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RESEARCH REPORT

Coenzyme Q_{10} and Pyridoxal Phosphate Deficiency Is a Common Feature in Mucopolysaccharidosis Type III

Dèlia Yubero • Raquel Montero • Mar O'Callaghan • Mercè Pineda • Silvia Meavilla • Veronica Delgadillo • Cristina Sierra • Laura Altimira • Plácido Navas • Simon Pope • Marcus Oppenheim • Viruna Neergheen • Arunabha Ghosh • Phillipa Mills • Peter Clayton • Emma Footitt • Maureen Cleary • Iain Hargreaves • Simon A. Jones • Simon Heales • Rafael Artuch

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Abstract Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders caused by deficiencies of lysosomal enzymes catalyzing degradation of glycosaminoglycans (GAGs). Previously, we reported a secondary plasma coenzyme Q_{10} (CoQ) deficiency in MPS patients. For this study, nine MPS patients were recruited in the Hospital Sant Joan de Déu (HSJD, Barcelona) and two patients in the Neurometabolic Unit, National Hospital (NMU, London), to explore the nutritional status of MPS type III patients by analyzing several vitamins and micronutrients in blood and in cerebrospinal fluid. Plasma CoQ and plasma and cerebrospinal fluid pyridoxal phosphate (PLP) content were analyzed by high-pressure liquid chromatography (HPLC) with electrochemical and fluorescence detection, respectively. We found that most MPS-III patients disclosed low plasma pyridoxal phosphate (PLP) values (seven out of nine) and also low plasma CoQ concentrations (eight out of nine). We observed significantly lower median values of PLP, tocopherol, and CoQ (Mann–Whitney U test, $p = 0.006$, $p = 0.004$, and $p = 0.001$, respectively) in MPS patients when compared with age-matched controls. Chi-square test showed a significant association between the fact of having low plasma PLP and CoQ values in the whole cohort of patients. Cerebrospinal fluid PLP values were clearly deficient in the two patients studied. In conclusion, we report a combined CoQ and PLP deficiency in MPS-III patients. These observations could be related to the complexity of the physiopathology of the disease. If our results are confirmed in larger series of patients, CoQ and PLP therapy could be trialed as coadjuvant therapy with the current MPS treatments.

Communicated by: Marc Patterson

Competing interests: None declared

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Introduction

Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders caused by deficiencies of lysosomal enzymes catalyzing degradation of glycosaminoglycans (GAGs) (Neufeld and Muenzer 2001). Among them, MPS type III (MPS-III) or Sanfilippo syndrome is an autosomal recessive lysosomal storage disease caused by mutations in

one of four genes which encode enzyme activities required for the lysosomal degradation of heparan sulfate. Four types have been recognized: heparan *N*-sulfatase is deficient in type A (OMIM #252900), α-*N*-acetylglucosaminidase in type B (OMIM #252920), acetyl-CoA α-glucosamide acetyltransferase in type C (OMIM# 252930), and *N*-acetyl glucosamine 6-sulfatase in type D (OMIM# 252940) (Neufeld and Muenzer 2001). This disorder primarily affects the central nervous system (Yogalingam and Hopwood 2001), being the only MPS with relatively minor somatic disease. It is characterized by speech delay, behavioral problems, progressive cognitive decline, dysmorphic facial features, loss of motor skills, and epilepsy. Due to the nonspecific clinical symptoms, a diagnostic delay is common in MPS-III (Neufeld and Muenzer 2001; de Ruijter et al. 2012). Over 300 mutations in the four genes (*SGSH*, *NAGLU*, *HGSNAT*, *GNS*) encoding for the enzymes have been described to date (Wijburg et al. 2013).

Coenzyme Q_{10} (CoQ) is an endogenously synthesized lipid present in all types of cells and in plasma associated with cholesterol transport lipoproteins. CoQ acts as an electron transporter inside the mitochondrial respiratory chain (MRC) and is necessary for ATP production. Among other biological properties, the reduced form of CoQ is an effective antioxidant for both cell membranes and plasma lipoproteins and also recycles α-tocopherol (Navas et al. 2007; Sohal 2004). Several secondary CoQ deficiencies related to different neurodegenerative conditions have been reported. In 2011, Delgadillo et al. reported for the first time plasma CoQ deficiency in different MPS patients monitored during genistein treatment (Delgadillo et al. 2011).

Owing to these preliminary findings, our aim was to explore the nutritional status of different MPS-III patients by analyzing several vitamins and micronutrients in blood. Additionally, we were able to analyze the CSF of two further MPS-III patients.

Material and Methods

Subjects

Patient samples were received by Hospital Sant Joan de Déu (HSJD, Barcelona) and the Neurometabolic Unit (NMU), National Hospital, Queen Square, London. In HSJD, nine MPS type III patients were studied (range age: 5–17 years; average 11 years) for nutritional assessment. Five out of nine patients were MPS-IIIA, three were MPS-IIIB, and one patient was type IIIC. At the time of the study, no patient was receiving a vitamin or CoQ supplement. Four out of nine patients were on treatment with genistein (100–150 mg/kg/day) following a previously reported protocol (Delgadillo et al. 2011). In five of them, seizures were being treated with a combination of different antiepileptics (lamotrigine, carbamazepine, and phenobarbital). Due to new onset movement disorders, cerebrospinal fluid (CSF) neurotransmitters and pyridoxal phosphate (PLP) concentration was assessed, by the NMU, from two patients with MPS-IIIA (4 and 16 years old).

Control population: All blood analyses results were compared with the age-dependent reference values established for each biochemical parameter in HSJD laboratory. Moreover, we selected an age-matched healthy pediatric population ($n = 9$; range age: 5–17 years; average = 11 years) to asses biochemical data in parallel with MPS patients. All controls were healthy subjects who came to the hospital for routine analysis: exclusion criteria were the diagnosis of inborn errors of metabolism or any other genetic or chronic condition, pharmacological treatments, or special diets. For assessment of pyridoxal phosphate status in CSF, results were compared to the NMU-established age-related reference intervals (Ormazabal et al. 2008).

Ethical issues: The study was approved by the Ethical Committee of the Hospital Sant Joan de Déu, and samples from patients and controls were obtained according to the Helsinki Declaration of 1964, as revised in 2001. CSF analysis was performed after informed consent and at the request of the clinical team.

Nutritional Study

Dietary questionnaires were completed by patient's parents and were performed to the 9 MPS-III patients selected. Nutritional status was followed up by the gastroenterology and nutrition department of the HSJD.

Laboratory Studies

Blood and urine samples were collected in the fasting state. Blood samples were centrifuged to 1,500 × *g* for 10 min at 4°C and the plasma/serum samples obtained were stored at −80°C up to the day of the analysis. Urine samples were collected and stored at −20°C until the moment of the analysis. CSF PLP was collected, stored at −80°C, and processed as previously described (Ormazabal et al. 2008).

In blood samples, routine parameter analyses (blood count, ions, glucose, hepatic and renal function, lipid and iron metabolism markers) were performed by standardized automated analysis. Amino acids were measured by ion-exchange chromatography with spectrophotometric detection after ninhydrin derivatization in a Biochom 30 analyzer (Chromsystems UK). For nutritional markers in blood (plasma/serum), we analyzed plasma CoQ content by reversed-phase high-pressure liquid chromatography (HPLC) with electrochemical detection (Montero et al.

2005), and vitamin E, vitamin A, vitamin B_1, and pyridoxal phosphate (PLP) were measured by HPLC with UV and fluorescence detection following previously reported procedures (Ormazabal et al. 2008; Moyano et al. 1997; Lu and Frank 2008). Serum folate and vitamins B_{12} and D were quantified by automated chemoimmunoluminescence procedures in an ARCHITECT analyzer (Abbot, USA). For trace element analysis (zinc, copper, and selenium), inductively coupled plasma mass spectrometry (ICP-MS) was applied, as reported (Tondo et al. 2010). Urinary total GAGs excretion in patients during the study was done by the automated-DMB spectrophotometric assay.

All patients were diagnosed with MPS-IIIA, B, or C on the basis of the demonstration of enzymatic activity deficiency of particular lysosomal hydrolases in leukocytes or skin fibroblasts (sulfamidase, α-*N*-acetylglucosaminidase, and heparin-α-glucosaminido-acetyltransferase). In all cases molecular studies were performed for identification of pathogenic mutations by automated DNA Sanger sequencing method (data available on request).

Statistical Studies

De Mann–Whitney *U* test was used to compare data for all the variables analyzed between MPS patients and age-matched controls. Chi-square test was applied to search for categorical association among the different biochemical parameters included in the study. Statistical differences were considered when p was <0.05. Statistical analysis was done with the SPSS 22.0 program.

Results

Nutritional Studies

Dietary assessments of the MPS patients confirmed that dietary intake was correct for their age according to recommended dietary allowances (data not shown). Moreover, all of them were able to feed normally and none required enteral feeding by gastric button or other feeding device.

Biochemical Results

Routine parameters (blood count, ions, glucose, hepatic and renal function makers, alkaline phosphatase, lipid and iron metabolism) and other nutritional biomarkers (amino acids; vitamins B_1, B_{12}, and A; folate and trace elements) were within the reference ranges in all MPS patients (data not shown). The urinary GAGs excretion was altered in all MPS patients at the time of the study (range 12.6–24.2 mg/mmol creat).

Main biochemical results are stated in Tables 1 and 2. The results of MPS patients and age-matched controls are expressed as range and average and as range for reference values established in our laboratory. Most MPS patients showed low plasma PLP values (seven out of nine) and also low plasma CoQ concentrations (eight out of nine) (Table 2). The other parameters stated in Table 1 were normal or slightly impaired in a minority of cases. Interestingly, CSF PLP values were below the appropriate reference range for both patients studied (Table 1).

When we compared the blood biochemical parameters in the two groups of subjects (MPS vs age-matched controls), we observed significantly lower median values of PLP, tocopherol, and CoQ (Mann–Whitney U test, $p = 0.006$, $p = 0.004$, and $p = 0.001$, respectively) in MPS patients (Table 1, Fig. 1). No differences were observed in total cholesterol values, alkaline phosphatase activity, total homocysteine, and the other parameters between the two groups. Chi-square test showed a significant association between the fact of having low plasma PLP and CoQ values in the whole cohort of patients and controls studied ($\chi^2 = 7.137$; $p = 0.008$), but not with other parameters analyzed. No association was found between both CoQ and PLP deficiency and the antiepileptic treatment.

Discussion

In a previous report, we studied 30 patients (2–23 years; 9.8 average) with diagnosis of different MPS (19 of them MPS-III) during the monitoring of genistein treatment (Delgadillo et al. 2011). In that report, decreased plasma CoQ values were a common feature. Thus, we have done a nutritional study in nine selected MPS-III patients, and CoQ deficiency was detected in most of them. Furthermore, not only plasma CoQ was decreased, but also a PLP deficiency was present in seven out of nine cases studied. Other nutritional parameters were assessed as normal or showed only minor differences when compared to reference values.

Concerning CoQ deficiency, we do not have yet an explanation for this finding. Total cholesterol values were normal, suggesting that the cause of such deficiency is probably unrelated to impaired cholesterol biosynthesis (and consequently CoQ biosynthesis). This statement would be supported by the data of Matalonga et al., who demonstrated a normal CoQ biosynthesis rate in cultured skin fibroblast from MPS patients (Matalonga et al. 2014). Plasma CoQ values depend on dietary sources (around 20% of total CoQ) and liver biosynthesis, but dietary CoQ deficiency is unlikely considering the adequate nutritional status of our patients and the normality of most of the other vitamins and trace elements analyzed. However, an absorption problem in the gut for CoQ cannot be ruled out at this

Table 1 Main plasma biochemical data of MPS patients and control subjects are stated. The results of MPS patients and age-matched controls are expressed as range and average and as range for reference values established in our laboratory. The number of patients displaying impaired results is also indicated

	MPS patients ($n = 9$) (5–17) 11 years	Age-matched controls ($n = 9$) (5–17) 11 years	Reference values
Alkaline phosphatase (UI/L)	(72–315) 207 0/9	(37–277) 167 0/9	<365
Total homocysteine (μmol/L)	(5.9–24.8) 9.6 3/9	(3.1–10.0) 5.5 0/9	<9.2 (12–20 years) <7.5 (5–11 years)
Total cholesterol (mmol/L)	(3.1–5.6) 4.3 1/9	(3.3–5.9) 4.4 2/9	2.4–5.2
Retinol (μmol/L)	(1.0–2.1) 1.3 0/9	(0.8–1.4) 1.1 1/9	0.9–2.1
Tocopherol (μmol/L)	(12–26) 17.3 1/9	(18–34) 25.4 0/9	13–36
Pyridoxal phosphate (nmol/L)	(8–99) 27 7/9	(32–105) 69 0/9	30–169
Coenzyme Q_{10} (μmol/L)	(0.20–0.53) 0.32 8/9	(0.41–0.76) 0.53 0/9	0.41–1.15
Coenzyme Q_{10}/cholesterol ratio (mmol/mol)	(47–102) 74 9/9	(100–141) 120 2/9	115–316
CSF PLP values (nmol/L)	9 (4 years) 7 (16 years)	Not available	16–44 10–37

CSF cerebrospinal fluid

Table 2 Individual plasma biochemical data of the nine MPS patients, age-matched controls, and reference values are stated. Results of age-matched controls are expressed as range and average and as range for reference values

Patient MPS type	Age (years)	Coenzyme Q_{10} (μmol/L)	Pyridoxal phosphate (nmol/L)	Tocopherol (μmol/L)	Total cholesterol (mmol/L)	Glycosaminoglycans (mg/mmol creatinine)
1. IIIA	10	0.31	19	17	5.0	13.3
2. IIIA	8	0.35	20	19	4.2	14.1
3. IIIA	10	0.31	22	14	4.3	24.2
4. IIIA	5	0.21	35	12	3.1	22.5
5. IIIA	16	0.20	15	26	4.3	15.9
6. IIIB	14	0.31	8	17	3.4	17.5
7. IIIB	11	0.53	22	15	5.2	16.5
8. IIIB	9	0.36	99	21	5.7	14.2
9. IIIC	17	0.29	8	15	3.5	12.6
Age-matched controls	(5–17) 11	(0.41–0.76) 0.53	(32–105) 69	(18–34) 25	(3.3–5.9) 4.4	
Reference values		0.41–1.15	30–169	13–36	2.5–5.2	<6.4 (5–12 years) <4.5 (13–17 years)

stage. Diarrhea is common in children with MPS-III and may be severe, and the mouse model has increased submucosal thickness with lysosomal storage of GAG in this region and in the lamina propria of the villus tip (Roberts et al. 2009). Increased CoQ consumption may also be a factor explaining this deficiency, since some authors suggested the possible involvement of the reactive oxygen species in the MPS type IIIB disease pathogenesis (Villani et al. 2009). Recently a positive effect has been observed in cultured fibroblasts from Sanfilippo A and B treated with CoQ and other antioxidants (Matalonga et al. 2014). Moreover, since CoQ participates in tocopherol recycling (Navas et al. 2007), the significantly lower median tocopherol values observed in our MPS patients when compared with age-matched controls might be related with the CoQ deficiency. The importance of these lower median tocopherol values is probably limited since in absolute values, most of the tocopherol results were normal when compared with the reference range.

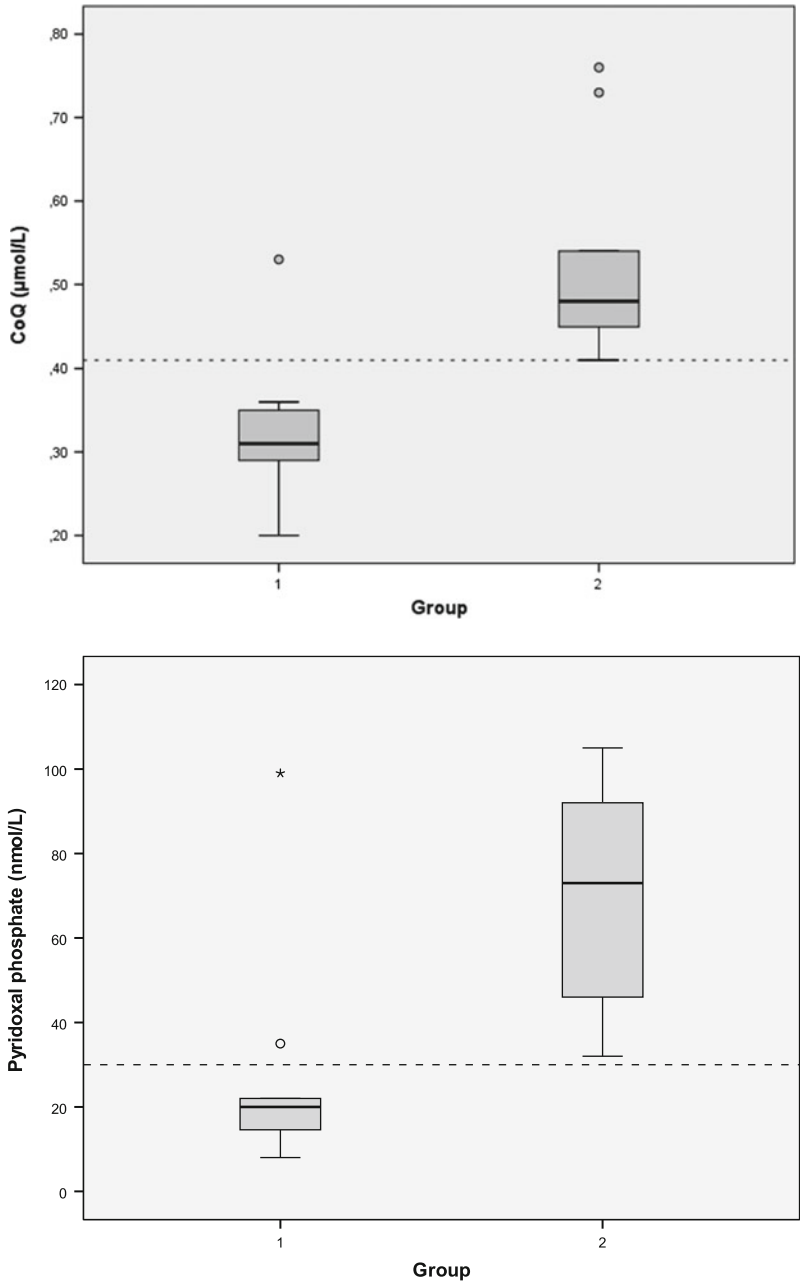

Fig. 1 The *box plot* represents PLP and CoQ values in group 1 (MPS patients) versus group 2 (age-matched controls). The *spot line* represents the minimum PLP and CoQ values in the reference population

As regards PLP deficiency, the explanation also remains elusive. The alkaline phosphatase activity (ALP) maintains the equilibrium needed to dephosphorylate PLP to pass through cell membranes. Increased ALP activity in plasma may lead to a secondary PLP deficiency. However, in our MPS patients, biochemical data supported a real PLP deficiency, since no increment of plasma ALP activity was demonstrated. The finding of PLP deficiency in both plasma and CSF might support a hypothesis of a transport defect affecting PLP movement across both the gut and the blood–brain barrier. Heparan sulfate and phosphorylated B_6 vitamers both bind to divalent cations, so it is conceivable that phosphorylated B_6 vitamers could become bound to HS.

An association between CoQ and PLP status has been reported (Spinneker et al. 2007). PLP is a versatile coenzyme and it is the essential cofactor for multiple reactions. It is involved in the transsulfuration pathway of homocysteine (and, although nonsignificant, some of our MPS patients showed some evidence of increased total homocysteine values at the time of the study), as well as in the metabolism of amino acids, neurotransmitters, and other

substrates (Spinneker et al. 2007). Furthermore, PLP is required for the formation of 4-hydroxyphenylpyruvic acid from tyrosine, the essential precursor of the benzoquinone ring of CoQ. In fact, it has been demonstrated that PLP deficiency is associated with low CoQ concentrations (Willis et al. 1999), which could explain CoQ deficiency as a secondary condition of low precursor availability.

The pathophysiology of the MPS disorders is complex, and the molecular basis and the sequence of events leading to neurodegeneration in MPS remain to be clarified. Several treatments have been designed for different types of MPS including enzyme replacement therapy, gene therapy, or substrate reduction therapy (Kakkis et al. 2001; Wraith et al. 2004; Piotrowska et al. 2006). These treatments lead the partial restoration of the enzyme activity or inhibition of GAG synthesis. However, such approaches have not been completely successful. PLP is the active form of vitamin B_6 and the cofactor of many enzyme reactions including neurotransmitter metabolism (dopamine, serotonin, and GABA among others). Primary and secondary PLP metabolism disturbances can produce refractory seizures in the newborn period and infancy that respond to this vitamin supplementation. Thus, PLP treatment seems advisable in this condition. On the other hand, the antioxidant properties of CoQ are well recognized. A chronic CoQ deficiency may lead to an increased oxidative stress that might in turn participate in neurodegeneration in MPS. Moreover, the demonstration of a beneficial effect of CoQ in GAGs accumulation in fibroblast further support that this therapy in combination with PLP would be advisable as coadjuvant treatment in MPS patients presenting PLP and CoQ deficiency.

Conclusions

We report for the first time a combined CoQ and PLP deficiency in MPS-III patients. These observations could contribute to the complexity of the physiopathology of the disease. After a careful evaluation of nutritional status in large series of MPS patients, both CoQ and PLP could be trialed as coadjuvant therapy with the current MPS treatments.

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Synopsis

Combined CoQ and PLP deficiency is common in MPS-III patients.

Compliance with Ethics Guidelines

Conflict of Interest

Dèlia Yubero, Raquel Montero, Mar O'Callaghan, Mercè Pineda, Silvia Meavilla, Veronica Delgadillo, Cristina Sierra, Laura Altimira, Plácido Navas, Simon Pope, Marcus Oppenheim, Viruna Neergheen, Arunabha Ghosh, Phillipa Mills, Peter Clayton, Emma Footitt, Maureen Cleary, Iain Hargreaves, Simon A. Jones, Simon Heales, and Rafael Artuch declare that they have no conflict of interest.

Informed Consent

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

Author Contributions

All co-authors have peer-reviewed the manuscript and there is a consensus agreement to submission. Thus, we confirm the absence of previous similar and simultaneous publications. Dr. Yubero, Montero, O'Callaghan, Heales, and Artuch had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Yubero, Montero, O'Callaghan, Pineda, Hargreaves, Heales, and Artuch
Acquisition of data: Yubero, Montero, O'Callaghan, Pineda, Meavilla, Delgadillo, Sierra, Altimira, Pope, Oppenheim, Neergheen, Ghosh, Mills, Clayton, Footitt, Cleary, Jones, and Heales
Analysis and interpretation of data: Yubero, Montero, O'Callaghan, Navas, Mills, Clayton, Hargreaves, Heales, and Artuch
Drafting of the manuscript: All authors
Critical revision of the manuscript for important intellectual content: All authors
Study supervision: Artuch

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CASE REPORT

Pitfalls in Diagnosing Neuraminidase Deficiency: Psychosomatics and Normal Sialic Acid Excretion

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Abstract Neuraminidase deficiency (mucolipidosis I, sialidosis types I and II, cherry-red spot myoclonus syndrome) is a lysosomal storage disorder with an expanding clinical phenotype. Here, we report the striking diagnostic history of late-onset neuraminidase deficiency in two sisters, currently aged 14 (patient 1) and 15 (patient 2).

Patient 1 was referred for evaluation of her vision after a traffic accident. During this examination, nummular cataract, macular cherry-red spot, and optic nerve atrophy were seen. Furthermore, tremors were noticed in her arms and legs. This combination suggested a lysosomal storage disorder. Her family history revealed an older sister, patient 2, who had a long history of unexplained neurologic symptoms; she was under unsuccessful treatment for conversion disorder. Patient 2 showed identical ophthalmological findings. In retrospect, she had presented with avascular osteonecrosis of the right femur head at age 9.

Urinary oligosaccharide patterns and enzyme activity revealed neuraminidase deficiency in both patients. Urinary-bound sialic acid levels were normal. Sequencing of *NEU1* demonstrated two known compound heterozygous mutations (c.1195_1200dup p.His399_Tyr400dup; c.679G>A, p.Glu227Arg).

The substantial time window between onset of typical symptoms and diagnosis in patient 2 suggests inadequate awareness of lysosomal storage disorders among clinicians. Of special interest is the observation that normal urinary sialic acid levels do not exclude neuraminidase deficiency. Urinary oligosaccharide screening is essential to diagnosis in such cases. In addition, patient 2 is the fourth case in the literature with a history of femur head necrosis. Bone defects might therefore be an early manifestation of late-onset neuraminidase deficiency.

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Introduction

Neuraminidase deficiency (mucolipidosis I, sialidosis types I and II, sialidase deficiency, cherry-red spot myoclonus syndrome, OMIM 256550) is an autosomal recessive lysosomal storage disorder (LSD) caused by mutations in the *NEU1* gene (Bonten et al. 2000). *NEU1* mutations result in diminished function of the enzyme exo-α-sialidase (EC 3.2.1.18), which leads to the buildup of sialic acid-containing glycoconjugates in tissues and oligosaccharidiuria. Based on the clinical presentation, two extremes of the phenotypic spectrum can be distinguished. The severe, early onset variant (type II) is characterized by coarse facial features, skeletal dysplasia, severe ataxia, and mental retardation (Lowden and O'Brien 1979). The

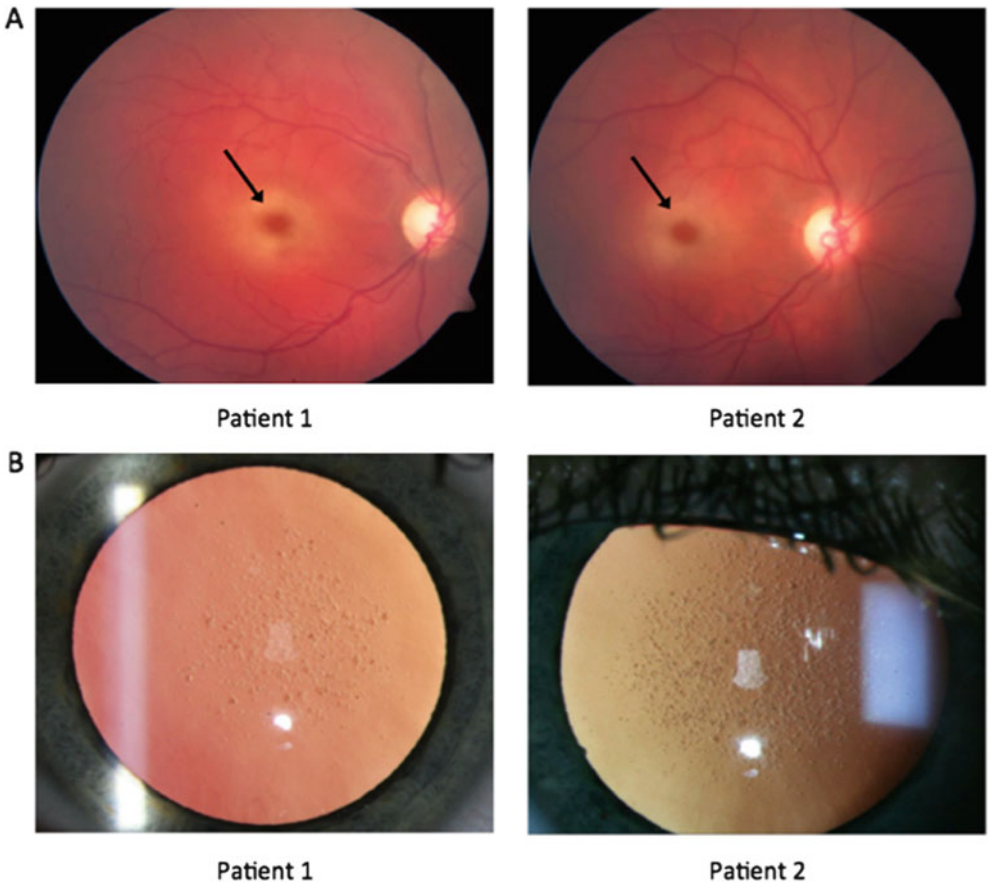

Fig. 1 (**a**) Fundoscopy in patients 1 and 2, at the age of 13 and 14, respectively, showed bilateral (only the *right eye* is shown) cherry-red spot in the macula (*arrow*) and atrophy of the optic nerve. (**b**) Slit lamp examination revealed bilateral nummular cataract in both patients

late onset variant (type I) generally presents between the age of 8 and 25 with progressive myoclonus, ataxia, and visual impairment. A macular cherry-red spot is often seen. Over time, some patients develop seizures and mild cognitive impairment. The clinical spectrum of this disorder is still expanding as genome-wide screening now allows identification of patients with a mild or atypical phenotype (Canafoglia et al. 2014).

Here, we report the diagnosis of late-onset neuraminidase deficiency in two sisters, currently aged 14 (patient 1) and 15 (patient 2). We describe their remarkable diagnostic history, which illustrates pitfalls in the diagnostic process and confirms osteonecrosis as an early manifestation of late-onset neuraminidase deficiency.

Case Reports

Patient 1, the index case, is a 14-year-old girl who first experienced loss of visual acuity at the age of 12. Specifically, impaired perception of contrast and depth led to frequent falls and recurrent trauma. At 13, her worsening eyesight led to a traffic accident resulting in fractures of the lower leg. Ensuing ophthalmological examination revealed impaired best-corrected visual acuity of 0.5, poor stereoscopic vision, macular cherry-red spot (Fig. 1a), partial paleness of the optic disk (Fig. 1a), nummular cataract (Fig. 1b), and upbeat nystagmus in both eyes. Optical coherence tomography of the macula showed clearly hyperreflective and thickened ganglion cell layer of the macula. Based on her history and clinical symptoms, a lysosomal storage disorder was suspected.

Her family history revealed an older sister, *patient 2* (now 15 years old), who was under treatment for conversion disorder. Subsequent examination of patient 2 showed ophthalmological findings identical to her sister (Fig. 1a, b). Her best-corrected visual acuity was limited to 0.3 and 0.5. In retrospect, she had presented with avascular osteonecrosis of the right femur head at the age of 9. Spontaneous revascularization occurred gradually, resulting in mild femur head deformation only (Fig. 2). Her hip pain, weakness of the right leg, unsteady gait, and tiredness, however, worsened gradually. From the age of 13, she experienced progressive loss of visual acuity because of

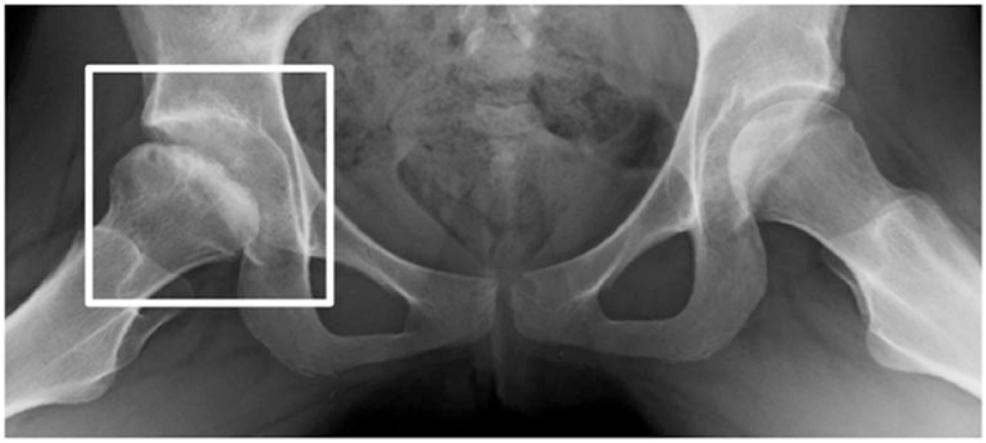

Fig. 2 X-ray of the pelvis in patient 2 at age 12 showed mild flattening and mild sclerosis of the right femur head as a result of avascular osteonecrosis (Legg–Calvé–Perthes syndrome) that started at age 9. Revascularization occurred spontaneously within 3 years, but pain and unsteadiness of the right hip remained

cataract and myopia. Concurrently, she developed mild ataxia and high-frequency intention tremor, affecting all four limbs. A cerebellar cause was suspected but could not be confirmed by neuroimaging or laboratory testing. In the absence of a somatic explanation, her neurologic symptoms were considered to be of psychosomatic etiology. The recent divorce of her parents and her history of osteonecrosis were together considered etiological for her movement disorder. Treatment for conversion disorder was started but proved ineffective. Within a year, walking without support and writing by hand became impossible because of worsening ataxia and myoclonus. Strikingly, a metabolic cause of her symptoms was only suspected upon mentioned ophthalmological examination of her younger sister. At evaluation in our center, neurologic examination of patient 2 showed downbeat nystagmus, severe ataxia, severe intention tremor, and sporadic myoclonus of the limbs and eyelids. The combination of typical ocular and neurologic signs prompted further investigation in both patients.

Biochemical Phenotype and Genotype

Brain MRI showed no intracranial pathology in either. Urinary-bound sialic acid levels measured by mass spectrometry were in the normal range (57 and 36 mmol/creatinine M for patient 1 and 2, respectively; normal range: 1.5–57) (van der Ham et al. 2007). However, urinary analysis by thin-layer chromatography revealed oligosaccharide patterns characteristic for (galacto)sialidosis in both patients (Fig. 3) (Holmes and O'Brien 1979). This finding prompted enzyme activity testing in cultured fibroblast. Activity of *N*-acetyl-neuraminidase was severely impaired (0.5 and 0.4 mmol/mg/h for patient 1 and 2, respectively; normal range: 15–45). Galactosialidosis was excluded by normal galactosidase activity (1,246 and 1,257 nmol/mg/h for patient 1 and 2, respectively; normal range: 600–1,650). Targeted next-generation sequencing (Nijman et al. 2014) of *NEU1* revealed two previously described compound heterozygous mutations (c.1195_1200dup p.His399_Tyr400dup; c.679G>A, p.Glu227Arg) in both patients, confirming neuraminidase deficiency (Bonten et al. 2000; Lukong et al. 2000). Nomenclature is according to HGVS guidelines and is based upon transcript NM 000434.3.

Clinical Management

Our current management focuses on adjustment and rehabilitation. As symptoms worsen during menstruation, both patients receive continuous oral contraception. Furthermore, both patients use clonazepam to reduce myoclonic jerks. No myoclonic seizures have occurred in either.

At present, both patients suffer from worsening eyesight, ataxia, tremor, and action myoclonus, threatening independent functioning.

Discussion

The combination of gait disturbance and loss of vision is a typical presentation for late-onset (type I) neuraminidase deficiency. Despite this typical presentation, the diagnostic delay in patient 2 was long and might have been even longer if distinctive ophthalmological signs such as macular cherry-red spot had not been found in the index patient. During these years, patient 2 was assessed by multiple specialists and underwent therapy for conversion disorder without success. Her diagnostic history shows that accepting a psychosomatic explanation for neurologic

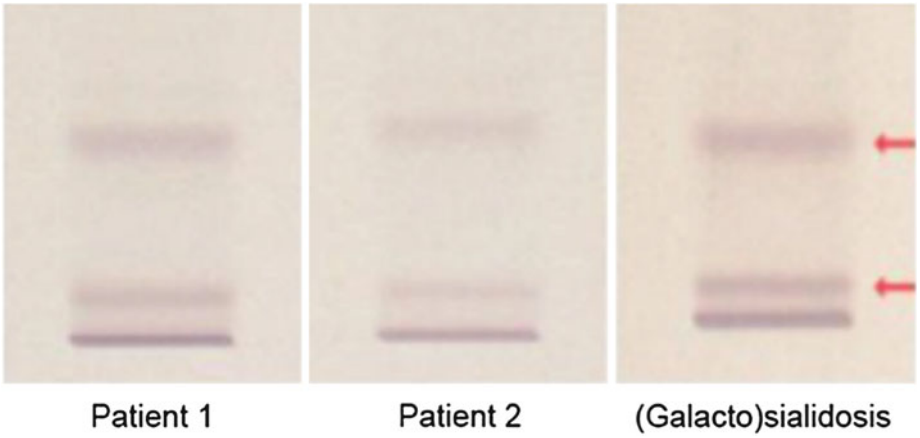

Fig. 3 Thin-layer chromatography of urinary oligosaccharides showed patterns compatible with galactosialidosis or neuraminidase deficiency (sialidosis) in both patients (Holmes and O'Brien 1979). The third panel serves as pathologic standard for both disorders. This method cannot distinguish galactosialidosis from neuraminidase deficiency. Enzyme activity testing in cultured fibroblasts is required for diagnosis

symptoms can postpone identification of a possible somatic etiology. Besides, it suggests inadequate awareness of metabolic disorders among clinicians.

Thin-layer chromatography of urinary oligosaccharides can be labor intensive (van der Ham et al. 2007) and difficult to interpret (Xia et al. 2013). Measurement of urinary sialic acid levels could be a convenient alternative. However, urinary sialic acid levels were not elevated in our patients, which has been reported before (van der Ham et al. 2007; Canafoglia et al. 2014). This underlines the fact that neuraminidase deficiency should be suspected even if sialic acid excretion in the urine is normal. In our patients, thin-layer chromatography of urinary oligosaccharides did show an abnormal pattern, which was key to diagnosis. This traditional diagnostic test should thus remain part of screening panels for storage disorders.

Avascular osteonecrosis is well described in a variety of LSDs, including Gaucher's disease (Aldenhoven et al. 2009) and to a lesser extent Fabry disease (Lien and Lai 2005; Sacre et al. 2010) and Niemann-Pick type B (Wasserstein et al. 2013). Thus far, it has not been considered a clinical sign of neuraminidase deficiency. Recent reports describe three neuraminidase-deficient patients with a history of osteonecrosis of the femur (Urbanski et al. 2014; Canafoglia et al. 2014). Patient 2 also presented with avascular femoral head osteonecrosis. Together this indicates that osteonecrosis can be an early clinical sign of late-onset neuraminidase deficiency.

Synopsis

Diagnosis of neuraminidase deficiency in two sisters, first misdiagnosed with conversion disorder, shows importance of urinary oligosaccharide screening when sialic acid excretion is normal and suggests osteonecrosis as an early symptom.

Compliance with Ethics Guidelines

Conflict of Interest

Imre Schene, Viera Ayuso, Koen van Gassen, Monique de Sain- van der Velden, Inge Cuppen, Peter M. van Hasselt, and Gepke Visser 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.

Details of the Contributions of Individual Authors

Imre Schene drafted the first manuscript.

Viera Kalinina Ayuso is the ophthalmologist who executed the ophthalmological examinations, contributed Fig. 1, and revised the manuscript.

Koen van Gassen executed the genetic investigation that demonstrated the compound heterozygous mutations in NEU1 and revised the manuscript.

Monique de Sain- van der Velden executed the metabolic investigations, contributed Fig. 3 and revised the manuscript.

Inge Cuppen is the pediatric neurologist who executed the neurological examination in our center and revised the manuscript.

Peter M. van Hasselt contributed to the follow-up of the patients and revised the manuscript.

Gepke Visser is the metabolic pediatrician of the patients and coordinated and revised the manuscript.

