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CASE REPORT

Reversible Keratopathy Due to Hypertyrosinaemia Following Intermittent Low-Dose Nitisinone in Alkaptonuria: A Case Report

R.M.K. Stewart • M.C. Briggs • J.C. Jarvis • J.A. Gallagher • L. Ranganath

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Abstract We describe a patient with ultra-rare disease, alkaptonuria, who developed tyrosine keratopathy following nitisinone therapy of 2 mg on alternate days. His vision became impaired approximately 7 weeks following the commencement of nitisinone and ophthalmological examination at week nine showed characteristic dendritic keratopathy associated with tyrosinaemia. The corneal lesion as well as his visual symptoms normalized completely following discontinuation of nitisinone. This is the first documented report of keratopathy due to acquired tyrosinaemia due to very low-dose nitisinone.

Introduction

Alkaptonuria (AKU) is a rare inherited metabolic disorder with severe premature spondyloarthropathy as a major manifestation (Phornphutkul et al. 2002). Although joint disease is a major feature, virtually all connective tissues are affected leading to a variety of clinical features and complications (Helliwell et al. 2008). A promising new agent, called nitisinone, is available for the treatment of AKU (McKiernan 2006). Early nitisinone therapy is likely to prevent morbidity but may only slow or arrest disease progression if started later. Nitisinone is not yet licensed for AKU; therefore, safety is an important issue and potential adverse effects are of interest to those involved in the management of metabolic disorders.

Alkaptonuria is a rare inborn error of metabolism characterized by high circulating homogentisic acid (HGA) due to a genetic defect in the enzyme homogentisate dioxygenase (HGD) (Phornphutkul et al. 2002). The main pathophysiological event is conversion of HGA to a polymeric melanin-like pigment and binding of this pigment to connective tissue, especially cartilage. This process takes many years and is known as ochronosis (Zannoni et al. 1969). The damaging effects of ochronosis include arthritis (especially in the spine and large weight bearing joints), stones (renal, prostatic, gall bladder and salivary), cardiac valve disease especially aortic (Goodfellow et al. 2005), ruptures (muscle, tendons and ligaments), osteopenia and fractures (O'Brien et al. 1963). Virtually all connective tissue is affected.

Nitisinone inhibits *p*-hydroxyphenyl pyruvate dioxygenase, the enzyme leading to the formation of HGA (Lock et al. 1998). In keeping with the mode of action of nitisinone, circulating tyrosine increases. The tyrosinaemia that occurs during nitisinone treatment resembles hereditary tyrosinaemia type 3. Adverse effects known to be associated with tyrosinaemia include corneal and dermal toxicity (Meissner et al. 2008). Therefore, skin rash and dendritic keratopathy might be expected in some patients with a nitisinone-induced tyrosinaemia.

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Nitisinone at a dose of 1–2 mg/kg/day has been widely used for more than 20 years in the treatment of a life-threatening condition called hereditary tyrosinaemia type 1 (HT-1) (Lindstedt et al. 1992). Corneal involvement is not part of the natural history in HT-1. Corneal lesions in medically managed HT-1 are reported as being rare (Masurel-Paulet et al. 2008; Gissen et al. 2003) with less than 7.4% frequency in one large series of 176 HT-1 patients treated with nitisinone (Holme and Linstedt 1998). Another report on 46 HT-1 patients noted 8.7% prevalence of keratitis with nitisinone treatment (Masurel-Paulet et al. 2008). A smaller series reported no eye symptoms or keratopathy in 11 HT-1 patients treated with nitisinone despite seeing high circulating tyrosine concentrations in several patients (Gissen et al. 2003).

In contrast to the quantities of nitisinone employed in HT-1 to prevent hepatic and renal pathology, the dose of nitisinone that decreases homogentisic acid in AKU by greater than 95% is only 2 mg daily, or approximately 5% of the dose used in HT-1. This is based on the experience of using nitisinone in the National Institutes of Health, USA (Phornphutkul et al. 2002; Suwannarat et al. 2005; Introne et al. 2011). However, one patient in the clinical trial of nitisinone in AKU developed corneal keratopathy that resolved fully with discontinuation of nitisinone (Introne et al. 2011).

Nitisinone is not licensed for the management of AKU at present. However, nitisinone continues to be used by physicians in AKU due to its plausible mode of action and efficacy in decreasing serum and urine HGA. Although the dose is much lower than that in HT-1, safety is clearly paramount when using an unlicensed product and we describe one patient with AKU on very low dose of nitisinone who developed symptomatic corneal keratopathy.

Case Report

A 21-year-old man (Mr X) was referred to the Liverpool for the management of his AKU. Apart from dark urine he did not have many symptoms of AKU, although he did experience back pain intermittently. The Department of Health National Specialised Services (NSCT) designated the Royal Liverpool University Hospital to host the National Alkaptonuria Service (NAS) in April 2012. The following case report data was collected as part of providing that service. The data from the NAS is being published as a clinical practice article. The NAS has been allowed to administer nitisinone up to 2 mg daily to people with AKU with all appropriate assessments and monitoring in place. Mr X attended the *NAS* in August 2012 and after baseline assessments that included measurement of visual acuity (6/5 unaided either eye), slit lamp examination of his eyes plus slit lamp photography to demonstrate clarity of the cornea and also document any scleral pigmentation (Fig. 1a), he was commenced on nitisinone 2 mg alternate days and discharged with a dietary and a safety monitoring plan. Approximately 7 weeks after commencing nitisinone 2 mg, he noticed increasing epiphora on alternate evenings while watching television; by the following morning the ocular symptoms had resolved. He did not admit any pain, redness of eye or significant blurring of vision. The symptoms progressed sufficiently for him to contact the NAS at week 9, when his nitisinone was discontinued. At this stage his visual acuity was noticed to be reduced in the right eye (6/9 unaided in the right eye, 6/5 unaided in the left) and slit lamp examination demonstrated a dendritic corneal opacity typical of tyrosine keratopathy (Fig. 1b). A blood sample collected at this time showed that his serum tyrosine was 941 μmol/L (reference range 30–90 μmol/L) (Table 2). Conjunctival swab for Herpes Simplex virus PCR was negative. During his assessments at this time he admitted that he was finding it difficult to modify his diet following nitisinone therapy despite having received dietetic counselling to decrease his protein intake. He was 86.9 kg in weight and 1.74 M in height with a body mass index of 28.7 kg/M^2. He also had an itchy urticarial skin rash at the same time his ocular symptoms and with the same diurnal variation (Fig. 2). The rest of the physical examination was within normal limits with a blood pressure of 116/70 mmHg and pulse rate of 70 bpm.

The patient was free-living and his diet was assessed by means of a 7-day food diary at baseline pre-nitisinone when he was consuming 85 g protein per day pre-nitisinone (1 g/kg body weight) and he was asked to reduce this to 0.8 g/kg body weight as he was commencing nitisinone. Once he developed keratopathy, following a washout of nitisinone over a month, he was asked to decrease his protein intake to 0.6 g/kg body weight before he could commence nitisinone. At year 1 review he was estimated to be consuming 0.8 g/kg body weight of protein based on a 7-day food diary analysis.

Following the discontinuation of nitisinone, his ocular symptoms, keratopathy and skin rash all resolved. Figure 1c shows a reduction of corneal opacity at 1 week following cessation of nitisinone and Fig. 1d shows no visible keratopathy at 1 year follow-up (visual acuity returned to 6/5 unaided). Figure 2b shows resolution of the skin rash.

He was trialled on nitisinone 2 mg once a week, which he was able to tolerate fully, with his diet and lifestyle. His liver and renal profiles were monitored and data is shown in Table 1. His transaminases increased to less than three times upper reference intervals and were simply monitored. The metabolic data is shown in Table 2.

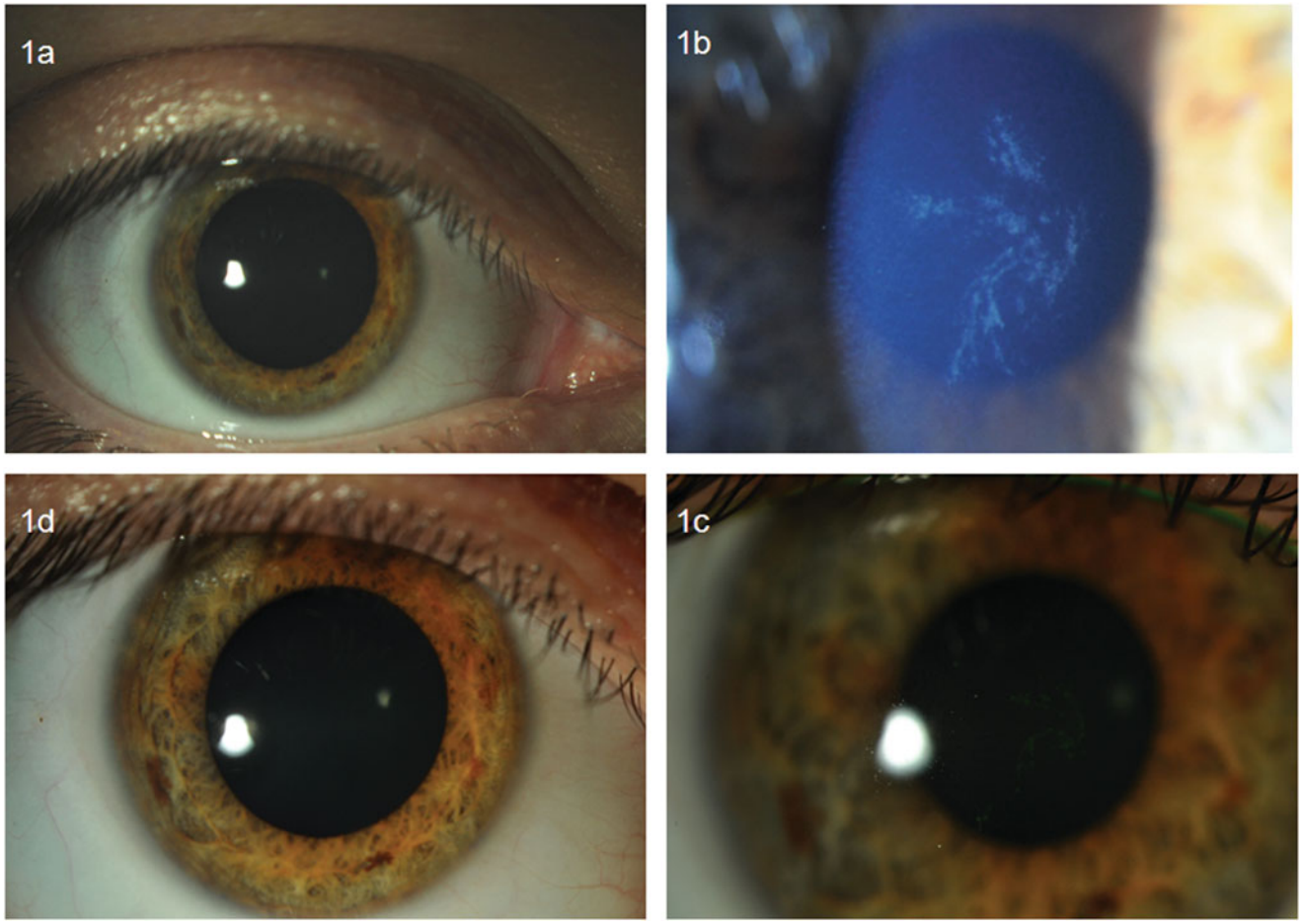

Fig. 1 Slit lamp examination of the eyes plus slit lamp photography showing (**a**) clarity of the cornea, (**b**) a dendritiform corneal opacity typical of tyrosine keratopathy, (**c**) a reduction of corneal opacity at 1 week following cessation of nitisinone and (**d**) no visible keratopathy at 1 year follow-up

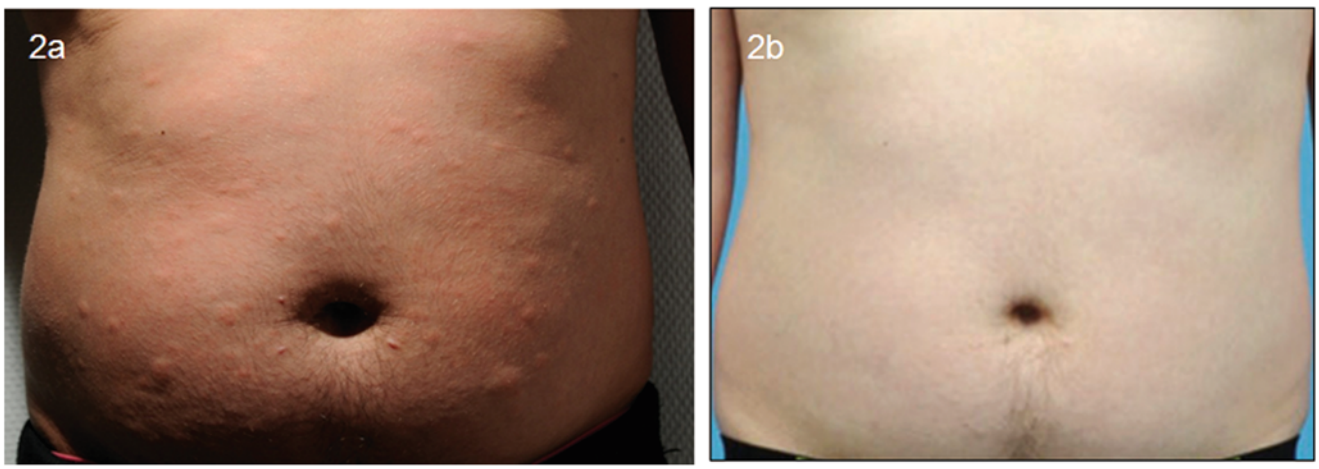

Fig. 2 (**a**) Itchy urticarial skin rash on nitisinone and (**b**) resolution of the skin rash following cessation of nitisinone

Table 1 Summary of liver and renal profiles

Analyte	Ref range	Pre-nitisinone	9 weeks post-nitisinone	Year 1 review
ALT	<35 μ/L	46	69	111
Alkaline phosphatase	35–125 μ/L	91	90	80
γGT	<50 μ/L	31	35	37
Urea	2.5–7.0 mmol/L	5.3	6.1	5.3
Creatinine	50–130 μmol/L	84	97	69
eGFR	>60 mL/min	>90	85	>90

Table 2 Metabolic data including serum tyrosine

	Urine homogentisic acid (mmol/day)	Serum homogentisic acid (μmol/l)	Serum tyrosine (μmol/l)
Baseline	14,246	33	42
2 days post nitisinone 2 mg	12,773	10.8	384
9 weeks 2 mg alt days	–	–	941
12 months 2 mg weekly	2,880[a] (80%↓)	16.7[a] (49%↓)	582

[a] $_{U}HGA_{24}$ and sHGA decreased at 4 weeks by 80 and 49% respectively compared with baseline

Discussion

This is the second published report of tyrosine keratopathy in AKU. The present case is remarkable in that the dose of nitisinone leading to the keratopathy was effectively 1 mg daily, given the very long half-life of nitisinone of 54 h (SPC for Orfadin™ 2 mg capsules: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000555/WC500049195.pdf). This is the lowest dose of nitisinone reported to have resulted in tyrosine keratopathy.

The previous report on tyrosine keratopathy in AKU employed 2 mg daily in the NIH clinical trial (Introne et al. 2011). A 48-year-old male developed corneal irritation 6 weeks after commencing nitisinone 2 mg daily. Slit lamp examination showed branching, sub-epithelial opacities consistent with the classic pattern previously seen with tyrosine crystal deposition. The patient was tried on a protein restricted diet but the ocular findings and symptoms were unchanged. Nitisinone was discontinued and the patient made a complete recovery. Two attempts were made to restart nitisinone; on each subsequent attempt, symptoms recurred. Nitisinone was stopped permanently. In the NIH study dietary protein was not restricted in the adults enrolled. Plasma tyrosine levels averaged 800 μM, with individual levels reaching as high as 1,500 μM; these levels were remarkably well tolerated. The single individual who developed a keratopathy classical for tyrosine toxicity approximately 6 weeks following initiation of oral nitisinone showed plasma tyrosine levels, measured while symptomatic, approximately 200 μM below the average for the treated cohort. Thus, the conclusion of the investigators in the NIH study was that about 5% of the AKU population might experience corneal toxicity as a result of nitisinone-induced tyrosinaemia, regardless of the specific plasma tyrosine level, suggesting that some predisposition to toxicity exists independent of the peak plasma tyrosine concentration.

The data in the present case shows that circulating tyrosine increases rapidly following the commencement of nitisinone counterbalancing the decrease in serum and urine HGA. Despite counselling, this patient was unable to decrease his protein intake sufficiently to minimize the increase in circulating tyrosine. The disappearance of symptoms and the improvement of his keratopathy and skin rash within a week of discontinuing nitisinone suggest that tyrosinaemia was the causal factor. There are published reports of poor compliance with diet leading to an exaggerated tyrosinaemia post-nitisinone when used in the treatment of HT-1 (Ahamad et al. 2002).

An interesting feature of the ocular symptoms was that these occurred in the evenings and also every other day. This may be consistent with the diurnal variation in the circulating tyrosine, known to be higher late in the day and lowest first thing in the morning (Fernstrom et al. 1979). A similar diurnal variation in ocular pain in the evenings has been reported previously (Schauwvlieghe et al. 2012). The symptoms on alternate days could be explained by the nitisinone dosing on alternate days; he could clarify whether his symptoms were on the day of administering nitisinone or the following.

The lack of correlation between serum tyrosine and keratopathy may reflect differences in tyrosine concentrations in different body pools, namely that the ocular anterior chamber tyrosine may be much higher than

circulating tyrosine as has been suggested previously (Lock et al. 1996), and local factors may be important in determining the size of each tyrosine pool.

Monitoring of circulating tyrosine is important and the aim is to try to maintain these below 700 μM wherever possible; low protein diet is reinforced to achieve levels of serum tyrosine below 500 μM wherever possible. It is noteworthy that other patients in the National AKU Centre with much higher circulating tyrosine levels do not show any ocular or skin symptoms. The one other report of keratopathy on 2 mg daily when used in AKU describes circulating tyrosine of 200 μM lower than the average values post-nitisinone reported as being 670–826 μM, i.e. 470–626 μM, although the exact value is not stated in the manuscript (Introne et al. 2011). Clearly factors other than just circulating tyrosine concentrations determine which patient on nitisinone develops ocular toxicity.

The important feature of keratopathy post-nitisinone is that it is fully reversible upon discontinuation, when identified and acted upon swiftly. With the potential for greater use of nitisinone in AKU, physicians need to be aware of this easily manageable complication, and further research may be required to identify an alternative treatment for ochronosis in these patients.

Our patient also had an itchy skin rash showing the same temporal relationship with the keratopathy. The close relationship would suggest that the causal factor is similar for both these complications, namely tyrosine. The published literature in this area is unclear on the relationship between the tyrosinaemia and skin lesions. Despite this cutaneous manifestations of tyrosinaemia have been described previously (Meissner et al. 2008).

We thus conclude that even very low doses of nitisinone, an inhibitor of a key enzyme in the tyrosine degradation pathway, can lead to tyrosine crystal keratopathy.

Compliance with Ethics Guidelines

This manuscript has not been published elsewhere.

All co-authors are aware and have agreed to this submission.

Stewart RMK, Briggs MC, Jarvis JC, Gallagher JA and Ranganath L declare that there are no conflicts of interest in submission of the paper.

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. In addition, the institutional review body (Royal Liverpool University Hospital) explicitly approved the National Alkaptonuria Service from which this data was generated.

Informed consent was obtained from all patients for being included in the study. This is being published as a clinical practice article and standard research ethics process is not therefore appropriate. The data from this patient have been completely anonymised to ensure he is not recognized from the publication of this manuscript. The data obtained were following standard clinical assessments upon referral to the National Alkaptonuria Service in Liverpool. Patients are informed verbally and through being handwritten materials about the activities of the National AKU Service. They are explicitly informed in the Patient information booklet of the National AKU Service that:

> We could publish results from the study but if we do, we will make sure you cannot be identified in any way. All data used for publicity or for other research purposes will ensure total anonymity. Please let us know when you are visiting Ward 9 B (where the National AKU Service is located) that you understand this and have no objection to it.

All the ocular photos were acquired during the standard assessments during the patient visit.

The skin rash photos were acquired following specific consent obtained by the medical photography department employing standard hospital guidance.

Contribution from Authors

LR Ranganath: Carried out patient assessments

MC Briggs and RMK Stewart: Performed all the ophthalmology examinations

JC Jarvis and JA Gallagher: Intellectual input and support, editing the manuscript

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CASE REPORT

Sports in LCHAD Deficiency: Maximal Incremental and Endurance Exercise Tests in a 13-Year-Old Patient with Long-Chain 3-Hydroxy Acyl-CoA Dehydrogenase Deficiency (LCHADD) and Heptanoate Treatment

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Abstract Exercise and subsequent catabolism is a potential trigger for creatine kinase (CK) concentration increase (rhabdomyolysis) in patients with LCHADD, therefore we evaluated the clinical and biochemical stability under physical exertion conditions at the age of 13 years in a currently 14-year-old LCHADD patient treated with heptanoate.

LCHADD was diagnosed during first decompensation at age 20 months. In the following 2 years, the patient had several episodes of rhabdomyolysis. Heptanoate 0.5–1 g/kg/day was started at 4 years, with no further CK elevations since. He is clinically stable, has retinopathy without vision impairment or polyneuropathy. Maximal incremental and endurance exercise tests were performed to evaluate both clinical and metabolic stability during and after exertion.

Physical fitness was adequate for age (maximum blood lactate 7.0 mmol/L, appropriate lactate performance curve, maximum heart rate of 196 bpm, maximum power 139 Watt = 2.68 Watt/kg body weight). There were no signs of clinical (muscle pain, dark urine) or metabolic derangement (stable CK, acyl carnitine profiles, blood gas analyses) – neither after maximal incremental nor endurance exertion.

This case illustrates that both under maximal incremental and endurance exertion, clinical and biochemical parameters remained stable in this currently 14-year-old LCHADD patient receiving heptanoate treatment.

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Introduction

LCHAD deficiency is an inherited metabolic disorder, involving the degradation of long-chain fatty acids. The acute complication is a metabolic decompensation with hypoketotic hypoglycemia, rhabdomyolysis, hepatopathy, cardiomyopathy, and loss of consciousness until coma (Arnold et al. 2009; Spiekerkoetter et al. 2009a). Biochemical diagnosis is established through acyl carnitine profile in dried blood spot/plasma (accumulation of long-chain acyl carnitines) and excretion of organic acids in urine during the metabolic crisis (accumulation of dicarbonic acids) (Lindner et al. 2010). Diagnosis is confirmed through enzymatic analysis showing enzyme deficiency in lymphocytes/fibroblasts and molecular analysis. Close to 85% of patients are homozygous for the common mutation c.1578G>C (Tyni et al. 2012), which also occurs in compound heterozygous state. Long-term complications include retinopathy and polyneuropathy. Both clinical manifestations are seen in the teenage years and can proceed into substantial morbidity (Gillingham et al. 2005). However, first signs of retinopathy may occur as early as 2 years of life, as was the case in our patient. These

complications are solely present in LCHAD and MTP deficiency, and not in the other long-chain fatty acid oxidation disorders. Therapy consists of a fat-defined (fat-reduced) diet and supplementation of medium-chain triglycerides (MCT) (Spiekerkoetter et al. 2009b; Roe and Mochel 2006; Roe et al. 2002, 2008). Therapy targeting long-term complications in patients with LCHAD deficiency includes approaches with a high supplementation of essential fatty acids (docohexaenic acid (DHA)) or anaplerotic substances, i.e. with heptanoate (Gillingham et al. 2005, 2009; Roe and Mochel 2006; Roe et al. 2002, 2008; Kinman et al. 2006). Both these therapeutic approaches lack a good parameter for follow-up and judgement of effectiveness. Follow-up on retinal background and nerve conduction velocity should be done routinely about once a year (Spiekerkoetter et al. 2009a).

We evaluated the clinical and biochemical stability under both incremental and endurance physical exertion conditions in a 13-year-old patient with LCHADD receiving heptanoate treatment for the last 9 years and a high caloric drink before exercise.

Case Report

The boy is the second child of healthy, non-consanguineous Caucasian parents, he has one older healthy sister. Until the age of 20 months his development was uneventful. At this point he suffered from a metabolic decompensation following an upper airway infection, showing coma, hepatopathy, and cardiomyopathy, leading to an intensive care unit admission and diagnosis. On eye examination, changes in retina were already visible, they have remained stable over the past 12 years since diagnosis. He does not suffer from polyneuropathy. Therapy consisting of a fat-defined diet (40% total energy intake, 50% MCT based) was started immediately. Up to the age of 4 years he repeatedly showed episodes with substantial elevation of creatine kinase (CK) concentrations and was metabolically instable (metabolic acidosis) during infections. Thus, in 2004 a therapy with heptanoate was started (currently: 2 x 15 mL in the morning and evening, equaling 0.6 g/kg/day). Heptanoate at 0.6 g/kg/day equals 15% of total energy. Total fat intake including heptanoate is 40% of total energy. Long-chain intake with 15 g/day is 0.3 g/kg/day and equals 8% of total energy. Protein intake is 1.25 g/kg/day, and kcal/kg/day are 30–35.

CK concentrations have been normal since then (see Fig. 1). It is unknown if this is the anaplerotic effect of heptanoate or if the stabilization came with increasing age (less infections). However, the patient, parents, and caretakers share the impression the boy is metabolically more stable since starting triheptanoate. At present, he is 14 years old and clinically asymptomatic. Besides following a fat-defined diet with heptanoate supplementation, he has no other medications or supplements. The boy is active, before exercise he consumes a high calorie drink (200 mL ProvideXtra® = 300 kcal, being a high energy food supplement with hydrolyzed protein and fiber, but fat, lactose and gluten free). On a regular basis, he performs karate training once a week and swimming once a week as well as being active in the local fire prevention department and the water-watch lifeguard organization. However, he has not undergone a pulse-controlled endurance training programme so far.

As exercise and subsequent catabolism is a potential trigger for metabolic derangement with CK concentration increase (=rhabdomyolysis) in LCHADD patients, we evaluated the clinical and biochemical stability under controlled physical exertion (both incremental and endurance) in this patient.

Applied Tests (Methods)

The proband first underwent a *standard incremental cycle test* (starting with 25 Watt, increasing by 25 Watt every 2 min) until exhaustion (Paridon et al. 2006). At the testing he was 13 years old, weighed 52 kg, and measured 167 cm (both values 90th percentile), BMI 18.6 kg/m^2, heart rate 78/min, temperature 36.6°C, oxygen saturation 100%.

Samples for blood glucose, lactate, and acyl carnitine profiles in dried blood spots were taken out of the hyperemic earlobe at the end of each increment, i.e. every two minutes; as well as a sample for analysis of urinary organic acids at 0, 4, 8, 16, and 24 h after the exercise test.

As the incremental exercise test showed normal results, 3 months later we performed an *endurance test* (Paridon et al. 2006; Beneke et al. 1996a, b, 2000a). To establish the maximal lactate steady state (anaerobic threshold), the patient underwent three endurance exercise sessions of 30 min each with a constant Watt performance of 80, 90, and 100 Watt, respectively (Beneke et al. 1996a, b, 2000b). Each test was done at the same daytime (afternoon) followed by a 48-hour resting period between each session. The heptanoate morning dose was given as usual, and thus, 6 h before the exertion test. Blood lactate and glucose were determined every 5 min during the 30 min of the endurance tests. Samples for blood glucose, lactate, creatine kinase, and acyl carnitine profiles in dried blood spots were taken regularly during each test and 1, 2, 4, 8, 16, 24, and 48 h after each test. Dietary intake remained unchanged during the whole testing period.

The anaerobic threshold, *maximal lactate steady state* (MLSS) refers to the exercise intensity where there is a dynamic equilibrium between lactate formation and elimination, i.e. there is no further net lactate production (Beneke

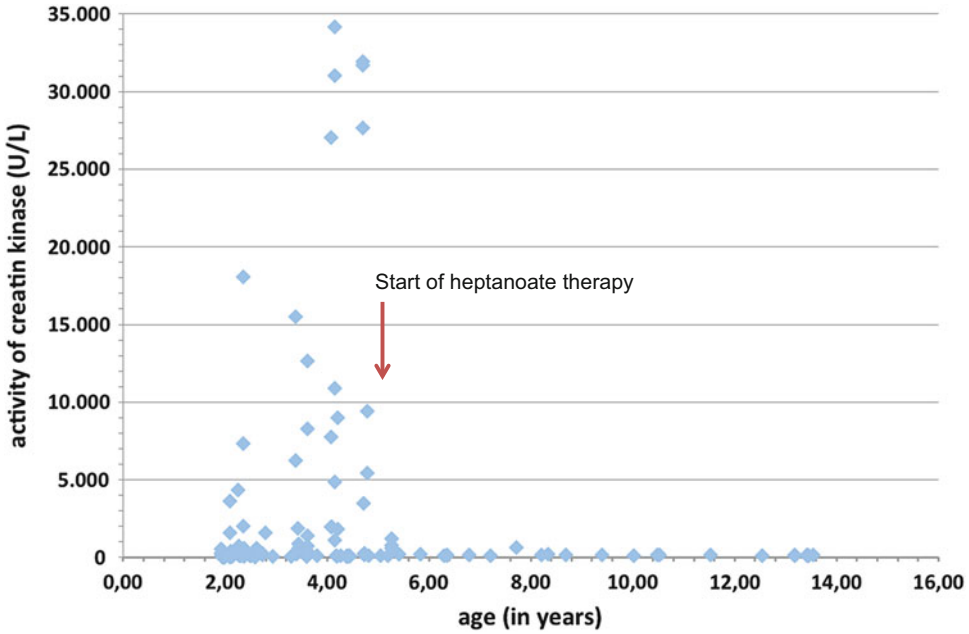

Fig. 1 Creatine kinase concentrations and start of heptanoate in the patient

et al. 1996a, b, 2000a, b). As it is known that the MLSS corresponds to about 70% of the maximal performance during the incremental exercise test, the patient's endurance exercise test series was started with 80 Watt, and then increased by 10 Watt each session.

Results

Standard Incremental Cycle Test

For this incremental exercise test lasting 11 min and 8 s, exhaustion was reached at 139 Watt, corresponding to a performance of 71% of the age-matched norm and equal to 2.68 Watt/kg body weight. During exercise he had adequate blood pressure and heart rate modulation (maximal heart rate 196/min at exhaustion) (Fig. 2). The ECG showed no signs of ischemia or arrhythmias. Subjectively, the patient had no symptoms.

Standard Endurance Cycle Test

The patient reached the maximal lactate steady state at 90 Watt (Fig. 3). Mean heart rate was 165/min.

Anaerobic Threshold, Maximal Lactate Steady State (MLSS)

The anaerobic threshold of the patient was tested to be at 90 Watt, his mean heart rate at the anaerobic threshold was 165/min. The MLSS, calculated as the mean from the last four samples of the 90 Watt endurance exertion test, was 2.2 mmol/L. Blood glucose concentrations remained stable (Table 1).

Metabolic Laboratory Tests

Neither during the incremental nor the endurance test, blood glucose, blood lactate, acyl carnitine profiles, or excretion of urinary organic acids showed any relevant fluctuation (data not shown). Acylcarnitine profiles as well as urinary organic acids did not show changes under the exercise tests, i.e. no increase in the amount of long-chain acylcarnitines or dicarboxylic acids.

In conclusion, the patient's physical fitness was adequate for age (maximum blood lactate 7.0 mmol/L, appropriate lactate performance curve, maximum heart rate of 196 bpm, maximum power 139 Watt = 2.68 Watt/kg body weight). There were no signs of clinical (muscle pain, dark urine) or metabolic derangement (stable CK, acyl carnitine profiles, blood gas analyses) – neither after maximal incremental nor endurance exertion.

