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CASE REPORT

False-Positive Newborn Screen Using the Beutler Spot Assay for Galactosemia in Glucose-6-Phosphate Dehydrogenase Deficiency

Grace Stuhrman • Stefanie J. Perez Juanazo • Kea Crivelly • Jennifer Smith • Hans Andersson • Eva Morava

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Abstract Classical galactosemia is detected through newborn screening by measuring galactose-1-phosphate uridylyltransferase (GALT) in the USA primarily via the Beutler spot assay. We report on an 18-month-old patient with glucose-6-phosphate dehydrogenase (G6PD) deficiency that was originally diagnosed with classical galactosemia. The patient presented with elevated liver function enzymes and bilirubinemia and was immediately treated with soy-based formula. Confirmatory tests revealed deficiency of the GALT enzyme, however, full-sequencing of *GALT* was normal, suggestive of a different ideology. The Beutler spot assay uses three other enzymatic steps in addition to GALT. A deficiency in either of these enzymes can result in suspected decreased GALT activity when using the Beutler assay. Congenital Disorders of Glycosylation screening for phosphoglucomutase-1 deficiency was negative. Quantitative analysis of G6PD enzyme in red blood cells showed a severe deficiency and a deletion in *G6PD*. Soy-formula, the standard treatment for galactosemia, has been reported to trigger hemolysis in G6PD deficient patients. G6PD and phosphoglucomutase-1 deficiencies should be considered when confirmatory tests are negative for pathogenic variants in *GALT* and galactose-1-phosphate level is normal.

Abbreviations

6PGD	6-Phosphogluconate dehydrogenase
ALT	Alanine transaminase
AST	Aspartate aminotransferase
CBC	Complete blood count
CDG	Congenital disorders of glycosylation
dl	Deciliter
G6PD	Glucose-6-phosphate dehydrogenase
Gal-1-P	Galacose-1-phosphate
GALT	Galactose-1-phosphate uridylyltransferase
Hb	Hemoglobin
hr	Hour
IU	International unit
μmol	Micromole
mg	Milligram
ml	Milliliter
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS	Newborn screening
PGM1	Phosphoglucomutase-1
U/g	Units per gram
UDP-glucose	Uridine diphosphate glucose

Communicated by: Daniela Karall

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Introduction

Newborn screening (NBS) for classical galactosemia (MIM 230400) in the USA has identified over 2,500 infants with this potentially lethal metabolic disorder (Pyhtila et al. 2014a). Classical galactosemia is inherited by autosomal recessive pattern and affects the metabolism of galactose as a result of a deficiency of the enzyme galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12). Symptoms can manifest within a few days after birth if dietary restriction of galactose is not initiated. These include

feeding difficulties, hypotonia, jaundice, failure to thrive, hepatosplenomegaly, sepsis, cataract, intellectual disability, and eventually death (Bosch 2006).

Multiple fluorescent and radioactive enzyme assays have been developed to screen for classical galactosemia (Li et al. 2010). However, the semiquantitative fluorescent Beutler spot assay is commonly used for diagnosis and mass screening in the USA (Fig. 1) (Fujimoto et al. 2000). This test, in addition to GALT, relies on phosphoglucomutase-1 (PGM1), glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (6PGD) in stepwise process to reduce $NADP^+$ to NADPH. The fluorescence of NADPH is measured to determine if an infant is presumptive positive for classical galactosemia. Therefore, the Beutler spot assay is influenced by the enzyme activity of PGM1, G6PD, and 6PGD to accurately quantify GALT (Fujimoto et al. 2000). Considering there are several enzymes in this pathway that can cause disorders when deficiencies are present, newborn screening using the Beutler method may lead to misdiagnosis for a select number of newborns with 6PGD.

Case Report

A Caucasian male born term, in the state of Louisiana, was reported as presumptive positive for galactosemia on his newborn screen. A routine newborn screen was completed on the second day of life. He had a GALT enzyme activity less than 2.5 U/g Hb (reference: >3.5 U/g Hb), and was referred to a biochemical geneticist in New Orleans. There was no known family history of jaundice, liver failure, sepsis, or metabolic disorders, and his parents were non-consanguineous. His birth history was unremarkable. He was breastfed from birth until 6 days of age when he was switched to a galactose free diet (soy-based formula) upon the report of his presumptive positive galactosemia newborn screen. His family was counseled on the biochemical background of galactosemia, the role of galactose-free diet, outcomes of galactosemia, and inheritance.

The initial confirmatory tests ordered included serum GALT enzyme, serum galactose-1-phosphate (Gal-1-P), galactosemia mutation panel for Q188R and N314D, and a liver profile. The GALT enzyme activity was 0.4 μmol/hr/ml blood (reference: 4.0–12.0 μmol/hr/ml blood) at less than 10% of residual activity. The initial Gal-1-P level was 0.3 mg/dl (reference: <1.0 mg/dl), but the child was on a lactose-free diet for 1 day prior to obtaining sample. The Gal-1-P level was undetectable after the child was on lactose-free diet for 5 days. The galactosemia mutation panel was negative for Q188R and N314D in the *GALT* gene. The liver profile revealed an elevated total bilirubin of 17.5 mg/dl (reference: <1.1 mg/dl) and a direct bilirubin of 0.4 mg/dl (reference: <0.4 mg/dl). Gal-1-P was measured again after 4 days on the soy-based formula and the level was undetectable.

The patient was scheduled for a follow-up visit 4 months later, but due to a miscommunication, was not seen until the following year. The patient's hemoglobin level was measured during a pediatric well-visit at 9 months of age at 8.3 g/dl (reference: 11.5–13.5 g/dl). At approximately 1 year of age, he was evaluated by genetics for a follow-up visit. His mom reported he had maintained a galactose-restricted diet and was still on a soy-based formula. The family was further counseled on implications of galactosemia and inheritance, and further genetic testing was recommended. Full-sequencing of the *GALT* gene was completed and the results showed no sequence pathogenic variants. These results triggered further testing to rule out possible PGM1 or G6PD deficiency. The tests included the modified Beutler assay, using specific substrates to target PGM1 and G6PD enzymes (Fig. 1), Congenital Disorders of Glycosylation (CDG) screening using transferrin isoelectric focusing, and quantitative measurement of G6PD. The modified Beutler assay measured PGM1 enzyme activity at <1 μmol/hr/ml (reference: 8–14 μmol/hr/ml) and G6PD enzyme activity at 2.5 μmol/hr/ml (reference: 4–16 μmol/hr/ml). Transferrin isoelectric focusing detected no abnormalities, ruling out PGM1 deficiency. Quantification of G6PD enzyme via kinetic assay was 13 IU/trillion RBC (reference: 146–376 IU/trillion RBC) at <5% of residual enzyme activity. Decreased G6PD enzyme activity and clinical features coinciding with G6PD deficiency warranted genetic testing for *G6PD* sequencing and deletion/duplication. Results suggested the patient was hemizygous in the *G6PD* gene for an in-frame duplication, c.1301–1318dup18 (predicted pathogenic variant). Lastly, sequencing in the *G6PD* gene was completed in the mother showing hemizygosity for the same unknown variant found in her son. The mother was clinically unaffected. At 19 months of age the patient had a fully normal development, but persistent elevated total bilirubin of 2.3 mg/dl, mild jaundice of the sclera, and slightly elevated liver function tests (AST: 99 (10–50 U/L), ALT: 227 (5–45 U/L). We counseled the parents on avoidance of oxidative drugs and fava beans and recommended removing soy products and switching to regular milk. Four months after removing soy from the patient's diet, a liver profile and complete blood count (CBC) revealed an improved total bilirubin of 1.7 mg/dl (reference: 0.1–2.0 mg/dl, direct bilirubin 0.5 mg/dl (reference: 0.1–0.4 mg/dl), and hemoglobin of 11.4 g/dl (11.5–13.6 g/dl).

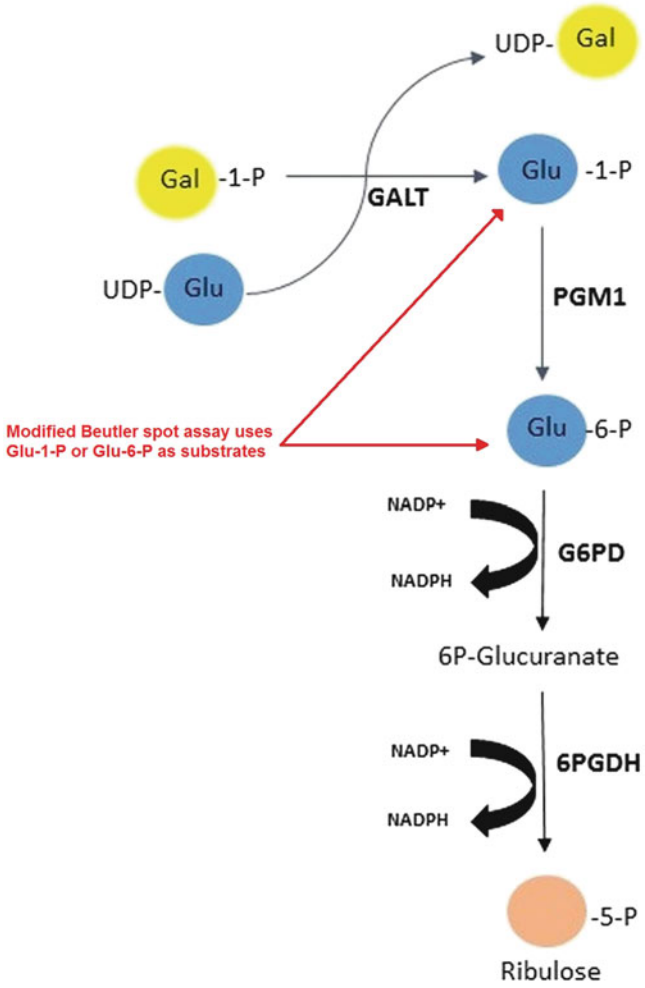

Fig. 1 In the Beutler spot assay, the substrates Gal-1-P and uridine diphosphate glucose (UDP-glucose) are part of the test reagent and breakdown into glucose-1-phosphate in the presence of GALT. Glucose-1-phosphate is then further metabolized, stepwise, to ribulose-5-phosphate by PGM1, G6PD, and 6PGD. The last two reactions are in conjunction with the reduction of $NADP^+$ to NADPH. The fluorescence of NADPH is measured to determine GALT deficiency. The Beutler spot assay can be modified to measure G6PD enzyme activity instead of GALT. This was achieved in our laboratory by adding glucose-6-phosphate as the main substrate. Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate. In presence of NADP, glucose-6-phosphate is oxidized by G6PD to generate 6-phosphogluconate. This reaction also generates NADPH by the reduction of NADP. The formation of NADPH produces fluorescence, which magnitude is proportional to the G6PD enzyme activity. By using glucose-6-phosphate, the preceding GALT and PGM1 enzymes present/involved in the pathway are avoided and G6PD enzyme is directly measured. This technique has been described previously (Beutler 1994)

Discussion

The Beutler spot assay is an inexpensive, efficient method for detecting classical galactosemia in mass newborn screening. Although there are other screening methods for galactosemia, predominantly measurement of total galactose (galactose and galactose-1-phosphate) in blood spots, there is a higher frequency of false-positives (Ohlsson et al. 2011). Several states use both measurements to screen for classical galactosemia. A study by Pyhtila et al. looked at the testing strategies of state labs in the USA in 2011–2012 (Pyhtila et al. 2014b). Of the 19 states that responded, 40% exclusively used GALT, 40% used GALT plus total galactose, and 20% only used total galactose if the GALT was low. In the state of Louisiana, newborn screening for classical galactosemia is conducted using the Perkin Elmer Neonatal GALT kit based on the Beutler spot assay. Presumptive positive newborn screens are based solely on GALT enzyme activity. The GALT cut-off is set at <3.5 U/g Hb. The current reporting is <2.5 U/g Hb is the lower limit of detection, between 2.5 and 3.0 U/g Hb is reported as presumptive positive, and 3.1−≤3.5 U/g Hb is reported as borderline and >3.5 IU is considered normal enzyme activity. Values were determined using the Perkin Elmer package insert and Louisiana in-state data. The Beutler spot assay measures fluorescent NADPH as the final product in a stepwise reaction (Fig. 1). Although GALT is the rate-

limiting enzyme, the Beutler spot assay requires normal enzyme activity of PGM1, G6PD, and 6PGD to avoid false-positive newborn screens for galactosemia (Fujimoto et al. 2000). Deficiency in any of these enzymes can result in a misdiagnosis of galactosemia when using the Beutler spot assay. PGM1 deficiency is a type of congenital disorders of glycosylation (CDG) caused by inborn errors of glycan metabolism (Scott et al. 2014). Only a few cases of 6PGD deficiency have been described (Caprari et al. 2001). Reported signs include hemolytic anemia and scleral jaundice.

The guidelines set by the American College of Medical Genetics recommend molecular testing for common pathogenic variants in the *GALT* gene in congruence with a quantitative GALT assay (American College of Medical Genetics 2001). Additional tests should be conducted if initial confirmatory testing reveals decreased GALT activity using the Beutler spot assay in combination with no detection of common *GALT* gene variants and a normal Gal-1-P. Additional testing should include full-sequencing of the *GALT* gene, transferrin glycoform analysis (CDG screening), and measure of G6PD. Interestingly, the Beutler spot assay can be modified to bypass the GALT and PGM1 enzymes, and therefore, measure G6PD enzyme activity (Fig. 1).

The USA currently does not recommend newborn screening for G6PD deficiency, an X-linked condition. Individuals with severe G6PD deficiency may be identified through newborn screening via the Beutler spot assay resulting in a false positive newborn screen for galactosemia (Frazier and Summer 1974). Deficiency in the G6PD enzyme occurs in at least 400 million people worldwide, making it the most common human enzyme deficiency. Over 400 variants in the *G6PD* gene have been identified. Variants associated with G6PD deficiency are divided into four classes based on the level of residual activity and the severity of phenotype ranging from severe (Class I) to mild (Class IV) (Frank and Maj 2005). The patient in discussion, although having a variant of unknown significance, had a residual G6PD activity of less than 10%. Despite G6PD being a very common disease, a severe deficiency in G6PD enzyme is required to trigger an abnormal NBS for galactosemia when using the Beutler spot assay. This underlines the possibility of identifying individuals with severe G6PD deficiency through newborn screening of classic galactosemia using the Beutler spot assay.

Treatment of galactosemia includes strict dietary avoidance of galactose. During infancy, this involves substituting breast milk or milk-based formula with a soy-based formula or other galactose-free formula. Treatment of G6DP deficiency includes avoidance of exogenous agents such as oxidative drugs and fava beans (Frank and Maj 2005). Fava beans are recognized to cause hemolysis in G6PD-deficient patients by an unknown oxidative compound, possibly vicine, convicine, isouramil, or divicine (Frank and Maj 2005; Chevion et al. 1982). In addition, other food items such as soy and legumes have been reported by the G6PD Deficiency Association to cause hemolysis based on increased hemoglobin levels. Although there are no scientific studies on the effect of soy and legumes on hemolysis in severe G6PD deficiency, it may be clinically relevant to remove these items from the individual's diet. If a severely G6PD deficient patient is reported as presumptive positive for galactosemia on their newborn screen, and, placed on soy-based formula, it may do more harm than good.

In conclusion, severe enzyme deficiency of G6PD can result in a presumptive positive newborn screen for classical galactosemia when using the Beutler spot assay. G6PD should be included in the differential diagnosis when confirmatory testing is negative for galactosemia variants and Gal-1-P level is normal. Under these circumstances, second tier tests such as a comprehensive metabolic panel, quantitative G6PD assay, and transferrin glycoform analysis should be conducted to rule out possible G6PD or PGM1 deficiencies.

Synopsis

Decreased GALT enzyme by Beutler spot assay can mask PGM1 or G6PD deficiencies and warrants further metabolic analysis in the absence of pathogenic variants in *GALT*. Soy-based formula intake can trigger hemolysis in G6PD deficiency.

Compliance with Ethics Guidelines

Conflict of Interest

Grace Stuhrman, Stefanie J. Perez Juanazo, Kea Crivelly, Jennifer Smith, Dr. Hans Andersson and Dr. Eva Morava declare that they have no conflicts of interest.

Informed Consent/Animal Rights

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

Details of the Contributions of Individual Authors

Grace Stuhrman drafted the initial manuscript and revised the manuscript.

Stefanie J. Perez Juanazo contributed to drafting the initial manuscript and conducted biochemical assays.

Jennifer Smith conducted biochemical assays.

Kea Crivelly, Eva Morava-Kozicz, and Hans Andersson critically reviewed and revised the manuscript.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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RESEARCH REPORT

Domains of Daily Physical Activity in Children with Mitochondrial Disease: A 3D Accelerometry Approach

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Abstract Feasible, sensitive and clinically relevant outcome measures are of extreme importance when designing clinical trials. For paediatric mitochondrial disease, no robust end point has been described to date. The aim of this study was to select the domains of daily physical activity, which can be measured by 3D accelerometry, that could serve as sensitive end points in future clinical trials in children with mitochondrial disorders.

In this exploratory observational study, 17 patients with mitochondrial disease and 16 age- and sex-matched controls wore 3D accelerometers at the upper leg, upper arm, lower arm and chest during one weekend. Using the raw data obtained by the accelerometers, we calculated the following outcome measures: (1) average amount of counts per hour the sensors were worn; (2) the maximal intensity; (3) the largest area under the curve during 30 min and (4) categorized activities lying, standing or being dynamically active. Measuring physical activity during the whole weekend was practically feasible in all participants. We found good face validity by visually correlating the validation videos and activity diaries to the accelerometer data-graphs. Patients with mitochondrial disorders had significantly lower peak intensity and were resting more, compared to their age- and sex-matched peers.

Finally, we suggest domains of physical activity that could be included when measuring daily physical activity in children with mitochondrial disorders, preferably using more user-friendly devices. These include peak activity parameters for the arms (all patients) and legs (ambulatory patients). We recommend using or developing devices that measure these domains of physical activity in future clinical studies.

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Ilse Dirks and Esmee van Mierlo contributed equally to this work.

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Introduction

Since lack of energy and fatigue are among the most burdensome complaints experienced by children with mitochondrial disease and their parents (Koene et al. 2013b), this symptom should be covered in future clinical trials. However, testing fatigue or fatigability in this paediatric population is challenging, since many children are not able to rate their fatigue using one of the widely used self-report fatigue questionnaires (Swanink et al. 1995; Gordijn et al. 2011) because of intellectual disabilities and most endurance tests (e.g. cycle ergometry (LeMura et al. 2001) are not feasible or too burdensome for children with mitochondrial disorders. Besides,

measuring performance in a laboratory situation may not always reflect the disabilities experienced in daily life since the performance in daily life may differ from the abilities of the child (Abel et al. 2003; Beenakker et al. 2005; Parreira et al. 2010).

In a recent review of all published studies in mitochondrial disease, an international expert panel recommended (amongst others) to use validated and clinically meaningful end points (Pfeffer et al. 2013). Only very few studies investigating outcome measures in paediatric mitochondrial disease have been published and most of the outcome measures studied are not generally applicable among children with mitochondrial disease. In a small group of children with non-proven mitochondrial disease, Martens et al. (2014) found lower physical activity level compared to healthy peers.

We hypothesized that measuring daily physical activity at home is a clinically relevant outcome measure and a good reflection of the fatigue experienced by children with mitochondrial disease in daily life. Physical activity is defined as "any bodily movement produced by skeletal muscle contraction that results in caloric expenditure" and includes sports, hobbies, playing, walking, cycling and activities of daily living (Caspersen et al. 1985). Physical activity has many domains, including the type, intensity, frequency and duration of the physical activity. It is currently not known which of these aspects is most affected in patients with mitochondrial disorders.

Daily physical activity in a home situation can be measured by using 3D accelerometry (Bjornson 2005; McDonald et al. 2005; Capio et al. 2010; Clanchy et al. 2011a, b; Jeannet et al. 2011; Koene et al. 2013a). Many commercially available activity monitors that are based on accelerometry measure only general domains of movement, such as the total amount of body activity, step count and position. Such monitors provide only the calculated parameters, and not the raw acceleration data. In this study, we aimed to select more detailed domains of physical activity that can be measured by accelerometry in future clinical trials. For this study, we selected an accelerometer (MOX) that provides raw accelerations in 3D, so we could design a tailored analysis.

Methods

This is an exploratory, observational study, aiming to select the domains of physical activity that should be measured by accelerometry in future clinical trials in patients with mitochondrial disease. The domains of physical activity were selected based on deviation from healthy age- and sex-matched peers. Before selecting these domains, the feasibility and face validity of the 3D accelerometer in this population was studied.

Accelerometer

For this study, we used a MOX accelerometer (MOX sensor, model MMOXX1.01, Maastricht Instruments BV, The Netherlands), that measures accelerations (Range ± 6G) in three degrees of freedom with a sample frequency of 25 Hz. The acceleration data was filtered with a Butterworth 0.025–7.5 Hz 4th-order high-pass filter to remove noise and movement artefacts. This accelerometer provides raw data which can be used for tailored analyses.

A set of four or five sensors was used. The accelerometers were attached to the chest, dominant lower arm and upper arm and to the leg using an attachment band (limbs) or a top (chest; Fig. 1). If the patient used a wheelchair, a fifth accelerometer was attached to the wheelchair. The wheelchair sensor was used to indicate passive moments of the child.