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CASE REPORT

New Cases of *DHTKD1* Mutations in Patients with 2-Ketoadipic Aciduria

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Abstract 2-Ketoadipic aciduria (OMIM 204750), a defect in the catabolic pathway of tryptophan, lysine, and hydroxylysine, is characterized by elevations in 2-ketoadipic, 2-aminoadipic, and 2-hydroxyadipic acids. Patients with the aforementioned biochemical profile have been described with a wide range of clinical presentations, from early-onset developmental delay, epilepsy, ataxia, and microcephaly to completely normal. This broad range of phenotypes has led some to question whether 2-ketoadipic aciduria represents a true disease state or if the biochemical abnormalities found in these patients merely reflect an ascertainment bias. We present four additional individuals from two families, with 2-ketoadipic aciduria with compound heterozygous or homozygous mutations in *DHTKD1*, three of which remain asymptomatic.

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Competing interests: None declared

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Introduction

Organic acid analysis has identified elevated 2-ketoadipic acid in patients with a wide variety of symptoms ranging from psychomotor retardation, hypotonia, epilepsy, ataxia, and failure to thrive to no clinical phenotype at all. To date over 20 individuals have been reported, about half of whom were asymptomatic (Przyrembel et al. 1975; Fischer and Brown 1980; Duran et al. 1984; Danhauser et al. 2012). With no known genetic etiology, 2-ketoadipic aciduria was thought to represent ascertainment bias with clinical symptoms being coincidental findings (Duran et al. 1984; Danhauser et al. 2012; Saudubray et al. 2012).

In 2012, whole-exome sequencing (WES) of a patient with a biochemical diagnosis of 2-ketoadipic aciduria identified compound heterozygous variants in dehydrogenase E_1 and transketolase domain-containing protein 1 (*DHTKD1*). Sanger sequencing of *DHTKD1* in a second unrelated patient identified a missense mutation on one allele and a nonsense variant on the other (Danhauser et al. 2012). These patients presented with hypotonia and variable degrees of psychomotor delay, speech delay, and attention deficit hyperactivity disorder with an otherwise unremarkable neurological examination. Due to the phenotypic variability associated with 2-ketoadipic aciduria, functional studies in primary fibroblasts from these patients were employed in order to elucidate the genetic etiology which revealed increased levels of 2-ketoadipic acid in cells and medium that was corrected with expression of wild-type *DHTKD1* (Danhauser et al. 2012).

We report mutations in two additional families and highlight the fact that while genetic abrogation of *DHTKD1* can lead to the accumulation of 2-ketoadipic, 2-aminoadipic, and 2-hydroxyadipic acids, this disruption does not always result in an observed clinical phenotype.

Table 1 Summary of the clinical findings of patients with DHTKD1 deficiency

	Patient 1	Patients 2–4	Patient 3 (Danhauser et al. 2012)	Patient 4 (Danhauser et al. 2012)
cDNA (NM_018706.5)	c.2143C>T	c.915G>C	c.1A>G	c.1228C>T
	c.2185G>A	c.915G>C	c.2185G>A	c.2185G>A
Protein (NP_061176)	p.Arg715Cys	p.Gln305His	p.Met1?	p.Arg410*
	p.Gly729Arg	p.Gln305His	p.Gly729Arg	p.Gly729Arg
Elevated urine/plasma metabolites	+	+	+	+
Motor delay	+	–	+	+
Speech delay	+	–	+	+
Microcephaly	+	–	–	+
Hypotonia	–	–	–	+
ADHD	+	–	+	–
Brain MRI abnormalities	–	N/A	–	–
Improvement over time	+	N/A	+	+

DHTKD1 dehydrogenase E1 and transketolase domain-containing protein 1, *ADHD* attention deficit hyperactivity disorder, *MRI* magnetic resonance imaging, *N/A* not applicable

Methods

Amino acid analysis in urine and plasma and urine organic acid analysis was performed in established biochemical genetics laboratories using proprietary methods.

Exome sequencing in both patients was performed prior to the discovery that mutations in *DHTKD1* cause 2-ketoadipic aciduria. WES for patient 1 was performed as a trio with both parents at UCLA Molecular Diagnostics Laboratories, Los Angeles, CA. WES for the second family was performed on the proband and both parents by BGI Americas and analyzed at the University of Colorado Denver. Identified *DHTKD1* mutations were evaluated for possible effects on protein structure and function by MutationTaster, SIFT, PROVEAN prediction, MutationAssessor, and PolyPhen-2. Variant frequencies were derived from the 1,000 genomes database.

Neuropsychological evaluations were performed at CHOC Children's Division of Psychology using NEPSY-II, Preschool Language Scale, 4th Edition; Adaptive Behavior Assessment System, 2nd Ed.; Behavior Assessment System for Children, 2nd Ed.; Behavior Rating Inventory of Executive Function, Preschool Version; Child Development Inventory, Sensory Profile, Wechsler Preschool and Primary Scale of Intelligence, 3rd Ed.; and Woodcock Johnson Tests of Achievement, 3rd Edition.

Case Reports

Our first patient, a girl, was born at full term after a pregnancy that was complicated by maternal hypertension with no postnatal complications to non-consanguineous parents of Filipino and Northern European ancestry. At 15 months of age, she presented with a history of failure to thrive (weight <3rd percentile), seizure-like episodes, and biochemical abnormalities consistent with 2-ketoadipic aciduria. Her height remained between the 10th and 25th percentile (length/weight ratio <3rd percentile) and head circumference was below the 3rd percentile. Of note, maternal head circumference was also below the 3rd percentile. A metabolic assessment revealed elevated levels of plasma 2-aminoadipate (37 μmol/L, nl < 4) with elevated 2-ketoadipate (434 mmol/mol creatinine, nl < 2) and 2-hydroxyadipate (28 mmol/mol creatinine, nl < 2) in urine (Table 1). Over time, she has suffered chronic episodes of headaches/migraines with persistent head tilting, nausea, and emesis. Neurological evaluation was performed and electroencephalogram, magnetic resonance imaging, and magnetic resonance angiogram of the brain were unremarkable. At 24 months of age, she began to show mild developmental delay. Initial neuropsychological evaluations measured less than the 10th percentile in verbal fluency and processing speed. Over the course of a 4-year follow-up, trends of improvement were noted in these areas; however, following the most recent evaluation, she is now below the 1st percentile for auditory attention and was given a diagnosis of a reading disorder.

WES revealed two heterozygous variants, c.2143C>T; p.Arg715Cys and c.2185G>A; p.Gly729Arg, in *DHTKD1*. While the latter variant, c.2185G>A; p.Gly729Arg, was previously reported in two patients with autosomal recessive 2-ketoadipic aciduria (Danhauser et al. 2012), the former variant, c.2143C>T; p.Arg715Cys, is novel. Both

variants have been observed in the general population with minor allele frequencies of 0.05% and 0.18%, respectively.

Our second patient, a girl, was born to consanguineous parents of Maltese origin in 1978. Routine urine screening performed at 6 weeks of age detected increased glutamate. Subsequent amino acid and organic acid testing showed elevated urinary 2-aminoadipic, 2-ketoadipic, and glutaric acids in the proband as well as in two elder brothers, ages 5 and 7. All three affected siblings were described as clinically normal when identified and have been followed over time and remain asymptomatic in adulthood (Wilcken et al. 1980; Wilcken 2014, personal communication).

Data analysis of the proband's exome identified a novel homozygous variant in *DHTKD1*, c.915G>C; p.Gln305His. Sanger sequencing showed that the proband's siblings are also homozygous for this variant and that each parent is a carrier. This variant has been observed in the general population with a minor allele frequency of 0.05%.

No other candidate genes were found during the WES analysis for either patient sequenced. All variants identified are predicted by several algorithms to be disease causing (MutationTaster), damaging to protein function (SIFT), deleterious to protein structure (PROVEAN), and probably damaging to protein structure and function (PolyPhen-2) and are predicted to have a high functional impact for c.2143C>T; p.Arg715Cys and c.915G>C; p.Gln305His and a medium functional impact for c.2185G>A; p.Gly729Arg (MutationAssessor).

Discussion and Conclusions

2-Ketoadipic acid is formed through three routes: from 2-aminomuconate in the oxidation of tryptophan, from 2-aminoadipic acid formed in the mitochondrial oxidation of lysine via the saccharopine pathway, and from 2-aminoadipic acid formed in the brain-specific, peroxisomal oxidation of lysine via the pipecolic acid pathway (Posset et al. 2015). The subsequent conversion of 2-ketoadipic acid to glutaryl-CoA, which is common to all three pathways and has long been assumed to involve a multienzyme complex similar to those that act on pyruvate, branched-chain keto acids and 2-ketoglutarate; indeed, the activities of the 2-ketoglutarate and 2-oxoadipic dehydrogenases in porcine heart could not be separated (Hirashima et al. 1967).

Several subjects have been described with 2-ketoadipic aciduria, including a 14-month-old girl with hypotonia, intermittent metabolic acidosis, and developmental delay (Przyrembel et al. 1975), a 14-year-old retarded boy and his intellectually normal sister (Wilson et al. 1975), a 9-year-old boy with a mild learning disability and his normal brother (Fischer et al. 1974; Fischer and Brown 1980), a 10-year-old retarded girl (Casey et al. 1978), a 9-year-old retarded boy with a history of seizures (Duran et al. 1984), a 7-year-old girl with cerebellar ataxia (Vianey-Liaud et al. 1985), two unrelated children with developmental delay (Danhauser et al. 2012), and three apparently normal siblings detected by a newborn screening program in Australia (Wilcken et al. 1980; Wilcken 2014, personal communication), whose genetic findings and outcome are presented here. Evidence for defects in the metabolism of 2-ketoadipic acid in these patients have included increased excretion of 2-ketoadipic and/or 2-hydroxyadipic acid in urine, often together with 2-aminoadipic acid, and delayed clearance of 2-aminoadipic and 2-hydroxyadipic after an oral lysine load. These studies are not strictly comparable because of the variability in biochemical testing among them. Recent studies on two unrelated patients with 2-ketoadipic aciduria revealed mutations in *DHTKD1*, a nuclear gene that encodes a protein similar to the E_1 component of a 2-ketoglutarate dehydrogenase complex (Danhauser et al. 2012). Our data extends these observations, showing mutations in the same gene in several additional patients with 2-ketoadipic aciduria. One of these patients has symptoms, but the other three are asymptomatic adult siblings.

The finding that all three patients with the same mutation, i.e., c.2158G>A; p.Gly729Arg (Danhauser et al. 2012; this paper), are symptomatic may suggest a relation to the clinical phenotype. The population frequency of this variant may approach one in 650 (188 of 120,740 chromosomes in the Exome Aggregation Consortium (ExAC) cohort as of April 2015). While this frequency seems high, it could be consistent as an underlying cause of mild developmental delay as observed in these patients. The other known sequence variants appear to be much less frequent; c.915G>C; p.Gln305His was present only once out of 121,316 chromosomes examined, and c.2143C>T; p.Arg715Cys was present in two of 121,298.

While 2-ketoadipic aciduria can occur without apparent clinical manifestations in childhood, and the relation to clinical manifestations may be due only to sampling bias, it is difficult to exclude the possibility that asymptomatic individuals will develop a clinical phenotype later in life or that particular mutations cause clinical disease and others do not. Indeed, knockdown of *DHTKD1* expression in a variety of cell lines suggests that this protein plays a role in mitochondrial function and energy production (Xu et al. 2013); and a proteomic/metabolomic study in mice implicated *DHTKD1* in glucose homeostasis through its connection to 2-aminoadipic acid (Wu et al. 2014). These studies suggest the possibility that further phenotypes, perhaps with a later onset, may be associated with genetic abrogation of *DHTKD1*. Of particular note is the implication of a c.1455T>G; p.Tyr485* mutation in *DHTKD1* as the cause

of dominantly inherited Charcot-Marie-Tooth disease in one family in China (Xu et al. 2012). The c.1228C>T; p. Arg410* mutation found in a 2-ketoadipic aciduria patient (Danhauser et al. 2012) might affect the E_1 protein in a similar manner as p.Tyr485*, and it will be of interest to determine if a similar neurological phenotype emerges in this patient or in the parent with the same mutation.

In summary, we present four additional patients with 2-ketoadipic aciduria with variants in *DHTKD1*. Three of these individuals have remained phenotypically normal in to adulthood, while the other shows clinical characteristics similar to the previously reported patients with 2-ketoadipic aciduria (Danhauser et al. 2012). These findings support the genetic etiology of 2-ketoadipic aciduria and continue to highlight the phenotypic variability historically seen in the reported patients.

Acknowledgments We thank the families for their steadfast commitment to participate in this study, the generous support of the Fry Family Foundation to CHOC Children's Metabolic Program, and Dr. Bridget Wilcken for her cooperation and support.

Synopsis

Our paper highlights that while genetic abrogation of *DHTKD1* leads to the accumulation of 2-ketoadipic, 2-aminoadipic, and 2-hydroxyadipic acids resulting in a biochemical diagnosis of 2-ketoadipic aciduria, this disruption does not always result in an observed clinical phenotype.

Compliance with Ethics Guidelines

Conflict of Interest

Ashlee Stiles, Leah Venturoni, Grace Mucci, Michael Woontner, Stephen Goodman, and Jose Abdenur declare that they have no conflict of interest.

Informed Consent

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

Animal Rights

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

Contributions of Individual Authors

Ashlee Stiles and Leah Venturoni contributed equally to the manuscript. Each helped to draft the case report presented.

Grace Mucci performed all neuropsychological testing and provided detailed summary of the data for the case report.

Naser Elbalalesy provided direct patient care, performed neurological examinations, and interpreted neurology test results.

Michael Woontner helped to perform data analysis of WES and edited the manuscript to provide critical feedback.

Steve Goodman and Jose Abdenur contributed equally to the manuscript. Each helped to interpret the biochemical test results and draft and review the case report providing critical feedback.

Competing Interests

The authors have no competing interests.

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RESEARCH REPORT

Screening Mucopolysaccharidosis Type IX in Patients with Juvenile Idiopathic Arthritis

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Abstract Mucopolysaccharidosis is a group of lysosomal disorders of a deficiency of specific enzyme required for glycosaminoglycan degradation. Mucopolysaccharidosis type IX is the rarest form of mucopolysaccharidosis. To date, only four patients have been reported. The first reported patient had mild short stature and periarticular soft tissue masses; the other reported patients are clinically indistinguishable from juvenile idiopathic arthritis. In the present study, we screened mucopolysaccharidosis type IX among patients with juvenile idiopathic arthritis with hyaluronidase enzyme assay. One hundred and eight patients with JIA and 50 healthy age-matched control subjects were enrolled in the study. Among all patients, none had deficient hyaluronidase activity. Though serum Hyal-1 activity was significantly increased in JIA patients, compared with control subjects ($p < 0.000$), no correlation was found between CRP, ESR, and Hyal-1 activity ($p = 0.187$). In conclusion, the data reported in our study indicates that systemic metabolic investigation for hyaluronidase activity is not recommended in all patients with JIA.

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Introduction

Mucopolysaccharidosis (MPS) is a rare group of lysosomal disorders of glycosaminoglycan (GAG) catabolism, caused by a deficiency of a specific enzyme required for GAG degradation (Wraith 1995). These disorders are associated with a progressive accumulation of different types of GAGs in the lysosomes of various organs compromising their function. The clinical features of MPS differ depending on the specific enzyme deficiency, but major clinical features are mainly facial dysmorphism; hepatosplenomegaly; cardiac, respiratory, and skeletal involvement; and neurological, hematological, and ocular symptoms (Neufeld and Muenzer 2001).

Mucopolysaccharidosis type IX is caused by the deficiency of enzyme hyaluronidase 1 (Hyal-1) which degrades hyaluronan (hyaluronic acid) (HA). MPS type IX is the rarest form of MPS, and to date only four patients were reported. The first patient was reported in 1996. She had periarticular soft tissue masses, mild short stature, and acetabular erosions without classical MPS features like neurological or visceral involvement (Natowicz et al. 1996). Other three patients were the children of consanguineous Middle Eastern parent, and all present as juvenile idiopathic arthritis (JIA) (Imundo et al. 2011).

All reported patients with MPS type IX were presented with joint and skeletal problems; therefore, MPS type IX can be easily misdiagnosed as JIA. There is no information in the literature concerning this prevalence investigation. The aim of the present study is to assess the prevalence of

MPS type IX among a group of Turkish patients diagnosed with JIA.

Material and Method

Study Design and Population

This was a cross-sectional study of 108 JIA patients attending the Outpatient Pediatric Rheumatology Clinic of Cerrahpasa Medical Faculty Children's Hospital. Patients' JIA diagnosis was confirmed by a child rheumatologist using ILAR criteria (Petty et al. 2004). The patients were selected by random sampling. The patients with other diagnoses except JIA, patients who were not definitely diagnosed with JIA, those diagnosed with systemic JIA, and those who refused to join the study were excluded from the study. Age, gender, age at diagnosis, detailed family history (including consanguinity, additional affected siblings), subtype of JIA, medications, and response to medical treatment were recorded. All patients underwent a careful physical examination including height, weight, arthritis, periarticular masses, scoliosis, hepatosplenomegaly, dysmorphic features, and ophthalmologic evaluation. Last laboratory investigations including complete blood count, glucose, liver transaminases, urea, creatinine, creatinine phosphokinase, C-reactive protein (CRP), erythrocyte sedimentation ratio (ESR), antinuclear antibodies (ANA), anti-double-stranded DNA (anti-ds-DNA), rheumatoid factor (RF), and HLA B27 were recorded.

Serum samples for hyaluronidase analyses from patients were collected at the time of attendance to outpatient clinic and were stored at −80°C until analysis. 50 age-matched healthy volunteers served as control group.

Hyaluronidase Assay

Serum Hyal-1 activity was assessed for both patient and control groups. Hyal-1 activity was measured as described by Natowitz and Wang (Natowicz and Wang 1996). Ten microliter serum was incubated with 250 µl buffered substrate solution (0.10 mol/l sodium formate, pH 3.9, containing 0.1 mol/l sodium chloride, 250 mg/l HA, and 1.5 mmol/l saccharic acid 1,4-lactone) for 4 h at 37°C. The enzyme reaction was specifically terminated by addition of 50 µl 0.8 mol/l potassium tetraborate at pH 9.1 to each sample. The tubes were heated for 3 min in a boiling water bath and cooled in tap water. 1.5 ml *p*-dimethylaminobenzaldehyde reagent, prepared as described by Reissig et al., was added, then vortexed, and heated at 37°C for 20 min, briefly centrifuged and read using a colorimeter at 585 nm (Reissig et al. 1955). Consequently, the amount of reaction product by reducing *N*-acetylglucosamine termini was determined. Blanks for the reaction consisted of tubes in which the buffered substrate was incubated for 4 h at 37°C in the absence of serum which subsequently received potassium tetraborate and then serum and were then treated as described above. A standard curve was formed by using known concentrations of *N*-acetylglucosamine. For this method, 1 unit of Hyal-1 activity was defined as the production of 1 µmol/min of reaction product (reducing terminal *N*-acetylglucosamine) at 37°C.

This study was approved by the Local Ethics Committee of the University of Istanbul Cerrahpasa Medical Faculty (protocol: 83045809/604/02-15538, 05/06/2014), and an informed consent was obtained from all parents of the children included in this survey.

Statistical Analysis

Data were expressed using descriptive statistics such as mean and SD for continuous variables and number and percentage for categorical variables. A one-sample *t*-test was carried out for the quantitative estimation of Hyal-1 activity. Comparison between the groups was carried out by independent sample *t*-test. Statistical analyses of the parameters were performed by using the Statistical Package for the Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA). *p*-value <0.05 was considered statistically significant.

Results

One hundred and eight patients with JIA were enrolled to the study. Patients' clinical and demographic characteristics are given at Table 1. Among all patients enrolled in the study, none had deficient hyaluronidase activity. Though serum Hyal-1 activity was significantly increased in JIA patients, compared with control subjects ($p < 0.000$), no correlation was found between CRP, ESR, and Hyal-1 activity ($p = 0.187$) (Table 2).

Discussion

In the present study, we screened for Hyal-1 deficiency for the diagnosis of MPS type IX among patients diagnosed with JIA. Among 108 patients with JIA, no patient totally lacked serum Hyal-1 activity, so we were unable to detect any patients with MPS type IX. Despite that, the abnormality found was the increased level of plasma Hyal-1 level related to the control group. These results suggest that the prevalence of screened MPS type IX in association with JIA is low.

Only four patients were reported with MPS type IX to date. Natowitz described the first patient in 1996 with

Table 1 Demographic and clinic characteristics of JIA patients and control subjects

Variables	Patients	Controls
Sex (male/female)	46/62	23/27
Age (months)	129.01 ± 54.51	129.67 ± 51.25
Oligoarticular JIA (%)	55/108 (52.3)	
Polyarticular JIA (%)	43/108 (39.8)	
Enthesitis-related JIA (%)	10/108 (9.2)	
Responsive to medication (%)	104/108 (96.2)	
Scoliosis (%)	4/108 (3.7)	
Recurrent otitis media (%)	3/108 (2.7)	
Minor dysmorphic appearance (%)	12/108 (11.1)	
Mental retardation (mild) (%)	3/108 (2.7)	

Table 2 Hyaluronidase enzyme activity of JIA patients and control group

	JIA patients	Control subjects
Age (month)	129.89 ± 53.6	133 ± 51.5
Patients	108	50
Hyaluronidase activity (mU/l)	3,127.7 ± 564.5	2,525.4 ± 669.3
p-value	$p < 0.000$	

Values are mean ± SD

multiple periarticular soft tissue masses, popliteal cysts, joint effusions, acetabular erosions, mild short stature, flat nasal bridge, bifid uvula, submucosal cleft palate, and recurrent episodes of otitis media (Natowicz et al. 1996). The other three patients, reported by Imundo et al. (2011), were siblings of consanguineous Middle Eastern parents. These patients were clinically indistinguishable from JIA; none of the patients had cutaneous swelling, otolaryngeal problems, or short stature (Imundo et al. 2011). Triggs-Raine demonstrated the molecular basis of MPS type IX that is caused by mutations in HYAL1, a gene encoding hyaluronidase (Triggs-Raine et al. 1999). Mouse models of human MPS type IX revealed that HYAL1 mutations resulted in osteoarthritis and not surprisingly that all mice appeared normal with no evidence of skeletal defects and no organomegaly (Martin et al. 2008). Another study revealed that HYAL2-deficient mouse exhibited skeletal and hematological abnormalities, especially on frontonasal and vertebral bone formations (Jadin et al. 2008). Because of the indistinguishable clinical features of the disease and lack of the diagnosing criteria of MPS type IX, all subtypes of JIA patients were included in the study, randomly. MPS type IX is the rarest form of MPS, but apparently the disease characterizes rheumatologic features, and none of the reported patients showed any of MPS findings. Although high consanguinity rate and some skeletal abnormalities including scoliosis were defined in our study group, we were unable to identify any patient with deficient Hyal-1 activity, therefore with MPS type IX.

Studies exploring plasma or synovial fluid HA levels can be a marker for rheumatoid arthritis (RA), especially as a reflection of synovial involvement and inflammation in adult patients. Deficient hyaluronidase enzyme activity was not detected in any of these patients (Goldberg et al. 1991; Paimela et al. 1991; Nagaya et al. 1999). Enhanced serum hyaluronidase activity was also described in patients with spondyloarthropathies that was most evident in psoriatic arthritis (Kunder 2010). In another study of 48 patients with bone or connective tissue abnormalities, some with specific diagnosis (mucopolysaccharidoses, Ehlers–Danlos syndrome, achondroplasia, osteogenesis imperfecta, etc.) and some without any specific diagnosis (osteoporosis, dysplasia, skeleton deformities, etc.) were tested for hyaluronidase enzyme levels, and deficient enzyme activity was not detected in these patients, either (Fiszer-Szafarz et al. 2005). Serum Hyal-1 activity was also enhanced in this survey, which was consistent with previous studies. We were unable to define any relationship between serum Hyal-1 activity and other laboratory or clinical findings including the activity, the subtype of JIA, or response to treatment.

Limitations of this study should be underlined. The failure to detect any MPS type IX in our group of patients might be due to the limited sample size; much larger study groups including those with other rheumatologic diseases with therapy-resistant joint and skeletal involvement would be warranted to detect extremely rare MPS type IX.

Conclusions

As being an extremely rare inherited progressive lysosomal storage disorder, there are no definite criteria about which patients should be screened. Ill-defined clinical findings of MPS type IX led us to investigate whether there are unrecognized patients along JIA patients, although only oligoarticular and acetabular involvement has been reported previously

Failure to describe new patients within JIA patients led us to conclude that screening of hyaluronidase activity should be more limited to patients with poor response to standard therapies, with mono-oligoarticular involvement, with additional skeletal manifestations, and having a family history of multiple individuals affected and a history of consanguinity before deciding for the population to be screened for further studies.

Take-Home Message

Systemic metabolic investigation for hyaluronidase activity is not recommended in all patients with JIA.

Details of the Contributions of Individual Authors

Ertugrul Kiykim serves as the guarantor for the article. He accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish. He has been involved in conception, design, analysis, and interpretation of the data and also drafting the article.

Kenan Barut has been involved in conception, design, analysis, and interpretation of the data.

Mehmet Serif Cansever has been involved in analysis and interpretation of the data.

Cigdem Aktuglu-Zeybek has been involved in conception, design, analysis, and interpretation of the data.

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Ozgur Kasapcopur has been involved in conception, design, and interpretation of the data and revising the article critically for important intellectual content.

Compliance with Ethics Guidelines

Conflict of Interest

Ertugrul Kiykim, Kenan Barut, Mehmet Serif Cansever, Cigdem Aktuglu-Zeybek, Tanyel Zubarioglu, Ahmet Aydin, and Ozgur Kasapcopur declare that they have no conflict of interest.

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

All procedures followed were in accordance with the ethical standards of the Local Ethics Committee of Cerrahpasa Medical Faculty and with the Helsinki Declaration of 1975, as revised in 2000.

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RESEARCH REPORT

The Pathobiochemistry of Gastrointestinal Symptoms in a Patient with Niemann-Pick Type C Disease

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Abstract The molecular basis of gastrointestinal intolerances in a severe case of Niemann-Pick type C disease was analyzed in an intestinal biopsy specimen. The enzyme activities of intestinal sucrase-isomaltase and maltase-glucoamylase are reduced in the patient, while that of lactase is comparable to the control. The association of SI with lipid rafts is reduced in the patient's biopsy as a consequence of altered composition of membrane microdomains. As association with lipid rafts influences the intracellular transport and the enzyme activities of sucrase-isomaltase and maltase-glucoamylase, these data explain reduced carbohydrate digestion in the intestinal lumen and delineate the effect of deficient cholesterol and sphingolipid homeostasis in development of gastrointestinal symptoms in NPC patients.

Introduction

Niemann-Pick type C (NPC; OMIM#257220) is a lysosomal storage disease (LSD) that is characterized by intracellular accumulation of unesterified cholesterol in the lysosomes and late endosomes (Patterson et al. 2001). The disease is caused by autosomal recessive mutations in the genes encoding the proteins NPC1 and NPC2, which act together in transporting cholesterol out of the lysosome into cellular membranes including ER, Golgi, and the plasma membrane. Cholesterol exerts many of its functions via membrane microdomains known as lipid rafts, which are enriched in cholesterol and sphingolipids (Lindner and Naim 2009). Accumulation of these lipids in the endosomes/lysosomes may elicit altered lipid raft composition and function in diverse cells in which these membrane structures play pivotal roles.

In many LSDs including NPC, gastrointestinal (GI) symptoms are early and common manifestations where the cellular homeostasis of lipids especially cholesterol and different sphingolipids is affected (Banikazemi et al. 2005; Bernstein et al. 2010). The GI symptoms include diarrhea, nausea, bloating, abdominal pain, and weight loss which resemble the symptoms in disaccharidase deficiencies (Jacob et al. 2000; Amiri and Naim 2012). We have previously shown that cholesterol- and sphingolipid-enriched lipid rafts are essential for polarized sorting of the GI enzymes sucrase-isomaltase (SI; EC 3.2.1.10/48; SUC for sucrase and IM for isomaltase) and dipeptidyl peptidase 4 (DPPIV or CD26; EC 3.4.14.5) to the apical surface in intestinal epithelium (Jacob et al. 2000; Alfalah et al. 2002). Noteworthy, the catalytic activities of SUC and IM within SI are substantially increased upon association of SI with lipid rafts (Jacob and Naim 2001).

This strongly suggests that an unbalanced lipid homeostasis in NPC or other LSDs can be associated with digestive and subsequent absorptive malfunction in the intestine due to impaired trafficking and reduced lipid raft association of SI and possibly other disaccharidases, such as maltase-glucoamylase (MGAM; EC 3.2.1.20/3). These effects could additionally increase the severity of the overall symptoms observed in patients who are subjected to treatment with miglustat or *N*-butyldeoxynojirimycin, an iminosugar analogue of glucose used in treatment of Gaucher disease and NPC. Miglustat inhibits glucosylceramide synthase and thus prevents glycosphingolipid (GSL)

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accumulation in lysosomes via substrate reduction therapy (Cox et al. 2000; Rosenbaum and Maxfield 2011). Due to its chemical structure, miglustat binds directly to the catalytic sites of intestinal α-glucosidases, such as SI and MGAM, and inhibits their activity at μM concentrations (Amiri and Naim 2014).

Materials and Methods

Materials

Disaccharides sucrose, isomaltose, maltose, and lactose; proteinase inhibitors pepstatin, leupeptin, antipain, and phenylmethanesulfonyl fluoride; and trypsin inhibitor aprotinin were purchased from Sigma (Steinheim, Germany) or Carl Roth GmbH (Karlsruhe, Germany). PVDF membrane and DTT were purchased from Carl Roth GmbH (Karlsruhe, Germany). Lubrol WX was obtained from MP Biomedicals (Eschwege, Germany). Molecular weight standards for SDS-PAGE and SuperSignal® West Femto Maximum Sensitivity Substrate ECL reagents were purchased from Thermo Scientific GmbH (Schwerte, Germany). Glucose oxidase-peroxidase reagent for colorimetric glucose detection was purchased from Axiom (Worms, Germany).

Antibodies

The monoclonal mouse antibodies against sucrase (HBB2/614/88) and isomaltase (HBB3/705/60) (Hauri et al. 1985) were generously provided by Prof. Dr. H. P. Hauri (Biocenter, Basel, Switzerland) and Prof. Dr. E. Sterchi (University of Bern, Bern, Switzerland). Monoclonal antibodies mlac6 and mlac10 (Maiuri et al. 1991) for immunoblotting of lactase-phlorizin hydrolase were generously provided by Prof. Dr. D. Swallow (University College London, United Kingdom). The secondary horseradish peroxidase-conjugated anti-mouse antibody was purchased from Thermo Fisher Scientific (Bonn, Germany).

Patient and Processing of Intestinal Biopsy Specimens

The NPC patient is a girl, born in 1999, with severe gastrointestinal symptoms. Using Sanger sequencing, compound heterozygosity for the mutations P1007A (c.3019C>G) and L1244P (c.3731T>C) in the gene encoding NPC1 was already identified. Control tissue was obtained from a non-NPC patient who underwent biopsy procedure for diagnostic purposes. The biopsy specimens (>10 mg) were taken under anesthesia and were frozen at −80°C immediately.

Protein Analysis

Biopsy specimens were homogenized in 0.55% Lubrol WX in PBS on ice using a Potter-Elvehjem homogenizer followed by passing through 26G needle for 20 times. The lysis was then continued by gentle rotation at 4°C for 1 h. After removing the cell debris by centrifugation at 1,000×*g*, a part of the supernatant was aliquoted as the total lysate, and the rest was subjected to ultracentrifugation at 100,000×*g* for 1 h at 4°C to isolated lipid rafts (pellet) from the non-lipid rafts (supernatant) fractions. Total lysates and raft/non-raft fractions were resolved by SDS-PAGE and the different disaccharidases were detected by immunoblotting.

The protein bands of the Western blots were quantified using the Quantity One® software from Bio-Rad Laboratories GmbH (Munich, Germany).

Enzymatic Activity Measurement

The biopsy specimens were processed according to Dahlqvist (Dahlqvist 1968) using sucrose, isomaltose, maltose, and lactose as substrates. Briefly, samples were incubated 1:1 with 0.056 M of the respective disaccharide for 1 h at 37°C. The amount of the glucose generated by disaccharidase activity was assessed by colorimetric method using glucose oxidase-peroxidase reagent from Axiom.

Results and Discussion

In this report we addressed the correlation between GI symptoms and the association of disaccharidases with lipid rafts in a biopsy specimen from an NPC patient. The patient is a 15-year-old girl, who is compound heterozygous for the mutations c.3019C>G and c.3731T>C within the NPC1 gene (NM_000271) that result in the amino acid exchanges P1007A and L1244P. She shows the late infantile type of the disease. Her development was normal until the age of 3.5 years, when she started stuttering and stumbling. Her motoric and cognitive skills regressed and she developed cataplexy and epilepsy at the age of 9. NPC was diagnosed 2 years later and she was directly treated with miglustat. The progression of the disease slowed down, but she developed severe GI symptoms that resemble those in carbohydrate malabsorption resulting in a strong weight loss. A duodenal biopsy specimen was taken to assess the basis of the GI symptoms. The activities of the enzymes SUC, IM, and MGAM were below the normal range, while that of lactase (lactase-phlorizin hydrolase; EC 3.2.1.68/108) was normal (Table 1). Western blotting revealed protein bands corresponding to SUC and IM

Table 1 Enzymatic activities and protein expression levels of major intestinal disaccharidases

Enzyme	Activity (IU/g)		Protein expression in NPC (% of control)
	Control range	NPC	
Sucrase	40–136	28	90
Isomaltase	35–123	15	80
Maltase-glucoamylase	140–298	94	–
Lactase	25–64	26	96

An intestinal biopsy specimen of the NPC patient was homogenized and used for determination of enzyme activities of sucrase, isomaltase, maltase-glucoamylase, and lactase. Expression levels of these enzymes were detected by immunoblotting (Fig. 1a) and quantified densitometrically

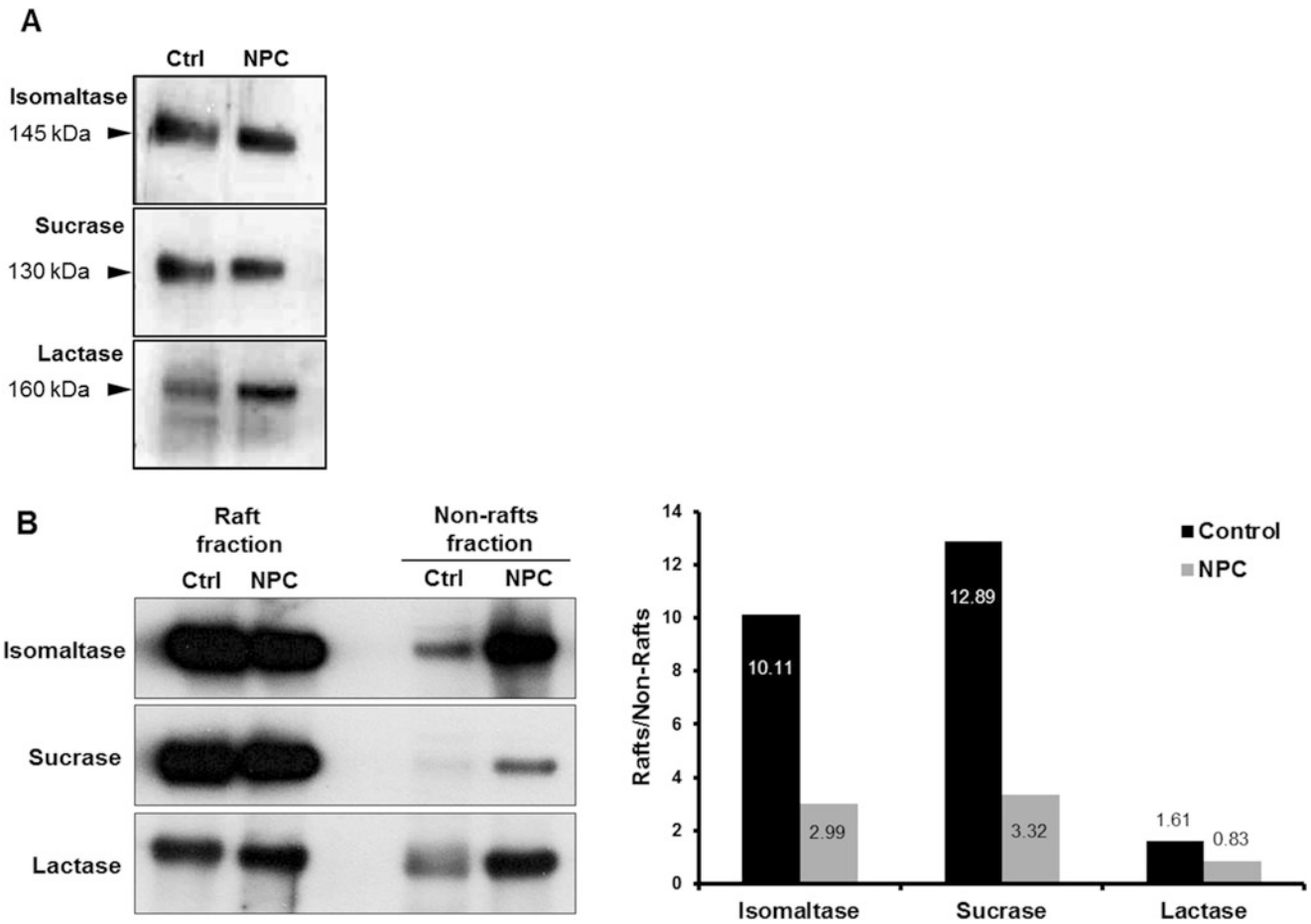

Fig. 1 Protein expression and lipid raft association of major intestinal disaccharidases. Immunoblot analysis of (**a**) total lysate and (**b**) lipid raft and non-raft fractions prepared from biopsy specimens of an NPC patient in comparison to a non-NPC control patient using Lubrol WX detergent. Lipid rafts were isolated by centrifugation at 100,000*g* for 1 h at 4°C. The ratio of raft/non-raft association of the analyzed disaccharidases in the NPC patient versus the control indicates a more substantial decrease in lipid raft association for sucrase and isomaltase than lactase

similar to those in the control counterparts (Fig. 1a), while their enzymatic activities were 30% and 57%, respectively, below the activity range in the controls (Table 1, Fig. 1a). This inconsistency suggests that a posttranslational processing event in the NPC patient may account for these variations.

