.

Hiroshi Yamamoto contributed conduct and reporting of the work.

Fuji Somura and Hiromi Goto contributed conduct for cardiovascular treatment to patients and checking the draft of report.

Author serving as guarantor for the article: Dr Kazuya Tsuboi

Details of funding: not applicable.

Competing interests statement: None

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CASE REPORT

Deep Brain Stimulation and Dantrolene for Secondary Dystonia in X-Linked Adrenoleukodystrophy

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Abstract Deep brain stimulation (DBS) has been used to treat secondary dystonias caused by inborn errors of metabolism with varying degrees of effectiveness. Here we report for the first time the application of DBS as treatment for secondary dystonia in a 22-year-old male with X-linked adrenoleukodystrophy (X-ALD). The disease manifested at age 6 with ADHD, tics, and dystonic gait, and deteriorated to loss of ambulation by age 11, and speech difficulties, seizures, and characteristic adrenal insufficiency by age 16. DBS in the globus pallidus internus was commenced at age 18. However, after 25 months, no improvement in dystonia was observed (Burke–Fahn–Marsden (BFM) scores of 65.5 and 62 and disability scores of 28 and 26, pre- and post-DBS, respectively) and the DBS device was removed. Treatment with dantrolene reduced skeletal muscle tone and improved movement (Global Dystonia Rating Scores from 5 to 1 and BFM score 42). Therefore, we conclude that DBS was a safe but ineffective intervention in our case with long-standing dystonia, whereas treatment of spasticity with dantrolene did improve the movement disorder in this young man with X-ALD.

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Competing interests: None declared

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Report

Deep brain stimulation (DBS) has demonstrated variable effectiveness in secondary dystonias caused by inborn errors of metabolism (e.g., homocystinuria, PKAN, GM1-gangliosidosis) (Andrews et al. 2010; Aydin et al. 2011). The present report of DBS in a now 22-year-old man with a diagnosis of X-linked adrenoleukodystrophy (X-ALD, OMIM# 300100) (Vidailhet et al. 2005) is its first reported application in X-ALD.

The patient presented at age 6 with ADHD, tics, and a dystonic gait. Neuroimaging revealed symmetrical occipito-parietal demyelination and abnormal signal intensities in the basal ganglia and thalamus (Fig. 1). Diagnosis was confirmed by elevated very long chain fatty acids in plasma. He lost the ability to ambulate within 5 years, and was wheelchair-bound by age 11. His speech deteriorated and was barely intelligible by age 16. Seizure disorder and adrenal insufficiency were effectively controlled with carbamazepine and clobazam, and hydrocortisone, respectively. Initially the dystonia affected only the lower extremities, but quickly generalized. At age 11 years, mild athetoid movements of the head, neck, and extremities were

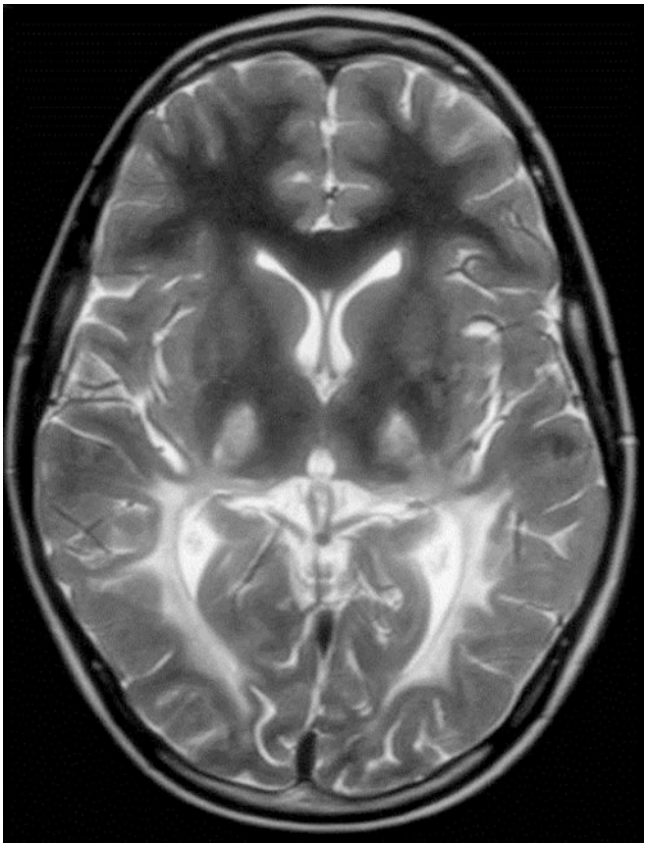

Fig. 1 MRI brain scan at 10 years of age. A representative image of the T2-weighted sequence is shown demonstrating changes in signal intensity within the posterior white matter in a bilateral, symmetric fashion as well as increased signal intensity within the thalami

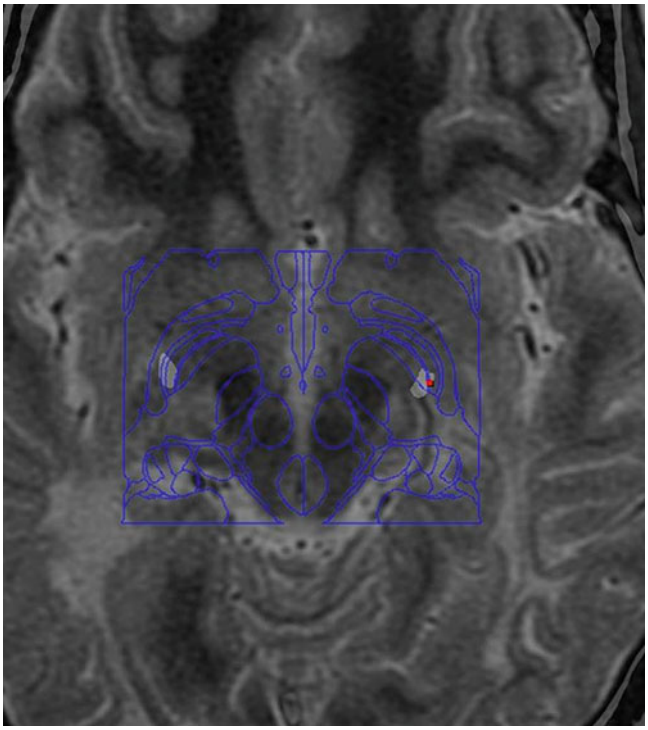

Fig. 2 Location of the DBS contacts. Preoperative T2-weighted MRI fused with postoperative CT (windowed to show metal contacts) with Schaltenbrand atlas superimposed using StealthStation S7 (Medtronic) software. Active contacts can be seen in the most ventral posterior pallidum bilaterally (approximately 2 mm anterior, 20 mm lateral, and 4 mm below the mid-commissural point)

observed. He held the neck in a flexed position, but was able to extend it on request. He had a tendency to elevate the right upper extremity, with his right wrist in a flexed position and fingers extended. There was mild intermittent flexion of the wrist. Knees were held in an extended position, with ankles plantar flexed and feet inverted (left greater than right). Tone was difficult to assess due to the dystonia. Deep tendon reflexes were brisk, especially at the knees; plantar responses were difficult to assess due to dystonia. Hoffman's sign was absent.

For dystonia, botox, levodopa/carbidopamine, trihexyphenidyl, pergolide, and pramipexole were ineffective. Intrathecal baclofen to reduce muscle tone was refused by the patient.

Repeat gadolinium magnetic resonance imaging revealed no evidence of active disease at age 16, indicating exhaustion of the disease process. The patient's Loes score was 15 and his IQ was <70 (WISC-III/IV). By then, he exhibited a pronounced cogwheel phenomenon, unresponsive deep tendon reflexes, and dystonic contractures, but was able to manipulate his wheelchair. Deep brain stimulation (DBS) was considered at the request of the patient and his family (Vidailhet et al. 2005); the benefits and risks (i.e., exacerbation of demyelination with external trauma) (Raymond et al. 2010) were carefully considered, and DBS was performed with informed consent at age 18.