Discussion

LCHAD deficiency is an inherited metabolic disorder, involving the degradation of long-chain fatty acids. Exercise and subsequent catabolism is a potential trigger for a

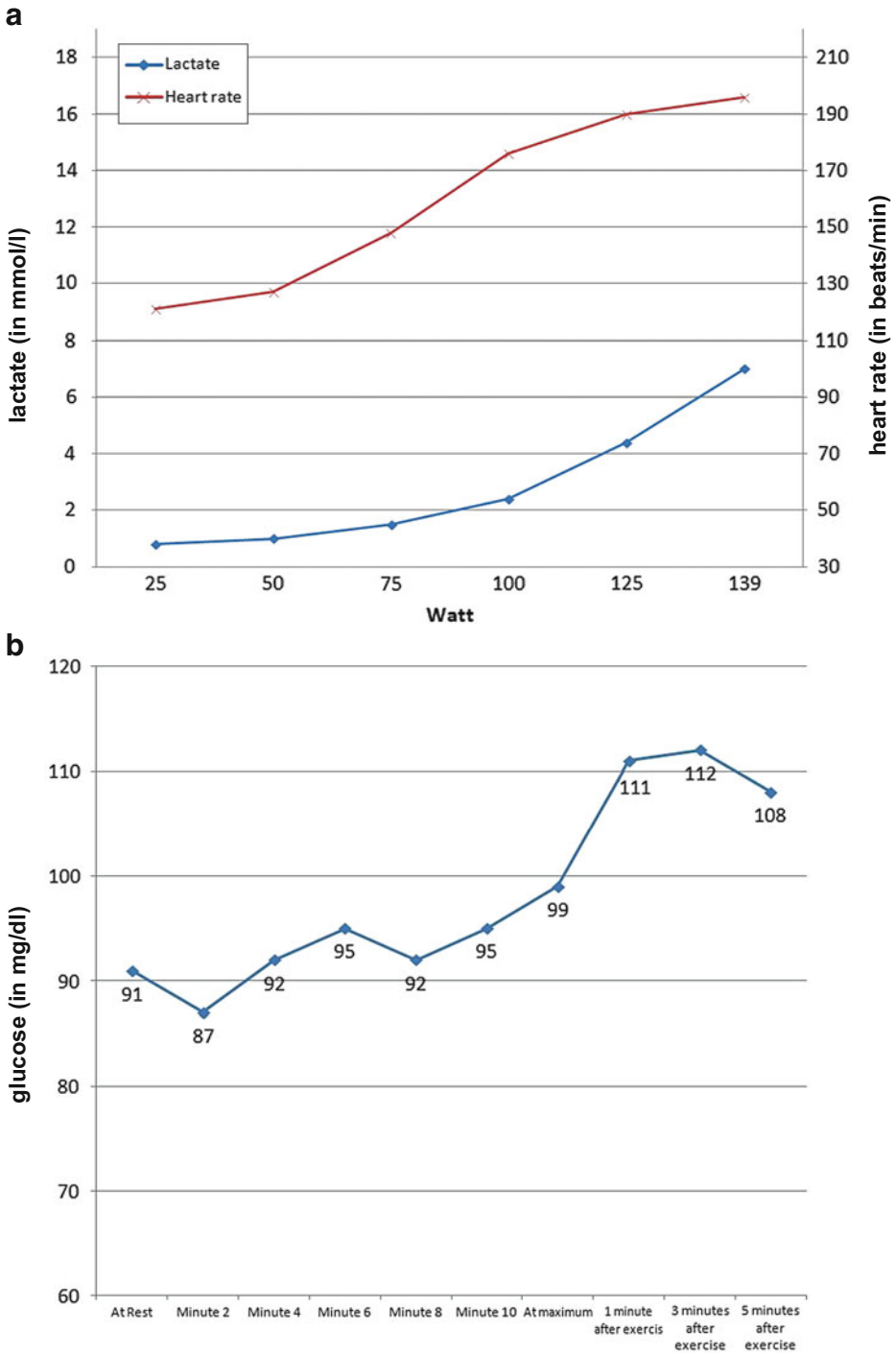

Fig. 2 (**a**) Blood lactate concentration and heart rate during the standard incremental cycle test – samples taken from earlobe. Lactate in mmol/L, heart rate in beats/minute. (**b**) Blood glucose concentration during the standard incremental cycle test – samples taken from earlobe (in mg/dL). *Y*-axis is blood glucose concentration in mg/dL

metabolic decompensation with rhabdomyolysis or increase of creatine kinase (CK) concentration. Other studies looking at exercise in long-chain fatty acid oxidation disorders have shown that (1) MCT given immediately prior to exercise improved exercise tolerance and decreased the risk of rhabdomyolysis in six LCHADD out of nine FAOD patients (Gillingham et al. 2006); (2) exercise significantly increased plasma 3-hydroxyacylcarnitines concentrations in six LCHADD out of eight FAOD patients; (3) MCT supplementation prior to exercise increased the

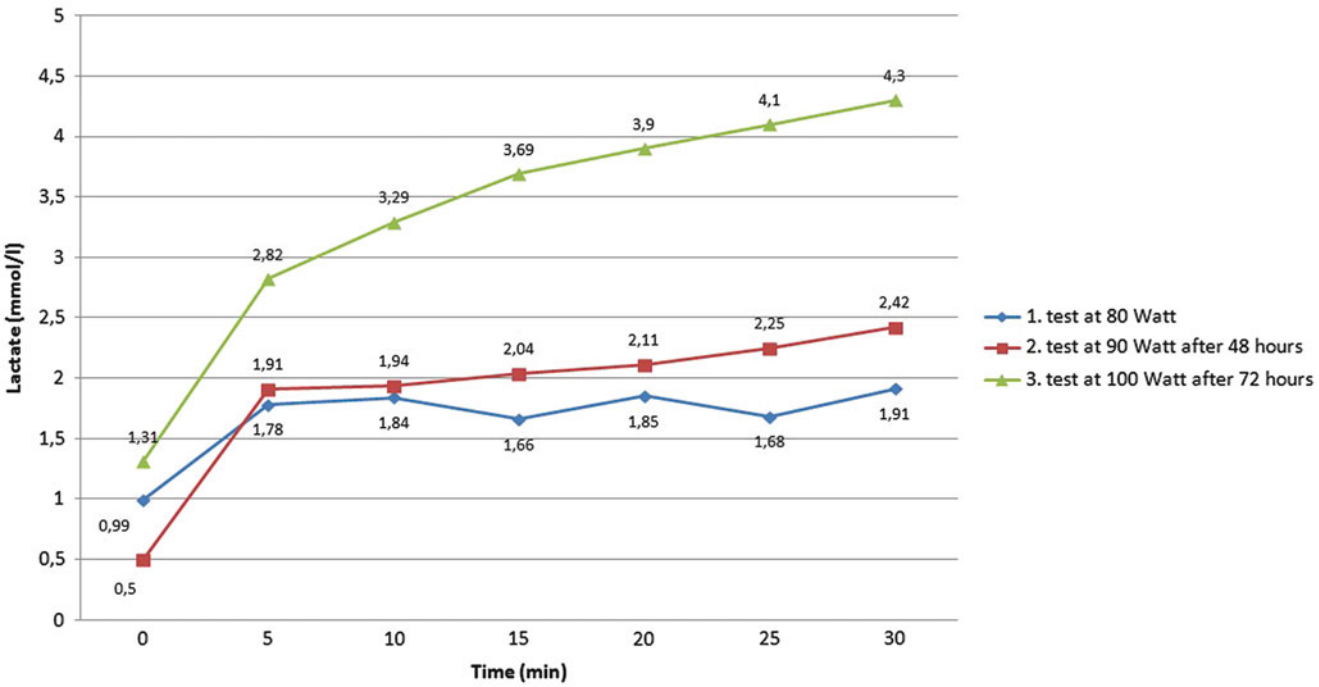

Fig. 3 Determination of maximal lactate steady state (MLSS) for the patient is at 90 Watt. MLSS is defined as the endurance performance where increase of blood lactate between 10 and 30 min of the endurance test is below or equal to 1 mmol/L

Table 1 Blood glucose concentrations in the patient during the endurance test (in mg/dL)

	Resting	Minute 5	Minute 10	Minute 15	Minute 20	Minute 25	Minute 30
80 Watt	111	107	109	109	101	106	106
90 Watt	109	100	94	102	103	104	105
100 Watt	109	102	101	102	108	111	105

oxidation of medium chain fats, decreased the oxidation of glucose, and lowered cardiac workload during exercise when compared with carbohydrate supplementation in eight LCHADD out of 11 FAOD patients (Behrend et al. 2012). However, these reports did not focus on the anaerobic threshold during incremental or endurance physical exertion tests in these patients.

We evaluated the clinical and biochemical stability under both incremental and endurance physical exertion conditions in a 13-year-old LCHADD patient treated with heptanoate for now nine years.

One goal was to determine the anaerobic threshold of the 13-year-old patient, i.e. exercise him at the highest lactate concentration at a steady state (maximal lactate steady state = MLSS). After he had uneventfully performed the incremental exercise test, for this purpose an endurance exercise test was added.

Both the incremental and the endurance exercise tests gave normal and stable results. Thus, they give a basis to develop a training programme for this LCHADD patient. Firstly, they are important results to evaluate the patients' physical fitness and metabolic stability under exertion, and secondly, they can be used to develop an individual training programme for the patient. That is, as a concrete suggestion for the patient, the measurement of on-site CK does not make sense as CK concentrations remained stable at all times during the exercise tests. A more useful recommendation could be a lactate monitoring during exercise (avoid an increase above 2.2 mmol/L lactate). As a conclusion, the best practicable and concrete recommendation for this patient is to monitor his pulse during endurance exercise and generally keep heart rate below 165 beats per minute.

Heptanoate has two effects: firstly, in LCHADD it can be used as an alternative substrate and secondly, it has

anaplerotic qualities. The boy has been on heptanoate treatment for 9 years. Heptanoate as an alternative substrate or its anaplerotic properties in this case seem to have had an impact on clinical stability and creatine kinase activity over the years.

In conclusion, this case illustrates that both under maximal incremental and endurance exertion, clinical and biochemical parameters remained stable in this 14-year-old LCHADD patient receiving heptanoate treatment.

Compliance with Ethical Standards

Conflict of Interest

Daniela Karall, Gerald Mair, Ursula Albrecht, Katharina Niedermayr, Thomas Karall, Wolfgang Schobersberger, and Sabine Scholl-Bürgi declare that they have no conflict of interest.

Informed Consent

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

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CASE REPORT

A Hunter Patient with a Severe Phenotype Reveals Two Large Deletions and Two Duplications Extending 1.2 Mb Distally to IDS Locus

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Abstract Mucopolysaccharidosis type II (Hunter syndrome, MPS II) is an X-linked lysosomal storage disorder caused by the deficit of iduronate 2-sulfatase (IDS), an enzyme involved in the glycosaminoglycans (GAGs) degradation. We here report the case of a 9-year-old boy who was diagnosed with an extremely severe form of MPS II at 10 months of age. Sequencing of the *IDS* gene revealed the deletion of exons 1–7, extending distally and removing the entire pseudogene *IDSP1*. The difficulty to define the boundaries of the deletion and the particular severity of the patient phenotype suggested to verify the presence of pathological copy number variations (CNVs) in the genome, by the array CGH (aCGH) technology. The examination revealed the presence of two deletions alternate with two duplications, overall affecting a region of about 1.2 Mb distally to *IDS* gene. This is the first complex rearrangement involving *IDS* and extending to a large region located distally to it described in a severe Hunter patient, as evidenced by the CNVs databases interrogated. The analysis of the genes involved in the rearrangement and of the disorders correlated with them did not help to clarify the phenotype observed in our patient, except for the deletion of the *IDS* gene, which explains *per se* the Hunter phenotype. However, this cannot exclude a potential "contiguous gene syndrome" as well as the future rising of additional pathological symptoms associated with the other extra genes involved in the identified rearrangement.

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Introduction

Mucopolysaccharidosis type II (MPS II or Hunter syndrome; MIM #309900) is an X-linked multisystemic progressive metabolic disorder caused by the deficit of activity of the iduronate 2-sulfatase (IDS) enzyme, which leads to the lysosomal accumulation of the glycosaminoglycans (GAG) heparan- and dermatan-sulfate. Albeit presenting as a continuum of pathological phenotypes, the disease mainly recognizes an attenuated and a severe form (Muenzer et al. 2009).

No strict genotype–phenotype correlation can be so far defined for the disease, nevertheless large deletions or insertions or important genomic rearrangements are always associated with severe phenotypes (Martin et al. 2008). Implications of large regions proximal or distal to the *IDS* gene have been previously described in some Hunter cases (Brusius-Facchin et al. 2012; Burruss et al. 2012; Probst et al. 2007; Honda et al. 2007; Dahl et al. 1995; Dahl et al. 1995; Beck et al. 1992; Clarke et al. 1992; Birot et al. 1996; Timms et al. 1997), mostly conferring to the MPS II phenotype other signs and symptoms associated with the extra genes implied in the rearrangement, such as *FMR1* and *MTM1* correlated with the Fragile X-syndrome (MIM#300624) and the X-linked myotubular myopathy (MIM#310400), respectively.

We here report the case of a 9-year-old boy who received an early biochemical and clinical diagnosis of MPS II. The difficulty of precisely defining the molecular variation affecting the *IDS* gene using PCR mapping suggested to extend the analysis to the whole X chromosome through the array CGH (aCGH) technology.

Materials and Methods

For mutation analysis genomic DNA was extracted from peripheral blood leukocytes using the commercial kit QIAmp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Informed consent was obtained from the parents. *IDS* exons and their flanking regions were PCR-amplified; specific sets of primers were used for selective amplification of *IDS* exon 2 and 3 versus *IDSP1* homologous exons (Villani et al. 1997). PCR products were purified by using Microcon YM 100 (Millipore) columns and sequenced with the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Sequence variations were confirmed by sequencing in both directions duplicate PCR products.

The presence of *IDSP1* gene was verified by applying the two PCR amplifications suggested by Lualdi (Lualdi et al. 2005) for the detection of the recombinants.

Array CGH analysis was carried out using a 244 K Agilent chip with a mean resolution of 30 kb (Agilent Technologies, Santa Clara, USA); the array was analyzed through an Agilent scanner (G2505C) and Feature Extraction software V.10.1.1.1. A graphical overview of the results was obtained using DNA Analytics software V.4.0.76. DNA sequence information refers to the public UCSC database [Human Genome Browser, February 2009, assembly hg19 (NCBI Build 37.5)].

For the interpretation of aCGH results, we followed the three-step workflow approach suggested by Poot (Poot and Hochstenbach 2010). To verify whether the same CNVs, or one partly overlapping with those under examination, had been previously described, we exploited the following publicly available databases: ISCA (The International Standards for Cytogenomic Arrays) database (https://www.iscaconsortium.org/), DECIPHER (Firth et al. 2009) (http://decipher.sanger.ac.uk), and the Database of Genomic Variants (Iafrate et al. 2004) (http://www.ncbi.nlm.nih.gov/dbvar/). For the undescribed CNVs, the genes mapping in the four genomic regions of the rearrangement were identified through the UCSC Genome Browser on Human Feb. 2009 (NCBI 37.5/hg19) assembly. The content of protein-coding genes was analyzed using OMIM, UniprotKB, and GeneCards for protein function and tissue expression; also the genes located in regions flanking the variation underwent the same analysis. The inheritance of the CNV was checked by evaluating the aCGH results of the patient's mother and sister.

Results

Clinical Description

Our 9-year-old patient was the second-born child of a non-consanguineous Italian couple of Caucasian ethnicity with no previous family history of neurological and/or metabolic disorders; however, the father is affected by hyperaldosteronism and chronically treated for hypertension. At the time of the patient's birth, the mother was 37 years old and the father was 41 and they had a healthy 11-year-old daughter. The patient was born at 36 weeks gestational age by cesarean section after a pregnancy complicated by threats of abortion. At birth, the patient weighed 2.5 kg (5th percentile) and he was 46 cm in length (5th percentile) with a head circumference of 33 cm (5–10th percentile). At two months of life, he presented with inguinal hernia which was surgically reduced. During the first year of life the child showed normal psychomotor development. Nevertheless, at 9 months of age he came to our attention for macrocephaly, mild facial dysmorphism, slight rigidity of the upper track, and claw hands. Suspecting a metabolic storage disease, biochemical and molecular genetics analyses were performed. Dosage of urinary GAG revealed a high urinary GAG/creatinine ratio equal to 914.41 mg/g (normal range values for the age: 30–300). IDS activity in cultured skin fibroblasts was 0.5 nmol/mg/h (n.v. 69.2), whereas other enzymes were normal, hence excluding multiple sulfatase deficiency and leading to the diagnosis of MPS II.

The first brain MRI examination performed at 11 months evidenced white matter lesions, ventricular expansion, and perivascular alterations.

At the age of 18 months the patient started enzyme replacement therapy (ERT) with idursulfase (Elaprase®) at the standard dosage of 0.5 mg/kg body weight per week. ERT was observed to be very well tolerated and safe, with no severe adverse events. Urinary GAG analysis performed just before the start of ERT detected a value of 493.19 GAG/g creatinine (normal range values for the age: 12–95); during ERT follow-up GAG levels reduced but never reached normal values, setting to an average level of about 300 mg GAG/g creatinine (normal range values for the age: 12–68) (data not shown). The search of anti-IDS, neutralizing antibodies performed during the follow-up of the patient resulted positive with a percentage of inhibition of 100% after 5 years of treatment.

Over the years the patient developed a progressive severe cognitive degeneration with a notable impairment of verbal communication. At present, cognitive functions are not testable; the adaptive behavior, evaluated by the Vineland Scale, showed in all domains an age equivalent to less than 1.5 years. At about 3 years of age, the patient started to receive occupational and speech therapies, in addition to similar services at school. At 4.5 years, the patient showed signs and symptoms of hypertensive hydrocephalus which was relieved through surgical derivation with a ventriculoperitoneal shunting. The frequent upper-respiratory tract infections, since the first months, were treated with antibiotic prophylaxis therapy. At 3.8 years he underwent adenotonsillectomy. The repeated ear infections lead to hearing problems with bilateral hypoacusia; at 4 years, he started to use hearing aids. A mild-moderate mitral insufficiency, which stabilized during the years, developed around 2 years of age. At 5 years he developed arterial hypertension and he started antihypertensive pharmacological therapy with good response and tolerance. The hepato-splenomegaly was present at the beginning of ERT but it slowly normalized 3.5 years post-therapy. He presented bone dysostosis with mild joint stiffness; up to today the patient did not require any surgical interventions or walking aids, but he uses wheelchair for long distances.

Array CGH Analysis

IDS mutational analysis allowed the sequencing of only exons 8 and 9, which did not carry any variations, and revealed the presence of an intragenic deletion. The absence of amplification of any fragments belonging to *IDSP1* pseudogene allowed to hypothesize that the deletion extended distally to this region. Several attempts to catch the telomeric boundary of the deletion through mapping PCR-methods, designing primers annealing to the region distal to *IDSP1*, failed. Thus, an in-depth analysis exploiting the aCGH technology was conducted to determine the extension of the deletion. This analysis revealed that the deletion extended for a total of 54.7 kb (148,568,786–148,623,440 bp). In addition, the aCGH analysis pointed out, in a telomeric position to the mentioned deletion, an 86.4 kb duplication (148,648,557–148,734,969 bp), a 198.7 kb deletion (148,830,445–149,029,121 bp), and a 644.6 kb duplication (149,105,821–149,750,457 bp) separated from one another by 3 regions spanning 25.1, 95.5, 76.7 kb, respectively (Fig. 1). The same rearrangement was detected in the patient's mother, but not in the patient's sister.

Discussion

Hunter syndrome is in most cases associated with point or small alterations of the *IDS* gene; only a little percentage of the patients present with intragenic or complete deletions of the gene or with complex rearrangements, including recombinational events between *IDS* and its pseudogene *IDSP1*. A suspect of "atypical rearrangement" should arise from the absence of PCR amplification of part of the exons (preferably the distal or the telomeric ones) or of the entire *IDS* gene and should lead to a deeper analysis of the nearby genes, included the *IDSP1* gene that should be investigated to verify the presence of recombinational events with the *IDS* gene. In case of negative results, the aCGH analysis should be afterwards recommended.

In the present paper we report the first case of a Hunter patient showing a complex rearrangement consisting of two deletions and two duplications extending far distally towards the telomere, involving on the whole a region of about 1.2 Mb of the X chromosome. To our knowledge, the entire complex rearrangement had never been reported so far in any of the publicly available databases.

A search for the CNVs partly or entirely overlapping with the single deletions in the ISCA database gave the following results: five variants previously described overlapping with the first deletion (four of them correlating with a pathogenic phenotype and one with an uncertain phenotype) and one pathogenic variant with the second deletion. The same search performed for the duplications gave an output of five cases overlapping with the first duplication (one pathogenic, three uncertain and one likely benign) and 11 cases with the second duplication of which four pathogenic, six uncertain, and one likely to be benign. Although none of the single CNVs analyzed gives an unambiguous phenotype as a result, on the whole the first deletion and the second duplication seem to have the most deleterious effect. This might be due to the presence in this region of three MIM-associated genes, *IDS*, *MAMLD1*, and *MTM1*, the only genes mapping in the rearranged region with which a specific pathology has been correlated (Table 1).

A search in the DECIPHER database, submitting the four CNVs separately evidenced a series of cases carrying deletions partly overlapping with the four variants detected by us. However, only one of them (case 256308) presented with a large deletion overlapping the entire chromosomal region involved in the rearrangement here described. The reported phenotype presented some symptoms in common with the subject described by us (delayed speech and language development, intellectual disability) along with other symptoms (soft skin, joint laxity and 2–3 toe syndactyly).

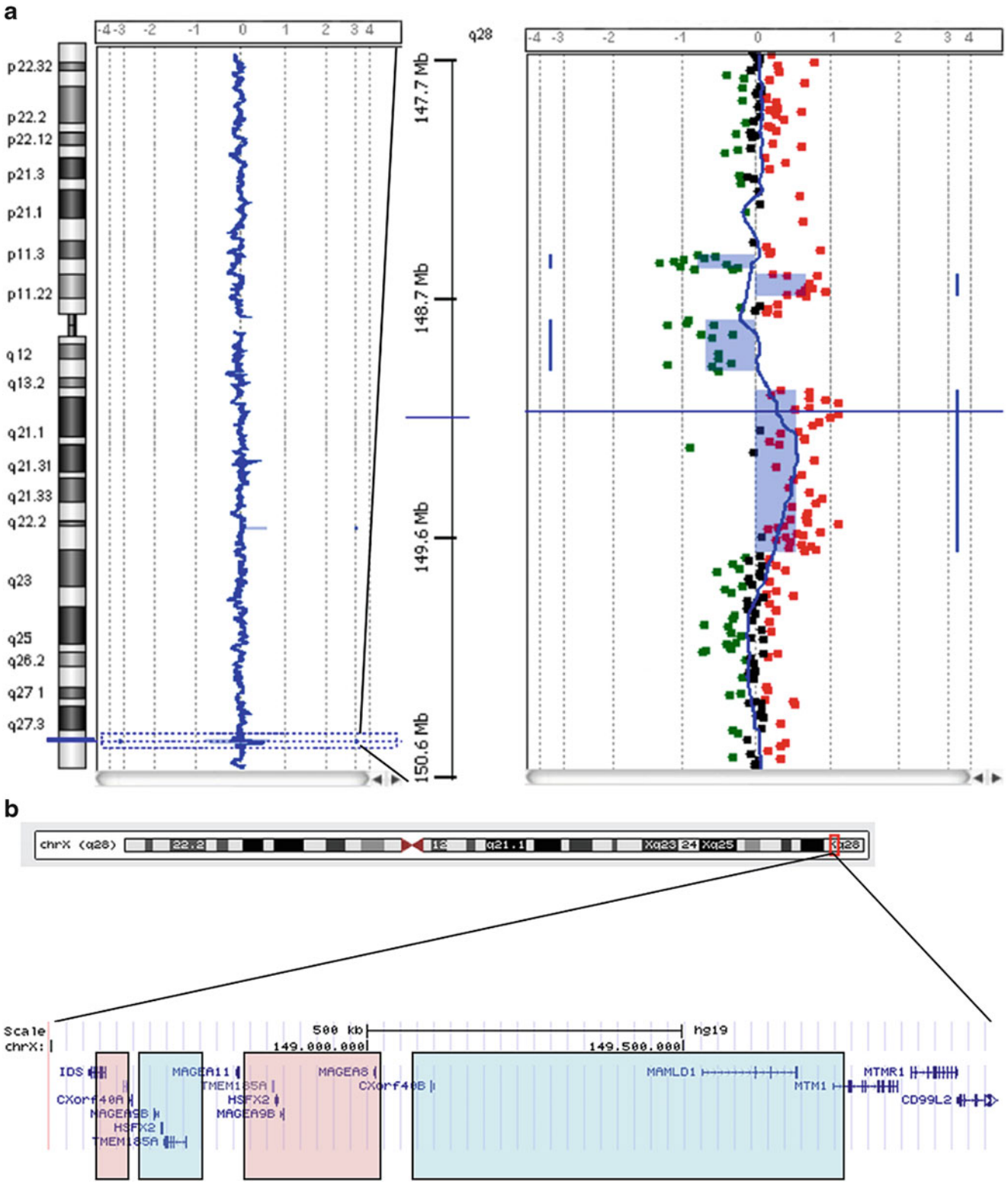

Fig. 1 (**a**) Array-CGH graphical output. Mapping of the rearrangement in position Xq28 and its magnification showing the details of the two deletions and of the two duplications; (**b**) UCSC Genome Browser graph of the protein-coding genes mapping in the X chromosome region (148,500,000–150,000,000 bp) including the described rearrangement (*red box*: deleted segment; *blue box*: duplicated segment)

Table 1 Summary of the genes mapping in the deleted/duplicated X chromosome regions in the rearrangement analyzed

Chromosomal region involved	Loci involved	Chromosomal position (first-last bp)	Status	Gene product	Function of the gene product	Observations
148,568,786–148,623,440	*IDS*	148,560,295–148,586,884	Del*	Iduronate 2-sulfatase	Required for the lysosomal degradation of heparan sulfate and dermatan sulfate	Mutations in this gene are associated with mucopolysaccharidosis type II also known as Hunter syndrome
	IDSP1	148,606,539–148,607,956	Del	Iduronate 2-sulfatase pseudogene 1	Unknown	–
	LOC100131434	148,609,130–148,621,312	Del	Uncharacterized LOC100131434	Unknown	–
	CXorf40A	148,622,519–148,632,086	Del*	Chromosome X open reading frame 40A	May have an important role of cell protection in inflammation reaction	Associated to intrahepatic cholangiocarcinoma, and cholangiocarcinoma
148,648,557–148,734,969	*MAGEA9B*	148,663,309–148,669,116	Dup	Melanoma antigen family A, 9 -like	Not known, though may play a role in embryonal development and tumor transformation or aspects of tumor progression	Associated with terminal osseous dysplasia, and enophthalmos
	HSFX2	148,674,172–148,676,974	Dup	Heat shock transcription factor family, X linked 2	Transcription factor	Associated with oligospermia, and male infertility
	TMEM185A	148,678,216–148,713,487	Dup	Transmembrane protein 185A	Transmembrane protein	This gene is best known for localizing to the CpG island of the fragile site FRAXF Associated with FRAXF syndrome and atypical autism
	LOC100420321	148,730,846–148,731,851	Dup	Melanoma antigen family A, 11 pseudogene	Unknown	Associated with melanoma
148,830,445–149,029,121	*LOC100420322*	148,839,670–148,840,322	Del	Melanoma antigen family A, 10, pseudogene	Unknown	Associated with melanoma
	LOC100420334	148,842,431–148,842,959	Del	Melanoma antigen family A, 11, pseudogene	Unknown	Associated with melanoma
	TMEM185AP1	148,850,544–148,854,484	Del	Transmembrane protein 185A pseudogene	Unknown	–
	HSFX1	148,855,726–148,858,517	Del	Heat shock transcription factor family, X linked 1	Transcription factor	Associated with oligospermia, and male infertility
	MAGEA9	148,863,600–148,869,399	Del	Melanoma antigen family A, 9	Not known, though may play a role in embryonal development and tumor transformation or aspects of tumor progression	Associated with terminal osseous dysplasia, and enophthalmos

(continued)

Table 1 (continued)

Chromosomal region involved	Loci involved	Chromosomal position (first-last bp)	Status	Gene product	Function of the gene product	Observations
	MAGEA7P	148,890,201–149,890,478	Del	Melanoma antigen family A, 7, pseudogene	Unknown	Associated with melanoma
	DUTP4	148,898,834–148,899,310	Del	Deoxyuridine triphosphatase pseudogene 4	Unknown	–
	MAGEA8	149,009,941–149,014,609	Del	Melanoma antigen family A, 8	Not known, though may play a role in embryonal development and tumor transformation or aspects of tumor progression	Associated with terminal osseous dysplasia, and enophthalmos
149,105,821–149,750,457	*CXorf40B*	149,100,415–149,106,716	Dup*	Chromosome X open reading frame 40B	Unknown	Associated with include gastric cancer
	LOC100272228	149,106,766-149,185,018	Dup	Uncharacterized LOC100272228	Unknown	–
	LOC643015	149,282,608–149,284,952	Dup	Nucleolar protein 11 pseudogene	Unknown	–
	MIR2114	149,396,239–149,396,318	Dup	MicroRNA 2114	Post-transcriptional regulation of gene expression	Associated with include ovarian cancer
	XRCC6P2	149,399,291–149,438,002	Dup	X-ray repair complementing defective repair in Chinese hamster cells 6 pseudogene 2	Unknown	–
	MAMLD1	149,531,551–149,682,448	Dup	Mastermind-like domain containing 1	Transactivates the HES3 promoter independently of NOTCH proteins. HES3 is a non-canonical NOTCH target gene which lacks binding sites for RBPJ	Mutations in this gene are the cause of X-linked hypospadias type 2
	MTM1	149,737,047–149,841,616	Dup*	Myotubularin 1	Lipid phosphatase which dephosphorylates phosphatidylinositol 3-monophosphate (PI3P) and phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2). Negatively regulates EGFR degradation through regulation of EGFR trafficking from the late endosome to the lysosome.	Mutations in this gene have been identified as being responsible for X-linked myotubular myopathy

For each gene the name, the position (based on NCBI 37.5/hg19 assembly), the deleted or duplicated status (Del = deleted gene; Dup = duplicated gene; * = partially deleted or partially duplicated gene) as well as the function of the gene product and the related observations are reported

Table 1 reports the genes mapping in each deletion and duplication detected in our case.

The first deletion (spanning region: 148,376,691–148,431,346 bp), partially matching with that previously defined by PCR mapping, leads to the removal of part of the *IDS* gene, of the entire pseudogene *IDSP1* and of a further locus whose function is still unknown (*LOC100131434*). The locus *CXorf40A* (chromosome X open reading frame 40A) is only partly affected by the deletion; such locus encodes for the endothelial-overexpressed lipopolysaccharide-associated factor 1 which might have an important role on cell protection in inflammation reaction.

The second deletion (148,638,257–148,789,779 bp) causes the elimination of five pseudogenes and three genes (*HSFX1*, *MAGEA9*, *MAGEA8*). *HSFX1* gene encodes for the heat shock transcription factor family X linked 1, whose function has not yet been cleared. The genes *MAGEA9* and *MAGEA8* are members of the *MAGEA* gene family, clustered at Xq28, coding for proteins with 50–80% sequence identity to each other, implicated in some inherited diseases. *MAGEA9* is one of the most frequently expressed cancer-testis (CT) genes in bladder tumors (Bergeron et al. 2009).

Among the genes mapping in the duplicated regions, we found another gene of the *MAGEA* cluster (*MAGEA9B*), *MAMLD1* and *MTM1*, along with other genes whose functions are still undefined. *MAMLD1* encodes a mastermind-like domain containing a protein that may function as a transcriptional co-activator. Mutations in this gene are the cause of the X-linked hypospadias type 2 (MIM#300758). *MTM1* gene encodes for the myotubularin protein, a phosphatase specifically acting on two types of phosphoinositides. Mutations in this gene are associated with the X-linked myotubular myopathy (Oliveira et al. 2013). In the case here described, *MTM1* gene encompasses the duplication boundary, thus being involved for 13% of its length.

Patients bearing large deletions involving the IDS gene have been previously described. Apart from the female cases, few reports described deletions encompassing the region proximal to the IDS locus, removing the fragile site mental retardation 1 (*FMR1*) and fragile site mental retardation 2 (*AFF2*) genes (Brusius-Facchin et al. 2012; Burruss et al. 2012; Probst et al. 2007; Clarke et al. 1992; Birot et al. 1996; Clarke et al. 1990), or extending both proximally and distally towards the telomeric sides of *IDS* (Brusius-Facchin et al. 2012; Honda et al. 2007; Birot et al. 1996; Timms et al. 1997). The phenotype observed in these cases spans from a severe to an early-onset severe form of Hunter disease presenting additional symptoms associated with the extra genes involved in the deletion, such as mental retardation associated with *FMR1* and *AFF2* genes and muscular hypotonia correlated with the *MTM1* locus. Among the deletions extending only distally to the *IDS* locus reported in the literature (Beck et al. 1992; Timms et al. 1997), recently Brusius-Facchin (Brusius-Facchin et al. 2012) characterized a molecular rearrangement consisting of a 3.9 Mb deletion removing *FMR1*, *AFF2* and *IDS* and a 3.1 Mb duplication encompassing *MAMLD1* and *MTM1* in a patient with a peculiar Hunter phenotype accompanied with severe intellectual disability and hypotonia.

Similarly to some of the cases reported in the literature, one of the peculiarities of the patient here presented is the extreme severity of the pathology, which led to a very precocious clinical diagnosis. During his life he developed most of the symptoms clinically associated with Hunter disease, including hydrocephalus and arterial hypertension.

Another feature is the mild response to enzyme replacement therapy observed in this subject. Urinary GAGs showed a fluctuating pattern during the follow-up but did not normalize, evidencing, after 6 years of treatment, a reduction of 24% with respect to pre-therapy value (data not reported). The detection of anti-IDS neutralizing antibodies, detected after 4 years of ERT, might in part explain the GAG pattern registered during the follow-up. On the opposite, hepato- and splenomegaly improved, although slowly, and they were no longer detectable after 4 years of treatment.

Although therapeutic inclusion criteria or efficacy of ERT are beyond the aim of our work, given that important genomic rearrangements have always been associated with severe forms of Hunter syndrome, we believe that these cases should be included in the discussion related to a possible discontinuation of the therapy for the severe forms of the disease, as suggested by Muenzer (Muenzer et al. 2012).

Although gene deletions are in most cases linked to the severe form of the pathology associated with each gene, as for total or partial duplications the prediction of the resulting phenotype is not so straightforward. Pathogenic gene duplications often involve dosage-sensitive genes or genes coding for proteins which tend to aggregate (Conrad and Antonarakis 2007). Moreover a partial gene duplication might cause in turn gene disruption leading to the production of a nonfunctional protein according to which region of the gene is involved. Although the second duplication detected in our patient involves part of *MTM1* gene, it does not seem to affect the phenotype observed, as the child does not present with hypotonia, the typical symptom associated with the X-linked myotubular myopathy.

Although carrier of the same genetic alteration, the mother, clinically examined, did not present any signs or symptoms associated with the deleted or duplicated genes involved in the described rearrangement.

Conclusion

In conclusion, even though the patient here described carries a complex composite rearrangement, extending 1.2 Mb from the *IDS* locus, only the first deletion appears to be the main genetic determinant of the disease phenotype evidenced in the subject so far. The extension of the rearrangement to several extra genes does not seem to affect the peculiar clinical Hunter phenotype as no additional symptoms associated with these genes have been evidenced up to now, through patient's regular monthly check-ups. This might be partly determined by the fact that while pathological phenotypes have been so far associated with mutations or deletions of the two principal disease-related genes here involved, *MAMLD1* and *MTM1*, no potential effects of their total or partial duplication have been so far described. Unfortunately, this does not allow to exclude the presence of a "contiguous gene syndrome" as well as the potential rising of future additional pathological symptoms associated with the other genes involved.

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This study makes use of data generated by the DECIPHER Consortium. A full list of centers who contributed to the generation of the data is available from http://decipher.sanger.ac.uk and via email from decipher@sanger.ac.uk. Funding for the project was provided by the Wellcome Trust.

One Sentence Take-Home Message

First case described of a Hunter patient with two deletions alternate with two duplications, involving exons 1–7 of the *IDS* gene and extending 1.2 Mb distally to it.

Details of the contributions of individual authors

AZ: conception and design of the study, performing of IDS molecular analysis, analysis and interpretation of molecular data, writing and critical revision of the manuscript. RT: conception and design of the study, clinical data collection, writing and critical revision of the manuscript. AR: clinical data collection and analysis, critical revision of the manuscript. CR: aCGH analysis, interpretation of aCGH data, critical revision of the manuscript. NG: urinary GAG analyses, critical revision of the manuscript. MCa: critical revision of the manuscript. MCl: critical revision of the manuscript. MS: conception and design of the study, critical revision of the manuscript, final approval of the manuscript.

Name of one author who serves as guarantor

Maurizio Scarpa.

Details of funding

No funding was raised to conduct this study.

Details of ethics approval

Ethics approval was not required to perform the described studies.

A patient consent statement

Informed consent for genetic analyses in the patient and relatives was obtained from the subject involved in the study, parents or tutors.

Conflict of interest

Maurizio Scarpa has received research grants and honoraria and travel support for speaking engagements from Actelion, Shire HGT, Genzyme Corporation, and BioMarin.

Alessandra Zanetti, Rosella Tomanin, Angelica Rampazzo, Chiara Rigon, Nicoletta Gasparotto, Matteo Cassina, and Maurizio Clementi declare no conflicts of interest.

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CASE REPORT

Widening Phenotypic Spectrum of AADC Deficiency, a Disorder of Dopamine and Serotonin Synthesis

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Abstract *Objectives*: Aromatic amino acid decarboxylase deficiency presents with prominent extrapyramidal and autonomic features and CSF monoamine deficiency with increased 3-*O*-methyldopa, a by-product of accumulated L-DOPA. Less than 100 cases have been identified. The disease is typically associated with a severe phenotype and worse prognosis in females. Gene transfer technology has been implemented using an adeno-associated virus encoding AADC in the putamen bilaterally.

Methods: We describe the phenotype/genotype in a cohort of five cases showing a heterogeneous phenotype and variably intact response to pharmacologic therapy.

Results: Five patients (age range 2–10 years, mean 5 years, 3M/2F) with confirmed AADC deficiency are described. Four (3M/1F) have had improvement on combinations of dopaminergic agonists, MAO inhibitors, pyridoxine/P5P, and folinic acid. Each presented with hypotonia, decreased voluntary movement, dystonia, irritability, and oculogyric crises. Two (1M/1F) are independently ambulatory and are not dependent on gastrostomy tube feedings; the 9-year-old girl is reading single words. One female has a severe phenotype including recurrent hypoglycemic events associated with bradycardia, although the latter have resolved with chronic anticholinergic therapy. One Taiwanese boy had the common homozygous mutation, and otherwise we describe five new DDC mutations.