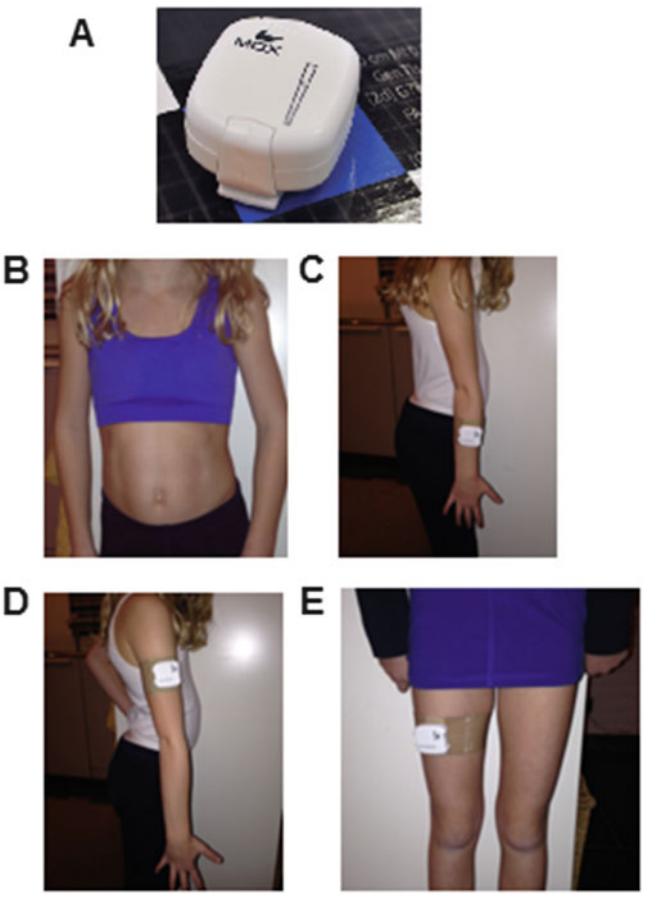

Fig. 1 The accelerometer localizations. (**a**) The MOX-accelerometer is approximately 4.5 × 4.0 × 1.4 cm in size and weighs 27 g; (**b**) Attachment of the accelerometer to chest; (**c**) to the lower (dominant) arm; (**d**) the upper arm and (**e**) the (dominant) upper leg

To estimate the amount of daily activity, various parameters were calculated from the acceleration data of each sensor, by using Matlab procedures that were developed and validated beforehand (Meijer et al. 2014). The first parameter is the activity counts, which was calculated by integrating the acceleration over 1-minute episodes and summing this outcome over all three axes. A constant acceleration of 1G (gravitational constant) over 1 min corresponds with 1,000 counts (Meijer et al. 2014). We used the following outcome measures: (1) average amount of counts per hour the sensors were worn (average counts (total amount of counts measured with the sensor/worn h; counts/h), also referred to activity level); (2) the maximal intensity (maximal amount of counts per min (counts/min)) and (3) the largest area under the curve (AUC) during 30 min (largest AUC during 1/2 h (counts)). The second outcome measure is an activity classification which categorizes the performed activities per second into lying, standing or being dynamically active. Lying and standing are classified depending on the gravitational angle acting on the posterior-anterior and cranial-caudal axes. Being dynamically active is classified when the integration of the acceleration over 1-second episodes is above a pre-defined threshold.

Study Protocol

Study Protocol for Patients

Patients were recruited at the Radboud Centre for Mitochondrial Medicine. Patients aged 4–18 years old with a confirmed mitochondrial disease, either based on pathological mutations in mtDNA or nuclear DNA or on mitochondrial dysfunction in fresh muscle as measured by routine biochemistry as applied in our centre (Rodenburg 2011), were eligible for inclusion. This group includes the full clinical spectrum of mitochondrial disorders, ranging from patients with exercise intolerance only to wheelchair bound patients with severe movement disorders. Exclusion criteria: (1) expected by the treating physician that travelling to the hospital would be too burdensome to the patient; (2) fever; (3) epilepsia continua; or (4) altered state of consciousness compared to normal at the time of inclusion. The number of children was determined by the number of children that could be included in the same season.

Patients were assessed at the outpatient clinic of the Radboud Center for Mitochondrial Medicine (RCMM) on Fridays. An experienced paediatric physiotherapist performed the Gross Motor Function Measure-88 (GMFM). After the two tests, parents were instructed how to attach the accelerometers. Wearing the sensors, patients were – if possible – instructed to follow a validation protocol (standardized activities, including waving, throwing a ball, lying down, sitting, standing, walking and running). In case of limited physical abilities, the position (orientation) of the arm, leg and chest was changed passively, if possible at low, middle and high velocity (intensity). The patient was videotaped with a synchronized camera during all tests, to be able to correlate specific movements (e.g. raising an arm, walking, movement disorders or epilepsy) to the data obtained by the accelerometer. By correlating these video images with the accelerometer data-graphs (correspondence of orientation and intensity for each sensor) we determined the face validity of the measurements in a laboratory situation.

After completion of the validation protocol, patients were asked to wear the sensors over the weekend, while the parents completed an activity diary. Parents were asked to complete the diary with the exact timing and a description of the activity (e.g. 12:36–13:18: Lunch, independently eating bread with knife and fork). For practical reasons (complexity of data analysis and logistics), we chose to only measure for 2 days, instead of the recommended 7 days (Cain et al. 2013). Patients were asked to wear the accelerometers at all waking hours, with the exception of bathing, showering and swimming. The reported activities were also correlated to the accelerometer data-graphs (correspondence of the intensity of the movements during the described activities) to determine face validity of the measurements at home.

On Monday, the parents were interviewed by phone for the feasibility and comfort of the accelerometers with a self-made questionnaire. Moreover, the parents were interviewed with the Pediatric Evaluation of Disability Inventory (PEDI), measuring the performance and capability in the activities of daily life (Custers et al. 2002; Vos-Vromans et al. 2005). The PEDI and the GMFM were used to determine the correlation between activity parameters and gross motor function (capability) and functional abilities (performance).

Study Protocol for Healthy Controls

The healthy controls were recruited at two regular schools in the surroundings of Nijmegen. Healthy controls were eligible for inclusion when they were healthy and aged between 4 and 18 years. Exclusion criteria: (1) confirmed diagnosis of Attention Deficit and Hyperactivity Disorder (ADHD); (2) symptoms of exercise intolerance, fatigue or muscle problems or (3) the child was under regular surveillance of a paediatrician. Controls were sex- and age-matched to a single patient.

Healthy controls were instructed in their home-environment in the same weekend as the age- and sex-matched patient. The attachment and localization of the accelerometers was the same as the patient protocol. Validation, using the validation protocol and videotaping, was also similar to the patient protocol. Healthy controls were also instructed to wear the accelerometer during waking hours and to keep

an activity diary. On Monday, the feasibility and comfort of the accelerometers was evaluated.

Analyses

Feasibility

Feasibility was tested using the parent reported complications of wearing the accelerometers and the quantity of the data obtained (% of subjects; the time the device collected data as a percentage of the intended measurement period (Saturday 0:00 to Sunday 23:59) and the time the device collected data as a percentage of the time the sensor was worn). Only patients in whom more than one sensor failed were excluded from the analyses. For the patients in which one sensor failed, only the available data are presented.

Face Validity

Face validity was assessed by visually correlating the videos with the obtained accelerometer data-graphs (correspondence of orientation and intensity for each sensor) during the validation protocol in each subject. Subsequently, the data from the diaries was correlated to the accelerometer data-graphs (correspondence of the intensity of the movements during the described activities). Only when the video images and described activities clearly did not correlate to the data-graphs, the data were excluded from the analyses. We assessed whether the percentage of dynamic activity and the total leg activity was lower in non-ambulatory children compared to ambulatory children. Finally, the functional abilities, assessed by the GMFM and the PEDI were correlated to the measurements.

Patients Versus Controls

We compared patients and their age- and sex-matched controls on each of the above-mentioned variables.

Subgroup Analyses

Based on the molecular finding in each patients, we've created three subgroups: (1) genetically confirmed primary mitochondrial disease; (2) genetically confirmed secondary mitochondrial disease (patients with a mutation in a non-mitochondrial gene with biochemically proven mitochondrial dysfunction, either with or without a proven link to mitochondrial processes) and (3) biochemically confirmed mitochondrial dysfunction.

Statistical Analyses

Because of the relatively small number of subjects included in our study, we used non-parametric tests to assess differences and correlations. We used a p-value of 0.05 for statistical significance; because of the exploratory character of this study, we did not use adjust critical p-values using the Bonferroni method. Missing data were not replaced. All analyses were performed using IBM's SPSS statistics software packages, version 20.0.0.1. Correlation coefficients were interpreted in accordance with the guidelines provided at the BMJ website (http://www.bmj.com/about-bmj/resources-readers/publications/statistics-square-one/11-correlation-and-regression).

Ethics

This study was approved by the regional Medical Research Ethics Committee (MREC NL50560.091.14). In accordance with the Helsinki agreement, written informed consent was obtained from participant's legal guardian and, where indicated, the participant.

Results

Study Population

Seventeen patients and 16 healthy age- and sex-matched controls were included in this study from February to May 2015. One healthy control withdrew his consent 1 day before the measurement would start and no other age- and sex-matched control was available for that weekend. The groups were comparable with respect to age, sex, BMI and sports- and highest education of parents, but – as expected – differed significantly with respect to height, weight, time spent at sports and the level of education of the child (Supplementary Table 1). There was a wide variability in the genetic, biochemical, clinical and functional abilities in the children with mitochondrial disease (Supplementary Table 2).

Feasibility

All participants, including patients with severe mental retardation, tolerated wearing the accelerometers for the duration of the measurement. The full study protocol was completed by 29 children (88% of total study population): 3 participants temporarily removed the sensors: 2 removed the top to ventilate after exercise and because of a party, 1 did not attach the chest-sensor on Sunday because of discomfort of the top and 1 boy lost his upper leg sensor during outdoor playing. Five sensors failed to record any data and the batteries of one sensor failed during the measurements (18% of all participants; 4% of all measurements). Due to these technical issues, 6% of the total measured time and 8% of the time the sensors were worn

was missing in 6 participants (4 patients and 2 healthy controls). One healthy control had to be excluded from the analyses because he lost his upper leg sensor and his upper arm sensor failed to record any data. Most subjects wore their sensors from the moment they awoke to the moment they undressed for bed; three participants removed all sensors after dinner (averagely sensors were worn 94% of the woken time). The time the sensors were taken off because of swimming, showering or bathing was 1.9%. The time the sensors were not worn because of lack of understanding or lack of motivation was 8.7%.

Face Validity

For all patients and healthy controls, the movements (orientation, intensity) at the videos corresponded to the acceleration data that was visualized in graphs (Fig. 2). Dynamic activity (i.e. walking) and the activity of arms and legs was higher in ambulatory compared to non-ambulatory patients. For these analyses, we excluded a boy who was not able to walk but had excellent abilities to move (on his buttock), but not to walk, from these analyses since we could not define in which group he belonged. We found a (very) strong and significant correlation between the motor abilities as measured with the GMFM and the resting percentage ($\rho = -0.82$), the largest amount of activity of the leg during half an hour ($\rho = 0.65$) and the peak-activity of the lower arm ($\rho = 0.67$; all $p < 0.0001$). The score on the mobility domain of the PEDI (functional abilities) also correlated very strongly with the resting percentage ($\rho = -0.87$) and strongly with the largest amount of activity of the leg during half an hour ($\rho = 0.70$) and the peak-activity of the leg ($\rho = 0.60$; all $p < 0.0001$; Supplementary Table 3).

Patients Versus Controls

We observed no difference in any of the activity variables between all children (including both healthy controls and patients) aged 12 years and older and children under 12 years of age ($p = 0.005$–0.815). We also observed no difference in any of the activity variables between patients under or above 12 years of age ($p = 0.05$–0.79). The same accounts for the difference between boys and girls ($p = 0.05$–1.00 and $p = 0.005$–1.00, respectively).

Almost all activity variables were significantly lower in patients with mitochondrial disorders compared to their age- and sex-matched controls (Table 1). We saw no difference in the quantity of arm activity between patients and controls. When comparing individual results, all but one patient had higher percentages of rest during the weekend, except for one girl matched to a healthy girl who had to study for her final exams during the whole weekend (couple 2 in Supplementary Fig. 1).

Subgroup Analyses

When comparing patients with genetically confirmed primary mitochondrial disease to their peers, we found a similar pattern of differences compared to all patients versus their matched controls. Only the maximal intensity of the upper- and lower arm and the largest AUC during half an hour for the upper arm reached significance in this small group. Patients with genetically confirmed secondary mitochondrial dysfunction differed significantly in their % of rest and standing, as well as their maximal intensity for the upper leg and lower arm. For patients with biochemically confirmed mitochondrial dysfunction, only the wear time differed significantly from their healthy peers, although the median peak activities were all (not significantly) lower. When comparing the latter two groups to the genetically confirmed primary mitochondrial disease patients, patients with genetically confirmed secondary mitochondrial disease (which are all non-ambulatory) are resting more and have a lower AUC during half an hour for the upper leg. Patients with biochemically confirmed mitochondrial dysfunction did not differ significantly from the patients genetically confirmed primary mitochondrial disease.

Not surprisingly, the leg activity of non-ambulatory patients was lower compared to ambulatory patients (Table 2). In fact, the activity of ambulatory patients did not deviate from healthy peers ($p = 0.05$–0.9), with the exception of the largest AUC in 30 min for the upper leg. The other peak intensity parameters did not reach significance, but showed substantially lower values compared to their peers. Non-ambulatory patients deviated from healthy controls – as expected – in all leg parameters and position parameters, but also in peak activity. The maximal intensity parameters for the arm were decreased to the largest extent and most significantly.

Patients with myopathy and encephalopathy were comparable with respect to all activity parameters ($p = 0.3$–0.9).

Recommendations

Based on the difference (both significance and the magnitude of the difference) between patients and healthy subjects, we made recommendations on which variables to use in future accelerometry studies in children with mitochondrial disease (Table 3). We recommend the use of maximal intensity and largest AUC in 30 min variables.

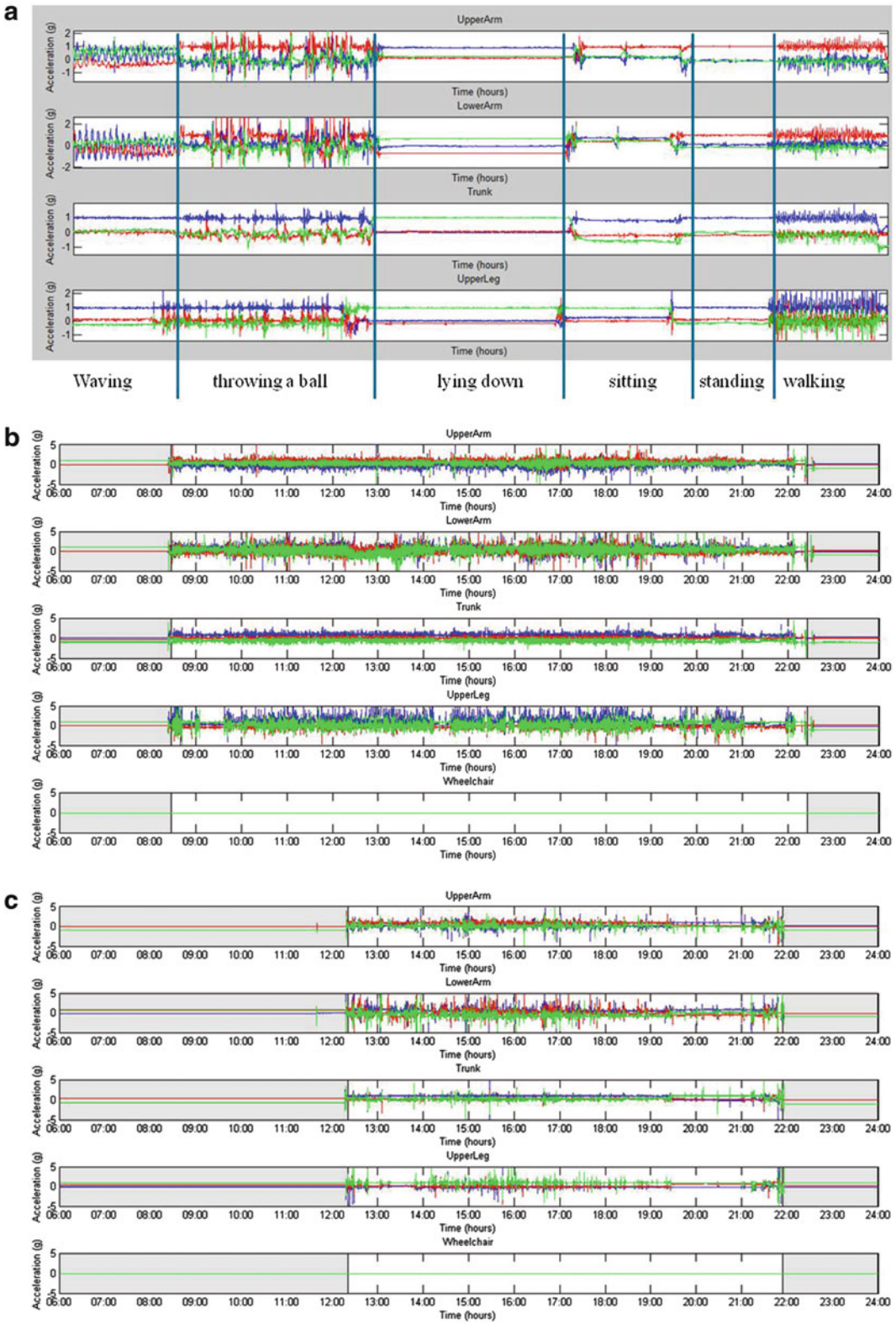

Fig. 2 Raw data. (**a**) Raw data representing the orientation and activity of the sensors during the validation protocol (patient 17). (**b**, **c**) Raw data representing the physical activity of two representative patients during the validation protocol in the laboratory. (**c**, **d**) Raw data representing the physical activity of one day for a matched couple. The couple (couple 3) with the most striking difference was selected, the healthy control is in panel (**c**) and panel (**d**) represents the patient

Table 1 Average activity over the weekend for children with mitochondrial disease compared to age- and sex-matched controls (including subgroup analyses for genetically confirmed primary mitochondrial disease, genetically confirmed secondary mitochondrial disease and biochemically confirmed mitochondrial dysfunction)

	Patients ($n = 17$)	Healthy controls ($n = 15$)		Patients with genetically confirmed primary mitochondrial disease ($n = 9$)	Matched controls ($n = 7$)		Patients with genetically confirmed secondary mitochondrial disease ($n = 4$)	Matched controls ($n = 4$)			Patients with biochemically confirmed mitochondrial dysfunction ($n = 8$)	Matched controls ($n = 7$)		
	Median Range	Median Range	*p*-value for difference between patients and healthy controls	Median Range	Median Range	*p*-value for difference between patients and matched controls	Median Range	Median Range	*p*-value for difference between patients and matched controls	*p*-value for difference between patients in this group and genetically confirmed primary mitochondrial disease patients	Median Range	Median Range	*p*-value for difference between patients and matched controls	*p*-value for difference between patients in this group and genetically confirmed primary mitochondrial disease patients
Wear time (h/day)	10 (8–13)	12 (6–15)	**0.004**	9 (8–13)	12 (9–14)	0.1	11 (10–12)	12 (10–15)	0.6	0.2	10 (8–12)	12 (6–14)	**0.03**	0.6
Average % rest	86 (44–99)	60 (2–89)	<**0.001**	75 (44–98)	59 (51–73)	0.07	98 (86–99)	57 (32–83)	**0.03**	**0.04**	86 (63–92)	69 (47–89)	0.2	0.5
Average % standing	4 (0–23)	18 (7–48)	**0.004**	15 (0–22)	21 (13–30)	0.05	0 (0–1)	18 (9–48)	**0.03**	**0.02**	4 (3–23)	12 (7–36)	0.2	0.5
Average % dynamic activity	13 (1–50)	18 (5–33)	**0.02**	13 (2–35)	20 (9–27)	0.1	7 (1–50)	22 (9–27)	0.5	0.6	9 (5–17)	17 (5–33)	0.2	1.0
Average counts upper leg (1,000 counts/h)	9.4 (3.1–29.6)	18.0 (7.0–27.2	**0.008**	10.9 (5.1–29.6)	15.0 (10.2–22.0)	0.2	4.7 (3.1–17.4)	17.1 (11.7–26.8)	0.1	0.2	11.7 (7.3–15.0)	15.8 (7.0–27.1)	0.5	1.0
Average counts upper arm (1,000 counts/h)	15.7 (11.3–36.2)	19.0 (10.5–30.2)	0.3	18.8 (14.2–32.2)	17.5 (14.5–25.9)	0.9	12.9 (11.2–36.3)	22.4 (15.7–30.2)	0.3	0.2	15.7 (13.8–21.8)	17.8 (10.5–22.9)	0.9	0.5
Average counts lower arm (1,000 counts/h)	21.5 (16.1–41.8)	27.1 (15.3–37.1)	0.3	25.3 (18.5–40.3)	26.1 (18.5–34.7)	1.0	20.1 (16.1–41.8)	31.3 (23.0–37.1)	0.3	0.5	20.5 (17.8–30.3)	23.9 (14.0–29.8)	0.9	0.4
Average counts wheel-chair (1,000 counts/h)	1.3 (0.7–1.4)	NA	NA	1.1 (0.8–1.3)	NA		1.1 (0.7–1.4)	NA			1.4 (1.3–1.5)	NA		
Maximal intensity upper leg (1,000 counts/min)	1.0 (0.5–2.6)	1.9 (0.9–2.6	<**0.001**	1.1 (0.7–2.6)	1.8 (1.2–2.4)	0.1	0.8 (0.5–1.0)	1.8 (1.1–2.4)	**0.03**	0.2	1.2 (0.9–1.6)	2.3 (0.9–2.6)	0.1	1.0
Maximal intensity upper arm (1,000 counts/min)	1.0 (0.8–1.9)	1.6 (0.9–2.1)	<**0.001**	1.0 (0.8–1.9)	1.7 (1.1–1.9)	**0.02**	0.9 (0.8–1.2)	1.4 (1.1–1.9)	0.06	0.4	1.1 (0.8–1.5)	1.6 (0.9–2.1)	0.2	0.9
Maximal intensity lower arm (1,000 counts/min)	1.2 (0.9–2.2)	1.9 (1.0–2.5)	<**0.001**	1.1 (1.0–2.2)	1.9 (1.3–2.5)	**0.03**	1.1 (1.0–1.4)	1.8 (1.5–2.4)	**0.03**	0.5	1.4 (0.9–1.8)	1.9 (1.0–2.5)	0.3	0.5
Largest AUC during 1/2 h upper leg (1,000 counts)	10.4 (6.4–37.3)	26.8 (11.4–66.2)	<**0.001**	18.9 (8.8–37.3)	27.5 (16.3–30.1)	0.2	7.2 (6.4–17.3)	30.3 (14.7–41.8)	0.06	**0.04**	12.5 (9.9–21.6)	24.0 (11.4–66.2)	0.1	0.8
Largest AUC during ½ h upper arm (1,000 counts)	15.8 (1.1–27.3)	25.3 (11.7–368)	**0.003**	15.7 (14.0–27.3)	26.2 (16.3–30.1)	**0.05**	12.9 (11.1–25.1)	25.5 (22.9–36.8)	0.1	0.2	17.9 (12.0–23.0)	20.7 (11.0–30.7)	0.8	0.7
Largest AUC during 1/2 h lower arm (1,000 counts)	21.2 (16.0–34.8)	30.8 (16.7–43.4)	**0.004**	21.9 (18.9–34.8)	30.8 (19.3–40.0)	0.1	18.7 (16.0–29.3)	32.3 (27.1–43.4)	0.06	0.3	25.6 (16.7–28.5)	25.2 (16.7–40.6)	0.5	0.8

A level of $p = 0.05$ was used for significance; significant values are indicated in *bold*
AUC area under the curve, *min* minutes

Table 2 Average activity over the weekend for children with mitochondrial disease compared to age- and sex-matched controls (including subgroup analyses for ambulatory and non-ambulatory patients and patients with myopathy or encephalopathy. We excluded a boy who was not able to walk but had excellent abilities to move (on his buttock), but not to walk, from the ambulatory/non-ambulatory analyses since we could not define in which group he belonged)