DBS leads (Medtronic model 3389-40) were inserted under general anesthesia with frame-based MRI-guided stereotaxy and macro-electrode stimulation and into the globus pallidus internus (GPi) and connected to an implantable neural stimulator (Kinetra). Figure 2 shows the location of the contacts used for stimulation. Continuous mono-polar stimulation began 6 weeks postoperatively with contact 1-positive (second most ventral left) and contact 5-negative (second most ventral right), pulse width 210 μs, 130 Hz, and 3.5 V. Parameters were adjusted on numerous occasions to maximize stimulation and avoid internal capsule response.

After 25 months, the patient had neither deteriorated nor shown improvement of dystonia. His Burke–Fahn– Marsden

(BFM) and disability scores were 62 and 26, respectively (baseline scores: 65.5 and 28). No change in either score occurred upon deactivation and removal of DBS (measured after 3 months).

Dantrolene was subsequently initiated at 12.5 mg orally once daily and gradually increased to 25 mg three times daily. Dantrolene sodium reduces skeletal muscle tone at the level of the muscle fiber rather than the neural level (Haslam et al. 1974). After 5 weeks, the patient's Global Dystonia Rating Scores were consistently 1 out of 10 (pre-treatment: 5 out of 10), and his BFM score was 42. Major improvements included increased flexibility of the right arm and lower limbs, and only occasional episodes of dystonic posturing. No side effects were reported; liver enzymes remained normal. However, his underlying neurometabolic condition has since deteriorated with cognitive decline and swallowing difficulties at age 22.

DBS was safe but ineffective in our patient. Treatment of spasticity may improve DBS outcomes in long-standing dystonia (Lumsden et al. 2013), particularly when supported by occupational and physiotherapies with individualized and goal-directed outcomes.

Acknowledgments We are grateful to Mr. Bryan Sayson for administrative assistance.

Synopsis

Deep brain stimulation was a safe but ineffective intervention for long-standing dystonia in X-ALD, whereas treatment with dantrolene improved movement and dystonia symptoms at the level of skeletal muscle.

Compliance with Ethics Guidelines

Conflict of Interest

Christopher Honey has received consulting fees from Medtronic, a commercial company that fabricates DBS instruments. The authors Clara van Karnebeek, Gabriella Horvath, Tyler Murphy, Jacqueline Purtzki, Kristin Bowden, Sandra Sirrs, and Sylvia Stockler have no conflict of interest to declare.

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). The patient provided informed consent for publication of this report.

Ethics

The institutional review board at BC Children's Hospital and the University of British Columbia approved the study protocol [H12-00067], and parents provided written consent for publication.

Details of Contributions of Individual Authors

CvK: Clinical biochemical geneticist who was responsible for organization of multidisciplinary meetings to discuss patient treatment and follow-up, data collection, and drafting of the first manuscript with subsequent coordination of authors' edits.

GH: Clinical biochemical geneticist currently following patient in the Adult Metabolic Clinic, responsible for the symptomatic management with dantrolene, and reviewed/edited the manuscript.

TM: Medical student who contributed to data collection and drafting of the manuscript.

JP: Physiatrist who performed pre- and post-DBS functional evaluation, and reviewed/edited the manuscript.

KB: Technical writer who provided critical revision of the manuscript.

SSirrs: Metabolic diseases specialist who managed and followed the patients in the Adult clinic around the time of DBS: Contributed to data collection, and reviewed/edited the manuscript.

SStockler: Neurometabolic diseases specialist who was responsible for the conception of the case report, contributed to data collection, and reviewed/edited the manuscript.

CRH: Neurosurgical consultant who coordinated surgery and DBS follow-up care, and reviewed/edited the manuscript.

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CASE REPORT

Cirrhosis and Liver Failure: Expanding Phenotype of Acid Sphingomyelinase-Deficient Niemann-Pick Disease in Adulthood

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Abstract Acid sphingomyelinase-deficient Niemann-Pick disease (ASMD) includes the severe neuronopathic type A, the non-neuronopathic type B, and rare intermediate cases. Here we report on such an atypical type B patient who died at 31 years of age from liver failure. This male subject was first seen in a paediatric department at the age of 3 years because of significant hepatosplenomegaly. Foam cells in bone marrow, interstitial pneumonitis, a slight facial dysmorphy and normal psychomotor development were additional findings. Acid sphingomyelinase studies in lymphocytes (and later *SMPD1* gene studies [c.151_154delGACT; c.1341-21_1341-18delAATG]) established the diagnosis of ASMD. Between the ages 6–27, he developed growth retardation, peripheral neuropathy, kyphoscoliosis, alopecia, and aortic valve insufficiency requiring valve replacement. Surgery for bilateral inguinal hernias was performed twice, when the patient was 10 and 21 years of age, respectively. At the age of 28, he was noted to have hepatosplenomegaly and follow-up investigations revealed ascites and gastric varices. Liver biopsy showed cirrhosis without areas of necrosis (A6 in Child-Pugh classification). He developed haematemesis and worsening encephalopathy leading to his death at age 31. In conclusion, cirrhosis should be considered as a possible complication of ASMD in adult patients, even if hepatic tests are normal.

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Introduction

Niemann-Pick disease (NPD) types A and B refer to an autosomal recessive lysosomal lipid storage disease caused by mutations in the *SMPD1* gene resulting in the deficient activity of the lysosomal enzyme acid sphingomyelinase (ASM, E.C. 3.1.4.12). "ASM-deficient NPD" should now be preferred, because this name allows inclusion of cases intermediate between the severe neuronopathic type A and the non-neuronopathic type B (Schuchman 2007), and also clearly differentiates this group from Niemann-Pick disease type C, a distinct entity (Vanier 2013). Most type A NPD (MIM# 257200) patients show a similar clinical course, with failure to thrive and prominent visceral involvement soon after birth, followed from 5 to 10 months of age by a progressive neurological deterioration. Death classically occurs between 1.5 and 3 years of life (McGovern et al. 2006). By contrast, typical type B NPD (MIM# 607616) is a chronic, non-neuronopathic form, with a widely variable degree of systemic involvement (McGovern et al. 2008; Hollak et al. 2012). The most typical symptoms are hepatosplenomegaly, interstitial lung disease, alteration of liver function, followed by joint/limb pain, bruising, headache, abdominal pain or diarrhoea. The age of diagnosis, typically in late infancy or childhood, may vary

from birth until late adulthood. A majority of typical type B patients survive until late adulthood, but some patients have a severe systemic disease, eventually leading to premature death, often by liver failure or cirrhosis (Labrune et al. 1991; Wasserstein et al. 2004; Pavlu-Pereira et al. 2005; McGovern et al. 2013). Recent studies indicate that liver cirrhosis may be more frequent than previously reported in type B NPD (Tassoni et al. 1991; Thurberg et al. 2012). Respiratory insufficiency is another important cause of morbidity.

We report here on a patient with atypical ASM-deficient NPD who died from liver failure in his 32nd year of age.