Conclusions: We report a wider phenotypic spectrum including intact response to pharmacologic management and milder outcome in a female, as well as five new mutations. Four of five patients have improved on combination therapy including a dopamine agonist, MAO inhibitor, pyridoxal-5′-phosphate, and folinic acid. The advent of viral-mediated gene therapy in AADC deficiency renders expanded knowledge of the outcome increasingly important.

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Introduction

Aromatic L-amino acid decarboxylase (AADC) deficiency (MIM 608643) is a neurotransmitter metabolism disorder inherited in an autosomal recessive fashion. AADC is a pyridoxal-5′-phosphate-dependent enzyme that functions in the catecholamine biosynthetic pathway (Pons et al. 2004; Hyland 2007). It is responsible for synthesizing dopamine and serotonin through decarboxylation of L-DOPA and 5-hydroxytryptophan. Mutations in *DDC* (MIM 107930) (Hyland 2007; Brun et al. 2010) lead to deficiency of serotonin and dopamine and their downstream metabolites, all components of the monoaminergic system (Lee et al. 2009; Hwu et al. 2012; Shih et al. 2013).

AADC deficiency is a rare condition that usually presents in infancy or early childhood. The most common symptoms are developmental delay, oculogyric crises, and dystonia (Hyland 2007; Brun et al. 2010). CSF studies are pathognomonic, showing reduced homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations, with increased L-DOPA and 3-*O*-methyldopa (Haliloğlu et al. 2012). Pyridoxal-5′-phosphate is a cofactor for

AADC, and a similar CSF profile can be seen in pyridoxal-5′-phosphate dependency (Mills et al. 2005). The diagnosis is confirmed through *DDC* gene sequencing or quantification of plasma AADC enzymatic activity.

Treatment strategy is based on a regimen compensating for lack of AADC activity through monoamine oxidase (MAO) inhibitors, dopaminergic agonists, and pyridoxine or the active form pyridoxal-5′-phosphate (PLP) (Allen et al. 2009; Brun et al. 2010). Folinic acid supplementation was added due to the possibility of cerebral folate depletion as a result of methylation of accumulated L-DOPA (Brun et al. 2010). Little to no improvement with therapy has been described (Swoboda et al. 2003) or even deterioration following initial response to therapy (Chang et al. 2004). It has been suggested that males respond to treatments better than females, based on a study that grouped responses to treatment into two groups: one who responded well to treatment (mostly males) and another who did not respond well to treatment (mostly females with one male) (Pons et al. 2004). The second group often developed treatment-related dyskinesias, and it was suggested that females are more dependent on dopamine levels (Pons et al. 2004). Transdermal rotigotine was efficacious in the early stages of AADC of an affected male; however, the treatment was not effective in his older, more impaired brother (Mastrangelo et al. 2013).

Recently, gene therapy has been used in cases of AADC deficiency (Hwu et al. 2012; Zwagerman and Richardson 2012; Chtarto et al. 2013; Lee et al. 2013). Gene transduction through adeno-associated virus (AAV) was used in a group of patients presented by Hwu et al. (Hwu et al. 2012). AAV2 vector-mediated delivery of the human *DDC* gene (AAV2-hAADC) into the putamen was used to promote motor activity, as the putamen is the major site of AADC activity in the brain. All four patients had gains in body weight and motor function, in addition to reduced oculogyric crises, 1 year after gene transfer (Hwu et al. 2012).

While Brun et al. (2010) presented a summary of 78 AADC patients published to date, less than 100 cases have been reported in the literature (Brun et al. 2010). In light of novel gene therapies for AADC deficiency, we present clinical details on a cohort of five patients with broad clinical variability, four novel mutations in *DDC*, and different responses to treatment.

Case Reports

Patient 1 is a 4-year-old boy with hypotonia noted at birth, gastroesophageal reflux at 2 months, and onset of prolonged oculogyric crises at 3 months. At 7 months of age, distal dystonic movements of the extremities were observed during episodes of upward gaze. Other manifestations were decreased head control, feeding dysfunction, hypersalivation, athetosis, hypokinesis, ptosis, excessive diaphoresis, and irritability.

CSF neurotransmitter profile showed decreased concentrations of HVA (88, normal range 294–1,115) and 5-HIAA (9, normal range 129–520), with elevated 3-*O*-methyldopa (754, normal range <300) and normal biopterin and neopterin levels. The plasma AADC activity level was undetectable. *DDC* sequencing showed compound heterozygosity with mutations at exon 3 (c.289delGfs +20X), leading to formation of a premature stop codon, and exon 6 (c.629C>T p.P210L). Upon diagnosis, the patient was treated with a combination of pramipexole, pyridoxal-5′-phosphate, folinic acid, and tranylcypromine with marked improvement in oculogyric crises and progressive mobility. The patient is now walking independently and has voluntary hand use, marking a dramatic improvement in functionality. Persistent symptoms at age four include moderate hypotonia, increased drooling, feeding difficulties (although feeds completely orally), and nonverbal status.

Patient 2 is a 10-year-old boy that first came to medical attention due to decreased head control and clenched hands shortly before his first birthday. He rolled over at 18 months and by 2 years had a vocabulary of about 40 words, but subsequently lost these skills. His initial diagnosis was cerebral palsy of unknown etiology. The patient had several hospitalizations for dehydration and hypoglycemia, once with a hypoglycemic seizure without recurrence. Other manifestations were ptosis, oculogyric crises, facial hypokinesia, diaphoresis, poor feeding, nasal stuffiness, and hypersalivation.

The diagnosis was established following a CSF monoamine profile of low HVA and 5-HIAA levels and plasma AADC enzymatic activity of 2.6 pmol/mL/min (normal range 36–129). *DDC* sequencing resulted in a novel homozygous missense mutation of exon 6 (c.665 T>C, p.L222P). On exam at 10 years, he is stable on combined therapy of pramipexole, pyridoxal-5′-phosphate, folinic acid, and tranylcypromine. Oculogyric crises have resolved. Dystonic movements, excessive drooling, and nasal stuffiness have improved. Significant bilateral ptosis persists, but the patient is able to take steps with support. Cognitive progress has been noted with intact reception for multistep commands although without expressive verbal language.

Patient 3 is a 4-year-old girl with unusual ocular movements observed in the first weeks of life. These evolved into oculogyric crises and episodic torticollis, associated with staring and orobuccal dyskinesias including lip smacking, by 2 months of age. She developed choreoathetosis and episodic unresponsiveness associated

with oxygen desaturation. Dysautonomic signs included unexplained swings in heart rate from low 50s to 180 beats per minute, and she did not appear to perceive pain. She had multiple hospitalizations the first 3 years of life due to intermittent hypoglycemia and bradycardia.

The patient was diagnosed with AADC deficiency following ascertainment of CSF HVA of 94 nM (normal range 294–1,115), 5-HIAA < 5 nM (129–520), 3-*O*-methyldopa elevated at 630 nM (<300), and AADC enzymatic activity level of 1.5 pmol/mL/min (36–129). Her examination at 14 months of age showed minimal voluntary movement and awareness other than apparent ability to recognize familiar voices. She has persistent hypotonia, ptosis, and dyskinesias and is gastrostomy tube dependent. Her episodic bradycardia stopped with chronic anticholinergic activity utilizing oral hyoscyamine and scopolamine patches. Her therapy otherwise includes ropinirole, leucovorin, and pyridoxal-5′-phosphate, but there has been minimal developmental progress.

Patient 4 is a 2-year-old male of Taiwanese descent who presented at 4 months of age due to developmental delay, decreased head control, and impaired visual tracking. At 7 months, he manifested generalized hypotonia, paroxysmal dystonia, and oculogyric crises. Diagnosis was established by undetectable levels of HVA and 5-HIAA on CSF neurotransmitters and elevated 3-*O*-methyldopa of 453 nM (<300). Plasma AADC enzymatic activity level was 0.53 pmol/mL/min ($N > 8$). The patient was homozygous for the IVS 6+4, A>T mutation.

The patient was first seen on ropinirole and pyridoxine therapy. Severe failure to thrive was treated after the first birthday with gastrostomy tube placement, and the medication program was transitioned to pramipexole, pyridoxal-5′-phosphate, and folinic acid. Within 4 months, the patient demonstrated improvement in motor tone, communication, and emotional stability. Persistent deficits at 2 years of age are poor head control, bilateral ptosis, less frequent and sustained but intermittent oculogyric crises, lower extremity dystonia, and nonambulatory status.

Patient 5 is a 9-year-old female who presented at 4 months of age with hypotonia and failure to gain motor milestones. Her diagnosis was established at age 7½ years following lumbar puncture indicated because of the presence of ataxia and proximal muscle weakness and a diagnostic label of cerebral palsy but without an identified etiology. Lumbar puncture showed CSF HVA of 72 (218–852), 5-HIAA 22 (66–33), and 3-*O*-methyldopa of 729 (<100). The AADC activity level in plasma was <1.5 (36–129), and the *DDC* gene sequencing showed compound heterozygosity with mutations at exon 3 (c.260C>T p.P87L) and exon 5 (c.446G>A p.S149T).

Exam at 9 years of age shows significant progress on combined treatment of bromocriptine and pyridoxine. She has no feeding dysfunction, improved ability to focus, intact reading ability of simple words, some speech (however, most interactions manifest by crying or tearing), and independent walking, albeit it for short distances. Persistent signs and symptoms are nasal stuffiness, mild ptosis, irritability, choreoathetosis, and hypersalivation. She has some unusual eye movements of upward gaze which are not sustained oculogyric crises.

Discussion

We present a cohort of five patients with AADC deficiency with a demonstration of broad phenotypic expression (Table 1). Of these, four (three males and one female) are doing relatively well and responding to therapy utilizing combinations of a dopaminergic agonist, MAO inhibitor, pyridoxine or P5P, and folinic acid. The prospect of gene therapy in AADC deficiency renders expanded knowledge of the phenotypic spectrum and natural history of the disorder increasingly important (Hwu et al. 2012). The Jake database reports 83 cases of AADC deficiency worldwide (JAKE database available at http://www.biopku.org/bioPKU_databasesJAKE.asp, accessed January 28, 2014). We contribute another five and report five new mutations in the *DDC* gene.

All five of our patients presented with characteristic features including hypotonia, decreased motor development and voluntary movement, dystonia, irritability, and oculogyric crises. Three of our patients, two males and one female, have intact gait and are not dependent on gastrostomy tube feedings. One female has a severe phenotype including recurrent hypoglycemic events associated with bradycardia, although the latter have resolved with chronic anticholinergic therapy. Hypoglycemia was an intermittent problem in two of our patients and has been described in the syndrome, attributed to a defect in synthesis of dopamine and hence catecholamines (Arnoux et al. 2013). One male of Taiwanese descent affected with the common IVS6+4 A>T mutation, reported in over 80% of Taiwanese patient populations (Lee et al. 2013; Shih et al. 2013), has a severe phenotype but has responded to combination medication therapy along with gastrostomy tube feedings. One 9-year-old girl on a combined regimen is now walking, verbalizing, and making developmental gains, which is relatively promising in light of prior reports reporting a worse prognosis in females (Tay et al. 2013).

Three of our five patients have improved on a combined therapy typically including a dopamine agonist, MAO inhibitor, and pyridoxal-5′-phosphate. AADC deficiency is characterized by decreased biogenic amines and their downstream metabolites and accumulation of 3-*O*-methyldopa as

Table 1 Clinical overview of AADC patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Current age (years)	4	10	4	2	9
Sex	Male	Male	Female	Male	Female
Age at diagnosis	16 months	7 years	10 months	13 months	7.5 years
Diagnostic confirmation	DDC sequencing: Exon 3: c.289delGfs +20X (Het)[a] Exon 6: c.629 C>T, p.P210L (Het)[a] AADC enzyme activity: <1.5 pmol/ mL/min	DDC sequencing: Exon 6: c.665T>C, p.L222P[a] (homozygous) AADC enzyme activity: 2.6 pmol/mL/min	AADC enzyme activity: 1.5 pmol/mL/min	DDC sequencing: IVS6+4 A>T (homozygous) AADC enzyme activity:0.53 pmol/ mL/min	DDC sequencing: Exon 3 c.260C>T, p.P87L (Het)[a]; Exon 5 c.446G>A, p.S149T (Het)[a] AADC enzyme activity:<1.5 pmol/ mL/min
Outcome	Ambulatory; feeds independently; nonverbal	Stands; takes steps with support; G-tube dependent; nonverbal	Severe; G-tube dependent; nonambulatory; nonverbal	Severe; G-tube dependent; nonambulatory; nonverbal	Ambulatory; feeds independently; verbal

[a] Newly reported *DDC* mutations

a by-product of accumulated L-DOPA. While the disorder has traditionally been associated with a severe presentation and poor responsiveness to medical intervention (Alfadhel and Kattan 2014), additional reports such as ours demonstrate a wider phenotypic spectrum. This must be a consideration in the selection of patients for future novel therapies including viral-mediated gene therapy.

Synopsis

Aromatic amino acid decarboxylase deficiency is a rare disorder with a heterogeneous phenotypic spectrum that must be taken into account in evaluating prospective gene transfer technologies.

Compliance with Ethics Guidelines

The authors, Guy Helman, Maria Belen Pappa, and Phillip Pearl, report no conflicts of interest. All patients were evaluated in good clinical practice. IRB approval was obtained (BCH protocol 3660). Guy Helman was responsible for data review and manuscript preparation and submission. Maria Belen Pappa provided data and manuscript review. Phillip Pearl was responsible for data collection and analysis, manuscript oversight, and ethical conduct oversight.

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RESEARCH REPORT

Antiepileptic Medications Increase Osteoporosis Risk in Male Fabry Patients: Bone Mineral Density in an Australian Cohort

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Abstract *Background*: Fabry disease (FD) is an inherited X-linked lysosomal storage disease with widespread clinical manifestations. Small prospective studies have shown increased osteopenia and osteoporosis in male FD patients. Limited information however exists about bone metabolism and osteoporosis risk factors within this group. We reviewed osteoporosis risk factors within our cohort.

Methods: A retrospective analysis of bone mineral density (BMD) results and fracture incidence in 44 patients (22 males and 22 females) was undertaken. Dual X-ray absorptiometry scans were performed at the lumbar spine, hip and femoral neck. The impact of risk factors including renal function, antiepileptic drug (AED), analgesia and vitamin D levels were assessed.

Results: Male FD patients had low *T* scores at all sites (spine -1.2 ± 1.06, hip -1.6 ± 0.9, femoral neck -2.23 ± 1.01). Female *T* scores showed more typical distribution (spine -0.07 ± 1.47, hip 0.02 ± 1.14, femoral neck -0.49 ± 1.31). A higher incidence of osteopenia and/or osteoporosis occurred in males versus females (spine 46.9% versus 31.8%, hip 75.5% versus 18.2% and femoral neck 86.4% versus 45.5%). Multiple regression analysis showed a 50.8% ($p < 0.001$) reduction in femoral neck BMD with AED usage, after adjustment for age, gender and renal function. Non-traumatic fractures occurred in 27.3% males over 205 patient-years versus 4.6% in females over 149 patient-years, $p = 0.095$.

Conclusions: Low bone density was highly prevalent in male patients with increased incidence of non-traumatic fractures. AED usage significantly reduces BMD. Treatment to prevent BMD deterioration will depend on determining the bone turnover status.

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Introduction

Fabry disease (FD) is an X-linked lysosomal storage disease resulting from deficiency of the lysosomal enzyme α-galactosidase A (OMIM 301500) (Desnick et al. 2003). Deficiency results in impaired glycosphingolipid metabolism with resultant intracellular accumulation of globotriaosylceramide (Gb3). This accumulation causes cellular dysfunction especially of vascular endothelium resulting in organ damage either directly or via inducing hypertrophy, fibrosis or inflammation (von Scheidt et al. 1991). Clinical manifestations are widespread, predominantly involving the heart, kidneys, cerebrovascular system, peripheral nerves and skin (Zarate and Hopkin 2008; Mehta et al. 2009). Prior to the availability of enzyme replacement therapy (ERT), male life expectancy was reduced to 40–50 years by cardiovascular and renal disease (Mehta et al. 2009). While the long-term impact of ERT on life expectancy is still being evaluated, therapeutic improvements expose FD patients to long-term disease sequelae including osteoporosis.

Osteoporosis is a common disease characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue. Postmenopausal women and elderly men are at highest risk, with disease spectrum ranging from asymptomatic bone loss to disabling hip or

vertebral fractures. Secondary causes of osteoporosis include Cushing's syndrome, hyperparathyroidism, diabetes, hyperthyroidism, cardiovascular disease, gastrointestinal tract disease and cancer (Hofbauer et al. 2010; Montalcini et al. 2013). Chronic disease may share a common but unidentified mechanism leading to osteoporosis either through inflammatory responses or malnutrition (Montalcini et al. 2013).

Increased incidences of osteopenia (up to 87%) and osteoporosis (up to 26%) have been reported in FD (Germain et al. 2005; Mersebach et al. 2007; Germain 2010a). Avascular necrosis of the hip has been attributed to FD (Ross and Kuwamura 1993; Germain 2010b). Normal bone development is dependent on heredity, exercise capacity, nutrition and hormone levels (Ferrari et al. 2012). Low BMD fractures in the general male population are associated with low body mass index (BMI) and reduced exercise (Ebeling 2008), increasing age, diabetes, hypogonadism, excessive alcohol intake, previous fractures and history of stroke or falls (Drake et al. 2012). Risk factors for osteopenia identifiable in FD include low BMI, reduced exercise, antiepileptic drug (AED) usage (Petty et al. 2007) and potentially poor gastrointestinal absorption of vitamin D and calcium. Additionally, FD patients are at risk of renal impairment, diastolic heart failure and peripheral neuropathy, all negatively correlated with bone density (Hofbauer et al. 2010).

Dual-energy X-ray absorptiometry (DEXA) is a validated, quick, safe and precise measurement of BMD (Johnell et al. 2005; Blake and Fogelman 2009). Low BMD, particularly at the hip, predicts osteoporotic fracture risk (Marshall et al. 1996; Stone et al. 2003; Johnell et al. 2005). *Z* scores, comparing BMD to age and gender-matched cohorts, are preferred in men age under 50 and premenopausal women (International Society of Clinical Densitometry (ISCD) 2013). The World Health Organization (WHO) and International Osteoporosis Foundation however defines osteoporosis as $\geq$2.5 standard deviations (SD) below average BMD value of young healthy women, or *T* score, (the NHANES (National Health and Nutrition Examination Survey) III values) at a single anatomical reference site (Kanis et al. 2011; Ferrari et al. 2012).

In this retrospective study, we assessed bone density and biochemistry of a heterogeneous cohort of FD patients.

Methods

Study Population

Patient records and the Fabry clinical research database from the Royal Melbourne Hospital from May 2000 to August 2013 were reviewed retrospectively. All patients that had undergone routine in-house bone density screening measurements were included. Follow-up bone density measurements taken, at least 4 years after initial assessment, were subsequently reviewed.

Informed consent was obtained for data analysis from all patients. Clinical information including BMI, renal function (radioisotope creatinine clearance and 24-h urine collection), smoking status, antiepileptic drug usage, fracture history and ERT usage were retrieved from the local data registry or patient contact.

Dual X-Ray Absorptiometry

The decision for bone density evaluation was based on clinical risk profile at the discretion of treating physician. All measurements were obtained on a single machine within the Royal Melbourne Hospital Bone Metabolism Unit. Standard measurements at the lumbar spine, hip and femoral neck were employed. Values were expressed as both *T* and *Z* scores using the WHO classification of BMD abnormalities [normal *T* score > -1 SD below the mean for young adults; osteopenia *T* score < -2.5 SD to < -1 SD; osteoporosis *T* score < -2.5; severe osteoporosis *T* score < -2.5 with spontaneous fracture (Kanis 1994)]. A *Z* score of ≤ -2.0 SD is defined as "below the expected range for age," and a *Z* score of > -2.0 SD is "within the expected range for age" (International Society of Clinical Densitometry (ISCD) 2013). *T* scores were used for further analysis, with osteoporosis grouped with or without fracture.

Biochemistry

Serum concentrations of calcium (normal 2.10–2.60 mmol/L), phosphate (normal 0.8–1.0 mmol/L), lactate dehydrogenase (normal 210–420 IU/L) and alkaline phosphatase (ALP) (normal 30–120 IU/L) were measured by standard biochemical methods. Serum levels of 25-hydroxy vitamin D (replete 55–108 nmol/L) were assayed using standard radioimmunoassay techniques.

Statistical Analysis

Descriptive statistics were presented as median with range or mean and standard deviation. Shapiro–Wilk test was used to assess all continuous variables for normality prior to data analysis. Pearson correlation was used to assess the correlations between continuous variables (e.g. vitamin D and BMI). The relationship between continuous variables and gender were assessed using either Student *T*-test or Wilcoxon Rank–Sum tests, and categorical variables were tested using Fisher's exact test. Multiple regression analysis was used to determine the impact of the use of AED on BMD measures while adjusted for age, gender, smoking

Table 1 Patient characteristics – baseline

	Male ($N = 22$)	Female ($N = 22$)
Age (years)	40.4 ± 9.1	45.6 ± 13.2
Renal replacement therapy (*n*)	7	0
Smoking (*n*)	7	6
AED (*n*)	13	2
Fracture prevalence	3	1
BMI (kg/m^2)	22.8 ± 3	28.5 ± 8.4, $p = 0.044$
^{51}Cr-EDTA GFR (ml/min/1.73 m^2)	87.3 ± 32.8 (13/22 Pt)	73.9 ± 13.2 (8/22 Pt)
CrCl (ml/min) – 24 h	131.6 ± 41.2 (15/22 Pt)	126.7 ± 34.6 (19/22 Pt)

AED anti-epileptic drug, *BMI* body mass index, *GFR* glomerular filtration rate, *CrCl* creatinine clearance

and renal replacement therapy (RRT). The analyses were performed using GraphPad Prism v6.0c for Mac OSX (1994–2013 GraphPad Software Inc.) and XLSTAT Version 2013.5.04 (1995–2013 Addinsoft). Results were considered significant when the p value was <0.05.

Results

Study Population

Of 78 adult patients receiving their primary Fabry care at our centre, 44 had at least one BMD measurement between May 2000 and August 2013. This consisted of 22 males (aged 40.4 ± 9.1 years) and 22 females (aged 45.6 ± 13.2 years). The median follow-up time was 11 years (range 1.8 to 13.6 years) for males and 7.6 years (range 1.2–12.7 years) females. At the time of 1st DEXA scan, 18 male and 8 female patients were receiving ERT. Patient characteristics are shown in Table 1.

Seven male patients reached chronic kidney disease stage 5 (CKD5) requiring haemodialysis, with five subsequently receiving a renal transplant, prior to the first BMD. This group was defined as receiving RRT. Thirteen male and two female patients required neuropathic pain relief with an AED, at the time of first BMD test. Carbamazepine (CBZ) was the initial AED selected, commencing at 200 mg daily, and titrated to effect with maximal dose of 800 mg. A single patient used phenytoin for neuropathic pain relief. Where analgesia with CBZ was ineffective, patients were changed to pregabalin at dose range 75–300 mg daily. Smoking incidence was similar in males and females. A significant difference in BMI existed between male and female groups (22.8 ± 3 versus 28.5 ± 8.4, $p = 0.044$). No correlation was evident between BMI and BMD. One male and one female patient with severe vitamin D deficiency were excluded from further analysis.

Dual X-Ray Absorptiometry

BMD was significantly lower in males than females at the spine, hip and femoral neck ($p = 0.011$, $p < 0.001$ and $p < 0.001$), respectively (see Table 2). Scatter plots of individual T scores, for males and females, at each site show large gender differences (see Fig. 1). The distribution of patients to normal, osteopenic and osteoporotic (with or without fracture) classification was according to the WHO T score guidelines (see Fig. 1). The incidence of osteopenia and/or osteoporosis was significantly different between males and females at the hip and femoral neck but not at the lumbar spine (male hip 75.5% versus female hip 18.2%, $p < 0.001$ and male femoral neck 86.4% versus female femoral neck 45.5%, $p = 0.004$). Z scores, at the femoral neck, were also significantly different, with 7/17 males less than 50 years old compared to 1/13 premenopausal women having osteoporosis, $p = 0.004$.

AED had a significant impact on BMD of male patients at all sites: spine ($p = 0.007$), hip ($p < 0.001$) and femoral neck ($p = 0.003$) (see Table 2). RRT in males was associated with lower bone density at the femoral neck ($p = 0.02$) and approached significance at the hip ($p = 0.056$) but not at lumbar spine ($p = 0.54$) (see Table 2). Multivariate analysis showed AED usage was the predominant factor associated with reduced BMD, with a 50.8% reduction in BMD at the femoral neck when adjusted for gender, age and RRT (see Table 3). The BMD results at the femoral neck had 68.1% correlation with spine and 92.6% with hip. Smoking had minimal effect on the bone mineral status. While only four male patients were not on ERT, the BMD scores at the femoral neck were comparable to those on ERT.

Table 2 BMD T scores total and male subgroup analysis

Patients	*N*	Spine	Hip	Femoral neck
Female	22	−0.07 ± 1.47	0.02 ± 1.14	−0.49 ± 1.31
Male	22	−1.2 ± 1.06[a]	−1.6 ± 0.9[a]	−2.23 ± 1.01[a]
Male subgroups				
AED use	13	−1.46 ± 0.92	−1.84 ± 0.86	−2.47 ± 1.03
Non-AED use	8	−0.39 ± 0.81[b]	−0.88 ± 0.34[b]	−1.54 ± 0.62[b]
RRT	7	−1.30 ± 1.24	−2.02 ± 0.68	−2.86 ± 0.72
Non-RRT	14	−1.00 ± 0.90	−1.27 ± 0.84[c]	−1.90 ± 0.73[c]
Smokers	7	−0.80 ± 0.49	−1.55 ± 1.05	−2.07 ± 1.15
Non-smokers	14	−1.25 ± 1.17	−1.51 ± 0.78	−2.20 ± 0.96

RRT renal replacement therapy, *AED* antiepileptic drug use for neuropathic pain
[a] Male versus female – spine $p = 0.011$, hip $p < 0.001$ and femoral neck $p < 0.001$
[b] AED versus non-AED – spine $p = 0.007$, hip $p < 0.001$ and femoral neck $p = 0.003$
[c] RRT versus non-RRT – hip $p < 0.056$ and femoral neck $p = 0.02$

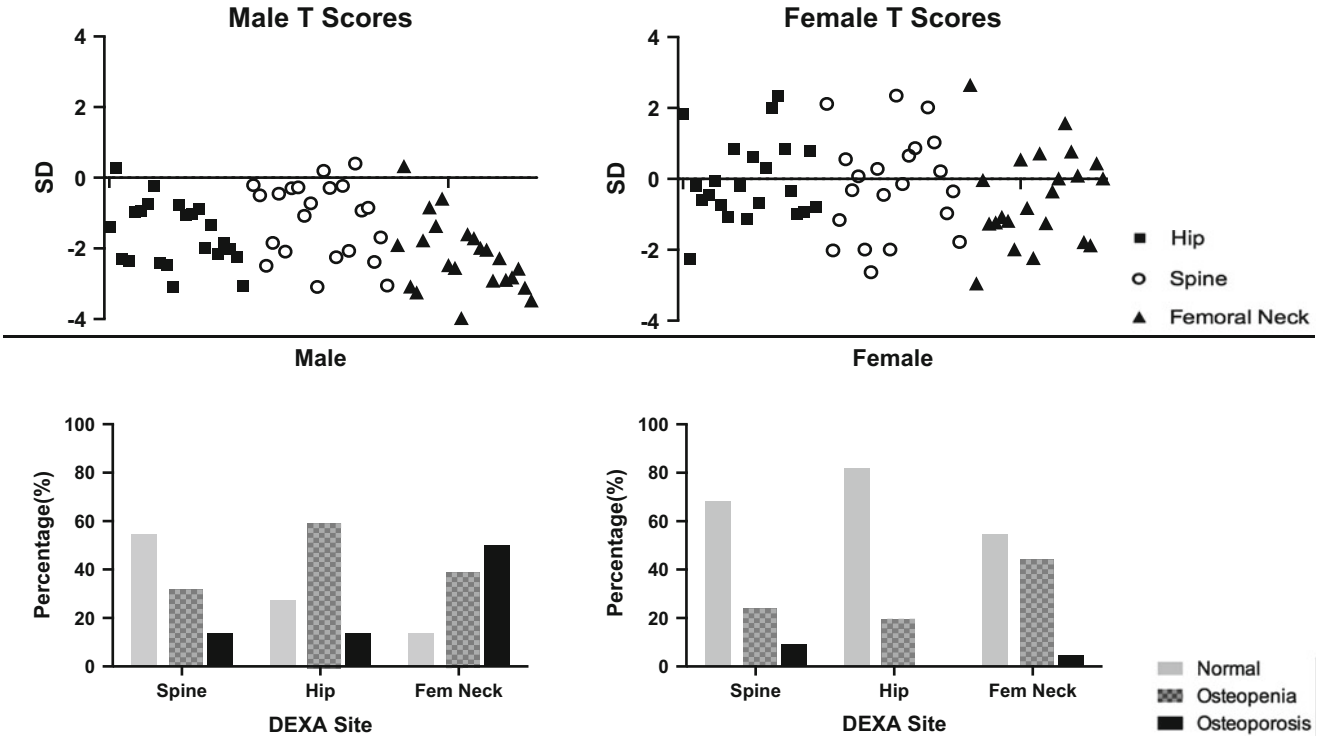

Fig. 1 Scatter plot of BMD *T* Scores and WHO BMD *T* score classification. First bone mineral density (BMD), measured by Dual X-ray absorptiometry, for each patient. Normal BMD > −1 SD below mean young adult, Osteopenia BMD <−1 to >−2.5 SD, Osteoporosis BMD <−2.5 SD (± fracture)

Biochemistry

Serum calcium (males 2.36 ± 0.10 versus females 2.38 ± 0.07 mmol/L, $p = 0.38$) and phosphate (males 1.01 ± 0.23 versus females 1.14 ± 0.12 mmol/L, $p = 0.03$) were both within normal ranges but lower in males than females at the time of first BMD. In contrast, serum alkaline phosphatase (males 92.95 ± 23.88 versus females 75.90 ± 28.73 IU/L, $p = 0.042$) and LDH (males 466.9 ± 156.9 versus females 444.3 ± 111.3 IU/L, $p = 0.61$) were higher in males than females. LDH was above the normal range but not significantly different

Table 3 Femoral neck multivariate analysis

Risk factor	Coefficient	95%CI	*p* value
AED	−1.508	−2.28 to −0.73	<0.001
Male	−0.82	−1.59 to −0.06	0.035
RRT	−0.55	−1.47 to 0.37	0.234
Age	−0.03	−0.06 to −0.001	0.041

RRT renal replacement therapy, *AED* antiepileptic drug use for neuropathic pain
Chronic AED usage reduces femoral neck bone mineral density by 50.8% after adjusting for age, gender and renal function

between males and females. Vitamin D levels were not available for all patients at baseline. A comparison of absolute nadir of vitamin D obtained for all patients during the study review showed the male having lower levels (males 40.4 ± 17.1 versus females 55.6 ± 28.6 nmol/L, $p = 0.051$). Furthermore, 15/19 males versus 11/21 females had vitamin D ≤55 nmol/L at some time point during the review period.

Patient Outcomes

Non-traumatic fractures were recorded as first incident fracture only. At baseline, three males had experienced non-traumatic fractures and only a single female. By the end of the follow-up period, a total of six males (27.3%) and a single female (4.6%) had experienced non-traumatic fractures. The total follow-up time was 205 patient-years for males versus 149 patient-years for females. Median age at time of fracture for men was 44.5 years (range 35–58 years) with four fractures occurring before 40 years of age. Of the seven patients with non-traumatic fractures, all were on AED and three were on RRT at the time of fracture. Neither gender difference nor presence of CKD5 altered the incidence of non-traumatic fractures ($p = 0.095$ and $p = 0.17$, respectively, Fisher's exact).

Follow-up BMD measurements were available in 12 patients (eight males and four females), all on ERT. The scans were performed at a median of 4.3 years (range 4–10 years) after initial scan. Repeat BMD showed no statistical change at the spine, hip or femoral neck; however, results were variable.

Discussion

This study revealed a very high prevalence of osteopenia and osteoporosis in the Fabry male population, in concordance with previous studies. We observed a higher prevalence of osteopenia and osteoporosis in males at the femoral neck (86% and 50%, respectively) than previously reported (Mersebach et al. 2007; Germain et al. 2005; Germain 2010a, b). In the seminal paper by Germain et al. (2005), examining BMD in 23 male Fabry patients, all site incidences of osteopenia and osteoporosis of 87% and 39%, respectively, were detected. However at the femoral neck alone, this was reduced to osteopenia 43.5% and osteoporosis 8.7%, respectively. Mersebach et al. (2007) compared 21 men and 32 women with FD and noted a significant difference in BMD between male patients and premenopausal women but no difference in males and postmenopausal women. In our study, however, the male FD cohort had much lower BMD scores than females at all sites, independent of menopausal status. Lower BMD, in males in our cohort, was also associated with increased fracture incidence independent of age and renal function.

A multivariate analysis showed a 50.8% ($p < 0.001$) reduction in femoral neck BMD with long-term usage of AED, after adjustment for age, gender and renal function. While low BMD appears attributable to FD, either directly or indirectly, AED use and to a lesser extent RRT have a significant impact. The requirement for AED and RRT are both consequences of disease severity. AEDs are usually initiated in childhood through to adolescence in males for analgesia secondary to Fabry-related neuropathic pain. This time period coincides with peak bone formation. Neuropathic pain severity may ease after young adulthood with reduction in AED usage. Additionally, ERT in Australia is not commenced until late adolescence, and this has been shown to reduce AED requirement. Whether the cause of lower BMD in male FD patients is due to impaired osteoclast and/or osteoblast function, poor nutrition status including vitamin D status, sex hormone level alteration or reduced exercise tolerance will need further elucidation.

Many FD patients require low dose AED, predominantly carbamazepine (CBZ), for neuropathic pain relief. AED use has been suggested to reduce absorption and utilization of vitamin D, alter parathyroid hormone (PTH) and calcitonin levels and reduce bioavailable serum oestrogens (Pack et al. 2011; Verrotti et al. 2010). Studies looking at the impact of CBZ on BMD and bone turnover markers are conflicting with some showing increased turnover (Verrotti et al. 2010), while others show no changes in BMD (Pack et al. 2011).

CBZ as a cytochrome p450 enzyme inducer has been suggested to accelerate vitamin D metabolism and thus reduce calcium absorption from the gut. More recent reviews however show inconsistent changes to vitamin D, calcium levels and PTH (Nakken and Taubøll 2010) in patients taking AEDs. Importantly, most reviews detailing AED use involve medication levels greater than those required for neuropathic pain relief. Cumulative drug load of AED has been reported as the dominant factor in fracture occurrence in epilepsy community (Petty et al. 2007). Additionally, neuropathic pain limits exercise tolerance and sunlight exposure, which may contribute to lower bone density and vitamin D deficiency in our cohort.

In the general male population, the predominant risks for osteoporosis are hereditary, sex steroid levels and chronic disease states (Kanis et al. 2011). Multiple secondary risk factors exist in men including excessive alcohol use, low calcium or vitamin D intake, smoking, low BMI, reduced exercise, AED use, liver or kidney disease (Ebeling 2008; Drake et al. 2012). The relationship between fracture risk and vitamin D level, as currently measured, remains inconclusive (Lips et al. 2010). Indeed, current routine monitoring of vitamin D for fracture prevention is unreliable, and the utility of routine vitamin D assay is disputed (Isenor and Ensom 2010). In our study, we found no association with vitamin D and BMD scores, although vitamin D levels in males were consistently lower than in females.