	Patients ($n = 17$)		Healthy controls ($n = 15$)			Ambulatory patients ($n = 9$)			Non ambulatory patients ($n = 7$)			Myopathy ($n = 7$)		Enchephalomyopathy ($n = 10$)		
	Median	Range	Median	Range	*p*-value for difference between patients and healthy controls	Median	Range	*p*-value for difference between ambulatory patients and healthy controls	Median	Range	*p*-value for difference between non-ambulatory patients and healthy controls	*p*-value for difference between ambulatory and non-ambulatory patients	Median	Range	Median	Range	*p*-value for difference between patients with myopathy and patients with encephalomyopathy
Wear time (h/day)	10	(8–13)	12	(6–15)	**0.004**	12	(9–14)	**0.04**	11	(9–12)	0.2	0.5	11	(8–13)	10	(8–12)	0.3
Average % rest	86	(44–99)	60	(2–89)	<**0.001**	73	(44–90)	0.2	98	(86–99)	**0.003**	**0.006**	77	(63–99)	89	(44–98)	0.6
Average % standing	4	(0–23)	18	(7–48)	**0.004**	16	(4–23)	0.6	1	(0–3)	**0.001**	<**0.001**	10	(0–23)	3	(0–22)	0.8
Average % dynamic activity	13	(1–50)	18	(5–33)	**0.02**	14	(3–34)	0.2	5	(1–50)	0.3	0.09	13	(1–50)	9	(5–35)	0.4
Average counts upper leg (1,000 counts/h)	9.4	(3.1–29.6)	18.0	(7.0–27.2	**0.008**	12.7	(8.2–29.6)	0.2	5.5	(3.1–17.4)	**0.02**	**0.03**	9.1	(3.1–16.9)	10.0	(5.1–29.6)	0.7
Average counts upper arm (1,000 counts/h)	15.7	(11.3–36.2)	19.0	(10.5–30.2)	0.3	18.9	(14.2–32.2)	0.9	14.3	(11.3–36.2)	0.5	0.1	16.5	(11.3–25.2)	15.5	(13.2–36.2)	0.6
Average counts lower arm (1,000 counts/h)	21.5	(16.1–41.8)	27.1	(15.3–37.1)	0.3	23.3	(18.9–40.3)	0.8	20.0	(16.1–41.8)	0.6	0.1	21.5	(16.1–31.8)	21.2	(17.8–41.8)	0.9
Average counts wheelchair (1,000 counts/h)	1.3	(0.7–1.4)	NA		NA	NA	0.8	NA	1.3	(0.7–1.5)	NA	0.6	NA	1.4	1.2	(0.7–1.5)	0.9
Maximal intensity upper leg (1,000 counts/min)	1.0	(0.5–2.6)	1.9	(0.9–2.6	<**0.001**	1.3	(0.7–2.6)	0.09	0.9	(0.5–1.0)	**0.006**	**0.03**	0.9	(0.5–1.7)	1.0	(0.9–2.6)	0.6
Maximal intensity upper arm (1,000 counts/min)	1.0	(0.8–1.9)	1.6	(0.9–2.1)	<**0.001**	1.1	(0.8–1.9)	0.08	0.9	(0.8–1.2)	**0.008**	0.06	1.0	(0.9–1.4)	1.0	(0.8–1.9)	0.8
Maximal intensity lower arm (1,000 counts/min)	1.2	(0.9–2.2)	1.9	(1.0–2.5)	<**0.001**	1.4	(1.0–2.2)	0.09	1.1	(0.9–1.4)	**0.009**	0.09	1.2	(1.0–1.8)	1.2	(0.9–2.2)	0.9
Largest AUC during 1/2 h upper leg (1,000 counts)	10.4	(6.4–37.3)	26.8	(11.4–66.2)	<**0.001**	20.2	(8.9–37.2)	**0.02**	9.9	(6.4–17.3)	**0.006**	**0.03**	9.5	(6.4–37.3)	13.9	(7.4–30.7)	0.4
Largest AUC during 1/2 h upper arm (1,000 counts)	15.8	(1.1–27.3)	25.3	(11.7–368)	**0.003**	17.9	(14.0–27.3)	0.07	14.6	(11.1–25.1)	**0.03**	0.09	15.5	(11.1–27.3)	17.0	(11.9–25.1)	0.5
Largest AUC during 1/2 h lower arm (1,000 counts)	21.2	(16.0–34.8)	30.8	(16.7–43.4)	**0.004**	25.6	(18.5–34.8)	0.05	18.2	(16.0–29.3)	0.2	**0.04**	20.4	(16.0–34.8)	22.9	(16.7–29.9)	0.5

A level of $p = 0.05$ was used for significance; significant values are indicated in *bold*
AUC area under the curve, *min* minutes

Table 3 Recommendations for future accelerometry studies in children with mitochondrial disease

	Ambulatory patients	Non-ambulatory patients
The percentage of the day spent resting or in dynamic activity	NR	NR
The level of activity of legs	NR	NR
The level of activity of arms	NR	NR
Maximal intensity of legs	H	NR
Maximal intensity of arms	R	R
Largest AUC in 30 min of legs	H	NR
Largest AUC in 30 min of arms	R	R

AUC area under the curve, *H* highly recommended, *NR* not recommended, *R* recommended

For non-ambulatory patients, arm activity variables should be used instead of leg variables.

Discussion

In this exploratory study, we aimed to select the domains of daily physical activity, measured by 3D accelerometry, that could be sensitive end points in future clinical trials. Although we experienced technical difficulties with the hardware in 18% of the subjects (4% of the measurements), we showed that measuring physical activity in a home-situation with 3D accelerometers was feasible and had good face validity in all seventeen children with mitochondrial disease. By comparing the children to an age- and sex-matched healthy peer who was measured within the same weekend, we selected domains of movement that deviated from normal in children with mitochondrial disorders (Table 3).

The percentages of rest, standing and dynamic activity were significantly different between patients and their healthy peers. However, since we observed no differences in these percentages between matched controls and non-ambulatory patients (who are sitting in a wheelchair), we don't recommend the use domains of physical activity for future studies. Arm activity levels (amount of activity) were comparable between patients and healthy controls. Our data could not confirm that this was due to compensatory use of arms in non-ambulatory children or due to high levels of unpurposeful arm activity in children with movement disorders such as ataxia. We advice not to include arm activity level as a domain of physical activity in children with mitochondrial disorders. The peak activity and largest area under the curve for both arms and legs showed the largest magnitude of difference and was significantly different between patients and controls for most parameters, also when only the nine patients with genetically confirmed primary mitochondrial disease were included. For ambulatory patients, the largest magnitude of change is observed in the peak activity of the upper leg, although a significant difference was observed only for the largest AUC in half an hour for leg activity. Both variables have a large spread across both healthy controls and patients. For non-ambulatory patients, the peak activity and the largest AUC of the arms were decreased to the same extent, but this decrease was most significant for the peak activity. All peak intensity parameters correlated moderately to gross motor function.

For this study, we selected the MOX-accelerometer, a device with opportunities to design a tailored data-analysis. It is known that measuring for longer periods decreased variability substantially in patients with cerebral palsy (CP) (Mitchell et al. 2015). However, we deliberately chose to measure physical activity only during the days in which the child was performing physical activity to their own desire and was not challenged with mental exercise (i.e. the weekend versus school week), since the aim of this study was to select domains of physical activity that deviated from the normal population and not to reliably quantify the amount of daily movement. We used 4–5 accelerometers instead of one, to be able to quantify movement patterns of the upper- and lower arm, the chest and the upper leg. This allowed us to draw conclusions about the orientation and the intensity of movements of these body parts, but is obviously less desirable from a patient perspective (Kirby et al. 2012).

Previously, Martens et al. (2014) showed that children with mitochondrial disease have a lower activity level compared to healthy controls and less time spent in moderate to vigorous activities, using a commercially available physical activity device. The resting percentage in the study by Martens et al. was lower compared to what we found, even in ambulatory patients only. This could be due to selection bias: Martens et al. studied ambulatory children without severe cognitive impairment and none of them had a genetically confirmed mitochondrial disease. Adults with mitochondrial disease also show lower habitual physical activity compared to matched controls (Apabhai et al. 2011). Interestingly, also the number of breaks in sedentary activity was reduced and longer periods of rest were observed. The resting percentage found in our study was comparable to the resting percentage in ambulatory normally weighted children with Down's-, William's- and Prader-Willi syndrome (Nordstrom et al. 2013) and patients with CP, when both patient groups were stratified based on their walking abilities (Gorter et al. 2012). Five young ambulatory boys (4–6 years) with Duchenne Muscular Dystrophy (DMD) (Jeannet et al. 2011), who were significantly younger than our study subjects, spent much less time resting, but had comparable levels of dynamic activity when compared to our ambulatory patients. In contrast to most previous studies, we also included non-ambulatory patients in our study. Measuring daily activity in

these patients is challenging, because accelerations elicited by the wheelchair are also measured by the other sensors. We found that the wheelchair accounted for about one fourth of the leg activity level in non-ambulatory children.

This study was performed on a relatively small and clinically heterogeneous group of patients but indicates abnormal domains of physical activity in patients with both high and limited abilities. Patients are compared to age- and sex-matched controls, who were measured in the same weekend to correct for weather conditions. Weaknesses include the substantial level of technical failures and the relatively short measurement time, which were caused by the use of the studied accelerometers and are not likely to play a major role in commercially available accelerometers. Other methodological issues inherent to measuring activity with accelerometers include: the lack of measurement of stable, sustained body positions and that the current data analysis is not able to differentiate between meaningful movements and aberrant movements, such as myoclonus or ataxia. Another weakness is that we included a heterogeneous population and that not all patients have a confirmed genetic mitochondrial disease: out of 17 patients, only 9 patients have a genetically confirmed "primary" mitochondrial disease. During analysis of this subgroup, we confirmed that also if only these patients were analysed, peak activity parameters had the highest magnitude and significance of difference. The activity parameters did not differ between patients with genetically confirmed primary mitochondrial disease and patients with biochemically confirmed mitochondrial dysfunction. Patients with genetically confirmed secondary mitochondrial disease did differ on three parameters, but these findings need to be interpreted carefully since all patients with genetically confirmed secondary mitochondrial disease were non-ambulatory. Since most patients were not able to reliably complete questionnaires regarding their level of fatigue, these were not included in this study.

This study provides insight into the domains of physical activity that are abnormal in children with mitochondrial disease. Since this approach is data-driven only, we are currently performing a more patient-centred approach in which the clinically relevant domains of movement are explored in close dialogue with patients and their families. Combining the results of both studies, we aim to select a simple, commercially available activity monitor, that provides insight in the clinically relevant aspects of daily physical activity of children with mitochondrial disease. This accelerometer should be validated in a larger and more homogeneous study population, with longer measurement period and standardization of family activities during the measurements, to provide representative activity patterns and reduce random variability. Validation should include at least the following aspects: acceptability, feasibility, test-retest reliability, including the influence of weather conditions and seasons, validity, especially in wheelchair bound patients, and responsivity.

In conclusion, accelerometry is a promising method to quantify the highly burdensome fatigue in children with mitochondrial disease in daily practice as well as natural history studies. The method is safe, feasible and well tolerated. Whether accelerometry is reliable and sensitive enough to detect changes during clinical trials needs to be studied in more detail.

Take Home Message

Children with mitochondrial disorders have lower peak activity rather than a lower amount activity compared to healthy peers.

Details of the Contributions of Individual Authors

Saskia Koene	Planning, supervising and reporting of study
Ilse Dirks	Execution and planning of study, critical review manuscript
Esmee van Mierlo	Execution of study, critical review manuscript
Pascal de Vries	Analysis of data, critical review manuscript
Anjo J. W. M. Janssen	Performance physiotherapeutic tests, critical review manuscript
Jan A. M. Smeitink	Supervision of study, critical review manuscript
Arjen Bergsma	Data analysis, critical review manuscript
Hans Essers	Data analysis, critical review manuscript
Kenneth Meijer	Critical review manuscript
Imelda J. M. de Groot	Supervision of planning of study, critical review manuscript

Name of One Author Who Serves as Guarantor

Saskia Koene.

A Competing Interest Statement

Imelda J. M. de Groot is consultant for Biomarin, PTC, Treat-NMD. She has collaborations in scientific projects on the development of technical aids (arm and hand exoskeletons) with Focal Meditech, Hankamp, BAAT Medical products, Summit, Laevo, Xsens, Maastricht Instruments. She has no financial interest in these firms. Jan Smeitink is the founding CEO of Khondrion BV.

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Details of Ethics Approval/A Patient Consent Statement

This study was approved by the regional Medical Research Ethics Committee (MREC NL50560.091.14). In accordance with the Helsinki agreement, written informed consent was obtained from participant's legal guardian and, where indicated, the participant.

Approval from the Institutional Committee for Care and Use of Laboratory Animals

Not indicated.

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RESEARCH REPORT

Preliminary Results on Long-Term Potentiation-Like Cortical Plasticity and Cholinergic Dysfunction After Miglustat Treatment in Niemann-Pick Disease Type C

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Abstract Niemann-Pick disease type C (NPC) is a rare autosomal recessive lysosomal storage disorder, which manifests clinically with a wide range of neurological signs and symptoms. We assessed multiple neurological, neuropsychological and neurophysiological biomarkers using a transcranial magnetic stimulation (TMS) multi-paradigm approach in two patients with NPC carrying a homozygous mutation in the *NPC1* gene, and in two heterozygous family members.

We assessed short-interval intracortical inhibition (SICI), intracortical facilitation (ICF), long-interval intracortical inhibition (LICI), short-latency afferent inhibition (SAI) and long-term potentiation (LTP)-like cortical plasticity with a paired associative stimulation (PAS) protocol.

Baseline SAI and LTP-like plasticity were impaired in both patients with NPC and in the symptomatic heterozygous *NPC1* gene mutation carrier. Only a limited decrease in SICI and ICF was observed, while LICI was within normal range in all subjects at baseline. After 12 months of treatment with miglustat, a considerable improvement in SAI and LTP-like plasticity was observed in both patients with NPC. In conclusion, these biomarkers could help to confirm the diagnosis of NPC, and may give an indication of prognostic outcomes in pharmacological trials.

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Introduction

Niemann-Pick disease type C (NPC) is a rare genetic disorder, which results in a wide range of neurological, visceral and psychiatric signs and symptoms, including cerebellar ataxia, cataplexy, seizures and dementia (Vanier 2010). This autosomal recessive lysosomal storage disorder (LSD) is caused by mutations of either the *NPC1* gene (in 95% of families) (Carstea et al. 1997) or the *NPC2* gene (Naureckiene et al. 2000). The incidence of NPC is estimated to be one in every 100,000 live births, although the late-onset phenotypes or variant forms may have a much higher incidence, ranging from 1:19,000 to 1:36,000 (Wassif et al. 2015).

Although the exact functions of the proteins derived from the *NPC1* and *NPC2* genes are still to be fully elucidated, the disease is characterized by clear abnormalities in intracellular transport of endocytosed cholesterol with sequestration of unesterified cholesterol in lysosomes and late endosomes, resulting in downstream effects on cholesterol homeostasis (Pentchev et al. 1985; Liscum et al. 1989). NPC is also characterized by the accumulation of beta amyloid (Yamazaki et al. 2001) and neurofibrillary tangles (Love et al. 1995), which are immunologically and ultrastructurally

similar to those seen in Alzheimer's disease (AD) (Auer et al. 1995). The similarities between NPC and AD extend to the cholesterol pathway, which has been repeatedly implicated in the pathogenesis of AD (Liu et al. 2013). This commonality between the two conditions is substantiated by observations that cholesterol levels can affect the processing of amyloid precursor protein (Bodovitz and Klein 1996) and accumulation of beta amyloid peptides (Simons et al. 1998), and is further supported by the notion that the ε4 isoform of apolipoprotein E promotes disease progression in both disorders (Corder et al. 1993; Fu et al. 2012).

Recently, abnormalities in cortical plasticity have been demonstrated in AD using non-invasive brain stimulation techniques, including transcranial magnetic stimulation (TMS) (Di Lorenzo et al. 2016). These studies were based on the strong evidence obtained from electrophysiological recordings in animal models of AD, which highlighted an impairment in hippocampal long-term potentiation (LTP) mediated by beta amyloid peptides and tau proteins (Palop and Mucke 2010). *Npc1*-mutant mice also show impaired hippocampal synaptic plasticity (D'Arcangelo et al. 2016), although in vivo evidence for impairment in LTP-like cortical plasticity in patients with NPC is currently lacking. In the most recent of these studies, miglustat (Zavesca®, Actelion Pharmaceuticals, Allschwil, Switzerland), a small iminosugar molecule that reversibly inhibits glycosphingolipid synthesis, was shown to rescue deficits in synaptic plasticity in *Npc1*-mutant mice (D'Arcangelo et al. 2016). Furthermore, an impairment in short-latency afferent inhibition (SAI), a measurement of sensorimotor integration obtained from TMS, has recently been observed in NPC, further supporting the link between NPC and AD (Manganelli et al. 2014). SAI is thought to be partially mediated by central cholinergic transmission; the restoration of SAI with the use of acetylcholinesterase inhibitors supports this hypothesis, and seems to correlate with the response to treatment (Di Lazzaro et al. 2005).

The aim of this study was to evaluate specific neurophysiological parameters, including SAI and LTP-like cortical plasticity, as well as short-interval intracortical inhibition (SICI), intracortical facilitation (ICF) and long-interval intracortical inhibition (LICI), in two previously described monozygotic twins homozygous for *NPC1* mutations (Benussi et al. 2015a), compared with those in two heterozygous family members and 18 healthy controls. SICI, ICF, LICI and SAI are TMS paired-pulse protocols which reflect specific intracortical circuits; SICI has been shown to reflect intracortical post-synaptic inhibition mediated by $GABA_A$ receptors, ICF to reflect excitatory glutamatergic circuits while LICI to be mediated by $GABA_B$ receptors (Benussi et al. 2015b).

Subjects heterozygous for *NPC1* mutations were evaluated because these individuals may exhibit neurophysiological manifestations of the signs and/or symptoms of NPC at a subclinical level (Josephs et al. 2004; Kluenemann et al. 2013). We further aimed to assess the effects of 12 months of treatment with miglustat on neurophysiological parameters in the two patients with overt NPC.

Materials and Methods

Subjects

Two monozygotic twins bearing a homozygous pathogenic mutation of *NPC1* (Patient 1 [P1] and Patient 2 [P2]) participated in the present study (Benussi et al. 2015a). One brother (Patient 3 [P3]) and the mother of the twins (Patient 4 [P4]), who were heterozygous for the *NPC1* mutation, as well as 18 healthy controls, were also included. P1 and P2 were evaluated before and after 12 months of treatment with miglustat.

Each subject underwent an extensive clinical work-up and neurological examination, as well as eye movement assessment (EyeSeeCam video-oculography [VOG] system, with the camera placed over the left eye).

Transcranial Magnetic Stimulation

TMS was performed with a figure-of-eight coil (each loop was 70 mm in diameter) connected to a Magstim BiStim^2 system (Magstim Company, Oxford, UK). Motor evoked potentials (MEPs) were recorded from the right first dorsal interosseous muscle (FDI) via surface Ag/AgCl electrodes placed in a belly-tendon montage and recorded using a BIOPAC MP150 electromyograph (BIOPAC Systems Inc., Santa Barbara, CA, USA).

rMT was defined as the minimal stimulus intensity needed to produce MEPs with an amplitude of at least 50 μV in 5 out of 10 consecutive trials during complete muscle relaxation. This was controlled by visually checking the absence of electromyography (EMG) activity at high-gain amplification.

SICI-ICF, LICI and SAI were studied at rest via a paired-pulse paradigm, delivered in a conditioning-test design, as previously reported (Benussi et al. 2016). For SICI-ICF, the conditioning stimulus was set at an intensity of 70% of the rMT, and the test stimulus was adjusted to evoke an MEP approximately 1 mV peak-to-peak in the relaxed FDI. Inter-stimulus intervals (ISIs) between the conditioning stimulus and the test stimulus of 1, 2, 3 and 5 ms were employed to investigate SICI, and ISIs of 7, 10 and 15 ms were used to investigate ICF.

LICI was elicited by applying two suprathreshold stimuli at long ISIs (50, 100 and 150 ms), with the conditioning stimulus set at 130% of the rMT preceding the test

stimulus, adjusted to evoke an MEP of approximately 1 mV peak-to-peak.

For SAI, the conditioning stimuli were single 200 μs-pulses of electrical stimulation applied to the right median nerve at the wrist through bipolar electrodes (cathode proximal). The intensity of the conditioning stimulus was set at just over the threshold for evoking a visible twitch of the thenar muscles, and the test stimulus was adjusted to evoke an MEP of approximately 1 mV peak-to-peak. ISIs were determined relative to the N20 component of the somatosensory evoked potential induced by stimulation of the right median nerve. The conditioning stimulus preceded the test stimulus by ISIs of −4, 0, +4 and +8 ms.

In all participants, 20 stimuli were delivered in a pseudorandomized sequence for each ISI, and for the test stimulus alone, for all stimulation paradigms (SICI-ICF, LICI and SAI). The amplitude of the conditioning MEPs was expressed as a ratio of the mean unconditioned response. The inter-trial interval was set at 5 s (±10%).

LTP-like effects were probed using a paired associative stimulation (PAS) protocol. PAS consisted of 200 electrical stimuli to the median nerve, paired with TMS stimuli over the contralateral motor cortex given at 0.25 Hz. The median nerve was stimulated at the wrist with standard bar electrodes (cathode proximally); pulses were constant current square waves with a width of 200 μs at an intensity of 300% of the perceptual threshold. Each TMS stimulus was adjusted to 1 mV MEP intensity and was preceded by the electrical conditioning stimulus at an ISI of 25 ms. Using an average of 30 responses, the mean MEP amplitude was determined at baseline and at 0, 10, 20 and 30 min after PAS from the right abductor pollicis brevis (APB) muscle, using the same stimulator output at all time points.

Complete muscle relaxation was monitored by audiovisual feedback throughout the experiment, where appropriate. If the quality of the data was degraded by movement of the patient, the protocol was recommenced and the initial data discarded.

Results

Two homozygous and two heterozygous *NPC1* mutation carriers, as well as 18 healthy controls were included in the study. Demographic and clinical characteristic for included patients are reported in Table 1.

Patient 1

P1 was a 25-year-old male monozygotic twin with moderate neurological impairment, including ataxic gait, limb dysmetria and supranuclear vertical gaze ophthalmoparesis, associated with splenomegaly from birth. Neuropsychological evaluation revealed global cognitive deficits, in particular in short- and long-term verbal memory, working memory and executive functions. Assessment of eye movements, using the EyeSeeCam VOG system over the left eye, revealed very slow and small vertical saccades (downward more than upward), mildly slowed horizontal saccades, and low-gain smooth pursuit, especially in the vertical plane. Molecular analysis revealed a homozygous c.2662 C>T (p.P888S) mutation in the *NPC1* gene (Benussi et al. 2015a).