Case Report

A Cambodian male patient, born at term from non-consanguineous parents, was seen at the age of 3 years with a 2-year history of hepatosplenomegaly and was noted to have facial dysmorphism, red brown colouration of the macula, and interstitial changes on a chest X-ray. The diagnosis of ASM-deficient NPD was considered and confirmed by showing deficiency of acid sphingomyelinase in lymphocytes (0.06 μkat/kg protein; normal values 1.83 ± 0.52), using ^{14}C-sphingomyelin as the substrate (Vanier et al. 1980). ALAT and ASAT were 76 and 110 mU/L, respectively, total bilirubin 8 mmol/L. Urinary glycosaminoglycans were measured and found normal. A repeated study of urinary glycosaminoglycans and oligosaccharides performed at the age of 27 years also concluded with normal results. A skin fibroblast culture was obtained when the child was 8 years old, which confirmed the profound acid sphingomyelinase deficiency, using the same method as above. A later study of the *SMPD1* gene revealed the presence of a frameshift mutation p.Asp51Leufs*25 (c.151_154delGACT) in exon 1 and of an intronic deletion c.1341-21_1341-18delAATG (IVS4-21_IVS4-18delAATG), plus three known polymorphisms (p.Val36Ala, p.Ala48_Leu49del and p.Gly508Arg), all in a heterozygous status. Segregation of the alleles could not be studied.

At the age of 6 years and 8 months, the patient's facial dysmorphism had increased, his voice was changed, the base of his thorax was enlarged. Examination showed a slight amyotrophy of the lower limbs. Neurological examination was normal except abolished tendon reflexes. Electromyography found a peripheral neuropathy. The interstitial pneumonitis was more pronounced than before.

Data between the age of 7 and 27 years are fragmentary, but the patient developed body growth delay, kyphosis, scoliosis, bowed fingers and alopecia. Surgery for bilateral inguinal hernias was performed twice, when the patient was 10 and 21 years of age, respectively.

At 20 years of age, because of aortic regurgitation, the patient underwent aortic valve replacement with mechanic prosthesis. Pathologic examination revealed severe atheroma of the aorta with calcifications. At that time, oral anticoagulant therapy was begun.

At 27 years of age, the patient had a short stature with kyphoscoliosis, dysmorphic features, and no apparent intellect alteration. Examination showed a marked hepatosplenomegaly, abolished deep tendon reflexes with minimal distal amyotrophy. His liver function tests were essentially normal, but he had a pronounced thrombocytopenia (47 G/L). Electromyography concluded with a severe demyelinating sensory-motor polyneuropathy of the four limbs, which, from a later study, remained stable for the following 3 years.

One year later, a liver biopsy was performed by transjugular procedure, revealing a 11 cm H_2O gradient in the supra-hepatic vein, a micronodular and macronodular cirrhosis without necrosis, without inflammation. Microvesicular steatosis was also found representing 70% of the total liver area (Fig. 1). Other classical factors of cirrhosis at young age – alcohol abuse, hepatitis B and C viruses, haemochromatosis and auto-immune hepatitis – were excluded.

At 30 years of age, clinical examination was unchanged. Dyspnoea on exertion was noticed; functional tests showed a mild respiratory insufficiency with FEV1/FVC at 81% and FVC at 77%. An ophthalmological examination showed a very low visual acuity (2/10 and 3/10), with absence of cataract but presence of a whitish deposit. Platelets had remained at a similar low level (49 G/L); liver tests were within normal range. Two months later, oesophageal and gastric endoscopy was performed, showing varices in the fundus, under the cardia. The patient was considered with cirrhosis (A6 in Child-Pugh classification).

When slightly over 31, the patient was referred to the hospital because of haematemesis. He also had lower limb oedema, chronic diarrhoea, abdominal pain with episodes of vomiting. Clinical examination showed short stature (1m30), liver and spleen enlargement, ascites and bowed fingers. Prothrombin level was 18%, TCA 75/34 under anticoagulant therapy, fibrinogen level 1.1 g/L (normal >2), factor V level 23%, albumin level 24.7 g/L, total bilirubin 74 μmol/L. Haematology showed a haemoglobin content of 120 g/L, platelet count 59 G/L, leucocytes 7.77 G/L (6.47 polynuclear cells, 0.77 lymphocytes). Haptoglobin level was <0.08 g/L associated with high LDH level (×1.5 normal). Liver tests were normal and lipase was mildly elevated (twice the upper normal level). A search for hepatitis B and C infection was negative. Alpha-foeto protein was normal. Urinalysis found no proteinuria. No monoclonal gammapathy was present, either in serum or in urine.

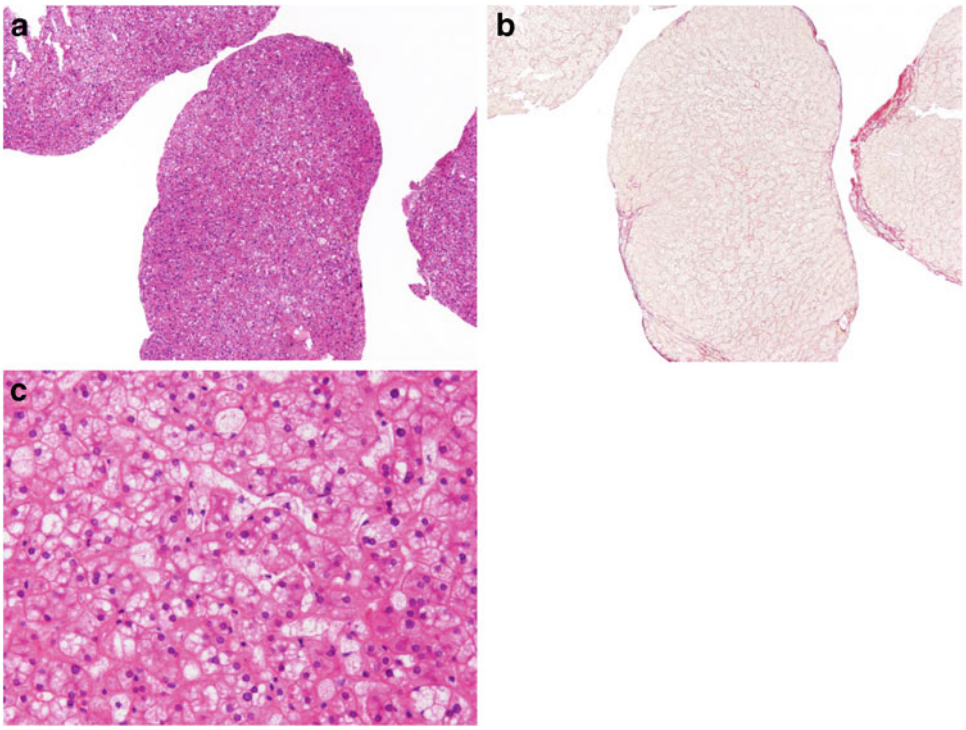

Fig. 1 Trans-jugular liver biopsy. (**a**) at low magnification, the biopsy is fragmented (H&E, ×40). (**b**) This staining highlights the fact that these fragments are cirrhotic nodules surrounded by fibrosis stained in *red* (Picrosirius-Hemalun, ×40). (**c**) Presence of hepatocytes and Kupffer cells with foamy cytoplasm probably due to the accumulation of lipids (H&E, ×200)

Abdominal ultrasonography showed a dysmorphic liver with multiple punctiform hyperechogenic lesions and an important enlargement of the spleen (21 cm), and ascites. Portal hypertension was suspected because of perisplenic venous derivations. Abdominal CT scan confirmed ascites and liver and spleen enlargement, portal hypertension and pancreatitis.

One month later, the patient developed encephalopathy. A new episode of haematemesis led to stop anticoagulant therapy. Encephalopathy worsened. Hepatic transplantation was considered but denied because of encephalopathy, mental retardation, heart and lung involvement, and due to absence of specific therapy for ASM deficiency. Laboratory data at this stage were as follows: total protein: 47 g/L; albumin: 22 g/L; bilirubin: 182 μmol/L; haemoglobin: 102 g/L; platelets: 40 G/L; factor V: <10%; fibrinogen: 0.6 g/L with monomers of fibrin >150 μg/mL (normal < 20), suggesting activation of coagulation.

After a new episode of haematemesis and worsening of encephalopathy, palliative therapy was considered. Ten days later, the patient died from refractory encephalopathy related to cirrhosis and hepatic failure.