Limited information exists on hormonal changes in FD patients, but normal testosterone (Hauser et al. 2005; Mersebach et al. 2007), oestradiol and sex hormone-binding globulin (SHBG) (Hauser et al. 2005) levels have been reported. Bioavailable oestrogens, e.g. oestradiol, involved in bone metabolism, are formed by the aromatization of testosterone in peripheral fat. Oestrogen levels are also dependent on SHBG levels. Male FD patients frequently have low BMI, possibly related to poor gastrointestinal absorption or increased metabolic demand, which may impact on peripheral oestrogen formation. Secondly, most AEDs induce liver enzymes that consequently cause increases in SHBG and hence reduce available oestradiol. Interestingly in the small study by Hauser et al. (2005), follicle-stimulating hormone and prolactin levels were elevated, while testosterone and luteinizing hormone were normal, which may suggest subclinical deficiency of sex hormones in FD patients.

Determinants of bone strength are the degree of mineralization, cumulative micro-damage and collagen cross-link formation (Nishizawa et al. 2013). Bone metabolic markers, both resorptive and formative, are the best non-invasive way to measure bone turnover and infer bone strength and may be a useful guide for measuring response to clinical therapies (Nishizawa et al. 2013). We found no utility in measuring ALP, calcium, phosphate or LDH as biomarkers for bone health in this cohort of FD patients. Bone resorption however can be accurately determined by measurement of type 1 collagen cross-linked C-telopeptide and tartrate-resistant acid phosphatase, while bone formation can be determined by measurement of bone-specific alkaline phosphatase and procollagen type 1 N-terminal pro-peptide (Nishizawa et al. 2013). Increased bone resorption is routinely managed with use of antiresorptive agents like bisphosphonates, while reduced bone formation with is treated with agents like recombinant PTH. Male FD patients are a high-risk group that would benefit from measuring bone turnover markers with view to designing an appropriate treatment.

There are limitations inherent in a retrospective observational study. Firstly, the population was small and heterogeneous in age and renal function. Secondly, AED use was dependent on neuropathic pain control and was thus variable. Thirdly, vitamin D levels and PTH were not available for most patients at first BMD measurement. Fourthly, sex hormone levels including testosterone, bioavailable oestrogens and SHBG were not measured. However, previous studies have questioned the role of testosterone replacement in men with osteoporosis (Kanis et al. 2011). Fifthly, an appropriate control group with a comparable chronic disease burden is difficult to determine. Comparison to other chronic diseases may reveal the impact on BMD of factors like exercise capacity, diet and depression. Finally, an assessment of ataxia and cerebrovascular events were not included in this review but both of which may be increased in advanced Fabry disease. These in turn can increase fall frequency and fracture incidence.

In conclusion, BMD identified male FD patients at significantly higher risk of osteoporosis than both female FD patients and unaffected males. AEDs significantly reduce BMD, but whether bone metabolism can be improved by reducing AED exposure, improving gastrointestinal absorption of vitamin D or calcium, or even by earlier ERT initiation is yet to be determined. ERT alone appears inadequate to improve bone mineral density based on preliminary findings in this review. In Australia, there are few funded treatment options available for osteoporosis in men, but all affected patients were treated with vitamin D supplementation. Trials using bone turnover markers to determine whether increased bone resorption or reduced bone formation predominate in Fabry patients, in combination with sex hormone status, are in progress and may direct appropriate treatment for this high-risk group.

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Take-Home Message

Osteoporosis is highly prevalent in male Fabry patients, and antiepileptic medications, used for neuropathic analgesia, increase the risk of osteoporotic fractures.

Compliance with Ethics Guidelines

Details of Contributions of Individual Authors

Andrew Talbot was primarily responsible for planning the study and conducting the analysis of all bone mineral data. He also performed the primary data interpretation, including statistical analysis and original manuscript preparation.

Joanna R Ghali contributed to data collection and interpretation including manuscript editing.

Kathy Nicholls consented all patients and was the primary clinician responsible for bone mineral examination and pathology requests and follow-up. She also contributed to data interpretation and original manuscript preparation.

Conflict of Interest

Andrew Talbot has received research support, speaker honoraria and travel assistance from Shire Corporation and Sanofi Corporation, speaker honoraria and travel assistance from Dainippon Sumitomo Pharma Co. and research support from Amicus Therapeutics and Protalix Biotherapeutics.

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Kathy Nicholls has received research support, speaker honoraria and travel assistance from Shire Corporation and Sanofi Corporation and research support from Amicus Therapeutics and Protalix Biotherapeutics.

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 data analysis of results included in the study.

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CASE REPORT

The Complexity of Newborn Screening Follow-Up in Phenylketonuria

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Abstract In the United States, and most developed nations, the newborn screening (NBS) panel covers many primary disorders of metabolism, including phenylketonuria (PKU). When an elevated phenylalanine level is identified, the infant is evaluated for PKU and should also be tested for tetrahydrobiopterin (BH4) deficiency. A neonate presented with a phenylalanine level of 254 μmol/L (reference range <138 μmol/L) on newborn screening. The infant's confirmatory phenylalanine was 118 μmol/L (reference range <77 μmol/L). Her urine pterin profile was normal, and initially she had no measurable activity of red blood cell (RBC) dihydropteridine reductase (DHPR). Subsequent study revealed normal levels of CSF tetrahydrobiopterin and neurotransmitter metabolites, and by 18 months of age, her RBC DHPR activity was detectable at 0.5 nmol/min/mgHgb (reference range 0.8–3.9). Sequencing of the QDPR gene for DHPR revealed c.1A>T nucleotide substitution in exon 3 expressed as "p.MET1?" Phenylalanine hydroxylase (PAH) gene sequencing revealed compound heterozygosity for L249F and A300S. Although initial testing suggested the child was affected with DHPR deficiency, further analysis, finding increasing levels of DHPR activity and PAH compound mutant heterozygosity, indicated that the primary disorder is mild hyperphenylalaninemia with carrier status for DHPR deficiency. This is an example of newborn screening results leading to confusing findings requiring extensive biochemical studies and genotyping in order to arrive at the appropriate diagnosis.

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Introduction

It is generally known that newborn screening (NBS) in the United States and many developed nations now includes screening for many primary metabolic diseases. What is not broadly understood are the complexities and uncertainties involved in the follow-up of many abnormal results. We present the case of a neonate detected with a mild elevation of phenylalanine on NBS which usually indicates mild hyperphenylalaninemia (MHP) but can point to a disorder of tetrahydrobiopterin (BH_4) metabolism. Consequently, initial follow-up for an elevated phenylalanine requires not only quantitative confirmation of the elevation and determination of either PKU requiring dietary therapy or MHP, which is benign, but also assessment of the urinary pterin profile and assay of red blood cell (RBC) dihydropteridine reductase (DHPR) activity. Differentiating PKU or MHP from BH_4 deficiency is critical since the management of these conditions is different.

In the case presented, evaluation following the newborn screen required plasma, urine, and CSF studies and full sequencing of both the PAH and QDPR genes in the proband and her parents to achieve a definitive diagnosis.

Clinical Report

The infant, born at 41 weeks gestation to a G1P0 mother, had a positive NBS suggestive of phenylketonuria (PKU), with a phenylalanine level of 254 μmol/L (reference range <138 μmol/L) at 34 h of age. On physical exam, the patient was a vigorous female neonate with a normal exam. Laboratory studies showed a phenylalanine level of 118 μmol/L (reference range <77 μmol/L) and 5 days later, 158 μmol/L. Since the hyperphenylalaninemia was less than 360 μmol/L, no treatment was initiated. At 9 days of age, the patient's RBC DHPR activity was reported as undetectable (reference range 0.8–3.9 nmol/min/mgHgb). Analysis of urine pterins revealed biopterin of 23% (reference range 11.5–50.7). Repeat urine pterins indicated 25.7% biopterin. Repeat RBC DHPR activity at 12 days of age was 0.2 nmol/min/mgHgb (reference range 0.8–3.9). These results suggested that a primary defect in BH_4 recycling was the cause of the initial phenylalanine elevation. Given these findings, a lumbar puncture was performed for the measurement of CSF neurotransmitter metabolites and concentration of BH_4. The result indicated normal levels of CSF neopterin, tetrahydrobiopterin, 5-methyltetrahydrofolate, and the neurotransmitter metabolites, homovanillic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA). However, neonates with DHPR deficiency, likely peripheral, can have normal CSF BH4 and neurotransmitter metabolites (Opladen et al. 2012). Sequencing of the QDPR gene for DHPR was performed and revealed a single c.1A>T nucleotide substitution in exon 3. This is expressed as "p.MET1?" with the '?' indicating unknown effect on protein translation. Deletion/duplication testing of the gene was negative. Sequencing of the PAH gene revealed two mutations: L249F and A300S, a genotype consistent with MHP (Guldberg et al. 1998). At 18 months of age, the infant's RBC DHPR activity had risen to 0.5 nmol/min/mgHgb (reference range 0.8–3.9). Parental testing revealed that the mother carried both the A>T alteration in the QDPR gene and the L249F mutation in the PAH gene. Her RBC DHPR activity was 0.8 nmol/min/mgHgb, at the lower end of the normal range. No changes were identified in the QDPR gene of the father, and he was found to carry the A300S PAH gene mutation. Deletion/duplication analysis of the DHPR gene was negative in both parents. At 2 years of age, growth and development in the infant has been normal with no signs of clinical disease.

We concluded that the phenylalanine elevation in the infant is due to the hypomorphic PAH genotype (MHP) and that given the increasing RBC DHPR enzyme activity over time and the presence of only a single QDPR mutation in the patient and her mother and no QDPR mutation in the father, the infant is only a carrier for DHPR deficiency.

Discussion

This experience suggests that in the immediate newborn period, a carrier for DHPR deficiency may have no detectable RBC DHPR activity and may be incorrectly diagnosed as having DHPR deficiency unless additional testing and careful follow-up are performed.

The initial NBS result in our patient suggested a diagnosis of mild hyperphenylalaninemia. However, a mild phenylalanine elevation such as this can also indicate a more serious condition caused by a primary defect in synthesis or recycling of BH_4. BH_4 is the obligatory cofactor not only for the hepatic PAH but also for two other hydroxylases, tyrosine hydroxylase and tryptophan hydroxylase, both required for neurotransmitter synthesis (Hyland et al. 2001). Thus, a deficiency of BH_4 due to a defect in its synthesis or recycling results not only in hyperphenylalaninemia but also in deficiencies of the neurotransmitters dopamine and serotonin with consequences of which can be neurologically devastating. Early diagnosis and treatment with BH_4 as well as with precursors of the neurotransmitters are critical in allowing an optimal neurological outcome (Jäggi et al. 2008).

This case represents several considerations: (1) it demonstrates the value of definitively assessing infants with an elevated phenylalanine level and absent or very low DHPR activity. Without the comprehensive follow-up testing, our patient could have been inappropriately treated for DHPR deficiency. (2) The PAH deficiency in this infant prevents determining whether the initial absence of DHPR activity per se would have led to the neonatal hyperphenylalaninemia. We believe that this is a possibility and may account for some instances of false-positive "PKU" results in NBS. (3) It is possible that our patient harbors a second QDPR mutation in trans as the unidentified mutation may be in the 5′ promoter region or deep within an intron. This would be especially plausible if the expression of this lesion were both tissue and time dependent in development resulting in no significant effect in brain tissue, more so in liver and with the highest impact in erythroid progenitors, yet an effect that diminishes with time. This would be akin to a "peripheral" deficiency of DHPR as reported by Opladen et al. (2012), the "peripheral" deficiency associated with 6-pyruvoyl-tetrahydrobiopterin synthase (PTPS) deficiency (Niederwieser et al. 1987). (4) It is also possible that the heterozygous state of DHPR deficiency due to the c.1 A>T gene mutation causes a "peripheral" deficiency. However, carrier status for DHPR deficiency seems unlikely to cause hyperphenylalaninemia since the mother of our patient is also heterozygous for mutant QDPR yet is normophenylalaninemic and was not noted to have a positive "PKU" test when she was born. (5) The combination of MHP and DHPR carrier status in the

patient that we present is presumably extremely rare, given the approximately 1:30,000 frequency of MHP and an approximate incidence of 1:612 for the carrier state of DHPR deficiency, the latter based on the frequency of 2% pterin defects among all hyperphenylalaninemia and DHPR deficiency accounting for approximately 1/3 of all pterin defects (Opladen et al. 2012). This calculates to one in approximately 41,000,000.

Newborn screening has expanded rapidly. As a result, children are being identified who are carriers or have mild variants of conditions once thought to only present as rapidly deteriorating disorders. It is thus critical that in the encounter of the family with the primary health-care provider prior to referral to a metabolic clinic, there be an opportunity to emphasize that further testing of an abnormal initial result may be needed not only to confirm the result but also to determine its significance. Guidance around follow-up of an abnormal result on NBS is essential in helping families better understand the process and anticipate what may be done during their initial encounter with the metabolic team.

Synopsis

This case illustrates the potential for misdiagnosis of DHPR deficiency when evaluating an infant detected with hyperphenylalaninemia on newborn screening.

Compliance with Ethics Guidelines

Conflict of Interest

Leah E. Hecht, Ann E. Wessel, Harvey L. Levy, and Gerard T. Berry declare they have no conflict of interest.

Informed Consent and Animal Rights

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

Leah E. Hecht drafted the initial manuscript and approved the final manuscript as submitted.

Ann E. Wessel reviewed and revised the manuscript and approved the final manuscript as submitted.

Harvey L. Levy assisted in drafting and revising and approved the final manuscript as submitted.

Gerard T. Berry assisted in drafting and revising and approved the final manuscript as submitted.

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RESEARCH REPORT

Revised Proposal for the Prevention of Low Bone Mass in Patients with Classic Galactosemia

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Abstract Decreased bone mass is frequently encountered in classic galactosemia, an inborn error of galactose metabolism. This decrease is most prominent in adults, but is already seen in prepubertal children with increased risk of osteoporosis and fractures later in life. Therefore, bone health in patients with classic galactosemia is increasingly monitored. Although the pathophysiological mechanism is still not fully understood, several factors could negatively affect bone metabolism in this disease. Patients are at risk of nutritional deficiencies due to the galactose-restricted diet. Primary ovarian insufficiency (POI) in female patients also contributes to decreased bone mass. Furthermore, patients with classic galactosemia might be less physically active due to motor or neurological impairments. A disease-specific intrinsic abnormality has been suggested as well. This revised proposal is an update of the 2007 recommendations. In this current approach, we advise comprehensive dietary evaluation, optimization of calcium intake if needed, monitoring and if necessary supplementation of vitamin D, hormonal status evaluation and hormone replacement therapy (HRT) consideration, as well as a regular exercise and assessment of skeletal deformities and clinically significant fractures. We propose bone mineral density (BMD) assessment by serial DXA scans of the lumbar spine, femoral neck, and total hip in adults and lumbar spine and total body less head (TBLH) in children.

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Abbreviations

$1,25(OH)_2D$	Calcitriol
25(OH)D	25-Hydroxyvitamin D
BMD	Bone mineral density
BMI	Body mass index
DXA	Dual-energy X-ray absorptiometry
HRT	Hormonal replacement therapy
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor binding protein 3
POI	Primary ovarian insufficiency
SD	Standard deviation
TBLH	Total body less head
WHO	World Health Organization

Introduction

Decreased bone mass is frequently encountered in classic galactosemia (OMIM 230400), an inborn error of galactose metabolism (Kaufman et al. 1993; Rubio-Gozalbo et al. 2002; Panis et al. 2004; Jumbo-Lucioni et al. 2012; Waisbren et al. 2012; Batey et al. 2013; Coss et al. 2013). This decrease in bone mass is most prominent in adults, but is already seen in prepubertal children (Kaufman et al. 1993; Rubio-Gozalbo et al. 2002; Panis et al. 2004). Since bone mass increases quickly during puberty and reaches its peak at the end of adolescence or early adulthood, a low bone mass at an early age predisposes to osteoporosis and an increased risk of fractures later in life (Eisman 1999). Therefore, bone health in patients with classic galactosemia is increasingly monitored.

Scarce data are available with regard to reduced bone mass and its clinical relevance in this patient population. The average reported bone mineral density (BMD) as assessed by dual-energy X-ray absorptiometry (DXA) in

adults with galactosemia is more than one standard deviation (SD) below the mean, and even about 25–30% of patients have a BMD more than 2 SD below the mean (Waisbren et al. 2012; Batey et al. 2013), whereas in pediatric patients with galactosemia, the average is between 0 and −1 SD (Rubio-Gozalbo et al. 2002; Panis et al. 2004). Fracture rates in adult patients with classic galactosemia have been reported between 31 and 63% (Waisbren et al. 2012; Batey et al. 2013), which is slightly higher than in the general population in which rates between 21 and 53% have been reported for this age category (van Staa et al. 2001). Only one study addresses the fracture rates in pediatric patients (Waisbren et al. 2012), and the reported prevalence of 18% is not higher than in the healthy pediatric population (Landin 1997).

Although the pathophysiological mechanism is still not fully understood, several factors could negatively affect bone metabolism in this disease. Due to the galactose-restricted diet, patients are at risk of developing nutritional deficiencies. The primary ovarian insufficiency (POI) with low estradiol levels in females with galactosemia predisposes affected females to decreased bone mass. A disease-specific intrinsic abnormality such as abnormal glycosylation of collagen or other glycoproteins involved in bone metabolism has also been suggested (Kaufman et al. 1993). Furthermore, the decreased IGF-I and IGFBP-3 concentrations that are found in galactosemia patients might be involved (Panis et al. 2007a).

For classic galactosemia, optimization of exogenous factors influencing the bone mass is considered the most important intervention. The latest recommendations regarding follow-up and treatment of reduced bone mass in this disease date from 2007 (Panis et al. 2007b). Since then, insights regarding different aspects of bone health have increased, and there is a need to update and expand the previous recommendations based on our experience and the current literature. This proposal shortly reviews the diagnostic procedure and recommendations for follow-up including DXA, dietary evaluation, biochemical markers, physical activity, and in females supplementation of estrogen.

Diagnostic Procedures

Dietary Evaluation and Bone Metabolism Markers

A balanced diet sufficient in energy, protein, and micronutrients is a criterion for achieving and maintaining a high bone mass. Recently, it has been shown that body weight and body mass index (BMI) correlate with bone mass in patients with classic galactosemia, emphasizing the need for monitoring caloric intake in this group (Batey et al. 2013). Evaluation of food records or food frequency questionnaires, preferably by a nutritionist, can be used to determine a patient's general nutrition status and to assess the completeness of a patient's diet.

Table 1 Recommended dietary allowances of calcium (Institute of Medicine 2011) and vitamin D (Holick et al. 2011) for different age groups in case of risk for low bone mass.

Age (years)	Calcium (mg/day)	Vitamin D (IU/day)
<1	200–260[a]	400–1,000
1–3	700	600–1,000
4–8	1,000	600–1,000
9–13	1,300	600–1,000
14–18	1,300	600–1,000
19–70	1,000 (M) or 1,200 (F)	1500–2,000
>70	1,200	1500–2,000

[a] Reflects adequate intake value, since a recommended dietary allowance value has not been established for this group
M value for males, *F* value for females

The galactose-restricted diet predisposes patients to nutritional deficiencies, including a calcium deficiency. Dietary calcium intake therefore needs to be monitored, as is also recommended for other metabolic diseases in which the bone health is often affected. The recommended daily dietary calcium intake, which varies slightly per country, can be used as reference (Table 1).

Adequate vitamin D status, assessed by dietary and laboratory evaluation, is also required for optimal bone health. The preferred marker for vitamin D status is the total 25-hydroxyvitamin D (25(OH)D) serum concentration. Measurement of $1,25(OH)_2D$ is not recommended, since these concentrations do not reflect vitamin D reserves and can even be normal or elevated in patients with vitamin D deficiency due to secondary hyperparathyroidism (Holick et al. 2011). It is advised to aim for a total 25(OH)D serum concentration higher than 30 ng/mL (75 nmol/L) in patients at risk of developing osteoporosis, and this requires a vitamin D intake of at least 400–1,000 IU/day (Holick et al. 2011) (Table 1).

The importance of vitamin K in acquiring adequate bone mass remains controversial. Several studies, including one study in patients with classic galactosemia, have reported that supplementation positively affects bone mass when combined with calcium and vitamin D. Panis et al. (2006) showed that supplementation of vitamin K increased the concentration of carboxylated osteocalcin, which is required for the incorporation of calcium and hydroxyapatite in the bone. Braam et al. (2003) found a slower bone loss in postmenopausal women receiving vitamin K in combination with calcium and vitamin D. However, there is

also evidence that vitamin K does not increase bone mass (Guralp and Erel 2014).

Other nutrients that might be important for bone metabolism include zinc, phosphorus, and magnesium. There have been concerns that soy formula might have a negative effect on bone metabolism and growth due to its high phytate content, which causes reduced absorption of zinc. However, in a recent meta-analysis, zinc serum concentrations were found to be similar in children who were fed soy infant formula during infancy compared to children fed human milk or cows' milk-based formula during infancy (Vandenplas et al. 2014). Therefore, soy-feeding-related zinc deficiency is unlikely and measuring zinc concentrations is not recommended. Deficiencies of phosphorus and magnesium are unlikely in a balanced diet, and therefore, routine monitoring is not advised for patients at risk of developing low bone mass. However, serum phosphate and serum magnesium require special attention when dietary assessment indicates an insufficient diet. Furthermore, there is some evidence that a high phosphorus intake, resulting from food additives, combined with a low or normal calcium intake may have adverse effects on bone metabolism due to a disturbed ratio of calcium-to-phosphorus intake (Calvo and Tucker 2013). However, further research in this field is required to define adequate phosphate concentrations.

Other biochemical markers for bone metabolism, including carboxylated and uncarboxylated osteocalcin (cOC, ucOC), bone-specific alkaline phosphatase (BAP), amino terminal telopeptide (NTX), and carboxy terminal telopeptide (CTX), are at this point only recommended in research settings.

Estrogen Supplementation

In females, hypergonadotropic hypogonadism needs to be assessed by measuring FSH, LH, and beta-estradiol concentrations. Estrogen is a powerful inhibitor of bone resorption via the alpha and beta estrogen receptors on osteoblasts and osteoclasts, and low levels negatively influence bone metabolism. Evaluation of these hormones and referral to a pediatric endocrinologist should start from the age of 10–12 years (Fridovich-Keil et al. 2011).

Physical Activity Evaluation

Sufficient physical activity is required to achieve optimal bone mass. The World Health Organization (WHO) recommends 60 min of moderate- to vigorous-intensity physical activity per day for children and 150 min/week for adults. However, this is often not achieved. Furthermore, patients with classic galactosemia might be less physically active due to motor dysfunction (Rubio-Agusti et al. 2013) and less participative in social activities (Gubbels et al. 2011). Regular evaluation of physical activity in patients with this disease is therefore important, and a standardized questionnaire, for instance, the Physical Activity Questionnaire for Children and Adolescents (PAQ-C/A), can be used for this (Janz et al. 2008).

Assessment of Spinal Deformities

Scoliosis and hyperkyphosis are seen in 1–5% of all children in the general population and seem to be partly related to reduced bone mass and neurological abnormalities (Altaf et al. 2013). Scoliosis is defined as a lateral curvature of the spine that is more than 10° on a conventional X-ray in standing position. A hyperkyphosis is defined as a kyphosis of more than 45°. We observed a prevalence of 29% of these spinal deformities in a cohort of patients with classic galactosemia ($n = 24$, 14 females and 10 males; age range 13–48 years with a mean of 22 years), which is surprisingly higher than reported in the general population (unpublished results). A relationship between galactosemia and deformities of the spine has not been reported. It is questionable whether the observation in our cohort represents a real phenotypical characteristic or whether it is a coincidental finding. Yet, we advise to perform physical examination of the spine.

Dual-Energy X-Ray Absorptiometry

The method of choice to evaluate the bone mass is with dual-energy X-ray absorptiometry (DXA) scans, since this is a safe and relatively easy method with low exposure to radiation and low costs. Recently, the 2013 recommendations of the International Society for Clinical Densitometry have been published (Schousboe et al. 2013; Gordon et al. 2014). For postmenopausal women and men aged 50 years or older, DXA scans of the lumbar spine, femoral neck, and total hip are advised and *T*-scores are preferred to express the decrease in BMD. However, in premenopausal women and men younger than 50 years of age, *Z*-scores taking into account age, gender, and ethnicity are preferred. Also for children *Z*-scores are preferred and the results should be adjusted in children with short stature or growth delay. Measurements of the BMD of the lumbar spine and the total body less head (TBLH) are recommended in children, since they yield the most reliable results. DXA scans of the femur have been proven to be unreliable in children, and total body scans with the head might give false-positive outcomes due to the relative large size of the head during childhood.

In postmenopausal women and men aged 50 years or older, a BMD *T*-score ≤ -1.0 standard deviation (SD) is diagnostic of low bone mass (formerly called osteopenia),

while a *T*-score ≤ -2.5 SD is diagnostic of osteoporosis. A BMD *Z*-score ≤ -2.0 SD in premenopausal women or males younger than 50 years of age is defined as a BMD below the expected range for age, whereas a *Z*-score > -2.0 SD represents a BMD within the expected range for age (Schousboe et al. 2013). In children the diagnosis of osteoporosis requires either the finding of one or more vertebral compression fractures or the presence of both a BMD *Z*-score ≤ -2.0 SD and a clinically significant fracture history, meaning two or more long bone fractures by age 10 years or three or more long bone fractures at any age up to 19 years. A *Z*-score ≤ -2.0 SD without a clinically significant fracture is classified as a low BMD (Gordon et al. 2014).

Follow-Up and Therapeutic Interventions

Dietary Evaluation and Bone Metabolism Markers

Batey et al. (2013) found that most adults with galactosemia do not routinely visit a nutritionist, which might have implications for their bone health. Since a balanced diet is important for maximizing bone accrual during adolescence as well as for maintaining bone mass during adulthood, regular dietary evaluation throughout life is desirable. We recommend yearly dietary assessment, preferably by a nutritionist, with special focus on energy, protein, and micronutrient requirements. In case a patient's diet is suboptimal, enhanced nutritional counseling is essential to optimize a patient's general diet taking into account limitations deriving from galactose restriction. Special focus on vitamin D and calcium intake is suggested, including yearly evaluation of total 25(OH)D serum concentrations. We recommend supplementation of calcium and vitamin D when intake is lower than the recommended daily intake and does not normalize despite dietary optimization. When vitamin D supplementation has been initiated, more frequent monitoring of 25(OH)D serum concentrations might be helpful in determining the optimal dosage. Supplementation of vitamin K might be beneficial when combined with an adequate intake of calcium and vitamin D, but currently there is not enough evidence to recommend the routine use of vitamin K supplements.

Estrogen Supplementation

If evaluation of hormone status indicates hypergonadotropic hypogonadism, initiation of hormonal replacement therapy (HRT) needs to be considered. The decision to start supplementation during adolescence will have to take into consideration growth, psychological factors, and bone measurements. Duration of HRT throughout adult life will depend on an individual assessment of the possible risks and benefits.

Physical Activity Evaluation

We advise physicians to assess a patient's physical activity and to encourage exercise when evaluation suggests that the patient does not meet the recommendations. Implementation of a regular routine of weight-bearing exercise is recommended, taking into consideration possible physical limitations.

Assessment of Spinal Deformities

For now, we recommend physicians to evaluate for possible spinal deformities during routine physical examination. In case of clinical suspicion, an X-ray of the entire spine with a Cobb angle is recommended, and subsequent referral to an orthopedist for extensive evaluation may be considered (Hresko 2013).

Dual-Energy X-Ray Absorptiometry

It is advised to assess BMD from an early age to ensure that optimal bone mass is reached in early adolescence. We recommend the first DXA to be performed at the age of 4 years, or as soon as the child is able to lie still, since a decreased bone mass can already be present at this early age. In children the minimum time interval between subsequent DXA scans is 6–12 months (Gordon et al. 2014). We suggest yearly DXA scanning when BMD is ≤ -2.0 SD and one DXA scan per 2 years when BMD is higher. In adults it is advised to repeat a DXA scan one year after initiation or change of therapy (Schousboe et al. 2013). The interval can be increasingly elongated once the BMD is stable.

Furthermore, we recommend regular assessment of fracture history in order to determine whether a pediatric patient meets osteoporosis criteria and to evaluate the clinical relevance of a patient's reduced bone mass. In patients with frequent clinically relevant fractures, additional pharmacological therapy needs to be considered.

Conclusion

Our recommendations regarding assessment and follow-up of decreased bone mass in patients with classic galactosemia (Fig. 1):

- Dietary evaluation to assess adequacy of a patient's diet.
- Optimization of calcium intake, either improvement of dietary intake (preferably) or supplements.

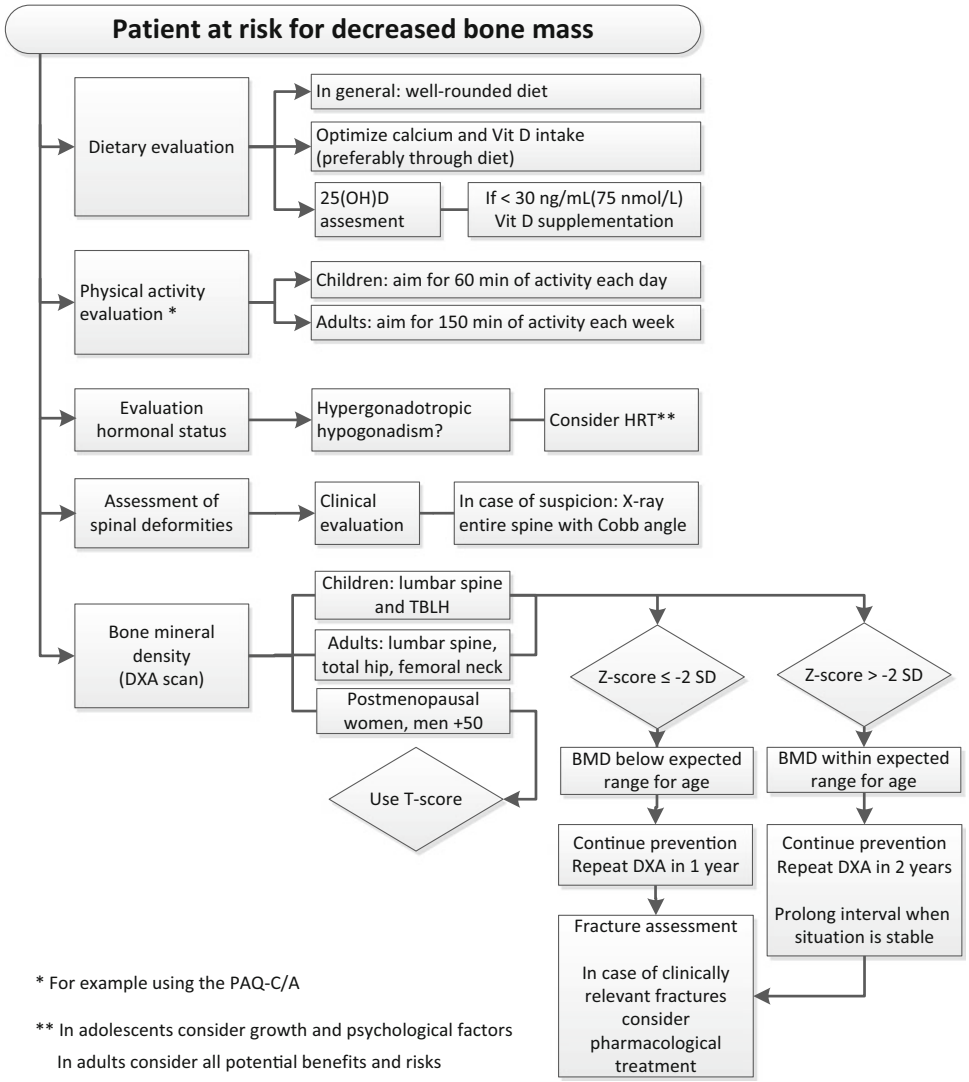

Fig. 1 Flow chart for the follow-up of bone health in patients with classic galactosemia

- Monitoring of vitamin D status by dietary assessment and laboratory measurement of total 25(OH)D concentrations. If total 25(OH)D serum concentrations are <30 ng/mL (<75 nmol/L), intake should be improved through diet or supplements.
- Hormonal status evaluation in females, in case of hypergonadotropic hypogonadism HRT, should be considered. Initiation and duration of treatment should take into consideration potential harms and benefits.
- Assessment of physical activity using standardized questionnaires. In case the WHO recommendations are not met, exercise needs to be encouraged.
- Evaluate for spinal deformities, since these might be more prevalent in this patient group, and fracture rate estimation.
- DXA as the tool to monitor BMD. DXA scans of the lumbar spine and TBLH are advised in children, whereas for adults DXA scans of the lumbar spine, femoral neck, and total hip are preferred.

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Synopsis

Bone health assessment in patients with classic galactosemia includes dietary evaluation, biochemical monitoring (vitamin D and estrogen status), assessment of physical activity, skeletal deformities and fractures, and serial DXA scans.

Compliance with Ethics Guidelines

Conflict of Interest

Britt van Erven, Myrna M.M. Römers, and M. Estela Rubio-Gozalbo declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

BvE, PhD on galactosemia, performed the study and drafted the manuscript. MR searched for relevant literature and helped to draft the manuscript. ER, PhD supervisor and lead of the galactosemia research group in Maastricht, designed, supervised, and coordinated the study and the final version of this manuscript. All authors have read and approved the final manuscript.

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CASE REPORT

m.8993T>G-Associated Leigh Syndrome with Hypocitrullinemia on Newborn Screening

Mari Mori · John R. Mytinger · Lisa C. Martin · Dennis Bartholomew · Scott Hickey

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Abstract Citrulline is among the metabolites measured by expanded newborn screening (NBS). While hypocitrullinemia can be a marker for deficiency of proximal urea cycle enzymes such as ornithine transcarbamylase (OTC), only a handful of state newborn screening programs in the United States officially report a low citrulline value for further work-up due to low positive predictive value. We report a case of a male infant who was found to have hypocitrullinemia on NBS. After excluding proximal urea cycle disorders by DNA sequencing, his NBS result was felt to be a false positive. At 4 months of age, he developed poor feeding, failure to thrive, apnea and infantile spasms with a progression to intractable seizures, as well as persistent hypocitrullinemia. He was diagnosed with Leigh syndrome due to a maternally inherited homoplasmic m.8993T>G mutation in the *ATPase 6* gene. His mother, who had previously been diagnosed with cerebral palsy, was concurrently diagnosed with neuropathy, ataxia, and retinitis pigmentosa (NARP) due to heteroplasmy of the same mutation. She had progressive muscle weakness, ataxia, and speech dyspraxia. The m.8993T>G mutation causes mitochondrial ATP synthase deficiency and it is hypothesized to undermine the synthesis of citrulline by CPS1. In addition to proximal urea cycle disorders, the evaluation of an infant with persistent hypocitrullinemia should include testing for the m.8993T>G mutation and other disorders that cause mitochondrial dysfunction.