We therefore examined whether the accumulation of cholesterol and sphingolipids in NPC influences the

association of SI with lipid rafts. Strikingly, these associations were substantially reduced by 3.9-fold for SUC and 3.4-fold for IM in comparison to the healthy control (Fig. 1b, c). Since the trafficking and sorting of SI to the apical membrane in epithelial cells depend on its association with cholesterol- and sphingolipid-enriched lipid rafts (Jacob and Naim 2001; Alfalah et al. 2002), the results shown here clearly indicate that sorting of SI is markedly impaired and its expression at the apical or microvillus membrane is greatly diminished. Unfortunately, the limited availability of biopsy material has hampered further investigation on the expression levels of SUC and IM in the brush border in the biopsy sample. Another role for the lipid rafts in conjunction with SI is regulatory relative to the activities of SUC and IM, which increase approximately threefold when SI is associated with lipid rafts (Wetzel et al. 2009). As shown here, the association of SUC and IM is markedly reduced, compatible therefore with a decrease in their activities. Altogether, we propose that the reduced delivery of a less-active SI to the microvillus membrane in the patient's enterocytes elicits carbohydrate malabsorption and appearance of GI symptoms in the NPC patient.

In biological membranes, cholesterol and sphingolipids are often associated with each other, and it has been suggested that metabolism of either one of these lipids in different LSDs results in an impaired localization and function of both of them (Aguilera-Romero et al. 2014). NPC disease offers a support to this view, since the impaired cholesterol transport due to mutations in the NPC1 gene leads to accumulation of cholesterol and subsequently sphingolipids in late endosomes and lysosomes (Parkinson-Lawrence et al. 2010). An imbalance in cholesterol and sphingolipid composition affects the overall function and transport of lipid raft-associated proteins such as SI and possibly also MGAM. Although MGAM association with lipid rafts is not demonstrated yet with certainty, its high level of structural, functional, and trafficking similarities with SI (Nichols et al. 2003) suggests that its function could be impaired as well due to aberrant lipid raft composition.

Finally, the association of lactase with lipid rafts was also reduced, but to a lesser extent than SUC and IM (2.0-fold) (Fig. 1b, c). However, since lactase does not depend on lipid rafts in its trafficking (Jacob and Naim 2001) or function, any alterations in the association of lactase with lipid rafts remain without consequences on enzymatic levels as shown here and on the patient's tolerance toward lactose.

In conclusion, the current study has delineated the pathobiochemical rationale behind occurrence of GI symptoms in LSDs like NPC, relative to the unbalanced lipid homeostasis in the intestinal epithelial cells. Based on these data, complementary assessments of intestinal disaccharidases in addition to low-carbohydrate diets for managing the GI intolerances in similar cases can be recommended. By virtue of our results, the patient described here was subjected to low-carbohydrate diet that resulted in a substantial improvement of her quality of life.

It should be noted that the GI side effects vary between different patients and variations in the responsiveness of LSD patients to treatment with miglustat have been observed. Therefore, future studies should focus on the elucidation of genotype/phenotype relationships and the extent of alterations in the intracellular lipid composition elicited by a particular phenotype in order to understand the basis for the severity of GI symptoms in NPC patients.

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Take-Home Message

Gastrointestinal symptoms observed in NPC patients can be due to reduced enzyme activities and lipid raft association of major intestinal disaccharidases.

Compliance with Ethics Guidelines

Conflict of Interest

Mahdi Amiri, Eva-Maria Kuech, Hadeel Shammas, Gabi Wetzel and Hassan Y. Naim 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.

Details on the Contributions of Individual Authors

Mahdi Amiri, Eva-Maria Kuech, Hadeel Shammas and Gabi Wetzel have equally contributed to designing and performing the experiments, analyzing the data and drafting the manuscript. Hassan Y. Naim has designed the concept of the study and written the final manuscript.

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RESEARCH REPORT

Improvement of Diffusion Tensor Imaging (DTI) Parameters with Decoppering Treatment in Wilson's Disease

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Abstract *Objective*: This study was undertaken to analyse serially the effects of decoppering therapy on the clinical features, disability and MRI brain including DTI metrics in patients with Wilson's disease.

Methods and Results: Thirty-five patients with clinically and serologically confirmed neuropsychiatric form of Wilson's disease (WD) on decoppering therapy were followed for a minimum duration of 1 year with serial assessment of their clinical features, disability status and serial MR imaging of the brain including DTI. The cohort included 18 treatment-naïve patients and 17 patients already on decoppering therapy (M/F = 2.18:1). The mean age at which they underwent baseline assessment for this study was 18.6 ± 7.6 years, and follow-up assessment was done after a mean duration of 23.5 ± 8.8 months (range, 12 to 45 months). Along with the overall clinical improvement noted at follow-up, the disability assessed using Chu staging and MSEADL showed significant reduction in the number of patients with severe disability and the mean NSS reducing from 9.74 to 6.37 ($p = 0.002$). The mean MRI scores showed significantly reduced disease burden from a baseline score of 5.9 (±4.2) to 4.9 (±4.7) in follow-up scans ($p < 0.05$). Voxel-wise comparison of serial DTI metrics on TBSS (tract-based spatial statistics) analysis showed that the entire cohort had significant ($p < 0.05$) improvement in all the four parameters (MD, FA, DA and RD) indicated by a decrease in MD, DA and RD values and increase in FA values. Comparison of whole-brain white matter DTI measures between pre- and posttreatment did not show any significant difference ($p < 0.05$).

Conclusion: Patients with Wilson's disease on decoppering therapy showed clinical improvement accompanied with improvement in DTI metrics. Quantitative DTI metrics may be used as surrogate markers of clinical status following initiation of medical therapy in Wilson's disease.

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Introduction

Wilson's disease (WD) is a rare disease of copper metabolism which shows neurological symptoms as a presenting feature in 40% of cases (Walshe 1962; Ala et al. 2007). Increased copper deposition in various brain structures causes cellular injury resulting in various clinical manifestations like motor and behavioural disturbances. The disease is diagnosed based on clinical features, the presence of KF ring along with biochemical markers including low serum level of ceruloplasmin, increased serum copper concentration and increased urinary copper excretion (Ala et al. 2007). MRI features in Wilson's disease have been well described in a number of studies (Prayer et al. 1990; Starosta-Rubinstein et al. 1987; Magalhaes et al. 1994; Sinha et al. 2006; van Wassenaer et al. 1996). The disease predominantly involves deep grey matter structures, corpus callosum and brainstem. Involvement of cerebral cortex and hemispheric white matter (WM) is relatively less common. White matter abnormal-

ities are usually seen in the frontal lobes and are associated with cortical abnormalities. The cerebellum is relatively spared (Sinha et al. 2006).

Diffusion tensor imaging (DTI) is a well-established technique for studying structural properties of neural tissue and is based on the properties of water diffusion (Le Bihan et al. 2001; Stejskal and Tanner 1965). In previous studies, abnormalities of diffusion have been noted in WD patients (Sener 2003a, b; Favrole et al. 2006). It has been found useful in studying WD patients in presymptomatic stage (Favrole et al. 2006). In our previous study, we found diffusion measurements are sensitive for detecting abnormalities in normal-appearing WM as well, and they also show correlation with the disability scores of patients (Jadav et al. 2013). However, in previous studies, effects of treatment on DTI measurements have not been evaluated. Effects of decoppering treatment on MRI have been studied using conventional MRI and MR spectroscopy (Sinha et al. 2007a, b; Kim et al. 2006; Tarnacka et al. 2008). Conventional MRI findings show improvement with prolonged treatment but are more often subjective. MRS has been found useful for serial evaluation; however, only a small part of the brain can be sampled by routinely available spectroscopy methods. Whole-brain spectroscopy is a good alternative, but it usually takes a very long time and is technically challenging.

To the best of our knowledge, the effect of decoppering therapy on DTI metrics has not been studied previously. We hypothesize that patients with clinical improvement will show improvement in their WM DTI metrics. Therefore, in this study, we aimed to evaluate the effect of decoppering therapy on clinical features, disability scores, conventional MRI and DTI metrics.

Patients and Methods

Patient Selection

The study included 35 patients with WD recruited from the outpatient services of the Department of Neurology at a tertiary care centre for neuropsychiatric patients. The diagnosis of WD was based on clinical features, the presence of corneal KF ring on slit lamp examination, low serum total copper and ceruloplasmin levels and elevated 24-h urinary copper excretion (Table 1). The details including demographics, history, family pedigree, duration of illness and phenotypic features were noted at the time of initial evaluation. Patients had a neuropsychiatric form of WD, and there was no active hepatic abnormality at the time of initial evaluation, though six patients had past history of subclinical liver dysfunction (elevated liver enzymes). During follow-up, none developed clinical evidence of liver dysfunction. The clinical severity and disability status were scored using the neurological symptom score (NSS), Chu staging and Modified Schwab and England Activities of Daily Living (MSEADL) scores (Schwab and England 1960; Chu 1986; Meenakshi-Sundaram et al. 2002). Ethical approval for the study was obtained from the NIMHANS institutional ethics committee. Written informed consent was obtained from the patients.

Table 1 Details of demographic and clinical features of patients with Wilson's disease

Demographic/clinical parameters	Values
M/F	24:11
Treatment status	
On treatment (%)	18 (51.4)
Drug naïve (%)	17 (48.6)
Age at baseline MRI (years)	18.6 ± 7.64 (R:8–37)
Age at follow-up MRI (years)	20.31 ± 7.58 (R:10–38)
Time period between MRIs (months)	23.48 ± 8.79 (R:12–45)
Involuntary movements (%)	20 (57.1)
Slowness of activities (%)	25 (71.4)
Dysphagia (%)	21 (60)
Walking difficulty (%)	21 (60)
Behavioural disturbance (%)	19 (54.3)
Seizure (%)	6 (17.1)
KF ring (%)	32 (91.4)
Dysarthria (%)	25 (71.4)
Bradykinesia (%)	12 (34.3)
Tremor (%)	14 (40)
Dystonia (%)	16 (45.7)
Chorea (%)	4 (11.4)
Athetosis (%)	2 (5.7)
Myoclonus (%)	2 (5.7)
Rigidity (%)	10 (28.6)
Spasticity (%)	1 (2.9)
Cerebellar signs (%)	7 (20)

Data Collection and Analysis

Their demographic and phenotypic characteristics were noted, and functional assessment was done using disability and impairment scales. Based on the treatment profile at initial evaluation, the cohort included 18 patients who were treatment naive and 17 patients on decoppering treatment for varying periods before the baseline MRI scan. Patients were then followed up longitudinally for a minimum of 1 year with recommended decoppering therapy. Subjects

underwent clinical evaluation and MRI of the brain again after a minimum of one year of decoppering therapy with routine and DTI sequences utilizing similar parameters. Routine investigations to rule out hepatic, renal, haematological and other organ involvement were performed at baseline and follow-up. Analysis of appearance or disappearance of signal changes with decoppering was performed.

Disability and Impairment Assessment

For serially assessing disability and impairment in ADL, three scales were applied at baseline and follow-up, viz. Modified Schwab and England Activities of Daily Living scores (MSEADL) (Schwab and England 1960), Chu staging (Chu 1986) and neurological symptom score (NSS) (Meenakshi-Sundaram et al. 2002).

MRI

MRI was obtained on a Philips Achieva MRI scanner with a superconducting magnet of 3.0 tesla field strength using a 32-channel head coil; standard protocols and methodology were utilized. Those who required sedation for MRI were administered midazolam or propofol. Conventional MR sequences used for the evaluation included T1-weighted (T1W), T2-weighted (T2W) and fluid-attenuated inversion recovery (FLAIR). T1-weighted images were acquired in axial plane (TR/TE = 650/14 ms, NEX-1). T2-weighted (TR/TE = 6,000/120 ms, NEX-1) images were acquired in axial and coronal planes. Fluid-attenuated inversion recovery (FLAIR) (TR/TE/TI = 11,000/120/2,600 ms, NEX-1) sequences were done in axial plane. The slice thickness was 5 mm for all the conventional imaging sequences. The DTI data was obtained from all individuals using single-shot spin-echo echo planar sequence. Imaging parameters included sensitivity-encoding (SENSE) reduction factor, 2.5; TR, 5 s; TE, 65 ms; acquired resolution, 2.2 mm isotropic; 32 noncoplanar gradient directions with b value of 800 s/mm2; and 2 repetitions. Scanning time per diffusion sequence acquisition was approximately 9 min.

Conventional MRI

MRI data was reviewed using a structured assessment for the presence or absence of signal intensity changes within the cerebral tissue on both DWI and FLAIR images. Severity of signal abnormalities on FLAIR images was analysed in those regions usually involved in WD (basal ganglia, thalamus, midbrain, pons, medulla, cerebellum and cerebral WM).

MRI Scoring

This grading system by King et al. (1996) provides a score with zero being a normal scan and a higher number in a scan with severe or marked changes. Structures assessed for grading included frontal, parietal, occipital and cerebellar WM and cortical changes.

DTI Analysis

Data analysis was carried out using FMRIB Software Library tools (www.fmrib.ox.ac.uk/fsl) version 4.1.6. Raw DTI images were preprocessed using "eddy current correction", to correct for distortions due to the gradient directions applied. Fractional anisotropy (FA) and mean diffusivity (MD) maps were generated using DTIFit, part of FMRIB's Diffusion Toolbox (http://www.fmrib.ox.ac.uk/fsl/fdt) that fits a diffusion tensor model at each voxel. All image analysis was performed using FSL 5.2. Group comparison of DTI data was performed by standard procedure of tract-based spatial statistics (TBSS). FA, AD, MD and RD maps were generated using FMRIB's Diffusion Toolbox, after preprocessing the DTI data. Preprocessing included eddy current correction and motion correction (by linear registering to the non-diffusion-weighted image using FLIRT). Group-wise voxel-based statistical analysis of FA was performed using TBSS. Here, individual subject's skull-stripped FA images were aligned to the MNI152 standard space using the nonlinear registration method, followed by the creation of group mean FA skeleton by thinning mean FA volumes (FA > 0.2 overlaid with the mean FA image). The mean FA skeleton represents the centres of all tracts common to the entire group of subjects. Each subject's aligned FA data were then projected onto the mean FA skeleton, and the resulting data was fed into voxel-wise paired sample testing. A voxel-by-voxel permutation nonparametric test (5,000 permutations) was used to assess group-related differences using threshold-free cluster enhancement, which avoids using an arbitrary threshold for the initial cluster formation. In addition to FA, MD, AD and RD were also compared using TBSS in an analogous fashion. The null distribution was built up over 5,000 permutations, and significance was tested at $p < 0.05$ corrected for multiple comparisons. A similar process of nonlinear registration and voxel-wise comparison was followed for determining the differences in MD, RD and DA maps. Results were expressed at $p < 0.051$ (family-wise error corrected) (Smith et al. 2006; Smith and Nichols 2009).

The whole-brain and whole-brain white matter DTI metrics such as FA, MD, RD and DA were extracted from

each subject's aligned FA data and projected skeletonized DTI image files, respectively, which were generated during the TBSS processing pipeline using command line operations as implemented in the FSL software programme.

Statistics

Data analysis was done using SPSS. Groups were compared using Student's *t*-test/Fisher's exact probability test. Follow-up data was analysed using paired *t*-test and McNemar's test.

Results

Demography and Clinical Features

Thirty-five patients of WD with predominant neurological features underwent evaluation initially. The details are mentioned in Table 1. There were 24 males and 11 females. The mean age at onset of disease of this cohort was 15.7 ± 6.6 years (median age, 14; range, 7–31 years). The mean age at which they underwent baseline MRI of the brain for this study was 18.6 ± 7.6 years, and they underwent a follow-up MRI of the brain at a mean age of 20.3 ± 7.6 years. The follow-up MRI was performed after 23.5 ± 8.8 months (range, 12–45 months) of follow-up.

None of the patients worsened during the follow-up period of almost 2 years: 32 patients (91.4%) had an improving course, and three (8.6%) had an unchanged course. Seventeen (48.6%) patients were drug naïve ($n = 17$), while 18 (51.4%) patients were already on decoppering therapy at the time of recruitment. All patients were given decoppering treatment with penicillamine and zinc individualized based on other organ involvement and tolerance. Twenty-four patients (68.6%) were both on D-penicillamine and zinc therapy, while 11 of them (31.4%) were only on zinc therapy.

Though there was improvement in the severity, the same symptoms dominated the clinical features during follow-up also. Corneal KF rings, noted in 32 patients initially, were detectable in 22 patients during follow-up. Sunflower cataract in one patient and arthritis in two patients were noted during baseline evaluation.

Disability Status

The Chu staging showed worse functional status in five patients (stage 3) and mild to moderate involvement (Chu stages 1 and 2) in 30 patients. At follow-up, the number of patients with mild to moderate involvement increased to 33, and only two patients had severely impaired functional status based on Chu staging. A 100% MSEADL score denoting no impairment was observed in 12 patients at baseline which increased to 21 at the time of follow-up scan. The NSS which includes assessment of 14 neurological features assigns a total score of 46 for the worst disability and 0 for a normal person. There was statistically significant improvement in mean NSS scores at baseline and follow-up from 9.74 to 6.37 ($p = 0.002$) indicating improvement in disease burden.

Neuroimaging

The mean MRI score at baseline was 5.9 (±4.2) which reduced to 4.9 (±4.7) in follow-up scans which signify decreased disease burden as observed in routine T2W/FLAIR sequences ($p < 0.05$).

TBSS Analysis ($p < 0.05$)

The serial DTI metrics showed that the entire cohort had significant ($p < 0.05$) improvement in all the four parameters (MD, FA, DA and RD). The improvement of DTI metrics was indicated by a decrease in MD, DA and RD values and increase in FA values. TBSS analysis comparing initial study and follow-up study showed multiple scattered areas with increased FA and decreased diffusivity distributed in both supra- and infratentorial white matter (Figs. 1 and 2).

Whole-brain DTI metric comparison between pretreatment and posttreatment studies showed no significant differences in the anisotropy and diffusivity measures ($p < 0.05$). Mean FA, MD, RD and AD values of the study cohort are tabulated in Table 2.

Discussion

In this longitudinal study, the changes in the WM integrity were studied using DTI in a cohort of patients with Wilson's disease (WD) who were receiving decoppering treatment for Wilson's disease. We found clinical improvement along with partial resolution of imaging changes in the conventional MRI. The DTI showed increased anisotropy and decreased diffusivity on follow-up imaging. Improvement in DTI metrics was paralleled by improvement in disability scores.

The DWI in patients of WD have shown both increased and decreased diffusivity in the focal lesions (Sener 2003a, b; Favrole et al. 2006; Jadav et al. 2013). Underlying pathological findings in WD include demyelination, inflammation, gliosis and spongiosis. Meenakshi-Sundaram et al. (2002) and Favrole et al. (2006) reported normal to decreased diffusivity in presymptomatic subjects, and increased diffusivity was seen in the symptomatic individ-

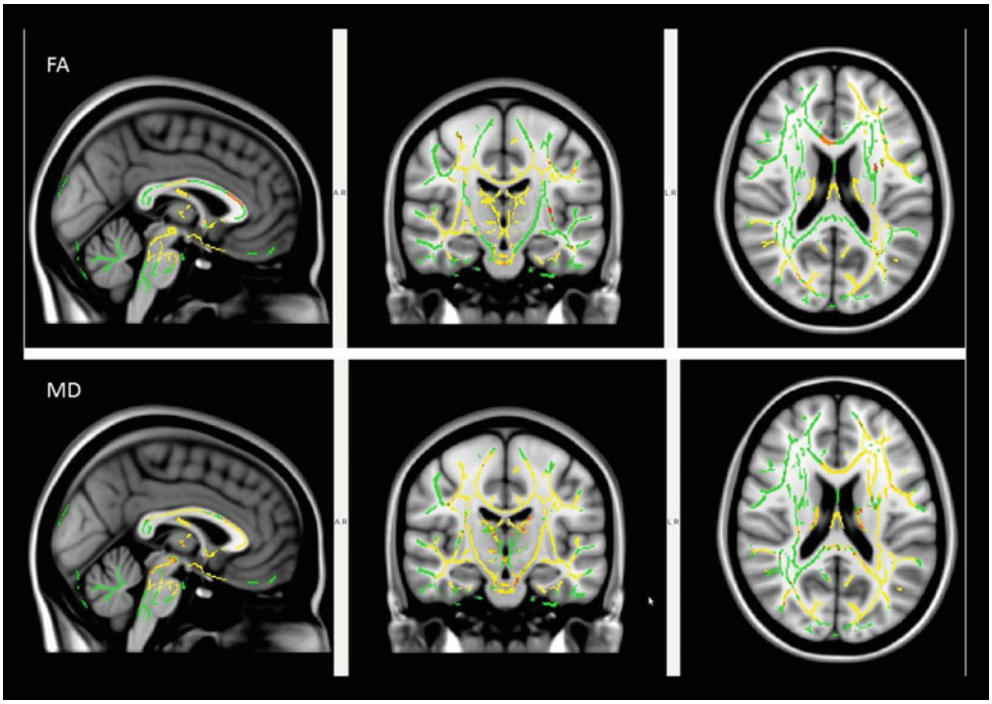

Fig. 1 TBSS analysis (*t*-test) comparing the initial and follow-up DTI imaging in patients with Wilson's disease. Voxels demonstrating significantly increased FA and decreased MD in the follow-up MRI are shown as *yellow red* in colour (FWE corrected $p < 0.05$). Results are shown overlaid on the MNI152-T1 template and the mean FA skeleton (*green*). *FA* fractional anisotropy, *MD* mean diffusivity, *FWE* family-wise error, *TBSS* tract-based spatial statistics

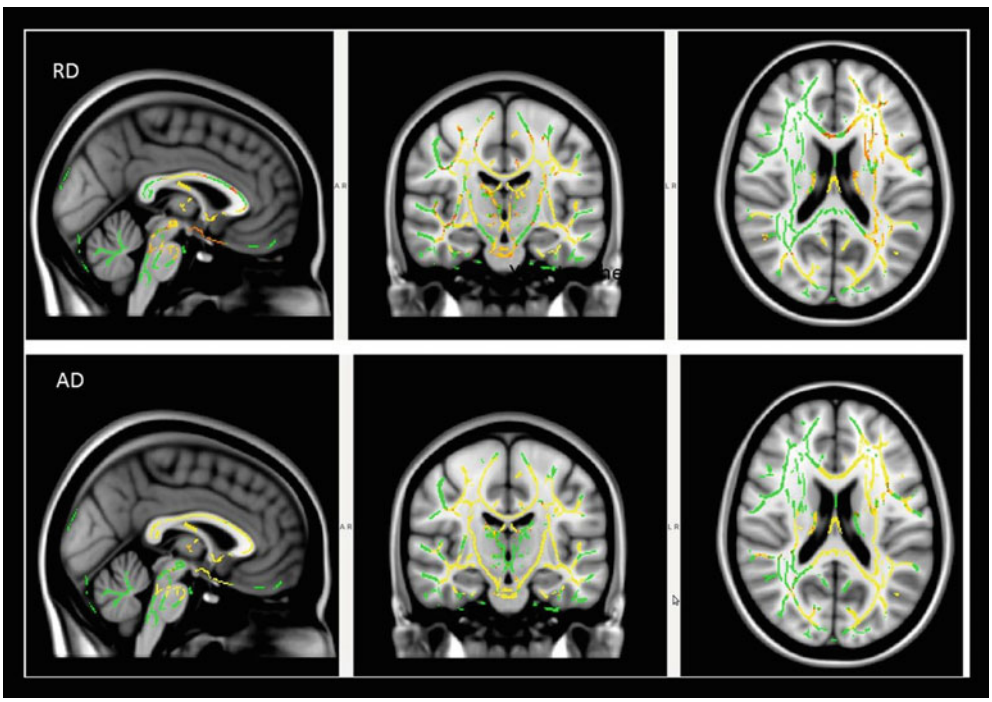

Fig. 2 TBSS analysis (*t*-test) comparing the initial and follow-up DTI imaging in patients with Wilson's disease. Voxels demonstrating significantly decreased AD and RD in the follow-up MRI are shown as *yellow red* in colour. Results are shown overlaid on the MNI152-T1 template and the mean FA skeleton (*green*) (FWE corrected $p < 0.05$). *RD* radial diffusivity, *AD* axial diffusivity, *FWE* family-wise error, *TBSS* tract-based spatial statistics

Table 2 Whole-brain WM mean values of fractional anisotropy and diffusivity measurements

	Posttreatment	Standard deviation	Pretreatment	Standard deviation
Mean_FA	0.42	0.02	0.43	0.04
Mean_MD	0.82	0.03	0.82	0.08
Mean_RD	0.61	0.03	0.61	0.09
Mean_DA	1.23	0.02	1.23	0.05

uals. This suggests changes in the diffusivity pattern with evolution of disease. In our previous study, we found increased diffusivity and decreased FA not only in the focal lesions but also in the normal-appearing hemispheric WM. Further correlation was observed between the disability scores and the DTI measurements (Jadav et al. 2013).

Treatment and its effects on clinical scores and MRI appearance have also been studied previously, and it was found that clinical disability scores and MRI appearance showed significant improvement following treatment. However, it was noted that patients who had extensive gliosis, diffuse white matter changes and significant atrophy showed poor response to the treatment (Kim et al. 2006). In another study, serial imaging was done for evaluating the pontine signal changes, and authors found significant resolution of these signal abnormalities after the start of decoppering therapy (Sinha et al. 2007a). Larnaout et al. reported one case which had extensive signal abnormalities of the subcortical WM at the initiation of therapy after 5 years of treatment that showed significant resolution of these pathological signal changes (Larnaout et al. 2008). However, most of these studies relied on serial changes in the signal abnormalities usually seen in WD patients. In a quantitative MRS study, authors reported significant improvement in the NAA/Cr ratio in patients who showed neurological improvement following treatment with decoppering therapy. Neurological deterioration was mirrored by fall in Glx/Cr and NAA/Cr ratios, while hepatic deterioration caused decreased mI/Cr and increased Glx/Cr ratios (Tarnacka et al. 2008). In our study, none of the patients reported significant neurological deterioration during the period of serial evaluation.

To the best of our knowledge, there are no studies which looked at the effect of treatment on DTI metrics in Wilson's disease patients. Pathological basis of improvement in WM DTI indices in WD remains speculative. Gliosis in WD possibly represents the irreversible WM damage. Reversible abnormalities include mitochondrial dysfunction, inflammatory changes and myelin disturbances. Reversible mitochondrial dysfunction is supported by the previous MRS study where increase in NAA/Cr ratio was noted following initiation of treatment. Presence of neuroinflammation has been reported to be present in the rat model of copper toxicity (Tarnacka et al. 2008). Demyelination and inflammatory changes have also been noted in the previous pathology study of Meenakshi-Sundaram et al. (2002). Some of these pathological changes possibly reverse following initiation of the decoppering therapy thus resulting in the radiological improvement in the form of increased NAA in MRS and increased FA and reduced diffusivity in DTI. This has been seen in the form of improved clinical disability scores as well as increased FA values and decreased diffusivity values. Another possible mechanism for these changes in the DTI metrics may be improvement in the underlying subclinical hepatic dysfunction.

Some of the limitations of the study include small sample size and variable follow-up duration. Another aspect which needs to be explained is the lack of significant improvement in the whole-brain white matter DTI metrics, while TBSS analysis showed areas with significant improvement. This discrepancy may be explained by understanding the fact that a large number of voxels are not showing any significant difference on voxel-wise analysis and they may be nullifying the effect of significant voxels when whole-brain WM metrics are considered together. Similarly, certain voxels of the brain might be showing worsening DTI metrics in spite of treatment like in the areas of significant gliosis, and these might be negating the effect of improving trends shown by other voxels. However, the use of sophisticated analytical methods like histogram analysis of whole-brain WM and use of parameters like kurtosis and skewness indices can show changes which may not be detectable by global means of anisotropy and diffusivity. In conclusion, our study shows clinical improvement which is paralleled by improvement in the disability scores and DTI metrics. Quantitative DTI metrics may be used as surrogate markers of clinical status following initiation of medical therapy in Wilson's disease.

Compliance with Ethics Guidelines

Conflict of Interest

A. Lawrence declares that he has no conflict of interest.
J. Saini declares that he has no conflict of interest.
S. Sinha declares that he has no conflict of interest.
S. Rao declares that he has no conflict of interest.
M. Naggappa declares that she has no conflict of interest.
P.S. Bindu declares that she has no conflict of interest.
A.B. Taly declares that he has no conflict of interest.

Contribution Declaration by Authors

"We declare that all the authors have been involved in (a) conception and design or analysis and interpretation of data and (b) drafting the article or revising it critically for important intellectual content".

Details of the Contributions of Individual Authors

A. Lawrence: planning, conduct and reporting of the work
J. Saini: planning, conduct and reporting of the work
S. Sinha: planning, conduct and reporting of the work
S. Rao: reporting of the work
M. Naggappa: conduct and reporting of the work
P.S. Bindu: reporting of the work
A.B. Taly: planning, conduct and reporting of the work

Informed Consent

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

Animal Rights

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

Financial Disclosure

None.

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RESEARCH REPORT

Vitamin E Improves Clinical Outcome of Patients Affected by Glycogen Storage Disease Type Ib

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Abstract *Background*: It has been suggested, on a few GSD1b patients, that vitamin E improves neutrophil count and reduces frequency and severity of infections.

The main objective of the present study was to investigate the efficacy of vitamin E on the neutropenia, neutrophil dysfunction and IBD in the entire Italian caseload of GSD1b patients.

Patients and methods: Eighteen GSD1b patients, median age at the time of the study protocol 14.5 (range, 0.6–42 years), were enrolled from four Italian referral centres for metabolic diseases. For the evaluation of the efficacy of vitamin E, neutrophil count and function, frequency of infections needing hospitalization and inflammatory bowel activity were evaluated periodically all over one year before and during vitamin E therapy.

Results: Frequency (1.5 ± 0.1 vs. 6.0 ± 0.6, $p = 0.003$) and severity of infections (2.2 ± 0.2 vs. 3.7 ± 0.4, $p = 0.003$) were lower and mean value of neutrophil count ($1{,}583 \pm 668$ vs. 941 ± 809, $p = 0.03$) higher during vitamin E supplementation. Neutrophil function results improved during vitamin supplementation. PCDAI showed a significant reduction in the inflammatory activity during vitamin E supplementation (9 ± 1.4 vs. 13 ± 1.2, $p = 0.006$). In seven patients G-CSF requirement decreased and the dose was reduced after the end of the study.

In conclusion, our study demonstrated the efficacy of vitamin E supplementation. Vitamin E has evident advantages as compared to G-CSF, as it can be assumed orally, and it has not been associated with severe side effects.

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Competing interests: None declared

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Introduction

Glycogen storage disease type 1 (GSD1) is an inborn error of carbohydrate metabolism. Two subtypes have been identified: GSD type 1a, caused by glucose-6-phospatase deficiency (G6Pase, G6PC gene), and GSD type 1b, due to a glucose-6-phosphate translocase defect (G6PT, SLC37A4 gene). It is characterized by hepatomegaly, fasting hypoglycaemia, lactic acidosis, hyperlipidaemia and hyperuricaemia. GSD1b patients also show neutropenia and impaired neutrophil function, recurrent infections and inflammatory bowel disease (Melis et al. 2005; Visser et al. 2012). These complications are highly debilitating and have a significant impact on quality of life (Melis et al. 2003).

The underlying cause of GSD1b neutropenia is an enhanced neutrophil apoptosis, but patients also manifest

neutrophil dysfunction of unknown aetiology. G6PT interacts with the enzyme glucose-6-phosphatase-β (G6Pase-β) to regulate the availability of G6P/glucose in neutrophils (Guionie et al. 2003; Jun et al. 2014). A deficiency in G6PT in neutrophils impairs both their energy homeostasis and function. Their energy impairment is characterized by decreased glucose uptake and reduced levels of intracellular G6P, lactate, adenosine triphosphate, and reduced NAD phosphate, whereas functional impairment is reflected in reduced neutrophil respiratory burst, chemotaxis, and calcium mobilization (Kim et al. 2006, 2008; Chou et al. 2010; Gorman et al. 2012; Visser et al. 2012).

Actually G-CSF is considered the only therapy for either neutropenia, neutrophil dysfunction and IBD in GSD 1b patients (Visser et al. 2000). However, this treatment is associated with severe side effects (Pinsk et al. 2002; Visser et al. 2002). Moreover, it has been shown that G-CSF addition to in vitro cultures does not rescue the GSD1b neutrophils from apoptosis (Kuijpers et al. 2003). It has been shown that antioxidants, for example, a soluble analogue of vitamin E (Trolox C), reduce the neutrophil apoptosis in GSD1b neutrophils (Leuzzi et al. 2003). Other studies supported the evidence that vitamin E plays an important role in protecting the cell from apoptosis and in enhancing the action and the production of G-CSF (Singh et al. 2009; Kulkarni et al. 2012).

We previously evaluated the efficacy of vitamin E supplementation in 7 GSD1b patients (Melis et al. 2009). Increased neutrophil counts, a significant decrease of frequency and severity of infections were detected during vitamin E supplementation. On the basis of this study, the role of vitamin E as additional therapy was proposed.

The aim of the current study was to evaluate the efficacy of vitamin E supplementation on neutrophil count, function and IBD in a larger cohort of patients and to analyse potential G-CSF adverse effects.

Patients and Methods

Patients

Eighteen GSD1b patients, median age at the time of the study protocol 14.5, (range 0.6–42 years), representing the entire Italian caseload, were enrolled from 4 Italian referral centres for metabolic diseases. Patients were coded by initials and date of birth to check for duplication. All patients with a diagnosis of GSD1b made either by enzyme studies showing the combination of deficient glucose-6-phosphatase activity in intact microsomes and (sub)normal glucose-6-phosphatase activity in disrupted microsomes or by mutation analysis of the glucose-6-phosphate transporter gene were enrolled in the study. The patients who were considered not able to comply with the protocol and with the therapy were excluded from the study. The G-CSF treatment was not considered an exclusion criterion. The study was conducted in the centres involved in the follow-up of each patient and the schedule form with follow-up programme to be filled in was sent to each centre.

Study Design

This study was designed as a prospective study. The study protocol was in accordance with the Italian regulations on privacy protection and with the Helsinki Doctrine for Human.

Experimentation was approved by the ethical committees of the participating centres. Before inclusion in the study, patients or their legal guardians signed a written informed consent. G-CSF treatment was not considered an exclusion criterion.

The study was carried out over a 3-year period; during the first year no vitamin supplementation was prescribed; during the second year patients were treated with vitamin E (600 mg/day were given to prepubertal patients and 900 mg/day to adults); during the third year vitamin E wash-out was performed.

Clinical and biochemical parameters were monitored and the results obtained during the second year were compared to both those of the first and third year. Neutrophil counts were obtained every three weeks throughout the study. A complete clinical assessment was made every six months, with particular attention to the presence of mouth ulcers, infections and signs suggestive of IBD.

Serum vitamin E levels, neutrophil function tests and ileocolonoscopy were performed once a year.

Manifestations compatible with adverse effects of G-CSF treatment, such as splenomegaly and osteopenia, when present, were monitored every 6 months.

Methods

Clinical Examination and Assessment of Infections

Patients were routinely examined in day hospital every 6 months or at the moment of any intercurrent infection. Physical examination included evaluation of weight, height and body mass index. The presence of nose, throat, lung and/or skin infections, abdominal pain, mouth ulcers and perianal lesions was recorded and evaluated according to a severity score index; in this scoring system the site of the infection, the need for therapy and/or hospital admission for observation and the duration of the disease were considered (Melis et al. 2009) .

Biochemical Investigations

Neutrophil count was measured by standard methods and neutropenia was defined as peripheral blood neutrophil count below 500×10^9/L. Neutrophil function tests included N-formyl-methionyl leucyl-phenylalanine (fMLP)-induced activation of respiratory burst and *E. coli*-induced respiratory burst and phagocytosis by flow cytometry. Serum vitamin E assay was performed by standard procedures.

Evaluation of the clinical and biochemical data associated to GSD1 and reflecting the metabolic control of the disease was also performed. In particular, the evaluation of the clinical and biochemical parameters of GSD1 included: serum glucose, triglycerides, cholesterol, lactic and uric acid levels. These parameters were expressed as mean value of all the determination obtained in each patient. Moreover, the frequency of hospital admissions for hypoglycaemia and the compliance to the dietary or medical treatment were recorded for all patients.

Assessment of Inflammatory Bowel Disease

Inflammatory bowel involvement was investigated by ileocolonoscopy at study entry and after one year of treatment. Inflammatory bowel activity was evaluated by the Paediatric Crohn's Disease Activity Index.

Evaluation of G-CSF Side Effects

Spleen size was evaluated by standard abdominal ultrasonography.

Bone mineral density (BMD) was measured by DEXA (Hologic QDR 1000, Hologic, Inc., Waltham, MA, USA). Measurements were taken at the L1-L4 vertebrae. *Z*-Scores were calculated by comparing BMD with age-matched (3–16 years) or age- and sex-matched (above 16 years) reference values according to the manufacturer's internal reference database.

Statistical Analysis

Data are expressed as mean ± SE. Statistical analysis was performed using Statistical Package for Social Science (SPSS 10 for Windows Update; SPSS Inc., Chicago, Illinois, USA). The comparisons between numerical variables were performed by Wilcoxon test. The comparison between categorical variables or associations between different parameters were performed using χ^2 test. The significance was set at 5%.

Results

Clinical Examination and Assessment of Infections

Frequency (1.5 ± 0.1 vs. 6.0 ± 0.6, $p = 0.003$) and severity of infections (score of 2.2 ± 0.2 vs. 3.7 ± 0.4, $p = 0.003$) were lower during the second year, when vitamin E supplementation was started. Most of them were bacterial infections. Vitamin E supplementation was the most important predictive factor of improvement of infections ($p = 0.03$). Infections' severity score results correlated with neutrophil count ($r = 0.92$, $p = 0.002$); conversely no correlation was observed between infections' severity score and parameters of metabolic control.

For 14 patients a third year of vitamin E withdrawal was available. During the vitamin E wash-out period, again the frequency and severity of infections increased (4.8 ± 0.3, 2.9 ± 0.2, respectively, $p = 0.01$).

Biochemical Investigations

Biochemical parameters of metabolic control were stable (Table 1) and no change in the dietary glucose requirements was recorded during vitamin E supplementation. The mean value of neutrophil count was significantly higher during vitamin E supplementation than during the period without vitamin E ($1{,}583 \pm 668$ vs. 941 ± 809, $p = 0.03$) (Fig. 1).

E. coli-induced respiratory burst (0.95 ± 0.01 vs. 0.85 ± 0.009, $p = 0.0001$), *E. coli*-induced phagocytosis (97.5 ± 0.9 vs. 73.6 ± 6.5, $p = 0.013$) and N-formyl-methionyl leucyl-phenylalanine (fMLP)-induced activation of respiratory burst (0.16 ± 0.02 vs. 0.10 ± 0.01, $p = 0.031$) were significantly higher during vitamin E supplementation. Mild but not significant increase of PMA-induced respiratory burst (1.98 ± 0.7 vs. 0.96 ± 0.09, $p = 0.15$) was detected during vitamin E supplementation (Fig. 2). Neutrophil function test results correlated with biochemical parameters indicative of metabolic control; *E. coli*-induced respiratory burst inversely correlated with insulin serum levels ($r = -0.85$, $p = 0.007$), N-formyl-methionyl leucyl-phenylalanine (fMLP)-induced activation of respiratory burst correlated with glucose serum levels ($r = 0.831$, $p = 0.02$), with bicarbonate serum levels ($r = 0.827$, $p = 0.027$), inversely correlated with both triglycerides ($r = -0.87$, $p = 0.005$) and lactic acid serum levels ($r = -0.72$, $p = 0.04$); *E. coli*-induced phagocytosis correlated with bicarbonate serum levels ($r = 0.925$, $p = 0.003$) and inversely correlated with lactic acid serum levels ($r = -0.88$, $p = 0.004$).