Discussion

The clinical spectrum of ASM-deficient NPD (ASMD) is wide and ranges from severe infantile neurovisceral form leading to death by 3 years of age (type A) to patients with a mild form of the purely systemic form (type B) with a normal lifespan. ASMD, like Gaucher disease, shows a continuum (Schuchman 2007). An intermediate phenotype has also been described, in which some patients are closer to type A, while others are closer to type B, with a variable degree of neurologic involvement, mild developmental delay, and cherry-red maculae, along with visceral manifestations. In recent large surveys, patients with an intermediate phenotype have been included in type B by leading authors in the field (Wasserstein et al. 2004; McGovern et al. 2013)

The acid sphingomyelinase deficiency is well established in our patient, from studies in leucocytes, cultured fibroblasts and gDNA (the pathogenicity of the intronic deletion is, however, not proven). However, his clinical phenotype, although in large part compatible with a type B ASMD, is clearly atypical. Abolition of deep tendon

reflexes from the age of 6, with severe demyelinating sensory-motor polyneuritis of the 4 limbs shown in the adult age, and a possible slight mental retardation are indeed seen in a small subset of patients still considered as type B (Wasserstein et al. 2004; McGovern et al. 2013). Cardiac abnormalities of various types have also been recently described in a few, otherwise typical, type B patients (Iaselli et al. 2011). Still, cardiac involvement was particularly severe in this patient, since it required aortic valve replacement at the age of 20. Above all, the prominent dysmorphic features and skeletal deformations seen in the present patient already from early childhood are highly unusual. Because of short stature with kyphosis, hand deformities and surgery performed for bilateral inguinal hernia, search for a concomitant mucopolysaccharidosis was performed in childhood and at the adult age. Urinary excretion of glycosaminoglycans was normal in both instances. To our knowledge, this phenotype is quite unique. At least it has not been seen in a cohort of over 150 ASMD patients diagnosed in France during the past 30 years.

On the other hand, there are several reports on liver fibrosis and cirrhosis in type B patients. Liver fibrosis studied in two patients was found to occur independently of age at onset or time of the liver biopsy: it was present in a young female but absent in an adult male patient (Takahashi et al. 1997). Fatal liver failure has also been reported in two children from the French cohort (Labrune et al. 1991). Cirrhosis and portal hypertension were further reported in a 33-year-old patient with NPD (Tassoni et al. 1991). Low factor V level of coagulation could have been related not only to liver disease but also to hypersplenism with resultant expansion of plasma volume (diluting the clotting factors) and increased intrasplenic utilisation of factors which has been described in NPD (Dewhurst et al. 1979). Nevertheless, higher levels of other factors of the coagulation despite oral anticoagulant therapy suggest hepatic failure in the case reported herein.

Recent studies performed during the phase 1 trial of enzyme replacement therapy with RhASM suggest that hepatic cirrhosis may have been underestimated in type B ASMD (Thurberg et al. 2012). Among the 17 adult patients who underwent a liver biopsy during baseline screening, two had biopsy-proven full-blown cirrhosis. Another publication from the same group studied morbidity and mortality in a cohort of 103 type B patients ranging from 1 to 72 years of age (McGovern et al. 2013). Nine patients had cirrhosis or liver failure requiring liver transplantation. Of note, six patients had fulminant liver failure and three of them had evidence of cirrhosis on liver biopsy. Of those with liver failure, two received successful orthotopic liver transplantation at 12 and 25 years of age.

The authors of the latter study propose that the massive sphingomyelin storage in lysosomes may provoke a fibrotic reaction in tissues, leading to progressive liver dysfunction, possibly compounded by inflammation. This appears somewhat in contradiction to the study by Thurberg and colleagues, where no correlation was found between sphingomyelin levels and either fibrosis grade or patient age.

The accumulation of the potentially toxic compound sphingosylphosphorylcholine, which shows a large increase in liver and spleen of ASMD patients (Rodriguez-Lafrasse and Vanier 1999), along with sphingomyelin, cholesterol and bis(monoacylglycero)phosphate, could also be contributing to liver dysfunction. Furthermore, ASM deficiency is associated with elevation in cytokines, which could also play a role in the development of liver fibrosis and cirrhosis. In a study in the ASM-deficient mouse model, it was also suggested that liver fibrosis could be related to cathepsin B overexpression (Moles et al. 2012)

In conclusion, our observation further demonstrates that liver failure and cirrhosis can occur in adult patients with ASMD. Thus, regular monitoring of liver status by biochemical tests, i.e. hepatic tests, haemostasis, hepatic ultrasonography and eventual subsequent histological study should be part of the follow-up for patients with type B ASMD. Because of liver failure or low platelet count, transjugular procedure should be considered. Histological study should be discussed on an individual basis, but probably included as a tool in the care of patients from the perspective of future enzyme replacement therapy.

Synopsis

Liver failure and cirrhosis are possible complications of acid sphingomyelinase deficiency in adult patients.

Conflict of Interest

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

Ethics Approval

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.

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RESEARCH REPORT

Dried Blood Spots Allow Targeted Screening to Diagnose Mucopolysaccharidosis and Mucolipidosis

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Abstract *Background*: As patients with different types of mucopolysaccharidosis (MPS) and mucolipidosis (ML) may present with overlapping clinical features – including coarse face, hepatosplenomegaly, bone dysplasia and claw-hand deformities, collectively also called 'MPS-like phenotype', enzymatic and/or molecular genetic analyses are indispensable for accurate diagnosis and applying specific therapy. In this prospective study, we screened patients with symptoms compatible with MPS for MPS I, II (males) and VI.

Methods: Dried blood spots/specimens (DBS) were collected from 200 patients with an MPS-like phenotype and analysed for activities of α-iduronidase (IDUA), iduronate-2-sulphatase (IDS), and arylsulphatase B (ARSB), the enzymes deficient in mucopolysaccharidosis (MPS) type I, II and VI, respectively. For the samples with pathologic enzyme activity, mutational analysis was carried out using the same DBS.

Results: Based on enzymatic analysis of 200 DBS samples, a total of 45 (22.5%) showed low activity; 17 for MPS I (8.5%), 11 for MPS II (5.5%) and 9 for MPS VI (4.5%). Enzyme activities were suggestive for ML II/III in 8 (4.0%) cases. For 41 (91.1%) samples, DNA could be extracted from the filter paper. Mutations were identified in 11 (64.7%), 11 (100%), 9 (100%) and 5 (62.5%) patients putatively diagnosed biochemically with MPS I, II, VI, and ML II/III, respectively.

Conclusions: DBS enzymatic analysis can be used to diagnose MPS/ML. Initial results should be confirmed by a second enzyme assay and/or by molecular genetic testing. Given the advantages of DBS over other sample types in terms of ease of collection, storage and transportation, DBS are particularly useful for screening patients with an MPS-like phenotype in regions lacking specialised laboratories. In order to ascertain the diagnosis in a large number of cases, patients should be assessed in parallel for at least MPS I, II and VI.