At baseline, before initiating treatment with miglustat, neurophysiological analysis with TMS revealed a clear impairment in SAI (mean 87.4% at ISI 0 and +4) and LTP-like plasticity (mean 116.1% after PAS protocol) compared with healthy controls. Furthermore, a limited impairment in SICI (mean 55.1% at ISI 1, 2, 3 and 5 ms) and ICF (mean 124.4% at ISI 7, 10 and 15 ms) was observed, while LICI (mean 31.4% at ISI 50, 100 and 150 ms) was similar to healthy controls (see Table 1 and Fig. 1).

Patient 2

P2 was the monozygotic twin of P1. Unlike his twin brother, he presented with only mild neurological and neuropsychological impairment, as well as splenomegaly. VOG revealed moderately slowed downward saccades, whereas upward saccades were minimally slowed, and horizontal saccades were normal, as was vertical and horizontal smooth pursuit.

As with P1, a homozygous c.2662 C>T (p.P888S) mutation in the *NPC1* gene was detected (Benussi et al. 2015a).

Neurophysiological analysis at baseline revealed a clear impairment in SAI (mean 81.4%) and LTP-like plasticity (mean 133.4%) compared with healthy controls but to a lesser extent than was observed in P1. SICI (mean 43.5%) and LICI (mean 27.0%) were within normal range, while ICF (mean 126.8%) results were similar to those of P1 (see Table 1 and Fig. 1).

Patient 3

P3 was the 34-year-old non-twin brother of P1 and P2. He was asymptomatic and the neurological examination was unremarkable. Eye movement assessment with VOG was also unremarkable. Molecular analysis revealed a heterozygous c.2662 C>T (p.P888S) mutation in the *NPC1* gene.

Neurophysiological analysis revealed only a very mild impairment in SAI (mean 60.9%) and LTP-like cortical plasticity (mean 146.9%) compared with healthy controls, while SICI (mean 37.2%), ICF (mean 145.8%) and LICI (mean 28.9%) were within normal range (see Table 1 and Fig. 1).

Table 1 Demographic and neurophysiological characteristics of enrolled patients

	HC ($n = 18$)	P1	P2	P3	P4
Age (years)	39.2 ± 15.7 (52.6)	25	25	34	57
rMT (%)	45.5 ± 7.1	40.0	47.0	44.0	45.0
Mean SICI 1, 2, 3, 5 ms (%)	33.6 ± 3.6	55.1	43.5	37.2	40.2
Mean ICF 7, 10, 15 ms (%)	152.0 ± 6.4	124.4	126.8	145.8	118.8
Mean LICI 50, 100, 150 ms (%)	26.5 ± 3.6	31.4	27.0	28.9	30.3
Mean SAI 0, 4 ms (% at baseline)	49.5 ± 23.7	87.4	81.4	60.9	75.5
Mean SAI 0, 4 ms (% after treatment)	–	70.8	60.3	–	–
LTP-like plasticity (% at baseline)	161.9 ± 32.3	116.1	133.4	146.9	127.5
LTP-like plasticity (% after treatment)	–	136.8	150.1	–	–

Demographic, clinical and neurophysiological characteristics are expressed as mean ± standard deviation, and (range) for age; rMT is expressed as percentage of the maximum stimulator output; SICI, ICF, LICI and SAI are expressed as percentage of mean MEP amplitude related to the control MEP; LTP-like plasticity is expressed as change (%) in mean MEP amplitude at 0, 10, 20 and 30 min after PAS protocol
HC healthy controls, *ICF* intracortical facilitation, *LICI* long-interval intracortical inhibition, *LTP* long-term potentiation, *MEP* motor evoked potential, *rMT* resting motor threshold, *SAI* short-latency afferent inhibition, *SICI* short-interval intracortical inhibition

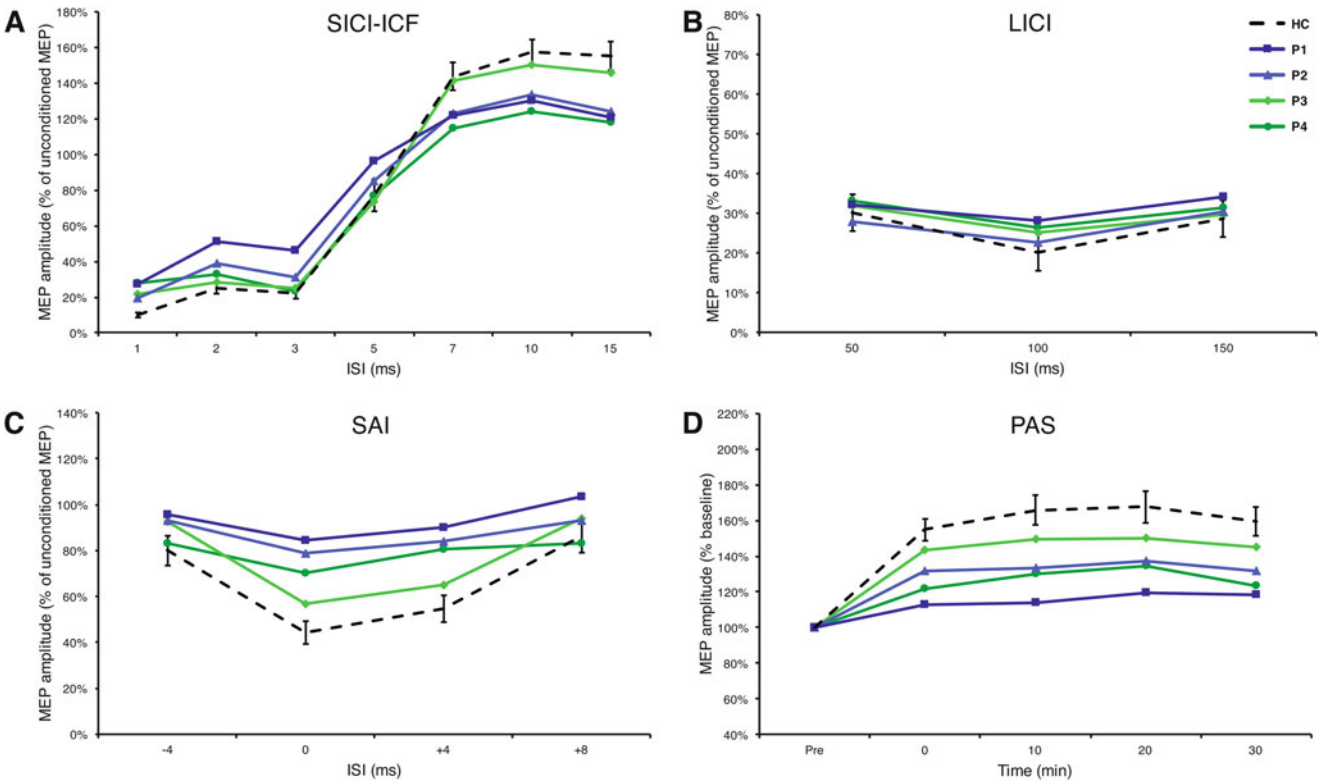

Fig. 1 SICI-ICF, LICI, SAI and LTP-like cortical plasticity in patients and HCs. (**a**) SICI-ICF, (**b**) LICI and (**c**) SAI at different ISIs. Data are plotted as a ratio to the unconditioned MEP amplitude. (**d**) PAS effect on corticospinal excitability, as measured by change in 1 mV MEP amplitude at various time points. Error bars represent standard errors. *HC* healthy controls, *ICF* intracortical facilitation, *ISI* inter-stimulus interval, *LICI* long-interval intracortical inhibition, *LTP* long-term potentiation, *MEP* motor evoked potential, *P1/2/3/4* Patient 1/2/3/4, *PAS* paired associative stimulation, *Pre* pre-PAS baseline, *SAI* short-latency afferent inhibition, *SICI* short-interval intracortical inhibition

Patient 4

P4 was the 57-year-old mother of P1, P2 and P3, and was initially evaluated at the Neurology ward of the University of Brescia Hospital (Brescia, Italy) for a resting tremor in her right arm. She presented with a history of mild spleen enlargement (bipolar diameter of 12 cm) for which numerous investigations were performed; all were inconclusive.

Neurological examination revealed an asymmetric extrapyramidal syndrome with a moderate rest tremor, bradykinesia and increased rigidity in the right upper limb, with a Unified Parkinson's Disease Rating Scale – part III (UPDRS-III) score of 15/108. No deficits in cranial nerves were detected and ocular movements were within normal range, although a slight dysmetria in ocular movements was detected with VOG. The patient did not report any Parkinsonian non-motor symptoms, such as hyposmia, constipation, orthostatic hypotension or rapid eye movement sleep behaviour disorder.

Routine laboratory evaluations, including free iron, ferritin, transferrin, free copper and ceruloplasmin levels, were within normal range.

Brain magnetic resonance imaging results were unremarkable, while ^{123}I-FP-CIT (DaTScan) single-photon emission computed tomography imaging revealed a symmetric decreased uptake in the striatum, with a greater impairment in the putamen.

Treatment with levodopa (levodopa/carbidopa 100 mg/25 mg 3 times daily for 1 week) ameliorated extrapyramidal signs and symptoms, with a significant improvement in the UPDRS-III score (from 15/108 to 10/108).

Molecular analysis revealed a heterozygous c.2662 C>T (p.P888S) mutation in the *NPC1* gene, while mRNA analysis with reverse transcription polymerase chain reaction, and copy number analysis of the *NPC1* and *NPC2* genes with multiplex ligation-probe amplification, did not identify an impairment in mRNA processing or in the number of copies of the analysed genes, respectively. Filipin test showed accumulation of unesterified cholesterol in perinuclear vesicles consistent with a "variant phenotype". Plasmatic oxysterols were significantly increased: cholestane-3β-5α-6β-triol of 42.35 ng/ml (normal values <20.45 ng/ml) and 7-ketocholesterol 109.92 ng/ml (normal values <45.58 ng/ml).

Neurophysiological analysis revealed a clear impairment in ICF (mean 118.8%), SAI (mean 75.5%) and LTP-like cortical plasticity (mean 127.5%) compared with healthy controls, while SICI (mean 40.2%) and LICI (mean 30.3%) were within normal range (see Table 1 and Fig. 1).

Effect of Miglustat Treatment on Neurophysiological Parameters

After baseline, neurological, neuropsychological and neurophysiological evaluation, P1 and P2 were initiated on miglustat treatment and were re-evaluated after 12 months. No notable side effects were reported except for gastrointestinal disturbances and diarrhea in P1.

Both subjects reported an improvement in neurological symptoms; in particular, P1 regained the ability to climb stairs unassisted and his gait visibly improved. P1 also reported a decrease in recurring intrusive thoughts that caused him significant distress and anxiety, which, in the past, he had tried to suppress with repetitive motor behaviors.

On neurological examination, clear improvement in dysarthria, ataxia, gait speed and balance, limb dysmetria, postural tremor and dysdiadochokinesia was observed in P1, while P2 displayed only a mild supranuclear vertical gaze ophthalmoparesis, only on downgaze, similar to pre-treatment conditions. Neuropsychological evaluation revealed a definite improvement in executive functions, calculation ability, and semantic and phonemic fluency in P1, while P2 showed an improvement in semantic and phonemic fluency and visuospatial abilities (see Table 2).

Neurophysiological evaluation after 12 months of treatment with miglustat revealed a considerable improvement in SAI and LTP-like plasticity in both P1 and P2 compared with baseline (see Table 1 and Fig. 2; for a scatterplot distribution of single patients regarding SAI and PAS see Supplementary Figure). No significant differences were observed in RMT and 1 mv MEP thresholds at 12 months.

Discussion

This study provides novel in vivo evidence of a substantial impairment in LTP-like cortical plasticity in symptomatic patients bearing homozygous mutations in the *NPC1* gene, and to a limited extent in heterozygous *NPC1* gene mutation carriers, when compared with healthy controls. Our findings also confirm and extend a previous report of impaired SAI in patients with NPC (Manganelli et al. 2014).

Numerous studies have highlighted impairment in LTP-like cortical plasticity in neurodegenerative disorders, including in AD and frontotemporal dementia (Di Lorenzo et al. 2016; Benussi et al. 2016). Using a PAS protocol that has previously been shown to induce LTP-like effects in healthy subjects (Wolters 2003), our study demonstrates

Table 2 Neuropsychological assessment after 12 months of miglustat treatment

Test	P1 Before treatment[a]	P1 Follow-up	P2 Before treatment[a]	P2 Follow-up
MMSE	**22**	27	30	30
MOCA	**21**	25	**25**	**25**
Babcock story recall test	**4**	**4**	10.5	10.5
Forward digit span	**3.75**	5.75	4.25	4.25
Backward digit span	3.42	**2.42**	3.42	3.42
Semantic fluency	**20**	33	30	36
Phonemic fluency	**11**	17	17	25
Clock drawing	10	10	10	10
Rey figure copy	29.5	30.5	**22**	29.5
Rey figure recall	10.75	13.75	11.5	16.5
Trial making A (s)	**116**	**104**	52	47
Trial making B (s)	**Np**	261	163	160
Raven's progressive matrices	33.75	32	35	35

Pathological scores are in boldface according to Italian normative data
MMSE Mini-Mental State Examination, *MOCA* Montreal Cognitive Assessment, *Np* not able to perform, *P1/2* Patient 1/2
[a] Data previously published (Benussi et al. 2015a)

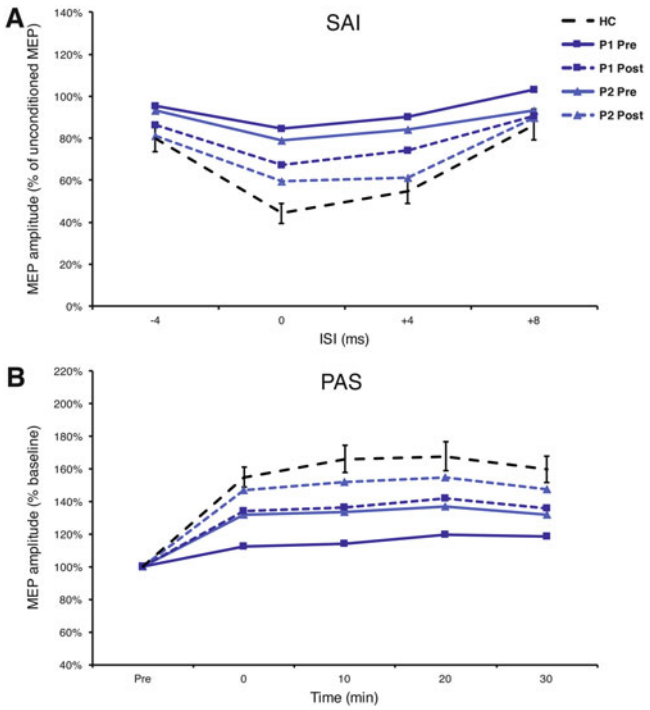

Fig. 2 Effect of miglustat treatment on SAI and LTP-like cortical plasticity. (**a**) SAI at different ISIs in P1 and P2 at baseline and after 12 months of miglustat treatment, and in HCs. Data are plotted as a ratio to the unconditioned MEP amplitude. (**b**) PAS effect on corticospinal excitability, as measured by change in 1 mV MEP amplitude at various time points, at baseline and after 12 months of miglustat treatment. Error bars represent standard errors. *HC* healthy controls, *ISI* inter-stimulus interval, *MEP* motor evoked potential, *P1/2* Patient 1/2, *PAS* paired associative stimulation, *SAI* short-latency afferent inhibition

lower motor cortex excitability after PAS in patients with mutations of the *NPC1* gene compared with healthy controls. The impairment in LTP-like synaptic plasticity observed in patients with NPC is supported by the notion that hippocampal LTP is impaired in *Npc1*-mutant mice (D'Arcangelo et al. 2016). On the other hand, only modest

alterations in SICI and ICF were observed in P1, P2 and P4. SICI is thought to reflect short-lasting postsynaptic inhibition, mediated by the γ-aminobutyric acid $(GABA)_A$ receptor at the local interneuron level, while ICF is thought to represent net facilitation of neuronal excitability, most likely mediated by glutamatergic *N*-methyl-D-aspartate (NMDA) receptors (Ziemann et al. 2015). Our findings therefore suggest mild impairments in $GABA_A$ receptor-mediated and NMDA receptor-mediated neurotransmission in patients with *NPC1* gene mutations. Although we did not identify a significant difference in LICI, which is thought to be mediated by $GABA_B$ receptors, generalized axonal dystrophy in GABAergic cell populations is a common finding in neuronal storage disorders (Gibson et al. 2008). Furthermore, defects in synaptic transmission at both GABAergic and glutamatergic synapses have been identified in mouse models of NPC, which may result, at least in part, from an impairment of synaptic vesicle trafficking (Byun et al. 2006). Together, these findings suggest that deficits in LTP-like plasticity and SICI-ICF may be early indicators of NPC, and could provide a potential explanation for key features of the disease, such as dystonia and seizures.

The clear impairments in SAI in this study seem to correlate with disease severity and with mutation status. Manganelli et al. (2014) recently reported similar impairments in SAI in three patients with NPC, providing evidence to support the hypothesis that cholinergic alterations may play a role in cognitive impairment in patients with this disease. This hypothesis is further substantiated by reports that patients with NPC suffer from grey matter atrophy in subcortical nuclei that are implicated in the central cholinergic pathway, including the thalamus, hippocampus and basal forebrain (Walterfang et al. 2011, 2013).

Another important finding in this study was the modest impairment in SAI and LTP-like plasticity in heterozygous family members, particularly in P4, who was symptomatic for Parkinson's disease. Individuals heterozygous for *NPC1* mutations are generally considered to be clinically unaffected, but it is uncertain whether *NPC1* haploinsufficiency can predispose *NPC1* mutation heterozygotes to intermediate and potentially subclinical NPC phenotypes. Biochemically, *NPC1* gene mutation carriers have an intermediate phenotype in terms of lipid regulation and metabolism. It has been reported that cultured skin fibroblasts from individuals heterozygous for *NPC1* gene mutations have an intermediate rate of cholesterol esterification, production of cholesteryl esters, and unesterified cholesterol storage level compared with cells from healthy individuals and those homozygous for *NPC1* gene mutations (Hung et al. 2016). Furthermore, in human *NPC1* gene mutation carriers, the concentrations of plasma oxysterols, 7-ketocholesterol and cholestane-3β-5α-6β-triol are significantly elevated compared with those in healthy individuals (Porter et al. 2010). Central nervous system abnormalities in heterozygous *Npc1*-mutant mice are suggestive of neurodegeneration with a significant loss of Purkinje cells, and increase in brain cholesterol and hyperphosphorylated tau (Mattsson et al. 2012). Consistent with these animal studies, case reports have described Parkinsonism or Lewy body pathology in *NPC1* gene mutation carriers (Josephs et al. 2004; Kluenemann et al. 2013; Chiba et al. 2014). Our results suggest that the notion that heterozygous *NPC1* gene mutation carriers are clinically unaffected may not always be accurate, and therefore, warrants further investigation. The case here described with a parkinsonian syndrome raises the possibility that NPC could be another storage disease in which mutations are a risk factor for PD. Potential mechanisms similar to the GBA association have been suggested, including the partial loss of enzyme function altering cytoplasmic lipid homeostasis or overwhelming the ubiquitin-proteasome pathway (Kluenemann et al. 2013). However, because PD is a relatively common neurologic disease it is possible that such associations are simply a rare coincidence.

The second aim of the present work was to evaluate the neurological, neuropsychological and neurophysiological status of patients after 12 months of treatment with miglustat. Whether or not miglustat can be beneficial for *NPC1* mutation carriers with a biochemical profile compatible with an LSD remains to be confirmed. After 12 months of treatment with miglustat, we observed a clear improvement in dysarthria, gait ataxia, limb dysmetria and dysdiadochokinesia in P1, while P2 remained stable upon neurological examination. Cognitive assessment revealed a clear improvement in calculation ability, semantic and phonemic fluency, and visuospatial abilities. More interestingly, along with clinical improvement, cholinergic impairment seemed to be reduced, and we observed an increase in LTP-like cortical plasticity after treatment with miglustat in both P1 and P2.

We acknowledge that the number of subjects included in this study was very small, owing to the fact that NPC is a rare disorder, and this raises the important issue of how we can generalize these results to other patients with NPC. Furthermore, differences in patients compared to controls could also be accountable to other unknown shared family genetics. Nevertheless, our findings provide novel evidence of reduced LTP-like cortical plasticity in patients with NPC, which seemed to be rescued by treatment with miglustat.

We conclude that impaired SAI and LTP-like plasticity may represent biomarkers that could further support the diagnosis of NPC, and may give an indication of prognostic outcomes in clinical trials.

Author Contributions

Conception and design of the study: AB, AP and BB. Acquisition and analysis of data: AB, MSC, MC, VB, MT, ES, AD, AP and BB. Drafting the manuscript and figures: AB, ES, AP and BB.

Conflicts of Interest

AB, MSC, MC, VB, MT, ES, AD, AP, BB declare that they have no conflict of interest.

Ethical Standards

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.

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RESEARCH REPORT

Prevalence of Mucopolysaccharidosis Types I, II, and VI in the Pediatric and Adult Population with Carpal Tunnel Syndrome (CTS). Retrospective and Prospective Analysis of Patients Treated for CTS

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Abstract *Background*: We wanted to investigate whether the prevalence of mucopolysaccharidoses (MPS) I, II, and VI was higher than expected in a selected cohort of patients with carpal tunnel syndrome (CTS). CTS is a common finding in patients with MPS, and therefore we screened patients who had undergone surgery for CTS for undiagnosed MPS.

Patients and Methods: Patients who had been operated for CTS were found in databases from two hospitals. Furthermore, patients who had undergone surgery for CTS when under the age of 18 were retrieved from the National Patient Registry. All included patients had a filter paper blood spot sample taken that was subsequently analyzed enzymatically for MPS I, II, and VI.

Results: 425 patients were included. 402 patients tested negative in the first test. 23 had inconclusive result whereof 18 was negative in a second test. The remaining five patients had two inconclusive tests each and were referred for further examination at the Center for Inherited Metabolic Diseases where the diagnosis was excluded. Thus, all included patients were negative for both MPS I, II and VI.

Discussion/Conclusion: Though our sample size is relatively small, results indicate that MPS is not prevalent in a cohort of adult patients with monosymptomatic CTS, and that screening is not indicated in this setting.