Table 1 Biochemical parameters indicative of metabolic control during vitamin E supplementation and in the year without supplementation

Parameters	Vitamin E supplementation	No vitamin	*p*
Glucose (mg/dl)	85	80	0.53
Lactic acid (mg/dl)	11.8	16.125	0.39
Uric acid (mg/dl)	5.53	5.92	0.73
Cholesterol (mg/dl)	119.66	109	0.41
Triglycerides (mg/dl)	244.26	153.06	0.09
Bicarbonate (mEq/l)	24.50	23.27	0.22
Haemoglobin (mg/dl)	10.41	10.49	0.89
ESR	41	62	0.07
CRP (mg/dl)	0.53	1.78	0.01

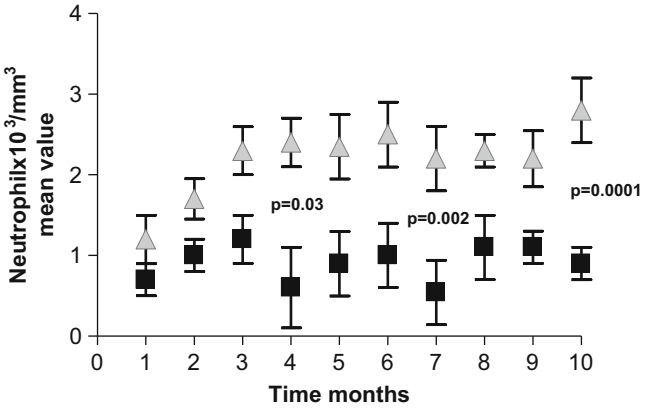

Fig. 1 Neutrophil count mean value during the first year, *filled square* (no vitamin supplementation), and second year, *grey shaded square* (vitamin supplementation)

Serum vitamin E levels significantly increased during vitamin supplementation ($p = 0.04$), from 750.8 ± 81.9 (first year) to 975 ± 70.3 (second year) to 784.8 ± 70 (third year). In most of the patients (10/14), vitamin E serum levels significantly decreased in the third phase. In these patients clinical features worsened during vitamin E wash-out including frequency and severity of infections, mouth ulcers and PCDAI. In the remaining patients vitamin E levels were stable. This might explain the nonsignificant reduction of vitamin E serum levels detected in the third phase.

No correlation was observed between parameters indicative of metabolic control and neutrophil count. Compliance to the dietary treatment did not correlate with neutrophil count, function and infections.

Inflammatory Bowel Disease Assessment

The presence of inflammatory bowel involvement was investigated in the seven patients followed at the Department of Pediatrics, Federico II University. Ileocolonoscopy was performed by an expert who was not aware of the treatment that patients were receiving. At study entry, ileocolonoscopy showed linear and aphthoid ulcers in three patients; histology showed severe infiltration of lymphocytes and monocytes in the lamina propria extending to submucosa and muscularis mucosa. In these patients, after one year of vitamin E supplementation, ileocolonoscopy showed an improved pattern with mild mucosal hyperaemia; histology showed foci of infiltration of lymphocytes in the lamina propria. In two additional patients, ileocolonoscopy initially showed ulcers, hyperaemia and friability of the mucosa, and histology demonstrated infiltration of lymphocytes and monocytes in the lamina propria. Normal pattern was detected both at ileocolonoscopy and histology at the end of the study.

PCDAI showed a significant reduction in the inflammatory activity during vitamin E supplementation (13 ± 1.2 vs. 9 ± 1.4, $p = 0.006$). The abdominal pain and extra-intestinal manifestations including arthritis and mouth ulcers were the most significantly changed items. Moreover CRP levels significantly decreased during vitamin E supplementation (1.78 ± 0.5; 0.53 ± 0.13, $p = 0.01$). Haemoglobin concentrations showed an increase during vitamin E supplementation; however, the changes did not reach statistical significance (10.49 ± 0.53; 10.41 ± 0.57, $p = 0.89$) (Table 1).

G-CSF Side Effect Evaluation

At the study entry, splenomegaly was present in 7/18 patients and was associated with hypersplenism in two of them. These patients initially got 3–5 mg/kg G-CSF dose each day. In these patients G-CSF dose was reduced at the end of the study. In particular, five patients got every-other-day treatment; in two patients everyday treatment was changed in twice-a-week therapy. G-CSF dose reduction was combined with vitamin E supplementation. Although G-CSF dose was reduced, neutrophil count was stable and the frequency and severity of infections did not increase after 1-year follow-up.

Bone DEXA was performed in 12/18 patients and demonstrated osteoporosis in five patients and osteopenia in one patient; normal values were observed in the remaining patient.

None of the patients showed the occurrence of malignancies.

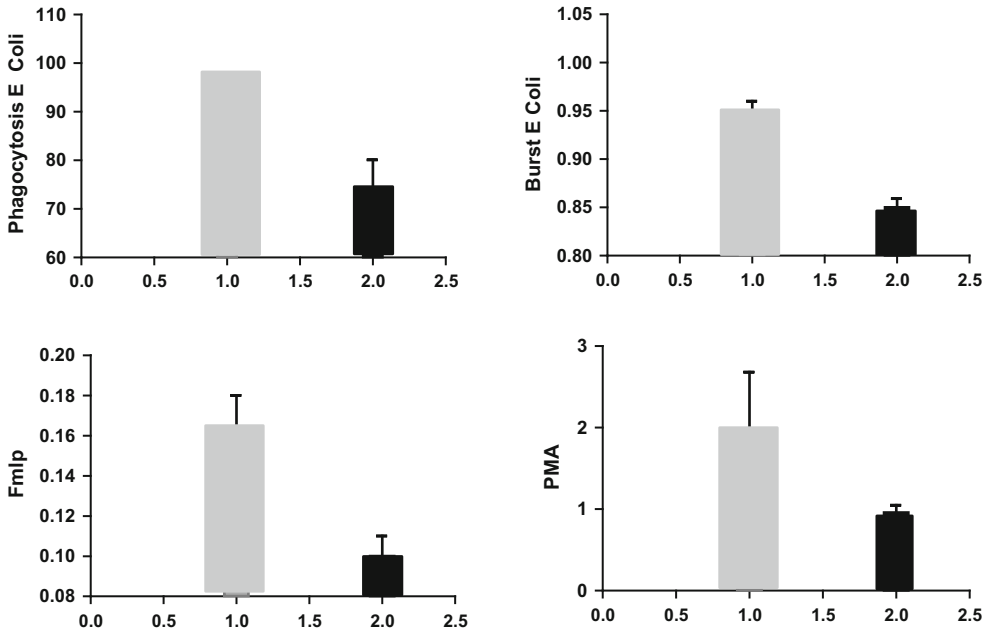

Fig. 2 Neutrophil function results during the first year, *filled square* (no vitamin supplementation), and second year, *grey shaded square* (vitamin supplementation)

Discussion

GSD1b patients' neutropenia is caused by enhanced neutrophil ER stress, oxidative stress and apoptosis arising from loss of G6PT/G6Pase-b activity (Jun et al. 2014). The G6P is the fuel for the pentose phosphate pathway that can generate NADPH by the hexose-6-phosphate dehydrogenase (Kim et al. 2008). In GSD1b neutrophils, the import of G6P into the endoplasmic reticulum is decreased, thus causing a local decrease in G6P dehydrogenase activity. This enzyme, which serves to produce NADPH, determines the cellular redox status by permitting regeneration of reduced glutathione, resulting in decreased sensitivity to direct or indirect apoptosis (Kim et al. 2006; Chou et al. 2010; Gorman et al. 2012). G-CSF treatment in GSD1b does not prevent the induction of apoptosis in circulating neutrophils (Kuijpers et al. 2003). Conversely, antioxidants reduce the neutrophil apoptosis in GSD1b neutrophils (Leuzzi et al. 2003). Moreover, it has been demonstrated that vitamin E plays an important role in protecting the cell from apoptosis and in enhancing the action and the production of G-CSF (Singh et al. 2009; Kulkarni et al. 2012).

We have previously investigated a possible role of vitamin E, a known antioxidant, on neutropenia, severity and frequency of infections and inflammatory bowel disease on a small number of patients. It is noteworthy that recurrent severe infections and inflammatory bowel disease are highly debilitating manifestations and impact on patients' quality of life and their overall health status.

In the current study, performed on the entire Italian caseload, we demonstrated that vitamin E supplementation increased the neutrophil count and improved neutrophil function. Increased and stable neutrophil counts during vitamin E supplementation correlated with significantly decreased frequency and severity of infections.

We hypothesized that vitamin E improves neutropenia by reducing the reactive oxygen species (ROS) and therefore apoptosis. Our results are in line with the observation that the specific G6PT-inhibitor S3484 increases apoptosis of neutrophils, which can be rescued by preincubation of cells with the reactive oxygen species (ROS) scavenger Trolox C or with the flavoprotein inhibitor diphenyleneiodonium (DPI).

We also detected an improvement of neutrophil function during vitamin E supplementation. Our results seem to confirm data already observed in elder patients. Both in vitro and in vivo studies demonstrated that vitamin E supplementation improves the lymphoproliferative capacity, neutrophil-mediated functions including phagocytic functions of PMN neutrophils and monocyte chemoattractant protein-1 production (Ventura et al. 1994; De la Fuente et al. 1998).

No correlation was detected among the results of biochemical parameters indicative of metabolic control and both frequency and severity of infections and neutrophil count. Conversely metabolic control seems to impact on neutrophil function; in fact increased lactic acid serum levels inversely correlated with *E. coli*-induced phagocytosis, *E. coli*-induced respiratory burst and N-formyl-

methionyl leucyl-phenylalanine (fMLP)-induced activation of respiratory burst results; surprisingly also insulin serum levels inversely correlated with *E. coli*-induced respiratory burst results. These results might suggest that both GSD1b-related metabolic disorder and excessive dietary treatment might impair neutrophil function.

We observed improved findings at ileocolonoscopy and bowel histology; a decrease of inflammatory activity was also observed by PCDAI. These effects are the most relevant for GSD1b patients and have strong implications for their quality of life and health status. Indeed, after withdrawal of vitamin E supplementation at the end of the study protocol, severe infections and low neutrophil counts were again observed and all patients asked to resume vitamin E. These results are in agreement with data observed in patients affected by ulcerative colitis (Seidner et al. 2005)

Concerning G-CSF-related side effects, splenomegaly was recorded in 7/18 patients and was associated with hypersplenism in two of them. During vitamin E supplementation, in these seven patients G-CSF dose was reduced after the end of the study and no significant reduction of neutrophil count neither increase of frequency and severity of infections were observed.

In conclusion, our study suggests the efficacy of vitamin E supplementation on improving clinical outcome of GSD 1b patients.

Moreover, during vitamin E supplementation, G-CSF doses or frequency of administration can be reduced with consequent reduction of G-CSF-related side effects. Vitamin E has evident advantages as compared to G-CSF, as it can be assumed orally, and it has not been associated with severe side effects.

The control of the metabolic disorder and the strict adherence to dietary prescription is highly recommended.

The efficacy of the vitamin E should also be evaluated in other forms of congenital neutropenias in which increased apoptosis has been reported, such as cyclic neutropenia, myelokathexis and congenital dysgranulopoietic neutropenia.

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Take-Home Message

Vitamin E supplementation improves clinical outcome of GSD1b patients and allows a G-CSF dose reduction with consequent reduction of G-CSF-related side effects.

Compliance with Ethics Guidelines

Conflict of Interest

We underline that: there are no prior publications or submissions with any overlapping information; the work is not and will not be submitted to any other journal while under consideration by JIMD; there are no potential conflicts of interest, real or perceived; Dr. D. Melis wrote the first draft of the manuscript and gave substantial contributions to conception and design, acquisition of data and analysis and interpretation of data; and neither an honorarium or grant or other forms of payment were given to anyone to produce the manuscript. All the authors listed on the manuscript take full responsibility for the manuscript; moreover, Dr. D. Casa, G. Minopoli, F. Balivo, G. Parenti, S. Paci and C. Dionisi-Vici gave substantial contributions to conception and design, acquisition of data and analysis and interpretation of data, revised the manuscript critically for important intellectual content, approved the submission of this version of the manuscript and take full responsibility for the manuscript. Dr. Marcolongo and Prof. Benedetti performed the biochemical investigation, namely, the neutrophil function test; gave substantial contributions to conception and design, acquisition of data and analysis and interpretation of data; revised the manuscript critically for important intellectual content; approved the submission of this version of the manuscript; and take full responsibility for the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Prof. Generoso Andria and Giancarlo Parenti gave substantial contribution to conception and design, acquisition of data and analysis and interpretation of data and critically revised the manuscript.

Daniela Melis, Giorgia Minopoli, Francesca Balivo, Paola Marcolongo, Rossella Parini, Sabrina Paci, Carlo Dionisi-Vici, Roberto Della Casa, Angelo Benedetti, Generoso Andria and Giancarlo Parenti declare that they have no conflict of interest.

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RESEARCH REPORT

Urine Beta2-Microglobulin Is an Early Marker of Renal Involvement in LPI

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Abstract *Objective*: Lysinuric protein intolerance (LPI) is a rare autosomal recessive disorder affecting the transport of cationic amino acids. It has previously been shown that approximately one third of the Finnish LPI patients have impaired renal function. The aim of this study was to analyse in detail urine beta2-microglobulin values, renal dysfunction, oral L-citrulline doses and plasma citrulline concentrations in Finnish LPI patients.

Methods and results: Of the 41 Finnish LPI patients, 56% had proteinuria and 53% hematuria. Mean plasma creatinine concentration was elevated in 48%, serum cystatin C in 62%, and urine beta2-microglobulin in 90% of the patients. Seventeen per cent of the patients developed ESRD, and five of them received a kidney transplant.

L-citrulline doses and fasting plasma citrulline concentrations were similar in adult LPI patients with decreased and normal GFR (mean ± SD 79.5 ± 29.2 vs. 82.4 ± 21.9 mg/kg/day, $P = 0.619$, and 80.3 ± 20.1 vs. 64.8 ± 23.0 μmol/l, $P = 0.362$, respectively).

Conclusions: Urine beta2-microglobulin is a sensitive early marker of renal involvement, and it should be monitored regularly in LPI patients. Weight-based oral L-citrulline doses and plasma citrulline concentrations were not associated with renal function. LPI patients with ESRD were successfully treated with dialysis and kidney transplantation.

Abbreviations

CKD-EPI	The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
LPI	Lysinuric protein intolerance
MDRD	Modification of diet in renal disease
SLC7A7	Solute carrier family 7, member 7
y+LAT-1	y+L amino acid transporter-1

Introduction

Lysinuric protein intolerance (LPI) is a rare autosomal recessive disorder affecting transport of dibasic cationic amino acids lysine, arginine and ornithine in the basolateral membrane in intestine and renal tubules (Perheentupa and Visakorpi 1965; Norio et al. 1971; Rajantie et al. 1980a). It is caused by mutations in the gene SLC7A7 (solute carrier family 7, member 7) which encodes the y+LAT-1 protein, the catalytic light chain subunit of the heteromeric amino acid transporter. All Finnish patients share the same homozygous mutation, a substitution of T for A at cDNA position 1181–2 (Borsani et al. 1999; Torrents et al. 1998, 1999). LPI is more prevalent in Finland than elsewhere in the world, but several patients have been reported from, e.g. Italy and Japan (Incerti et al. 1993; Koizumi et al. 2000).

Because of reduced intestinal absorption and renal reabsorption of dibasic cationic amino acids, plasma

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concentrations of lysine, arginine and ornithine are low in patients with LPI. It is believed that the lack of arginine and ornithine causes secondary dysfunction of the urea cycle, resulting in protein aversion and hyperammonemia after dietary protein loads. Lysine deficiency is supposed to have an influence on immune system and growth.

LPI was first described as late as in 1965, and thus the knowledge of the natural progression of the disease is still limited. Newborns with LPI are usually asymptomatic until the amount of dietary protein increases. Later, the principal symptoms are nausea, vomiting, failure to thrive, growth retardation, hepatosplenomegaly, muscular weakness and osteoporosis. In addition, hypercholesterolemia, hypertriglyceridemia, hematologic abnormalities and deficient B cell function have been reported (Simell et al. 1975; Lukkarinen et al. 1999; Simell 2001; Tanner et al. 2010). Life-threatening complications include nephropathy, pulmonary fibrosis and alveolar proteinosis, the mechanisms of which are still unclear (DiRocco et al. 1993; Parto et al. 1994; Tanner et al. 2007).

Treatment of LPI consists of dietary protein restriction, oral L-citrulline supplementation to boost the urea cycle, L-lysine hydrochloride to correct lysine deficiency and, in some patients, sodium benzoate or sodium phenylbutyrate to scavenge ammonia (Simell 2001). Some patients may also require L-carnitine supplementation (Tanner et al. 2008).

In 2007, we reported (Tanner et al. 2007) that one third of the Finnish patients with LPI had signs of renal dysfunction, in some of them leading to end-stage renal disease (ESRD). The causes of this complication have remained unknown. In this study, we describe in detail the urine beta-2-microglobulin values, renal involvement, oral L-citrulline doses and plasma citrulline concentrations of all Finnish LPI patients in our cohort.

Patients and Methods

The follow-up of Finnish LPI patients is centralised to the Department of Pediatrics at the Turku University Hospital. The patients visit our outpatient clinic 1–2 times per year. The current study cohort consists of 41 patients (26 female). Mean age of the patients was 37.3 years (range 3 to 69 years), and six patients were under 18 years of age. Two patients died during the follow-up in 2007–2013 period.

In this study, we analysed retrospectively medical records and laboratory tests of the patients from 2007 to 2013. Hypertension, renal function tests, urinary protein and amino acid excretion, and plasma citrulline concentrations were the particular objects of interest. The development of renal dysfunction was investigated by observing changes in plasma creatinine, serum cystatin C and urine beta2-microglobulin levels over time.

Sitting blood pressure was measured annually after 15 min rest using average values of three consecutive blood pressure measurements from right arm using an oscillometric noninvasive blood pressure monitor (GE Dinamap Carescape V100 monitor). Size of the cuff was chosen according to the size of the right arm. All laboratory analyses were performed using standard clinical laboratory methods. Urine amino acids were measured from morning spot urine and plasma amino acids in fasting plasma with HPLC. Urine beta-2-microglobulin was analysed in morning spot urine using chemiluminescence detection. Proteinuria was measured from 24 h urine and also by using urine dipstick test (positive if proteins ++ or +++). Hematuria was defined using urine dipstick test. Glomerular filtration rate (GFR) was calculated using Cockcroft-Gault formula, 4-variable MDRD formula and CKD-EPI formula for adults and Schwartz formula for patients under 18 years of age. The data were analysed using IBM SPSS Statistics 22.0 software. Only adults were included in the statistical analyses of weight-based citrulline doses and fasting plasma citrulline concentrations. One patient had not used L-citrulline regularly and was excluded from these analyses. The patients with ESRD have been analysed separately.

This study was approved by the joint Ethics Committee of the University of Turku and Turku University Hospital.

Results

Characteristics of 41 patients with LPI are presented in Table 1. At the time of this study, 20 of the 36 patients (56%) had proteinuria (urine protein >0.1 g/24 h or urine albumin >30 mg/24 h) and nineteen of them (53%) had hematuria (positive urine dipstick test). Twenty-four hour urine was collected from 24 patients for protein excretion measurements. Seventeen of them (71%) had albuminuria, 46% had microalbuminuria (urine albumin 30–300 mg/24 h) and 25% had macroalbuminuria (urine albumin >300 mg/24 h). Plasma creatinine concentration was elevated in 48% and serum cystatin C in 62% of the patients. Urine beta2-microglobulin was measured from 31 patients and was elevated in 28 of them (90%). 4-variable MDRD formula (normal >60 ml/min/1.73 m^2), Cockcroft-Gault formula (normal >90 ml/min) and CKD-EPI formula (normal >90 ml/min/1.73 m^2) showed decreased GFR in 18, 28 and 25 patients, respectively. There was a correlation between beta2-microglobulin and GFR ($r = -0.69$, $P < 0.001$) (Tables 2 and 3). Table 4 shows that in some LPI patients, a rapid elevation of urine beta2-microglobulin preceded decrease of GFR. There was no correlation between urine beta2-microglobulin and urine albumin

Table 1 A summary of the follow-up data of the 41 patients with LPI at Turku University Hospital in 2007–2013

Patient number	Sex	Age at study	Proteinuria	Hematuria	Hypertension (RR > 140/90 mmHg)	Estimated GFR			Elevated serum cystatin C	Elevated urine beta2-microglobulin	Bicarbonate supplementation	Phospate supplementation	ESRD[b]
						4v-MDRD[a]	Cockcroft-Gault	CKD-EPI					
1	F	3				162							
2	M	10				289				+			
3	F	10				250				+			
4	F	16		+		104				+			
5	F	16				109				+			
6	F	17				112			+	+			
7	F	18	+			74.7			+	+	+	+	
8	F	20				111	116	126		+			
9	F	21	+	+		31	32	34	+	+	+	+	
10	M	22	+	+		46	43	47	+		+	+	
11	F	24	+	+		60	74	69	+	+		+	
12	F	28				80	79	93		+			
13	F	29	+		+	79	64	93			+		+
14	M	29	+	+	+	32	38	35	+	+		+	+
15	M	30				79	109	90		+			
16	F	32			+	84	159	99	+	+		+	
17	F	33	+	+		46	51	52	+	+			
18	M	36		+	+	99	81	111					
19	M	38		+	+	77	99	87		+			
20	F	39		+		82	102	96		+			
21	F	39	+	+		8	9	8	+	+			+
22	F	40				72	61	83	+	+			
23	M	41	+	+	+	59	85	66	+	+			
24	F	43				29	32	32	+		+		
25	M	44	+	+	+	122	116	115					
26	M	45	+	+	+	20	25	21	+	+	+		
27	F	46	+	+	+	71	68	81		+			
28	F	46	+	+	+	45	44	50	+	+			
29	F	47	+	+		36	48	39	+	+			
30	M	48			+	32	50	34	+	+			
31	M	49	+	+		79	71	78		+			

(continued)

Table 1 (continued)

Patient number	Sex	Age at study	Proteinuria	Hematuria	Hypertension (RR > 140/90 mmHg)	Estimated GFR			Elevated serum cystatin C	Elevated urine beta2-microglobulin	Bicarbonate supplementation	Phospate supplementation	ESRD[b]
						4v-MDRD[a]	Cockcroft-Gault	CKD-EPI					
32	F	49	+		+	59	57	66	+	+	+	+	+
33	M	50	+		+	66	76	73	+	+			
34	F	53	+		+	68	52	76	+				
35	M	55	+	+	+	11	11	11	+	+	+		+
36	F	57	+	+		38	53	41	+	+			
37	M	58			+	87	69	94					
38	F	58	+		+	41	34	44	+	+			
39	F	60	+	+	+	7	8	7	+			+	+
40	F	62	+	+	+	74	61	80	+	+			+
41	M	69	+	+	+	55	61	56		+			

[a] <18 years old with Schwarz formula
[b] Five of the patients with ESRD had received a kidney transplant

Table 2 Urine beta2-microglobulin, GFR and urine albumin in LPI patients: correlations between urine beta2-microglobulin, GFR and urine albumin

	Urine beta2-microglobulin	GFR[a]
GFR[a]	−0.69[b]	
Urine albumin	0.09	−0.12

[a] GFR was calculated using CKD-EPI formula
[b] Correlation is significant at the 0.01 level

Table 3 Urine beta2-microglobulin, GFR and urine albumin in LPI patients: correlations between urine beta2-microglobulin and GFR in 2011–2013

		GFR[a] in		
		2011	2012	2013
Urine beta2-microglobulin in	2011	−0.61[b]	−0.50[c]	−0.54[c]
	2012		−0.57[b]	−0.56[c]
	2013			−0.69[b]

[a] GFR was calculated using CKD-EPI formula
[b] Correlation is significant at the 0.01 level
[c] Correlation is significant at the 0.05 level

Table 4 Urine beta2-microglobulin, GFR and urine albumin in LPI patients: urine beta2-microglobulin and changes in GFR in LPI patients in 2011–2013

Patient number	Urine beta2-microglobulin[a]			GFR[b]		
	2011	2012	2013	2011	2012	2013
19	0.004	1.64	2.20	88	95	87
20	1.40	2.28	5.50	102	106	96
22	7.96		6.90	93	105	83
27	7.08	2.44	8.49	98	99	81
33	0.58	2.13	13.7	96	78	73

[a] Normal value <0.25 mg/l
[b] GFR was calculated using CKD-EPI formula

(Table 2). Base excess (BE) was measured from twenty-six patients, and it was decreased (<−2.5) in 21 of them (81%). Nineteen patients (46%) had elevated blood pressure, and 16 of them were treated with antihypertensive drugs. Twelve patients have received treatment with ACE inhibitors. Twelve patients (29%) needed oral supplementation of bicarbonate, phosphate or both (Table 1). Eight patients needed specific phosphate supplementation due to tubular wasting of phosphate.

During this follow-up, 29 of the 41 patients (70%) had developed renal dysfunction (i.e. proteinuria, decreased GFR, hypophosphatemia and/or decreased bicarbonate level); two of them were children. A total of seven patients (18%) have progressed to end-stage renal disease (ESRD, GFR <15 ml/min/1.73 m^2), two of them already in their early twenties. In 2007, only one patient was treated with peritoneal dialysis, and three had received a kidney transplant. In 2013, two patients were in peritoneal dialysis, and a total of five patients had received a kidney transplant at mean age of 39.4 years (range 20–55 years) (Table 5). Most of the renal transplantations were initially successful (first one in 2005). However, three patients experienced episodes of graft rejection, and one patient lost her transplant. The reason of rejections is still obscure. Allograft biopsies were not routinely performed because of patients' bleeding tendency. Also, plasma concentrations of the immunosuppressive drugs of one of the patients remained below the therapeutic level though the dosage was increased. After the transplantations, anaemia and recurrent or chronic infections have remained a problem. Four patients had proteinuria since transplantation. GFR (CKD-EPI) remained normal in only one patient. Other recipients had decreased GFR (MDRD, range 32–57; Cockcroft-Gault, range 38–64; CKD-EPI, range 35–93). One patient lost her transplant three years after the operation, was subsequently treated with peritoneal dialysis and died 8 years after transplantation from sequelae of kidney failure at the age of 60 years. In addition, one patient with renal dysfunction died of the complications of the disease at the age of 16 years.

We were also interested in L-citrulline doses and plasma citrulline concentrations (Table 6). The amount of L-citrulline supplementation depended on individual protein intake. Therefore, daily doses varied widely, i.e. from 28 to 229 mg/kg. Mean adult dose was 82.9 mg/kg (including all adults), while children had higher mean weight-adjusted citrulline dose (122.7 mg/kg). Mean adult L-citrulline dose was 79.5 ± 29.2 mg/kg in patients with decreased renal GFR (CKD-EPI) and 82.4 ± 21.9 mg/kg patients with normal GFR ($p = 0.619$). Mean daily L-citrulline dose was 94.3 ± 29.2 mg/kg in patients with ESRD. Fasting plasma citrulline concentration was analysed in 20 adult patients. Mean concentration was 76.3 μmol/l (range from 38 to 118 μmol/l), 80.3 ± 20.1 μmol/l in patients with decreased GFR and 64.8 ± 23.0 μmol/l in patients with normal GFR ($p = 0.362$). Fasting plasma citrulline concentration was analysed in one patient with ESRD, and it was 62.0 μmol/l.

Urine amino acids were measured from 33 patients. Urine arginine was elevated in 31 patients (94%). Mean urine arginine was 149.7 ± 132.4 μmol/mmol creatinine (normal range 0–5 μmol/mmol creatinine). Mean urine citrulline was 55.2±78.4 μmol/mmol creatinine (normal

Table 5 A summary of the data of the five LPI patients with a renal transplant

Patient number	Sex	Age			Complications after the transplantation				Immunosuppressive drugs	
		At study	At start of dialysis	Transplantation	Infections	Anaemia	Graft rejection	Graft loss	After the transplantation	At study
13	F	29	20	24	+	+	+		CsA	TAC, MPA
14	M	29	20	20	+	+	+		MPA, TAC	CsA
32	F	49	45	46	+	+			CsA, MPA	CsA, MPA
39	F	60	51	52	+	+	+	+	CsA, MPA	–
40	F	62	53	55	+	+			CsA, MPA	CsA, MPA

CsA cyclosporin, *MPA* mycophenolic acid, *TAC* tacrolimus

Table 6 Oral L-citrulline doses in LPI patients with or without nephropathy in 2013

	Normal GFR		Decreased GFR[a]		
	N (female)	Mean ± SD	*N* (female)	Mean ± SD	*P*-value[b]
Age	8 (4)	36 ± 12	20 (12)	42 ± 13	0.055
GFR[c]	8 (4)	103 ± 13	20 (12)	57 ± 20	<0.001
Weight-based citrulline dose (mg/kg)	8 (4)	82.4 ± 21.9	19 (11)	79.5 ± 29.2	0.619
Fasting plasma citrulline (μmol/l)	4 (2)	64.8 ± 23.0	15 (10)	80.3 ± 20.1	0.362

[a] Patients with ESRD were not included
[b] *P*-value by using non-parametric tests: independent samples
[c] GFR was calculated using CKD-EPI formula

Table 7 Correlations between urine arginine, urine citrulline, urine amino acids, plasma citrulline and weight-based citrulline dose

	Urine arginine	Urine citrulline	Urine amino acids	Plasma arginine	Plasma citrulline
Urine citrulline	0.64[a]				
Urine amino acids	0.41[b]	0.49[b]			
Plasma arginine	0.40[b]	0.35	0.13		
Plasma citrulline	0.07	0.18	0.15	0.06	
Citrulline dose	0.15	0.07	−0.19	0.29	−0.18

[a] Correlation is significant at the 0.01 level
[b] Correlation is significant at the 0.05 level

range 1–15 μmol/mmol creatinine). Plasma arginine was measured from 32 patients, and mean plasma arginine was 26.4 μmol/l ± 11.5 (normal range 15–185 μmol/l). There was no correlation between urine citrulline or urine arginine and weight-based oral citrulline doses (Table 7).

Discussion

Renal insufficiency in Finnish patients with LPI was first reported in 2007. At that time, 10% of Finnish LPI patients had ESRD and 59% had impaired renal function (Tanner

et al. 2007). Since then, renal dysfunction has become more frequent in LPI patients: 18% of the patients had ESRD and 70% impaired renal function despite regular follow-up and careful treatment. At the time of the study, almost all patients over 45 years of age had developed renal problems, and only twelve patients had normal renal function.

Beta2-microglobulin is a component of the major histocompatibility class I molecule (MHC I) and is presented in all nucleated cells (Creswell et al. 1974). It is eliminated by glomerular filtration and is, thus, elevated in renal dysfunction. Muscle mass, body weight and gender do not affect its plasma concentrations. It has been shown that serum beta2-microglobulin increases more and earlier than serum creatinine (Bianchi et al. 2001). Therefore, it might represent an ideal marker of GFR in patients with renal diseases (Wibell et al. 1973; Trollfors and Norrby 1981; Acchiardo et al. 1989; Shea et al. 1981; Bianchi et al. 2001). Furthermore, beta2-microglobulin is reabsorbed almost completely in renal tubules, and therefore, increased urinary excretion is a sign of decreased tubular reabsorption and damage of tubular structures (Gauthier et al. 1984). In our study, urine beta2-microglobulin was elevated in 90% of the patients. In some patients, it started to elevate before any changes in GFR were detected and while plasma creatinine and serum cystatin C were still within the reference range. We feel that it is currently the most sensitive early marker of renal disease in subjects with LPI.

Pathogenesis of renal disease in LPI is still poorly understood. Histological data are limited, but immune complex-mediated glomerulonephritis has been detected in some patients, and glomerular lesions have been similar to those in systemic lupus erythematosus (SLE). Also, antinuclear antibodies have been measured in some patients (Parto et al. 1994; Kamoda et al. 1998). However, renal insufficiency may perhaps be a part of natural progression of the LPI disease, but the role of L-citrulline therapy has also been considered (Zager et al. 1983). L-citrulline is used to improve the function of the urea cycle and, subsequently, protein tolerance. As a neutral amino acid, it uses a different transport route than arginine and ornithine and is readily absorbed, causing high peak plasma concentrations (Rajantie et al. 1980b, 1981). The Finnish patients were originally treated with arginine monohydrochloride during years 1965–1976 and subsequently with more effective and better tolerated L-citrulline supplementation (Awrich et al. 1975). However, it seems that renal problems have become more common in LPI patients during the citrulline therapy, and even children with nephropathy have been observed during the last three decades. Nephropathy in children was not seen during the arginine therapy (Lukkarinen et al. 2006). One must, however, remember that natural history of untreated LPI is yet very poorly characterised, and it is possible that in the past many patients have died before renal involvement was detected.

We found no difference in weight-based L-citrulline doses and plasma citrulline concentrations between the patients with normal and decreased renal function. However, in theory, it is possible that high citrulline concentrations might have a role in the development of nephropathy. High concentrations of especially cationic amino acids are nephrotoxic in animals (Zager et al. 1983). Large amounts of citrulline increase the intracellular synthesis of arginine, which may cause damage and apoptosis in tubular, glomerular and mesangial cells via increased production of nitric oxide (Sebastio et al. 2011; Alderton et al. 2001; Mori 2007; Morris 2007; Ogier de Baulny et al. 2012). On the other hand, patients with citrullinemia have very high citrulline concentrations but have not been reported to have renal problems. One of our patients with ESRD neglected citrulline therapy for many years, but his renal function still decreased rapidly. However, due to equivocal role of citrulline in renal function in LPI, we have during the last few years deliberately slightly reduced L-citrulline doses of the patients to minimise the possible risks. At the beginning of the 2000s, mean weight-based oral citrulline dose was up to 110 mg/kg, but it has recently been reduced to 80–90 mg/kg. Many LPI patients use sodium benzoate and/or sodium phenylbutyrate to increase nitrogen excretion and thus reduce the need of L-citrulline.

In conclusion, renal insufficiency has become more common in Finnish LPI patients. We suggest that urine beta2-microglobulin is the most sensitive early marker of renal problems, and it should be monitored regularly in LPI patients. Urine beta2-microglobulin was elevated in 90% of our patients. We also calculated GFR with three different formulas, and of those, the CKD-EPI formula seems to be the most reliable in LPI patients who typically have low muscle mass. In this study, we did not find significant correlations between weight-based L-citrulline doses and renal function. However, due to possible role of citrulline in renal problems, we have slightly reduced L-citrulline doses and monitored plasma citrulline concentrations regularly. More investigation is clearly needed in this issue. In Finland, a total of six LPI patients have been treated with peritoneal dialysis and five of them have received a kidney transplant. One patient lost her transplant. Considering the overall situation, the prognosis after transplantation has been satisfactory.

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Synopsis

Urine beta-2-microglobulin is an early marker of renal complications affecting the majority of Finnish LPI patients.

Compliance with Ethics Guidelines

Conflict of Interest

Mari Kärki declares that she has no conflicts of interest.

Laura M. Tanner declares that she has no conflicts of interest.

Harri Niinikoski declares that he has no conflicts of interest.

Kirsti Näntö-Salonen declares that she has no conflicts of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

Mari Kärki has been responsible for collecting and analysing the data and writing the manuscript.

Laura M. Tanner has been responsible for planning the present study design as well as drafting the manuscript.

Harri Niinikoski has been responsible for examining and treating the study subjects as well as drafting the manuscript.

Kirsti Näntö-Salonen has been responsible for examining and treating the study subjects, planning the present study design as well as drafting the manuscript.

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RESEARCH REPORT

The Spectrum of Krabbe Disease in Greece: Biochemical and Molecular Findings

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Abstract Krabbe disease is an autosomal recessive neurodegenerative lysosomal storage disease caused by the deficiency of β-galactocerebrosidase. This deficiency results in the impaired degradation of β-galactocerebroside, a major myelin lipid, and of galactosylsphingosine. Based on the age of onset of neurological symptoms, an infantile form (90% patients) and late-onset forms (10% patients) of the disease are recognized. Over 130 disease-causing mutations have been identified in the β-galactocerebrosidase gene. We present the biochemical and molecular findings in 19 cases of Krabbe disease, 17 of them unrelated, diagnosed in Greece over the last 30 years. β-Galactocerebrosidase activity assayed in leukocyte homogenates using either the tritium-labeled or the fluorescent substrate was diagnostic for all. Increased plasma chitotriosidase activity was found in 11/15 patients.

Mutational analysis, carried out in 11 unrelated cases, identified seven different mutations, four previously described (p.I250T, c.1161+6532_polyA+9kbdel, p.K139del, p.D187V) and three novel mutations (p.D610A, c.583-1 G>C, p.W132X), and seven distinct genotypes. The most prevalent mutation was mutation p.I250T, first described in a patient of Greek origin. It accounted for 36.4% (8/22) of the mutant alleles. The second most frequent mutation was c.1161+6532_polyA+9kbdel that accounted for 22.7% (5/22) of the mutant alleles. The observed frequency was lower than that described in Northern European countries and closer to that described in Italian patients.

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Introduction

Krabbe disease, also known as globoid leukodystrophy (GLD, OMIM # 245200), is a rare autosomal recessive demyelinating disorder that belongs to the group of lysosomal storage diseases. It is caused by the deficiency of β-galactocerebrosidase (GALC, E.C. 3.2.146) which impairs the degradation of galactocerebroside, a major myelin lipid, and of galactosylsphingosine or psychosine (Suzuki 1998; Wenger et al. 2013). Accumulation of galactocerebroside elicits the formation of the characteristic multinucleated macrophages known as globoid cells. Psychosine is cytotoxic to oligodendrocytes and Schwann cells, arresting myelination and contributing to the progressive demyelination observed. According to the "psychosine hypothesis," it is the main contributor to the disease pathology (Suzuki 1998; Svennerholm et al. 1980; Tanaka et al. 1989).

Based on the age of onset of neurological symptoms, an infantile form and late-onset forms of the disease are recognized. It has been reported that approximately 90% of the patients have the former and 10% the latter forms of the disease (Wenger 2008); however, a higher incidence for the later-onset forms is suggested in a recent report (Duffner et al. 2012). The galactocerebrosidase gene (*GALC*; MIM # 606890) was cloned by two groups more than 20 years ago (Chen and Wenger 1993; Sakai et al. 1994). It is localized

on chromosome 14q31, has 17 exons and 16 introns, and spreads over 58 kb (Luzi et al. 1995). It encodes a 669-amino-acid (80 kDa) mature protein containing six potential glycosylation sites, which is proteolytically cleaved into 30 and 50 kDa forms that comprise the active form of the enzyme (Chen and Wenger 1993).