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Introduction

Mucopolysaccharidoses (MPS) represent a group of rare inborn lysosomal storage disorders (LSDs), with an incidence of 0.05–1.89 per 100,000 live births (for recent review, see Muenzer 2011). To date, 7 different MPS types have been identified, and some of them have been subdivided into several subtypes. Each (sub)type is characterised by the deficiency of a specific lysosomal enzyme involved in the degradation of glycosaminoglycans (GAGs). Loss of enzyme activity results in accumulation of incompletely degraded GAGs in lysosomes and progressive cellular damage as well as dysfunction of multiple tissues and organs (Muenzer 2011). Patients typically appear normal at birth, but gradually develop a plethora of rather unspecific clinical manifestations, which vary according to the severity of the disease. All MPS types but type II, that is X-chromosomal, are inherited in an autosomal recessive manner. Despite the biochemical and

genetic heterogeneity, there is a significant overlap in the clinical presentation among the different MPS types. For instance, MPS I, II and VI share many clinical features called 'MPS-like' phenotype. This phenotype includes, among others, coarse facial features, hepatosplenomegaly, joint and skeletal dysplasia (dysostosis multiplex, dwarfism, claw-like hand), cardiorespiratory problems, and vision and hearing impairment (Chamoles et al. 2001b; Muenzer 2011). In addition, patients with mucolipidosis (ML) II (I-cell disease) and III (pseudo-Hurler polydystrophy) (Van Hoof 1974) may also show a phenotype similar to that of patients with MPS. The latter disorders are characterised by the deficiency of one of the enzymes that play a role in generating the mannose-6-phosphate recognition marker on lysosomal enzymes. Consequently, missorting of multiple lysosomal enzymes leads to a general lysosomal dysfunction (Kollmann et al. 2010). Given these overlapping features between MPS and ML types, it is difficult to distinguish between them based on clinical findings alone.

Therefore, next to clinical evaluation, laboratory tests are indispensable for an accurate and timely diagnosis. These diagnostic tests may include measurement of urinary GAG levels, enzyme activity assays and molecular genetic analysis (Muenzer 2011). Although urinary GAG analysis can be useful for screening, it is not sufficiently sensitive to rule out MPS in case of a negative test result. Consequently, measurement of enzyme activities – conventionally on leukocytes or cultured fibroblasts – is currently the gold standard for diagnosing a specific MPS disorder (Muenzer 2011; Muenzer et al. 2009; Scarpa et al. 2011; Wood et al. 2012). However, enzyme activities in leukocytes are very sensitive to prolonged shipping or unfavourable environmental conditions, while analysing cultured fibroblasts requires skin biopsy for sample collection and an expensive and lengthy procedure, taking a long time before results become available (for recent review see Wood et al. 2012). Therefore, dried blood spots/specimens (DBS) – obtained by drying a small blood volume on filter paper – represent a convenient alternative sample type, offering advantages in terms of ease of collection, storage and transportation (Reuser et al. 2011).

So far, several studies have shown the feasibility of the enzymatic diagnosis of MPS I, II or VI using DBS (Blanchard et al. 2008; Chamoles et al. 2001a; Chamoles et al. 2001b; Dean et al. 2006; Duffey et al. 2010a; Duffey et al. 2010b; Fuller et al. 2011; Wang et al. 2007; Wolfe et al. 2011). However, none of these studies had a prospective design, i.e. only patients with a confirmed diagnosis of MPS were included.

Enzymatic diagnosis should always be complemented by sequencing of the disease gene to confirm the diagnosis of MPS, especially if enzyme replacement therapy or haematopoietic stem cell transplantation is planned (Wang et al. 2011). DNA extracted from blood or saliva samples is most commonly used for this purpose (Wood et al. 2012). However, some recent studies have shown that genotyping is also feasible on DNA extracted from a DBS (Harahap et al. 2012; Hollegaard et al. 2011; Quraishi et al. 2012). Although this method has already been applied successfully to detect mutations involved in Fabry disease, an X-chromosomal LSD (Hagège et al. 2011; Tai et al. 2012), its usefulness remains to be shown for other LSDs such as MPS and ML II/III. Therefore, the current prospective study aimed additionally at testing whether DBS could be applied to ascertain the diagnosis of MPS in patients with an 'MPS-like' phenotype, using the same DBS for both enzymatic and molecular genetic analyses.

Methods

Dried Blood Spots

After obtaining informed consent, DBS were collected from patients with a suspicion of MPS based on clinical symptoms, mainly including coarse facial features, hepatosplenomegaly, corneal clouding, gibbus, developmental delay, short stature, frequent respiratory illness and/or umbilical hernia. In order to ascertain the diagnosis in a large number of patients with MPS, selection was based rather on the "overall" phenotypic impression than on the total number of symptoms. Many samples were submitted from patients with coarse face as the leading symptom. Patients were not selected on the bases of MPS-specific laboratory investigations and were sent for analysis to the Metabolic Laboratory (Hamburg University Medical Centre, Germany) by courier mail at room temperature between July 2011 and March 2012. The average age of the patient population was 9.3 a, while the median is 2.9 a. The age range of the population varies between 0 a (newborn) to 75.5 a. Approx. 60% were males. After reception, samples were stored with desiccant at −20°C to avoid degradation of enzymes until the analysis was completed. For this study consisting of enzymatic and molecular genetic assays, the first 200 samples were chosen without any bias.

Measurement of Enzyme Activity

To analyse the activity of the enzymes α-iduronidase, iduronate-2-sulphatase, arylsulphatase B and β-galactosidase in DBS, previously described methods were slightly modified (Civallero et al. 2006; Chamoles et al. 2001b). For each test, a 3 mm DBS was mixed with elution liquid and substrate in a 96-well plate (for details: see Table 1). For α-iduronidase and arylsulphatase B as well as β-galactosidase

Table 1 Reagents used for analysis of enzyme activity

Enzyme	Elution liquid	Substrate buffer
α-L-iduronidase	20 µL 10 mM D-saccharic acid 1,4-lactone (Sigma-Aldrich, Taufkirchen, Germany) (inhibitor) 110 µL 0.05 M sodium phosphate buffer pH 3.5 (0.1 M citric acid and 0.2 M Na_2HPO_4 dihydrate (Merck, Darmstadt, Germany)	20 µL 4-methylumbelliferyl-α-L-iduronidase (Toronto Research Chemicals, North York, Canada) in 0.05 M sodium phosphate buffer pH 3.5
Iduronate 2-sulphatase	40 µL 0.1 M sodium acetate/0.1 M acetic acid buffer pH 5.0	20 µL 1.25 mM methylumbelliferyl-α-L-iduronide-2-sulphate (Moscerdam, Rotterdam, Netherlands) in 0.1 M sodium acetate/0.1 M acetic acid buffer pH 5.0 (Merck, Darmstadt, Germany)
Arylsulphatase B	30 µL dH_2O water +20 µL 15 mM lead acetate in sodium acetate buffer pH 5.0 (Merck, Darmstadt, Germany)	50 µL 0.01 M 4-methylumbelliferone sulphate (Sigma-Aldrich, Taufkirchen, Germany) in 0.05 M sodium acetate buffer pH 5.0
β-galactosidase	100 µL 0.1 M citric acid buffer	100 µL 3.4 mg 4-methylumbelliferyl-β-D-galactoside (Sigma Aldrich, Taufkirchen, Germany) in 5 mL 0.1 M citric acid buffer pH 4.4 (Merck, Darmstadt, Germany)

dH_2O demineralised water, *EC* Enzyme Commission

Table 2 Statistical data for the fluorometric MPS tests using dried blood specimens

	Controls (nmol/punch*21 h)			Patients (nmol/punch*21 h)		
Test	Median (1st/99th percentile)	Range	*n*	Median	Range	*n*[a]
MPS I	0.08 (0.05/0.16)	0.04–0.26	106	0	0–0.01	15
MPS II	0.06 (0.03/0.1)	0.02–0.25	112	0	0–0.01	15
MPS VI	0.53 (0.3/0.86)	0.2–1.41	104	0.03	0–0.1	12