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Introduction

Mucopolysaccharidoses (MPS) are a group of inherited diseases caused by deficiencies of lysosomal enzymes, causing progressive accumulation of glycosaminoglycans in the lysosomes and thereby cellular damage. Accumulation develops in all tissues and organs resulting in progressive multiorgan involvement. (Beck et al. 2007) MPS diseases are clinically characterized by a broad phenotypic continuum. Some patients present early with severe manifestations and other later with attenuated symptoms. These latter patients may be difficult to diagnose, partly because their symptoms may be mistaken for signs of more common diseases. MPS is not part of the newborn screening program in most countries including Denmark, and together with the difficult diagnosis this makes the presence of undiagnosed MPS patients in the population probable. Considering this, we investigated whether the prevalence of MPS I, II, and VI is higher than expected in a selected cohort of patients with carpal tunnel syndrome. In Denmark the observed prevalence of all MPS disorders is 6.03 in 1,000,000 inhabitants. The observed prevalence in Denmark of MPS I, MPS II, and MPS VI is 0.74, 0.91, and 0.37 in 1,000,000 inhabitants, respectively (Malm et al. 2008).

CTS is caused by impingement of the median nerve in the carpal tunnel of the wrist. The symptoms include paraesthesia and pain (Katz and Simmons 2002). The symptoms can be reversible when treated sufficiently early with surgical decompression of the median nerve. There is no study that estimates the incidence of CTS in the Danish population. Sweden has a population comparable to the Danish and in 2011 the annual incidence there of CTS in women and men was 428 and 182/100,000 adults, respectively, peaking among women aged 45–54 years (Atroshi et al. 2011). CTS in children and adolescents is rare and often results from trauma. The most common causes of non-traumatic CTS in this age group are mucopolysaccharidoses and mucolipidoses (al-Qattan et al. 1996). CTS is a common symptom in patients with MPS, but diagnosis may be difficult and late because of the limited symptoms being reported by the patients. Previous studies have shown a prevalence of CTS up to 67% in patients with Scheie's syndrome (Thomas et al. 2010), 18% in patients with MPS II (Mendelsohn et al. 2010), and in 6 out of 7 patients with MPS VI (Haddad et al. 1997).

In order to resolve whether focused MPS screening in a selected population with CTS is worthwhile, we decided to screen patients who had had surgery for CTS for MPS.

Patients and Methods

This study was designed with a retrospective and a prospective part. The study was approved by the health research ethics committee (journal number H-2-2011-096) in Copenhagen, Denmark.

In the prospective part, adult patients referred for CTS operation at Nørmark Private Hospital (NPH), Denmark, from 2011 to 2015 were included. If the surgeon found surgical indication at the first consultation, the patient was informed about the project. If the patient consented, they were included in the study, and a filter paper blood sample for MPS testing was taken on the day of the operation.

The patients in the retrospective part already had undergone operation for CTS. One group consisted of patients, who were treated at NPH in the period from 1997 to 2011. A second group consisted of similar patients operated at Køge hospital, Denmark. A third group consisted of patients operated for CTS at an age below 18 years from 2002 to 2012 in Denmark. These patients were retrieved from the National Patient Registry (NPR) using as search criteria the procedure code "decompression of the median nerve."

All patients who met the inclusion and exclusion criteria (had been or was about to be operated for CTS and were below age 60 years when operated) were sent a letter with information and an invitation to participate. If they did not respond, they were either contacted by phone or had a new letter send. If a response was not received after this second approach, no further attempt at contacting the patient was done.

All patients who consented were included and had a filter paper blood spot sample taken. The tests were sent by courier post at room temperature to the Metabolic Laboratory at Hamburg University Medical Center and analyzed enzymatically for activities of α-iduronidase (IDUA), iduronate-2-sulfatase (IDS), and arylsulfatase B (ARSB), the enzymes deficient in mucopolysaccharidosis (MPS) type I, II, and VI, respectively (Cobos et al. 2014).

If the tests had an inconclusive result, a new filter paper blood spot sample was taken. If the test was positive or if the second test was inconclusive, the patient was referred to the Centre for Inherited Metabolic Diseases at Rigshospitalet (RH), Copenhagen for confirmatory testing of MPS I, MPS II, or MPS VI using clinical examination (AML) and determination of urine glucosaminoglycans and leucocyte enzyme activities of the respective enzymes.

Results

In the prospective study, 114 patients were included and in the retrospective 311. All groups had similar demographics apart from the NPR patients who were younger (Table 1).

In the prospective study 115 patients were asked to participate only 1 declined, 78 (68%) of the participants were women and the average age at first test was 47 [20–59].

42% of invited patients from NPH, 30% from Køge Hospital, and 10% from NPR consented to and completed the study (Table 1).

All patients included were negative for all three diseases tested, MPS I, MPS II, and MPS VI. Five patients had two inconclusive tests but all were negative for MPS after further examinations at RH including clinical examination and determination of urine glycosaminoglycans and leucocyte enzyme activities. 95% of the patients had MPS ruled out by the first test (Table 2). In all we did 448 blood spot tests and 28 (6%) were inconclusive. In 19 cases the tests were inconclusive because the activity of arylsulfatase B was below its reference range and could be in agreement with MPS VI, in 4 cases the activity of alpha-iduronidase was below its reference range and could be in agreement with MPS I, in 1 case the activity of iduronate-2-sulfatase was below its reference range and could be in agreement with MPS II, in 3 cases the enzyme activities was in general below their respective reference ranges and in 1 case the activity of both alfa-iduronidase and arylsulfatase B was below their reference ranges probably due to preanalytical problems.

Table 1 Patients invited to participate in the retrospective study ($n = 4,897$)

	Included patients [*n* (%)]						
		Women [*n* (%)]	Average age at first test	Did not want to participate [*n* (%)]	Not possible to contact [*n* (%)]	Not operated for CTS [*n* (%)]	No response [*n* (%)]
Nørmark private hospital	281	227 (81%)	54 [26–73]	139	31	3	216
Køge Sygehus	10	8 (80%)	52 [38–61]	3	0	2	18
National patient registry	20	16 (80%)	25 [19–32]	23	11	40	100
Total	311 (35)			165 (18)	42 (5)	45 (5)	334 (37)

Table 2 Overview of test results

	Total number of included patients: 425		
	1st test negative	2nd test negative	Negative tests at RH
Retrospective study	294	13	4
Prospective study	108	5	1
Total	402	18	5

Discussion and Conclusion

CTS has a high prevalence in the general population, but patients with MPS are affected much more frequently. In this study we examined the clinical utility of screening for MPS I, II, and VI among patients who had undergone surgery for CTS as a high-risk population screening. We screened a total of 425 patients, including 20 who had undergone surgery when under the age of 18. All patients were negative for all tested MPS disorders. As judged by CTS and MPS single Danish prevalence as well as published data on MPS occurrence in patients with CTS and vice versa, we made a rough, overall estimate not to expect a frequency above 0.5% of MPS in patients with CTS. Thus, we had to screen around 800 patients with CTS to be able to find 1–4 patients. We had hoped to include more patients, but it proved difficult to make patients join especially in the retrospective study. It came to our knowledge that many of the patients from the NPR were not operated for CTS. The procedure code "decompression of the median nerve" was also used when patients had undergone surgery for wrist fractures and deep cut injuries in the wrist region among others. Therefore, they could not be included in the study. This probably also explains why 52% of the NPR patients did not respond to our letters. One of the patients from NPR was already diagnosed with MPS and was therefore not included. Furthermore, a number of patients were operated many years ago and may have forgotten about it. The patients were from all over Denmark and some of the patients found it difficult to come to Copenhagen. Some of the patients had the blood sample taken in their own home, but it was not possible in every case. Others may be true monosymptomatic cases, who may find it irrelevant to get tested. Only few patients were below age 18 years, among whom one child was already diagnosed with MPS. This is a weakness of the study in two ways: one is that most MPS disorders would be expected to present in childhood making our cohort less representative, though it should be kept in mind that our aim was to make conclusions about screening for the late onset attenuated forms of MPS; another is that we only found this one case with MPS, when more would be expected as judged from known operations for CTS among Danish MPS children. Thus, our search strategy or Danish national coding practice may be insufficient.

Previously published results on high-risk screening in populations of patients with symptoms compatible with MPS (Table 3) come from seven studies.

In one study they screened two groups of patients (Ly-Pen et al. 2010). One group with CTS under the age of 30 and another group consisting of children and young adults with joint stiffness, flexion contractures, claw hand, trigger fingers, thenar atrophy, poor hand function, and hand paresthesia. From a population of 600,000 they included 12 patients and found low α-L-iduronidase activity compatible with MPS I in the dried blood spot sample in two patients (Haddad et al. 1997).

Nathalie Van Meir and Luc De Smet did a review of 35 articles on pediatric CTS published from 1989 to 2005. They found 163 cases of pediatric CTS and 95 (58%) of the cases were due to MPS. The subtypes were not defined (Van Meir and De Smet 2005). Davis et al. found 13

Table 3 Overview of high-risk studies focusing on symptoms compatible with MPS

	Number of screened persons	MPS	MPS I	MPS II	MPS VI
Lukacs et al. (2014)	–	–	6.1%	3.3%	4.8%
Lukacs (2013)	602	–	8%	5%	4%
Coelho et al. (1997)	9,901	–	8.3%	–	–
Ly-Pen et al. (2010)	12	–	16.7%	–	–
Davis and Vedanarayanan (2014)	13	7.7%	–	–	–
Potulska-Chromik et al. (2014)	11	9.1%	–	–	–
Wang et al. (2006)	58	–	1.7%	–	–

patients with CTS in the age between 2 and 17 years and 1 (7.7%) of them had MPS (Davis and Vedanarayanan 2014). Potulska-Chromik found 1 (9.1%) patient with MPS in 11 children with CTS (Potulska-Chromik et al. 2014).

Wang et al. screened 58 patients under the age of 18 with cardiomyopathy and found one patient with MPS I (1.7%) (Wang et al. 2006).

To conclude, the results of this study cannot support screening for MPS among patients with monosymptomatic CTS, at least in adults though a bigger sample size would have strengthened the conclusion. In young patients with CTS, it cannot be excluded that screening for MPS could be beneficial because of our limited population in this age group. The abovementioned studies may indicate that children with CTS without prior trauma should be tested for MPS, but for a majority of the studies inclusion criteria are vaguely described making the results difficult for clinical use. To diagnose the majority of MPS patients, a more general approach like newborn screening for MPS is probably needed.

Take Home Message

Screening of MPS in adult patients with monosymptomatic CTS is not indicated.

Conflict of Interest

The study was supported by a research grant from Genzyme, Shire, and Biomarin. The authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsors.

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Nanna Kjaer has a received a grant from the sponsors.

Informed Consent

All procedures followed were done in accordance with the Helsinki Declaration of 1975 and were approved by the health research ethics committee (journal number H-2-2011-096) in Copenhagen, Denmark. Informed consent was obtained from all participants to be included in the study.

Animal Rights

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

Details of the Contributions of Individual Authors

MBN was involved in designing the study, contacted the patients, collected, and analyzed the data and drafted the manuscript.

AML was involved in designing the study, gave advice on data collection and analysis and critically revised the manuscript.

NK was involved in contacting the patients and collecting data.

All authors approved the final version of the manuscript.

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RESEARCH REPORT

Primary Carnitine Deficiency: Is Foetal Development Affected and Can Newborn Screening Be Improved?

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Abstract Primary carnitine deficiency (PCD) causes low levels of carnitine in patients potentially leading to metabolic and cardiac symptoms. Newborn screening for PCD is now routine in many countries by measuring carnitine levels in infants. In this study we report Apgar scores, length and weight in newborns with PCD and newborns born to mothers with PCD compared to controls. Furthermore we report how effective different screening algorithms have been to detect newborns with PCD in the Faroe Islands.

Results: Newborns with PCD and newborns born to mothers with PCD did not differ with regard to Apgar scores, length and weight compared to controls. Newborns with PCD and newborns born to mothers with PCD had significantly lower levels of free carnitine (fC0) than controls. Screening algorithms focusing only on fC0 had a high rate of detection of newborns with PCD. Sample collection 4–9 days after birth seems to result in a higher detection rate than the current 2–3 days.

Conclusion: The clinical status at birth in infants with PCD and infants born to mothers with PCD does not differ compared to control infants. Screening algorithms for PCD should focus on fC0, and blood samples should be taken when the maternal influence on fC0 has diminished.

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Introduction

The Faroe Islands are a small group of islands in the North Atlantic with a population of only 50,000 inhabitants of British and Norse descent (Jorgensen et al. 2002, 2004; Als et al. 2006). Primary carnitine deficiency (PCD, OMIM #212140) is a prevalent inherited disorder in the Faroe Islands with a prevalence of 1:300 compared to an estimated prevalence of 1:20,000–120,000 around the world (Koizumi et al. 1999; Wilcken et al. 2003; Magoulas and El-Hattab 2012; Rasmussen et al. 2014a, b). PCD (also known as, e.g., CTD and CUD) is an autosomal recessive disorder of fatty acid oxidation caused by a dysfunctional carnitine transporter (OCTN2) coded by the *SLC22A5* gene on chromosome 5 (Nezu et al. 1999). Patients continually lose carnitine in urine because of impaired renal reabsorption of filtered carnitine, which causes low levels of carnitine in patients (Scaglia et al. 1998; Longo et al. 2006; Rasmussen et al. 2015). Symptoms range from no symptoms to fatigue, cardiomyopathy and even fatal cardiac arrhythmia. A majority of the cases reported in the literature of symptomatic PCD patients have been among children (Tein 2003; Stanley 2004; Longo et al. 2006; Magoulas and El-Hattab 2012; Rasmussen et al. 2013). However, it is not known if newborns with PCD or newborns born to mothers with PCD are affected by abnormally low carnitine levels during foetal development

and at birth. Neonatal screening for PCD was routinely implemented in the Faroese newborn screening programme in 2009, but was preceded by a pilot screening study from 2002 to 2009 (Lund et al. 2012). The fear that there were undiagnosed PCD patients in the population led to the implementation of a nationwide screening programme in 2009 to uncover as many patients as possible (Rasmussen et al. 2014a). The screening programme had two parts, one being an offer to all inhabitants to have their blood carnitine levels determined and another being a retrospective newborn screening programme analysing all dried blood spot (DBS) samples from the newborn screening 1986–2001, which were available from the Danish Neonatal Screening Biobank (Rasmussen et al. 2014a). The extensive and ongoing screening programmes have led to the diagnosis of approximately 160 patients with PCD in the Faroe Islands, including children and adults.

In this study we investigate how newborns with PCD and newborns born to mothers with PCD compare to controls with regard to birth weight, length and Apgar scores. Furthermore we show how effective the newborn screening programmes have been detecting newborns with PCD and what role differences in screening algorithms and cut-off values have played.

Materials and Methods

Patients verified by genetic analysis to have PCD and children of mothers with PCD were included, as well as controls with normal free carnitine (fC0) levels matched by gender – all born between 1979 and 2011. Data on birth weight, length, Apgar scores, parity and gestational age were collected from birth charts filled out by midwives from 1979 to 2011 (Apgar 1953; Finster and Wood 2005).

fC0 levels from the included subjects measured unbutylated by tandem mass spectrometry in the population-based voluntary screening programme from 2009 and onwards were collected when available (Rasmussen et al. 2014a).

Prospective Newborn Screening

Carnitine levels in DBS samples from the prospective newborn screening were either analysed as described previously as part of a pilot project of extended newborn screening in babies from 2002 to 2008 or as part of the routine newborn screening from 2009 and onwards (Lund et al. 2012). Analyses were done by tandem mass spectrometry using an fC0 cut-off, which from 2002 to 2008 was adjusted when necessary according to medians and percentiles of measured carnitine levels and varied from 5.7 to 6.3 μmol/L, while being 5 μmol/L from 2009. DBS samples for newborn screening were obtained 4–9 days after birth and analysed butylated until 2009 when the recommended time to obtain a sample was changed to 2–3 days after birth and the analysis was done unbutylated, which remains unchanged until today (Lund et al. 2012).

During most of the pilot period 2002–2008 a lower cut-off value for acetyl-carnitine (C2) was also used alongside fC0 when evaluating the samples for PCD.

Retrospective Screening

DBS samples from Faroese newborns collected for neonatal screening are stored at minus 24°C in the Danish Newborn Screening Biobank (Norgaard-Pedersen and Simonsen 1999; Norgaard-Pedersen and Hougaard 2007). All DBS samples from children born in the Faroe Islands dating back to 1986 and onwards, which had not previously been analysed for carnitine levels, were also retrospectively analysed unbutylated in order to determine carnitine levels and reveal subjects born with abnormally low levels of carnitine. The stored DBS samples were analysed using tandem mass spectrometry as previously described, but the lower cut-off value for fC0 was set higher at 9 μmol/L to adjust for possible hydrolysis of acylcarnitines to free carnitine known to occur on storage of DBS (Lund et al. 2012; Strnadova et al. 2007).

Statistics

Data analysis was performed using IBM® SPSS® Statistics Version 19 (SPSS Inc., Chicago, IL, USA). All continuous variables were expressed as mean (standard deviation). One-way Anova was used to test for a significant difference in mean between the three groups – Tukey HSD test was used to test the difference between individual groups. Level of significance was set at $p < 0.05$.

Results

The study included 79 PCD patients, 56 subjects born to untreated mothers with PCD and 313 controls (Table 1). A total of 212 females and 236 males were included.

Length, Weight, Gestational Age and Parity

There was no significant difference in length ($p = 0.98$) and weight ($p = 0.38$) between the groups of newborns with PCD, newborns born to mothers with PCD and controls (Table 1). The groups neither differed with regard to gestational age (overall mean 39.7 weeks, $p = 0.49$) and parity (overall mean 1.38 live births, $p = 0.56$).

Table 1 Length, weight, Apgar score at 1 and 5 min, free carnitine (fC0) from the newborn screening programme and fC0 from the voluntary screening programme compared between patients with PCD, patients born to mothers with PCD and controls

		n	Length cm	Weight gram	Apgar 1 min	Apgar 5 min	fCO newborn scr. µmol/L	fCO voluntary scr. µmol/L
Patients w. PCD	Male	44	53.9 (2.4)	3,727 (611)	9.1 (1.2)	9.8 (0.5)	7.9 (5.6)	3.6 (1.9)
	Female	35	52.3 (2.7)	3,462 (589)	9.2 (1.1)	9.9 (0.3)	8.4 (7.0)	3.1 (1.1)
	Total	79	53.2 (2.6)	3,612 (611)	9.2 (1.2)	9.9 (0.4)	8.1 (6.2)	3.4 (1.6)
Patients born to mothers w. PCD	Male	26	54.4 (2.4)	3,977 (599)	8.8 (1.7)	9.6 (1.0)	11.4 (6.0)	14.0 (2.6)
	Female	30	52.4 (3.1)	3,526 (638)	9.0 (1.4)	9.9 (0.3)	11.8 (6.8)	12.3 (3.5)
	Total	56	53.3 (3.0)	3,726 (654)	8.9 (1.5)	9.7 (0.8)	11.6 (6.3)	13.0 (3.2)
Controls	Male	166	53.6 (2.1)	3,796 (515)	9.5 (0.9)	9.9 (0.3)	44.1 (22.3)	22.2 (5.8)
	Female	147	52.7 (2.1)	3,627 (517)	9.1 (1.7)	9.8 (0.9)	43.8 (19.6)	20.8 (5.4)
	Total	313	53.2 (2.1)	3,717 (522)	9.3 (1.3)	9.9 (0.7)	43.9 (21.0)	21.5 (5.6)

Mean (SD). Note that fC0 was determined in plasma in the newborn screening prg., while in whole blood in the voluntary screening prg.

Table 2 Three different newborn screening strategies during different time periods

Period	Years *n*	A PCD patients in total *n*	B PCD patients included in the newborn screening prog. *n*	C Patients found by newborn screening *n*	C in percentage of B %
1986–2001	16	35	27	23	85.2
2002–2008	7	19	8	2	25
2009–2014	6	9[a]	5[b]	5	100

[a] The total number of patients is lower than expected, likely due to a lack of detection of patients with other genotypes than c.95A>G/c.95A>G
[b] The four remaining newborns had received L-carnitine before the blood test

Apgar

There was no significant difference between the groups in Apgar scores at 1 ($p = 0.18$) and 5 min ($p = 0.48$) (Table 1). Apgar scores at 1 min ranged from 5 to 10 in newborns with PCD and newborns born to mothers with PCD compared to 1 to 10 in controls. Apgar scores at 5 min ranged from 8 to 10 in newborns with PCD, 7 to 10 in newborns born to mothers with PCD and 3 to 10 in controls.

Free Carnitine, fC0

There was a significant difference in mean fC0 between controls and the other groups in both the newborn screening and the voluntary screening programmes ($p < 0.01$) (Table 1). There was though no significant difference in mean fC0 between newborns with PCD and newborns born to mothers with PCD measured in the newborn screening programme ($p = 0.34$) – while fC0 measured in the same groups later in the voluntary screening programme differed significantly ($p < 0.01$) (Table 1).

1986–2001, Retrospective Screening

A total of 35 PCD patients were born in the period 1986–2001 – of whom 27 (77%) had neonatal DBS samples available in the biobank. Twenty-three of the 27 PCD patients were identified by the retrospective screening of the stored DBS (85.2%) (Table 2).

2002–2008, Pilot Project

Nineteen PCD patients were born during the pilot project from 2002 to 2008, but only two of the eight patients, who participated in the pilot extended newborn screening programme, were identified by the screening algorithm used at that time (25%) (Table 2).

2009–2014, Routine Newborn Screening

Nine PCD patients were diagnosed during 2009–2014, of whom all five patients, who had not already received L-carnitine supplementation before the DBS was taken, had fC0 below the cut-off level and were thus screen positive for PCD (Table 2).

Table 3 Comparing the three different newborn screening strategies with regards to detection of c.95A>G homozygous newborns

Period	Years *n*	A c.95A>G/C.95A>G patients in total *n*	B c.95A>G/c.95A>G patients included in the newborn screening prog. *n*	C c.95A>G/c.95A>G patients found by newborn screening *n*	C in percentage of B %
1986–2001	16	16	12	12	100
2002–2008	7	12	5	1	20
2009–2014	6	8	4[a]	4	100

[a] The four remaining newborns had received L-carnitine before the blood test

c.95A>G Homozygotes

The retrospective screening of the stored DBS samples identified all known 12 patients homozygous for the severe c.95A>G mutation born between 1986 and 2001 (Table 3). However the pilot newborn screening programme only uncovered one of five known c.95A>G homozygous patients born in the period 2002–2008 (20%) (Table 3). In the current screening programme all four patients homozygous for the c.95A>G mutation born between 2009 and 2014, who had not received L-carnitine prior to the blood test being taken, were revealed (100%) (Table 3).

Discussion

We have shown that length, weight and Apgar scores at birth are not significantly affected in newborns with PCD and newborns born to mothers with PCD compared to controls. Newborns with PCD and newborns born to mothers with PCD seem to have a normal foetal development and respond and function normally at birth, even though their carnitine levels are low. Symptoms of cardiomyopathy and metabolic decompensation seem to develop beyond neonatal age in those children with PCD, who develop symptoms (Stanley 2004; Magoulas and El-Hattab 2012).