Up-to-date 131 mutations have been identified in the *GALC* gene, 128 of which have been reported to be the cause of Krabbe disease (Human Gene Mutation Database, HGMD professional 2015.1; Graziano and Cardile 2015; Wenger et al. 2001) that include missense, nonsense, deletion, and insertion mutations. Although genotype-phenotype correlations have been reported (Tappino et al. 2010 ; Wenger et al. 1997; Xu et al. 2006), the interpretation of the effects of mutations is complicated by the concomitant presence of polymorphisms (Harzer et al. 2002; Luzi et al. 1996; Wenger et al. 2001).

In Greece, the Institute of Child Health is the only center offering laboratory diagnosis for lysosomal storage diseases, including Krabbe disease. We report the biochemical and molecular findings in 19 cases of Krabbe disease diagnosed in our center over the last 30 years.

Patients and Methods

Patients

The present paper includes 19 patients with Krabbe disease, originating from all over Greece. They were referred for diagnosis to the Institute of Child Health on the basis of their clinical evaluation and neuroimaging studies.

Patients 8 and 9 and 15 and 16 are siblings. It is also reported that the family of patient 6 had lost a boy at the age of 10 months with psychomotor retardation, regression, and neurological disease.

A brief description of the patients is shown in Table 1. The study was approved by the Ethics Committee of the Institute of Child Health.

Methods

β-Galactocerebrosidase activity was assayed in leukocyte homogenates using tritium-labeled [H^3] galactosylceramide (ARC; American Radiolabeled Chemicals, Inc.) or 6-hexadecanoyl-4-methylumbelliferyl-β-D-galactopyranoside (Glycosynth) as substrates (Wiederschain et al. 1992; Young et al. 1972).

Chitotriosidase activity in plasma was measured using 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose as substrate (Michelakakis et al. 2004).

Molecular Analysis

DNA analysis was carried out by PCR amplification of all the exons and flanking regions of the *GALC* gene followed by automate sequencing. Mutations were confirmed by restriction enzyme analysis when appropriate or by resequencing. RNA extraction and cDNA analysis were performed as previously described (Rodriguez-Pascau et al. 2009). Primer sequences are available on demand. The novel missense mutation p.D610A was confirmed by restriction analysis with the *HphI* enzyme. It was analyzed in samples from 50 control individuals and was not found. All mutations are described according to the current mutation nomenclature guidelines (http://www.hgvs.org/mutnomen), ascribing the A of the first ATG translational codon as nucleotide +1.

Results: to – Discussion

Krabbe disease is a panethnic rare neurodegenerative autosomal recessive disorder. Its estimated overall prevalence is 1:100,000 births, while a very high prevalence is found in two separate inbred Druze and Arab Muslim communities in Israel (Foss et al. 2013; Rafi et al. 1996). Assuming a birth rate of 100,000/year, a rough incidence estimate for Krabbe disease in Greece would be 0.63/100,000 births. However, this is most probably an underestimate since underdiagnosis, especially of adult-onset forms, is highly probable.

The Institute of Child Health is the only center in Greece providing the diagnosis of lysosomal storage diseases. This is the first study on a large number of Greek Krabbe patients. The cases presented here account for 4.4% of the total number of patients diagnosed with a lysosomal storage disorder in our center. The patients originated from different parts of Greece including the Ionian and Aegean Sea islands (7/19) and Northern and Central Greece (11/19), whereas one patient was of Gypsy origin (#11).

The clinical phenotypes of Krabbe patients range from the classical infantile form, typically with onset $\leq$6 months of age and rapid progression, to late-onset forms (6 months–$\geq$9 years) with varying age of onset and rate of progression (Graziano and Cardile 2015).

According to the information available to us, at least 13/19 patients reported had onset of symptoms $\leq$6 months of age (Table 1). A much later onset was clearly observed in three patients (#7, #15, #16). The two latter are siblings, and they are still alive at the ages of 37 and 42 years, respectively, ages at which the diagnosis was established. The time between onset and diagnosis in our cohort ranged

Table 1 The clinical signs on referral along with the results of the biochemical investigations and DNA studies

Patient	Sex	Age of onset	Age at diagnosis	Symptoms on referral	GALC activity (fluorescent substrate; nmol/mg protein/h) normal: 0.4–3.8 units	GALC activity (radioactive substrate; nmol/mg protein/h) normal: 0.1–0.97 units	Chitotriosidase activity (nmol/ml/h) normal: 0–150 units	Genetic variations: mutations/polymorphisms
1	F	6 months	9 months	Psychomotor regression, muscular hypertonia, myoclonic jerks, irritability, megalencephaly, ↑CSF protein level	–	0.0	434	c.1161+6532_polyA +9kbdel/c.1161 +6532_polyA+9kbdel *hom p.R184C* (*c.550C>T*)
2	M	Unknown	4 months	Seizures, irritability, feeding difficulties ↑CSF protein level	–	0.03	23	–
3	F	6 months	10 months	Psychomotor retardation, axial hypotonia, ↑CSF protein level	–	0.0	–	–
4	M	6 months	8 months	Psychomotor regression, hypotonia, ↑CSF protein level, normal brain CT scan	–	0.0	34	–
5	M	5 months	5.5 months	Psychomotor retardation, cerebral palsy, hypertonia, ↑CSF protein level, normal brain CT scan	–	0.0	–	–
6	M	26 months	26.5 months	Spastic tetraplegia, loss of vision, abnormal MRI	–	0.04	0.0	–
7	M	3 years 1 month	3 years 5 months	Psychomotor regression, speech, walking and vision impairment, abnormal MRI	–	0.01	800	–
8	M	3 months	6.5 months	Infantile seizures, irritability, hypotonia, megalencephaly, psychomotor retardation, loss of vision, abnormal MRI	–	0.03	75	p.I250T (c.749T>C)/ p.I250T (c.749T>C)
9	F	4.5 months	6.5 months	Irritability, hypertonia, opisthotonus, loss of vision, abnormal MRI	–	0.03	398	p.I250T (c.749T>C)/ p.I250T (c.749T>C)
10	M	Unknown	10 months	Psychomotor retardation, hypertonia, ↑CSF protein level, abnormal MRI	–	0.05	276	p.I250T (c.749T>C)/ c. 583-1G>C[a] *het p.I562T* (*c.1685T>C*)
11	M	5 months	7 months	Irritability, regression, feeding difficulties ↑CSF protein level, abnormal MRI, CT scan, NCV	–	0.04	404	p. W132X (c.396G>A)[a]/ p. W132X (c.396G>A)[a]
12	M	6 months	8 months	Psychomotor retardation, hyperpyrexia, spastic tetraplegia,	–	0.0	994	c.1161+6532_polyA +9kbdel /p.D187V

(continued)

Table 1 (continued)

Patient	Sex	Age of onset	Age at diagnosis	Symptoms on referral	GALC activity (fluorescent substrate; nmol/mg protein/h) normal: 0.4–3.8 units	GALC activity (radioactive substrate; nmol/mg protein/h) normal: 0.1–0.97 units	Chitotriosidase activity (nmol/ml/h) normal: 0–150 units	Genetic variations: mutations/polymorphisms
				↑ CSF protein peripheral hypertonia, abnormal MRI				(c.560A>T) *het p. R184C* (*c.550C>T*)
13	F	4 months	11 months	Regression, mild axial hypotonia, hypertonia of the extremities, opisthotonus, ↑ CSF protein, abnormal MRI	–	0.0	380	p.I250T (c.749T>C)/ p.I250T (c.749T>C)
14	M	6 months	1.5 years	Psychomotor regression, abnormal MRI, abnormal NCVs	–	0.0	217	p.I250T (c.749T>C)/ p.I250T (c.749T>C)
15	M	7 years	37 years	Spastic paraparesis, optic atrophy, demyelinating peripheral neuropathy, abnormal MRI	0.0	0.015	11	p. D610A (c.1829A>C)[a]/ p. K139del (c.411-413delTAA)
16	F	Unknown	42 years	Abnormal MRI, behavioral problems	0.0	0.01	33	p. D610A (c.1829A>C)[a]/ p. K139del (c.411-413delTAA)
17	M	3.5 months	6 months	Generalized hypertonia, developmental delay, abnormal MRI, decreased NCVs	0.0	0.003	1,556	p. K139del (c.411-413delTAA)/p. K139del (c.411-413delTAA)
18	M	Neonatal period	12 months	Psychomotor retardation, infantile seizures, ↑ CSF protein level, abnormal MRI	0.0	0.0	209	p. I250T (c.749T>C) c.1161+6532_polyA +9kbdel *het p. R184 C* (*c.550C>T*)
19	M	3 months	5 months	Psychomotor retardation, regression, hypotonia, seizures, abnormal MRI	0.0	0.007	653	*c.1161+6532_polyA +9kbdel*/unknown *het p. R184C* (*c. 550C >T*) *hem p. I562T* (*c. 1685T>C*)

[a] Novel mutation
↑increased
NCV nerve conduction velocity. Patients 8 and 9 and 15 and 16 are siblings

from 15 days to 30 years, the longer delay being observed for patient 15.

This delay is in agreement with previous reports, suggesting a substantial difficulty in the diagnosis of the late-onset forms of the disease due to their variable phenotype (Duffner et al. 2012 ; Kolodny et al. 1991). Assaying of β-galactocerebrosidase, irrespective of the substrate used, was diagnostic for all cases. As previously reported, assaying of residual GALC activity could not reliably differentiate earlier and later-onset patients (Wenger et al. 2014).

Chitotriosidase is an enzyme produced by activated macrophages. Increased plasma activity, which is at least 100-fold elevated compared to controls, is observed in Gaucher disease (Hollak et al. 1994), whereas more modest increases have been reported in other lysosomal storage diseases (Michelakakis et al. 2004).

Plasma chitotriosidase activity was assayed in 16/19 patients. One patient (#6) had zero activity, apparently belonging to the approximately 6% of the general population that does not synthesize enzymatically active enzyme (Boot et al. 1998). In 11 of the remaining 15 patients, an increase that ranged from 1.4× to 10.4×, the upper normal limit, was observed. It is possible that the increase in chitotriosidase activity observed in Krabbe disease patients reflects the psychosine-induced activation of peripheral immune cells described in the disease (Parsqui et al. 2007; Formici et al. 2007). Although further studies will be needed to investigate the mechanism and significance of the observed increase in the plasma chitotriosidase activity, our results are in agreement with previous observations and strongly suggest that assaying this enzyme activity can be useful in the diagnosis of Krabbe disease (Michelakakis et al. 2004; Wajner et al. 2006).

DNA was available from 11 of the 17 unrelated patients (and for two siblings). Mutational analysis resulted in the identification of 21/22 mutant alleles. They included four previously described mutations and three novel ones (Table 1).

The most frequent mutation was the missense mutation p.I250T (c.749T>C). It was first described in homozygosity in a Greek female patient, born to consanguineous parents, with disease onset at 28 months of age (De Gasperi et al. 1996). Recent data confirm that the mutation is associated with a negligible level of residual activity and strongly suggest that it leads to altered processing of the 80 kDa precursor resulting in an undetectable 50–54 kDa N-terminal GALC fragment (Lee et al. 2010). In our cohort it was identified either in homozygosity (three unrelated patients – and the sib of one of them) or heterozygosity (two patients). It accounts for 36.4% (8/22 alleles, considering only unrelated individuals). No information regarding possible consanguinity of the parents of the homozygous patients was available. However, the parents of the siblings #8 and #9, homozygous for the mutation, originated from the same village of an Ionian island. Furthermore, no common geographical origin for this mutation was identified since the patients came from Northern, Central, and Western Greece. All the patients of our series bearing this allele had a much earlier onset of the disease than the described for the original patient. Two of the patients bearing the p.I250T mutation in heterozygosity are also carriers of either the p.I562T (c1685T>C) or the p.R184C (c.550C>T) polymorphisms.

The second most frequent mutation in our cohort was the large deletion c.1161+6532_polyA+9kbdel. The mutation has probably originated in Sweden where Krabbe disease has recently been reported to be the most frequent lysosomal storage disorder (Hult et al. 2014). It is found all over Europe but also in other countries like Mexico and India. It has been described to account for 44% of the alleles of American patients with Northern European ancestry and for 52% of unrelated Dutch cases. An overall 35% frequency is described in patients from different European countries. Recently it has been reported to account for 18% of the disease alleles in an Italian cohort of patients. In this study it accounted for 22.7% of the disease alleles, a frequency lower than that described in Northern European countries and closer to that described in Italian patients. It is a severe mutation that in the homozygous state results in the classic infantile form or, depending on the nature of the second mutation, in later-onset disease when in compound heterozygosity. It is invariably found to be associated with the transition c.550C>T (p.R184C) which is present on the same allele (Debs et al. 2013; Kleijer et al. 1997; Tappino et al. 2010; Luzi et al. 1995; Rafi et al. 1995).

In agreement with the above, in our cohort, the mutation was identified either in homozygosity (one patient) or heterozygosity (three patients) in four patients with severe early-onset disease. In all the cases, the genotyping results are consistent with its association with the c.550C>T transition.

p.K139 del (c.411–413 del TAA) was the third most frequently identified mutation. It was found in homozygosity in a patient (#17) with severe early-onset disease and in heterozygosity in siblings #15 and #16, both with late-onset slowly progressing disease. The sibs and the homozygous patient originated from Northern Greece pointing to a possible common origin. The mutation leads to the deletion of a lysine residue that is highly conserved in eukaryotes (http://www.ensebml.org). It has recently been reported at the heterozygous state in a Turkish patient with onset of disease reported to occur at the age of 25 years (Debs et al. 2013). It appears then that in homozygosity the mutation results in a severe early-onset form, whereas in heterozygosity,

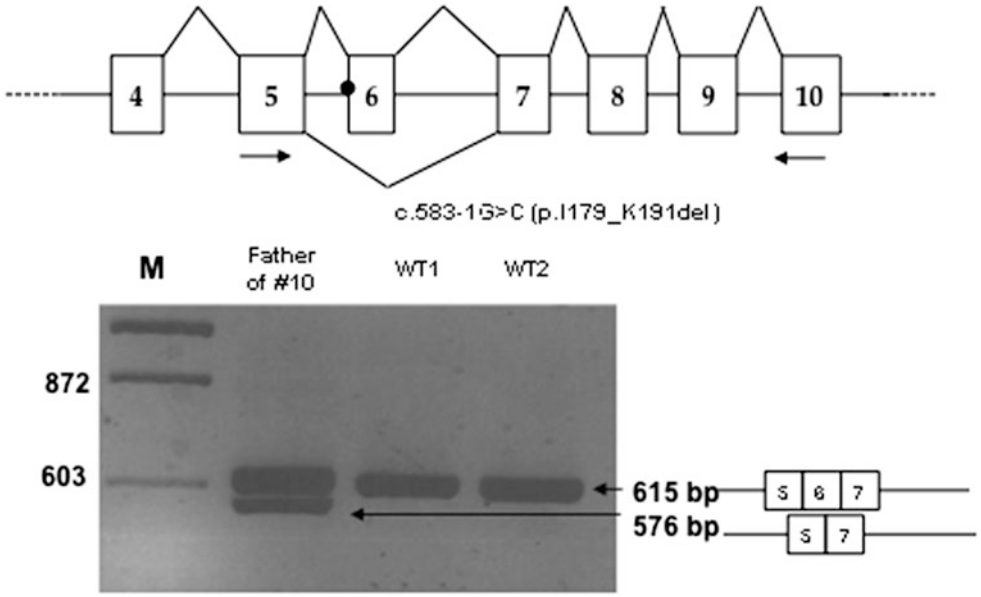

Fig. 1 *Top*: scheme of the splicing pattern of exons 4–10 of the GALC gene in the wt allele (above the figure) and in the allele bearing the c.583-1G>C mutation (below the figure, only the skipping of exon 6 is indicated). *Arrows* correspond to primers used for the PCR shown below. *Bottom*: PCR amplification of cDNAs from the father of patient #10 and from two wild-type individuals. A scheme of exons 5–7 is indicated. *M* molecular weight markers

apparently depending on the second mutation, it can be associated with later-onset forms of the disease. Interestingly, deletion of the previous, equally conserved, lysine residue (p.K138del) has been reported in a Greek patient; however, no details regarding the phenotype or the origin of the patient are available (Wenger et al. 2001).

The novel mutation p.D610A (c.1829A>C) was identified in heterozygosity with the p.K139del in two siblings, patients #15 and #16, with a late-onset form. The missense mutation in exon 16 changes aspartate, an acidic amino acid, to valine, a neutral branched-chain amino acid. Its impact on the enzyme structure is not clear; however, since it was found in patients with late-onset forms in heterozygosity with the apparently severe mutation p.K139del, it could be argued that it predisposes to later-onset forms of the disease.

Two other novel mutations were found in two patients with early-onset disease. The first one, c.583-1G>C, observed in heterozygosity with p.I250T, is a splicing mutation. We were able to show that it results in the skipping of exon 6 (Fig. 1) and the synthesis of a shorter protein (p.I179- K191del). The second is the nonsense mutation p.W132X (c.396G>A) leading to premature termination of synthesis of the enzyme. It was found in homozygosity in a patient of Gypsy origin whose parents were third cousins.

Finally, the p.D187V (c.560A>T) mutation, previously described in an adult patient (Luzi et al. 1996), was found in heterozygosity with the c.1161+6532_polyA+9kbdel in an early-onset case.

Only one allele of this series remained unidentified. It is the case of patient 19, who bears the c.1161+6532_polyA+9kbdel mutation in the paternal allele. We performed the analysis of the maternal cDNA and gDNA, since no sample from the patient was available. No mutation was detected, neither at gDNA nor at cDNA level. However, her cDNA was homozygous for several polymorphisms that were heterozygous at genomic level. These polymorphisms were p.Q328Q, c.984G>A (exon 9); p.S450S, c.1350C>T (exon 13); and p.I562T, c.1685C>T (exon 15). Some level of recovery of the heterozygosity was observed upon cycloheximide (CHX) treatment. This suggests that the mother is transmitting an allele that suffers nonsense-mediated decay, but the causing mutation was not found. Alternatively, it could be a problem of expression (in spite of the CHX results). We have analyzed part of the promoter and found no change. A deletion of part of the gene could be the causing mutation. This deletion should include one or more exons and generate a frameshift. The exons involved should not be the last 11–17 because the patient bears these exons and his paternal allele does not. Moreover, exons 5 and 9 should not be deleted because of heterozygosity of at least one SNP in the mother (exon 9) or in the patient (exon 5). Southern blot was performed, but no clear results were obtained. Further studies will be performed to characterize this allele.

In conclusion, in our cohort of patients, a considerable delay in the diagnosis of late-onset cases was observed. Sequencing analysis identified seven different mutations, three of which were novel, which accounted for 25/26 alleles studied (21/22, if only unrelated patients are considered), and seven distinct genotypes. The prevailing mutation was the missense mutation p.I250T (c.749T>C) that accounted for about 36% of the mutated alleles. Both p.I250T and the c.1161+6532_polyA+9kbdel were identified either in homozygosity or in heterozygosity in severe early-onset forms of the disease.

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Take Home Message

In 11 Krabbe patients we identified seven mutations (three novel) and seven genotypes; chitotriosidase activity can be of value in diagnosing Krabbe disease.

Compliance with Ethics Guidelines

Conflict of Interest

Evangelia Dimitriou, Monica Cozar, Irene Mavridou, Daniel Grinberg, Lluïsa Vilageliu, and Helen Michelakakis declare that they have no conflict of interest.

Contribution of Authors

Monica Cozar performed the molecular analysis and Lluïsa Vilageliu and Daniel Grinberg supervised the work and revised the MS. Evangelia Dimitriou and Irene Mavridou were involved in the diagnosis of patients and the collection of data. Helen Michelakakis planned the study, supervised the diagnostic work, and wrote the manuscript.

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RESEARCH REPORT

Exercise Intolerance and Myoglobinuria Associated with a Novel Maternally Inherited *MT-ND1* Mutation

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Abstract The most common clinical phenotype caused by a mtDNA mutation in complex I of the mitochondrial respiratory chain is Leber hereditary optic neuropathy. We report a family with a novel maternally inherited homoplasmic mtDNA m.4087A>G mutation in the ND1 gene (*MT-ND1*) associated with isolated myopathy, recurrent episodes of myoglobinuria, and rhabdomyolysis. DNA from blood in seven family members and muscle from four family members were PCR amplified and sequenced directly and assessed for the m.4087A>G variation in *MT-ND1*. Mitochondrial enzyme activity in all muscle biopsies was measured. PCR and direct sequencing of the *MT-ND1* genes from blood showed that all seven family members were homoplasmic for the m.4087A>G mutation (NC_012920.1:c.781A>G). The mutation predicts a threonine to alanine substitution at position 261 (p.T261A). The same mutation was found in muscle of all four family members available for muscle biopsy, and biochemical analyses revealed an isolated complex I defect in muscle of all family members (range 22–52% of normal). Muscle morphology showed severe myopathic changes with internal nuclei in multiple fibers of all family members. Monosymptomatic myopathy with recurrent myoglobinuria is a rare phenotype of mitochondrial myopathies. We report this phenotype in a family affected by a novel homoplasmic mutation in *MT-ND1*. It is the first time such a phenotype has been associated with complex I gene mutations and a homoplasmic mutation of mtDNA.

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Introduction

Complex I, the largest of the mitochondrial respiratory chain (RC) complexes, consists of at least 46 subunits of which seven are encoded by mitochondrial DNA (mtDNA). The remaining 39 subunits are encoded by nuclear DNA (Wong 2007). The most common clinical phenotype caused by a mtDNA mutation in complex I is Leber hereditary optic neuropathy (LHON), a condition associated with subacute visual failure, which is often caused by a mutation in the mitochondrial ND1 subunit gene (Schapira 2006). Mutations in mtDNA can be homoplasmic (all mtDNA copies carry the mutation) or heteroplasmic (mutated mtDNA coexists with wild-type mtDNA) (Taylor and Turnbull 2005). Most patients with LHON are homoplasmic for mtDNA mutations, and the penetrance of the mutations is variable among family members (Schapira 2006; Taylor and Turnbull 2005). We report a family with a novel maternally inherited homoplasmic mtDNA m.4087A>G mutation in the ND1 gene (*MT-ND1*) associated with a new phenotype.

Patients and Methods

Case Report

The proband is a 41-year-old man with an isolated myopathy. He had congenital ptosis, which is unusual for patients with mitochondrial disease, but not without precedence. He had rigid spine, increased CK and lactate

levels, and episodic myoglobinuria. Throughout childhood he felt physically weaker than his peers. At age 17, he was hospitalized due to a physical collapse. His CK values were highly elevated, and a muscle biopsy showed nonspecific myopathic changes, but no specific diagnosis was reached. At age 38, he developed rhabdomyolysis and muscle stiffness following injection of suxamethonium during surgery for appendicitis. On physical examination, he had decreased eye movements in all directions and he had previously undergone bilateral surgery for ptosis. He had pronounced rigid spine, scapula alata, and reduced proximal strength of the upper extremities (strength 4; MRC scale) but normal strength in the lower extremities.

Other Affected Family Members

The sister of the proband had an episode of rhabdomyolysis in her thigh muscles, at age 20, during biking while working as a mail delivery person. At the age of 43, she was hospitalized with severe pain and rhabdomyolysis. CK was elevated to 35,000 U/l (normal values: <210 U/l). On physical examination, she did not have ptosis or rigid spine like her brother. Her 16-year-old daughter frequently experienced exercise-induced muscle cramps and pain. The sister's 11-year-old seemingly unaffected son was clinically examined, and a blood sample was secured from him. A 71-year-old maternal aunt had hearing impairment since age 50 years. In her youth, she experienced muscle cramps and physical collapse after strenuous work but did not seek medical advice. The mother of the proband aged 74, who is in a nursing home because of brain damage following Herpes encephalitis, and the grandmother, who recently passed away at age 101, were both unavailable for clinical examination, but blood samples were secured from both. The grandmother and aunt wore hearing aids.

Muscle Biopsies

Percutaneous needle muscle biopsies were performed in the vastus lateralis muscle of the proband, his sister, niece, and the maternal aunt. The biopsies were frozen immediately in isopentane cooled by liquid nitrogen and kept in a freezer at −80°C until histochemical and biochemical analyses. Serial frozen 10 μm sections of muscle tissue were stained with hematoxylin and eosin (H&E), Gömöri trichrome, COX/SDH, and ATPase. Mitochondrial enzyme activity in all muscle biopsies was measured at 37°C as previously described (Larsen et al. 2012).

Western Blotting

Western blot analysis was performed essentially as previously reported (Ostergaard et al. 2007). The membrane was incubated with an antibody against ND1 (1:1000, H00004535-A01, Abnova.) and an antibody against Porin (Proteintech, Chicago, IL, USA), which was used as a loading control. The secondary antibodies were goat anti-mouse/goat anti-rabbit (Dako, Glostrup, Denmark). The ND1 antibody was raised against amino acids 21–71 (YP_003024026).

DNA Analysis and HUMARA Assay

Total DNA was isolated from blood and muscle samples by standard methods. The mtDNA-encoded complex I genes were PCR amplified and sequenced directly using template DNA derived from muscle of the proband. Whole blood-derived DNA from the proband, his maternal grandmother, his mother, his maternal aunt, his sister, and his niece and nephew were subsequently assessed for the m.4087A>G variation in *MT-ND1*. The remaining complex I mtDNA genes were PCR amplified and sequenced directly on DNA derived from muscle of the proband to exclude other potential pathogenic variations.

To test for a potential X-linked mode of inheritance, we carried out a HUMARA assay (Allen et al. 1992) to assess potential biased X-chromosome inactivation in the sister of the proband.

Bicycle Ergometry Test

The patient underwent an exercise test on a cycle ergometer. The patient has a slight enlargement of the ascending aorta, and is missing and aortic valve, and for that reason he was not pushed to his limit during exercise testing. Instead he performed a cycle test at 69% of VO_2 max. We withdrew a blood sample before, during, and after the bicycle test for lactate analysis.

Results

Muscle Biopsies and Blood Tests

Biochemical analyses revealed an isolated complex I defect in the muscle of the family members (Table 1). Histochemical data from the proband and his sister showed a severe myopathic muscle with internal nuclei in almost all fibers and an increased variability in fiber diameter (Fig. 1a). The muscle biopsy of the aunt also showed multiple centrally nucleated fibers (11%). There were no dystrophic changes or histological signs of congenital myopathy in any of the biopsies. No COX-negative or ragged red fibers were found in any of the family members (Fig. 1b–d). The blood samples of the proband and his sister showed increased values of CK (1,100 and 953 U/l

Table 1 Activities of mitochondrial enzymes in skeletal muscle

	Proband	Sister	Daughter	Aunt	Normal values
Complex I	0.10	0.09	0.08	0.19	0.34 ± 0.09 (0.18–0.58)
Complex II	0.52	0.24	0.41	0.42	0.39 ± 0.07 (0.32–0.62)
Complex III	1.50	0.68	1.49	1.05	1.20 ± 0.21 (0.75–1.71)
Complex IV	5.3	3.3	4.6	5.0	3.8 ± 0.7 (2.5–5.7)
Citrate synthase	239	345	307	212	309 ± 83 (181–468)
Complex I/II	0.19	0.39	0.20	0.46	0.87 ± 0.21 (0.48–1.36)

Enzyme activity is expressed as milliunits per milliunits citrate synthase. Citrate synthase activity is expressed as milliunits per milligrams protein. Normal values are from 29 age-matched controls and expressed as mean ± SD (ranges in parentheses)

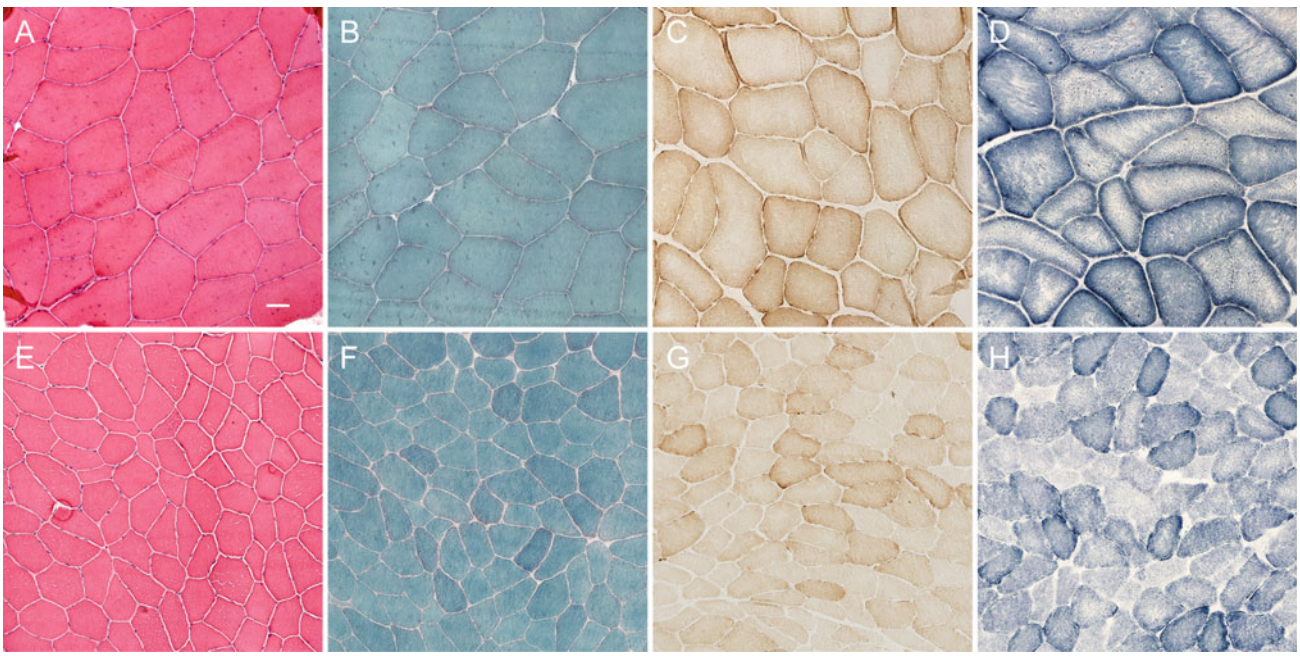


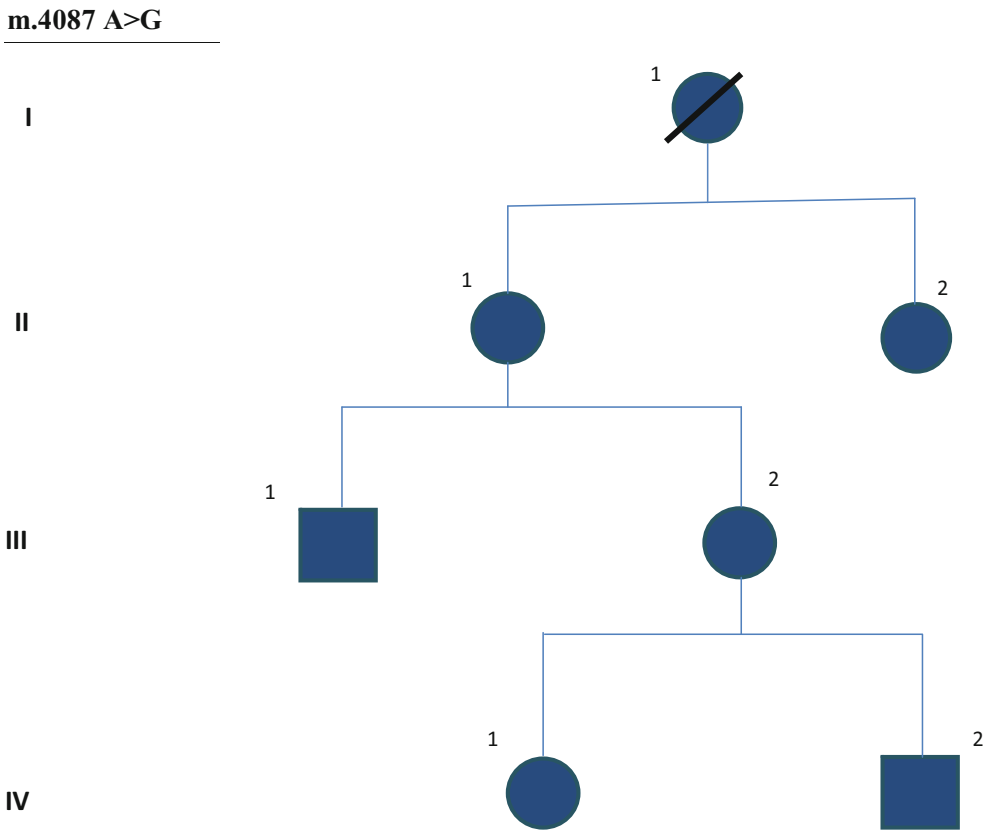
Fig. 1 Muscle sections from the proband (**a**–**d**) and a control (**e**–**h**). (**a**) H&E dye showing multiple centrally nucleated fibers in the muscle fibers of the proband. (**b**–**d**) Gömöri trichrome, COX, and SDH showing no COX-negative or ragged red fibers in the muscle fibers of the proband. The *white bar* is 50 μm

(normal values: 35–210 U/l)) and myoglobin (139 and 144 μg/l (normal values: 19–49 μg/l)). The sister had normal lactate values and the blood sample of the niece and aunt showed normal values. An acyl-carnitine analysis of the proband was in the normal range. Western blot analysis showed that the proband had normal amounts of ND1 protein in the muscle (data not shown).

DNA Analysis and HUMARA Assay

PCR and direct sequencing of the *MT-ND1* genes from the blood showed that all family members were homoplasmic for a novel m.4087A>G mutation (Fig. 2). The mutation was found homoplasmic in the muscle and blood of the proband, his maternal aunt, his sister, and her daughter. Since the mutation was found to be homoplasmic in four generations, and in two embryonically different tissues (blood and muscle), we did not find it necessary to test for the mutation in other tissues. The mutation predicts a threonine to alanine substitution at position 261 (p.T261A). The threonine at position 261 is not highly conserved, and the physiochemical difference between threonine and alanine is only moderate. No other mutations were detected in the seven complex I mtDNA genes.

To exclude a possible X-linked inheritance of an unknown mutation, the sister of the proband was assessed



I: 1 Maternal grandmother (blood)

II: 1 Mother (blood) 2 Maternal aunt (blood + muscle biopsy)

III: 1 Proband (blood + muscle biopsy) 2 Sister (blood +muscle biopsy)

IV: 1 Niece (blood +muscle biopsy) 2 Nephew (blood)

Fig. 2 Family pedigree. All shown family members carried 4087 A>G mtDNA mutation in *MT-ND1*

with the HUMARA assay. She displayed a slightly biased ratio (18:82), which is not uncommon, arguing against an X-linked inheritance.

Bicycle Ergometry Tests

During the bicycle ergometry test, the proband reached a VO_2 level of 22 ml O_2/kg/min at a workload of 64 watt. Since the patient cycled at predicted 69% of VO_2 max, VO_2 max was calculated to be 32 ml O_2/kg/min, which is 60% of expected for age (Swain et al. 1994). The lactate levels in the blood sample before and at the end of cycling were 0.6 and 2.7 mmol/l, respectively. On another occasion his blood lactate levels at rest had been slightly elevated (2.7 mmol/l).

Discussion

Here, we report a family with a novel, homoplasmic m.4087A>G mutation in *MT-ND1*, which expands the genotypic spectrum of mtDNA-related diseases and provides a new phenotype for aberrations in *MT-ND1* and for homoplasmic mtDNA mutations in general. We have sequenced the *MT-ND1* gene in an excess of 80 samples from an ethnically matched population and never encountered the m.4087A>G change before. Moreover, m.4087A>G is absent from MitoMap.org, a highly comprehensive database compiling all published mtDNA variations.

The symptoms of myopathy and exercise intolerance in affected family members, and the ophthalmoplegia in the

proband, are all common symptoms in mitochondrial myopathies. Less common are painful exercise-induced muscle cramp accompanied by high levels of CK and episodic myoglobinuria and rhabdomyolysis. Recurrent episodes of myoglobinuria have only been described in a few other mitochondrial myopathy cases, particularly in cases with mutations in genes encoding structural complex III and IV genes and some mtDNA-tRNA genes (Vissing et al. 2013). Recurrent episodes of myoglobinuria caused by mutations in mtDNA complex I subunit genes have not been described before, and neither has a homoplasmic mtDNA mutation with a myopathic phenotype. Only one (heteroplasmic) mtDNA mutation of a complex I gene (m.11832G>A mutation in *MT-ND4*) has been reported to cause myopathy, but not myoglobinuria (Taylor and Turnbull 2005).

The *MT-ND1* mutation was present in all family members, and accordingly complex I activity in skeletal muscle was significantly decreased in all tested individuals (22–52% of mean control when expressed as a ratio relative to complex II activity) and muscle histology showed myopathic changes. An acyl-carnitine analysis of blood from the proband showed normal values, indicating intact beta-oxidation of fatty acids. A western blot showed normal amounts of ND1 protein. Since complex I is a very large protein complex, and we only targeted it with a single antibody, the finding of normal protein expression does not exclude a complex I deficiency. In fact, biochemical analyses showed a clear defect of complex I activity in the muscle of all tested family members.

The proband performed a bicycle ergometry test with a calculated VO_2 max of 32 ml O_2/kg/min. This is 60 % of what is expected in age-matched controls (Astrand 1960) showing that the patient has exercise intolerance and supports an oxidative defect. The lactate levels before and at the end of cycling were in the normal range. We have previously showed that lactate measurements cannot be used as a reliable test to diagnose mitochondrial myopathy (Jeppesen et al. 2003).

The pedigree could theoretically be compatible with an X-linked mode of inheritance, and we therefore carried out the HUMARA assay to assess for potential biased X-chromosome inactivation. The sister to the proband displayed a slightly biased ratio (18:82), which is not uncommon. Thus, the HUMARA assay does not support an X-linked mode of inheritance.

It is common to see a great variability in phenotype among affected family members with homoplasmic mtDNA mutations, e.g., in LHON, where only 50% of males and 10% of females develop impaired vision, indicating that nuclear genetic factors play an important role in the clinical expression (Taylor and Turnbull 2005), as well as environmental factors (cigarette smoking, alcohol, and certain drugs) (Yu-Wai-Man et al. 2011). Other homoplasmic complex I mtDNA mutations are associated with a spectrum of phenotypes from subtle symptoms to severe multisystem disorders. As an example, the homoplasmic m.14459G>A mutation in the *MT-ND6* gene can cause LHON, dystonia, and Leigh-like disease but can also be found in asymptomatic individuals within the same family (Wong 2007). The family in our study expresses a new phenotype for homoplasmic mutations in *MT-ND1* ranging from mild myopathic symptoms and reduced complex I activity to myopathic features on muscle biopsy and recurrent episodes of myoglobinuria, rhabdomyolysis, and muscle cramps.

Synopsis

We present a novel maternally inherited *MT-ND1* mutation associated with exercise intolerance and myoglobinuria.

Compliance with Ethics Guidelines

Competing Interest

Dr. Vissing has received research and travel grants from Genzyme Corporation and serves on the scientific global advisory board for Pompe disease for Genzyme Corporation.