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All states in the United States have implemented expanded NBS using tandem mass spectrometry (MS/MS) which permits a rapid analysis of metabolites based on mass-to-charge ratio. Almost all states screen for the 20 core metabolic conditions recommended by the American College of Medical Genetics and Genomics (ACMG) using MS/MS (ACMG 2006). These include distal urea cycle disorders such as citrullinemia and argininosuccinate aciduria detected by a high citrulline level. A low citrulline level may be incidentally detected by this method and may be used as a marker for deficiencies of a proximal urea cycle enzyme such as *N*-acetylglutamate synthase (NAGS), carbamoyl phosphate synthetase 1 (CPS1), and ornithine transcarbamylase (OTC). The ACMG recommends that a significantly low citrulline level be reported to rule out OTC or CPS1 deficiency (ACMG 2006), though the two conditions are not included in the recommended universal NBS panel due to a high false-positive rate of hypocitrullinemia (Cavicchi et al. 2009).

We report a case of a male infant who was found to have hypocitrullinemia on NBS and who later was diagnosed with Leigh syndrome due to a maternally inherited homoplasmic m.8993T>G mutation in the *MT-ATP6* encoding subunit 6 of mitochondrial ATP synthase. The 24-year-old mother was diagnosed with NARP caused by

the same mutation but a reduced mutant load in the peripheral blood of >95%. She also had hypocitrullinemia. Patients with m.8993T>G may benefit from early diagnosis in regard to prognosis and presymptomatic treatment with citrulline, coenzyme Q10, and, perhaps, EPI-743, a promising medication in the pipeline(Enns et al. 2012; Martinelli et al. 2012).

Case Report

A Caucasian male born in Florida at term to a 24-year-old mother after an uncomplicated pregnancy with a birth weight of 3.3 kg was referred to a local metabolic clinic at 5 days of age due to an abnormal NBS result with a low citrulline level (3.37 μmol/L at age 24 h, reference range 4–50 μmol/L), raising concern for a proximal urea cycle disorder. He failed his newborn hearing screen in the right ear. The family denied consanguinity. The mother had been diagnosed with cerebral palsy in childhood attributed to in utero bradycardia/anoxia. An evaluation in Florida included serum amino acid analysis, urine organic acid analysis, and serum ammonia. Serum amino acid analysis revealed persistently low citrulline (3 μmol/L, reference range 9–38), low arginine (24 μmol/L, reference range 29–134), and high alanine (537 μmol/L, reference range 139–474) levels. The patient was on regular formula at the time of the specimen collection. Urine organic acid analysis and serum ammonia levels were normal. The patient was started on protein-restricted formula (2 g/kg/day) and L-citrulline supplement (170 mg/kg/day) for a presumed urea cycle disorder. The patient and the mother relocated to live with the maternal grandmother during the work-up and transferred the care of the child to our service. Sequencing and deletion/duplication analysis of the *OTC* gene for OTC deficiency, *CPS1* gene for CPS I deficiency and the *NGS* gene for NAGS deficiency were negative for deleterious mutations. The patient was asymptomatic with normal growth and development at the age of 3 months. He was concurrently taken off diet restriction and L-citrulline after the negative genetic test results and a normal citrulline level of 20 μmol/L.

The patient had been doing well until 4 months of age, when he was noted to be losing weight by his primary care provider. He was admitted for an evaluation of failure to thrive and hypotonia. A brain MRI and an EEG were obtained for a concern for seizure-like activities. The EEG was initially normal. A brain MRI revealed abnormal signal and restricted diffusion in bilateral lenticular nuclei, the medial thalami and the caudate nuclei, as well as encephalomalacia (Figs. 1 and 2). An MR spectroscopy showed a lactate peak in the left anterior basal ganglia (Fig. 3). These findings were concerning for an underlying

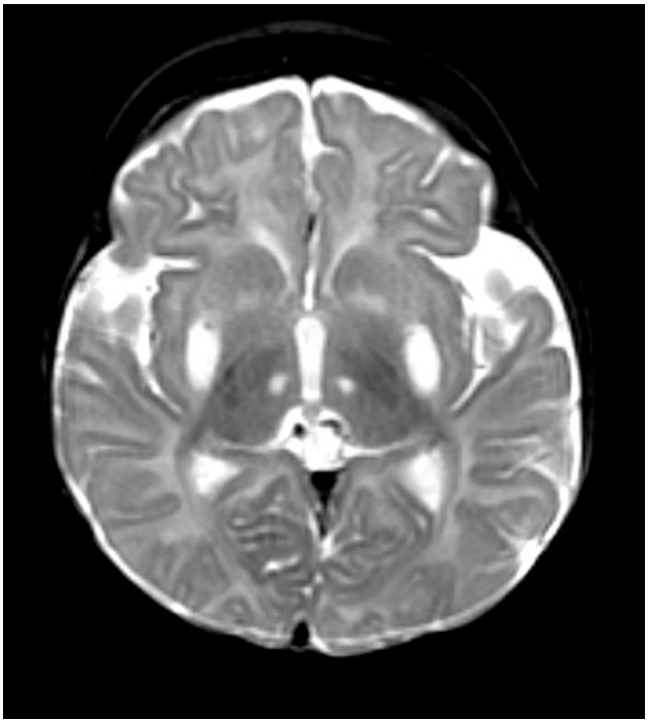

Fig. 1 Axial T2. Abnormal increased signal in subganglionic tissues, lenticular nuclei, and medial thalamic nuclei

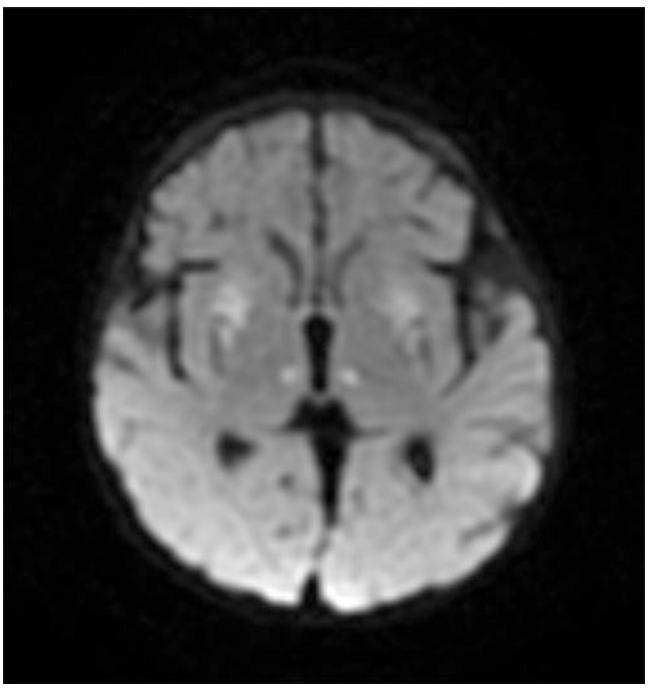

Fig. 2 Diffusion restriction in subganglionic tissues, lenticular nuclei, and medial thalamic nuclei

mitochondrial disorder, although a hypoxic-ischemic event was also a possibility. A muscle biopsy showed nonspecific changes in SDH, COX, and NADH stains. Electron transport chain enzyme spectrophotometry assay on snap

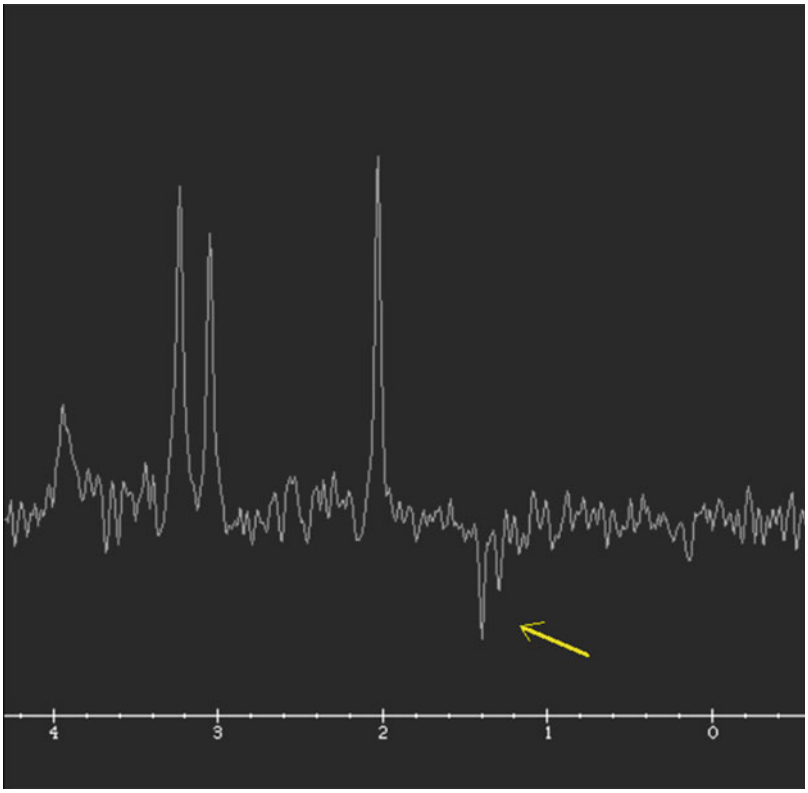

Fig. 3 MR spectroscopy TE 144 confirms inverted lactate duplet

frozen quadriceps muscle was unremarkable except for a reduced enzyme activity of the complex II (31% of mean). Respiratory chain complex V (ATP synthase) activity was not measured due to a technical limitation. A mitochondrial DNA mutation screening panel (leukocytes) was ordered due to the MRI findings and revealed homoplasmic m.8993T>G mutations in the *MT-ATP6* gene encoding ATP synthase, leading to the diagnosis of Leigh syndrome. The mutant load was confirmed by real-time allele refractory mutation system (ARMS) quantitative PCR analysis. Mild persistent lactic acidosis with lactate levels in the range of 3–7 mol/L was consistent with the diagnosis. A therapy with coenzyme Q10 was started for the treatment of Leigh syndrome. L-citrulline was restarted at an increased dose of 1.1 g/day (200 mg/kg/day), as serum citrulline level had decreased to 5 μmol/L. An ophthalmologic eye exam revealed bull's eye maculopathy consistent with the underlying diagnosis (Laird et al. 2006).

The patient required a G-tube for feeding for hypotonia and a failed swallow study, Nissen fundoplication for severe reflux and intermittent urinary catheterization for a neurogenic bladder. He remained on therapy with L-citrulline and coenzyme Q10 (100 mg/kg/day) supplement. He continued to have seizure-like activities. Long-term monitoring EEG at 5 months of age revealed frequent focal seizures arising from each hemisphere. He developed intractable seizures requiring multiple admissions with mechanical ventilation despite being on three antiseizure medications. Due to frequent apneic episodes, he was on home oxygen therapy. At 6.5 months of age, he developed infantile spasms. The EEG showed amplitude suppression in the bilateral anterior head regions, a disorganized and slow background, posteriorly dominant multifocal spikes, and infantile spasms associated with a broad high amplitude slow wave and electrodecrement. His infantile spasms resolved within one day of starting vigabatrin. Although his background abnormalities and multifocal spikes persisted, an overnight EEG performed three weeks after starting vigabatrin confirmed the clinical remission of infantile spasms. However, the frequency of his focal seizures increased, he became increasingly lethargic, and the family agreed to hospice care. He died at 11 months of age at home related to ongoing seizures and apnea. He was enrolled for a safety and efficacy clinical trial of EPI-743 designed for children with Leigh syndrome (NCT01721733) when he was 10 months of age, but he died before the therapy was started.

Testing of the mother with real-time ARMS quantitative PCR revealed that she had the m.8993T>G mutation with a mutation load of >95% in the blood; she was diagnosed with NARP based on the mutation and clinical presentation. The maternal grandmother was also tested for the familial

mutation, but no mutation was detected in a blood specimen by real-time ARMS quantitative PCR. Neither the patient nor the mother has any siblings. The mother was a product of an uncomplicated pregnancy and perinatal hypoxia. She was diagnosed with cerebral palsy at the age of 14 months. She did not walk until 15–16 months and required special education throughout school. At the time of diagnosis with NARP, the mother was found to have low levels of multiple amino acids in the serum including citrulline (3 μmol/L), and L-citrulline supplement was started. She did not tolerate coenzyme Q10 due to headaches. An ophthalmologic eye exam did not reveal any evidence of pigmentary retinopathy. A brain MRI revealed increased signals in the bilateral basal ganglia in FLAIR and diffusion sequences. Her lactate, alanine, and proline levels were within the normal range. Since giving birth to the proband, she has demonstrated progressively worsening neurologic symptoms including deterioration in speech, swallowing function, and ambulation.

Discussion

Although citrulline level is measured in NBS to detect distal urea cycle disorders leading to hypercitrullinemia (ACMG 2006), an NBS protocol for low hypocitrullinemia is not established because of low sensitivity of hypocitrullinemia for proximal urea cycle disorders (Cavicchi et al. 2009). Persistent hypocitrullinemia initially identified by NBS warrants a work-up beyond proximal urea cycle disorders.

Persistent hypocitrullinemia has been seen in patients with mitochondrial disorders (Atkuri et al. 2009) including patients with Pearson syndrome (OMIM 557000) (Ribes et al. 1993), m.8993T>G-associated Leigh syndrome/NARP (OMIM 516060) (Rabier et al. 1998; Parfait et al. 1999; Enns et al. 2006; Debray et al. 2010; Henriques et al. 2012), and mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes syndrome (MELAS, OMIM 540000) (Perry et al. 1989; Koga et al. 2005; Naini et al. 2005). Hypocitrullinemia is also associated with secondary mitochondrial respiratory chain dysfunction caused by organic acidemias (Atkuri et al. 2009), deficiency of mitochondrial pyrroline-5-carboxylate synthase (P5C) (Rabier and Kamoun 1995; Baumgartner et al. 2000), or intestinal malrotation in newborns (Cavicchi et al. 2009). Citrulline is synthesized in the mitochondrial matrix of enterocytes in the small intestine catalyzed by pyrroline-5-carboxylate synthase (P5C), CPS1, and OTC (Naini et al. 2005). Hepatocytes also synthesize citrulline, but they cannot export the product. Hypocitrullinemia is thought to be a nonspecific marker of impaired oxidative phosphorylation in the enterocyte. Reduced mitochondrial ATP or enterocyte dysfunction can lead to reduced activity of P5C or CPS1 in enterocytes, leading to reduced synthesis of circulating citrulline (Parfait et al. 1999; Munnich and Rustin 2001). Thus, persistent citrullinemia can be a marker for dysfunction of enterocytes, mitochondrial ATP production, or mitochondrial P5C and urea cycle enzymes. The prevalence of hypocitrullinemia among infants with mtDNA disorders including m.8993T>G is unknown and the predictive power of hypocitrullinemia for these disorders cannot be determined. In a review of six children with Leigh syndrome due to the m.8993T>G mutation, only one child was found with hypocitrullinemia (Morava et al. 2006).

Although there is no current cure for mitochondrial disorders or Leigh syndrome, coenzyme Q10 may have beneficial effects related to its antioxidant properties. A coenzyme Q10 derivative EPI-743 is believed to be more potent than coenzyme Q10, readily crosses the blood–brain barrier (Enns et al. 2012), and has shown promising results with no side effects in four patients (Enns et al. 2012). A randomized clinical trial of EPI-743 for children with Leigh syndrome is underway (NCT01721733).

When hypocitrullinemia is detected in NBS and persists in a confirmatory test, differential diagnoses should not be limited to proximal urea cycle disorders but also include m.8993T>G as well as other disorders that can impair P5C or CPS1 function including MELAS and Pearson syndrome. Serum lactate level may be high in these disorders but is not a sensitive marker, especially when the patient is asymptomatic.

Conclusions

We described a case of m.8993T>G-associated Leigh syndrome who initially presented with hypocitrullinemia on NBS. Persistent hypocitrullinemia detected on NBS with no defect in NAGS, CPS1, or OTC may result from other disorders causing respiratory chain dysfunction. Although additional study is needed, a presymptomatic diagnosis of mitochondrial disorders may be of therapeutic benefit. Further studies are needed to establish a protocol for hypocitrullinemia including a cut-off value.

Synopsis

When hypocitrullinemia is detected in NBS and persists in a confirmatory test, differential diagnoses should not be limited to proximal urea cycle disorders but also include m.8993T>G as well as other disorders that can impair P5C or CPS1 function including MELAS and Pearson syndrome.

Compliance with Ethics Guidelines

Mari Mori, John R. Mytinger, Lisa C. Martin, Dennis Bartholomew, and Scott Hickey declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

Contribution of Authors

Mari Mori: Dr. Mori cared for the patient during hospital admittance and in the genetics clinic. She enrolled the patient in the EPI-743 clinical study. Dr. Mori drafted the initial draft and approved the final manuscript as submitted.

Scott E. Hickey: Dr. Hickey diagnosed the patient with Leigh syndrome and his mother with NARP, cared for the patient during hospital admittance and in the genetics clinic, aided with enrollment into the EPI-743 clinical study, aided with draft revision, and approved the final manuscript as submitted.

Dennis Bartholomew: Dr. Bartholomew reviewed the newborn screening results, performed the initial work-up, aided in draft revision, and approved the final manuscript as submitted.

Dr. John R. Mytinger: Dr. Mytinger cared for the patient during hospital admittance and in neurology clinic, aided in draft revision and approved the final manuscript as submitted.

Dr. Lisa C. Martin: Dr. Martin interpreted the brain MRI in radiology, aided in preparing the figure, and approved the final manuscript as submitted.

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RESEARCH REPORT

Urge Incontinence and Gastrointestinal Symptoms in Adult Patients with Pompe Disease: A Cross-Sectional Survey

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Abstract *Objective*: To determine the frequency and impact of gastrointestinal symptoms, and bowel and urinary incontinence, as this is currently unknown in adults with Pompe disease.

Methods: Adult German Pompe patients and age- and gender-matched controls were asked about symptoms in the upper and lower intestinal tract as well as urinary incontinence using the Gastrointestinal Symptoms Questionnaire and the International Consultation on Incontinence Questionnaires for Bowel Symptoms and Urinary Incontinence.

Results: The overall response rate was 78%; 57 patients and 57 controls participated. The mean age of the patients was 48.3 years ±14.7 (28 female, 29 male). 84% of patients were receiving enzyme replacement therapy. Stool urgency, diarrhoea, and urinary urge incontinence were reported significantly more frequently in patients compared to the age- and gender-matched controls (55%, 56%, 33% vs. 20%, 18%, 7%). 20% of Pompe patients used loperamide daily against diarrhoea. No other gastrointestinal tract-related symptoms were reported to occur more frequently in Pompe patients than in controls.

Conclusions: Compared to age- and gender-matched controls, both urinary and bowel incontinence occur in a higher frequency in adults with Pompe disease and have a major impact on daily life.

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Introduction

Pompe disease (synonyms: glycogen storage disease type II, acid maltase deficiency) is an autosomal recessive disorder caused by deficiency of the lysosomal enzyme acid alpha-glycosidase (GAA) due to mutations in the *GAA* gene. Enzyme replacement therapy (ERT) with alglucosidase alfa (Myozyme®) for patients with classic infantile and late-onset Pompe disease has been available since 2006 (Toscano and Schoser 2013). The main clinical and prognostic features in adult patients with Pompe disease are proximal and axial paresis in the limb girdles and respiratory insufficiency due to glycogen storage in the skeletal muscles (van der Beek et al. 2012). ERT was shown to have a beneficial effect on these symptoms and to delay progression of skeletal muscle symptoms in adolescent and adult patients with Pompe disease (Regnery et al. 2012; Laforêt et al. 2013; Güngör

et al. 2013; Schneider et al. 2013; de Vries et al. 2012; Toscano and Schoser 2013).

The awareness of multisystemic symptoms as well as long-term complications and the response of signs and symptoms to ERT is currently a major issue in the follow-up of adult patients with Pompe disease. Gastrointestinal symptoms are well known in storage disorders like Fabry disease (e.g. abdominal pain, poor weight gain, chronic diarrhoea, postprandial discomfort) and Gaucher disease (weight loss, cachexia, abdominal pain) and were reported to respond well to ERT (Verderese et al. 1993; Banikazemi et al. 2005). The lysosomal accumulation of glycogen in smooth muscles in different organs was shown in a *GAA* knockout mouse model (Bijvoet et al. 1999), in infantile Pompe disease (Winkel et al. 2003) and in autopsies (including the organs of the gastrointestinal tract and the urinary tract; Swash et al. 1985; van der Walt et al. 1987; Kobayashi et al. 2010; Hobson-Webb et al. 2012), in biopsies of the arrector pili muscle (Katona et al. 2014), and in imaging studies of cerebral vessels of adult Pompe disease cases (Sacconi et al. 2010; Hobson-Webb et al. 2012).

With respect to gastrointestinal symptoms, the International Pompe Registry and guidelines for the treatment of Pompe disease focus mainly on the registration and management of malnutrition and dysphagia in infantile patients (https://www.registrynxt.com/Pompe/Pages/Home.aspx; Bembi et al. 2008). In adult patients, observations regarding the nutritional status only included changes of body mass index under ERT (van der Beek et al. 2009; Ravaglia et al. 2010a; Ravaglia et al. 2010b; Kobayashi et al. 2010; Papdimas et al. 2010; Bernstein et al. 2010; Regnery et al. 2012; Schüller et al. 2012). Recent reports on small cohorts addressed the issue of gastrointestinal symptoms in adults with Pompe disease as well as symptoms such as chronic diarrhoea, bowel urge incontinence, meteorism, gastrointestinal reflux, and obstipation (Bernstein et al. 2010; Sacconi et al. 2010; Remiche et al. 2012). In 20 patients with late-onset Pompe disease, 25% had incontinence definitely attributable to Pompe disease (Remiche et al. 2012). The application of ERT led to a variable reduction of lysosomal glycogen in the smooth muscles (Bijvoet et al. 1999; Winkel et al. 2003) and to a reduction of gastrointestinal symptoms including incontinence (Bernstein et al. 2010; Sacconi et al. 2010; Remiche et al. 2012). In addition, urinary incontinence was reported less frequently in patients with adult Pompe disease than bowel incontinence (Chancellor et al. 1991; Remiche et al. 2012).

In the present multicentre cross-sectional study in adults with Pompe disease, we systematically surveyed by questionnaires the occurrence of symptoms of the upper and lower gastrointestinal tract and in depth the frequency and impact of both urinary and bowel incontinence and compared this with age- and gender-matched controls from the general population.

Patients and Methods

Patients

Seventy-three patients with biochemically and genetically confirmed adult Pompe disease were recruited by the German Pompe centres. Patients were asked to complete the questionnaire when they attended the neuromuscular clinics for follow-up and/or ERT. The following German centres participated: Berlin ($n = 9/10$), Bochum ($n = 7/7$), Bonn ($n = 6/9$), Halle ($n = 13/15$), Mainz ($n = 13/20$), and Munich ($n = 10/12$) (number of participants/number of patients invited to participate). Controls were recruited in a biased manner: Controls, whose age was approximately the same as that of the patients with Pompe disease, were either accompanying acquaintances, partners, or relatives of patients with other nonhereditary neuromuscular diseases (e.g. amyotrophic lateral sclerosis, diabetic neuropathy, myasthenia gravis; exclusion criterion: history of colon cancer). All participants signed an informed consent form the Local Ethics Committee of the Martin-Luther-University Halle (Saale) and all the other participating institutions approved the study.

Questionnaires

General Information

The general information included questions about onset and severity of Pompe disease, the use and start of ERT, and age, gender, and body mass index (BMI) of each participant. Furthermore, the participants were asked whether they had ever undergone a gastroscopy or colonoscopy and whether they take drugs to relieve gastrointestinal symptoms.

Gastrointestinal Symptoms Questionnaire

The validated Gastrointestinal Symptoms Questionnaire is a standardised checklist and allows screening for the severity of both upper and lower gastrointestinal tract symptoms during the previous 4 weeks (Bovenschen et al. 2006; see also for English version). The severity was rated on a 0–6-point Likert scale, where 0 meant “no complaints” and 6 represented the worst imaginable severity of the symptom. The questionnaire has been standardised and validated in primary care patients referred for Helicobacter pylori urea breath testing and has also been used in myotonic dystrophy type 2 (Tieleman et al. 2008). We

used the German equivalent of this questionnaire (Supplementary Fig. 1), but we also added questions about the frequency of symptoms using an unstandardised method.

Since this questionnaire only rates the severity, questions about the frequency of these symptoms of the upper and lower GI tract were included (1: < two times a week, 2: 2–5 times a week, 3: > 5 times a week).

International Consultation on Incontinence Questionnaire-Bowel-Long Form (ICIQ-B-LF)

For the in-depth survey about bowel incontinence and its impact on quality of life, the validated ICIQ-B-LF was used (Cotterill et al. 2011). The self-report ICIQ-B-LF contains 21 questions arranged in five scored domains: bowel pattern, bowel control, other bowel symptoms, sexual impact, and quality of life. The German equivalent is depicted in Supplementary Fig. 2.

International Consultation on Incontinence Questionnaire-Urinary Incontinence-Short Form (ICIQ-UI-SF)

The validated self-report ICIQ-UI-SF for urinary incontinence was used to score frequency and amount of urinary incontinence and its impact on the quality of life. In addition, the questionnaire asks for the situations in which incontinence occurred (Timmermans et al. 2013). A German questionnaire has been validated previously (http://www.awmf.org/uploads/tx_szleitlinien/084-001k_S2_Harninkontinenz).

Statistics

Descriptive statistics are presented as mean ± 1 standard deviation. All variables were analysed to evaluate their normality using the Kolmogorov–Smirnov test. Differences between groups were analysed using unpaired Student's *t*-test or Mann–Whitney rank sum test, χ^2 test, or Fisher exact test. Spearman's rank correlation coefficient was determined to investigate correlations (SPSS 17, IBM Software Group, USA).

Results

Patient Characterisation

Out of 73 adult Pompe patients, 57 patients responded to all questionnaires (response rate 78%, 28 women, 29 men) in contrast to age- and gender-matched controls from the general population (response rate 84%). Details are given in Table 1. Forty-nine patients (84%) were currently receiving ERT. There was no difference in BMI of Pompe patients and controls (24.2 ± 4.7 vs. 25.7 ± 4.8).

Information About Endoscopy and Drugs for Gastrointestinal Symptoms

Equal numbers of patients and controls had undergone endoscopy of the upper and/or lower gastrointestinal tract in their life (20.7–26.9%). There was no statistical difference in the numbers of patients and controls who used drugs for heartburn, flatulence, and obstipation. However, statistically significant more patients than controls required drugs against diarrhoea and urge incontinence ($p < 0.01$: Table 1). This comprised loperamide 1–3 times a day in 12 patients (21%). Only one control reported taking loperamide about three times a year.

Gastrointestinal Symptoms Questionnaire

Detailed results of this questionnaire are given in Table 2. When the severity of symptoms of the upper and lower gastrointestinal tract was analysed, patients had significantly higher scores than controls in the items "diarrhoea" and "stool urgency". Diarrhoea was reported by 23 patients (14 women, 9 men, median age: 51 years, range: 23–73). Stool urgency was reported by 23 patients (10 women, 13 men, median age: 53 years, range: 23–73). Statistically significant higher numbers of controls than patients reported heartburn, bloating, postprandial fullness, and flatulence. However, when the severity of these symptoms was asked for, no statistically significant differences for these symptoms between controls and patients were found. Additionally, analysing the nonstandardised part of the questionnaire, there was no difference in the frequency of symptoms between patients and controls (data not shown).

International Consultation on Incontinence Questionnaire-Bowel-Long Form (ICIQ-B-LF)

Bowel control was analysed in detail using the ICIQ-B-LF (Table 3). The scores for both frequency and interference with daily activities for the domains "bowel pattern", "bowel control", and "quality of life" were statistically higher in patients than controls. For the domain "other bowel symptoms", only the interference with daily life but not the frequency was scored higher in patients than controls. The differences in the domain "sexual impact" did not reach statistical significance. There was a mostly high linear correlation between frequency and interference

Table 1 Demographic and clinical data, including frequency of colonoscopy and gastroscopy and frequency of drug intake for different gastrointestinal complaints in adults with Pompe disease and in age- and gender-matched controls (i.e. accompanying spouses and partners)

	Pompe patients ($n = 57$)	Age- and gender-matched controls ($n = 57$)	p
Gender (female/male)[b]	28/29	28/29	
Age at examination (years)[a]	48.3 ± 14.7 (18–73)	52.3 ± 15.8 (23–78)	n.s.
Disease duration (years)	17.1 ± 11.9 (2–50)	n.a.	
Wheelchair bound [*n* (%)]	12 (20.7)	n.a.	
Non-invasive ventilation [*n* (%)]	19 (32.8)	n.a.	
ERT [*n* (%)]			
Currently receiving [*n* (%)]	49 (84)	n.a.	
Interrupted [*n* (%)]	4 (6.7)	n.a.	
Never [*n* (%)]	4 (6.7)	n.a.	
BMI[a]	24.2 ± 4.7 (15.4–35.7)	25.7 ± 4.8 (18.0–41.0)	n.s.
Colonoscopy [*n* (%)][c]	15 (26.9)	12 (20.7)	n.s.
Gastroscopy[c]	13 (22.4)	13 (22.4)	n.s.
Drug intake for			
Gastrointestinal complaints [*n* (%)][c]	17 (29.3)	15 (26.9)	n.s.
Heartburn [*n* (%)][c]	5 (8.6)	9 (15.5)	n.s.
Flatulence [*n* (%)][c]	8 (13.8)	7 (12.1)	n.s.
Obstipation [*n* (%)][c]	3 (5.2)	3 (5.2)	n.s.
Diarrhoea/urge incontinence [*n* (%)][c]	12 (20.7)	1 (1.7)	0.0046

Data are given as mean ± 1SD (range). Groups were compared using unpaired Student's *t*-test[a] or χ^2 test[b] and Fisher exact test[c]
n.a. not applicable, *n.s.* nonsignificantly

for all five domains using the Spearmen Rank Coefficient: bowel pattern $r^2 = 0.73$, bowel control $r^2 = 0.88$, other bowel symptoms $r^2 = 0.54$, sexual impact $r^2 = 0.85$, quality of life $r^2 = 0.88$.

International Consultation on Incontinence Questionnaire-Urinary Incontinence-Short Form (ICIQ-UI-SF)

The differences in the frequency of urinary incontinence in patients compared to controls did not reach statistical significance (Table 4). However, patients were statistically more disturbed in their daily life than controls. When only those individuals who reported urinary incontinence were compared, the statistically significant difference between patients and controls was even more pronounced. Significantly, more patients than controls experienced urinary incontinence before they reached the toilet. Urinary urge incontinence was reported by 18 patients (12 women, 6 men, median age: 52 years, range: 27–73). Thirteen patients reported both urinary urge incontinence and stool urgency and/or diarrhoea (9 women, 4 men, median age: 52 years, range: 29–73). Significantly, more controls than patients reported urinary incontinence during coughing and pressure. These controls were mostly women ageing >45 years (12/15).

Symptoms in Cachectic Patients

Five out of 57 patients (8.8%, 1 woman, 4 men, age range: 23–57 years, disease duration range: 9–19 years, all received ERT for 2–7 years) had a BMI <18 (equals cachexia). All patients reported diarrhoea, three reported stool urgency, and one reported urinary urge incontinence. Three patients took regularly loperamide.

The ages of the patients with stool urgency, diarrhoea, and urinary urge incontinence were not significantly different from those patients who did not report these symptoms (age: mean 50.7 years ± 11.9 median 51 years (23–73) vs. mean 45.4 years ±17.0, median 50 years (18–74); $p = 0.21$). The duration of Pompe disease (time from the onset of the first symptoms of the skeletal muscles, i.e. pain, weakness, dyspnoea) in patients with stool urgency, diarrhoea, and urinary urge incontinence was not significantly different from those patients who did not report these symptoms (disease duration: mean 18.3 years ± 11.8, median 16 years (3–51) vs. mean 15.6 years ± 12.7, median 10 years (3–43); $p = 0.47$). Of the eight patients who had either interrupted ERT or had never been treated with ERT, two reported diarrhoea and another one urinary urge incontinence, but none stool urgency.

Table 2 Severity and frequency of abdominal and epigastric symptoms in adult patients with Pompe disease and in age- and gender-matched controls (i.e. accompanying spouses and partners) as analysed by the Gastrointestinal Symptoms Questionnaire

Symptom	Pompe patients reporting symptoms [*n* (%)]	Controls reporting symptoms [*n* (%)]	p^b	Severity Pompe patients ($n = 58$)	Severity Age- and gender-matched controls ($n = 58$)[a]	p^a
Abdominal pain						
General	2/41 (4.5)	2/49 (4.1)	n.s.	0.22 ± 0.88	0.06 ± 0.32	n.s.
Postprandial	8/41 (20)	8/43 (19)	n.s.	0.34 ± 0.73	0.23 ± 0.52	n.s.
Fasting	2/39 (6.9)	1/40 (2.5)	n.s.	0.18 ± 0.72	0.03 ± 0.16	n.s.
Persistent after stools	3/38 (7.9)	0/41 (0)	n.s.	0.26 ± 0.72	0	n.s.
Epigastric pain						
General	1/39 (2.6)	1/49 (2.0)	n.s.	0.13 ± 0.66	0.02 ± 0.14	n.s.
During daytime	7/39 (18)	6/43 (14)	n.s.	0.28 ± 0.79	0.19 ± 0.55	n.s.
At night/asleep	2/37 (5.4)	2/41 (4.8)	n.s.	0.14 ± 0.48	0.12 ± 0.56	n.s.
Heartburn	5/49 (10)	14/51 (27)	0.04	0.30 ± 0.71	0.37 ± 0.69	n.s.
Regurgitation	4/48 (8.3)	10/50 (20)	n.s.	0.20 ± 0.61	0.24 ± 0.52	n.s.
Abdominal rumbling	14/51 (27)	23/50 (46)	n.s.	0.84 ± 1.05	0.58 ± 0.70	n.s.
Bloating	17/52 (1.9)	36/53 (68)	0.04	1.32 ± 1.24	1.04 ± 0.91	n.s.
Empty feeling	7/48 (15)	5/50 (10)	n.s.	0.40 ± 0.79	0.16 ± 0.55	n.s.
Nausea	7/46 (15)	3/51 (5.9)	n.s.	0.20 ± 0.50	0.19 ± 0.71	n.s.
Vomiting	4/48 (8.3)	3/51 (5.9)	n.s.	0.06 ± 0.24	0.16 ± 0.70	n.s.
Loss of appetite	4/48 (8.3)	2/51 (3.9)	n.s.	0.23 ± 0.66	0.12 ± 0.71	n.s.
Postprandial fullness	8/46 (17)	20/53 (38)	0.03	0.80 ± 1.13	0.58 ± 0.95	n.s.
Belching	4/45 (8.9)	18/51 (35)	0.003	0.51 ± 0.76	0.41 ± 0.61	n.s.
Flatulence	6/43 (14)	30/51 (59)	0.01	1.07 ± 1.24	0.83 ± 0.79	n.s.
Hematemesis	1/44 (2.3)	1/50 (2.0)	n.s.	0	0.02 ± 0.14	n.s.
Dysphagia						
Liquid food	7/44 (16)	5/51 (9.8)	n.s.	0.39 ± 0.84	0.21 ± 0.63	n.s.
Solid food	6/46 (13)	6/51	n.s.	0.23 ± 0.67	0.23 ± 0.67	n.s.
Stools						
Melaena	1/37 (2.7)	0/41 (0)	n.s.	0	0	n.s.
Bloody	1/39 (2.6)	2/41 (4.5)	n.s.	0.05 ± 0.22	0.05 + 0.22	n.s.
Mucous	2/38 (5.2)	2/41 (4.5)	n.s.	0.15 ± 0.57	0.15 + 0.57	n.s.
Frequent hard	2/39 (5.1)	12/43 (28)	n.s.	0.34 ± 0.62	0.35 + 0.65	n.s.
Diarrhea	26/41 (63)	7/40 (18)	<0.001	0.93 ± 1.21	0.28 + 0.64	0.02
Alternately solid or loose	10/44 (23)	28/48 (58)	n.s.	0.95 ± 0.93	0.83 + 0.81	n.s.
Constipation	4/40 (10)	10/41 (24)	n.s.	0.35 ± 0.70	0.34 + 0.76	n.s.
Frequently with pain	1/39 (2.6)	6/40 (15)	n.s.	0.26 ± 0.59	0.25 + 0.74	n.s.
Stool urgency	23/42 (55)	8/41 (20)	0.0014	1.26 ± 1.50	0.29 + 0.68	<0.01
Incomplete	6/39 (15)	2/40 (5.0)	n.s.	0.38 ± 0.85	0.08 + 0.35	n.s.
Steatorrhea	2/38 (5.2)	1/40 (2.5)	n.s.	0.11 ± 0.39	0.03 + 0.16	n.s.