[a] Confirmed patients

duplicates were run, while for iduronate-2-sulfatase, we ran samples in single and repeated the analysis of samples with low enzyme activity in duplicate because of the higher cost of that particular assay. In total, 9–14 DBS punches (3 mm) are necessary for analysis of the full MPS panel. Enzyme activities were compared with laboratory-specific reference ranges determined using unaffected controls (around 100 newborns and 20–30 older individuals). After shaking the plate for 45 min, all reactions were incubated for 21 h at 37°C. For α-iduronidase, arylsulphatase B and β-galactosidase, the enzymatic reaction was stopped by adding 200 µL 0.17 M glycine carbonate buffer (12.8 g glycine and 18 g sodium carbonate in 200 mL demineralised water – Merck, Darmstadt, Germany). For iduronate-2-sulphatase, the reaction was stopped by adding 40 µL Pi/Ci stop buffer (0.4 M sodium phosphate/0.2 M citric acid pH 4.5 (Merck, Darmstadt, Germany)) and 10 µL of lysosomal enzyme solution (Moscerdam, Rotterdam, Netherlands), followed by a second incubation for 24 h at 37°C, which was stopped with 200 µL 0.17 M glycine carbonate buffer. For all samples in all assays, a blank was run in parallel to correct for fluorescence quenching by haemoglobin. For these blanks, the DBS was only added to the well after stopping the incubation, followed by 30 min shaking at room temperature. All plates also contained a positive and a negative control (i.e. samples from a healthy volunteer and from patients with confirmed diagnosis, respectively). For all enzyme assays, calculation of results was based on a standard curve with different dilutions of 0.05 M 4-methylumbelliferone (Sigma-Aldrich, Taufkirchen, Germany) in 0.17 M glycine carbonate buffer. Fluorescence was read on a Victor D instrument (Wallace Oy, Turku, Finland). Enzyme activities were expressed as nmol per 21 h and per punch and compared with laboratory-specific reference values (Müller et al. 2010; Wood et al. 2012). Details on the individual ranges are shown in Table 2.

Molecular Genetic Analysis

Molecular genetic analysis was carried out on the DNA extracted from DBS used previously for enzymatic analysis. DNA-samples were isolated with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Mutation analysis was performed by exon-specific PCR amplification of individual

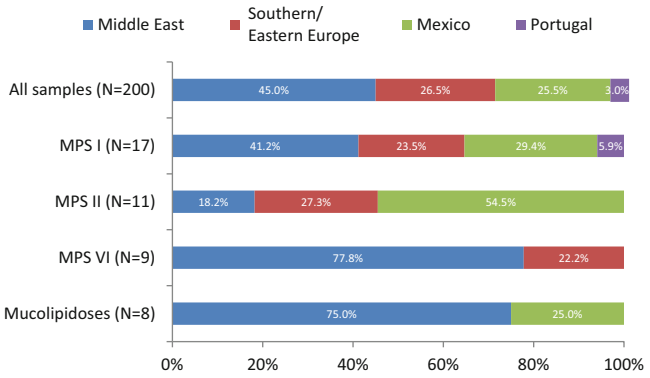

Fig. 1 Geographic distribution of all analysed dried blood spots and of those samples that were putatively positive for mucopolysaccharidosis (MPS) I, II, VI or potential mucolipidoses II/III. Turkey, Saudi Arabia, Kuwait, United Arab Emirates, Syria and Israel were considered as countries in the Middle East, while Romania, Russia, Kazakhstan, Croatia, Armenia, Bulgaria, Lithuania, Latvia, Serbia and Slovenia were considered as Eastern/Southern European countries

exons together with short flanking intronic sequences, followed by conventional Sanger sequencing (using GeneAnalyzer 3500/Applied Biosystems).

Results

Demographics

In total, 200 DBS were analysed. About 50.0% originated from countries in the Middle East, mainly Turkey (18.0%) and Saudi Arabia (16.0%), while about 25% originated from Eastern and Southern European countries and another quarter from Mexico (Fig. 1).

Enzymatic and Molecular Genetic Analyses on Dried Blood Spots

All DBS were analysed for activities of the enzymes α-iduronidase, iduronate-2-sulphatase and arylsulphatase B. In addition, activity of a reference lysosomal enzyme, β-galactosidase, was measured to confirm sample integrity (Gasparotto et al. 2009). If α-iduronidase, iduronate-2-sulphatase or arylsulphatase B activity was below the normal reference range and with a value consistent with enzyme activities of patients with established diagnosis, samples were considered as putatively positive for MPS I, MPS II or MPS VI, respectively. If activities of at least two of the three enzymes tested were elevated or in the upper reference range, while β-galactosidase activity was within the normal range, patients were considered potentially having ML II/III (Gusina and Tsukerman 1988; Wood et al. 2012). To rule out multiple sulphatase deficiency, activities of both sulphatases (and possibly other sulphatases) were determined in parallel (Scarpa et al. 2011; Wood et al. 2012).

In total, 45 DBS (22.5%) tested putatively positive: 17 (8.5%) for MPS I, 11 (5.5%) for MPS II, 9 (4.5%) for MPS VI and 8 (4.0%) for ML (Tables 3, 4, 5, and 6). For the positive samples, mutation analysis of the corresponding disease gene was carried out.

MPS I

For 17 DBS, α-iduronidase activity was well below the reference range, while β-galactosidase activity was normal (Table 3). The samples belonged to 12 males and 5 females, with a median age of 2.0 years at the time of DBS collection. The geographic origin of the patients was similar to that of the total group of patients (Fig. 1).

Genotyping of the α-L-iduronidase gene (*IDUA*) was performed for 13 DBS (76.5%), whereas there was no material left over after enzymatic analysis of 4 samples to do a molecular genetic test (Table 3). For 11 patients (84.6%), a total of 10 different mutations of the *IDUA* gene were detected: eight patients were homozygous, two patients carried two different heterozygous mutations each, and in one patient, only one heterozygous mutation was detected. Remarkably, only four of the mutations identified have been previously reported (Table 3). Of the six novel mutations, the two intronic splice site mutations, the nonsense mutation, and the 52 base pair insertion are very probably disease causing, whereas the pathogenic significance of the two missense mutations needs to be proven in the future. For two patients (15.4%), no mutations were found in the *IDUA* gene.

MPS II

Enzymatic analysis revealed a lower-than-normal activity of iduronate-2-sulphatase for 11 DBS (all from male patients, Table 4). Normal β-galactosidase and arylsulphatase B

Table 3 Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots of 17 patients diagnosed with mucopolysaccharidosis type I

No	Origin	Age (years)	Gender	α-Iduronidase (nmol/21 h*punch)	β-Galactosidase (nmol/ 21 h*punch)	Mutation *IDUA* gene[a]	
						Mutation 1	Mutation 2
1	Armenia	6	M	0	0.74	p.Met1?[b,1] (c.1A>C)	p.Met1?[b,1] (c.1 A>C)
2	Kuwait	1.5	M	0	0.54	p.Arg628* (c.1882C>T)	p.Arg628*[b,2] (c.1882C>T)
3	Latvia	2	M	0	0.89	p.Ser633Trp (c.1898C>G)	p.Gln70*[b,3] (c.208C>T)
4	Mexico	4	F	0	0.85	p.Ser633Trp (c.1898C>G)	p.Ser633Trp (c.1898C>G)
5	Mexico	59	M	0	0.58	–	–
6	Mexico	2	M	0	0.72	p.Ser633Trp (c.1898C>G)	p.Ser633Trp (c.1898C>G)
7	Mexico	5	M	0	0.5	NA	NA
8	Mexico	2	M	0.01	1.35	NA	NA
9	Portugal	54	F	0	0.53	–	–
10	Russia	21	M	0.01	1.09	NA	NA
11	Saudi Arabia	4	M	0	1.0	c.1525-1G>C	c.1525-1G>C
12	Saudi Arabia	0.5	M	0	0.5	p.Leu623Pro (c.1868T>C)	p.Leu623Pro (c.1868T>C)
13	Saudi Arabia	5	F	0.01	1.04	p.Trp402*[b,4] (c.1206G>A)	–
14	Saudi Arabia	1	F	0.01	2.2	c.1598_1599ins52	c.1598_1599ins52
15	Serbia	1	M	0.01	0.7	p.Pro22Ser (c.64C>T)	c.1829-1G>A
16	Turkey	0.5	F	0	1.08	p.Trp68* (c.203G>A)	p.Trp68* (c.203G>A)
17	Turkey	2	M	0	1.12	NA	NA
Reference range				0.04–0.26	0.5–3.2		

NA not assessed due to insufficient material, – mutation not found
[a] Nomenclature: http://www.hgvs.org/mutnomen/
[b] Mutations already reported
[1] Lee-Chen and Wang (1997), [2] Beesley et al. (2001), [3] Clarke and Scott (1993), [4] Scott et al. (1992)

activities (assessed for all DBS with sufficient material) excluded multiple sulphatase deficiency (MSD; data not shown). Consequently, these samples were considered positive for MPS II. Their median age was 4.0 years at the time of DBS collection. Remarkably, more than 50% of these patients originated from Mexico (Fig. 1).