Jabin Rafiq, Morten Duno, Elsebet Østergaard, Kirstine Ravn, Christoffer Vissing, and Flemming Wibrand declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

Mrs. Rafiq – study concept and design, acquisition of data, and drafting of the paper

Dr. Vissing – study concept and design, acquisition of data, study supervision, and critical revision of the manuscript

Dr. Duno – analysis and interpretation and critical revision of the manuscript

Dr. Wibrand – analysis and interpretation and critical revision of the manuscript

Dr. Østergaard – acquisition of data, analysis, and interpretation of data

Mr. C.R. Vissing – acquisition of data, analysis, and interpretation of data

Dr. Ravn – acquisition of data, analysis, and interpretation of data

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CASE REPORT

PNPO Deficiency and Cirrhosis: Expanding the Clinical Phenotype?

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Abstract We report the case of a 4-year-old boy with pyridoxamine 5-phosphate oxidase deficiency, now the second reported case to develop hepatic cirrhosis. He presented with an encephalopathy in the first 1.5 h of life and received a first dose of PLP at 40 h of life. *PNPO* gene sequencing identified homozygosity for a novel variant in exon 7, c.637C>T (p.Pro213Ser). Persistent elevations in alanine transferase and aspartate transferase combined with an echogenic liver on ultrasound prompted performance of a liver biopsy which demonstrated hepatic cirrhosis. This is the second reported case of hepatic cirrhosis in PNPO deficiency. The pathogenesis is unclear but may be related to epigenetic activation of purinergic signaling in the hepatic stellate cells. PNPO deficiency may in time prove to be a suitable candidate for consideration of therapeutic orthotropic liver transplantation in select patients.

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Introduction

Pyridoxamine 5-phosphate oxidase (PNPO) deficiency (OMIM 610090) is a rare autosomal recessive inborn error of metabolism (IEM). To date there have been less than 50 cases reported in the medical literature (Veerapandiyan et al. 2011; Mills et al. 2005, 2014; Hoffmann et al. 2007; Clayton 2006; Bagci et al. 2008; Ruiz et al. 2008; Schmitt et al. 2010; Ware et al. 2014; Porri et al. 2014). The initial reported clinical phenotype of PNPO deficiency included prematurity, early-onset neonatal encephalopathy, and seizures resistant to conventional anticonvulsants and pyridoxine. Those who have survived the neonatal period have had significant neurodevelopmental disorders in the form of ongoing seizures, developmental delay, and microcephaly (Mills et al. 2005; Hoffmann et al. 2007; Clayton 2006; Bagci et al. 2008; Ruiz et al. 2008; Schmitt et al. 2010). As is often the case with rare diseases, the clinical phenotype broadens as more cases are diagnosed with time, as demonstrated in recent reports of PNPO deficiency patients whose seizures respond to pyridoxine and who have seizure onset as late as 5 months of age (Mills et al. 2014; Plecko et al. 2014). We now report the second case of a patient with PNPO deficiency in whom hepatic cirrhosis has been identified in early childhood.

Case Report

Our patient is currently 4 years of age and was briefly been reported in the literature (Mills et al. 2014). He was delivered at term via emergency lower uterine segment cesarean section under general anesthetic after a failed induction of labor for preeclampsia at 37 weeks' gestation. There was no documented fetal distress. He was cyanosed

and bradycardic at birth but responded well to initial bag and mask ventilation and had APGAR scores of 5, 8, and 8 (at 1, 5, and 10 min). Birth weight was 3.39 kg, length 50 cm, and head circumference 34 cm.

At 1.5 h of age, he was noted to be encephalopathic and in status epilepticus. He required intubation and ventilation. Loading doses of phenobarbitone, phenytoin, and midazolam did not result in seizure control. The EEG showed a burst suppression pattern and multifocal sharp wave activity. Intravenous pyridoxine 100 mg was administered during this EEG recording (at 40 h of age) with no effect on EEG or seizure activity. A trial of oral pyridoxal 5′-phosphate (PLP) 100 mg TDS (30 mg/kg/day) was commenced at 40 h of age. In the 12 h following PLP administration, the EEG showed ongoing ictal events, the majority manifesting clinically as abnormal eye movements and multifocal, erratic myoclonic jerks. EEG improvement was seen by 3 days of treatment with PLP; seizures were controlled and neurological examination was normalized.

Withdrawal of PLP was attempted at 4 months of age. On reducing the dose to 50 mg once daily, seizure activity returned. The *PNPO* gene (OMIM 603287) was sequenced utilizing automated florescence sequencing methods and showed homozygosity for a novel variant in exon 7, c.637C>T (p.Pro213Ser). Both parents were also heterozygous for this change. Software analysis predicted this to be a pathogenic alteration in the *PNPO* gene (http://sift.jcvi.org, http://genetics.bwh.harvard.edu/pph/).

At 2 years of age, the PLP dose required to prevent seizures was 50mg/kg/day divided into four doses and given at carefully managed, specific intervals (200 mg at 05:00 h, 125 mg at 11:45 h, 125 mg at 16:00 h, and 250 mg at 19:15 h). The PLP has been prepared in a consistent fashion; 50 mg capsules have been opened to release the powder within which was then dissolved in apple juice, with immediate administration. However, seizures that have included apnea continued to be observed during dosing interruption, e.g., vomiting illnesses, or even with delayed dose delivery during "steady state." On follow-up at 4 years of age, this patient is developing well, with a normal neurological examination.

Prior to the performance of a percutaneous liver biopsy, a FibroScan liver stiffness measure was performed returning an abnormal result of 8.5 Kpa. Persistently elevated serum transaminases were noted from 2 years of age. Serial measurements demonstrated alanine transferase (ALT: 205, 132, 89 U/L, reference range 5–20) and aspartate transferase (AST: 47, 75, 112 U/L, reference range <48). Alkaline phosphatase, gamma-GT, and bilirubin levels were always normal. Indices of hepatic synthetic function remained normal, including blood glucose, albumin, and coagulation studies. An abdominal ultrasound at 3 years of age demonstrated a mildly enlarged liver and spleen and mild increase in liver parenchymal echogenicity.

Percutaneous liver biopsy demonstrated early cirrhosis with disturbed hepatic architecture, portal–central linking fibrosis, early nodule formation, and marked sinusoidal fibrosis. There was no evidence of inflammation or steatosis. Occasional hepatocytes demonstrated an induced (i.e., ground glass) appearance. There was no stainable iron, alpha1-antitrypsin globules, copper-associated protein, or hepatitis B surface antigen.

Discussion

PNPO deficiency is a rare disorder with less than 50 reported cases in the literature. Common clinical features in reported cases thus far include (1) neonatal encephalopathy with seizures resistant to multiple anticonvulsants (Mills et al. 2005; Schmitt et al. 2010), (2) burst suppression EEG pattern (Veerapandiyan et al. 2011), (3) nonresponsiveness to pyridoxine (Clayton 2006), (4) complete or partial responsiveness to PLP (Pearl et al. 2013), (5) prematurity (Veerapandiyan et al. 2011), and (6) neonatal lethality if the diagnosis was not suspected and P5P administered (Khayat et al. 2008). Recently three groups of PNPO deficiency clinical phenotypes have been postulated: (1) early-onset neonatal seizures responsive to PLP, (2) infantile spasms in infancy, and (3) seizures beginning in the first three months of life responsive to pyridoxine (Mills et al. 2014). The early diagnosis and treatment with PLP has been linked with improved neurodevelopmental outcomes (Hoffmann et al. 2007).

Recently an 8-year-old Australian patient with PNPO deficiency was described with hepatic cirrhosis and early portal hypertension (Sudarsanam et al. 2014). He was initially managed with 100 mg/kg/day of PLP. At 2 years of age, similar hepatic derangement was observed to that seen in our patient. AST and ALT in this patient decreased numerically with PLP dose reduction to 50–60 mg/kg/day. Hepatic levels of B6 vitamers, pyridoxal and pyridoxic acid, were 40 times greater than two control samples. The authors postulated that cirrhosis might have developed secondary to high doses of PLP (~100 mg/kg/day) or chemical instability of PLP when delivered in the aqueous form. The parents of our patient reported that exposure of the PLP to light and sunlight led to discoloration of the medication and that they had learned to administer it immediately after it was prepared. Mild elevations in AST and ALT have been reported in another patient with PNPO deficiency being treated with 50 mg/kg/day of PLP, although that patient has not been reported to have undergone a liver biopsy (Porri et al. 2014).

Liver cirrhosis is an end point of multiple potential insults such as infection, toxin ingestion, drug-induced liver injury, autoimmune inflammation, and IEM. Despite the heterogeneity in potential triggers and potentiators, the molecular pathways that lead to fibrosis remain constant. The hepatic stellate cell (HSC) is the main fibrogenic cell type which orchestrates the deposition of extracellular matrix material (ECM) in the liver (Friedman 2000). HSCs are located in the perisinusoidal cells in the subendothelial space between hepatocytes and sinusoidal endothelial cells (Blomhoff and Wake 1991). After a fibrogenic stimulus, HSCs are activated into a myofibroblast-like state which upregulates the secretion of collagen, fibronectin, and extracellular matrix proteins (Dranoff et al. 2007).

The process of HSC activation has been demonstrated to be under epigenetic control including aberrant DNA methylation, noncoding RNA expression, and histone posttranslational modification (Yao and Li 2015; Bian et al. 2013; Mann 2014). Collagen and procollagen gene expression can be regulated by a number of signal pathways. The dominant stimulus occurs via the cytokine, transforming growth factor beta (TGF-B) (Friedman 2000). Other signaling pathways leading to fibrosis include Toll-like 4 receptor (Wnt, Ying Ying), peroxisome proliferator-activated receptor gamma, and purinergic receptors (Yao and Li 2015). Upregulation of the purinergic receptors P2Y on the HSC has been linked with collagen production, suggesting these receptors may be appropriate targets for antifibrotic agents (Dranoff et al. 2004, 2007). Of interest, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) is a synthetic inhibitor of P2 receptors (Dranoff et al. 2007). In vitro studies of PPADS demonstrated their ability to reduce HSC proliferation and fibrogenesis. PPADS analogues have been proven to act on P2 receptors in both antagonist and agonist roles, for example, PLP and pyridoxine-alpha 4,5-monophosphate, respectively (Brown et al. 2001). Aberrant purinoceptor activation of HSC due to PLP metabolites or degradation products may be a potential cause of hepatic fibrosis in PNPO deficiency.

Our case is now the second patient with PNPO deficiency in whom hepatic cirrhosis has been demonstrated. Our patient has never received "high dose" PLP, rather a dosage range of 30–50 mg/kg/day. These two cases highlight the broadening phenotype of PNPO deficiency and the occurrence of significant liver disease, cirrhosis, in early childhood. Surveillance for evidence of cirrhosis should be part of the clinical management of these patients. However, the most suitable form of monitoring is unclear. Cystic fibrosis liver disease (CFLD) is a common cause of morbidity and mortality in children with cystic fibrosis. Clinical modalities employed to monitor for CFLD, such as hepatic ultrasound and biochemical indices, are not predictive for their risk of clinically significant hepatic fibrosis (Lewindon et al. 2011). Clinically significant CFLD can exist in the presence of normal biochemical parameters, AST, ALT, and ultrasound. The gold standard test for identifying hepatic fibrosis is a liver biopsy but is not without clinical risk (Lewindon et al. 2011). To date only two PNPO deficiency patients have undergone a liver biopsy, so the true incidence of hepatic fibrosis is unknown in this cohort; however, reasonable hepatic cirrhosis surveillance in the PNPO deficiency cohort may be yearly ultrasound examinations and liver function testing.

These two cases of cirrhosis have led to consideration of the role of orthotopic liver transplantation (OLT) in the future management in our patient, but also as a potential treatment alternative for the IEM, as is the case for numerous IEM. The urea cycle defects and organic acidurias are the most suitable for OLT (Mazariegos et al. 2014) in carefully selected patients. OLT can generate a metabolic level of protection from the morbidity and mortality associated with a metabolic decompensation. Increasing reports of semi-elective OLT have emerged for "treatable IEM" such as glycogen storage disease type 1a where the disease burden associated with the nutritional management has a major impact on the quality of life (Boers et al. 2014). Crigler-Najjar disease is a non-life-threatening defect in bilirubin conjugation in which OLT is now an established option to improve the quality of life, reducing the requirement for phototherapy that is typically required for >16 h/day (Tu et al. 2012). PLP is a cofactor for numerous enzymes associated with neurotransmitter function, i.e., aromatic amino acid decarboxylase (synthesis of dopamine and 5-hydroxytryptamine), branched chain amino acid 2-oxoglutarate aminotransferase (synthesis of glutamate), glutamate decarboxylase (conversion of glutamate to GABA), GABA transaminase (breakdown of GABA and regeneration of glutamate), glycine cleavage system (catabolism of glycine), L-serine racemase (formation of D-serine), and histamine decarboxylase (synthesis of histamine) (Clayton 2006). The principal manifestation of the disorder is thus likely to be due to a deficiency of PLP in the brain leading to disordered neurotransmission and hence seizures, although we cannot be absolutely certain that accumulation of other vitamers such as pyridoxamine phosphate in the brain does not contribute to impaired function of PLP-dependent enzymes. A liver transplant should result in normal conversion of dietary pyridoxine, pyridoxamine, and their phosphates to PLP in the liver and thus lead to correction of circulating PLP deficiency. However, it will not correct the abnormality of the salvage pathway in the brain, which may be important for conversion of pyridoxamine phosphate back to PLP in that organ. On balance, it seems likely that OLT would lead to improved seizure control and a reduced requirement for PLP and/or the possibility of giving extra B6 in the form of

pyridoxine with possibly a lower risk of liver damage/cirrhosis in the graft.

In some PNPO patients, there is an emerging phenotype of normal neurodevelopmental outcomes (Mills et al. 2014), but this comes with a high disease burden and the need for constant vigilance. Insufficient P5P (e.g., dose interruption or delay) can result in life-threatening seizures and severe neurological morbidity. In our patient, even a delay of 15 min in usual dose timing has led to life-threatening seizures with apnea. The "round-the-clock" medication requirement to reduce the risk of catastrophe produces a significant disease burden for the family. While an OLT in PNPO deficiency would not cure the disease, we propose that a steady-state production of hepatic-derived PLP might lower the risk of seizures with delayed dosing and minimize the potential risk of long-term P5P-related complications. To this end we postulate that PNPO deficiency may be a suitable IEM for the consideration of OLT in carefully selected cases.

Compliance with Ethics Guidelines

Conflict of Interest

David Coman, Peter Lewindon, Peter Clayton, and Kate Riney declare that they have no conflicts of interest.

Informed Consent

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

Author Contributions

A/Prof David Coman is a metabolic physician and involved in the care of the patients and has coordinated the manuscript development and design.

A/Prof Peter Lewindon is a pediatric gastroenterologist who is involved in the patients' care and has been involved in the manuscript development.

Professor Peter Clayton is a metabolic physician who has provided invaluable clinical advice on the patients and has been involved in the manuscript development.

Dr. Kate Riney is a pediatric neurologist, is the primary caregiver for the patient, and has been involved in the manuscript development.

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RESEARCH REPORT

Liver Fibrosis Associated with Iron Accumulation Due to Long-Term Heme-Arginate Treatment in Acute Intermittent Porphyria: A Case Series

Barbara Willandt · Janneke G. Langendonk · Katharina Biermann · Wouter Meersseman · François D'Heygere · Christophe George · Chris Verslype · Diethard Monbaliu · David Cassiman

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Abstract Acute intermittent porphyria (AIP) is an autosomal dominant disorder of heme biosynthesis due to a mutation in the porphobilinogen deaminase gene. The mutation causes a deficiency in the porphobilinogen deaminase enzyme, thereby causing an accumulation of heme precursors (δ-aminolevulinic acid and porphobilinogen). These neurotoxic heme precursors elicit acute neurovisceral attacks, which can be treated with heme-arginate infusions. Some patients require heme-arginate infusions on a regular basis for many years, which ultimately leads to an iron accumulation (increased serum ferritin and iron accumulation in the liver, spleen, and bone marrow on MRI). We report three AIP patients, who developed iron accumulation (with serum ferritin up to 7,850 microgram/liter) due to multiple heme-arginate infusions. We report for the first time that the iron accumulation in these patients was associated with fibrosis on liver histology.

Conclusion: Regular heme-arginate treatment in AIP does not only lead to increased serum ferritin but may also induce liver fibrosis. This should be taken into account, when weighing the risks and benefits of repeated heme-arginate treatment against the risk and benefits of treating refractory AIP by liver transplantation.

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Introduction

Acute intermittent porphyria (AIP) is an autosomal dominant disorder of the heme biosynthesis pathway, resulting from mutations in the gene of the porphobilinogen deaminase (PBGD), also called hydroxymethylbilane synthase (HMBS; OMIM #609806). Patients suffer from acute neurovisceral attacks with abdominal pain, nausea, vomiting, and neuropsychiatric symptoms. These acute attacks, which can be life-threatening, are commonly treated with carbohydrate solutions and intravenous administration of heme-arginate preparations (Puy et al. 2010). Heme-arginate molecules contain the central iron molecule, the reactive center of the heme molecule. Less than 10% of patients develop recurrent attacks (Puy et al. 2010) and therefore require heme-arginate preparations on a regular basis for several years, which can result in progressive iron overload. We present three AIP cases developing a distinct iron overload secondary to the heme-arginate treatment, complicated with the development of liver fibrosis, confirmed on pathology.

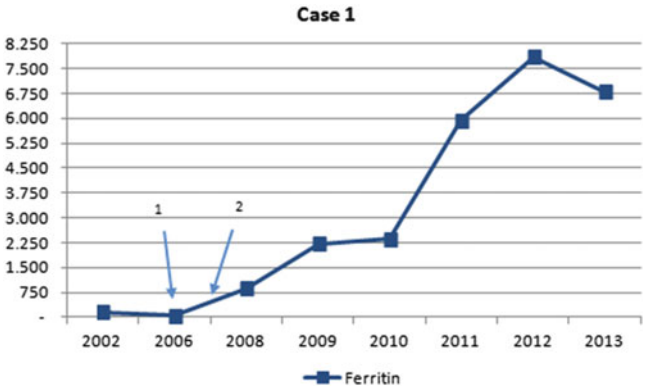

Fig. 1 Evolution of serum ferritin levels in case 1 (expressed in μg/L – normal values: 13–150 μg/L). *Arrow 1* introduction of heme-arginate infusions. *Arrow 2* introduction of weekly infusions

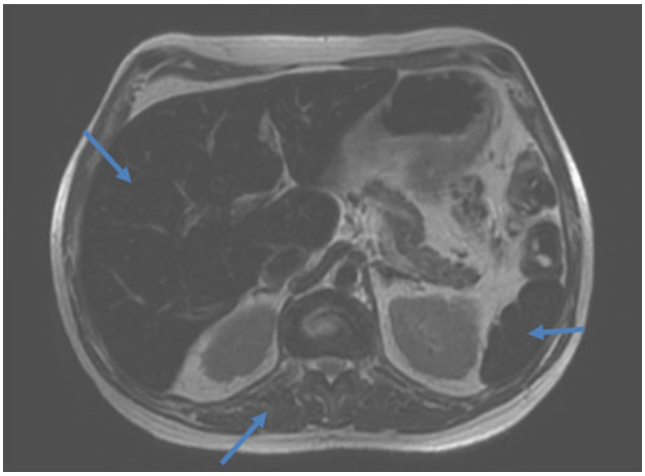

Fig. 2 T2-weighted image with signal intensity of the liver and spleen lower than the signal in the paravertebral muscle, pointing to iron accumulation in the liver and spleen

Cases

Case 1

In 2000, a 42-year-old male patient was diagnosed with AIP, based on clinical signs and increased urinary δ-aminolevulinic acid (ALA) and porphobilinogen (PBG), later on confirmed by genetic analysis (HMBS mutations c.517 C>T, p.173 P>W). Initially he suffered from recurrent acute attacks every few months. In the following years, he experienced a gradual increase of attack frequency, with attacks occurring every 2–3 weeks by the year 2006. Acute attacks were treated with glucose and heme-arginate infusions (3 mg/kg/day for 4 days). In May 2007 prophylactic treatment with heme-arginate infusions was introduced on a weekly basis, which initially led to an improvement of symptom control. Despite this regular medical treatment, he experienced a further increase in frequency of attacks the following years, severely affecting his quality of life. As a result of the chronic treatment with heme-arginate infusions, he developed a progressive iron overload, with increasing ferritin levels (Fig. 1) and on MRI reduced signal intensity of the spleen and liver on T1- and T2-weighted images as sign of secondary hemochromatosis (Fig. 2). Echocardiography showed a borderline left ventricular function, but without evidence of myocardial iron accumulation on MRI. Liver transaminases were slightly elevated (<2× ULN) and liver synthetic capacity remained normal (normal albumin and INR). Underlying mutations in the HFE1 gene (C282Y, H63D) on chromosome 6p21 were excluded, pointing to the absence of a genetic predisposition to hereditary hemochromatosis.

Treatment with phlebotomies was introduced in April 2009 in between acute attacks but was poorly tolerated (recurrence of attacks immediately after the phlebotomies). Eventually the patient was referred to our hospital for orthotopic liver transplantation because of poor quality of life, based on the frequent recurrence of acute attacks despite maximal medical treatment and progressive hyperferritinemia. In January 2014 he received a liver transplant. Histological review of the explant liver showed diffuse deposition of iron in the Kupffer cells and the hepatocytes but surprisingly also showed a septal stage of fibrosis. Currently the patient is doing well, without significant posttransplantation complications.

Case 2

A female patient was diagnosed with AIP in 1973, at the age of 38 years. In 2001 her medical history was complicated with a breast carcinoma, which was treated with a mastectomy followed by chemotherapy. Diagnosis of AIP was made when she presented with severe back pain and dark-colored urine. The diagnosis was confirmed by genetic analysis (HMBS mutations c.181 ins G). Because of a high frequency of acute attacks, she was started on weekly prophylactic heme-arginate infusions in 1996 (200 mg/day). Despite this intensive treatment, severe acute attacks continued at high frequency. In addition, there was a progressive increase in ferritin levels (Fig. 3) and transferrin saturation, with signs of bone marrow iron deposition on MRI of the spine. The latter was performed because of predominant back pain in a patient with previous breast carcinoma. No evidence of metastasis was found, but a clear iron deposition was detected. Liver function remained normal. Underlying mutations in the HFE1 gene (C282Y, H63D, S65C) on chromosome 6p21 were excluded. Heme-arginate administration was reduced and monthly phlebotomies were started in 2010. Because of persisting disabling attacks, the patient was eventually transplanted in August 2012. Pathological review of the explant liver showed excessive iron deposition in the hepatocytes and

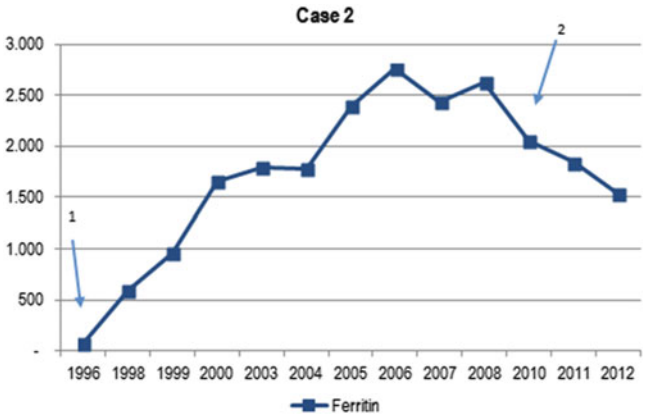

Fig. 3 Evolution of serum ferritin levels in case 2 (expressed in µg/L – normal values: women 10–140 µg/L). *Arrow 1* introduction of heme-arginate infusions. *Arrow 2* start of phlebotomies

Kupffer cells as well as advanced fibrosis with portoportal fibrous septa (Fig. 4). The posttransplantation period was complicated by a thrombosis of the hepatic artery, which was treated with an immediate surgical thrombectomy, but eventually she required a second transplant in November 2013. The patient is currently doing well.

Case 3

Another female patient was diagnosed with AIP in 1987 at the age of 45 years, confirmed by urinary and genetic analysis (HMBS mutations c.cgg-346-tgg, p.arg-116-trp, R116W). Treatment with heme arginate was administered on a regular basis for recurrent neurovisceral attacks from 2005, at 3 mg/kg/day (lifelong dose estimated at 40,000 mg). Biochemical follow-up revealed an asymptomatic elevation of ferritin levels up to 1904 µg/L. Mutations in the HFE1 gene (C282Y, H63D, S65C) were excluded. In 2010, the patient was diagnosed with hepatocellular carcinoma and treated with chemoembolization, followed by a partial hepatectomy (segments IV, V, and VIII) in 2011. Liver function was normal up until this complication. Histological review of the resection specimen showed excessive iron deposition in the hepatocytes and advanced fibrosis. Eventually the patient died in 2013, at the age of 70 years, of metastatic disease.

Discussion

We collected data of three illustrated cases of AIP patients presenting with acute neurovisceral attacks on an almost weekly basis and in need of frequent medical treatment. All patients were treated during attacks with the classical dose of 3–4 mg/kg of heme-arginate infusions per day and also periodically received preventive treatment, for many years. Secondary to this iron-loaded treatment, they developed secondary hemochromatosis, both biochemically and on imaging. For the first time, we report here that these ferritin increases were associated with advanced fibrosis on liver histology.

AIP is an autosomal dominant disorder of the heme biosynthesis pathway. Mutations result in an enzyme activity of approximately 50% of normal. More than 200 different mutations have been described in the gene encoding porphobilinogen deaminase (PBGD), also called hydroxymethylbilane synthase (HMBS) (Siegesmund et al. 2010). Prevalence of a mutant AIP gene is estimated at 1 per 500, but the penetrance of the disorder is incomplete and the prevalence of symptomatic disease is estimated at only 1–2 per 100,000 in Europe (Elder et al. 2013). The reduced PBGD enzyme activity causes accumulation of heme precursors ALA and PBG, which are neurotoxic and cause the acute neurovisceral attacks. These acute attacks are debilitating and may become life-threatening if unrec-

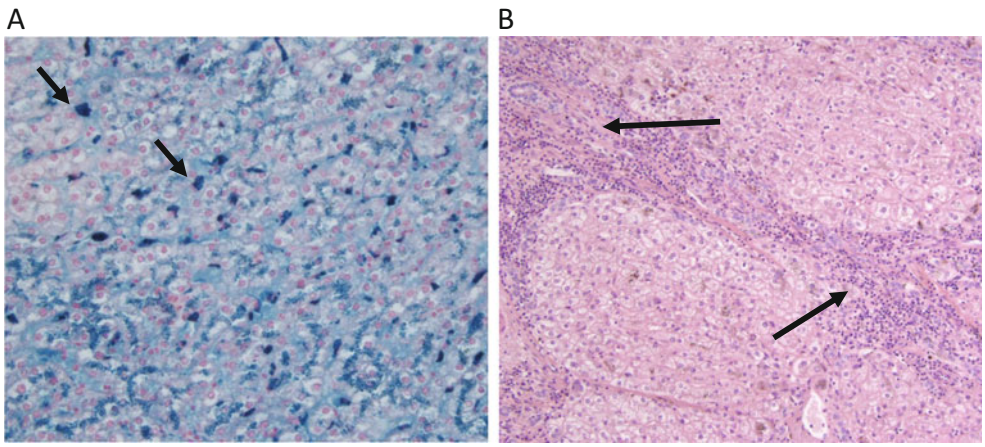

Fig. 4 (**a**) Iron staining on liver resection specimen from case 2 (*arrows*: iron deposits in Kupffer cells). (**b**) Fibrosis on liver resection specimen from case 2 (*arrows*: fibrous septum running from the upper left portal tract to the lower right portal tract)

ognized or left untreated. Most commonly patients present with abdominal pain, but pain can also occur in the back or thighs. Gastrointestinal dysmotility symptoms such as nausea, vomiting, and constipation are also highly prevalent. In addition, a variety of neurological and psychiatric symptoms can be associated with the attacks.

Diagnosis of AIP is based on clinical suspicion, with first-line confirmation through high urinary ALA and PBG during or shortly after acute attacks. Direct DNA sequencing has become the gold standard for diagnostic confirmation and defining the underlying genetic defects. This also allows counseling of family members, thereby preventing attacks in those with latent disease (Puy et al. 2010; Herrick and McColl 2005).

Treatment is primarily based on avoidance of precipitating factors that either induce production of ALA and PBG or increase the demand for heme synthesis in the liver and bone marrow. These precipitants include hormonal fluctuations in women, porphyrinogenic drugs, infections, alcohol use, smoking, and caloric deprivation (Badminton and Elder 2002; Herrick and McColl 2005). In case of an acute attack, treatment consists of intravenous administration of carbohydrates (minimum of 300 g/day), heme-arginate preparations (3 mg/kg heme arginate once daily for 4 days), and further supportive measures (e.g., pain treatment with safe drugs). The intravenously administrated heme inhibits the upregulation of ALA synthase in the liver resulting in a dramatic reduction of urinary and plasma ALA and PBG (Puy et al. 2010). Liver transplantation has become a valid treatment option in selected patients with recurrent attacks and significant disabling symptoms with poor quality of life (Seth et al. 2007; Singal et al. 2014).

Less than 10% of patients show frequently recurring attacks requiring medical treatment. In some of these patients, prophylactic use of heme arginate – e.g., every few weeks – is applied to prevent further attacks and improve symptom control and therefore also quality of life. In the literature, few complications of treatment with heme-arginate administration have been reported. Aside from anaphylactic reactions and local irritation of blood vessels (with thrombophlebitis and progressive loss of venous access), there are a few rare complications reported such as renal failure and acute liver failure due to overdose (Anderson et al. 2005; Frei et al. 2012). However, knowing that a single dose of human heme arginate contains 22.7 mg of iron (Puy et al. 2010; Siegesmund et al. 2010), there is also a high risk of developing iron overload and secondary hemochromatosis with related complications in the subgroup of patients receiving repeated administration of heme arginate over several years. Current literature is limited on this matter, but AIP seems not to be directly associated with fibrosis or cirrhosis in itself. Even in AIP patients developing HCC, cirrhosis is rarely reported (Innala and Andersson 2011; Stewart 2012). Based on the current literature, it is even suggested that patients diagnosed with HCC in a non-cirrhotic liver should be screened for acute intermittent porphyria (Deybach and Puy 2011). In the patients we report here, the liver fibrosis we observed is most likely due to the secondary hemochromatosis induced by years of treatment with heme-arginate preparations. Other underlying causes of liver fibrosis, such as chronic viral hepatitis, were excluded on serology and on biopsies. To our knowledge, these are the first patients described with fibrosis due to medical treatment of acute AIP attacks with heme arginate. This implies the necessity for close monitoring of ferritin levels in patients receiving regular heme arginate and the need to screen for related complications such as liver fibrosis/cirrhosis as well as for extrahepatic complications (cardiac involvement, diabetes, etc.). It remains unclear when treatment and screening for complications should be started, although applying the same guidelines as for hereditary hemochromatosis and other forms of iron overload seems the most appropriate (Siddique and Kowdley 2012; European Association for the Study of the Liver 2010).

Conclusion

Chronic administration of heme arginate over many years in AIP patients can result in secondary hemochromatosis with liver fibrosis. Recurrence of acute neurovisceral attacks in patients with AIP necessitates regular heme-arginate infusion in less than 10% of AIP patients. This subgroup appears to be at risk for evolution to secondary hemochromatosis with associated complications, such as liver fibrosis. Close monitoring of ferritin levels is needed during follow-up, as well as screening for complications of iron overload. Increasing ferritin levels are to be taken into account when evaluating these patients for liver transplantation, in addition to the standard considerations as are the presence of repeated life-threatening acute attacks, failure of medical therapy, and poor quality of life.

Synopsis

Regular treatment with heme arginate in AIP may lead to iron overload and liver fibrosis.

Compliance with Ethics Guidelines

Conflict of Interest

Barbara Willandt, Janneke G. Langendonk, Katharina Biermann, Wouter Meersseman, François D'Heygere,

Christophe George, Chris Verslype, Diethard Monbaliu, and David Cassiman declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

Barbara Willandt collected the data and wrote the first report. Janneke G. Langendonk, Katharina Biermann, Wouter Meersseman, François D'Heygere, Christophe George, Chris Verslype, Diethard Monbaliu, and David Cassiman were all involved in planning and executing the clinical follow-up and hence data collection. All authors contributed in writing or correcting/improving the final manuscript.

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CASE REPORT

GM2-Gangliosidosis, AB Variant: Clinical, Ophthalmological, MRI, and Molecular Findings

Deborah Renaud · Michael Brodsky

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Abstract GM2-gangliosidosis, AB variant is a very rare form of GM2 gangliosidosis due to a deficiency of GM2 activator protein, associated with autosomal recessive mutations in GM2A. Less than ten patients, confirmed by molecular analysis, have been described in the literature.

A 12-month-old Hmong girl presented to the neurometabolic clinic for evaluation of global developmental delay, hypotonia, and cherry red spots. The parents were not known to be consanguineous. Her examination was remarkable for hypotonia with hyperreflexia and excessive startling. The head circumference was normal. An extensive neurometabolic evaluation was negative.

Developmental regression began at 14 months of age. Retinal examination at 16 months of age disclosed 4+ cherry red/black spots with "heaped up" ring of whitish infiltrate surrounding both foveae but no evidence of optic atrophy or peripheral retinal abnormalities. Repeat magnetic resonance imaging (MRI) scan at 17 months of age revealed delayed but interval myelination associated with abnormal signal intensity of the bilateral thalami presenting as T2 hyperintensity of the posterior thalami in the region of the pulvinar nuclei and T2 hypointensity in the anterior thalami. Sequencing of the GM2A gene revealed a homozygous c.160 G>T mutation, predicted to result in a premature protein termination p. Glu54*.

Communicated by: Nicole Wolf, MD PhD

Competing interests: None declared

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Introduction

The GM2 gangliosidoses are a group of disorders characterized by progressive neurological deterioration associated with the excessive accumulation of GM2 ganglioside and related glycolipids in the lysosomes, primarily of neuronal cells. GM2-gangliosidosis, AB variant (OMIM # 272750) is a very rare form of GM2 gangliosidosis due to a deficiency of GM2 activator protein, associated with autosomal recessive mutations in GM2A. The clinical phenotype of the AB variant is very similar to the classic infantile form of Tay-Sachs disease. Hexosaminidase A (Hex A) and B (Hex B) levels are normal (Gravel et al. 2014). Less than ten patients with AB variant, confirmed by molecular analysis, have been described in the literature.

We describe the clinical, ophthalmological, magnetic resonance imaging (MRI), and molecular findings in a patient of Hmong descent. The finding of a homozygous mutation present in a previous patient of Hmong descent suggests a founder mutation.

Case Report

A 12-month-old Hmong girl presented to the neurometabolic clinic for evaluation of global developmental delay, hypotonia, and cherry red spots.

She was the product of her mother's fifth pregnancy. Her mother had experienced three early miscarriages. She was born at term by spontaneous vaginal delivery following an uncomplicated pregnancy apart from mild gestational diabetes requiring dietary management. The first concern was at 3–4 months of age, when she developed roving eye movements and decreased visual fixation. By 6 months of age, hypotonia was noted and global developmental delay

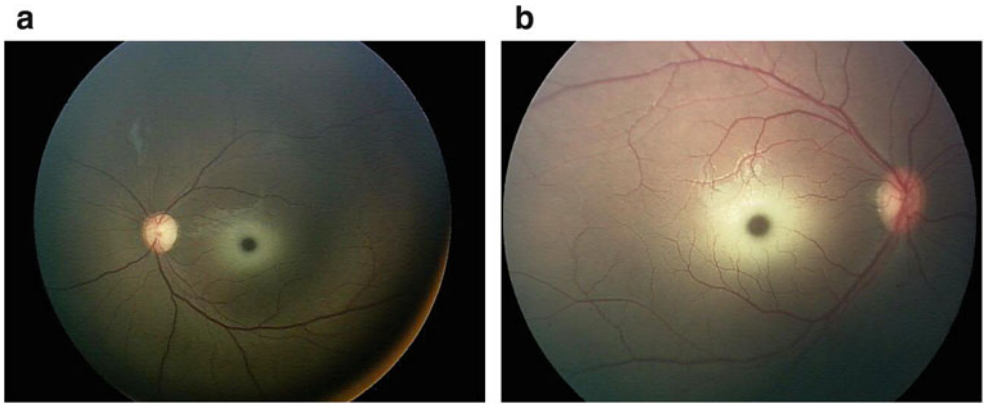

Fig. 1 Retinal examination disclosed 4+ cherry red with a ring of "heaped up" whitish infiltrate surrounding both fovea with no optic atrophy or peripheral retinal abnormalities

was noted at 10–11 months. She started to crawl just before 12 months of age and was still gaining skills at the time of her first assessment. Excessive startling and irritability were noted and myoclonic jerks at sleep onset also developed. She was otherwise healthy. The family history was negative and the parents were not known to be consanguineous. Her examination was remarkable for global developmental delay and hypotonia with hyperreflexia and excessive startling. The head circumference was normal. Bilateral cherry red spots were present. An extensive neurometabolic evaluation including beta-hexosaminidase A activity and evaluation for other causes of cherry red spots including GM1-gangliosidosis, Krabbe disease, cherry red spot myoclonus, and Niemann-Pick type C were negative.

Developmental regression began at 14 months of age. Progressive hypotonia developed over the subsequent 2 months. By 16 months of age, she was no longer able to crawl, roll, or sit independently. Her grasp became weaker and her fine motor skills declined. She started to have difficulty with swallowing and choking on solids and liquids. Retinal examination at 16 months of age disclosed 4+ cherry red spots with a ring of "heaped up" whitish infiltrate surrounding both foveae but no evidence of optic atrophy or peripheral retinal abnormalities (Fig. 1).

Repeat magnetic resonance imaging (MRI) scan at 17 months of age revealed delayed but interval myelination associated with abnormal signal intensity of the bilateral thalami presenting as T2 hyperintensity of the posterior thalami in the region of the pulvinar nuclei and T2 hypointensity in the anterior thalami (Fig. 2)

Molecular analysis of the GM2A gene revealed a homozygous c.160 G>T mutation, predicted to result in a premature protein termination p. Glu54*.

Discussion

The GM2 gangliosidoses are a group of disorders characterized by the excessive accumulation of GM2 ganglioside and related glycolipids in the lysosomes, primarily of neuronal cells. Three forms of GM2 gangliosidosis have been described. Tay-Sachs disease and its variants result from mutations in the HEXA gene and are associated with deficient hex A activity but normal hex B activity. Sandhoff disease and its variants result from mutations in HEXB and are associated with a deficiency of both Hex A and Hex B activity. GM2-gangliosidosis, AB variant is a very rare form of GM2 gangliosidosis due to a deficiency of GM2 activator protein, associated with autosomal recessive mutations in GM2A. Hexosaminidase A and B levels are normal. The clinical phenotype of the classic infantile form of GM2 gangliosidosis is characterized by normal early development followed by developmental regression, progressive weakness, exaggerated startle, vision loss with cherry red spots, and seizures (Gravel et al. 2014). All patients with GM2-gangliosidosis, AB variant to date have presented with the classic infantile clinical phenotype.