Data are given as mean ± 1SD. Groups were compared using Mann–Whitney rank sum test[a] and Fisher exact test[b]
Frequencies were compared using Mann–Whitney rank sum test
n.s. nonsignificantly

Table 3 Bowel pattern questionnaire in adult patients with Pompe disease and in age- and gender-matched controls (i.e. accompanying spouses and partners) as analysed by ICIQ-B-LF

	Pompe patients ($n = 57$)	Age- and gender-matched controls ($n = 57$)	p
Bowel pattern			
Frequency	6.2 ± 3.5	4.2 ± 2.4	<0.001
Interference	7.5 ± 9.0	2.5 ± 4.4	<0.001
Bowel control			
Frequency	5.6 ± 5.6	1.5 ± 2.0	<0.001
Interference	13.5 ± 16.2	1.9 ± 3.1	<0.001
Other bowel symptoms			
Frequency	4.4 ± 2.3	3.8 ± 1.4	n.s.
Interference	5.0 ± 7.1	1.3 ± 2.7	<0.01
Sexual impact			
Frequency	0.3 ± 1.1	0.02 ± 0.13	n.s.
Interference	0.4 ± 1.6	0.02 ± 0.13	n.s.
Quality of life			
Frequency	3.7 ± 4.3	1.5 ± 2.2	0.02
Interference	11.5 ± 15.4	1.9 ± 5.0	<0.001

Data are given as mean ± 1SD. Groups were compared using Mann–Whitney rank sum test
n.s. nonsignificantly

Table 4 Urinary incontinence in adult patients with Pompe disease and in age- and gender-matched controls (i.e. accompanying spouses and partners) as analysed by ICIQ-UI-SF

	Pompe patients ($n = 58$)	Age- and gender-matched controls ($n = 58$)	p
People reporting urinary incontinence [n (%)][b]	23/55 (42)	14/56 (25)	n.s.
Frequency of urinary incontinence[a]	0.98 ± 1.45	0.43 ± 0.93	n.s.
Quantity of urinary incontinence[a]	1.15 ± 1.58	0.57 ± 1.06	n.s.
Interference with daily life[a]	2.09 ± 3.15	0.48 ± 1.33	0.01
Interference with daily life in those patients reporting urinary incontinence[a]	5.00 ± 2.95	2.14 ± 2.07	0.004
Situations in which urinary incontinence occurs [n (%)][b,c]			
Never	32 (58)	37 (66)	n.s.
Before the toilet is reached	18 (33)	4 (7.1)	0.002
During coughing and pressure	3 (5.5)	15 (27)	0.0021
During sleeping	2 (3.6)	0	n.s.
During physical exertion	4 (7.3)	4 (7.1)	n.s.
After urinating and dressing	4 (7.3)	2 (3.6)	n.s.
Without explicable cause	3 (5.5)	2 (3.6)	n.s.
Permanently	0 (0)	0 (0)	n.s.

Data are given as mean ± 1SD. Groups were compared using Mann–Whitney rank sum test[a] and Fisher exact test[b]. [c] More than one answer possible
n.s. nonsignificantly

Discussion

This is the first multicenter cross-sectional study which systematically analysed symptoms of the upper and lower gastrointestinal tract as well as bowel and urinary incontinence using validated questionnaires in a large representative cohort of adult patients with Pompe disease.

The comparison with a population-based age- and gender-matched control group revealed that stool urgency and diarrhoea did occur more frequently in Pompe patients than in controls. These symptoms/signs were present in more than half of the Pompe patients. Twenty percent of the patients self-treated these symptoms/signs with loperamide indicating that they have an impact on the activities of daily living in Pompe patients. Stool urgency and diarrhoea were reported in equal frequencies in men and women. Other signs and symptoms of the upper and lower gastrointestinal tract did not occur more frequently in Pompe patients than in controls. Some symptoms of the GI tract (heartburn, bloating, postprandial fullness, flatulence) were reported statistically more significantly in controls than in patients. However, a statistically significant difference was not found, when we asked for the severity of these symptoms. The frequency of these symptoms in both our Pompe patient group and our control group is within the reported range (Bovenschen et al. 2006; Hollenz et al. 2002). Our controls were recruited in a slightly biased manner, but we think that they represent a sound population-based control group. The nonstandardised part of the "Gastrointestinal Symptoms Questionnaire" we had added to analyse the frequency of the symptoms should be omitted in future studies.

Urinary urge incontinence was present in one third of the Pompe patients, statistically more often in Pompe patients than in matched controls. Twenty-three percent of patients reported both symptoms of urinary and stool urge incontinence. This symptom was reported twice as frequently in women than men. Functional bladder tests in those Pompe patients reporting urinary urge incontinence are warranted.

Most of our patients had a disease course much longer than the duration of their individual period of ERT. Therefore, we do not know whether and how frequently urge symptoms occur as initial disease symptoms.

Bowel and urinary incontinence has probably been an under-reported symptom in Pompe patients. However, our study shows that these symptoms are physically disabling and have an important impact on the patients' daily life.

To avoid a recall bias, we have not asked for the onset of both urinary and bowel incontinence. Therefore we cannot judge how these symptoms are related and whether the symptoms responded to ERT. Forty-nine out of 57 of our Pompe patients were regularly treated with ERT. Thus, we cannot rule out that alglucosidase alfa might cause diarrhoea and stool urgency as a side effect. However, intravenously administered alglucosidase alfa (ERT) did not seem to be associated with any other gastrointestinal symptoms in the present study. Follow-up studies with ERT naïve patients are required in the future to clarify this issue. The frequent occurrence of both urinary and bowel incontinence suggests that these symptoms are due to smooth muscle dysfunction related to Pompe disease and not side effects of the ERT. Nevertheless, in most patients, urge symptoms persisted and did not seem to respond to ERT. An accumulation of glycogen in the smooth muscles of the lamina muscularis in all parts of the gastrointestinal tract but also in the urinary bladder has been frequently demonstrated in the mice models of glycogen storage disease type II and also in autopsies of patients with infantile and adult Pompe disease (Van der Walt et al. 1987; Bijvoet et al. 1999; Winkel et al. 2003; Kobayashi et al. 2010; Hobson-Webb et al. 2012). Therefore, evidence suggests that disturbed smooth muscles cause the symptoms of bowel and urinary incontinence. Gastrointestinal symptoms, such as diarrhoea and abdominal pain, are even more prevalent in Fabry disease (alpha-galactosidase A deficiency), another storage disorder, but were reported to be ameliorated by ERT (Keshav 2006; Hoffmann et al. 2007). Pathophysiologically, in contrast to Pompe disease, these symptoms may be caused by a combination of enteric neuropathy and a myopathy of the intestinal smooth muscle (Keshav 2006). Lipid accumulation in the smooth gastrointestinal muscles occurs in the lipid storage disorder Gaucher disease type 1. Gastrointestinal symptoms were reported to improve upon ERT (Verderese et al. 1993). Other myopathies with multisystemic involvement including gastrointestinal symptoms include myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2) due to a myopathy of the intestinal smooth muscles causing dysphagia, delayed gastric emptying, obstipation, and other gastrointestinal motility problems (Tieleman et al. 2008; Tanaka et al. 2013). In mitochondrial myopathies (another group of diseases with multisystemic affection), gastrointestinal symptoms have been less well studied, but it has been suggested that they are due to autonomous neuropathy (Chinnery et al. 2001; Pfeffer et al. 2011).

In conclusion, urinary and bowel incontinence are frequent symptoms in adults with Pompe disease and are often socially disabling.

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Synopsis

In adults with Pompe disease, urinary and bowel incontinence are frequent symptoms and are often socially disabling.

Compliance with Ethical Guidelines

Conflict of Interest

Nesrin Karabul, Cornelia Kornblum, Rudolf A. Kley, Eugen Mengel, Matthias Vorgerd, Marcus Deschauer, Benedikt Schoser, and Frank Hanisch have received lecturer honoraria and travel fees from Genzyme, a Sanofi company. Frank Hanisch has also received lecturer honoraria and travel fees from Astellas and Biomarin Incorp.

Anika Skudlarek, Janine Berndt, Stephan Wenninger, Nikolaus Tiling, and Ursula Plöckinger 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. Written informed consent was obtained from all patients included in the study.

Statement About the Contribution of Every Co-author

Nesrin Karabul – principal investigator, participated in the design of the study, recruited patients, contributed to data analysis, major participation in drafting the manuscript

Anika Skudlarek – major participation in collection and analysis of data, participation in drafting the manuscript

Janine Berndt – participation in statistical analysis

Cornelia Kornblum – recruited patients, participation in drafting the manuscript

Rudolf A. Kley – recruited patients, participation in drafting the manuscript

Stephan Wenninger – recruited patients

Nikolaus Tiling – recruited patients, participation in drafting the manuscript

Eugen Mengel – participation in drafting the manuscript

Ursula Plöckinger – participation in drafting the manuscript

Matthias Vorgerd – participation in drafting the manuscript

Marcus Deschauer – participation in drafting the manuscript

Benedikt Schoser – participation in drafting the manuscript

Frank Hanisch – principal investigator, designed the study, recruited patients, performed statistical analysis, major participation in drafting the manuscript

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CASE REPORT

A Rare Cause of Elevated Chitotriosidase Activity: Glycogen Storage Disease Type IV

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Abstract Human chitinolytic enzyme named "chitotriosidase" takes part in the defense mechanism against pathogens and the homeostasis of innate immunity. Chitotriosidase was firstly reported to be markedly high in plasma of patients with Gaucher disease. Abnormal lipid laden macrophages are thought to be responsible for stimulating the secretion of chitotriosidase in Gaucher disease. Subsequently, various disorders have also been found to be associated with elevated levels of chitotriosidase. Chronic liver diseases that are also related with macrophage activation may have elevated chitotriosidase activity. We report the second case of the literature with glycogen storage disease (GSD) type IV that presented with high chitotriosidase levels. GSD type IV should be taken into consideration in case of elevated chitotriosidase levels, stigmas of chronic liver disease, and inconsistency of lysosomal storage diseases.

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Competing interests: None declared

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Abbreviations

GSD	Glycogen storage disease
LSD	Lysosomal storage disease
NPD	Niemann-Pick disease
PAS	Periodic acid-Schiff

Introduction

Chitin is an abundant polysaccharide molecule that consists of homopolymers of β-1,4-linked *N*-acetylglucosamine units and appears in the cell walls of fungi, exoskeleton of arthropods, and structures of parasites (Eide et al. 2013). Chitinases, the enzymes that are responsible for hydrolyzing chitins, were previously identified in bacteria, fungi, insects, plants, and nematods. Humans were thought to be incapable of processing chitin due to absence of chitinases. However, in 1994, Hollak et al. reported the first discovered mammalian chitinase – chitotriosidase – that was found to be significantly elevated in the serum of patients with Gaucher disease (Hollak et al. 1994; Gorzelanny et al. 2010). Chitotriosidase is mainly produced, stored, and secreted by activated macrophages and neutrophils (Kanneganti et al. 2012). Primary indication for studying chitotriosidase activity is screening for lysosomal storage diseases (LSD), especially Gaucher and Niemann-Pick disease (NPD) A/B (Sheth et al. 2010).

Elevated chitotriosidase activities have also been reported in various diseases related with macrophage activation (Michelakakis et al. 2004; Kanneganti et al. 2012; Tumer et al. 2013).

Previously, a patient with glycogen storage disease (GSD) type IV was reported with high chitotriosidase activity (Michelakakis et al. 2004). Herein, we also present

a patient with elevated chitotriosidase activity who had been investigated for LSD and finally was diagnosed with GSD type IV.

Case Report

A 3.5-year-old girl presented to the emergency department with complaints of pallor, malaise, and abdominal distension. Abdominal distension had initially been recognized at 7 months of age. She was investigated for hepatosplenomegaly and anemia in another hospital 2 years ago. On admission to our hospital paleness, fatigue, doll's face appearance, telangiectasia on the face, tachycardia with cardiac murmur (II/VI), severe abdominal distention due to ascites, enlarged firm liver (6 cm below costal margin), and spleen (8 cm below costal margin) were determined. Neurologic examination and development stages were appropriate for her age. Initial laboratory evaluation revealed hemoglobin: 4.2 g/dL, white blood cell: 7.3×10^3/ μL platelet: 113×10^3/ μL, alanine aminotransferase (ALT): 124 U/L (<39), aspartate aminotransferase (AST): 381 U/L (<52), gamma-glutamyl transferase: 92 U/L (<23), alkaline phosphatase: 301 U/L (<269), direct bilirubin: 0.4 mg/dL, total bilirubin: 1.2 mg/dL, albumin: 4.3 g/dL, total protein: 6.8 g/dL, glucose: 200 mg/dL. International normalized ratio (INR) was 1.9 and was unresponsive to parenteral vitamin K. Hematological tests for hemolysis were normal and occult blood in stool was negative. Triglyceride, cholesterol, creatine kinase, alpha1 antitrypsin, ceruloplasmin levels, and metabolic investigations (tandem mass spectrometry and urine organic acid profile) were normal. Serology of hepatitis B, hepatitis C, and parvovirus PCR was negative. Abdominal ultrasonography showed hepatosplenomegaly and coarse echo pattern of the liver. She was also investigated for lysosomal storage disease. Despite elevation of plasma chitotriosidase (1,209 nmol/h/mL, reference range: 0–90 nmol/h/mL) and two foamy cells on bone marrow aspiration, enzyme levels in white blood cells for Gaucher disease [beta glucosidase: 206 (200–2,000 pmol/spot*20 h)] and NPD A/B [acid sphingomyelinase: 454 (200–3,500 pmol/spot*20 h)] were normal. Mutation analysis for NPD type C was negative. Echocardiography was normal.

Due to bleeding diathesis, a transjugular liver biopsy was performed. Diastase-resistant periodic acid-Schiff (PAS) positive-stained intracytoplasmic material in hepatocytes and extensive periportal and parenchymal fibrosis with nodule formation revealed the diagnosis of GSD type IV with micronodular cirrhosis. Appropriate size biopsy sample for enzymatic study could not be obtained due to liver stiffness. Finally, a homozygous mutation in the GBE1 gene [p.K521E (c.1561A>G)] confirmed GSD type IV.

Discussion

Under normal conditions, chitinases in human are presumed to take part in degrading the structure of chitin in pathogens as well as organizing the homeostasis of innate immunity. Besides this, chitotriosidase activity is also known to be directly associated with acute or chronic inflammatory conditions (Kanneganti et al. 2012). In Gaucher disease, storage of glucocerebroside in macrophages causes proinflammatory activation and finally elevation of chitotriosidase. Similar mechanism is also observed in NPD. Massive increased activity of chitotriosidase is a hallmark of Gaucher disease (10–1,000-fold), while other conditions have less elevated levels (Sheth et al. 2010; Kanneganti et al. 2012). Limited reports are available about chitotriosidase levels in other LSD (Michelakakis et al. 2004; Sheth et al. 2010). Sheth et al. (2010) indicated that 76.8% of the patients with LSD such as Gaucher disease, NPD type A/B, Morquio, mucopolysaccharidosis type VI, Tay-Sachs and Sandhoff diseases, metachromatic leukodystrophy, and GM2 gangliosidosis had statistically significant elevated levels of plasma chitotriosidase. NPD type C, Krabbe disease, GM1 gangliosidosis, Wolman and cholesterol ester storage disease, fucosidosis, and galactosialidosis were also reported to have elevated chitotriosidase levels (Michelakakis et al. 2004).

Some original articles and case reports have stated that various inherited or acquired conditions related with inflammation and macrophage activation may also be associated with high chitotriosidase levels. Fungal and granulomatous infections like tuberculosis and leishmaniasis, malaria, β-thalassemia, sarcoidosis, Wegener's granulomatosis, cerebral adrenoleukodystrophy, atherosclerosis, nonalcoholic liver disease, diabetes mellitus, multiple sclerosis, Alagille syndrome, GSD type I and IV, lung and prostate cancer are the diseases in the literature that have been related with elevated plasma chitotriosidase activity and inflammation (Hollak et al. 1994; Altarescu et al. 2002; Michelakakis et al. 2004; Malaguarnera 2006; Orchard et al. 2011; Kanneganti et al. 2012; Tumer et al. 2013).

In our case, NPD was thought to be a provisional diagnosis due to the following findings: hepatosplenomegaly, early onset chronic liver disease, a few foamy cells in bone marrow aspiration, and elevated chitotriosidase level. However, normal sphingomyelinase level and negative mutation analysis for NPD type C helped us to exclude NPD type A/B and C. The second provisional diagnosis was GSD type IV. Diastase-resistant PAS positive-stained intracytoplasmic material in hepatocytes with micronodular cirrhosis and homozygous mutation in the GBE1 gene [p.K521E (c.1561 A > G)] confirmed the exact diagnosis. Despite being a novel mutation, SIFT score (0, damaging), mutation taster score (disease causing, prob: 0.999985371153983), and PolyPhen2 score (0.998- probably damaging) supported its capability about disease formation.

This mutation is placed on a conserved area in different species and there have also been other reported mutations around the position of the mutation. For example, Arg515Cys and Arg524Gln mutations were referred to as disease-causing variations in HGMD-public (Human Genome Mutation Database). This finding strongly suggests that this variation is placed on a critical region for enzyme activity.

GSD type IV (Andersen disease) is characterized by deficiency of glycogen-branching enzyme (amylo-1, 4 to 1, 6-transglucosidase) that results in accumulation of unbranched and abnormal glycogen in the liver, heart, muscle, nervous system, and skin (Ozen 2007; Escobar et al. 2012). The most common form that is named "classical hepatic form" rapidly progresses to cirrhosis in the first 18 months of age and results in hepatosplenomegaly, end-stage liver disease, and finally death due to liver failure between 3 and 5 years of life. Besides histological examination of the liver, enzyme deficiency and the result of mutation analysis of GBE1 gene confirm the exact diagnosis (Ozen 2007).

Elevated chitotriosidase activity in GSD type IV was previously stated in only one study (Michelakakis et al. 2004). In two chronic liver diseases, nonalcoholic steatohepatitis and GSD type I, lipid accumulation, and peroxidation in hepatocytes trigger activation of resident macrophages of the liver (Kupffer cells). As a consequence, this activation induces proinflammatory cytokines and results in secretion of chitotriosidase (Malaguarnera 2006; Tumer et al. 2013). Besides this, Kupffer cells also activate hepatic stellate cells that synthesize several extracellular matrix components and also induce hepatic fibrosis and ultimately liver cirrhosis. In GSD type IV, similar mechanisms may act in the elevation of chitotriosidase and fibrosis (Malaguarnera 2006; Kanneganti et al. 2012).

In conclusion, the diagnosis of GSD type IV should be taken into consideration in patients with chronic liver disease and elevated chitotriosidase activity, once Gaucher disease and NPD have been ruled out.

Take Home Message

Elevation of chitotriosidase activity is not only determined in lysosomal storage diseases but also in other macrophage activation-related conditions such as glycogen storage diseases.

Compliance with Ethics Guidelines

Conflict of Interest

Hayriye Hizarcioglu-Gulsen, Aysel Yuce, Zuhal Akcoren, Burcu Berberoglu-Ates, Yusuf Aydemir, Erdal Sag, and Serdar Ceylaner declare that they have no conflict of interest.

Informed Consent

An informed consent was obtained from the parents of the patient.

Animal Rights

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

Details of the Contributions of Individual Authors

Hayriye Hizarcioglu-Gulsen is the corresponding author. The draft of the manuscript was prepared and written by her and she is the guarantor for this report.

Aysel Yuce also participated in designation of the case report and revised it critically.

Zuhal Akcoren was responsible for histological assessment.

Burcu Berberoglu-Ates, Yusuf Aydemir, and Erdal Sag were responsible for clinical follow-up of the patient and they also participated in the drafting the manuscript.

Serdar Ceylaner performed the genetic analysis of the GBE1 gene.

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RESEARCH REPORT

Cirrhosis Associated with Pyridoxal 5′-Phosphate Treatment of Pyridoxamine 5′-Phosphate Oxidase Deficiency

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Abstract We report the case of an 8-year-old boy with pyridoxamine 5′-phosphate oxidase (PNPO) deficiency. He developed seizures at 24 h of age that were refractory to standard anticonvulsant therapy and a trial of pyridoxine but responded to pyridoxal phosphate (PLP) at 28 days of life. Genetic testing identified compound heterozygous mutations in the PNPO gene. Management of encephalopathic episodes required escalation of PLP dose to 100 mg/kg/day by 2 years of age. Routine blood tests at this time showed significantly deranged liver function tests (LFTs). A wedge liver biopsy showed early cirrhosis with marked elevation of pyridoxal and pyridoxic acid levels in the liver sample. Despite extensive investigation, no cause other than PLP therapy could be identified for the cirrhosis. The PLP dose was weaned to 50 mg/kg/day before episodes of encephalopathy recurred. Concurrent with the reduction of his PLP dose, LFTs showed improvement. However, at 8 years of age, there is persistent evidence of hepatic fibrosis and early portal hypertension. We hypothesise that hepatic toxicity due to PLP or its degradation products is the cause of cirrhosis in this boy. Until further evidence becomes available, we would suggest that people with PNPO deficiency are treated with the minimum dose of PLP required to prevent episodes of encephalopathy.

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Background

Pyridoxal 5′-phosphate (PLP) is the active member of the pyridoxine vitamer family. The first case of neonatal epilepsy responsive to PLP but not pyridoxine was reported in 2002 (Kuo and Wang 2002). Mills and colleagues subsequently described the genotype and phenotype of neonatal epileptic encephalopathy due to pyridoxamine 5′-phosphate oxidase (PNPO) deficiency (Mills et al. 2005). The treatment dose of PLP has largely been empirical, guided by clinical response with reported doses varying from 30 to 60 mg/kg/day (Mills et al. 2005; Hoffmann et al. 2007; Bagci et al. 2008). PLP is not licensed as a drug for the treatment of epilepsy outside of Asia.

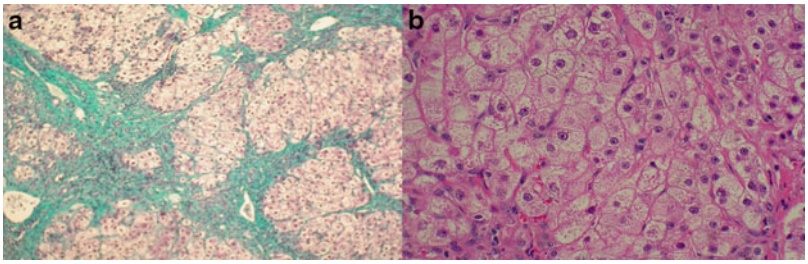

Fig. 1 Liver biopsy specimens (2006) age 2 years and 2 months. (**a**) Wedge liver biopsy (2006): *Masson's trichrome stain* shows marked disruption of architecture with extensive fibrosis, portal to portal and central bridging with focal nodules of hepatocyte surrounded by fibrous tissue consistent with early cirrhosis. (**b**) Wedge liver biopsy: *Haematoxylin and eosin stain* shows swollen hepatocytes with ballooning degenerative changes in the cytoplasm which have a granular appearance. Stains for glycogen and fat were negative

Case

We report an eight-year-old boy with PNPO deficiency who developed cirrhosis while being treated with high-dose PLP.

The initial clinical details have been previously reported (Hoffmann et al. 2007; Schmitt et al. 2010). In summary, this boy was born at term and developed seizures at 24 h of age. Despite multiple anticonvulsant medications and a trial of pyridoxine, his seizures proved refractory. At 28 days he was trialled on PLP 50 mg four times a day. His seizures ceased immediately. All antiepileptic medications were successfully withdrawn. He was maintained on PLP and made good developmental progress. His liver function tests (LFTs) were normal at 1 month of age.

A trial of withdrawal of PLP at 7 months resulted in encephalopathy and EEG changes within 7 h which resolved within 20 min of administration of 200 mg of PLP. Genetic studies revealed he was compound heterozygous for a missense mutation, D33V (c.98A>T), and a single base pair deletion, c.246delT, in the PNPO gene.

Over the following 18 months, there were recurrent episodes of PLP-responsive encephalopathy, especially during periods of illness, requiring escalation of the dose of PLP to 1,500 mg/day (~100 mg/kg/day) given in four divided doses. Routine LFTs at 2 years of age were markedly elevated aspartate transaminase (AST): 292 U/L (normal: 10–50 U/L), alanine transaminase (ALT): 169 U/L (normal: 0–45 U/L), gamma-glutamyl transaminase (GGT): 131 U/L (normal: 0–45 U/L) and alpha fetoprotein (AFP): 635 IU/mL (normal: 0–6 IU/mL). The clotting profile was mildly deranged – prothrombin time (PT) 19 s (normal: 11–15 s). There was palpable hepatosplenomegaly (confirmed on ultrasound). The trough whole blood PLP level (6 h post-dose) measured using high-performance liquid chromatography (HPLC) was markedly elevated – 6,090 nmol/L (normal: 35–110 nmol/L).

A wedge liver biopsy (age 2 years 2 months) showed extensive fibrosis and focal nodules of hepatocytes surrounded by fibrous tissue consistent with early cirrhosis (Fig. 1). All investigations for viral and metabolic causes of cirrhosis were negative. Respiratory chain enzyme analyses in the muscle and liver were normal.

Assays of hepatic levels of B6 vitamers were performed on the liver samples and samples of two control patients. Liver samples were weighed, immediately homogenised with 25% trichloroacetic acid and centrifuged and the supernatant analysed by HPLC/fluorescence to measure non-phosphorylated forms of the B6 vitamers. Because this process was rapid, we believe that there was no significant hydrolysis of phosphorylated vitamers. Levels of pyridoxal and pyridoxic acid in the liver were approximately 40 times greater than two control specimens (Table 1). We feel it is likely that this technique measured non-phosphorylated forms of the vitamers and pyridoxic acid.

We hypothesised that high doses of PLP accounted for his liver dysfunction and weaned the dose of PLP to ~50 mg/kg/day. Dose frequency was changed from 4 to 6 hourly to smaller 3 hourly doses in the hope that lower peak levels might protect against hepatotoxicity. Vitamin C was added to help with potential oxidative stress. His LFTs improved substantially but did not entirely normalise (Fig. 2).

Managing him at this dose resulted in heightened susceptibility to seizures or encephalopathic episodes. The maintenance dose has since been increased to 60 mg/kg/day. His parents give an extra 400 mg orally for episodes of encephalopathy, and a rectal dose of PLP (400 mg) is used if the episode does not respond to oral PLP (only used on five occasions during his life).

Increases in the dose of PLP beyond 60 m/kg/day have been associated with transient increases in his transaminase levels above his baseline level (Fig. 2). He has persistent mild hepatosplenomegaly, with evidence of hepatic fibrosis and mild portal hypertension.

Currently he has infrequent episodes of encephalopathy usually triggered by missing a dose of PLP or with illness. At the age of 8 years, he is an academically gifted student in a mainstream school.

Table 1 Levels of pyridoxine and its metabolites (non-phosphorylated forms) in the liver tissue of two controls and our patient

	Control liver 1 (μg/g)	Control liver 2 (μg/g)	Patient (μg/g)
Pyridoxamine	0.1	0.3	0.4
Pyridoxine	0.2	0.3	0.8
Pyridoxal	0.03	0.02	1.3
Pyridoxic acid	0.02	0.02	1.0

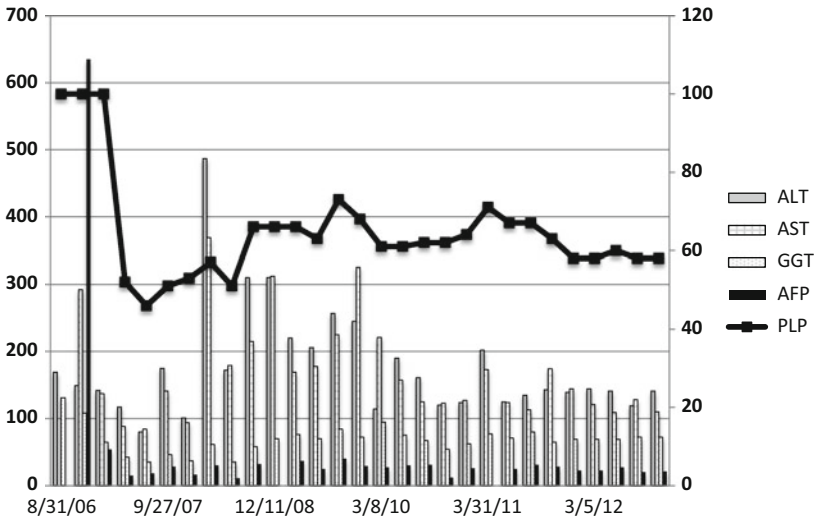

Fig. 2 Serial levels of liver enzyme (*left axis*) and dose of PLP (*right axis*). AST (U/L) – normal range 10–50 U/L, ALT (U/L) – normal range 0–45 U/L, GGT (U/L) – normal range 0–45 U/L, AFP (U/L) – normal range 0–6 IU/mL, PLP – PLP dose (mg/kg/day)

Discussion

Vitamin B6 consists of three related pyridine derivatives: pyridoxine, pyridoxamine and pyridoxal and their 5′-phosphate esters. PLP is the active form of vitamin B6. After passive intestinal absorption, vitamin B6 is converted to the phosphorylated derivatives in the liver. Pyridoxine-5′-phosphate (PNP) and pyridoxamine-5′-phosphate (PMP) are oxidised to PLP by PNPO. PLP re-enters the circulation bound to albumin. Delivery of active cofactor to the tissues requires hydrolysis of circulating PLP to pyridoxal. Pyridoxal crosses the blood–brain barrier (and enters other tissues) but then needs to be re-phosphorylated to produce the active cofactor (Clayton 2006).

Although most PLP-catalysed reactions regenerate PLP, some enzymes can generate PMP. Thus PNPO plays a role as a metabolite repair enzyme recycling PLP. The turnover of PLP enzymes may also generate PMP. When on treatment with B6, patients with PNPO deficiency have higher levels of pyridoxamine and PMP than patients with antiquitin deficiency (Footitt et al. 2013). The role of PNPO in recycling and trafficking PLP probably explains why PNPO deficiency is much more difficult to treat than antiquitin deficiency.

Compared with other patients with PNPO deficiency reported in the literature, this boy has had a very good neurological outcome. We hypothesise that this results from early introduction of PLP, prompt management of encephalopathy and perhaps the biochemical effects of his mutation. His management entailed the use of very high doses of PLP (to our knowledge the highest dose/weight reported).

There has been only one previous reported case of liver toxicity secondary to high-dose PLP (Yoshida et al. 1985) in a 7-year-old boy with homocystinuria who developed hepatitis with elevated LFTs within 4 days of an increase in his dose of PLP to 1,000 mg/day. Another study reported transient elevation of LFTs in 14/28 patients with infantile spasms treated with PLP (dose range 30–50 mg/kg/day (Takuma and Seki 1996)). The deranged LFTs returned to normal within 2 weeks of reduction or withdrawal of PLP.

In our case, liver dysfunction was identified after the child had been on high doses of PLP for almost 2 years. The improvement in LFTs on reducing the dose of PLP suggests a dose-related response; however, the persistent

abnormalities of liver function suggest that there is ongoing hepatic inflammation.

The cause of this toxicity is not known. It is possible that degradation products of PLP in solution contributed to this boy's liver toxicity. Initially PLP was given as a powder dissolved in milk/yoghurt/water and left in solution before being administered. The solution showed colour change raising concerns about the chemical stability of the preparation. In aqueous solution, PLP is vulnerable to degradation induced by exposure to light and oxygen. In aerobic conditions, the principal breakdown (oxidation) products include 4-pyridoxic acid-5′-phosphate and the dipyridyl α-diketone. In anaerobic conditions, the acyloin is produced (Morrison and Long 1958; Wei et al. 1972; Bazhulina et al. 1974).

In view of the above, we recommend that PLP be consumed as a whole capsule where possible and that any suspension or solution is taken immediately. Until further evidence becomes available, we suggest that the dose of PLP is carefully titrated against symptoms with close monitoring of liver function especially when doses greater than 50 mg/kg/day are used.

Synopsis

Treatment for PNPO deficiency.

Compliance with Ethics Guidelines

Annapurna Sudarsanam, Harry Singh, Bridget Wilcken, Michael Stormon, Susan Arbuckle, Bernhard Schmitt, Peter Clayton, John Earl and Richard Webster declare that they have no conflict of interest.

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Informed Consent

Informed consent has been obtained from the patient and his family for the publication of this paper.

Details of Contributions of Individual Authors

Annapurna Sudarsanam, Harry Singh and Richard Webster were involved with drafting the article. Richard Webster, Harry Singh, Bridget Wilcken, Michael Stormon, Susan Arbuckle, Bernhard Schmitt, Peter Clayton and John Earl were all involved with the management of the case and critically appraised and revised the article.

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CASE REPORT

Hypertrophic Cardiomyopathy in Pompe Disease Is Not Limited to the Classic Infantile-Onset Phenotype

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Abstract Pompe disease is a genetic disorder caused by a deficiency of acid α-glucosidase (GAA). Patients with classic infantile-onset Pompe disease usually present with hypertrophic cardiomyopathy and die before 1 year of age, if not treated with enzyme replacement therapy (ERT). In comparison, patients with late-onset Pompe disease typically do not have hypertrophic cardiomyopathy. However, here we describe five patients who presented with hypertrophic cardiomyopathy but did not fit the criteria of classic infantile-onset Pompe disease. Their ages at diagnosis of cardiomyopathy were 1 month in two patients following detection of an audible cardiac murmur and 2–3 years in the three remaining patients. All patients survived for 5–8 years without ERT. Three patients died before the advent of ERT from causes other than congestive heart failure. One patient had a good response to ERT starting at 5 years of age. The sibling of one patient, who did not receive ERT and died at age seven, was diagnosed prenatally. At 3 months of age, the sibling had hypertrophic cardiomyopathy, and a muscle biopsy at that time revealed glycogen accumulation.

This case series demonstrates that Pompe disease is a continuum of disease, and the development of cardiomyopathy is not limited to classic infantile-onset Pompe disease. These patients do not fit into the discrete phenotypes of infantile- or late-onset Pompe disease, which may suggest reconsidering the nomenclature of Pompe disease.