A (probably) disease-causing mutation of the iduronate 2-sulphatase gene (*IDS*) was identified in all 11 DBS. Most of these mutations have already been reported in MPS II patients (Table 4). Of the six patients originating from Mexico, two carried the same mutation, i.e. p.Arg468Gln. Of the four novel mutations, three are very probably pathogenic, whereas further studies will be needed to assess the missense variant (p.Ser349Arg).

MPS VI

Nine patients showed arylsulphatase B activities far below the reference range and β-galactosidase activity within normal limits, compatible with MPS VI (Table 5). Wherever possible, iduronate-2-sulphatase activity was also determined to exclude MSD (data not shown). Of the nine MPS VI patients, five were males and four were females, with a median age of 2.0 years at the time of DBS collection. The majority of these patients (seven patients; 77.8%) came from countries in the Middle East (Fig. 1).

Molecular genetic analysis of the DBS revealed mutations in the arylsulphatase B gene (*ARSB*) in all nine patients: seven were homozygous, while two patients carried two different heterozygous mutations each. Remarkably, 7 out of 14 disease alleles (50%) of patients from the Middle East carried the p.Tyr251* mutation (three homozygous, one heterozygous). Only three of the mutations found by us have previously been reported (Table 5). Of the five novel mutations, two are very probably disease causing, whereas future studies are needed concerning the pathogenic significance of the three novel missense mutations.

Mucolipidoses

For eight DBS, belonging to four males and four females with a median age of 2.0 years, enzymatic analysis

Table 4 Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots of 11 patients diagnosed with mucopolysaccharidosis type II

No	Origin	Age (years)	Gender	Iduronate-2-sulphatase (nmol/21 h*punch)	β-Galactosidase (nmol/21 h*punch)	Mutation *IDS* gene[a]
1	Armenia	19	M	0	0.5	p.Ala346Val[b,1] (c.1037C>T)
2	Armenia	23	M	0	0.5	p.Ala346Val[b,1] (c.1037C>T)
3	Lithuania	1.5	M	0	0.79	p.Arg468Gln[b,2] (c.1403G>A)
4	Mexico	7	M	0	0.68	c.1463delT
5	Mexico	5	M	0	0.9	p.Gly340Asp[b,3] (c.1019G>A)
6	Mexico	2	M	0	0.57	p.Arg468Gln[b,2] (c.1403G>A)
7	Mexico	1	M	0	0.54	p.Gln75*[b,4] (c.223C>T)
8	Mexico	2.5	M	0	0.67	p.Ser349Arg (c.1047C>A)
9	Mexico	12	M	0	0.6	p.Arg468Gln[b,2] (c.1403G>A)
10	Saudi Arabia	4	M	0.01	0.78	Rearrangement[c]
11	Turkey	2	M	0	0.62	c.213_215del13[d]
Reference range				0.02–0.25	0.5–3.2	

[a] Nomenclature: http://www.hgvs.org/mutnomen/
[b] Mutations already described
[c] Complex rearrangement between gene and pseudogene
[d] c.213_225delCCTCCTCTTCCAG
[1] Li et al. (1995), [2] Whitley et al. (1993), [3] Karsten et al. (1998), [4] Kato et al. (2005)

Table 5 Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots in nine patients diagnosed with mucopolysaccharidosis type VI

No	Origin	Age (years)	Gender	Arylsulphatase B (nmol/21 h*punch)	β-Galactosidase (nmol/21 h*punch)	Mutation *ARSB* gene[a]	
						Mutation 1	Mutation 2
1	Kuwait	1	M	0.03	0.61	p.Tyr251* (c.753C>G)	p.Tyr251* (c.753C>G)
2	Russia	8	F	0.04	0.7	p.Thr92Lys[b,1] (c.275C>A)	p.Leu98Pro[b,2] (c.293T>C)
3	Russia	2	F	0.04	0.52	c.691-1G>A	c.691-1G>A
4	Saudi Arabia	1	M	0.03	0.7	p.Tyr251* (c.753C>G)	p.Tyr251* (c.753C>G)
5	Saudi Arabia	1.5	M	0.04	0.84	p.Tyr251* (c.753C>G)	p.Tyr251* (c.753C>G)
6	Saudi Arabia	3	M	0.06	0.78	p.Gly56Asp (c.167G>A)	p.Gly56Asp (c.167G>A)
7	Syria	4	M	0.02	0.55	p.Tyr251* (c.753C>G)	p.Gly308Glu (c.923G>A)
8	Turkey	1	F	0.03	0.5	p.Leu321Pro[b,3] (c.962T>C)	p.Leu321Pro[b,3] (c.962T>C)
9	Turkey	14	F	0.03	0.54	p.Val376Glu (c.1127T>A)	p.Val376Glu (c.1127T>A)
Reference range				0.2–1.41	0.5–3.2		

[a] Nomenclature: http://www.hgvs.org/mutnomen/
[b] Mutations already reported (www.hgmd.cf.ac.uk)
[1] Karageorgos et al. (2007), [2] Voskoboeva et al. (2000), [3] Isbrandt et al. (1994)

suggested ML II/III: activities of α-iduronidase, iduronate-2-sulphatase and/or arylsulphatase B were in the upper reference range or above, while β-galactosidase activity fell within the normal range (Table 6). The majority (75.0%) of these samples originated from the Middle East (Fig. 1).

Genotyping of *GNPTAB*, one of the genes implicated in ML II/III revealed probably disease-causing mutations in five of the samples. All these patients carried different alterations, all in apparently homozygous state. None of these mutations have previously been associated with ML II/III (Table 6). Of the five novel mutations, three are very probably pathogenic. As to the two novel missense variants, one changes arginine to cysteine whereas the other one cysteine to arginine.

Table 6 Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots in eight patients with the possible diagnosis of mucolipidoses II/III

No	Origin	Age (years)	Gender	α-Iduro-nidase (nmol/ 21 h*punch)	Iduronate-2-sulphatase (nmol/21 h*punch)	Arylsulphatase B (nmol/ 21 h*punch)	β-Galactosidase (nmol/ 21 h*punch)	Mutation *GNPTAB* gene[a]	
								Mutation 1	Mutation 2
1	Mexico	1	F	0.45	NA	1.69	0.59	c.616_619delACAG	c.616_619delACAG
2	Mexico	2	M	0.45	1.37	1.55	0.53	–	–
3	Saudi Arabia	1	F	0.21	0.93	1.06	0.70	p.Arg46* (c.136C>T)	p.Arg46* (c.136C>T)
4	Saudi Arabia	5	M	0.5	1.15	2.06	0.44	–	–
5	Turkey	0.5	F	0.29	0.25	1.77	0.75	–	–
6	Turkey	3	F	0.33	NA	1.29	0.50	p.Glu279* (c.835G>T)	p.Glu279* (c.835G>T)
7	Turkey	4	M	0.23	1.47	1.28	0.64	p.Arg986Cys (c.2956C>T)	p.Arg986Cys (c.2956C>T)
8	UAE	2	M	0.74	NA	1.54	0.51	p.Cys523Arg (c.1567T>C)	p.Cys523Arg (c.1567T>C)
Reference range				0.04–0.26	0.02–0.25	0.2–1.41	0.5–3.2		

NA not assessed due to insufficient material on dried blood spot, *UAE* United Arab Emirates, – mutation not found
[a] Nomenclature: http://www.hgvs.org/mutnomen/

Discussion

This study shows that DBS are very useful samples for diagnosing many forms of MPS, because they can be used for both enzymatic and molecular genetic analyses and generally facilitate sampling, shipping and handling of the samples.