Carnitine Levels in Newborns

Our study showed that mean fC0 levels in newborns with PCD and newborns (PCD carriers) born to mothers with PCD did not differ a few days after birth (Table 1) – but when measured later in the voluntary screening programme there was a significant difference. Our data indicate that maternal carnitine levels during pregnancy significantly reduce the level of fC0 in newborns, as was also demonstrated by Novak et al. in the 1980s, who showed a direct correlation between maternal and foetal carnitine levels (Novak et al. 1981). Pasquali and Longo also showed that carnitine levels measured within 2 days from birth in infants with PCD fell when measured again after 14 days while carnitine levels in infants born to mothers with PCD increased after 14 days (Pasquali and Longo 2013).

Screening Programmes

It is remarkable that the retrospective screening programme (1986–2001) identified 85.2% of all PCD patients and 100% of the patients homozygous for the severe c.95A>G mutation while the pilot screening programme (2002–2008) only uncovered 25% and 20%, respectively (Tables 2 and 3). The reason lies in the screening algorithm for PCD used in the pilot screening programme between 2002 and 2008. Instead of looking only for a low level of fC0 to find potential PCD patients, the algorithm also required that acetyl-carnitine C2 was below a certain cut-off. This proved to be wrong because retrospective data analysis shows that if only the fC0 cut-off had been used in the algorithm then the pilot screening programme (2002–2008) would have uncovered seven out of eight PCD patients and all five c.95A>G homozygous patients. The bulk of known PCD patients were diagnosed through the population-based voluntary screening programme established in 2009 (Rasmussen et al. 2014b).

Differing Genotype Detection

Although the rate of detection in the newborn screening programme between 2009 and 2014 seems to be complete it is remarkable that all but one newborn with PCD diagnosed during the period was homozygous for the severe c.95A>G mutation (Tables 2 and 3). Thus compared to genotype distribution seen in the voluntary screening programme, there seems to be a reduced detection of subjects with other genotypes than c.95A>G/c.95A>G (Rasmussen et al. 2014c). The patients homozygous for the severe c.95A>G mutation have the lowest fC0 levels of all Faroese PCD patients, which is likely why they seem to be better detected

in the current screening programme (Rasmussen et al. 2014c). The retrospective screening programme based on DBS samples from 1986 to 2001 seems to have been more sensitive with regard to identifying other genotypes as well (Table 2). This might be because the lower cut-off level was set higher than absolutely needed to compensate for a higher mean fC0 because of acylcarnitine degradation over time. The reason could also be the fact that the maternal influence on fC0 measured in newborns was weaker before 2009 due to later blood sampling after birth, 4–9 days versus the current 2–3 days. Furthermore the highly increased public attention concerning PCD in the Faroe Islands since 2009 has led to the use at an unknown extent of self-administered over-the-counter supplements with L-carnitine by pregnant women, which may increase the neonatal level of fC0 and thus make the current neonatal screening for PCD less efficient.

Improve Detection in the Faroe Islands

Increasing the lower cut-off value of fC0 would likely increase the sensitivity of the current neonatal screening programme, but it would also increase the number of false positives and would probably not ensure a full detection of PCD patients because of the strong maternal influence on fC0 levels at the time of birth (Strnadova et al. 2007; Fingerhut et al. 2009). Instead a strategy of either an immediate repeated test of a new DBS if the screening result was borderline or an additional routine screening test with measurement of unbutylated fC0 at, e.g., 4 weeks after birth could be proposed (Longo et al. 2006). A third option is genetic screening for known PCD-related mutations in all Faroese newborns. Considering the high prevalence and public focus on PCD with a high likelihood of unprescribed supplementation with L-carnitine in the Faroe Islands the latter or third option would be the most obvious to ensure the highest possible detection of PCD in newborns.

Conclusion

Newborns with PCD and newborns born to mothers with PCD do not differ in length, weight and Apgar scores compared to controls. Current neonatal screening for PCD with testing only 2–3 days after birth in the Faroe Islands does not capture all patients with PCD that may benefit from preventive treatment. A strategy of a second screening a few weeks after birth measuring unbutylated free carnitine or genetic screening for mutations in the *SLC22A5* gene may be advantageous.

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Compliance with Ethics Guidelines

Conflict of Interest

Jan Rasmussen, David M. Hougaard, Noreen Sandhu, Katrine Fjællegaard, Poula R. Petersen, Ulrike Steuerwald and Allan M. Lund all declare that they have no conflict of interest.

Contribution of Individual Authors

Jan Rasmussen: First author and responsible for planning, conducting and reporting the work in the chapter.

David M. Hougaard: Second author and responsible for planning and critically reviewing the work in the chapter.

Noreen Sandhu: Third author and responsible for planning, conducting and critically reviewing the work in the chapter.

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Allan M. Lund: Last author and responsible for planning, conducting and critically reviewing the work in the chapter.

Take-Home Message

Primary carnitine deficiency does not affect the health status of newborns and free carnitine should be measured when performing newborn screening.

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RESEARCH REPORT

The Spectrum of Niemann-Pick Type C Disease in Greece

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Abstract Niemann-Pick type C disease (NPC) is a neurovisceral lysosomal storage disease caused by mutations in either the *NPC1* or the *NPC2* gene. It is a cellular lipid trafficking disorder characterized by the accumulation of unesterified cholesterol and various sphingolipids in the lysosomes and late endosomes, and it exhibits a broad clinical spectrum. Today, over 420 disease-causing mutations have been identified in the *NPC1* and the *NPC2* genes. We present the clinical, biochemical, and molecular findings in 14 cases diagnosed in Greece during the last 28 years. Age at diagnosis ranged from 2.5 months to 48 years. Systemic manifestations were present in 7/14 patients. All developed neurological manifestations (age of onset 5 months to 42 years). Six patients are still alive (age: 5–50 years). Classical filipin staining pattern was observed in all but four patients (3 NPC1, 1 NPC2). The rate of LDL-induced cholesteryl ester formation was severely reduced in 4/7 and significantly reduced in 3/7 patients studied. Increased chitotriosidase activity was observed in 9/12 patients. Mutation analysis in 11 unrelated patients identified 12 different mutations in the *NPC1* gene: eight previously described p.E1089K (c.3265G>A), p.F284Lfs*26 (c.852delT), p.A1132P(c.3394G>C), del promoter region and exons 1-10, p.R1186H (c.3557G>A), p.P1007A (c.3019C>G), p.Q92R(c.275A>G),p.S940L (c.2819C>T), and four novel ones: (p.N701K fs*13 (c.2102-2103insA), p.K1057R (c.3170A>G), IVS23 +3insT(c.3591+3insT), p.C1119*(c.3357T>C); and the previously described *IVS2+5G>A(c.190+5G>A)* mutation in the *NPC2* gene. All patients were of Greek origin. Assuming a birth rate of 100,000/year, a rough incidence estimate for NPC disease in Greece would be 0.5/100,000 births.

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Introduction

Niemann-Pick type C disease (NPC) (OMIM ID: 257220, 607625) is a rare, autosomal recessive, neurovisceral lysosomal storage disease, characterized by the accumulation of unesterified cholesterol and various sphingolipids in the lysosomes and late endosomes caused by mutations in either the *NPC1* (95% of cases) or the *NPC 2* (5% of cases) gene (Vanier 2010). The *NPC1* gene encodes a large transmembrane glycoprotein (1278 amino acids, 13 transmembrane domains) residing mainly in the late endosome/lysosome compartment (Carstea et al. 1997; Davies and Ioannou 2000; Higgins et al. 1999). On the other hand, the *NPC2* gene (MIM ID: 601015)

encodes a small (151 amino acids) soluble lysosomal protein (Naureckiene et al. 2000; Vanier and Millat 2004). Both the NPC1 and the NPC2 proteins are required for the egress of endocytosed cholesterol from late endosomes/lysosomes (Vance and Karten 2014; Vanier 2015). Up to date more than 400 and 20 mutations have been reported in the *NPC1* and *NPC2* genes, respectively (HGMD professional 2016.2). NPC is a panethnic disease with an estimated incidence at diagnosis of 1:100,000–120,000 live births (Patterson et al. 2012; Vanier 2010; Jahnova et al. 2014). However, the extensive phenotypic heterogeneity of NPC that shows a broad clinical spectrum ranging from a neonatal rapidly fatal form to an adult onset chronic neurodegenerative disease, along with the relative difficulty of laboratory testing, makes the estimation of true prevalence difficult. While recent data from massive parallel sequencing are in fairly good agreement with these estimations for "classical" clinical forms of the disease, they also suggest a high incidence of unrecognized, mild adult onset forms (Wassif et al. 2016). In Greece, the Institute of Child Health is the only center offering laboratory diagnosis for lysosomal storage diseases, including NPC. We present the clinical, biochemical, and molecular findings in 14 cases with NPC disease diagnosed in Greece during the last 28 years, including three previously reported cases (Mavridou et al. 2014; Rodriguez-Pascau et al. 2012).

Patients and Methods

The demographic and clinical characteristics of 14 patients (11 unrelated) with NPC, originating from all over Greece, are shown in Table 1. The majority was referred to the Institute of Child Health for the diagnosis of a lysosomal storage disease and only 2 were referred specifically for the investigation of NPC. The report also includes four previously reported patients (patients #2, #3, #6, #7) (Mavridou et al. 2014; Rodriguez-Pascau et al. 2012; Zafeiriou et al. 2003). Patients #2 and #7 belong to the same family tree (Mavridou et al. 2014), patients #4 and #5 are second cousins, and patients #12 and #13 are siblings. Five patients are on Miglustat treatment (#5, #7, #11, #12, and #13).

Chitotriosidase activity was measured in plasma using the 4-methylumbelliferyl β-D-*N, N′, N″*-triacetylchitotrioside substrate (Michelakakis et al. 2004). The filipin staining and LDL-induced cholesteryl ester formation tests were performed in cultured fibroblasts as described (Vanier et al. 1991). Genomic DNA amplification and sequencing of exons and corresponding intronic flanking regions were performed for the detection of mutations in the *NPC 1* and *NPC 2* genes. The detection of the large deletion in patient 3 was described in Rodriguez-Pascau et al. (2012). The study was approved by the Ethics Committee of the Institute of Child Health.

Results and Discussion

NPC is a panethnic disorder showing autosomal recessive inheritance, for which two isolates have been described. The first and most important one was described in an Acadian Nova Scotian community originating from Normandy and it is characterized by the *NPC1* p.G992W mutation (Crocker and Farber 1958; Winsor and Welch 1978; Greer et al. 1998). The second one was described in Hispanics from southern Colorado and New Mexico (Wenger et al. 1977). The latter carried the *NPC1* p.I1061T mutation, which is otherwise frequent in patients of Western European descent (Millat et al. 1999; Millat et al. 2001a, b; Imrie et al. 2015). Recently we reported a hot-spot of NPC1 in a Greek island (Mavridou et al. 2014). Estimates of birth prevalence have been published for different countries, based on diagnoses in authors' laboratories. Over the period 1984–2002 they ranged from 0.66 to 0.83 for France, UK, and Germany (Patterson et al. 2001; Vanier and Millat 2003). Birth prevalences varying between 0.35 and 2.20 were reported for Australia, the Netherlands, and Portugal (Vanier 2010). More recent data indicate values of 0.96 for France and 0.93 for Czech Republic (Vanier 2010; Jahnova et al. 2014).

The Institute of Child Health is the only Center in Greece providing the diagnosis of lysosomal storage diseases and this is the first study on a large number of Greek NPC patients. In agreement with previous reports the majority of patients (13 patients, 92.9%) carried mutations in the *NPC1* gene and only one (7.1%) in the *NPC2* gene. The cases presented here account for 3% of the total number of patients diagnosed with a lysosomal storage disorder in our center. Assuming a birth rate of 100,000/year, a rough incidence estimate for NPC disease in Greece would be 0.5/100,000 births. However this is most probably an underestimate. In that context it is worth mentioning that the family of patient #11 had lost another child with very similar clinical manifestations to their diagnosed daughter. Furthermore, 2 maternal first cousins of patient 14 are also reported to have died with clinical symptoms compatible with NPC disease. Also, only two siblings presented with an adult neurological onset, making the contribution of this disease form much lower than described in recent large cohorts from three countries (Jahnova et al. 2014; Stampfer et al. 2013; Imrie et al. 2015). This strongly suggests decreased awareness regarding NPC among adult neurologists, which is in good agreement with probable underdiagnosis of mild and late-onset forms (Wassif et al. 2016).

NPC exhibits a broad clinical heterogeneity and clinical manifestations include a range of systemic and neurological manifestations that appear at different ages and progress at different rates (Patterson et al. 2001; Vanier 2010). This is also illustrated in our cohort (Table 1). Age at diagnosis of the patients reported here ranged from 2.5 months to

Table 1 Demographic and clinical characteristics of the NPC patients studied

Patient/ sex	Age of diagnosis	Perinatal/neonatal period	Neurological manifestations/age	Alive/age of death
NPC 1				
1/M	2.5 months	PJ, LD, HS, ↑FP	Moderate psychomotor retardation: 9 months	Alive (5 years)
2/F	6 years	PJ	Mild early developmental delay: psychomotor regression: 4 years Tremor, gait disturbance, ↑tendon reflexes, mental retardation (+ mild HS): 6 years VSGP, dystonic movements, axial hypotonia, swallowing difficulties, no speech, unable to stand or walk, epileptic seizures (no HS): 13 years	14 years[a]
3/F	7 months	FA (resolved until birth), HS, PJ, FT, LD, ascites	Hypotonia, dystonia, developmental delay: 5 months VSGP: 8 months Able to sit: 18 months Never sat up or walked (Pulmonary proteinosis and respiratory failure)	26 months[a]
4/M	2 years	PJ	Mild psychomotor retardation, decreased muscular tone, walking difficulties, normal brain CT scan: 2 years	3.5 years[a]
5/F	2 months	HS, PJ, LD	Mild hypotonia: 3.5 years	Alive (12 years)
6/M	3.5 months	HS, PJ, LD	Speech and behavioral changes, frequent falls, ataxia, VSGP: 2.8 years	5.5 years[a]
7/F	4.5 years	HS, LD	Mild early developmental delay Ataxic gait: 2 years; VSGP, brain MRI normal, drooling, mild global retardation, speech problems: 4.5 years	Alive (10.5 years)
8/M	9 months	HS, FT, PJ, LD	Generalized hypotonia: 3 months	Lost to FU
9/F	1.5 years	Unknown	Unable to walk: 1.5 years (HS, ascites: 1 year)	Lost to FU
10/M	6 months	HS, Interstitial pneumonitis, LD, PJ	Regression, no speech, tremor, unsteady gait: 4 years	5 years[a]
11/F	12 years	PJ	Learning difficulties: 5 years Mental deterioration, emotional disturbance, epileptic seizures, pyramidal signs, normal brain MRI, VSGP: 8 years Dysarthria, dystonia, no walking, psychiatric symptoms: 9 years	Alive (22 years)
12/F	45 years	Uneventful	Unsteady gait, speech problems: 39 years Ataxia, mental deterioration, VSGP, brain atrophy (MRI), depression and anxiety: 45 years	Alive (47 years)
13/M	48 years	Uneventful	Unsteady gait: 42 years Ataxia, mental deterioration, dysarthria, dystonia, VSGP, brain atrophy (MRI), depression and anxiety: 48 years	Alive (50 years)
NPC 2				
14/F	14 years	Uneventful	Mild early developmental delay Behavioral disturbances, mental regression: 6 years Seizures (+HS): 7 years No speech, unable to walk, further deterioration, increase in seizure frequency, brain atrophy (CT scan): 10 years	14 years[a]

F female, *M* male, *PJ* prolonged jaundice, *LD* liver disease, *HS* hepatosplenomegaly, *FP* alpha feto-protein, *FA* fetal ascites, *FT* failure to thrive, *VSGP* vertical supranuclear gaze palsy, *FU* follow-up

[a] Age of death

48 years, with a median of 1.5 years. Data on perinatal/neonatal manifestations of the disease were available in 13/14 patients. 10/13 (77%) patients had systemic manifestations of the disease that included prolonged jaundice (9/10; 90%), hepatosplenomegaly (7/10; 70%), liver disease (6/10; 60%), failure to thrive (2/10;20%), ascites (1/10;10%), and interstitial pneumonitis (1/10;10%). The three patients reported to be free of any perinatal manifestations of the disease included the two siblings with the adult onset of neurological signs and the only NPC2 patient of our cohort. The presence of systemic manifestations was the trigger for the diagnosis of the disease in patients #1, #5, #6, and #10, and they preceded the appearance of any neurological involvement. On the other hand, diagnosis for

the rest of the patients was achieved following the appearance of neurological symptoms, with a delay of 0–8 years for children and about 6 years for the two adults.

All patients eventually developed neurological manifestations with an age of onset ranging from 5 months to 42 years. Systemic manifestations persisted in 3/10 patients. One patient (#11) developed psychiatric signs and another one (#14) behavioral changes. Six patients are still alive (age: 5–50 years) (Table 1). Three patients (#1, #3, #9; all having shown perinatal manifestations of the disease) had early infantile (<2 years) onset of neurological manifestations (hypotonia, dystonia, moderate psychomotor retardation, developmental delay) which progressed so that during follow-up further regression and additional symptoms were noted. Age of diagnosis in this group ranged from 2.5 months to 1.5 years. One patient in this group is still alive (5 years of age), one died (26 months), and one was lost to follow-up.

In eight patients (#2, #4, #5, #6, #7, #10, #11, #14) the onset of neurological manifestations occurred between 2.0 and 6 years of age (mild psychomotor retardation, decreased muscular tone, walking difficulties, mild hypotonia, speech and behavioral changes, frequent falls, ataxia, VSGP, regression, tremor, unsteady gait, learning difficulties). Perinatal manifestations were present in all but one, for whom relevant information was available. Diagnosis was made between the age of 2 months and 14 years, the earliest diagnoses being made based on the perinatal manifestations. Four patients died (3.5–14.0 years old), three are still alive (10.5, 12, and 22 years old) and one was lost to follow-up.

Finally two siblings had an adult onset of neurological disease (unsteady gait, speech problems). None had any perinatal and/or systemic manifestations and they were both diagnosed with a delay of 6 years following the onset of neurological symptoms. They are currently alive with progressing disease.

Neuroradiological changes are not specific and it is generally agreed that they are not contributive to the diagnosis (Vanier 2010). Imaging data were available in six of our patients. At the time they were performed they were either normal (#4, #7, #11) or showed brain atrophy (#12, #13, #14).

The laboratory diagnosis of NPC is an overall complex, challenging, and costly process. A critical review of the currently available diagnostic approaches has recently been published (Vanier et al. 2016).

The diagnostic algorithm in our patients involved chitotriosidase assays in plasma, filipin staining test, and LDL-induced cholesteryl ester formation test in cultured fibroblasts, as well as molecular genetic testing. This being a retrospective study, cholestane triol could only be validly analyzed in plasma from cases #12 and #13, who showed elevated values (0.065 and 0.186 ng/μl, respectively, compared to concentrations <0.05 in control subjects).

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 12/14 patients (Table 2). Increased activity, ranging from 1.2 to 8× the upper normal value, was observed in 9/12 patients. Minimal or no increase was observed in the two adult onset cases, in good agreement with previous reports. The use of chitotriosidase in the diagnostic algorithm of NPC has been criticized as lacking specificity and sensitivity. However, despite its limitations, our results indicate its usefulness and we advocate keeping this assay in the diagnostic algorithm, at least until analysis of the newer biomarkers is established locally.

Filipin is a fluorescent polyene antibiotic that specifically binds to non-esterified cholesterol. The filipin test was developed about 30 years ago and, till recently, it was considered the gold standard method for diagnosing NPC (Vanier et al. 2016). It is used in fibroblasts loaded with LDL to demonstrate the impaired intracellular transport of endocytosed cholesterol through the fluorescent staining of non-esterified cholesterol accumulated in the late endosome/lysosome compartment (Vanier and Latour 2015). The majority of cases (80–85%) show a “classical” staining pattern characterized by the presence of numerous strongly fluorescent perinuclear vesicles that are cholesterol loaded. In the remaining cases a less intense and variable, “variant,” staining pattern, that can be a source of confusion, is observed (Vanier et al. 2016). In our cohort of patients the filipin test was performed in 14/14 patients. “Classical” filipin staining pattern was observed in 10/14 and “variant” in 4/14 patients. The latter included patient #11 with onset of neurological symptoms at the age of 5 years and still alive at the age of 22 years, the two siblings (#12, #13) with an adult-onset disease, and the NPC2 patient (#14).

The lipoprotein-derived cholesterol esterification test evaluates the re-esterification rate of exogenous cholesterol by acyl-CoA: cholesterol acyltransferase after its egress from the late endosome/lysosome compartment. Very low rates are described in patients with the “classical” filipin staining pattern with the “variant” phenotype showing a moderate or nonsignificant impairment. Although the test was till recently used as a secondary diagnostic test, the above observation along with the complexity and the cost of the test have practically limited its use to research settings (Vanier et al. 2016). The rate of LDL induced cholesteryl ester formation, studied in nine of our patients (Table 2) was diagnostic for all, although with variations, in accordance with previous reports. A severe reduction was

Table 2 Laboratory findings and origin of the NPC patients studied

Patient	Plasma chitotriosidase activity (nmol/ml/h)[a]	Filipin staining	LDL-induced cholesteryl ester formation (pmol/mg prot/4.5 h)[b]	Genotypes	Origin
NPC 1					
#1	192	Classical	nd	p.E1089K (3265G>A)/**p.N701K fs*13 (c.2102-2103insA)**	Peloponnese-Central Greece
#2	572	Classical	30	p.A1132P (c.3394G>C)/p.A1132P (c.3394G>C)	South Aegean
#3	247	Classical	nd	p.F284Lfs*26 (c.852delT)/del promoter region and exons 1-10	Central Greece
#4	1200	Classical	50	**IVS23+3insT(c.3591+3insT)**/not identified	South Aegean
#5	391	Classical	20	**IVS23+3insT (c.3591+3insT)/p.K1057R (c.3170A>G)**	South Aegean Minor Asia
#6	240	Classical	50	p.R1186H (c.3557G>A)/p.R1186H (c.3557G>A)	Central Greece
#7	110	Classical	nd	p.A1132P (c.3394G>C)/p.A1132P (c.3394G>C)	South Aegean
#8	246	Classical	<10	p.Q92R (c.275A>G)/**p.C1119* (c.3357T>C)**	Central Greece
#9	573	Classical	150	p.R1186H (c.3557G>A)/p.R1186H (c.3557G>A)	North Greece North Greece
#10	nd	Classical	220	p.R1186H (c.3557G>A)/**IVS23+3insT (c.3591+3insT)**	South Aegean Peloponnese - Central Greece
#11	17	Variant	530	p.P1007A (c.3019C>G)/p.S940L (c.2819C>T)	Peloponnese
#12	176	Variant	nd	p.P1007A (c.3019C>G)/p.P1007A (c.3019C>G)	North Greece
#13	100	Variant	nd	p.P1007A (c.3019C>G)/p.P1007A (c.3019C>G)	North Greece
NPC 2					
#14	nd	Variant	425	IVS2+5G>A (c.190+5G>A)/IVS2+5G>A (c.190+5G>A)	Central Greece

Patients #11 and #12 are siblings

[a] Normal range 0–150 nmol/ml/h

[b] Normal values: 2950 ± 1200 pmol/mg prot/4.5 h. Novel mutations are shown in bold. Patient #4 is assumed to be heterozygous for the mutation IVS23 + 3insT(c.3591 + 3insT), identified in his paternal second cousin

observed in five patients with classical filipin staining, and a significant reduction in four patients (highest values being observed in those with "variant" filipin).