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Introduction

Pompe disease is a lysosomal storage disorder in which a deficiency of acid α-glucosidase (GAA) causes lysosomal glycogen accumulation in multiple tissues and cell types, most notably cardiac, skeletal, and smooth muscle cells (Hirschhorn and Reuser 2001). Pompe disease has been classified into two phenotypes: classic infantile onset (symptoms develop before 1 year of age with hypertrophic cardiomyopathy) and late onset (symptoms develop at or after 1 year of age without hypertrophic cardiomyopathy) (Kishnani et al. 2006b). About 75% of patients with classic infantile-onset Pompe disease die before 12 months of age with a median age at death of 8.7 months (Kishnani et al. 2006a). Patients with the heterogeneous and more slowly progressive late-onset Pompe disease, which includes childhood-, juvenile-, and adult-onset subgroups (Kishnani et al. 2012), typically present with muscle weakness and respiratory failure but no cardiac manifestations (Hirschhorn and Reuser 2001; Kroos et al. 2007). However, Pompe disease is a continuum of disease with variable age of onset, organ involvement, and degree of myopathy, and therefore, not all patients can be categorized according to the aforementioned classification.

Enzyme replacement therapy (ERT) with recombinant human alglucosidase alfa (Myozyme®, Genzyme, Cambridge, MA) is the only approved therapy for Pompe disease. ERT prolonged overall survival and ventilator-free

survival and reversed cardiomegaly in infantile-onset Pompe disease (Kishnani et al. 2007). Motor function was maintained when infants with classic infantile-onset Pompe disease were diagnosed through newborn screening and received very early treatment with ERT (Chien et al. 2008; Chien et al. 2009). Primarily based on data from juvenile- and adult-onset patients, ERT for late-onset Pompe disease has been associated with improved motor capability and stabilized pulmonary function (Winkel et al. 2004; Case et al. 2008; van Capelle et al. 2008; Strothotte et al. 2010; van der Ploeg et al. 2010). There is limited data about the effect of ERT in children with Pompe disease (Strothotte et al. 2010; van der Ploeg et al. 2010), who present with symptoms far earlier than juvenile-onset Pompe patients. In these children early symptom presentation is possibly associated with a poor treatment response.

In children, Pompe disease may include at least 3 groups of patients who survive more than one year: (1) nonclassic infantile-onset Pompe disease with cardiomyopathy (Winkel et al. 2005), (2) nonclassic infantile-onset Pompe disease without cardiomyopathy (Spiridigliozzi et al. 2012), and (3) juvenile-onset Pompe disease with delayed motor developmental milestones during childhood. In a review of 225 published cases, survival for nonclassic infantile-onset Pompe disease with cardiomyopathy was usually only 2 to 3 years without ERT (Winkel et al. 2005). However, we observed a group of patients who presented with cardiomyopathy and survived well beyond 1 year without ERT. This finding significantly broadens our understanding about childhood-onset Pompe disease and demonstrates a need to monitor the cardiac status of these children.

Case Presentations

During the first Asia-Pacific Pompe Disease Expert Meeting, held in Taipei in June 2013, cases of infants and young children with an atypical clinical course were noted. Subsequently, we performed a survey to further understand the characteristics of this group of patients. Criteria for enrollment were (1) disease onset during late infancy or early childhood; (2) presence of hypertrophic cardiomyopathy, as defined by a thickened left ventricular wall or interventricular septum; and (3) survival without ERT for a few years. In this case series, we describe five such patients and one sibling who was diagnosed prenatally. The characteristics of these patients are described in Table 1.

Patient 1

Patient 1 is a 13-year-old male who displayed poor feeding and decreased activity in the first month after birth (Jeon et al. 2007). He had difficulty with standing at 2 years of age. Upon physical examination for a common cold, at the age of 2 years and 6 months, a cardiac murmur and cardiomegaly were discovered. He was diagnosed with Pompe disease at age 4 based on low GAA activity and the presence of two *GAA* gene mutations [c.875A>G (p.Tyr292Cys) and c.1857C>G (p.Ser619Arg)]. At the time of diagnosis, he showed Gowers' sign and waddling gait. His serum creatinine kinase (CK) level was 1,235 U/L (normal < 165 U/L). A chest X-ray revealed cardiomegaly (Fig. 1a), and an echocardiogram revealed hypertrophic cardiomyopathy. The interventricular septal diastolic dimension was 24.8 mm (normal 3.3–6.3 mm). Left ventricular mass index (LVMI) was 484 gm/m^2 (normal <65 g/m^2). Alglucosidase alfa, 20 mg/kg qow, was started at 5 years of age. After 10 weeks of ERT, he could walk downstairs without using the handrail. He could walk for 1 h after 40 weeks of ERT. Currently, he has been treated for 8 years.

Patients 2A and 2B

Patient 2B is a 2.5-year-old male who was diagnosed with Pompe disease through prenatal screening. His elder sister (patient 2A) was noted to have elevated liver enzymes since birth. Elevated CK levels and mild cardiomegaly were documented in patient 2A when she was 2 years old. Patient 2A was prone to falls from the age of 5 years, and she died suddenly in her sleep at age 7. At that time, she had dysarthria (she was hardly understood by her teachers) and walked with support with a myopathic gait. Consequently, the mother received prenatal testing during her pregnancy with patient 2B, which confirmed the presence of two *GAA* gene mutations [c.872T>C (p.Leu291Pro) and c.1798C>T (p.Arg600Cys)] in the fetus. At birth, a chest X-ray revealed that patient 2B had normal cardiac size. However, at 3 months of age, patient 2B presented with hypotonia, absence of head control, and macroglossia. His CK level was 744 U/L (normal <200 U/L). A chest X-ray revealed cardiomegaly (Fig. 1b), and echocardiography revealed hypertrophic cardiomyopathy. LVMI was 126 g/m^2 (normal <65 g/m^2). ERT was initiated immediately. After 30 months of ERT, his cardiac size decreased, and improvements in motor function were observed across all motor scales.

Patients 3A and 3B

Patient 3A is a 15-year-old female who was noted to have a cardiac murmur at 1 month of age (Kim et al. 2009). Echocardiography revealed hypertrophic cardiomyopathy. She started to walk at 15 months of age with her right leg lagging. A follow-up echocardiography revealed asymmetric septal hypertrophy and systolic anterior motion of the

Table 1 Characteristics of patients with Pompe disease

No.	Sex	Age at detection of cardiomegaly	Age at onset of weakness	Creatine kinase level (U/L)	Age at start of ERT	Status at start of ERT	Current age (age died)	Current status (at death)	Mutations
1	M	2 years 6 months	2 years	1,235	5 years	Walk with support	13 years	Walk freely	c.875A > G/ c.1857C > G
2A	F	2 years	5 years	Elevated	–	–	(7 years)	(Walk with support)	c.872T > C/ c.1798C > T*
2B	M	3 months	–	744	3 months	Normal	2.5 years	Walk freely	c.872T > C/ c.1798C > T*
3A	F	1 month	1 year 3 months	741	8 years	Walk with support	15 years	Unable to walk	c.2407_13del/ c.1316T > A*
3B	M	1 month	5 years	562	–	–	(6 years)	(Walk with support)	c.2407_13del/ c.1316T > A*
4	F	3 years	3 years	104	–	–	(7 years)	(Unable to walk)	c.1082C > T*/ c.1432G > A*

*reported mutation

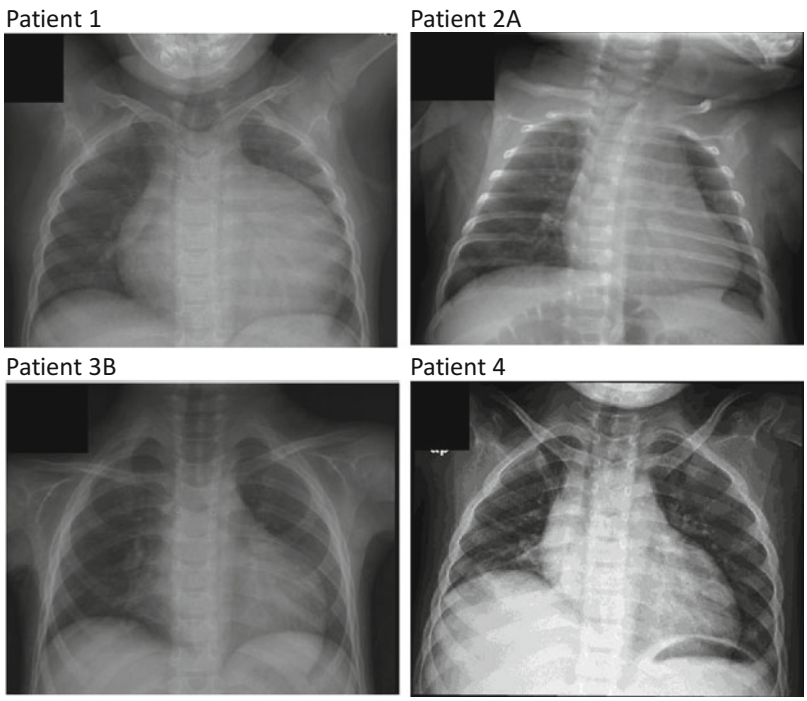

Fig. 1 Chest X-ray pictures of four Pompe patients revealed cardiomegaly at ages: (**a**) 4 years (patient A), (**b**) 3 months (patient 2A), (**c**) 1 year and 3 months (patient 3B), and (**d**) 3 years (patient 4)

mitral valve. LVMI was 94 g/m^2 (normal <65 g/m^2). Pompe disease was confirmed at 8 years of age based on low GAA activity and the presence of two *GAA* gene mutations [c.2407_13del and c.1316T>A (p.Met439Lys)]. Her CK level was 741 U/L (normal <165 U/L). At diagnosis, she walked with difficulty due to continued lagging of the right leg, and she could not run. Patient 3A has been receiving ERT since 8 years of age. She is wheelchair bound.

Patient 3B is patient 3A's younger brother who was found to have Pompe disease at 5 years of age after the diagnosis of his older sister. Although a cardiac murmur was heard at 1 month of age, and CK elevation and hypertrophic cardiomyopathy were noted at 15 months of age (Fig. 1c), LVMI was 142.2 g/m^2 (normal <65 g/m^2). At the time of diagnosis of Pompe disease, he showed Gowers' sign, waddling gait, and CK elevation (562 U/L, normal <165 U/L). He died at 6 years of age after an infection.

Patient 4

Patient 4 is a female who started to walk at 15 months of age, but fell easily starting at 3 years of age. At that time, chest X-ray revealed cardiomegaly (Fig. 1d), and echocardiogram and magnetic resonance imaging demonstrated left ventricular hypertrophy with left ventricular outflow tract obstruction. LVMI was 188 g/m^2 (normal <95 g/m^2). Her CK level was 104 U/L (normal < 170 U/L), and she had two *GAA* gene mutations [c.1082C>T (p.Pro361Leu) and c.1432G>A (p.Gly478Arg)]. She was able to walk 100 m at 5 years of age but could walk only 30 m at 6 years of age with coarse breath sounds, bilateral wheezing, and decreased oxygen saturation (80%) in room air. She was unable to walk at 7 years of age and died suddenly at age 7 years and 3 months.

Discussion

In this report, we describe a group of children with Pompe disease who presented with cardiomyopathy. Our findings support the concept that, in addition to classic infantile-onset Pompe disease, older children with Pompe disease can also present with cardiomyopathy. These patients do not fit into the discrete phenotypes of infantile- or late-onset Pompe disease, but may be called nonclassic, nontypical, or atypical infantile-onset Pompe disease.

The presence of cardiomyopathy in older children with Pompe disease may not necessarily indicate a poor prognosis similar to classic infantile-onset Pompe disease. In a review of nonclassic Pompe disease, the mean age of death in 32 patients with symptom onset at 0–1 year was 6.1 (0.9–24) years, and 12 of the patients presented with hypertrophic cardiomyopathy (Winkel et al. 2005). Although the cardiomyopathy in our patients occurred as early as 1 month of age, no patients died of congestive heart failure. However, arrhythmias are still a risk and could have contributed to the acute death of patients 2A and 4.

The current nomenclature used to describe individual patients with Pompe disease describes discreet phenotypes like infantile- or late-onset Pompe disease. These phenotypic designations are oversimplified since patients with infantile-onset Pompe disease can either present with cardiomyopathy before 6 months of age and die before 1 year of age (classic infantile-onset Pompe disease) or present with only weakness after 6 months of age and survive well beyond 1 year of age (nonclassic infantile-onset Pompe disease). The nomenclature for nonclassic infantile-onset Pompe disease is also variable. Slonim et al. defined "nontypical" infantile-onset Pompe disease as those with less severe cardiomyopathy and longer survival of 1–2 years (Slonim et al. 2000). Others have used "atypical infantile-onset Pompe disease" to characterize patients who have symptom onset before 1 year of age but with no cardiomyopathy (Bembi et al. 2008; Kishnani et al. 2012). Gungor and Reuser suggested using the term "childhood" Pompe disease to cover the gap between "classic infantile" and "adult" Pompe disease (Gungor and Reuser 2013).

In conclusion, cardiomyopathy cannot be used as a single criterion for the classification of Pompe disease in infants or children. For prospective follow-up of asymptomatic patients with Pompe disease, like those discovered by newborn screening, cardiomegaly needs to be monitored even after 1 year of age.

Take-Home Message

Hypertrophic cardiomyopathy may present in childhood in patients with Pompe disease.

Compliance with Ethics Guidelines

Conflict of Interest

Yin-Hsiu Chien has received research grants from Genzyme, a Sanofi company.

Wuh-Liang Hwu has received research grants and traveling funds from Genzyme, a Sanofi company.

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Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained as part of the Pompe Registry study in Soonchunhyang University Hospital and National Taiwan University Hospital. Informed consent was waived for retrospective medical information with no identifying information about patients from Xinhua Hospital included in the article.

Author Contribution to the Manuscript

Dong-Hwan Lee and Wuh-Liang Hwu planned and conducted this research, and Wen-Juan Qiu, Jeongho Lee, and Yin-Hsiu Chien contributed to the cases.

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CASE REPORT

Clinical, Biochemical, and Molecular Presentation in a Patient with the *cblD*-Homocystinuria Inborn Error of Cobalamin Metabolism

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Abstract Disorders of intracellular cobalamin (vitamin B_{12}) metabolism result from deficient synthesis of the coenzymes derived from vitamin B_{12}: adenosylcobalamin and methylcobalamin. Disturbances of cobalamin-cofactor synthesis result in elevated levels of homocysteine and/or methylmalonic acid. Nine defects of intracellular cobalamin metabolism have been defined. The most common of these disorders is *cblC* (combined methylmalonic aciduria and homocystinuria). The *cblD* disorder is rare with fewer than twenty cases reported in the literature. Some *cblD* patients have combined methylmalonic aciduria and homocystinuria (referred to as "*cblD* original," "*cblD*-combined," or herein "*cblD*-MMA/HC"); some have isolated homocystinuria (referred to as "*cblD*-variant 1" or herein "*cblD*-HC"); and others have isolated methylmalonic aciduria (called "*cblD*-variant 2" or herein "*cblD*-MMA"). Only six cases of *cblD*-HC have been defined thus far. We report the 7th case of *cblD*-HC. The clinical manifestations, biochemical profile, genetic mutation, and plausible ancestry are discussed.

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Introduction

Disorders of intracellular cobalamin (vitamin B_{12}) metabolism result from deficient synthesis of two coenzymes derived from vitamin B_{12}, namely, adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl). AdoCbl is required for activity of the mitochondrial enzyme methylmalonyl-CoA mutase, which catalyzes the conversion of methylmalonyl-CoA (generated during catabolism of branched-chain amino acids, odd-chain fatty acids, and cholesterol) to succinyl-CoA which enters the Krebs cycle. MeCbl is needed for activity of the cytoplasmic enzyme methionine synthase, which catalyzes the methylation of homocysteine to form methionine (Watkins and Rosenblatt 2011).

Inborn errors of cobalamin-cofactor synthesis are a group of rare disorders affecting multiple steps between the lysosomal release of cobalamin and the subsequent generation of mitochondrial AdoCbl and cytosolic MeCbl. Disturbances of cobalamin-cofactor synthesis result in elevated levels of homocysteine and/or methylmalonic acid, depending on which step is affected in the pathway. To date, nine distinct defects have been identified using *in vitro* somatic complementation and molecular genetic analysis. The disorders have been designated *cblA, cblB, cblC, cblD, cblE, cblF, cblG, cblJ,* and *mut* (Watkins and Rosenblatt 2011; Coelho et al. 2012; Yu et al. 2013). The clinical manifestations can be highly variable even within a single complementation group and can span the prenatal period through adolescence and adulthood. The clinical phenotype

is believed to be influenced by the severity and location in the pathway of the defect. Clinical manifestations include intrauterine growth retardation, microcephaly, congenital heart disease, feeding difficulty, megaloblastic anemia/cytopenia, global developmental delay, hypotonia, seizures, neuropsychiatric symptoms, and thromboembolic complications. The prototype and best understood is *cblC*, which is also the most common of these disorders. The *cblC* disorder causes combined methylmalonic aciduria and homocystinuria.

The *cblD* disorder has been among the rarest of the disorders of intracellular cobalamin metabolism. Some *cblD* patients have combined methylmalonic aciduria and homocystinuria (referred to as "*cblD* original," "*cblD*-combined," or herein "*cblD*-MMA/HC"); some have isolated homocystinuria (referred to as "*cblD*-variant 1" or herein "*cblD*-HC"); and others have isolated methylmalonic aciduria (called "*cblD*-variant 2" or herein "*cblD*-MMA") (Suormala et al. 2004). Goodman et al. (1970) described the first *cblD* disorder cases in a sibship in 1970 with combined methylmalonic aciduria and homocystinuria. No additional patients with the *cblD* disorder were reported until 2004 when Suormala et al. (2004) described two patients with *cblD*-HC and one patient with *cblD*-MMA using complementation analysis. To date, eighteen *cblD* patients have been reported; including five patients with the classic form of the disorder, six with *cblD*-HC, and seven with *cblD*-MMA (Coelho et al. 2008; Miousse et al. 2009; Parini et al. 2013; Stucki et al. 2011). Mutations at the *MMADHC* gene have been shown to underlie the *cblD* disorder in all of these patients. This manuscript describes the seventh patient to be reported with the *cblD*-HC disorder. The clinical presentation, biochemical profile, and genetic mutation that underlie the *cblD*-HC disease in this patient are also discussed.

Case Presentation

Clinical Presentation

The patient is a girl of East Indian descent who came to our attention at the age of 5 years. The parents are first cousins and in good health. Her only sibling, an 11-year-old sister, was also in good health. The patient was born at term in India following a pregnancy that was significant for hypertension and hyperemesis. The birth weight was 3.25 kg. Other measurements at birth were not available. Her neonatal course was unremarkable.

Beyond the neonatal period, the patient was generally well with the exception of hypotonia and microcephaly (according to the parents). Reportedly, by 18 months of age, she was deemed to have global developmental delay. She did not achieve good head control until 18 months of age. She sat independently by 2 years. She walked by 2 1/2–3 years. By 5 years of age, the time of immigration of the family to Canada, she was still unable to pick up finger foods with a pincer grasp. She also had profound delay in her expressive and receptive speech and language skills (at 15-month level). Additionally, she displayed multiple repetitive/stereotypical mannerisms. She was noted to have significant deficits in her social and emotional reciprocity. She lacked ability to spontaneously seek shared enjoyment (joint attention). She could not maintain eye-to-eye gaze. She was unable to maintain any peer relationships as she preferred solitary play. She had profound difficulty with transitioning as she would throw a temper tantrum when her routine was broken. Hence, she was deemed to be on the autistic disorder spectrum.

Biochemical and Radiological Parameters

While in India (at 18 months of age), her serum homocysteine (total) was elevated at 71 μmol/L (upper limit of reference range 10 μmol/L). Methionine was decreased at 1 μmol/L (reference range 11–16 μmol/L). Urine organic acids were not available. Hematological parameters were also not available. A brain MRI at 15 months of age revealed frontotemporal and corpus callosum atrophy. Hypomyelination in peripheral white matter of bilateral cerebral hemispheres was also noted. The electroencephalogram was normal. She was diagnosed with an inborn error of cobalamin metabolism. She was commenced on vitamin B_{12} injections (the exact type, dose, and frequency were not known to us). Following 3 months of treatment, her serum homocysteine dropped to 32 μmol/L.

At the time of referral to our center (at 5-years of age), serum homocysteine was elevated at 45 μmol/L, methionine was elevated at 33 μmol/L, and methylmalonic acid was within the reference range. The acylcarnitine profile was unremarkable. There was no evidence of megaloblastic anemia or cytopenia. Her white blood cells (WBC) were 7.9×10^9/L (normal $4–10 \times 10^9$/L), hemoglobin (Hgb) was 121 g/L (normal 120–160 g/L), mean corpuscular volume (MCV) was 81.4 fL (normal 77–96 fL), hematocrit (Hct) was 0.357 (normal 0.36–0.48), and platelets (Plt) were 309×10^9/L (normal $150–400 \times 10^9$/L). The brain MRI was normal. She was commenced on daily hydroxycobalamin 1 mg intramuscular injections, betaine, and folic acid. Homocysteine dropped to 18 μmol/L. Hydroxycobalamin injections were, however, discontinued after 3 months due to significant administration difficulties encountered by the parents due to her difficult behavior. She remained on betaine and folic acid. While off treatment, her homocysteine levels have varied between 25 and 40 μmol/L, and her hematological profile remained

essentially unchanged (WBC 12 × 10^9/L, Hgb 121 g/L, MCV 81.1 fL, Hct 0.37, and Plt 384 × 10^9/L). Recently, hydroxocobalamin injections were restarted weekly at a dose of 5 mg. There have been progressive gains in her development with the support of multidisciplinary input. Currently, at 10 years of age, she is at grades 1–2 level with vocabulary consisting of approximately 100 words and using 2–3 word sentences. Her behavior has improved significantly with increasing attention and fewer temper tantrums.

Studies of Cultured Patient Fibroblasts

Skin fibroblasts were obtained from the patient. Ability of her fibroblasts to incorporate label from [1-^{14}C] propionate and 5-[^{14}C]methyltetrahydrofolate into trichloroacetic acid-precipitable cellular macromolecules (measures of function of methylmalonyl-CoA mutase and methionine synthase, respectively) was performed as described previously (Miousse et al. 2009). The patient had decreased methyltetrahydrofolate incorporation (54 pmol/mg protein/18 h; reference 225 ± 165 pmol/mg protein/18 h), indicating decreased function of methionine synthase. Function of methylmalonyl-CoA mutase was within the reference range.

Synthesis of the two cobalamin coenzyme derivatives, AdoCbl and MeCbl, was assessed by incubation of her fibroblasts in medium containing [^{57}Co] cyanocobalamin bound to transcobalamin followed by extraction and separation of cobalamin derivatives by high-performance liquid chromatography (Miousse et al. 2009). The proportion of intracellular cobalamin present as MeCbl was 3.5% (reference 58.0 ± 6.7). AdoCbl levels were within the reference range. Somatic cell complementation classified this patient as *cblD*.

Molecular Diagnosis

Genomic DNA was isolated from patient fibroblasts with the QIAamp DNA minikit (Qiagen) and exons and flanking intronic sequence of the *MMADHC* gene amplified using previously described primers (Coelho et al. 2008). Sequencing of PCR amplicons identified a homozygous missense mutation: c.746A>G (p.Tyr249Cys).

Discussion

The clinical manifestations of disorders of intracellular cobalamin metabolism can be highly variable even within a single complementation group. The *cblC* disorder (combined methylmalonic acidemia and homocystinuria due to mutations in *MMACHC*) is the most common inherited disorder of cobalamin metabolism, with over 550 patients reported. The *cblD* disorder has been rare with only eighteen cases reported in the literature (a single sibship identified before 2004). This disorder includes patients with the classic presentation of combined methylmalonic acidemia and homocystinuria (*cblD*-MMA/HC), isolated homocystinuria (*cblD*-HC), and isolated methylmalonic acidemia (*cblD*-MMA).

Mutations in the *MMADHC* (methylmalonic aciduria, *cblD* type, and homocystinuria) gene on chromosome 2q23.2 have been reported in all identified *cblD* patients (Coelho et al. 2008; Miousse et al. 2009; Parini et al. 2013; Stucki et al. 2011). The MMADHC protein has sequence homology with a bacterial ATP-binding cassette transporter and contains a mitochondrial targeting sequence (Coelho et al. 2008). It does not appear to bind cobalamin. The MMADHC protein is present both in the cytoplasm, where it binds the cobalamin chaperone protein MMACHC (mutated in the *cblC* inborn error) and in the mitochondria (Deme et al. 2012; Gherasim et al. 2013; Mah et al. 2013). It is not understood how the protein directs cobalamin to different cellular compartments. The identification of mutations in *cblD* patients has allowed investigation of the relationship between the genotypes and the three biochemical phenotypes of this disorder. Coelho et al. (2008) examined the mutations in seven unrelated patients with *cblD* disorder. The group reported that mutations found in the patients with *cblD*-MMA were located toward the N-terminal part of the protein and consisted of a nonsense mutation, a duplication, and a frame-shift deletion. Mutations found in the patients with *cblD*-HC were located toward the C-terminal part of the protein and consisted of missense mutations. Mutations found in the patients with the combined phenotype were located toward the C-terminal and consisted of a nonsense mutation, a splice-site deletion, and a frame-shift duplication. This correlation between the location of the mutations and the biochemical phenotype was further confirmed by a recent study by Jusufi et al. using mutant constructs of the MMADHC protein (Jusufi et al. 2014). It was postulated that the N-terminal domain, which contains a mitochondrial targeting sequence, was required for the synthesis of AdoCbl, and the C-terminal domain was required for MeCbl synthesis. Mutations predicted to result in a nonfunctional protein resulted in combined homocystinuria and methylmalonic aciduria (Coelho et al. 2008). Subsequently identified *MMADHC* mutations have been consistent with this hypothesis (Miousse et al. 2009; Parini et al. 2013).

Although mutations in six *cblD*-HC patients have been reported, clinical findings have been reported in only three cases (Suormala et al. 2004; Miousse et al. 2009). The first clinical description of a patient with *cblD*-HC was

published by Suormala et al. (2004), a boy of Irish descent who was diagnosed at the age of six. He was the product of an uneventful pregnancy and labor/delivery to related parents and suspected "high-grade consanguinity." At 6 years of age, he presented with global developmental delay, learning disability, spastic ataxia, and deterioration of gait. Brain MRI analysis revealed cerebral and cerebellar atrophy. Visual evoked potentials were delayed. The mean corpuscular volume (MCV) was elevated at 94 fL (normal range 70–87 fL). Hemoglobin (Hgb) was normal at 11.9 g/dL (reference range not given for the authors' laboratory). Homocysteine was elevated at 9 μM (normal not detectable) and methionine was low at 14 μM (normal 15–40 μM). Methylmalonic acid was not detectable in the urine. Treatment consisted of hydroxocobalamin 1 mg intramuscular daily and then weekly injections. After 1 week on therapy, the patient was reported to be more alert with improved muscle tone. Six months later, he was able to walk and became more verbal.

The second patient with *cblD*-HC was also described by Suormala et al. (2004), a boy of Italian heritage who was diagnosed at the age of 3 months. He was the product of an uneventful pregnancy and labor/delivery to unrelated parents. At 3 months of age, he presented with severe hypotonia, nystagmus, dystonic movements, and seizures refractory to anticonvulsants. Brain MRI analysis revealed reduced myelination and a small cerebellar vermis. Megaloblastic anemia was found (MCV 105 fL and Hg 8.5 g/dL). Homocysteine was elevated at 128 μM and methionine was decreased at 4 μM, with undetectable methylmalonic acid in the urine. He was treated with hydroxocobalamin 1 mg intramuscular daily injections. Seizures disappeared within 10 days and the hematological status normalized. By 4 years of age, his gross and fine motor skills were reported as normal. Speech delay was still present. Brain MRI was normal.

The clinical description of the third patient with *cblD*-HC was reported by Miousse et al. (2009), a girl of Mexican background whose parents are first cousins. At 4 months of age, she was admitted to hospital with respiratory syncytial virus pneumonia and was noted to have developmental delay (inability to roll, inconsistent visual tracking, and limited responsiveness to voice), macrocephaly, and hair loss. A computed tomography scan revealed nonobstructive hydrocephalus. Homocysteine was 52.2 μmol/L (normal 0–14 μmol/L), methionine was not detected, and plasma methylmalonic acid level was normal. MCV was normal at 97.5 fL (normal 74–108 fL). The patient was started on a regimen of hydroxocobalamin (details not provided), folate, and betaine. Although the biochemical parameters improved, the clinical status did not. The patient developed a nearly obstructive vena cava clot. Medical support was then withdrawn due to progressive hydrocephalus and prolonged ventilatory support.

All six patients with *cblD*-HC had missense mutations affecting the C-terminal domain of *MMADHC*: two patients homozygous for c.737A>G (p.D246G), two patients homozygous for c.746A>G (p.Y249C), one patient homozygous for c.776T>C (p.L259P), and one patient heterozygous for c.746A>G and c.545C>A (T182N). Here, we report the seventh case of *cblD*-HC in a girl of East Indian origin homozygous for the c.746A>G nucleotide change. As reported previously, the 746A>G mutation occurred in a region of the *MMADHC* gene that is highly conserved among species and is sufficient to cause deficient synthesis of methylcobalamin (Coelho et al. 2008). Interestingly, although our patient was born in India to East Indian consanguineous parents, her last name is of Portuguese origin. Given that the c.746A>G mutation was previously reported in a European (Italian) boy, it is plausible that there is a shared ancestry for the mutated allele in these two patients.

With the recognition of the *cblD*-HC and *cblD*-MMA types of *cblD* disease and with the advances in the understanding of the molecular basis of *cblD* and the causative role of the *MMADHC* gene, the clinical, biochemical, and molecular diagnosis of *cblD* disease has improved considerably. Although *cblD* can be diagnosed early in life, through expanded newborn screening, cases can be missed as screening programs do not screen for low methionine levels seen in the classic and *cblD*-HC form. Given that patients generally respond well to treatment with vitamin B_{12}, clinicians must be aware of this inborn error of cobalamin metabolism.

Compliance with Ethics Guidelines

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible.

Committee on human experimentation (institutional and national) and with the Helsinki

Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of the patient for being included in the study. It can be available upon request.

Proof that informed consent was obtained can be available upon request.

Additional informed consent was obtained from the parents of the patient for whom identifying information is included in this article.

Conflict of Interest

Celia Atkinson, Isabelle R. Miousse, David Watkins, David S. Rosenblatt, and Julian AJ Raiman declare that they have no conflict of interest.

Details of Contributions of Authors

Dr. Celia Atkinson: Wrote the manuscript and participated in clinical care and clinical diagnosis of the patient.

Dr. Isabelle R. Miousse: Revised the manuscript and participated in the biochemical and molecular diagnosis of the patient.

Dr. David Watkins: Revised the manuscript and participated in the biochemical and molecular diagnosis of the patient.

Dr. David S. Rosenblatt: Revised the manuscript and participated in the biochemical and molecular diagnosis of the patient.

Dr. Julian AJ Raiman: Revised the manuscript and participated in the clinical care and clinical diagnosis of the patient. GUARANTOR.

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RESEARCH REPORT

Uncertain Diagnosis of Fabry Disease in Patients with Neuropathic Pain, Angiokeratoma or Cornea Verticillata: Consensus on the Approach to Diagnosis and Follow-Up

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Abstract *Introduction*: Individuals with neuropathic pain, angiokeratoma (AK) and/or cornea verticillata (CV) may be tested for Fabry disease (FD). Classical FD is characterised by a specific pattern of these features. When a patient presents with a non-specific pattern, the pathogenicity of a variant in the α-galactosidase A (GLA) gene may be unclear. This uncertainty often leads to considerable distress and inappropriate counselling and treatment. We developed a clinical approach for these individuals with an uncertain diagnosis of FD.

Materials and Methods: A document was presented to an FD expert panel with background information based on

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clinical experience and the literature, followed by an online survey and a written recommendation.

Results: The 13 experts agreed that the recommendation is intended for individuals with neuropathic pain, AK and/or CV only, i.e. without kidney, heart or brain disease, with an uncertain diagnosis of FD. Only in the presence of FD-specific neuropathic pain (small fibre neuropathy with FD-specific pattern), AK (FD-specific localisations) or CV (without CV inducing medication), FD is confirmed. When these features have a non-specific pattern, there is insufficient evidence for FD. If no alternative diagnosis is found, follow-up is recommended.

Conclusions: In individuals with an uncertain diagnosis of FD, the presence of an FD-specific pattern of CV, AK or neuropathic pain is sufficient to confirm the diagnosis of FD. When these features are non-specific, a definite diagnosis cannot (yet) be established and follow-up is indicated. ERT should be considered only in those patients with a confirmed diagnosis of FD.

Introduction

Fabry disease (OMIM 301500; FD) is an X-linked multisystem lysosomal storage disorder caused by deficient activity of α-galactosidase A (αGalA, E.C. 3.2.1.22). The estimated birth prevalence has originally been reported to be between 1:40.000 and 170.000 (Meikle et al. 1999; Poorthuis et al. 1999; Desnick et al. 2007). More than 600 variants/mutations in the α-galactosidase A (GLA) gene have been described (Garman 2007; HGMD 2014), most of which are private variants/mutations. For consistency, the term "variant" will be used throughout this article for all variations in the GLA gene, being either pathogenic, nonpathogenic or a genetic variant of unknown significance (GVUS); in the latter, an individual has an uncertain diagnosis of FD.

Fabry disease is generally divided into "classical" and "nonclassical" phenotypes. The first phenotype is usually characterised by a specific pattern of neuropathic pain (related to small fibre neuropathy, SFN), angiokeratoma and cornea verticillata (CV), while some or all of these features are usually absent in the latter (Elleder et al. 1990; Nakao et al. 1995). For definitions, see Table 1.

Since the availability of enzyme replacement therapy (ERT) with recombinant human α-galactosidase A (agalsidase alpha, Shire HGT and agalsidase beta, Genzyme Corp., a Sanofi company), an increasing number of screening studies in high-risk populations as well as newborn screening studies have been performed (e.g. Bekri et al. 2005; Spada et al. 2006; Brouns et al. 2007; Lin et al. 2009; Wallin et al. 2011; Dubuc et al. 2012; Mechtler et al. 2012). These screening studies have revealed a high number of individuals with variants in the GLA gene (Kobayashi et al. 2012; Terryn et al. 2013; van der Tol et al. 2014). While the pathogenicity of some GLA variants is well described, the subjects identified by these screening studies often have a GVUS in the GLA gene, i.e. an uncertain diagnosis of FD. Interestingly, most male patients with a GVUS demonstrate residual enzyme activity, in contrast to the absent or near absent enzyme activity in classically affected males. Also, previous studies have shown that patients with a nonclassical phenotype often only show a slight increase of lysoGb3 in plasma, while classically affected males invariably have very high levels (Aerts et al. 2008; Rombach et al. 2010; Gold et al. 2013; Lukas et al. 2013).

The reason to test for FD is usually a non-specific symptom such as stroke, chronic kidney disease or left ventricular hypertrophy in the absence of other causes. However, individuals with neuropathic pain, angiokeratoma or CV – in the absence of symptomatic involvement of the heart, brain or kidney – may also be tested for the presence of a variant in the GLA gene. These solitary features may be present in a pattern that differs from what is usually seen in FD patients with a classical phenotype and is therefore considered non-specific. For example, angiokeratoma may be scattered instead of clustered, or neuropathic pain may not be related to an SFN and have started at a much older age than expected in the context of a classical FD phenotype. CV may be present in individuals who used medication that may induce CV. If in such an individual a variant in the GLA gene is found, while there is residual enzyme activity (for males) and normal or only slightly increased Gb3 and lysoGb3, the pathogenicity of this variant is generally unclear; the variant is a GVUS. The subsequent uncertain diagnosis may cause considerable distress for the patient and the family and may also lead to inappropriate counselling and initiation of treatment with expensive enzyme replacement therapy. Thus, it is of great importance to achieve a correct diagnosis.