The MRI findings in patients with GM2-gangliosidosis, AB variant, have not been well characterized. Chen et al. (1999) describe abnormal MRI signal intensity in the periventricular white matter and basal ganglia in a patient with AB variant. The MRI in Tay-Sachs demonstrates diffuse dysmyelination of hemispheric white matter and bilateral symmetric signal change in the thalami with hyperintensity on T1 and hypointensity on T2 and FLAIR images (Sharma et al. 2008). Magnetic resonance imaging of our patient at 17 months of age revealed delayed but interval myelination associated with abnormal signal

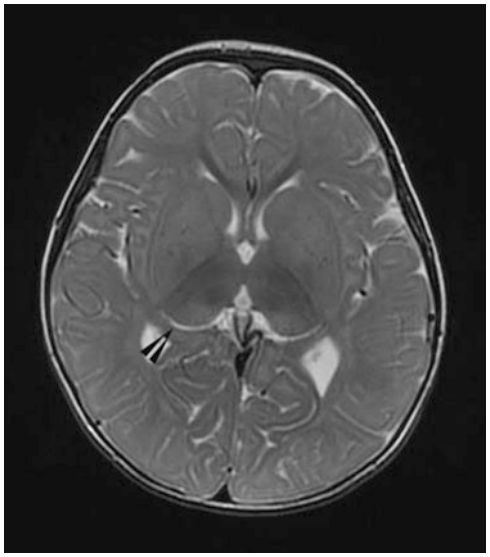

Fig. 2 Magnetic resonance imaging (MRI) scan at 17 months of age revealed delayed but interval myelination associated with abnormal signal intensity of the bilateral thalami presenting as T2 hyperintensity of the posterior thalami in the region of the pulvinar nuclei (*arrowhead*) and T2 hypointensity in the anterior thalami

intensity of the bilateral thalami presenting as T2 hyperintensity of the posterior thalami in the region of the pulvinar nuclei and T2 hypointensity in the anterior thalami (Fig. 1).

The first case confirmed by molecular analysis was a black female diagnosed on the basis of autopsy findings suggestive of GM2 gangliosidosis in the setting of normal hexosaminidase A enzyme activity. A homozygous c.412T>C missense mutation was found in the GM2AB gene resulting in a single amino acid substitution, p. Arg169Pro (Schroder et al. 1991; Xie et al. 1992). Another missense mutation, c.506G>C, described by Schroder et al. (1993) leads to a substitution of proline for Arg169 resulting in premature degradation of the mutant GM2 activator protein. Schepers et al. (1996) described two small homozygous intragenic deletions in patients presenting with a family history of known consanguinity. The first in a patient of Saudi origin had a three base pair in frame deletion (AAG 262-264) with the deletion of lysine 88, and the other in a patient of Spanish origin had a single base deletion (A410) resulting in a frame shift beginning at codon 137 and leading to a stop codon at 170. A recent review of 73 cases of GM2 gangliosidosis in the United Kingdom included 2 cases of AB variant (one of Caucasian and the other of Pakistani origin); however, molecular findings were not described (Smith et al. 2012).

Chen et al. (1999) described a boy of Laotian Hmong ancestry with no known consanguinity. He was normal until 5 months of age when he developed delayed motor milestones and increasing weakness associated with cherry red spots. His hexosaminidase A activity was normal. Molecular analysis of the GM2A gene revealed a homozygous c.160 G>T mutation, predicted to result in a premature protein termination p. Glu54*. This mutation is the same as our patient who is also the product of non-consanguineous parents of Hmong ancestry, suggesting a founder mutation.

Take-Home Message

Typical clinical symptoms and MRI findings associated with cherry red spots in the setting of normal beta-hexosaminidase A activity should lead to sequencing of GM2A for AB variant, a rare, but underdiagnosed, form of GM2 gangliosidosis.

Compliance with Ethics Guidelines

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

Author Contributions

Deborah L. Renaud, M.D., is the primary author and corresponding author.

Michael Brodsky, M.D., contributed to the writing and editing of the manuscript and provided Fig. 1.

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RESEARCH REPORT

Successful Domino Liver Transplantation from a Patient with Methylmalonic Acidemia

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Abstract Liver transplantation has been reported in patients with methylmalonic acidemia (MMA), but long-term outcome is controversial. Many patients with other approved indications for liver transplantation die before donor grafts are available. A 28-year-old man with MMA underwent cadaveric liver transplantation. His liver was used as a domino graft for a 61-year-old man with primary sclerosing cholangitis, who had low priority on the transplant waiting list. Surgical outcome was successful, and after transplantation both patients have excellent graft function. The patient with MMA showed substantial decrease in methylmalonate in urine (from 5,277 ± 1,968 preoperatively to 1,068 ± 384 mmol/mol creatinine) and plasma (from 445.9 ± 257.0 to 333.3 ± 117.7 μmol/l) over >1-year follow-up, while dietary protein intake increased from 0.6 to 1.36 ± 0.33 g/kg/day. The domino recipient maintained near-normal levels of plasma amino acids but did develop elevated methylmalonate in blood and urine while receiving an unrestricted diet (peak plasma methylmalonate 119 μmol/l and urine methylmalonate 84–209 mmol/mol creatinine, with 1.0–1.9 g/kg/day protein). Neither patient demonstrated any apparent symptoms of MMA or metabolic decompensation during the postoperative period or following discharge.

Conclusion: Liver transplantation substantially corrects methylmalonate metabolism in MMA and greatly attenuates the disease. In this single patient experience, a liver from a patient with MMA functioned well as domino graft although it did result in subclinical methylmalonic acidemia and aciduria in the recipient. Patients with MMA can be considered as domino liver donors for patients who might otherwise spend long times waiting for liver transplantation.

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Introduction

Methylmalonic acidemia (MMA) is a biomarker for a family of disorders in which the activity of methylmalonyl-CoA mutase is defective (Nyhan et al. 2012). Deficiency of the mutase apoenzyme (mut^0) is the most severe form, often leading to death in infancy or severe neurologic disability (Matsui et al. 1983). The mutase is involved in the catabolism of four essential amino acids, as well as odd chain fatty acids and the side chain of cholesterol (Fig. 1), so dietary restriction of the sources of methylmalonate is demanding. Excessive metabolite accumulation from diet or catabolic stresses can precipitate ketosis and major metabolic decompensation, which can be fatal without successful treatment. Failure to thrive and developmental delay are regular features. Kidney disease, including renal tubular acidosis and interstitial nephritis, may develop and lead to renal failure which may require renal transplantation (Walter et al. 1989).

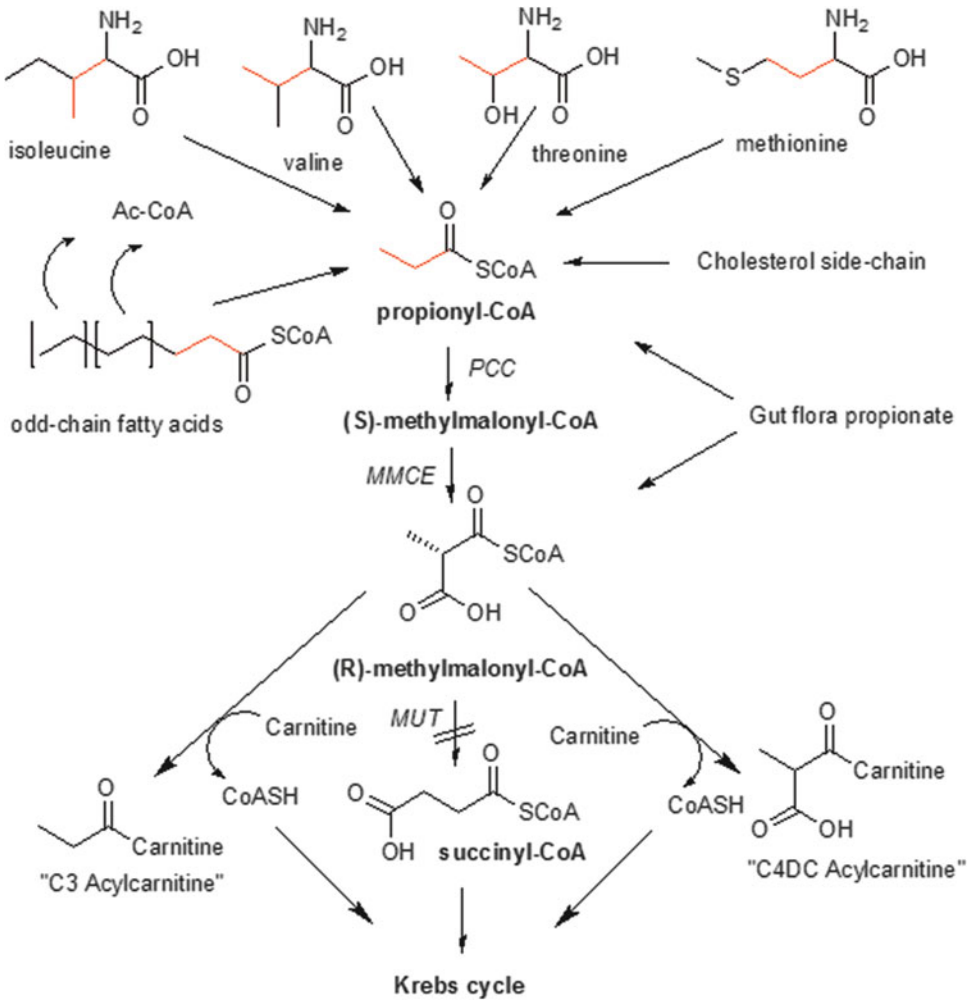

Fig. 1 Human propionate metabolism. The three carbon groups of the metabolic sources of propionyl-CoA are shown in *red*. Enzymes are labeled (*PCC* propionyl-CoA carboxylase, and *Mut* methylmalonyl-CoA mutase, *MMCE* methylmalonyl-CoA epimerase). The acylcarnitine species are labeled according to their common designations (C3-acylcarnitine = propionylcarnitine, C4DC-acylcarnitine = methylmalonylcarnitine)

Liver transplantation (LTx) in MMA has been found to eliminate life-threatening recurrent ketoacidosis in some, but not all, cases (Leonard et al. 2001; Kayler et al. 2002; Nagarajan et al. 2005; Morioka et al. 2007; Chen et al. 2010). The mutase remains deficient in extrahepatic tissues, so, not surprisingly, disease manifestations may persist. Late-onset kidney and neurologic disease progression is not prevented, and cerebrospinal fluid concentrations of methylmalonate remain high (Leonard et al. 2001; Nyhan et al. 2002; Kaplan et al. 2006). Patients with coexisting kidney disease have undergone simultaneous liver–kidney transplantation with good results (Nagarajan et al. 2005). It is clear that LTx can be lifesaving, but its long-term value in MMA is still debated (Morioka et al. 2007; Chapman et al. 2012).

Nearly 15,000 patients are on the waiting list for liver transplantation in the United States, and fewer than 6,000 are transplanted each year. Several strategies such as the use of partial and split grafts, and of non-heart-beating and other "marginal" donors, have been used to increase graft availability. Domino transplantation (transplantation of an organ removed from the prospective recipient of another organ) was first performed with heart transplantation in the late 1980s (Yacoub et al. 1990). Domino liver transplantation (DLTx) was first performed in the late 1990s (Ando et al. 1997; Furtado et al. 1997; Azoulay et al. 1999; Furtado 2000) for familial amyloid polyneuropathy (FAP) and has found wider indications (Kitchens 2011), including familial hypercholesterolemia (FH) (Popescu et al. 2009; Liu et al. 2010; Popescu and Dima 2012) and maple syrup urine disease (MSUD) (Barshop and Khanna 2005; Khanna et al. 2006). In each of these conditions, the "trait" is transplanted, but the donor disease either does not develop in the domino recipient (as in MSUD) or, if it does, is manageable for a period of time exceeding the patient's expected survival from end-stage disease (as in FAP and FH).

We first reported DLTx using the liver from a patient with MSUD (Barshop and Khanna 2005; Khanna et al. 2006). This experience established that, in certain conditions, the recipient of the domino liver does not manifest the disease phenotype since the deficient enzyme is substantially extrahepatic. Since then, other centers have also performed DLTx using livers from donors with MSUD (Mazariegos et al. 2012; Badell et al. 2013).

This report describes the first experience with DLTx using the liver from a patient with MMA as a domino graft. Overall, favorable results were observed in both patients.

Methods

Patients

Patient 1 (domino donor) was a 28-year-old man with MMA, diagnosed in infancy during an episode of ketoacidosis and hyperammonemia. At birth, he required resuscitation due to umbilical cord prolapse and developed persistent metabolic acidosis and neutropenia, and elevated urine methylmalonate was found. He had a trial of cyanocobalamin (1,000 μg/day IV for >1 month), but showed no lowering of methylmalonate. His fibroblasts were shown to be in the mut^0 complementation group in the Rosenblatt laboratory (Raff et al. 1991), and he was subsequently documented to be homozygous for the c.322C>T (p.Arg108Cys) mutation in the *MUT* gene, previously shown to cause MMA (Worgan et al. 2006). For several years he was well controlled with management including dietary restriction of propiogenic amino acids and large intravenous doses of carnitine. He grew to adulthood and had relatively good quality of life with school, working, and driving. After the age of 21, he became inconsistent with diet and began to experience metabolic decompensation and episodes of altered mental status including paranoia and agitation and a decreasing functional status. Over the 3 years prior to LTx, he developed episodes of metabolic decompensation requiring multiple admissions to hospital with ketoacidosis. He also had increasing neurologic disability including seizures, altered gait, and slower speech. His biological MELD score was 10 (INR = 1, bilirubin = 0.7 mg/dl and creatinine 1.15 mg/dl) and was granted 30 MELD exception points after presentation to the United Network for Organ Sharing (UNOS) regional review board. The patient was lean, had no evidence of insulin resistance, and did not have elevated triglycerides, cholesterol, or liver enzymes, although liver biopsy at the time of explant for the domino procedure showed steatosis, as often seen in MMA (Fujisawa et al. 2013). The donor for patient 1 was an unrelated 30-year-old woman who suffered brain death, and the liver was procured locally.

Patient 2 (domino recipient) was a 61-year-old man with primary sclerosing cholangitis and biliary cirrhosis. He had a history of ulcerative colitis for which he underwent cholecystectomy and proctocolectomy 15 years previously. He had mild proctitis that was quiescent on medications, but had several episodes of bacterial cholangitis in the preceding year and poor quality of life. Bile duct brushings were negative for cancer. The patient had a MELD score of 16, which was far less than the average score at transplant for his blood type for our region (>25). He could not get extra points from the UNOS regional review board and had no living donor available and accordingly was offered the domino transplant. The Liver Transplant and Biochemical Genetics teams, including an independent donor advocate unassociated with the transplant program, spoke with him about domino liver transplantation, disease details in the donor, the metabolic condition, and the possibility of developing symptomatic MMA. Approval was obtained from the institutional ethics committee and the institutional review board.

Perioperative Courses

The liver of patient 1 (MMA patient/domino donor) was removed using the standard method, clamping the supra- and infrahepatic cava and conserving as much of the hepatic veins, portal vein, and hepatic artery as possible. Liver biopsy (Fig. 2) showed 25–30% macrosteatosis, but the transplant team felt that this was suitable for use as a domino graft. The liver transplant was completed in a piggyback fashion using well-described techniques.

Suprahepatic vena caval anastomosis was performed on patient 2 between the donor vena cava and recipient confluence of the middle, left, and right hepatic veins. Hepatic arterial anastomosis was performed between donor iliac artery jump graft and recipient common hepatic artery at the level of the gastroduodenal takeoff. A Roux-en-Y choledochojejunostomy was performed to reconstruct the biliary system because of history of primary sclerosing cholangitis.

Patient 1 (MMA patient, domino donor, and recipient of the cadaveric liver transplant) had excellent graft function but developed ascites and pleural effusion post-liver transplant. Imaging showed narrowing of the suprahepatic cava, probably related to clamp injury. An angiogram confirmed the presence of hemodynamically significant gradient, and segmental balloon angioplasty was performed with excellent recovery. The postoperative period was also complicated by acute kidney injury, deep venous thrombosis of the subclavian vein, and pulmonary embolism which responded to anticoagulation. He was initially discharged from the hospital on postoperative day (POD) 84. He was readmitted to hospital on POD 104–106 for the management

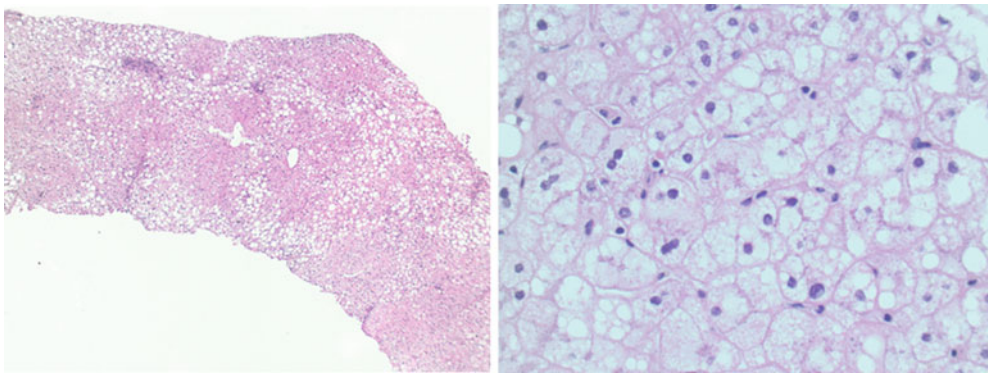

Fig. 2 Liver biopsy of explant from domino donor. Images (H&E stain, *left*: 40×, *right*: 400× magnification) show approximately 30% macrovesicular steatosis and an additional 10–20% microvesicular steatosis which is azonal, with focal ballooning degeneration and rare foci of lobular inflammation. There was no fibrosis identified on trichrome stain (not shown)

of renal insufficiency (including hemodialysis) and subsequently recovered. He was treated in hospital on POD 132–136 because of elevated liver enzymes, and biopsy indicated mild rejection that was treated to resolution. He had a seizure episode and purulent meningitis of unknown etiology was documented by lumbar puncture, which responded to an empiric course of antibiotics and antifungal and antituberculosis therapy, including hospitalization (POD 183–191). A pseudomonas pneumonia was diagnosed, and treatment was completed during another hospitalization (POD 206–219). He was initially given tacrolimus for immunosuppression, subsequently changed to cyclosporine following the seizure episode, and then to sirolimus, because of compromised renal function. Throughout his postoperative period, despite these complications, he showed no sign of metabolic decompensation or ketoacidosis. He has remained stable at home performing activities of daily living and has maintained normal liver chemistry and metabolic stability.

Patient 2 (domino recipient) had excellent graft function and was discharged on postoperative day 16 with tacrolimus for immunosuppression. He was admitted again (POD 153–156) because of febrile illness when a pulmonary opacity was identified and then readmitted (POD 158–165) when bronchoscopy confirmed *Nocardia brasiliensis* pneumonia. He responded well to a prolonged course of trimethoprim–sulfamethoxazole and moxifloxacin. He was noted to have nephrolithiasis and was hospitalized (POD 183–184) for management of a ureteral stone. At no point was there evidence of metabolic acidosis or ketonuria.

Biochemical Testing

Quantitative evaluation of urine and plasma organic acids and plasma carnitine and acylcarnitine profile was performed prior to and following transplantation at regular intervals during clinic visits and hospitalizations. Plasma samples were analyzed on an automated amino acid analyzer. Urine organic acids were analyzed by gas chromatography–mass spectrometry (Hoffmann et al. 1989).

Results

Domino Donor

Following LTx, dietary protein intake for the domino donor (MMA patient, liver transplant patient 1, recipient of the cadaveric liver transplant) was liberalized in small increments, and the time course is shown in Fig. 3, along with the course of laboratory test results. Plasma propionylcarnitine (C3-acylcarnitine) rose initially during the period of early postoperative complications and then settled at a plateau slightly lower than pretransplantation levels (63.2 ± 25.4 μmol/l preoperatively, 234.6 ± 71.8 over the first 50 postoperative days, and 58.3 ± 30.1 μmol/l beyond day 90). There was a drop in plasma methylmalonate from the pretransplantation level of 445.9 ± 257.0 μmol/l ($N = 3$) to 76.0 μmol/l in the postoperative period when protein was severely limited, and as protein was reintroduced, the level of plasma MMA was moderately elevated (801.4 ± 344.4 μmol/l from POD 7–40, while the patient had postoperative complications) and then reached a plateau (333.3 ± 117.7 μmol/l from 3 to 11 months posttransplant). Pretransplantation urinary methylmalonate was 5,277 ± 1,968 mmol/mol creatinine (reference range 0–5) and 1,068 ± 384 mmol/mol creatinine from month 3 to 11 (Fig. 3) despite liberalization of diet to 1.0–1.9 g/kg/day (daily average 1.4 ± 0.3, equivalent to >45 g/day), an amount which could not be considered previously.

The domino donor had normal liver enzymes and liver function but impaired renal function. Preoperative eGFR was >60, and serum creatinine was 1.10 ± 0.32 mg/dl,

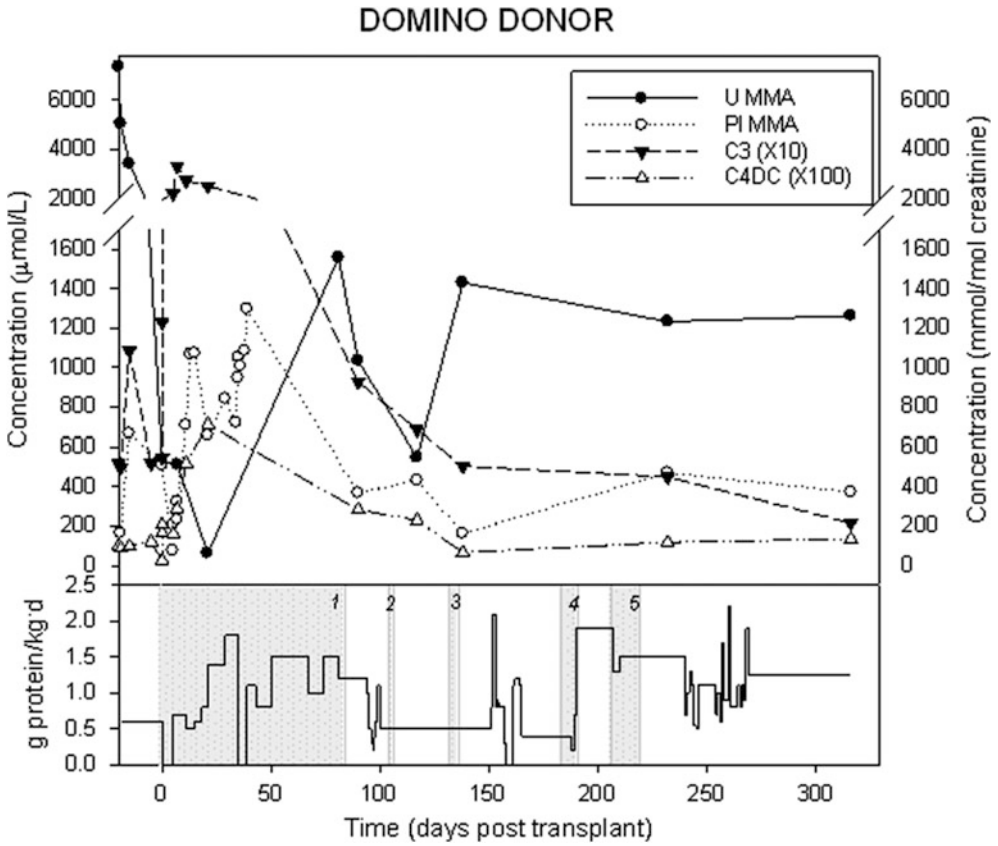

Fig. 3 Diet and laboratory results in the domino liver transplant donor. The *shaded regions* represent periods of hospitalization (see text). *Note*: methylmalonylcarnitine values are shown multiplied by 100 to facilitate viewing

changing to 2.91 ± 1.61 over the first 100 postoperative days with estimated GFR as low as 29 ml/min/1.73 m^2 on POD 24, but the renal function improved progressively (creatinine 2.86 ± 1.08 mg/dl and eGFR 29.9 ± 10.8 over POD 100–200, creatinine 2.40 ± 0.50 mg/dl and eGFR 33.2 ± 8.2 over POD 200–300, and creatinine 1.67 ± 0.50 mg/dl with eGFR 51.0 ± 12.1 ml/min/m^2 beyond POD 300).

Domino Recipient

Following LTx, the domino recipient (liver transplant patient number 2) developed appreciable plasma methylmalonate levels, undetectable preoperatively, 32 µmol/l (normal 0–0.01 µmol/l) in the immediate postoperative period, and 63.5 ± 32.8 µmol/l during the months following LTx (Fig. 4). Pretransplantation urine methylmalonate was ≤2 mmol/mol creatinine. Following LTx urine methylmalonate increased to 161 mmol/mol creatinine in the immediate postoperative period and averaged 128 ± 53.5 during the next 9 months. Plasma propionylcarnitine (C3-acylcarnitine) was 0.21 ± 0.02 µmol/l prior to surgery and 8.0 ± 4.9 µmol/l over 9 months of follow-up. The domino recipient maintained normal renal and liver functions, has resumed his normal activities of daily living, and is following an unrestricted diet.

Discussion

Methylmalonic acidemia is a disease in which death and/or mental retardation is common, and liver transplantation has been used in an attempt to prevent the characteristic potentially lethal metabolic decompensation. In that respect, transplantation has been largely successful, and most patients have not had ketoacidosis following transplantation. Positive outcomes and improved quality of life have been reported (Kayler et al. 2002; Nagarajan et al. 2005), but limited impact on clinical outcome or progressive neurologic disorder has been reported in some cases, so the role of liver transplantation in MMA is debated (Kaplan et al. 1996, 2006; Chakrapani et al. 2002; Nyhan et al. 2002; Kasahara et al. 2006; Chen et al. 2010).

Our previous experience with domino liver transplantation for MSUD led us to speculate that transplanting a methylmalonic acid domino graft would not cause symptomatic methylmalonic acidemia in the recipient, but this

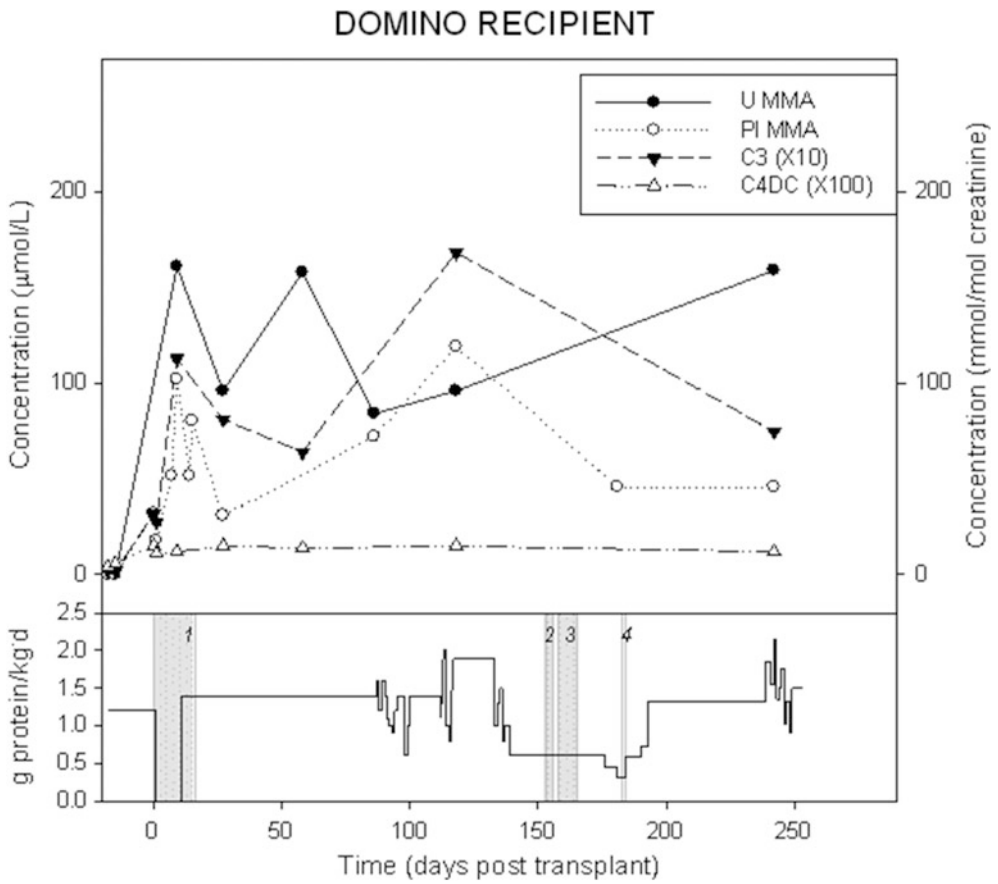

Fig. 4 Diet and laboratory results in the domino liver transplant recipient. The *shaded regions* represent periods of hospitalization (see text). *Note*: methylmalonylcarnitine values are shown multiplied by 100 to facilitate viewing

transplant sequence had never been performed previously or documented in the peer review literature. The recipient of the domino organ from the patient with MSUD maintained near-normal levels of plasma amino acids and normal levels of urine branched-chain keto and hydroxy acids, and he did not develop symptomatic MSUD. As in the case of the branched-chain keto acid dehydrogenase in MSUD, methylmalonyl-CoA mutase is expressed widely in body tissues. We found that domino LTx from our patient with MMA led to appreciable levels of methylmalonate in the recipient, but the levels were much lower than to those of the domino donor.

The observation that methylmalonate levels continue to be quite elevated in patients with methylmalonic acidemia following LTx has been made previously (Kasahara et al. 2006; Chen et al. 2010). It is expected that in the domino recipient, peripherally produced methylmalonate is broken down normally since all extrahepatic cells contain active mutase. However, elevated methylmalonate levels suggest that the amount produced by the mutase-deficient liver exceeds the metabolic capacity of peripheral cells.

Our domino donor developed seizures following LTx that may have been caused or exacerbated by the dosing of tacrolimus or an infection. It is true that LTx has been reported to be less than curative for MMA and posttransplant CSF methylmalonate levels continue to be elevated (Kaplan et al. 2006); metabolic stroke has been reported as much as 5 years following LTx (Chakrapani et al. 2002).

Tubulointerstitial nephritis and progressive renal function impairment have been associated with MMA (Rutledge et al. 1993). There are several reports of combined liver–kidney transplantation in patients with MMA (Nagarajan et al. 2005; Kasahara et al. 2006). In a recent review, reduction in methylmalonate levels to $13.8 \pm 9.2\%$ of preoperative levels was observed following LTx, but 4/18 cases had renal insufficiency and 3/18 had postoperative neurologic disability (Kasahara et al. 2006). Patients with MMA who have had LTx can subsequently develop cyclosporine or tacrolimus nephrotoxicity superimposed on the preexisting tubulointerstitial nephritis. Our MMA patient developed transient renal failure during the postoperative period and required dialysis transiently. However, eGFR returned to >50 ml/min. He will require ongoing monitoring to determine if kidney function worsens. Total follow-up duration at the time this manuscript was finalized is more than 2.5 years.

In conclusion, domino liver transplantation from patients with MMA is feasible. In this case, the course of the domino donor was stabilized, and the domino recipient clearly benefited. In carefully selected patients it is a valuable strategy and a ready resource for a donor liver for a select group of patients who would otherwise die waiting for an organ.

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Preliminary results from this study were presented orally at the Society for the Study of Inborn Errors of Metabolism Annual Symposium in Birmingham, England, 5 September, 2012.

Compliance with Ethics Guidelines

- This manuscript has been circulated among the coauthors and approved by them.
- There is no previous similar or simultaneous publication of this information.
- All coauthors contributed substantially to the work (in conception and design, analysis and interpretation of data, drafting the article, and/or critically revising the manuscript for important intellectual content).
- All coauthors have agreed to this submission.
- Ajai Khanna, Robert Gish, Susan Winter, William Nyhan, and Bruce Barshop 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.

Details of the Contributions of Individual Authors

Dr. Bruce Barshop was responsible for the conception and planning of this project, did the majority of writing, and prepared the graphical figures.

Dr. Ajai Khanna was responsible for the conception and planning of this project, performed the surgery, and substantially contributed to the writing.

Dr. Robert Gish assisted in planning this project, performed medical management, and contributed to the writing.

Dr. Susan Winter provided clinical information and contributed to the writing.

Dr. William Nyhan assisted in planning this project and contributed to the writing.

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RESEARCH REPORT

Reduction of Plasma Globotriaosylsphingosine Levels After Switching from Agalsidase Alfa to Agalsidase Beta as Enzyme Replacement Therapy for Fabry Disease

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Abstract *Introduction*: Agalsidase alfa and agalsidase beta, recombinant enzyme preparations for treatment of Fabry disease (FD), have different approved dosing schedules: 0.2 mg/kg and 1.0 mg/kg every other week (EOW), respectively.

Methods: This open-label, multicenter, exploratory phase 4 study evaluated plasma globotriaosylsphingosine (lyso-GL-3) and plasma and urine globotriaosylceramide (GL-3) levels at baseline and 2, 4, and 6 months after the switch from agalsidase alfa (0.2 mg/kg EOW for ≥12 months) to agalsidase beta (1.0 mg/kg EOW) in 15 male patients with FD. Immunoglobulin (Ig)G antidrug antibody titers were assessed, and safety was monitored throughout the study.

Results: Plasma lyso-GL-3 concentrations decreased significantly within 2 months after switch and reductions continued through month 6 (mean absolute changes, −12.8, −16.1, and −16.7 ng/mL at 2, 4, and 6 months, respectively; all $P < 0.001$). The mean percentage reduction from baseline was 39.5% ($P < 0.001$) at month 6. For plasma GL-3, the mean absolute change from baseline (−0.9 μg/mL) and percentage reduction (17.9%) at month 6 were both significant ($P < 0.05$). Urine GL-3 measurements showed intra-patient variability and changes from baseline were not significant. No clinical outcomes were assessed in this 6-month study, and, therefore, no conclusions can be drawn regarding the correlation of observed reductions in glycosphingolipid concentrations with clinically relevant outcomes. There were no differences in IgG antidrug antibody titers between the two enzymes. The switch from agalsidase alfa to agalsidase beta was well tolerated.

Conclusion: Plasma lyso-GL-3 and GL-3 levels reduced after switching from agalsidase alfa to agalsidase beta, indicating that agalsidase beta has a greater pharmacodynamic effect on these markers at the recommended dose. These data further support the use of agalsidase beta 1.0 mg/kg EOW as enzyme replacement therapy in FD.

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Introduction

Fabry disease (FD) is an X-linked disorder that affects both males and females and is caused by deficient activity of the lysosomal enzyme α-galactosidase A (α-Gal A) (Germain 2010). This leads to the accumulation of globotriaosylceramide (GL-3, Gb_3), predominantly in the lysosomes of multiple cell types, and the elevation of globotriaosylsphingosine (lyso-GL-3, lyso-Gb_3), the more water-soluble deacylated form of GL-3, in the plasma (Aerts et al. 2008). In vitro studies suggest that these

elevations trigger a cascade of pathological processes, including inflammatory and fibrotic responses that cause progressive damage to multiple organs (Germain 2010). In males with classic FD, early signs and symptoms, including neuropathic pain, hypohidrosis, and gastrointestinal dysmotility, usually appear in early childhood (Hopkin et al. 2008), and life-threatening renal, cardiac, and cerebrovascular complications typically develop by the fourth or fifth decade of life (Germain 2010).

FD is treated with recombinant α-Gal A enzyme replacement therapy (ERT). In the USA, agalsidase beta (Fabrazyme®; Genzyme, a Sanofi company) is the only ERT approved to treat FD. Two ERTs are licensed in the European Union: agalsidase beta and agalsidase alfa (Replagal®; Shire Human Genetic Therapies) (Schaefer et al. 2009). Agalsidase alfa and agalsidase beta have identical amino acid sequences and are functionally equivalent (Lee et al. 2003). However, agalsidase beta contains more fully sialylated oligosaccharides and more mannose-6-phosphate than agalsidase alfa (Lee et al. 2003; Togawa et al. 2014); cellular uptake of recombinant agalsidases is mediated by mannose-6-phosphate receptors. Greater uptake of agalsidase beta than of agalsidase alfa has been suggested in vitro (in skin fibroblasts from patients with FD) (Keslová-Veselíková et al. 2008; Lee et al. 2003) and in vivo (in the kidney and heart after administration of the same dose of the two enzymes in a mouse model of FD) (Desnick and Schuchman 2012; Sakuraba et al. 2006). Although not adequately powered, the results from a study in patients with FD suggest that the two agents have similar clinical effects when administered at the same dose of 0.2 mg/kg every other week (EOW) (Vedder et al. 2007).

However, based on the doses used in the clinical development programs for these agents, the recommended doses of the two agents differ by a factor of 5 – agalsidase alfa is 0.2 mg/kg EOW (Replagal® Summary of product characteristics, last updated September 2014), whereas agalsidase beta is 1.0 mg/kg EOW (Fabrazyme® Summary of product characteristics, last updated October 2014; Fabrazyme® Prescribing information, last updated May 2010). The optimal dose of agalsidase has long been a matter of debate (Desnick 2004), and identification of the biochemically and clinically most effective dose has been hampered by the slowly progressive nature of FD that lacks reliable, predictive biochemical or clinical surrogate end points. Additionally, the clinical presentation of FD is highly heterogeneous and the timing of symptom onset highly variable (Eng et al. 2007; Hopkin et al. 2008). There have been no randomized, double-blind trials of patients matched for sex, age, and disease severity to allow a direct comparison of the effectiveness of agalsidase beta and agalsidase alfa at their approved doses (Desnick and Schuchman 2012). Recent data from the Canadian Fabry Disease Initiative, where agalsidase alfa and beta have been used head-to-head, have shown no difference in end points between patients treated with agalsidase alfa (0.2 mg/kg) and patients receiving agalsidase beta (1.0 mg/kg), although patients were not randomized for variables known to affect prognosis, and GL-3 and lyso-GL-3 data were not reported (Sirrs et al. 2014). In addition, as this study was underpowered, a definite conclusion about comparability of enzymes at equal or licensed dose is still unresolved.

GL-3, a substrate for α-Gal A, is routinely measured in blood and urine and occasionally in biopsy samples. The level of GL-3 is used as a surrogate marker to assess treatment efficacy, and clearance of GL-3 from the tissues, mainly the liver and kidneys, has been used in several studies as an outcome measure (reported as relative to normal and/or percentage decrease from baseline) (Eng et al. 2001; Schiffmann et al. 2000). Short-term therapy has been shown to rapidly decrease plasma GL-3 levels in adult and pediatric male patients with FD (Clarke et al. 2007; Schiffmann et al. 2010). Recently, lyso-GL-3 has emerged as a more sensitive biomarker that may correlate with disease severity and/or organ involvement in FD (Rombach et al. 2010). Untreated patients with classic FD typically have highly elevated plasma levels of lyso-GL-3 (Aerts et al. 2008; Togawa et al. 2010; Niemann et al. 2014), and plasma lyso-GL-3 decreased within 3 months of initiation of treatment in naive FD patients treated with either agalsidase alfa or agalsidase beta, with significantly larger reductions with agalsidase beta 1.0 mg/kg versus agalsidase alfa and agalsidase beta 0.2 mg/kg (van Breemen et al. 2011). Recurrent elevations in plasma lyso-GL-3 have been reported in patients whose agalsidase dose was reduced. These patients either changed to a lower dose of agalsidase beta (from 1.0 to 0.5 mg/kg EOW or 0.5 mg/kg per month) or switched from agalsidase beta to agalsidase alfa (0.2 mg/kg EOW) (Smid et al. 2011), leading to the hypothesis that ERT using agalsidase reduces plasma lyso-GL-3 concentration in a dose-dependent manner regardless of the type of agalsidase (beta or alfa) administered.