The advantage of using DBS for enzymatic diagnosis of MPS and ML II/III has been demonstrated (Chamoles et al. 2001b; Chamoles et al. 2001a). In the past years, fluorometric methods, immunocapture enzyme assays and even high-throughput tandem mass spectrometry assays have been developed and optimised to allow (large-scale) screening for LSDs (Blanchard et al. 2008; Dean et al. 2006; Duffey et al. 2010a; Duffey et al. 2010b; Fuller et al. 2011; Reuser et al. 2011; Wang et al. 2007; Wolfe et al. 2011). However, all studies using DBS were feasibility studies, including only patients with a confirmed diagnosis of MPS. In contrast, in our study, DBS of 200 patients with a vague clinical suspicion of MPS, i.e. an 'MPS-like phenotype', were analysed for the first time in a prospective way. Patients were screened for α-iduronidase activity and also for iduronate-2-sulphatase and arylsulphatase B activities because there is a significant overlap in clinical manifestations between the corresponding MPS types. Indeed, of the 200 patients, 8.5% were tested positive for MPS I, 5.5% for MPS II and 4.5% for MPS VI. Remarkably, eight patients were classified as potentially having ML II or III, as the latter disorders are characterised by elevated activities of acid hydrolases in plasma – and therefore potentially also in DBS. Indeed, in ML II or III, the mannose-6-phosphate tag on the lysosomal enzymes – which ensures sorting to the lysosome – is missing, leading to the excretion of the enzymes into the plasma. This yields elevated in vitro plasma enzyme activities, which can also be found in DBS (Gusina and Tsukerman 1988; Fuller et al. 2011; Wood et al. 2012).

Based on the data of this study, we recommend that patients with an 'MPS-like phenotype' should be tested for at least MPS I, II and VI. As soon as it will be possible to reliably perform DBS testing for MPS IVA, this disease should also be included. Leading clinical features of MPS III are neurological symptoms whereas coarse face has rarely been described in this group of patients. Therefore we think that it is justified not to include MPS III in the targeted screening. In contrast, MPS VII may present with a Hurler-like phenotype. However, given the rarity of this condition, we have decided not to include it in our program.

Our strategy may allow a timely diagnosis of three MPS types – and potentially even ML II/III and multiple sulphatase deficiency – at a relatively low additional cost. Urinary GAG analysis (quantitative and qualitative) as the first tier testing followed by enzyme determination may currently cost in our laboratory approximately 120 Euros, whereas the immediate analysis of the three enzymes on DBS costs approximately 80 Euros.

Genotyping of DNA extracted from filter paper has already been successfully performed in a number of diseases, including Fabry disease (Hagège et al. 2011; Tai et al. 2012), an X-chromosomal LSD. Our study also demonstrates that DBS can also be used for molecular genetic analysis of the genes involved in MPS I, II, VI and ML II/III.

For two DBS with pathologically decreased activity of α-iduronidase (MPS I), no *IDUA* mutation was found (Table 3, patients 5 and 9). This might be due to the fact that certain mutations – e.g. exon-spanning inversions, duplications, or deep intronic mutations – cannot be detected by conventional sequencing. This might also be the case in the patient with putative MPS I, in which only a heterozygous mutation was found (Table 3, patient 13). Alternatively, the lack of identifiable mutations may be due to false positive results in the initial enzymatic analysis. Indeed, based on our experience, α-iduronidase appears to be less stable in DBS than the other enzymes tested. As such, the enzyme may be more sensitive to unfavourable preanalytical conditions (during sampling, shipping, etc.), leading to diminished α-iduronidase activity while the activities of the other enzymes remain within the normal range, as might be the case in the two patients. Consequently, confirmatory testing of an independently obtained second DBS is important to validate the initial results of the enzymatic analysis (Wood et al. 2013). As no second DBS has been received for the two putative MPS I patients without identifiable mutations in the *IDUA* gene, the results of their enzymatic tests should be considered tentative.

Our experience of performing both enzymatic and molecular genetic analysis on DBS for diagnosing MPS or ML II/III has important practical consequences because DBS offer many advantages over other sample types, such as leukocytes, cultured fibroblasts, whole blood or saliva. Indeed, sample collection is easy and minimally invasive, requiring only a small blood volume (Reuser et al. 2011). Furthermore, we show that a single specimen can be used for both biochemical and molecular genetic testing. However, results of a single DBS enzyme assay are not sufficient for diagnosis and should always be accompanied by further confirmatory tests, which can be either a second enzyme test or molecular genetic studies. If protected from humidity, DBS can be stored easily for long time with only minimal losses in enzyme activity (Reuser et al. 2011) or stability of genomic DNA (Chaisomchit et al. 2005; Hollegaard et al. 2011). In addition, DBS can be shipped via regular mail at room temperature – and thus at a low cost – and pose little biohazard risk to the handlers. Consequently, DBS may be especially useful for diagnosing

MPS or ML II/III in regions of the world that lack specialised laboratories. In such regions, it may not be feasible to transport whole blood or other tissue samples, in order to confirm the DBS-based diagnosis. In the latter case, a second independently collected DBS would be needed to confirm the diagnosis (Wood et al. 2013). To elucidate the diagnostic efficacy of the DBS method to detect affected individuals, a study for MPS I, MPS II, MPS VI and ML II/III is necessary with patients of confirmed diagnosis, especially as the sensitivity of our screening assay remains unknown because of the small group of patients diagnosed.

Conclusions

Our prospective study shows that DBS can be used successfully for both enzymatic and molecular genetic diagnosis of MPS I, II, and VI, and ML II/III in patients with a clinical suspicion of MPS. Given the advantages of DBS over other sample types in terms of ease of collection, storage and transportation, DBS are particularly useful for screening patients in regions lacking specialised laboratories. We suggested that patients should always be screened in parallel for at least α-iduronidase, iduronate-2-sulphatase and arylsulphatase B (MPS I, II, and VI), i.e. by a multitasking assay that also provides a potential for diagnosing ML II/III and MSD.

Take-Home Message

In patients with suspicion of mucopolysaccharidosis (MPS) based on clinical features, enzymatic analysis on dried blood spots (DBS) may allow diagnosis of MPS I, II, VI and mucolipidosis II/III and it is suggested to corroborate the initial findings by molecular genetic testing from the same DBS or by another enzyme measurement.

Authors' Contributions

Paulina Nieves Cobos was involved in designing the study, carried out the biochemical measurements and revised the manuscript.

Cordula Steglich carried out the genetic analysis.

René Santer was involved in the design of the study, review of the data and revised the manuscript.

Andreas Gal was responsible for the molecular genetic testing. He was involved in drafting the manuscript and revising it critically for important intellectual content.

Zoltan Lukacs was involved in designing the study and collecting, analysing and interpreting the data. He was involved in drafting the manuscript and revising it critically for important intellectual content. Zoltan Lukacs accepts full responsibility for the work and the conduct of the study, had access to all data and controlled the decision to publish.

All authors have given final approval of this version to be published.

Competing Interests

Paulina Nieves Cobos has no competing interests.

Cordula Steglich has no competing interests.

René Santer has no competing interests.

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Ethical Approval

Ethical approval was not required for this study.

Patient Consent Statement

All patients included in the study or their parents/caretakers gave informed consent.

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