To address diagnostic dilemmas with regard to FD, we initiated "The Hamlet study: Fabry or not Fabry" to valorise clinical and laboratory assessments in order to improve the diagnosis of FD [Dutch trial register www.trialregister.nl NTR3840 and NTR3841]. For individuals with an uncertain diagnosis of FD, diagnostic algorithms are developed based upon literature data and international expert consensus through a modified Delphi procedure. As part of this study, we developed the approach to aid in the diagnostic pathway, counselling and follow-up for individuals with an uncertain diagnosis of FD, who present with cornea verticillata, angiokeratoma or neuropathic pain, with a GVUS in the GLA gene, but without a classical FD phenotype (for definitions, see Table 1). The approach is based on the current available evidence and international consensus.

Table 1 Criteria for a definite diagnosis of FD, classical (Smid 2014) with permission for reprint

A definite diagnosis of classical FD	
Male	Female
A variant in the GLA gene	
And	And
Severely decreased or absent leukocyte AGAL activity (<5% of the normal mean) combined with a minimum of 1 of the following criteria:[a]	A minimum of 1 of the following criteria:
Fabry neuropathic pain, cornea verticillata, angiokeratoma, increased plasma lysoGb3 or Gb3 in the range of "classical" FD males	Fabry neuropathic pain, cornea verticillata, angiokeratoma, increased plasma (lyso)Gb3 in the range of "classical" FD males
Or	Or
An affected family member with a definite diagnosis according to the criteria above	An affected family member with a definite diagnosis according to the criteria above
Uncertain FD diagnosis	
The individual does not fit the criteria for a definite diagnosis of classical FD. Further evaluations are needed, following the diagnostic algorithm	
Gold standard	
The gold standard for a diagnosis of FD in patients with an uncertain FD diagnosis, a GVUS and a non-specific FD sign is the demonstration of characteristic storage of the affected organ (e.g. heart, kidney, aside from skin) by electron microscopy analysis, according to the judgment of an expert pathologist	

This definition was made to select those patients in whom there is no doubt that FD is present. If this definition is not met, FD cannot be ruled out, but further evaluation is needed to avoid labelling this individual with the wrong diagnosis

[a] *Definitions*:

Fabry neuropathic pain fits the "FD-specific criteria" if there is neuropathic pain related to small fibre neuropathy in hands and/or feet, starting before age 18 or increasing with heat, fever. Quantitative sensory testing (QST) reveals a decreased cold detection threshold and the intraepidermal nerve fibre density (IENFD) is decreased. There is no other cause for Fabry neuropathic pain

Angiokeratomas fit the "FD-specific criteria" if they are clustered and present in characteristic areas: bathing trunk area, lips and umbilicus. There is no other cause for angiokeratoma

Cornea verticillata fits the "FD-specific criteria" if there is a whorl-like pattern of corneal opacities. There is no other cause (medication induced, among others, amiodarone, chloroquine)

Materials and Methods

Panel and Delphi Procedure

FD experts were invited to participate in the study panel through email. A consensus document was compiled and presented to the experts with an explanation of the rationale of the study as well as literature references and the applicable adopted results from the previous consensus procedure on general diagnostic criteria for FD; see Table 1.

The modified Delphi procedure consisted of an online survey round (round 1), after which a written recommendation was created for review by the panel (round 2). In round 1, virtual case histories were presented with neuropathic pain, angiokeratoma and/or CV. These case histories contained information on clinical symptoms and biochemical findings (enzyme activity in leukocytes, Gb3 and lysoGb3) were given. The panellists were asked to indicate whether or not the available information was sufficient to confirm or reject FD in the particular case on a 5-point Likert scale and were invited to add comments and suggestions. Anonymised results were presented to the panel (absolute scores and comments) after round 1. Clarification and additional data were provided. The consensus document for round 2 that was subsequently created represented the opinion of the expert panel as assessed in round 1. This document was thereafter reviewed and discussed by the expert panel via personal communication. A final version was drafted, and all participants agreed on the recommendations presented herein.

Adopted Definitions

The criteria for a definite and an uncertain diagnosis of FD were adopted from a previous consensus procedure; see Table 1 (Smid 2014). Strict definitions of the FD-specific clinical features (neuropathic pain, angiokeratoma, CV) were applied. If these strict definitions are fulfilled, the specificity for FD is very high (i.e. there is no differential diagnosis). These criteria were created to select classical FD patients in whom there is no doubt about the diagnosis.

Results/Recommendations

See Fig. 1 for the diagnostic algorithm that was constructed based on the following results.

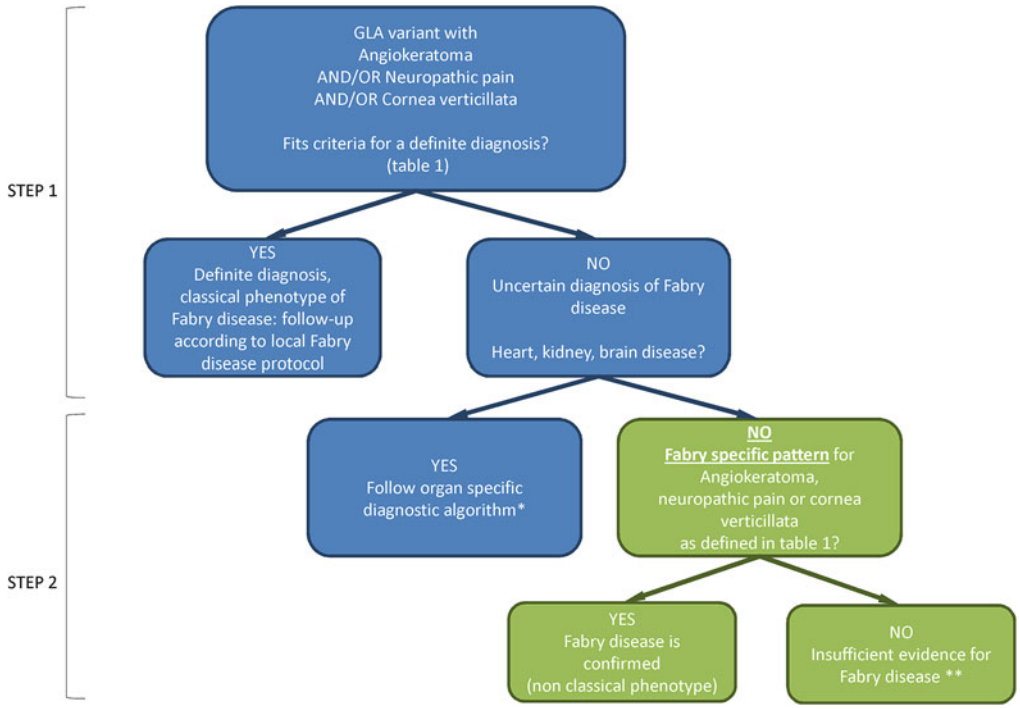

Fig. 1 Diagnostic algorithm. *Green*: individuals with a GLA GVUS (uncertain diagnosis of FD) with angiokeratoma, SFN or cornea verticillata without heart, kidney or brain disease, the subjects of the current study. Step 1: apply criteria for a definite diagnosis, Table 1. Step 2: approach to individuals with an uncertain diagnosis of FD. *Organ-specific algorithm will be published in separate articles (Smid 2014; Van der Tol 2014a, b). **Follow-up in an expert centre for FD could be considered; ERT is not (yet) indicated

Panel

Thirteen FD experts were invited and consented to participate. The panel consisted of four general FD specialists in internal medicine (RL, GL, DC, MJ), two paediatricians (FW, UR), two neurologists (CS, AM), a cardiologist (FW), three nephrologists (ES, CT (paediatric nephrologist), MW) and a medical geneticist (GH).

The panel agreed on the following 2-step approach:

Step 1: An Individual with a GLA Variant, First Evaluation (Adopted from (Smid 2014))

The panel agreed that all individuals with a GLA mutation need to undergo a full assessment of all organ systems that are involved in FD, and extensive biochemical analyses should be pursued, including αGalA enzyme activity in leukocytes, plasma lysoGb3 (if available), plasma Gb3 and urine Gb3. The presence of Fabry-specific neuropathic pain, AK and/or CV should be thoroughly assessed. A complete family history should be taken. An expert on FD should interpret clinical and biochemical assessments. The criteria to identify the patient with a definite diagnosis of FD can subsequently be applied (Table 1).

Step 2: An Individual with a GLA Variant and an Uncertain Diagnosis of FD (i.e. He or she has a GVUS in the GLA gene)

If heart, kidney or brain disease is present, the respective organ-specific algorithm – developed in separate projects as part of the Hamlet study – should be applied. For these algorithms, left ventricular hypertrophy, chronic kidney disease and stroke/TIA are defined according to internationally accepted definitions. For example, kidney disease is defined as chronic kidney disease according to the 2012 international guideline for kidney disease (KDIGO, http://kdigo.org/home/guidelines/ckd-evaluation-management/), with a GFR and urinary protein excretion as measures of kidney disease (Van der Tol 2014a). In individuals who present with neuropathic pain, AK or CV, but in whom heart, kidney or brain involvement is absent, the panel agreed on the following:

Cornea Verticillata

In an individual who presents with cornea verticillata, in the absence of medication use that may induce cornea verticillata (Table 2), and in whom a GLA variant is found, there are no known alternative diagnoses but FD.

Table 2 Medication that may induce cornea verticillata (for review on drug-induced corneal complications, see Hollander and Aldave (2004))

Medication that may induce cornea verticillata	Comment
Amiodarone	Well documented to induce cornea Verticillata
Aminoquinolines (chloroquine, hydroxychloroquine, amodiaquine)	Limited evidence, mainly case reports
Atovaquone	–
Clofazimine	–
Gentamicin (subconjunctival)	–
Gold	–
Mepacrine	–
Monobenzone (topical skin ointment)	–
NSAIDs (ibuprofen, naproxen, indomethacin)	–
Perhexiline maleate	–
Phenothiazines	–
Suramin	–
Tamoxifen	–
Tilorone hydrochloride	–

However, in an individual who presents with cornea verticillata only but who has used medication that may induce cornea verticillata at any time during the medical history (Table 2), there is insufficient evidence for a diagnosis of FD, despite the presence of a GLA variant.

Angiokeratoma

In an individual with a variant in the GLA gene and clustered angiokeratoma in the bathing trunk area, umbilicus and/or perioral region, there are no known alternative diagnoses but FD.

In an individual with *scattered* (i.e. not clustered) angiokeratoma, there is insufficient evidence for a diagnosis of FD, despite the presence of a GLA variant. The differential diagnosis of angiokeratoma should be considered (Table 3).

A skin biopsy of an angiokeratoma may be considered. A biopsy with characteristic storage on EM confirms the diagnosis of FD. However, the pretest likelihood of finding storage in an angiokeratoma or general skin biopsy is unknown for individuals with a nonclassical phenotype of FD, but is considered to be very low (see also Sect. 3.2). For review on the differential diagnosis of angiokeratoma, see Zampetti et al. (2011).

Neuropathic Pain

The differential diagnosis of neuropathic pain and SFN in particular is broad. The neuropathic pain that is caused by FD has a characteristic presentation and is related to the presence of SFN. In 95% of male and 75% of female FD patients, an abnormal heat detection threshold for cold and/or diminished intraepidermal nerve fibre density is found (Biegstraaten et al. 2011, 2012; Uceyler et al. 2011). In case of a variant in the GLA gene and the presence of neuropathic pain in hands and feet starting at childhood and increasing with heat/fever (Biegstraaten et al. 2012; Uceyler et al. 2013), there is no known alternative diagnosis. However, in all patients with pain as the only symptom, neurophysiological tests, quantitative sensory testing and a skin biopsy for intraepidermal nerve fibre density are needed to confirm the presence of isolated SFN, preferably before testing for FD is performed to rule out other causes.

Table 3 Differential diagnosis of angiokeratoma

Angiokeratoma	Preferred localisation
Angiokeratoma of Fordyce	Scrotal/vaginal
Angiokeratoma of Mibelli	Fingers and toes
Angiokeratoma corporis circumscriptum	Trunk/extremities
Angiokeratoma circumscriptum naeviforme (rare, easily confused with melanoma)	Neck
Idiopathic	All localisations
Other lysosomal storage disorders[a]	Related to the corresponding disease

[a] Other lysosomal storage disorders that present with angiokeratoma, such as fucosidosis, Schindler's disease and sialidosis, are rare and have a distinct clinical pattern, dissimilar to FD. In the clinical context, another LSD disease will not likely be mistaken for FD, but additional testing for lysosomal storage disorders can be considered

In individuals with a GVUS in the GLA gene and neuropathic pain related to SFN that does not fit the characteristic Fabry neuropathic pain description, there is insufficient evidence for a diagnosis of FD, despite the presence of a GLA variant. The differential diagnosis for SFN should be considered involving an expert on SFN; see Table 4.

Pathology

In addition to the 2-step approach, the feasibility to perform biopsies in individuals with neuropathic pain, AK and/or CV was discussed. In previous consensus procedures it was agreed that characteristic storage on electron microscopy (EM) of an affected organ (i.e. heart or kidney) should be considered as the gold standard for FD (Fogo et al. 2010; Leone et al. 2012; Smid 2014; Van der Tol 2014a, b). The panel is convinced that in the group of patients discussed here (i.e. patients with non-specific neuropathic pain, AK or CV but without heart or kidney involvement), a skin biopsy

Table 4 Differential diagnosis to be considered in cases of SFN and an uncertain diagnosis of FD

Isolated SFN	
Idiopathic (approximately 40%)	
Diabetes mellitus/impaired glucose metabolism	Sarcoidosis
Toxin: medication/alcohol/drug induced	HIV
Hypothyroidism	Coeliac disease
Sjögren's disease	Postinfection
Monoclonal gammopathy	Hyperlipidaemia
Amyloidosis	Hereditary sensory and autonomic neuropathy
Vasculitis	"Burning feet" syndrome
Erythromelalgia	

with characteristic storage on electron microscopy (EM) could confirm the diagnosis of FD.

The presence of characteristic storage in the skin has been well documented in most classical male FD patients (Eng et al. 2001; Thurberg et al. 2004), while reports on skin biopsies in nonclassical FD patients, i.e. who have confirmed storage in a kidney or heart biopsy but not fulfilling the criteria for a definite classical diagnosis of FD (Table 1), are lacking. Thus, it is unknown if a nonclassical phenotype of FD will also coincide with characteristic storage in the skin. Since the prevalence of characteristic skin storage is unknown in these individuals, we do not recommend to perform a skin biopsy in all patients with non-specific neuropathic pain, AK and/or CV and an uncertain diagnosis of FD.

Discussion

In case of an FD-specific pattern of cornea verticillata, angiokeratoma and/or neuropathic pain, and in the absence of other causes for these features, there is no alternative diagnosis than FD. Because of the major implications of an FD diagnosis, it is of great importance to ensure that the feature closely meets the criteria of an FD-specific feature (see Table 1). Yet in case of a GLA GVUS (an uncertain diagnosis of FD) and with cornea verticillata, angiokeratoma and/or neuropathic pain that are non-specific, there are currently no diagnostic tools to confirm or reject the diagnosis of FD. Thus, in these cases there is insufficient evidence for a diagnosis of FD. The expert panel advises to explain to the individual and family members that based upon current knowledge, FD is an unlikely diagnosis. Alternative diagnoses should be considered carefully. This line of argumentation is depicted in the algorithm in Fig. 1.

If no alternative diagnosis is made, it remains uncertain, but still very unlikely, that FD disease plays a role in the development of the clinical feature that was the reason to test for FD. Follow-up in an expert centre for FD could be considered on an individual basis. In case heart, kidney or brain disease develops, the diagnosis should be re-evaluated by applying the respective diagnostic algorithm, e.g. with a kidney biopsy in case of chronic kidney disease (Smid 2014; Van der Tol 2014a, b).

The expert group stressed the need for adequate counselling for these individuals and their family members to avoid unnecessary burden of a chronic illness.

In patients with heart or kidney involvement and an uncertain diagnosis of FD, histological evidence of characteristic storage by electron microscopy in an affected organ is the current gold standard for FD. In the patients who are the subjects of the current study, the heart and kidney are, by definition, not involved. Skin biopsies will, most likely, also yield negative results in the majority of cases. In classical male FD patients, characteristic storage in kidney cells is already present at a young age and in the absence of proteinuria (Tondel et al. 2008). Therefore, it may be postulated to perform a kidney biopsy even in the absence of any clinical signs of kidney disease. However, in case of nonclassical FD without chronic kidney disease, the prevalence of characteristic storage in the kidney (or heart) is currently unknown and expected to be low. Furthermore, Houge et al. reported on a male patient with characteristic storage in the kidney, while kidney biopsies of family members with the GLA variant did not show deposits, illustrating intra-familial differences (Houge et al. 2011). Because of the low expected yield and the inability to exclude future FD-associated organ involvement when storage is absent, a kidney biopsy is currently not recommended.

In individuals with a persisting uncertain diagnosis of FD, based upon clinical judgment, regular follow-up can be considered on an individual basis. If indeed kidney, heart or brain disease develops, a confirmation of the diagnosis should first be made following the subsequent organ-specific diagnostic algorithm, frequently involving a biopsy.

This recommendation does not serve to encourage screening for FD of groups or individuals (van der Tol et al. 2014). However, individuals with a GLA GVUS and thus an uncertain diagnosis of FD are frequently identified. With the recommendations in this study, unnecessary burden, inadequate counselling and unnecessary treatment with costly ERT can be avoided for these individuals and family members. Further studies may indicate new diagnostic tools, and the algorithm may subsequently be updated.

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Synopsis

In patients with an α-galactosidase A gene variant and neuropathic pain, cornea verticillata or angiokeratoma, who have an uncertain diagnosis of Fabry disease, detailed characterisation of these features is essential before the diagnosis of Fabry disease can be confirmed.

Guarantor Marieke Biegstraaten

Ethics approval was not required for this study.

Key words: Fabry disease; Diagnosis; Genetic variant; Consensus

Contributions

L van der Tol, CE Hollak and M Biegstraaten were involved in the design of the study, development of the consensus document, the survey rounds and analyses.

D Cassiman, G Houge, M Janssen, RH Lachmann, GE Linthorst, U Ramaswami, C Sommer, C Tøndel, ML West, F Weidemann, FA Wijburg and E Svarstad and AT Møller took part in the survey rounds as expert panellists.

All authors took part in writing and revising the manuscript.

Conflict of Interest

Linda van der Tol has received travel support and reimbursement of expenses from Actelion, Shire HGT or Genzyme.

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CASE REPORT

Improvement of Cardiomyopathy After High-Fat Diet in Two Siblings with Glycogen Storage Disease Type III

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Abstract Glycogenosis type III (GSD III) is an autosomal recessive disorder due to amylo-1,6-glucosidase deficiency. This disease causes limit dextrin storage in affected tissues: liver, skeletal muscles, and heart in GSD IIIa and only liver in GSD IIIb. Cardiomyopathy is quite frequent in GSD IIIa with variable severity and progression of manifestations. It is not clear if diet manipulation may interfere with cardiomyopathy's progression. Recent case reports showed improvement of cardiomyopathy following a ketogenic diet.

Two siblings (girl and boy), 7- and 5-year-old, both affected with GSD IIIa, developed severe and rapidly worsening left ventricular hypertrophy in the first years of life, while treated with frequent diurnal and nocturnal hyperproteic meals followed by orally administered uncooked cornstarch. Subsequently they were treated with high-fat (60%) and high-protein (25%), low-carbohydrate (15%) diet. After 12 months exertion dyspnea disappeared in the girl and biochemical blood tests, cardiac enzymes, and congestive heart failure markers improved in both (CK 3439→324, 1304→581 U/L; NT-proBNP 2084→206, 782→135 pg/mL, respectively); ultrasound assessment in both patients showed a relevant reduction of the thickness of interventricular septum (30→16, 16→11 mm, respectively) and left ventricle posterior wall (18→7, 13→8 mm, respectively) and an improvement of the outflow obstruction. A diet rich in fats as well as proteins and poor in carbohydrates could be a beneficial therapeutic choice for GSD III with cardiomyopathy. Future research is needed to confirm the beneficial effect of this treatment and to design treatment strategies with the aim to provide alternative source of energy and prevent glycogen accumulation.

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Glycogen storage disease type III (GSD III) is an inherited recessive disease, due to a defect of amylo-1,6-glucosidase or glycogen debranching enzyme (MIM 232400). This disorder causes limit dextrin storage in affected tissues: liver, skeletal muscles, and heart in GSD IIIa and only liver in the less frequent GSD IIIb. Clinically patients show fasting hypoglycemia, hyperlipidemia, growth delay, enlargement of liver and both skeletal muscles, and heart involvement. Cardiomyopathy with left ventricular hypertrophy is a relatively common finding although with variable severity and progression. It may be associated with potential risk of serious arrhythmia and symptomatic heart failure (Austin et al. 2012). Functionally these patients have only partial glycogenolysis, while glycolysis and gluconeogenesis are preserved. Standard treatment consists of frequent diurnal and nocturnal hyperproteic meals followed by orally administered uncooked cornstarch, with the aim to maintain normal blood glucose (Kishnani et al. 2010). These measures are effective in maintaining the metabolic control although growth retardation, liver, and cardiac and muscular complications may still occur in the long-term follow-up even in well-controlled patients (Kishnani et al. 2010). Recent reports regarding one infant and one adult patient showed an improvement of cardiomyopathy following a ketogenic diet (Valayannopoulos

Table 1 Biochemical and clinical data of the two siblings before and after 1 year of high-fat diet

	Girl		Boy	
	Before	12 months later	Before	12 months later
Clinical data				
Age (years)	7	8	5	6
Weight (kg), centile	24.4, 75–90th	25.3, 50–75th	18.5, 50th	18.3, 25th
Height (cm), centile	115.8, 50th	119, 25th	101, 10th	107, 10th
Hepatomegaly (cm from rib cage)	6	6	8	8
Diet				
Row cornstarch (g/kg ideal body weight/day)	6	0	5	0
Glucose (mg/kg ideal body weight/min)	6.5	1.9	4.8	1.9
Proteins (% total energy; g/kg ideal body weight)	24; 3.5	26; 3.3	33; 5	25; 4.2
Lipids (% total energy)	12	59	16	60
Carbohydrates (% total energy)	64	15	52	15
Kcal/day (kcal/kg ideal body weight)	1,100; 59	1,120; 51	1,025; 60	1,050; 70
Biochemical data				
Preprandial glucose levels (mg/dL)	90–100	80–100	80–90	87–94
1 h postprandial glucose levels (mg/dL)	70–90	70–90	120–125	70–90
Lactate (normal range 0.44–2.22 mmol/L)	1.9–2.1	0.8–2.2	0.6–1.1	0.4–0.8
Triglycerides (normal range 50–200 mg/dL)	117–179	127–161	155–224	246–273
Cholesterol (normal range 130–200 mg/dL)	114–176	170–171	170–204	164–175
Creatine kinase (normal range 20–180 U/L)	3,439–4,473	324–698	1,304–1,868	581–1,006
Aspartate transaminase (normal range <32 U/L)	303–413	264–303	557–1,583	286–324
Alanine transaminase (normal range <33 U/L)	444–544	280–653	521–1,298	447–693
NT–pro-brain natriuretic peptide (normal range <125 pg/mL)	1,907–2,262	206	649–917	135
Troponin T (normal range 0–14 ng/L)	25	21	49	21
Myoglobin (normal range 28–72 ng/mL)	172	39	153	61
Echocardiogram				
Maximum thickness of the septum (mm)	30	16	16	11
Maximum thickness of the posterior wall (mm)	18	7	13	8
Outflow tract obstruction (max/medium Doppler gradient at rest, mmHg)	90–70/30–25	50/21	32–23/10–7	No gradient

et al. 2011; Sentner et al. 2012). This result was obtained with addition of synthetic ketone bodies as D,L-3-hydroxybutyrate in one case (Valayannopoulos et al. 2011) and with a high protein very-low-calorie diet in the other case (Sentner et al. 2012). Another adult patient (Dagli et al. 2009) improved increasing proteins and reducing carbohydrates intake.

We present two siblings (girl and boy), 7- and 5-year-old, both affected with GSD IIIa who had rapid deterioration of left ventricular hypertrophy. Twelve months after beginning a high-fat and high-protein diet, clinical symptoms ameliorated and biochemical tests as well as echocardiograms showed a clear improvement of cardiomyopathy.

Case Reports

Two siblings from consanguineous Tunisian parents were affected by GSD III and carried the homozygous mutation p.G1087R. The girl was diagnosed at 1.4 year on a clinical basis (hepatomegaly with hyperechogenic tissue at ultrasound, fasting hypoglycemia, hypertriglyceridemia), while the brother at birth. Both were treated with frequent hyperproteic feedings, followed by row cornstarch, because of fasting hypoglycemia. Cardiac hypertrophy was diagnosed at 2.7 years of age in the girl and at 9 months in the boy. The girl started propranolol (1 mg/kg/day) at the age of 2.7 years and the boy at 1.6 years.

Biochemical and clinical data at baseline and 1 year after starting high-fat diet are shown in Table 1.

At 5.8 years the girl showed a heart systolic ejection murmur. Electrocardiogram recording revealed increase of hypertrophy. Two-dimensional echocardiogram showed a marked thickening of the interventricular septum (IVS) and left ventricular posterior wall (PW) (Fig. 1), with dynamic left ventricular outflow tract obstruction and mitral regurgitation (Table 1 and Fig. 1). Cardiomyopathy deteriorated

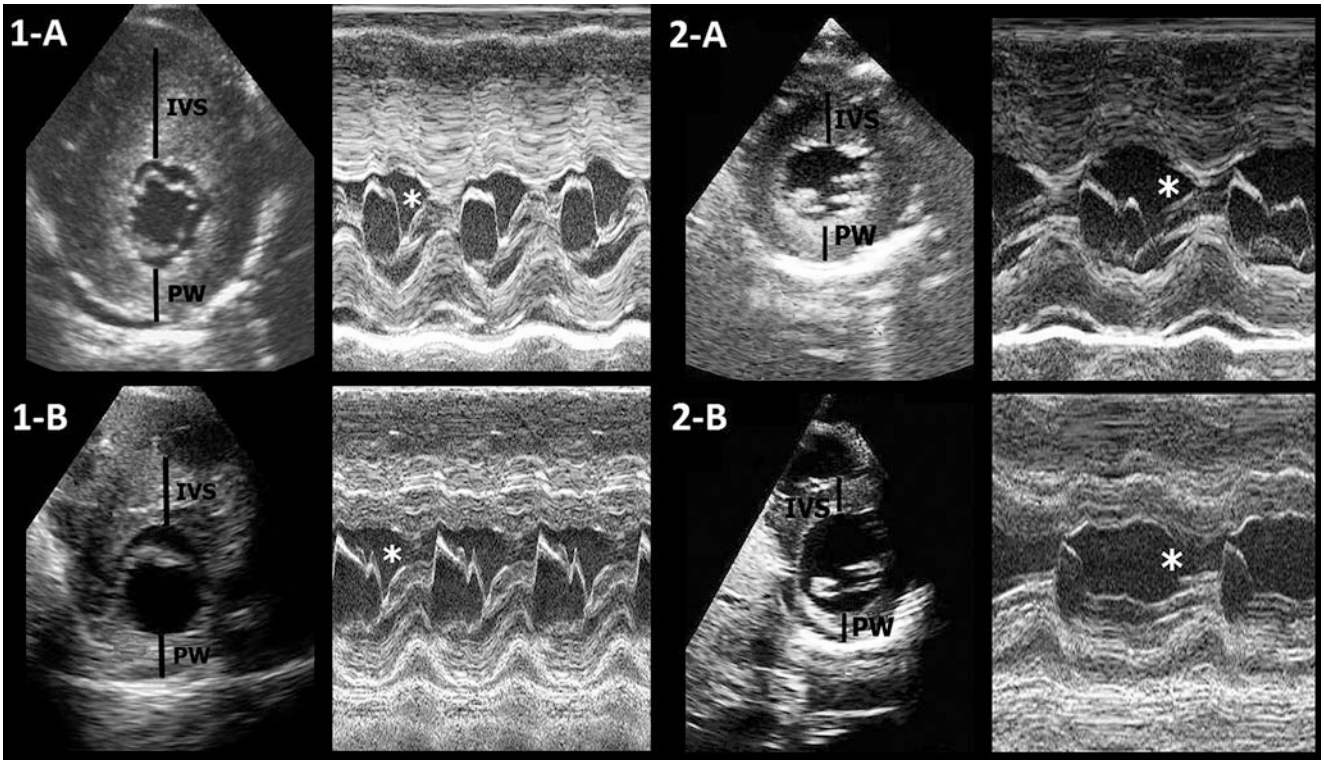

Fig. 1 2D and M-mode echocardiography of girl (1-A and 2-A) and boy (1-B and 2-B) before and after the diet manipulation. *IVS* interventricular septum, *PW* posterior wall of the left ventricle. Note the typical systolic anterior motion of the mitral valve (SAM), responsible of outflow obstruction (*asterisk*) and the reduction of the thickness and the amplitude of SAM after the diet

rapidly with the girl complaining of weakness and exertion dyspnea. As propranolol was increased (3 mg/kg/day) without success, at 6.6 years bisoprolol was started (0.3 mg/kg/day). We observed a slight decrease of left ventricular outflow obstruction without any change of IVS and left ventricular PW thickness (Table 1). At this time heart transplantation was considered as a possible choice for the girl. A heart MRI was performed which showed a left ventricular mass index of 232 g/m^2 (normal values: mean 58 g/m^2, 95th centile confidence interval 42–84 g/m^2).

At two years old, also the boy showed reduced physical activity associated to a rapid worsening of cardiomyopathy, with mild left ventricular obstruction, despite increase of propranolol therapy (3 mg/kg/day). At 2.8 years bisoprolol was started (0.3 mg/kg/day) and later ultrasound examination showed a slight increase of myocardial thickness (Table 1 and Fig. 1).

A high-fat (60%), low-carbohydrate (15%), high (unchanged)-protein (25%) diet was started at age seven for the girl and five for the boy (Table 1). Cornstarch was progressively reduced and eventually stopped. Fats rich in polyunsaturated fatty acids were preferred over saturated fats (Fuehrlein et al. 2004) and only extra-virgin olive oil as relish was recommended. Additional protein powders were used to increase protein intake without high saturated fat addition.

After 12 months a clinical, biochemical and echocardiographic improvement was observed in both siblings: clinical symptoms disappeared in the girl and both children became more active than before; alanine transaminase, aspartate transaminase, creatine kinase, and NT-probrain natriuretic peptide decreased substantially (Table 1); and a relevant reduction of the thickness of IVS and p of left ventricle PW was seen as well as a reduction of the outflow obstruction in both patients (Table 1 and Fig. 1). During withdrawal of cornstarch the pre- and postprandial glucose levels were maintained in the normal ranges and the patients showed good metabolic balance.

Discussion

The two patients had a severe progressive cardiomyopathy with ventricular hypertrophy and ventricular outflow tract obstruction, notwithstanding a high-protein diet as recommended by Kishnani et al. (2010). The pharmacological treatment with beta-blockers was only partially effective and also not free of risk in these patients because it might mask symptoms of hypoglycemia (Kishnani et al. 2010).

It was much impressive to observe that with the new diet they had a dramatic improvement of their clinical conditions and biochemical and echocardiographic findings.

The dietetic management of GSD III is controversial (Dagli et al. 2009): few cases showed an improvement of cardiac hypertrophy after high-protein diet (Dagli et al. 2009; Sentner et al. 2012). The rationale is that high-protein diet might reduce limit dextrin accumulation in myocardial cells and increase the use of proteins through gluconeogenesis (Sentner et al. 2012). Higher dietary protein intake might also enhance muscle protein synthesis (Kishnani et al. 2010). Another patient, 2 months old, had an improvement of cardiomyopathy after a ketogenic diet (Valayannopoulos et al. 2011). This treatment not only forced the activity of gluconeogenesis with a high protein diet but also facilitated ATP generation from fatty acid oxidation and ketolysis as alternative source of energy.

Our patients started a high-protein diet from diagnosis, but as it was not enough to maintain normoglycemia, addition of cornstarch was needed to reach a good homeostasis. With the increase of fats this addition was no more needed. Fats were increased in the diet of our children in a softer way than suggested by Valayannopoulos et al. (2011), without reaching a formal ketogenic diet, with the aim of providing them with a more comfortable diet, which could be followed for a long period of time. In our clinical experience, the complete ketogenic diet is hampered by bad compliance, being much difficult to follow in practice for a long time.

The clinical evolution of these two siblings shows that a high-fat and high-protein diet with limited carbohydrate intake could be a beneficial therapeutic choice for GSD III pediatric patients with cardiomyopathy. The patients reported in the literature (Dagli et al. 2009; Sentner et al. 2012; Valayannopoulos et al. 2011) and our present cases have in common an increased use of caloric sources different from carbohydrates. In fact the patient reported by Valayannopoulos et al. (2011) had a reduction of carbohydrates sources with a ketogenic diet, the adult reported by Dagli et al. (2009) increased protein intake to 30% and was able to halve cornstarch assumption, and the other adult reported by Sentner et al. (2012) improved on a hyperproteic severely hypocaloric diet which, although containing 61% carbohydrates, was ketogenic "per se" due to the calories limitation (900 kcal/day).

Why a high carbohydrate diet may sustain the development of cardiomyopathy in GSD III is not clear. Hyperinsulinism might play a role: glycogen storage disease patients who are excessively fed with carbohydrates might manifest lipolysis inhibition due to high insulin secretion leading to reduction of energy availability for the heart (Valayannopoulos et al. 2011).

According to these findings, it might be possible to say that the row cornstarch should not be administered to GSD III patients, whose diet should be manipulated introducing enough proteins and fats to maintain normoglycemia.

The main limitation of this clinical report is that it is not a case–control study; however each child served as its own control. Further studies are needed to confirm these results. These two siblings should also be followed longitudinally in the next years, in order to assess if the current improvement is sustained and if they do not develop side effects. Future research is needed to design alternative treatment strategies to prevent glycogen accumulation in GSD III.

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Compliance with Ethics Guidelines

Conflict of Interest

Alessandra Brambilla, Savina Mannarino, Roberta Pretese, Serena Gasperini, Cinzia Galimberti and Rossella Parini declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

All authors are involved in the planning of the different treatment and conception and design of the paper; Alessandra Brambilla analyzed the data and prepared a draft of the article; Savina Mannarino analyzed and interpreted the cardiac data and revised the draft critically; Serena Gasperini, Roberta Pretese, and Cinzia Galimberti revised the draft critically; and Rossella Parini revised the draft critically, accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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RESEARCH REPORT

Erratum to: Widening Phenotypic Spectrum of AADC Deficiency, a Disorder of Dopamine and Serotonin Synthesis

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Change in Nomenclature to DDC Mutation.
It was learned subsequent to publication of the series of patients with AADC deficiency that the nomenclature of the second lesion in Patient 5, given as Exon 5 c.446G>A, p.S149T, should have been c.446G>C, p.S149T. The laboratory issued an amended report, and the authors regret any inconvenience.

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