Mutation analysis was carried out in 11 unrelated patients. Ten of them were found to carry mutations in the *NPC1* and one in the *NPC2* gene (Table 2). In the NPC1 patients, 12 different mutations and nine different genotypes were identified. Eight of the mutations had been previously described: p.E1089K (3265G>A) (Sun et al. 2001), p.F284Lfs*26 (c.852delT) (Fancello et al. 2009), p.A1132P(c.3394G>C) (Mavridou et al. 2014), del promoter region and exons 1-10 (Rodriguez-Pascau et al. 2012), p.R1186H(c.3557G>A) (Carstea et al. 1997), p.P1007A (c.3019C>G) (Greer et al. 1999), p.Q92R(c.275A>G) (Ribeiro et al. 2001), and p.S940L(c.2819C>T) (Greer et al. 1999). The mutations p.A1132P(c.3394G>C) and the large deletion of the promoter region and of exons 1-10 have only been described in Greek patients (included in this report as #2, #3, #7). In addition, 4 novel *NPC1* mutations were identified: p.N701Kfs*13 (c.2012-2013 ins A), a frame shift mutation leading to a premature stop codon on the sterol-sensing domain of the NPC1 protein; IVS23 +3insT (c. 3591+3insT), a splicing mutation leading to the loss of exon 23; p.C1119*(c.3357 T>C), a nonsense mutation leading to a premature stop codon at the TM9 domain of NPC1 protein; and p.K1057R (c.3170 A>G), a missense mutation on the cysteine-rich luminal loop of the NPC1 protein which is predicted to be benign in silico by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). All new mutations occurred in compound heterozygosity and,

with the exception of mutation IVS23+3insT (c. 3591 +3insT) identified in two unrelated patients, were only identified in one single patient. The most frequent mutation was p.R1186H (c.3557G>A) which accounted for 22.7% of the identified alleles of unrelated patients. It is a missense mutation located in the 6th small cytoplasmic loop, between the TM 11 and 12 domains of the NPC1 protein, altering an amino acid that is phylogenetically conserved (Carstea et al. 1997; Millat et al. 2001a). In our cohort it was identified in the homozygous and heterozygous state, and always associated with a classical filipin staining pattern. Interestingly, this mutation was also the most frequent one in the historical cohort of Czech patients (Jahnova et al. 2014). Mutation p.P1007A (c.3019C>G) has been described to occur with high frequency in NPC1 patients with the variant filipin staining pattern. Millat et al. (2001a) reported a prevalence of this allele of 22.7% among 33 patients with this biochemical phenotype. In our cohort of patients the mutation was also identified either in homozygosity or heterozygosity in three patients all with the variant filipin staining pattern. In homozygosity it was associated with the adult form of the disease in siblings #12 and #13, in good accordance with other studies (Ribeiro et al. 2001; Stampfer et al. 2013) whereas in heterozygosity with the missense mutation p.S940L(c.2819C>T) (Greer et al. 1999) it was associated with juvenile onset of disease (Patient #11). Patient #14 was shown to be homozygous for the mutation IVS2+5G>A (c.190+5G>A) in the *NPC2* gene. This splice mutation, first described in homozygosity in an Algerian patient, was shown to lead to the synthesis of multiple abnormal mRNAs and no detectable protein (Millat et al. 2001b). This patient, who was highly contributive in the delineation of the NP-C2 complementation group, also showed intermediate values for filipin and cholesteryl ester formation tests (#27 in Vanier et al. 1996). She and her affected sister started to show neurological symptoms when 6–7 years of age, but had a slower rate of disease progression compared to our case; they were still alive at 24 and 30 years of age. None of these 3 NPC2 patients suffered from specific respiratory disease.

DNA was available from the parents of 9 NPC1 patients (#1, #2, #5, #7, #8, #9, #10, and #11). In all but one, heterozygosity for one of the mutations identified in their offsprings was confirmed. The exception was the parents of Patient #1, in which case the mother carried the mutation p.N701Kfs*13(c.2102-2103insA) but the father was not found to carry the second mutation p.E1089K (c.3265G>A) identified in his son. Paternity was confirmed by DNA analysis of polymorphisms (16 polymorphisms, paternity probability: 99.999997%). One possible explanation of the above finding would be that the p.E1089K (c.3265G>A) is a de novo mutation, however germinal mosaicism cannot be excluded. Fibroblast cultures were also available from the parents of patients #4 and #9. Filipin staining showed accumulation of fluorescent perinuclear vesicles in some of the cells from the parents of the former and a significant number of positive cells in the cells from the parents of the latter patient. In all of them, the rate of LDL-induced esterification of exogenous cholesterol was normal (Table 3). These data are in agreement with previous reports and highlight the problems that may be encountered in the filipin staining test.

In conclusion we presented the clinical, biochemical and molecular findings in a cohort of 14 Greek NPC patients that highlight the diversity of the disorder in Greece.

Take Home Message

In 13 NPC1 and 1 NPC2 patients we identified 13 different mutations (4 novel) and ten genotypes; classical filipin staining was observed in eight patients.

Table 3 Laboratory findings and origin of the parents of two NPC1 patients

Carriers	Filipin staining	LDL-induced cholesteryl ester formation (pmol/mg prot/4.5 h)[a]	Mutation	Origin
#4				
Father	Some cells show accumulation of fluorescent perinuclear vesicles	2960	[b]IVS23+3insT(c.3591+3insT)	South Aegean
Mother	Similar pattern	1075	Unknown	South Aegean
#9				
Father	A significant number of positive cells after LDL loading	2000	p.R1186H(c.3557G>A)	North Greece
Mother	Similar (typical heterozygote) pattern	2390	p.R1186H(c.3557G>A)	North Greece

[a] Normal values: 2950 ± 1200 pmol/mg prot/4.5 h
[b] The father of patient #4 is assumed to carry the mutation IVS23 + 3insT(c.3591 + 3insT), identified in his cousin

Contribution of Individual Authors

Mavridou I.: Fibroblast cultures; filipin staining; mutation analysis by PCR; writing of the paper

Dimitriou E.: Assay of chitotriosidase activity

Vanier MT: Filipin staining; LDL-induced cholesteryl ester formation test; writing of the paper

Vilageliu L., Grinberg D.: Mutation analysis by DNA sequencing, writing of the paper

Latour P: Mutation analysis by DNA sequencing

Xaidara A, Lycopoulou L, Bostantjopoulou S, Zafeiriou D: Attending physicians

Michelakakis H: Coordinated the study; writing of the paper

Guarantor: Helen Michelakakis PhD

Conflict of Interest

MT Vanier has received honoraria and travel reimbursements from Actelion Phamaceuticals Ltd. as a member of advisory committees and speaking engagements, as well as honoraria from Shire for participating in a DSM Board. D. Zafeiriou has received research and travel grants, as well as honoraria for lecturing and participating at Advisory Boards from Genzyme-Sanofi, Shire, Actelion and Biomarin. H. Michelakakis has received honoraria and travel reimbursements from Genzyme-Sanofi, Actelion, research grant from Actelion. E. Dimitriou has received travel reimbursements from Genzyme-Sanofi and Shire. I. Mavridou has received travel reimbursement from Genzyme-Sanofi, Actelion, and Shire.

The study was approved by the Ethics Committee of the Institute of Child Health.

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RESEARCH REPORT

What Is the Best Blood Sampling Time for Metabolic Control of Phenylalanine and Tyrosine Concentrations in Tyrosinemia Type 1 Patients?

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Abstract *Background*: Treatment of hereditary tyrosinemia type 1 with nitisinone and phenylalanine and tyrosine restricted diet has largely improved outcome, but the best blood sampling time for assessment of metabolic control is not known.

Aim: To study diurnal and day-to-day variation of phenylalanine and tyrosine concentrations in tyrosinemia type 1 patients.

Methods: Eighteen tyrosinemia type 1 patients aged >1 year (median age 7.9 years; range 1.6–20.7) were studied. Capillary blood samples were collected 4 times a day (T1: pre-breakfast, T2: pre-midday meal, T3: before evening meal, and T4: bedtime) for 3 days. Linear mixed-effect models were used to investigate diurnal and day-to-day variation of both phenylalanine and tyrosine.

Results: The coefficients of variation of phenylalanine and tyrosine concentrations were the lowest on T1 (13.8% and 14.1%, respectively). Tyrosine concentrations did not significantly differ between the different time points, but phenylalanine concentrations were significantly lower at T2 and T3 compared to T1 (50.1 μmol/L, 29.8 μmol/L, and 37.3 μmol/L, respectively).

Conclusion: Our results indicated that for prevention of too low phenylalanine and too high tyrosine concentrations, measurement of phenylalanine and tyrosine pre-midday meal would be best, since phenylalanine concentrations are the lowest on that time point. Our results also indicated that whilst blood tyrosine concentrations were stable over 24 h, phenylalanine fluctuated. Day-to-day variation was most stable after an overnight fast for both phenylalanine and tyrosine. Therefore, in tyrosinemia type 1 patients the most reliable time point for measuring phenylalanine and tyrosine concentrations to enable interpretation of metabolic control is pre-breakfast.

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Introduction

Hereditary tyrosinemia type 1 (McKusick 27670, HT1) is a rare inherited metabolic disease caused by fumarylacetoacetate hydrolase (FAH) deficiency in the liver and kidney. FAH is the final enzyme in tyrosine catabolism. Without treatment, patients develop liver failure, hepatocellular carcinoma, porphyria-like neurological episodes, renal tubulopathy, and cardiomyopathy (Mayorandan et al. 2014). Increased blood and urine succinylacetone (SA) are diagnostic indicators (de Laet et al. 2013).

Treatment of HT1 consists of the combination of prescription of NTBC (2-(2 nitro-4-3 trifluoro-methylben-

zoyl)-1, 3-cyclohexanedione) and dietary restriction of phenylalanine (Phe) and tyrosine (Tyr) (de Laet et al. 2013). NTBC has led to remarkable improvement in the outcome of HT1 (Larochelle et al. 2012; de Laet et al. 2013). It inhibits the enzyme 4-hydroxyphenylpyruvate dioxygenase at a stage before the metabolic defect, but increases tyrosine concentrations as a consequence, necessitating a diet restriction of tyrosine and its indispensable precursor phenylalanine. Monitoring and adjustment of treatment is based on measurements of NTBC, SA, and phenylalanine and tyrosine concentrations (de Laet et al. 2013).

Neurocognitive delay is reported in HT1 (Masurel-Paulet et al. 2008; de Laet et al. 2011; Bendadi et al. 2014; van Vliet et al. 2014), suggesting a relation with both low phenylalanine and high tyrosine concentrations (de Laet et al. 2011; van Vliet et al. 2014) and/or Phe:Tyr ratios (de Laet et al. 2011). Therefore, regular and reliable measurement of both phenylalanine and tyrosine concentrations could be important. However, what is the predictive value of a single concentration of phenylalanine and tyrosine in treated HT1 patients taken at a specific time point during the day?

Two studies have paid attention to the variation of phenylalanine and tyrosine concentrations in HT1 patients (Wilson et al. 2000; Daly et al. 2012). The first was an intervention study in five HT1 patients and one tyrosinemia type II patient showing that phenylalanine concentrations decreased during the day in five patients (Wilson et al. 2000). The second study was a retrospective study in eleven HT1 patients showing lower blood phenylalanine concentrations in the afternoon (Daly et al. 2012). Notwithstanding of clear interest, these studies leave the predictability of a single blood sample of phenylalanine and tyrosine in a 24 h time period and from day-to-day to be studied.

Therefore, the aim of this prospective study was to investigate diurnal and day-to-day variation of phenylalanine and tyrosine concentrations in HT1 patients treated with NTBC and dietary restriction of phenylalanine and tyrosine. The results should enable reliable recommendations about the optimal timing of phenylalanine and tyrosine for safe dietary changes in HT1.

Methods

Subjects

Patients aged $\geq$1 year with HT1 were recruited. Recruitment took place at the University Medical Center Groningen (UMCG), Beatrix Children's Hospital (the Netherlands), and Birmingham Children's Hospital (United Kingdom). Inclusion criteria included: (1) treatment with NTBC (Orfadin, Swedish Orphan International AB, Stockholm, Sweden); (2) tyrosine restricted diet and phenylalanine, tyrosine free L-amino supplements; (3) good metabolic control defined as having 60% of the tyrosine concentration measurements $<$500 μmol/L in the 12 months to entering the study. Patients with liver transplants, co-morbidities, or additional phenylalanine supplements were excluded. Patient characteristics are shown in Table 1. Written informed consent was obtained from all participants and/or caregivers. The medical ethics committee of the UMCG provided approval. At Birmingham Children's Hospital a favorable ethical opinion was obtained from the local research ethics committee.

Assessment of Dietary Intake

The participants continued their usual tyrosine restricted diet including phenylalanine and tyrosine free L-amino acid supplements. Participants or caregivers kept a written record of all food, drinks, and L-amino acid supplements during the 3 days of the study period. The nutritional analysis of food intake of the Dutch participants was calculated using a hospital computer program based on the Dutch food composition database (NEVO 2010) (Westenbrink and Jansen-van der Vliet 2010). The nutritional analysis of food intake of the British participants was calculated using the Microdiet computer program based on McCance and Widdowson's The Composition of Foods series, with supplementary analysis data provided by manufacturers and added to the database.

Body weight measurements were performed by participants or caregivers at home 1 week before the start of the study, at the beginning of the study, and the day after the study to monitor that a stable body weight was maintained.

Blood Sampling

All participants or caregivers collected blood spot samples from finger punctures on filter paper (Grade TFN 179 g/m^2. Sartorius. Göttingen, Germany) and stored at −20°C till analysis. This was performed 4 times a day (pre-breakfast: 7–8 a.m., pre-midday meal: 12–1 p.m., before evening meal: 5–6 p.m. and bedtime) for 3 days with 12 samples per participant. Phenylalanine and tyrosine concentrations of all blood specimens were analyzed by LC-MS/MS in the laboratory of Metabolic Diseases of the UMCG in Groningen following a standardized protocol taking into account differences such as the spot size and localization of the punch (Holub et al. 2006; Lawson et al. 2016).

Statistical Analysis

Descriptive statistics were applied for baseline characteristics. The normality of the data was assessed by analyzing the P–P plots. Data were represented as median and range.

Table 1 Patient characteristics

				Average of 3 days		
No.	M/F	Age	Weight	Total protein (g)	Natural protein (g)	Protein substitute (g)
1	M	15.3	74.7	96	36	60
2	M	15	57	92	32	60
3	M	11.2	27.7	75	15	60
4	F	10.5	31	74	14	60
5	M	9.8	24	86	26	60
6	M	8.1	26.9	79	19	60
7	F	8	31.2	66	16	50
8	M	7.9	45.2	86	26	60
9	F	7.5	21.4	59	14	45
10	M	7.3	23.2	66	26	40
11	M	6.5	21.4	56	11	45
12	F	3.1	12.4	35	17	18
13	F	2.2	10	34	13	21
14	F	1.6	11.5	35	5	30
15	M	7.2	31	47	13	34
16	M	6.4	24.5	36	12	24
17	M	20.7	58	88	28	60
18	M	13.8	59	63	20	44

No. number

For categorical data, proportions are shown. To investigate the mean difference of phenylalanine and tyrosine concentrations between the separate time points (diurnal variation) and to account for intra individual correlations between multiple measurements, a linear mixed-effect model was applied. With this repeated measures analysis, all available data points were used.

The coefficient of variation (CV) of tyrosine and phenylalanine per subject per time point was used as a measure of day-to-day variation. To investigate the difference in CV of both phenylalanine and tyrosine between the separate measurement moments, linear mixed-effect models were applied. In both linear mixed-effect models the individual variation in daily intercept per subject was accounted for by estimating a random effect. Two sided significance tests were used ($\alpha < 0.05$). Statistical analyses were performed using IBM SPSS statistical software, version 22.0 (IMB Corporation, Armonk, NY, USA).

A blood phenylalanine concentration <30 μmol/L was considered clinically suboptimal as well as a blood tyrosine >400 μmol/L.

Results

Patient Characteristics

Eighteen participants (12, 66.7% males) were included with a median age of 7.9 years (range 1.6–20.7). Five patients were recruited from UMCG. Two participants were diagnosed by newborn screening, the others after development of clinical symptoms. Thirteen patients were recruited from Birmingham Children's Hospital. Four patients diagnosed due to previous family diagnosis, and two diagnosed via screening (incidental findings). All others were diagnosed after development of clinical symptoms. Nitisinone was prescribed in a median dose of 0.95 (range 0.65–2) mg/kg/day.

Blood Concentrations

Before assessing the diurnal and day-to-day variation, we investigated blood tyrosine and phenylalanine concentrations during the study period in relation to the current

treatment recommendations for HT1 patients. In total we had 215 samples (18 subjects × 12 blood spots = 216, 1 missing value). Blood tyrosine concentrations were within the aimed range 200–400 μmol/L in 128 (59.5%) of the samples. Tyrosine concentrations were <200 μmol/L in 9 (4.2%) samples (range 135–181), ≥400 μmol/L in 78 (36.3%) samples (range 400–791), and ≥600 μmol/L in 6 (2.8%) samples (range 600–791). Phenylalanine concentrations were <20 μmol/L in 20 (9.3%) samples (range 8–19), <30 μmol/L in 57 (26.5%) samples (range 8–29), <40 μmol/L in 108 (50.2%) samples (range 8–39), and ≥40 μmol/L in 107 (48.8%) samples (range 40–143).

Diurnal and Day-to-Day Variation of Blood Tyrosine Concentrations

To determine the optimal time point for measuring blood tyrosine concentrations to indicate metabolic control, both the diurnal variation and the CV per time point as a measure of day-to-day variation of blood tyrosine concentrations were assessed by a linear mixed-effect model. Mean tyrosine concentrations (μmol/L) per time point per subject are shown graphically in Fig. 1a.

Assessing diurnal variation, the linear mixed-effect model for predicting tyrosine concentrations showed that the tyrosine concentrations were expected to be 371.9 μmol/L at T1. Tyrosine concentrations were lower at T2 and T3 compared to T1 with 13.9 μmol/L and 13.1 μmol/L, respectively. Tyrosine concentrations at T4 were expected to be 18.2 μmol/L higher compared to T1. Differences in tyrosine concentrations were not significant (Table 2). In post-hoc analysis it was shown that tyrosine concentrations at T2 and T3 were significantly lower than at T4 ($p = 0.002$) with, respectively, 32.1 and 31.3 μmol/L (data not shown).

The linear mixed-effect model for predicting the day-to-day variation of tyrosine concentrations showed that the CV of tyrosine was expected to be 13.8% at T1. CV of tyrosine concentrations on T2 is expected to be the same as on T1. CVs of tyrosine concentrations at T3 and T4 were expected to be 1.6% and 3.1% higher than at T1, respectively. None of these differences were significant (Table 3).

Diurnal and Day-to-Day Variation of Blood Phenylalanine Concentrations

To determine the optimal time point for measuring blood phenylalanine concentrations to indicate metabolic control, both the diurnal variation and the CV per time point as a measure of day-to-day variation of blood phenylalanine concentrations were assessed by a linear mixed-effect model. Mean phenylalanine concentrations (μmol/L) per time point per subject are shown graphically in Fig. 1b.

Assessing diurnal variation, the linear mixed-effect model for predicting phenylalanine concentrations showed that the phenylalanine concentrations at T2 and T3 were expected to be significantly lower ($p < 0.001$) than at T1 with 20.3 μmol/L and 12.8 μmol/L, respectively. Phenylalanine concentrations at T4 were expected to be 3.7 μmol/L lower than at T1; this difference was not significant (Table 2).

The linear mixed-effect model for predicting the day-to-day variation of phenylalanine concentrations showed that the CV of phenylalanine was expected to be 14.1% on T1. CV of phenylalanine concentrations at T2 and T4 are expected to be 11.0 and 6.5% higher than on T1. These differences are significantly different ($p = 0.024$ and $p = 0.002$, respectively). CV of phenylalanine at T3 is expected to be 6.5% higher than at T1; this difference was not significant (Table 3).

Discussion

This is the first study investigating the optimal time to take blood samples to reliably monitor metabolic control in HT1 patients. Addressing the optimal time of blood sampling includes two discussions: (1) the optimal time point to detect a clinically relevant phenylalanine and/or tyrosine concentration; (2) the most reliable time of measurement giving the least day-to-day variation. The clinically most optimal moment for blood sampling may be the moment where phenylalanine concentrations are the lowest and tyrosine concentrations are the highest. Our results indicated that for that purpose measurement of phenylalanine and tyrosine pre-midday meal would be best, since phenylalanine concentrations are the lowest on that time point. However, our results also indicated that whilst blood tyrosine concentrations were stable over 24 h, phenylalanine fluctuated. Day-to-day variation was most stable after an overnight fast for both phenylalanine and tyrosine. For comparison, in healthy individuals amino acids such as phenylalanine and tyrosine concentrations rise during the day due to intake and decrease during the night (Maher et al. 1984; Farquhar et al. 1985). In contrast, in patients with phenylketonuria (PKU) phenylalanine and tyrosine concentrations decrease rather than increase during the day with meals and nett-protein synthesis, while they tend to increase during periods of fasting in which there is a nett-catabolism of natural protein as shown for phenylalanine in (PKU) (Farquhar et al. 1985; van Spronsen et al. 1993). This difference is due to the fact that the intake of phenylalanine and tyrosine is decreased in the diet of the patients.