The INFORM study was designed to confirm this hypothesis by monitoring the changes in plasma lyso-GL-3 levels in patients with FD whose agalsidase dose was increased by a switch from agalsidase alfa administered at 0.2 mg/kg EOW to agalsidase beta administered at 1.0 mg/kg EOW (when investigational agalsidase alfa treatment became unavailable in the USA). Fifteen male FD patients were recruited to the study, and the effects of the dose change were followed for 6 months, during which patients' plasma and urine concentrations of glycosphingolipids (plasma lyso-GL-3 and urinary and plasma GL-3) were evaluated. Antibody titers to both agalsidase preparations were also investigated.

Methods

Patients

Adult and pediatric male patients with a diagnosis of FD, confirmed by α-Gal A activity assay and/or *GLA* gene mutation analysis in accordance with local standards, were eligible for the study if they had received agalsidase alfa (0.2 mg/kg EOW) for ≥12 months (Fig. 1). Patients who had switched to agalsidase beta before the start of the study who met the eligibility criteria and had provided blood and urine samples at protocol-specified time points (at baseline and 2 and 4 months after switching to agalsidase beta) were also included.

Patients who were on dialysis or had undergone renal transplantation, patients with end-stage cardiac disease, and patients (or their guardians or parents) who were unable to fully comply with study requirements were excluded from the study.

Study Design

INFORM was an open-label, multicenter, exploratory phase 4 study conducted at six sites in the USA between April 2012 and March 2013. After baseline samples were collected, eligible patients received agalsidase beta at 1.0 mg/kg EOW via intravenous infusion (Fig. 1). No patient had a treatment hiatus between his last agalsidase alfa infusion and his first agalsidase beta infusion.

Assessments

Assessments were conducted at baseline and months 2, 4, and 6 after the switch from agalsidase alfa to agalsidase beta. The end points were the mean percentage change and absolute change from baseline in plasma lyso-GL-3, plasma GL-3, and urine GL-3 at months 2, 4, and 6.

Concentrations of plasma GL-3, plasma lyso-GL-3, and urine GL-3 were assessed in samples obtained at baseline and months 2, 4, and 6. Plasma GL-3 concentrations were quantified using a validated liquid chromatography–tandem mass spectrometry (LC/MS/MS) method as previously described (Nelson et al. 2004; Roddy et al. 2005; Wilcox et al. 2004). Urine GL-3 was extracted from whole urine with chloroform/methanol and measured by LC/MS/MS. Urine GL-3 levels were normalized to creatinine and expressed as micrograms of GL-3 per 1 mmol creatinine. Plasma lyso-GL-3 was measured using a validated LC/MS/MS method. Briefly, 100 µL of plasma was treated with acidified acetonitrile containing dimethyl psychosine (Avanti Polar Lipids Inc.; Alabaster, AL, USA) as internal standard and filtered in a 96-well lipid-removal filtration plate. The eluent was dried down and reconstituted for LC/MS/MS analysis. The lower limit of quantification (LLOQ) was defined as the lowest concentration of the analyte that could be measured with accuracy (80–120% recovery) and precision (percentage coefficient of variation ≤20%). The LLOQ was 2.0 µg/mL for plasma GL-3, 0.2 µg/mL for urine GL-3, and 5.0 ng/mL for plasma lyso-GL-3. All assays were validated and performed in a clinically compliant laboratory environment. Normal values for plasma GL-3 and plasma lyso-GL-3 were <7.0 µg/mL (n = 203, from 104 males and 101 females) and <5 ng/mL (n = 100, from 50 females and 50 males), respectively. The normal range for urine GL-3 was <81 µg/mmol creatinine (n = 50 normal subjects).

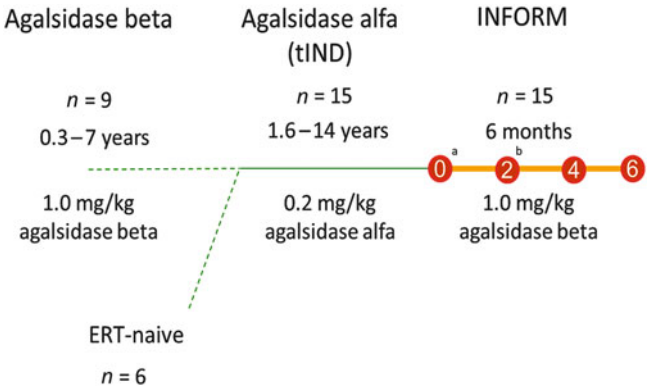

Fig. 1 Enzyme replacement therapy before and during INFORM study. [a]Sixteen patients screened for 15 eligible patients. [b]One patient voluntarily discontinued on day 64 of treatment as he no longer wished to participate in the study. *tIND* treatment Investigational New Drug

Antidrug IgG antibodies against agalsidase beta and agalsidase alfa were measured in serum samples obtained at baseline, and serum IgG antibodies against agalsidase beta were measured at months 2, 4, and 6. A specific enzyme-linked immunosorbent assay (ELISA) method to assess IgG antibodies to agalsidase beta or agalsidase alfa was used. The method for agalsidase beta was adapted for agalsidase alfa, and modifications were made to achieve similar assay sensitivity. Briefly, IgG antibody to agalsidase beta or agalsidase alfa was screened and confirmed. Both assays were used for baseline sample analysis, and only agalsidase beta ELISA was used for posttreatment sample analysis. Confirmed positive samples were further analyzed to determine titers for all of the posttreatment samples. Serum was serially diluted twofold starting at an initial 1/100 dilution. The titer was reported as the reciprocal of the last dilution above the assay cutoff point.

A potential neutralizing effect of IgG antibody formation was assessed in a post hoc analysis. The neutralizing capacity of enzyme activity was expressed as micrograms

of recombinant human α-Gal A inhibited by 1 mL of patient sera. In addition, inhibition of enzyme uptake was assessed in all IgG antibody-positive patients.

Safety was assessed by recording the incidence and type of adverse events (AEs) associated with protocol-related procedures and other AEs not considered associated with agalsidase beta treatment. AEs associated with agalsidase beta treatment were also recorded. AEs were classified according to the *Medical Dictionary for Regulatory Activities*, version 16.0, and grouped by preferred term and system organ class. Each AE or serious AE (SAE) was judged, in the opinion of the study investigators, to be not related, unlikely related, possibly related, or related to agalsidase beta treatment; the investigators graded the severity of the AE or SAE as mild, moderate, or severe. No clinical efficacy outcomes were assessed.

Statistical Methods

Sample size was chosen on the basis of a study that reported a statistically significant median increase in lyso-GL-3 concentration in a group of male patients switched from agalsidase beta to agalsidase alfa or a lower dose of agalsidase beta (Smid et al. 2011). Based on the assumption that a reverse effect on lyso-GL-3 concentrations in patients who switched from agalsidase alfa (0.2 mg/kg EOW) to agalsidase beta (1.0 mg/kg EOW) would be observed, a cohort of 20 patients would provide >90% power to detect changes in lyso-GL-3 concentration of the same magnitude. Plasma lyso-GL-3, rather than plasma GL-3, was chosen to power the study, as levels are more pronouncedly increased in untreated classically affected males (Aerts et al. 2008), which makes it a more suitable discriminator of a dose effect.

Demographic data and baseline characteristics were summarized using descriptive statistics. Patient-level plasma lyso-GL-3, plasma GL-3, and urine GL-3 concentrations at baseline and months 2, 4, and 6 after switching to agalsidase beta are presented graphically. Changes from baseline were summarized as mean percentage change and absolute change by using descriptive statistics for each visit. Changes in plasma lyso-GL-3 and plasma GL-3 concentrations were tested using one-sample *t*-tests. Urine GL-3 concentration showed considerable fluctuations between visits, and changes were not normally distributed. Therefore, changes were tested using a one-sample test of medians (sign test) and described as median changes from baseline. Statistical tests were performed and the corresponding *P*-values are reported. Statistical reports were generated by using SAS® version 9.2 in a secure and validated environment. AE data were also summarized descriptively.

Results

Patient Demographics

Patient demographics and *GLA* mutations are summarized in Table 1. Fourteen of the 15 male patients (93.3%) who enrolled completed the study. One patient (patient 5) voluntarily withdrew consent from the study on day 64 of treatment for reasons unrelated to safety.

The majority (86.7%) of subjects included in the study was Caucasian, with a mean age (standard deviation [SD]) at enrollment of 28.5 (16.1) years. The median duration of FD at baseline (from initial diagnosis to first study treatment) was 9.3 years (range 2–19 years). Prior to agalsidase alfa treatment, nine patients received agalsidase beta treatment for a mean of 3.5 years (range 0.3–7 years), and six were ERT-naive. Patients had received agalsidase alfa (0.2 mg/kg EOW) for a mean of 3.7 years (range 1.6–14 years) before switching to agalsidase beta (1.0 mg/kg EOW) (Fig. 1).

Among the patients with known *GLA* gene mutations, 12 patients had mutations previously reported to be associated with a classical presentation of FD, and two had mutations reported to be associated with later-onset FD (patients 9 and 13) (references are presented in Table 1).

Plasma Lyso-GL-3

The patient-level plasma lyso-GL-3 concentrations according to Clinical visit after the switch to agalsidase beta at 1.0 mg/kg EOW are presented in Fig. 2.

Plasma lyso-GL-3 concentration decreased significantly within 2 months of the switch to agalsidase beta 1.0 mg/kg EOW, and the reduction continued at months 4 and 6. The mean absolute change in plasma lyso-GL-3 concentration was −12.8 ng/mL ($P < 0.001$) at month 2, −16.1 ng/mL ($P < 0.001$) at month 4, and −16.7 ng/mL ($P < 0.001$) at month 6. The mean (SD) percentage reduction from baseline in plasma lyso-GL-3 concentration was 39.5% (23.57) ($P < 0.001$) at month 6.

In most cases, the plasma lyso-GL-3 concentration decreased consistently at each successive assessment for each patient (Fig. 2). The two patients with a *GLA* mutation associated with later-onset FD (patients 9 and 13) had lyso-GL-3 concentrations below quantitative limits at study baseline and throughout the study.

Plasma GL-3

The patient-level plasma GL-3 concentrations according to Clinical visit after the switch to agalsidase beta at 1.0 mg/kg EOW are presented in Fig. 3.

Table 1 Characteristics and individual *GLA* mutations at baseline for the 15 patients enrolled in the study

Patient number (random)	*GLA* gene mutation	Associated phenotype, as reported in the literature	Reference	Age category[a]	Duration of Fabry disease (years)[b]	Leukocyte α-Gal A activity (U/mg)/plasma α-Gal A activity (U/mL)
1	358Edel	Classic	Blanch et al. (1996)	B	11.5	0.9/1.0
2	c.256delT	Classic	Topaloglu et al. (1999)	C	14.1	NA/1.0
3	c.717_718delAA	Classic	Blanch et al. (1996)	E	NA	0.1/0.0
4	R220X	Classic	Meaney et al. (1994)	C	10.0	1.4[c]/NA
5	D322E	Classic	Lee et al. (2010)	E	2.7	NA/NA
6	NA	–	–	D	18.8	3.5[c]/NA
7	R227Q	Classic	Eng et al. (1993)	C	11.6	6.8[c]/0.1
8	C56X	Classic	Shabbeer et al. (2006)	A	8.1	NA/NA
9	g.IVS4 +919G>A	Late onset	Chien et al. (2013) and Eng et al. (1994)	A	5.9	5.1[c]/NA
10	R112C	Classic	Shabbeer et al. (2006)	C	3.1	0.4/0.2
11	C202Y	Classic	Eng et al. (1997)	E	1.9	NA/0.1
12	R227Q	Classic	Eng et al. (1993)	D	8.6	0.1/0.1
13	R112H	Late onset	Chien et al. (2013) and Eng et al. (1994)	B	10.7	1.7[c]/0.1
14	R227Q	Classic	Eng et al. (1993)	B	8.3	0.1/0.1
15	R301X	Classic	Eng et al. (1994)	F	13.2	ND/0.4
Mean (SD)				28.5 (16.1)	9.2 (4.7)[d]	
Median (range)				24.0 (5–61)	9.3 (1.9–18.8)	

α-*Gal A* α-galactosidase A, *NA* not available, *ND* not detectable, *SD* standard deviation

[a] Age was calculated from the date of birth to the date of the first study infusion of agalsidase beta. To protect patients' data privacy, age categories are used: A, >0 to ≤10 years; B, >10 to ≤20 years; C, >20 to ≤30 years; D, >30 to ≤40 years; E, >40 to ≤50 years; F, >50 years

[b] Duration of Fabry disease was calculated from the date of initial diagnosis of Fabry disease to the date of the first study infusion

[c] In nmol/h/mg protein

[d] $n = 14$; the date of diagnosis was not available for one patient

The mean absolute change from baseline in plasma GL-3 concentration was −0.5 μg/mL at month 2, −0.7 μg/mL at month 4, and −0.9 μg/mL at month 6. The mean absolute change from baseline at month 6 was statistically significant ($P < 0.05$). The mean percentage change in plasma GL-3 concentration at month 6 was −17.9% ($P < 0.05$).

Urine GL-3

The patient-level urine GL-3 concentrations according to Clinical visit after the switch to agalsidase beta at 1.0 mg/kg EOW are presented in Fig. 4. The two patients with a *GLA* mutation associated with later-onset FD (patients 9 and 13) had urine GL-3 concentrations below quantitative limits at study baseline and throughout the study.

The median absolute change from baseline in urine GL-3 concentration was −7.6 μg/mmol at month 2, −9.9 μg/mmol at month 4, and −11.1 μg/mmol at month 6. None of these median changes from baseline were statistically significant.

The median percentage change from baseline in median urine GL-3 concentration was −33.8% at month 6. None of the median percentage changes from baseline in urine GL-3 were statistically significant.

Serum Anti-agalsidase Beta IgG Antibody Titers

Prior to first agalsidase beta infusion, eight patients were positive for serum IgG antibodies to both agalsidase alfa and agalsidase beta when the same assay format was used (patients 1, 2, 3, 4, 6, 8, 11, and 15); patients 1, 3, and 8 had never been treated with agalsidase beta (patient 8 had the highest pre-agalsidase beta antibody titer) (Fig. 5). Seven patients were seronegative for IgG antibodies to both agalsidase beta and agalsidase alfa, four of whom had been treated with both agalsidase alfa and agalsidase beta (patients 7, 9, 10, and 12). No patient was positive for antibodies against one ERT and negative for the other ERT. Titers of antibodies to agalsidase alfa and agalsidase beta at baseline were not significantly different (no difference of

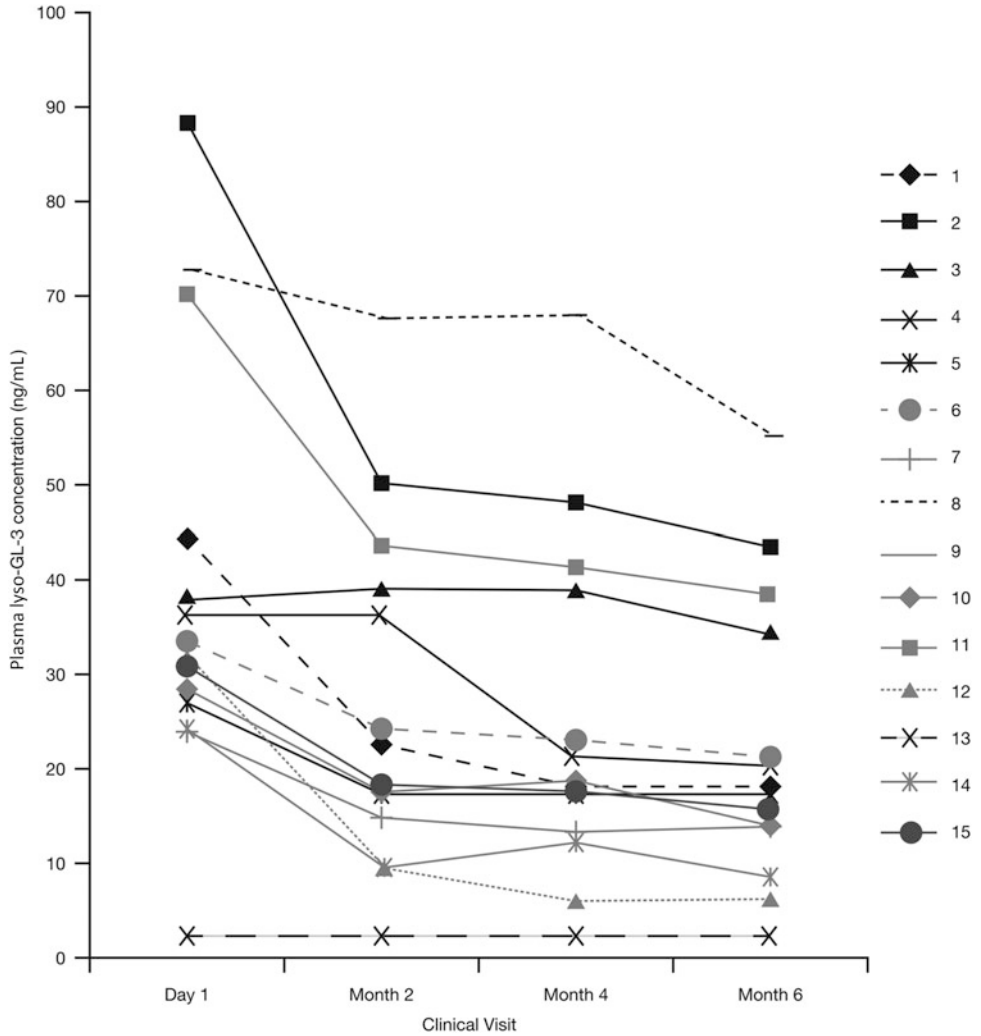

Fig. 2 Patient-level plasma lyso-GL-3 concentrations according to Clinical visit after the switch to agalsidase beta at 1.0 mg/kg every other week. Figure includes three of the four patients (patients 2, 3, 8, and 11) who had the highest agalsidase antibody titers before switch. Symbols overlap for two patients (9 and 13) whose lyso-GL-3 concentrations were below quantitative limits at baseline and throughout the study. These patients have *GLA* gene mutations reported to be associated with a later-onset form of Fabry disease. *Lyso-GL-3* globotriaosylsphingosine

>1 dilution), even in the six patients who had not yet been exposed to agalsidase beta (patients 1, 3, 5, 8, 13, and 14). Patient 14 was the only seronegative patient who seroconverted during the agalsidase beta study treatment period. He seroconverted by month 2 but had low antibody titers (maximum antibody titer 1:400).

In patients who were positive for agalsidase beta antibodies, there was no discernible pattern between the titer measured at any time point during the study and concentration of lyso-GL-3, plasma GL-3, or urine GL-3; patients who were negative for antibodies at baseline were among those with the lowest plasma concentrations of lyso-GL-3 and GL-3.

Patient 8, who had a baseline antibody titer of 102,000, was found to be positive in the neutralizing activity and enzyme uptake inhibition assays. Prior to enrollment into the trial, he had received agalsidase alfa for 1.6 years and had not been exposed to agalsidase beta. His enzyme activity inhibition was >100% and the uptake assay was positive. The level of neutralizing activity (expressed in micrograms of recombinant human α-Gal inhibited by 1 mL of patient sera) decreased from 538 to 389 at 6 months, and lyso-GL-3 decreased by 23% over the course of 6 months.

None of the other patients demonstrated uptake inhibition, and only one additional patient (patient 11) had some inhibition of enzyme activity at 2 months, which subsequently decreased. This patient's lyso-GL-3 level decreased significantly at 2 months of agalsidase beta treatment (37%) and continued to further decrease at 4 months (41%) and 6 months (45%).

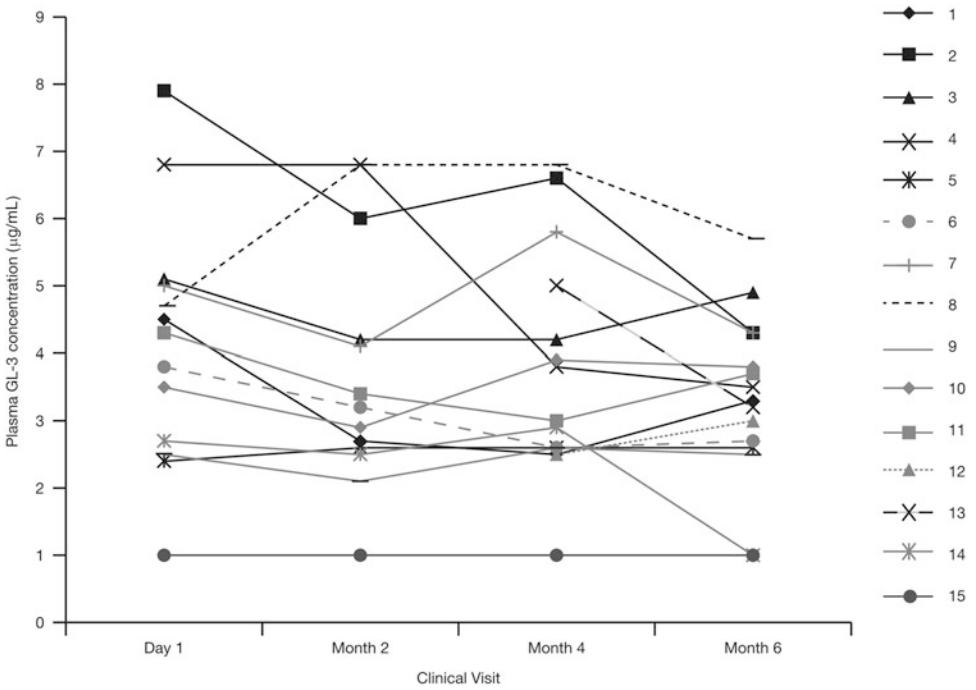

Fig. 3 Patient-level plasma GL-3 concentrations according to Clinical visit after the switch to agalsidase beta at 1.0 mg/kg every other week. For two patients (patients 12 and 13), baseline and month 2 values were not available. *GL-3* globotriaosylceramide

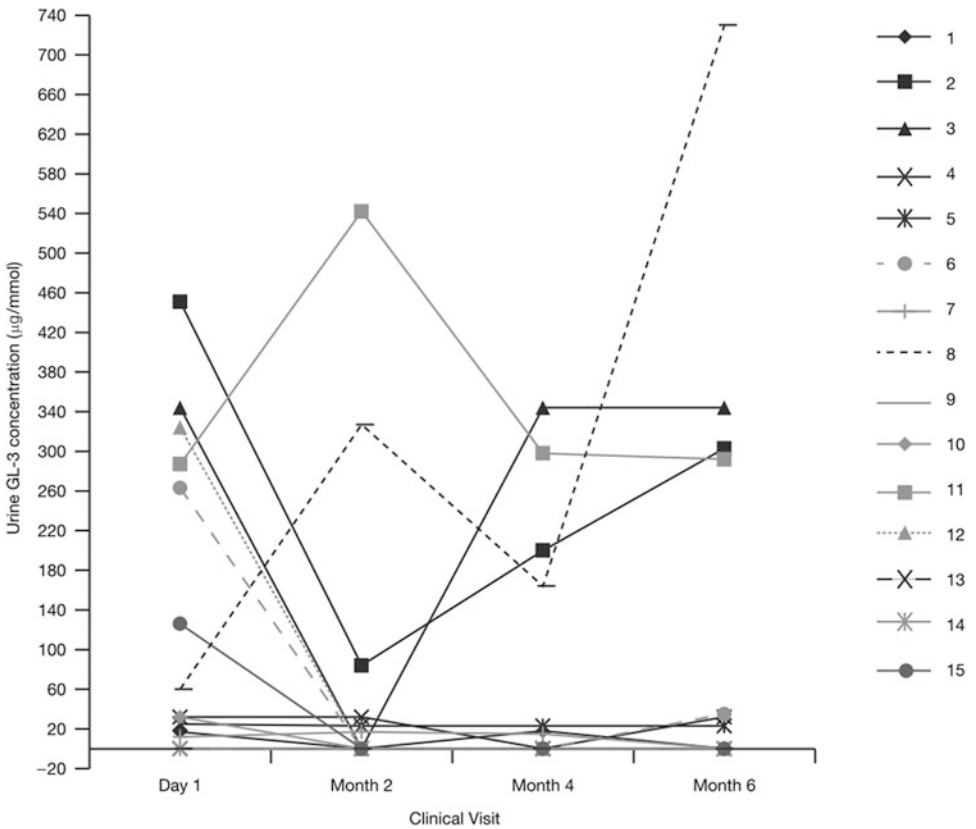

Fig. 4 Patient-level urine GL-3 concentrations according to clinical visit after the switch to agalsidase beta at 1.0 mg/kg every other week. *GL-3* globotriaosylceramide

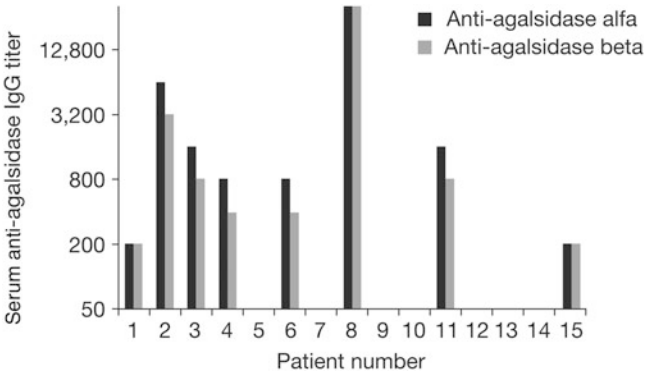

Fig. 5 Serum anti-agalsidase IgG titers at baseline. Titers of antibodies to agalsidase alfa and agalsidase beta at baseline were not significantly different even in patients who had not yet been exposed to agalsidase beta (patients 1, 3, 5, 8, 13, and 14). *Ig* immunoglobulin

Adverse Events

There were no SAEs reported during the study. Only one of the 15 patients (7%) experienced a non-SAE (an infusion-associated reaction) following completion of the first agalsidase beta infusion after switching. The AE resolved after 2 days and the patient continued treatment without experiencing any further AEs. The serum sample drawn prior to the first agalsidase beta infusion was positive for agalsidase IgG antibodies (titer 1:3,200).

Discussion

This study demonstrates that, in male patients with FD, increasing the dose of ERT by switching from agalsidase alfa at 0.2 mg/kg EOW to agalsidase beta at 1.0 mg/kg EOW can further reduce plasma lyso-GL-3 and GL-3 concentrations beyond reductions previously achieved with agalsidase alfa. These reductions were evident 2 months after increasing the dose of agalsidase. The reductions provide further evidence that the in vivo activity of agalsidase alfa at its recommended dose is below that of agalsidase beta at its recommended dose. These results underscore the importance of dose in the treatment of FD and corroborate findings by other groups (Smid et al. 2011; Tøndel et al. 2013; van Breemen et al. 2011; Weidemann et al. 2014). In previously untreated patients, plasma lyso-GL-3 decreased, reaching almost stable levels within 3 months of initiation of treatment with either agalsidase alfa or agalsidase beta administered at 0.2 mg/kg, and with 1.0 mg/kg of agalsidase beta (van Breemen et al. 2011). However, the reduction of plasma lyso-GL-3 was significantly greater for patients treated with agalsidase beta 1.0 mg/kg versus patients who received agalsidase alfa or agalsidase beta at a dose of 0.2 mg/kg. Plasma lyso-GL-3 concentrations tended to be higher in antibody-positive males when treated with agalsidase alfa or agalsidase beta at 0.2 mg/kg, compared with antibody-negative males. This was not the case after 12 months of agalsidase beta 1.0 mg/kg; the lyso-GL-3 reduction at this dose was similar for antibody-positive and antibody-negative patients (van Breemen et al. 2011), suggesting that a dose increase can compensate for potentially reduced efficacy in patients with anti-agalsidase antibodies. Another study reported a significant increase in lyso-GL-3 concentration in adult male patients who switched from agalsidase beta 1.0 mg/kg to agalsidase alfa 0.2 mg/kg or received a reduced agalsidase beta dose (one group subsequently switched to agalsidase alfa), suggesting recurrence of disease activity (Smid et al. 2011). The observed relapse in plasma lyso-GL-3 elevation in almost all patients within a few months after agalsidase dose reduction in that cohort led experts to conclude that there is an ERT dose effect on plasma lyso-GL-3 levels and that the biochemical response to ERT is suboptimal at an agalsidase dose of 0.2 mg/kg EOW in the majority of classic FD patients (Smid et al. 2011; Ferraz et al. 2014). Systematic and complete data collections from 105 FD patients showed that patients receiving agalsidase beta at 1.0 mg/kg EOW had a stable disease course, whereas patients whose agalsidase beta dose was reduced and patients who were switched to agalsidase alfa experienced worsening of renal function and deterioration of Fabry-related symptoms (Warnock and Mauer 2014; Weidemann et al. 2014). Moreover, the magnitude of GL-3 clearance in podocytes (terminally differentiated cells) in young patients (median age 16.5 years) was dependent on the cumulative dose of agalsidase beta or alfa administered over time (Tøndel et al. 2013).

An important consideration of ERT with agalsidase is the patient's immunological response to the enzyme. In this study, titers of serum IgG antibodies to both agalsidase alfa and agalsidase beta were measured using the same assay format, and the findings suggest that agalsidase alfa and agalsidase beta have epitopes in common; over half of the patients who had never been exposed to agalsidase beta were positive for agalsidase beta antibodies at baseline. Importantly, no relationship was apparent between IgG antibody titers and concentration of plasma lyso-GL-3, plasma GL-3, or urine GL-3 in patients receiving agalsidase beta 1.0 mg/kg EOW. In contrast, as noted above, patients with antibodies receiving 0.2 mg/kg of either agalsidase alfa or agalsidase beta tended toward smaller correction in plasma lyso-GL-3 concentrations compared with patients receiving agalsidase beta 1.0 mg/kg, who experienced a reduction similar to patients without antibodies (van Breemen et al. 2011). It is important to note that, to date, a comparison of antibody test results obtained from different laboratories and different studies has been hampered by the

lack of a standardized assay and reference standard (Schellekens 2008). The number of samples that test positive for neutralizing activity and/or inhibition of uptake is dependent on the sensitivity and specificity of the method (Fabrazyme® Prescribing information, last updated May 2010). Thus, it is not surprising that neutralizing antibody activity results from this study differ from results reported by other groups (Linthorst et al. 2004; Lenders et al. 2015). Studies have demonstrated that both agalsidase alfa and agalsidase beta are structurally equivalent and, on a milligram basis, induce similar, fully cross-reactive, antibody responses in vivo (Blom et al. 2003; Lee et al. 2003; Linthorst et al. 2004), refuting the suggestion that variation in glycosylation patterns between agalsidase beta and agalsidase alfa may have implications for the long-term safety of ERT (Barbey et al. 2008). The perceived difference in immunogenicity of the two enzymes could be based on differences in analytical testing.

Our observations further support the suggestion that plasma lyso-GL-3 could be a sensitive pharmacodynamic biomarker for dose–response effects of ERT in FD. Of the glycosphingolipids examined in this study, lyso-GL-3 showed more consistent changes in concentration than did plasma or urine GL-3. Lyso-GL-3 concentration was significantly reduced from baseline at each successive Clinical assessment, and individual patient values of plasma lyso-GL-3 concentration fluctuated less than did those of plasma and urine GL-3 concentrations (data not shown). Although during the past decade attention has largely focused on the pathogenic role of GL-3 in FD (DeGraba et al. 2000; Park et al. 2011; Shen et al. 2008), the importance of lyso-GL-3 in the FD process has become evident. For example, exposure of smooth muscle cells in vitro to lyso-GL-3 at concentrations similar to those seen in patients with FD (e.g., in the patients treated for 12 months with agalsidase alfa or beta at 0.2 mg/kg [van Breemen et al. 2011]) stimulates proliferation of the smooth muscle cells (Aerts et al. 2008), a finding consistent with the increased intima–media thickness observed in patients with FD (Barbey et al. 2006).

Lyso-GL-3 has also been proposed to have a role in renal injury by causing podocyte injury and glomerular accumulation of extracellular matrix – key characteristics of glomerulosclerosis (Sanchez-Niño et al. 2011). In vitro, human podocytes exposed to lyso-GL-3 increased their expression of mediators of glomerular injury (transforming growth factor [TGF]-β1, the extracellular matrix proteins fibronectin and type IV collagen, and CD74) in a dose- and time-dependent manner, and the effect of lyso-GL-3 on extracellular matrix production was mediated by TGF-β1 (Sanchez-Niño et al. 2011).

It has not yet been unequivocally demonstrated that lyso-GL-3 contributes to manifestations of FD in vivo. However, studies in patients with classic FD have explored correlations between levels of plasma lyso-GL-3 (a potential pathogenic factor inducing vascular dysfunction [Rombach et al. 2012]) and disease manifestations (Rombach et al. 2010; Rombach et al. 2012). One study found that lifetime exposure to plasma lyso-GL-3 correlates with severity of FD manifestations (assessed by Mainz severity scoring index), and plasma lyso-GL-3 was reported to be an independent risk factor for development of cerebrovascular white matter lesions in male patients and left ventricular hypertrophy in female patients with classic FD. No correlation was observed between plasma lyso-GL-3 and renal function (Rombach et al. 2010). Other studies found significant correlations between carotid intima–media thickness (previously reported to be predictive of stroke [Chambless et al. 2000]) and plasma lyso-GL-3 levels in female patients with classic FD, and between exposure to lyso-GL-3 and cold detection threshold and thermal sensory limen at the upper limb (Biegstraaten et al. 2012).

No clinical outcomes were assessed in our 6-month study because FD is a chronic disease and progresses slowly, and, therefore, no conclusions can be drawn from this short study regarding the correlation of observed reductions in plasma lyso-GL-3 with clinically relevant outcomes. As acknowledged by others, following the biochemical response may prove to be a valuable tool in clinical management of FD patients if treatment is started early, before irreversible damage has occurred (van Breemen et al. 2011), and is probably the most sensitive way to evaluate the effects of a change in agalsidase dose in short-term studies (Smid et al. 2011). The switch from agalsidase alfa to agalsidase beta in our study was safe and well tolerated.

Of note, the methods used to measure lyso-GL-3 in the literature vary. Standardization of these methods would allow comparison of lyso-GL-3 data from different studies. Moreover, discussing our results in the context of results of recently published agalsidase beta – agalsidase alfa switch studies is hampered by the fact that these studies did not report plasma lyso-GL-3 or GL-3 data (Pisani et al. 2013), included Fabry-variant patients with normal or low lyso-GL-3 levels at baseline (six out of nine patients) (Lin et al. 2014), and included many female patients with low lyso-GL-3 at baseline (seven out of 11 patients) (Tsuboi and Yamamoto 2014).

The small number of patients and the lack of long-term clinical follow-up are limitations of our study. However, although fewer patients were included in the analysis than required by the statistical power calculation, a statistically significant difference in lyso-GL-3 clearance was still detected. Although a two-arm crossover design with a switch from agalsidase alfa to agalsidase beta followed by a crossover to alternate ERT product would have been a more robust design than our open-label, single-arm study, this would have been challenging to conduct after termination

of the US treatment Investigational New Drug permit for agalsidase alfa in June 2012. Given that the long-term clinical benefits of ERT for patients with FD are still unclear, the study was not intended to assess clinical outcomes related to dose differences and only measured GL-3 and lyso-GL-3 concentrations. The lack of pre-study data is another limitation of this study. However, as already discussed, the lack of a standardized assay and reference standard precludes comparisons of glycosphingolipid and antibody test results across studies. With regard to the impact of ERT on plasma lyso-GL-3 levels, it is important to note that lyso-GL-3 levels in classic FD patients have been reported to decrease rapidly after start of ERT in a dose-dependent manner, reaching almost stable levels within 3 months of treatment (van Breemen et al. 2011; Ferraz et al. 2014). Therefore, it was expected that the patients in the current study should have reached a maximal reduction in plasma lyso-GL-3 levels during the agalsidase alfa (0.2 mg/kg EOW) treatment period (mean 3.7 years, range 1.6–14 years) prior to switching to agalsidase beta (1.0 mg/kg EOW).

In conclusion, the reduction in plasma lyso-GL-3 and GL-3 observed in patients after switch from agalsidase alfa to agalsidase beta indicates that the recommended dose of agalsidase beta at 1.0 mg/kg EOW has a greater pharmacodynamic effect on these markers. Plasma lyso-GL-3 appears to be a biomarker to assess the initial response to ERT in patients with FD. Structural similarities between the two ERTs may suggest similar immunogenic profiles. Additionally, despite a fivefold increase in ERT dose, switching to a higher-dose ERT (agalsidase beta) is safe and well tolerated. Further studies with a larger sample size are needed to determine the clinical implications of lyso-GL-3 as an indicator of disease severity and progression and the clinical response to treatment.

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We dedicate this manuscript to the memory of John A. Barranger who acted as the principal study investigator but sadly passed away.

Synopsis

Agalsidase beta at a dose of 1.0 mg/kg administered EOW may further reduce plasma and urine glycosphingolipid concentrations beyond reductions previously achieved with agalsidase alfa in patients with Fabry disease.

Compliance with Ethics Guidelines

Conflict of Interest

Ozlem Goker-Alpan has received research support (Actelion, Shire HGT, Genzyme, Amicus, Pfizer-Protalix Biotherapeutics), payments for consultancy (Actelion, Shire HGT, Pfizer-Protalix Biotherapeutics), and speaker bureaus (Actelion, Shire HGT, Genzyme). Daniel J. Gruskin and Larry Blankstein are Genzyme employees. Neal J. Weinreb receives travel reimbursements and/or honoraria and/or research support from Shire HGT, Genzyme, Pfizer Corporation, and Actelion Corporation. Michael J. Gambello, Gustavo H.B. Maegawa, and Khan J. Nedd declare that they have no conflict of interest.

The study is registered at www.ClinicalTrials.gov under the identifier NCT01650779 and was sponsored by Genzyme, a Sanofi company.

Patient Consent Statement

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

Details of the Contributions of Individual Authors

Daniel J. Gruskin and Larry Blankstein were involved in the study planning and coordination of statistical analyses. Ozlem Goker-Alpan, Michael J. Gambello, Gustavo H.B. Maegawa, Khan J. Nedd, and Neal J. Weinreb were involved in the study conduct. All authors contributed to the first draft of the manuscript, were involved in the critical review and revision of subsequent drafts, and approved the final draft for submission.

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