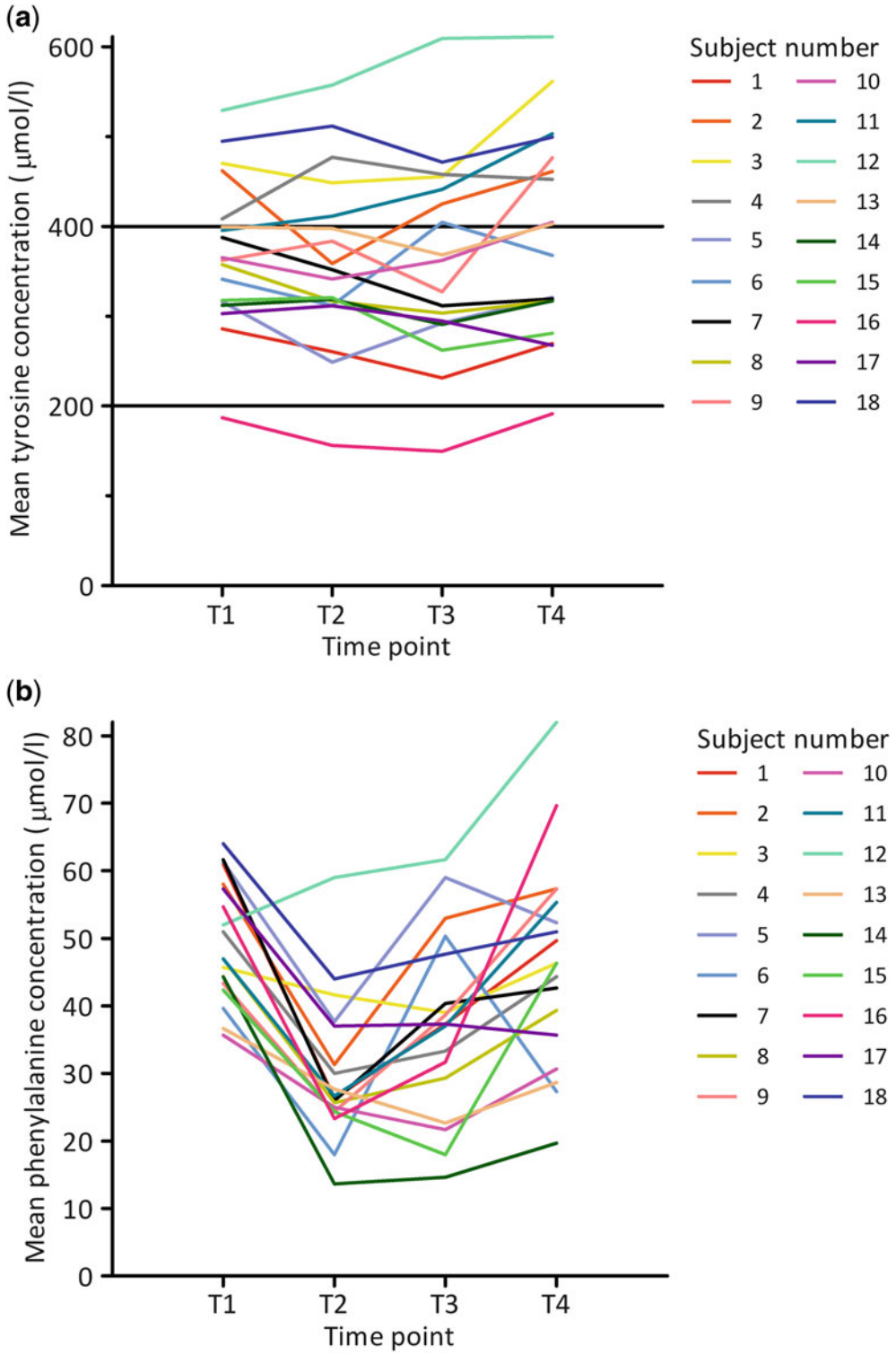

Fig. 1 Mean tyrosine concentrations (µmol/L) (**a**) and mean phenylalanine concentrations (µmol/L) (**b**) per time point per subject. The *black horizontal lines* indicate the upper and lower reference value of tyrosine (de Laet et al. 2013). T1 = pre-breakfast, T2 = pre-midday meal, T3 = before evening meal, T4 = bedtime

Table 2 Results of mixed-effect model for predicting tyrosine en phenylalanine concentrations in blood in tyrosinemia type 1 patients

	Tyrosine (μmol/L)			Phenylalanine (μmol/L)		
Variable	*β*	95% CI	*p*	*β*	95% CI	*p*
Intercept	371.9	341.4; 402.4	<0.001	50.1	45.9; 54.3	<0.001
Time point						
T1 before breakfast	Reference value			Reference value		
T2 before lunch	−13.9	−33.6; 5.8	0.165	−20.3	−25.2; −15.5	<0.001
T3 before dinner	−13.1	−32.7; 6.5	0.188	−12.8	−17.6; −7.9	<0.001
T4 before bedtime	18.2	−1.4; 37.8	0.068	−3.7	−8.6; 1.1	0.130

CI confidence interval, *β* effect differences in μmol/L compared to T1 (intercept)

Table 3 Results of mixed-effect model for predicting the day-to-day variation of tyrosine en phenylalanine concentrations in blood in tyrosinemia type 1 patients

	CV tyrosine (%)			CV phenylalanine (%)		
Variable	*β*	95% CI	*p*	*β*	95% CI	*p*
Intercept	13.8	9.8; 17.9	<0.001	14.1	7.3; 20.8	<0.001
Time point						
T1 before breakfast	Reference value			Reference value		
T2 before lunch	0.0	−4.6; 4.6	1.000	11.0	1.5; 20.5	0.024
T3 before dinner	1.6	−3.0; 6.1	0.488	6.5	−3.0; 16.0	0.177
T4 before bedtime	3.1	−1.5; 7.6	0.184	15.2	5.7; 24.7	0.002

CV coefficient of variation, *CI* confidence interval, *β* effect differences in μmol/L compared to T1 (intercept)

Methodological Issues

Before addressing the results in more detail, the following methodological issues are discussed. First, we did not study patients with clinically proven deficiencies of amino acids as all our patients showed acceptable growth curves and also met the Dutch recommendations for total protein intake for patients who use amino acid supplementation as part of daily protein intake. Second, amino acid analysis was done in blood spots. Measurements of amino acids in blood spots are influenced by factors such as humidity, hematocrit, and size and location of punches in the blood spot (Holub et al. 2006; Lawson et al. 2016). Patients' hematocrit was not measured, but can be considered normal as no illness or trauma occurred in our patients during the time of the study. Size and location of punches in the blood spot was standardized, so that the influence of such blood spot related issues can be regarded to be as minimal as possible. Third, the study was performed at home to maintain normal patient routine and not in a research environment in which intake is standardized. As a consequence we had to assume that participants followed the study protocol in the correct manner. However, we instructed each participant/caregiver before study commencement and maintained regular contact during the study period and all participants/caregivers returned blood samples according to protocol. Fourth, we did measure in blood spots in a protocolled way rather than in plasma or serum. The advantage is that the discussion on differences between measurement in blood spot versus plasma as shown in various articles is not of importance for this study. On the other hand, we do not know exactly how to translate our data and conclusions for measurements in plasma, knowing that the debate on the comparison between blood spot and plasma is ongoing (Gregory et al. 2007; Kand'ár and Zakova 2009; De Silva et al. 2010; Mo et al. 2013; Groselj et al. 2015).

Optimal Metabolic Control Is Less Clear for Blood Phenylalanine than Blood Tyrosine

Thus far, there is no clear consensus about the lower acceptable limit for blood phenylalanine. The lower end of the reference values are between 26 and 46 μmol/L, also

depending on age (Pasquali and Longo 2014). Concentrations <20 μmol/L being considered to be very low (Wilson et al. 2000). It is often advised to keep phenylalanine plasma concentrations >40 μmol/L (Chakrapani et al. 2012). This is why we reported phenylalanine levels <20, <30, <40, and ≥40 μmol/L. For tyrosine, upper target concentrations are reported as 400, 500, 600 μmol/L, while it also is advised to keep tyrosine plasma concentrations between 200 and 400 μmol/L (Wilson et al. 2000; Chakrapani et al. 2012; de Laet et al. 2013). The advised concentrations are based on plasma while phenylalanine and tyrosine concentrations of this study are measured in blood spots. Therefore, we reported concentrations between <200, between 200 and 400, ≥400 and ≥600 μmol/L. To decide on clinical significance of a variation in phenylalanine and/or tyrosine concentrations we decided to define a variation significant when for phenylalanine one of the samples showed a result of <30 μmol/L, while the others were >30 μmol/L, while for tyrosine one of the samples showed a result of >400 μmol/L when the others were <400 μmol/L.

Day-to-Day Variation Is Comparable for Phenylalanine and Tyrosine, While Diurnal Variation Only Plays a Role for Phenylalanine

When considering the optimal time of blood sampling in HT1 patients, this includes two discussions: (1) the most reliable time of measurement giving the least day-to-day variation; and (2) the optimal time point to detect a clinically relevant phenylalanine and/or tyrosine concentration. With respect to the first issue, our study demonstrated that CVs of both phenylalanine and tyrosine concentrations were the lowest at T1 (13.8% and 14.1%, respectively). With respect to the second issue, phenylalanine concentrations were both statistically and clinically significantly lower at T2 and T3 compared to T1, showing levels <30 μmol/L. This indeed is of clinical importance since too low phenylalanine concentrations in PKU are related to impaired growth and development (Rouse 1966; Smith et al. 1990; Teissier et al. 2012), and possibly also to physical and mental development in HT1 (de Laet et al. 2011; van Vliet et al. 2014).

In contrast, we found the highest tyrosine levels at T4, being 18.1 μmol/L higher than at T1, but still within an acceptable range of variation. Therefore, this difference is not considered clinically significant.

Clinical Implications Regarding the Optimal Timing of Blood Sampling

Until now, little attention has been paid to the optimal timing of blood sampling for metabolic control in HT1 and leaving us without recommendation. Most knowledge on the most reliable time of phenylalanine and tyrosine as metabolic control is available from PKU in which phenylalanine is much higher and tyrosine lower (Blau et al. 2010). In PKU, phenylalanine also shows a decrease during the day, being the highest just before breakfast, and tyrosine showing large variation during the day (van Spronsen et al. 1993; van Spronsen et al. 1996; MacDonald et al. 1998). In HT1 our data add to the data of Wilson et al. (2000) and Daly et al. (2012) showing that phenylalanine can decrease during the day, while tyrosine is more or less stable. So, when concluding on the most optimal time for blood sampling for metabolic control in practice, the exact moment does not matter when tyrosine is regarded. In contrast for phenylalanine, we have to choose between the moment that is the most consistent (pre-breakfast) and the moment that may give the largest clue to a clinically significant low level (pre-midday meal). As consistency is very important, we would advise to choose for blood spot sampling prior to breakfast taking into account that it is important to take a decrease into account that may easily be around 20 μmol/L. Considering the clinical consequences of (cerebral) phenylalanine deficiency, we suggest that in HT1 the fasting lower target reference for phenylalanine concentrations in blood spots should be at least 50 μmol/L with the basic thought that phenylalanine concentrations should not be lower than 30 μmol/L during the day. If there is any suggestion of clinical phenylalanine deficiency (e.g., impaired growth, impaired growth of nails and hair), a second blood sample taken later on the same day after a meal may be warranted to check blood phenylalanine concentrations. If phenylalanine concentrations are consistently low, phenylalanine supplementation should be added to maintain blood concentrations within target range.

Conclusion

In conclusion, the most reliable moment for measuring metabolic control in HT1 patients in regular patient care is in a fasting state prior to breakfast. This study also confirms that phenylalanine concentrations may drop significantly post breakfast by an average of 20.3 μmol/L. Therefore, we recommend that metabolic control in HT1 patients is measured in fasting state, and that the fasting lower target reference for phenylalanine concentrations in blood spots should be at least 50 μmol/L. Future research should investigate whether the diurnal and day-to-day variation of blood phenylalanine and tyrosine concentrations is different in HT1 patients receiving phenylalanine supplementation.

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Take Home Message

We recommend blood sampling for metabolic control in tyrosinemia type I pre-breakfast.

Compliance with Ethics Guidelines

Conflict of Interest

Esther van Dam has received advisory board fees from Biomarin and Merck Serono.

Anne Daly has received research funding and lecture fees from Nutricia and Vitaflo.

Gineke Venema-Liefaard has no conflicts of interest to declare.

Margreet van Rijn has received research grants, consultancy fees, and advisory board fees from Merck Serono and Nutricia Research, speaker honoraria from Merck Serono, Nutricia Research and Orphan Europe, and expert testimony fees from Merck Serono.

Terry G. J. Derks has received speaker honoraria from Danone, Nutricia, Recordati Rare Diseases and Vitaflo, research grants from Sigma Tau and Vitaflo, and consultancy and advisory board fees from Dimension Therapeutics.

Patrick J. McKiernan has received advisory board fees and speaker honoraria from Sobi.

M. Rebecca Heiner-Fokkema has no conflicts of interests to declare.

Anita MacDonald received research funding and lecture fees from Merck Serono, Nutricia, and Vitaflo, and advisory board fees from Arla Foods, Biomarin, Merck Serono and Nutricia.

Francjan J. van Spronsen has received research grants, advisory board fees, and speaker honoraria form Merck Serono, Nutricia Research and Sobi, speaker fees from Vitaflo, and advisory board fees from Arla Foods and Biomarin.

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 and/or their parents/caregivers before being included in the study.

Details of the Contributions of the Authors

Esther van Dam collected data, drafted the initial manuscript, revised the manuscript, and approved the final manuscript as submitted.

Anne Daly included patients in Birmingham, collected data, co-wrote the manuscript, and approved the final manuscript as submitted.

Gineke Venema-Liefaard included patients in Groningen, collected data, critically reviewed the manuscript, and approved the final manuscript as submitted.

Margreet van Rijn co-wrote the manuscript and approved the final manuscript as submitted.

Terry G. J. Derks critically reviewed the manuscript and approved the final manuscript as submitted.

Patrick J. McKiernan critically reviewed the manuscript and approved the final manuscript as submitted.

M. Rebecca Heiner-Fokkema was responsible for the biochemical analyses, critically reviewed the manuscript, and approved the final manuscript as submitted.

Anita MacDonald included patients in Birmingham, collected data, co-wrote the manuscript, and approved the final manuscript as submitted.

Francjan J. van Spronsen initiated the study and wrote the study draft, co-wrote the manuscript, and approved the final manuscript as submitted.

Competing Interests

All authors have indicated that they have no financial relationships relevant to this article to disclose.

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RESEARCH REPORT

A Homozygous Mutation in GPT2 Associated with Nonsyndromic Intellectual Disability in a Consanguineous Family from Costa Rica

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Abstract Intellectual disability is a highly heterogeneous disease that affects the central nervous system and impairs patients' ability to function independently. Despite multiples genes involved in the etiology of disease, most of the genetic background is yet to be discovered. We used runs of homozygosity and exome sequencing to study a large Costa Rican family with four individuals affected with severe intellectual disability and found a novel homozygous missense mutation, p. 96G>R, c. 286G>A, in all affected individuals. This gene encodes for a pyridoxal enzyme involved in the production of the neurotransmitter glutamate and is highly expressed in the white matter of brain and cerebellum. Protein modeling of GPT2 predicted that the mutation is located in a loop where the substrate binds to the active site of the enzyme, therefore, suggesting that the catalytic activity is impaired. With our report of a second mutation we fortify the importance of *GPT2* as a novel cause of autosomal recessive nonsyndromic intellectual disability and support the premise that *GPT2* is highly important for the neurodevelopment of the central nervous system.
Synopsis: The mutation p. 96G>R c. 286G>A in GPT2, located in a loop where the substrate binds to the active site of the enzyme, fortifies the importance of GPT2 in the pathogenesis of nonsyndromic intellectual disability.

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Intellectual disability (ID) is a disease that affects the central nervous system and it is diagnosed by three major criteria: IQ less than 70, deficits in at least two adaptive behaviors (communication, personal care, ability to live independently, work, health and/or personal safety) and the symptoms should present themselves before the age 18 (American Psychiatric Association 2013). ID is the most frequent disability in children and young adults (Leonard and Wen 2002). ID can be classified according to the IQ level (mild, moderate, severe, or profound) or according to the etiology of the disease, whether it is known (syndromic) or unknown (nonsyndromic).

Epidemiological studies have determined that about 25% of ID cases have a genetic background and this number increases with the severity of the disease (McLaren and Bryson 1987). Despite that numerous candidate genes have been involved in the etiology of the disease it is noteworthy that for about half of the patients it is still not possible to establish the cause of the disease. Nevertheless, recently, with the combination of exome sequencing and microarray technology it has become possible to identify rare genetic variants associated with this disease especially in consanguineous families.

The aim of this study was to combine autozygosity mapping and exome sequencing to determine the genetic

cause of intellectual disability in a Costa Rican family with four affected siblings and whose parents are first-degree cousins. We report a novel homozygous missense mutation in *GPT2* – a reversible enzyme involved in the production of neurotransmitter glutamate. These findings contribute to elucidating genes that are critical for the adequate functioning of the central nervous system and understanding how mutations in these genes translate in different phenotypes.

Materials and Methods

Following informed consent genomic DNA was extracted from whole blood of all family members using the sucrose-phenol-chloroform method. We first excluded known microdeletions associated with intellectual disability syndromes using MLPA P245-A2 kit and MLPA P297-B2 kit, a Fragile X syndrome and pathogenic CNVs using high-resolution chromosomal Affymetrix Cytoscan HD microarrays (Affymetrix, Santa Clara, California). Then we used polymorphic markers from the Cytoscan HD Arrays to perform a genome scan, followed by a linkage analysis considering an autosomal recessive pattern of inheritance, based on the disease segregation observed in the family. Other parameters were: a disease-gene frequency of 0.0001, uniform marker-allele frequencies and 100% penetrance. 10,000 SNPs were considered, and the EasyLINKAGE-plus v.5® (Hoffmann and Lindner 2005) Allegro® (Gudbjartsson et al. 2005) and Haplopainter® (Thiele and Nürnberg 2005) software were used. In order to make the linkage analysis possible, only data from all the affected individuals and three unaffected siblings were included. Genome positions were taken from the NCBI36/hg18 assembly (Karolchik et al. 2004, https://genome.ucsc.edu/). These SNPs were also taken into account to identify possible runs of homozygosity (ROH). The runs of homozygosity were calculated with PLINK v.1.07. (Purcell et al. 2007, http://pngu.mgh.harvard.edu/purcell/plink/) and were defined as genomic regions larger than 1 Mb with at least 1 SNP every 50 Kb. ROH shared only by affected individuals were considered candidate regions.

We used 5500xl of Applied Biosystems (Applied Biosystems™, Foster City, California) for whole exome sequencing of two of the patients. Only non-synonymous, gain of function, insertions, and homozygous deletions were considered with special attention given to those inside the candidate region. We excluded common polymorphisms reported in dbSNP132, 1000 genome project (February 2012), 5400 Exome sequencing project (NHLBI), and 230 controls from the Institute of Human Genetics in Erlangen. A special attention was given to genes expressed in the central nervous system.

Confirmation and validation were performed through bidirectional direct sequencing, using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI3730 capillary sequencer (Applied Biosystems). Sequence traces were evalutated using the DNAStar software package.

A crystal structure is available for human GPT2 (EC 2.6.1.2). However, this structure was crystallized without substrate and therefore the loops 95–104 and 143–152 are not resolved in the structure. To allow interpretation of the G96R and S153R mutations, the structure of barley GPT2 (PDB code: 3TCM; Duff et al. 2012) was used instead. This structure contains a PLP-cycloserine inhibitor and all active site residues are resolved in the crystal structure. Barley and human GPT2 exhibit 65% sequence similarity and the sites of mutation are conserved between both species. Modeling of the mutations was done with Swiss-PdbViewer (Guex and Peitsch 1997) and RasMol (Sayle and Milner-White 1995) was used for structure analysis and visualization.

Each affected individual was assessed for social competence by a neurologist using Vineland Social Maturity Scale. A complete clinical evaluation was also performed. Due to the severity of phenotype no common IQ evaluations could be performed. Structural brain abnormalities were evaluated by magnetic resonance imaging (MRI).

Results

We report a family with four patients, without any previous perinatal noxa, who could not reach an appropriate social level and had a severe delay in reaching different basic developmental milestones (Table 1). At the neurological exam they showed spastic diplegia, exalted osteotendinous reflexes, and bilateral extensor plantar reflex, suggesting pyramidal dysfunction. The two younger siblings affected had history of one status epilepticus during early childhood and presented a more severe phenotype, according to physical examination (i.e., sialorrhea and aggressive behavior) and lower social quotient (Table 2). Using MRI we found cortical atrophy thinning of corpus callosum and cerebellum and mild cerebellum atrophy; no other major structural findings were detected (Fig. 1).

The linkage analysis identified a locus on chromosome 16, segregating fully with the disease. Maximum two-point LOD scores were obtained for marker SNP_A-2159072 and others in the same region, Z_{max} (0.00) = 3.3803. In addition, we found a ROH on chromosome 16 in all of the affected individuals but not in any of the healthy family members (chr16: 27157613–32483006). Nevertheless, after homozygosity mapping, this region appeared to be more extense chr16: 27177795–48202503 and contained 226 genes of which 56 were noncoding genes (microRNA and pseudogenes) and 11 were reported as expressed in the brain. The WES coverage for this region was 80%, capturing 256 exons corresponding to 171 coding genes. After filtering and validating the exome sequencing results in this region,

Table 1 Vineland Social Maturity Scale measures and summary of important developmental milestones in the patients affected with intellectual disability of the consanguineous Costa Rican family

Characteristics of patients	PZ43	PZ35	PZ33	PZ26
Perinatal history	AGA, no perinatal noxa	AGA, no perinatal noxa	AGA, no perinatal noxa	AGA, no perinatal noxa
Vineline Social Maturity Scale				
Age equivalent (years)	5.6	5.6	3.7	2.3
Estimated social age (years)	22.4	22.4	14.8	9.2
Age of independent walking (years)	>2 (drags feet)	>2 (tiptoes and kicks)	>3 (stagger)	>3 (stagger)
Seizures, age, further treatment	No	No	One status epilepticus at 7 years old, no treatment	One status epilepticus at 8 months old, no treatment
Sialorrhea	No	No	Yes	Yes
Language				
First words age	>5	>5	>5	>5
Simple phrases (age in years)	>10	>10	>10	>10
Comprehension	Limited	Limited	Very limited	Extremely limited
Sphincter control age in years	4	4	6	Failed
Awareness of danger	Yes	Yes	No	No
Aggressive behaviors	No	No	Yes	Yes
Loss of acquired skills	No	No	No	No

AGA appropriate for gestational age

Table 2 Summary of clinical signs found on physical examination in the patients affected with intellectual disability of the consanguineous Costa Rican family

Clinical signs	PZ43	PZ35	PZ33	PZ26
Facial dysmorphism				
Incisors protrusion	+	+	+	+
High arched palate	+	+	+	+
Left double antihelix	–	–	–	+
Adduct thumb	–	Left	Both, pred. left	Both, pred. left
Fine motor skill				
Voluntary use of hand as clamp	+	+	+	+
Cutaneous stigmata				
Hypomelanosis guttata	+	+	+	+
café au lait spots	+	–	–	–
Becker nevus	–	+	–	+
Others				
Mitral systolic murmur	+	–	–	–
Mild right scoliosis		+	+	+
Cranial nerves	NAD	NAD	NAD	NAD
Motor activity				
Spastic diplegia	+	+	+	+
Frequent falls	–	–	–	+
Exalted osteotendinous reflexes	+	+	+	+
Plantar reflex	Bilateral extensor	Bilateral extensor	Bilateral extensor	Bilateral extensor
Cerebellum	NAD	NAD	NAD	NAD

NAD no abnormality detected

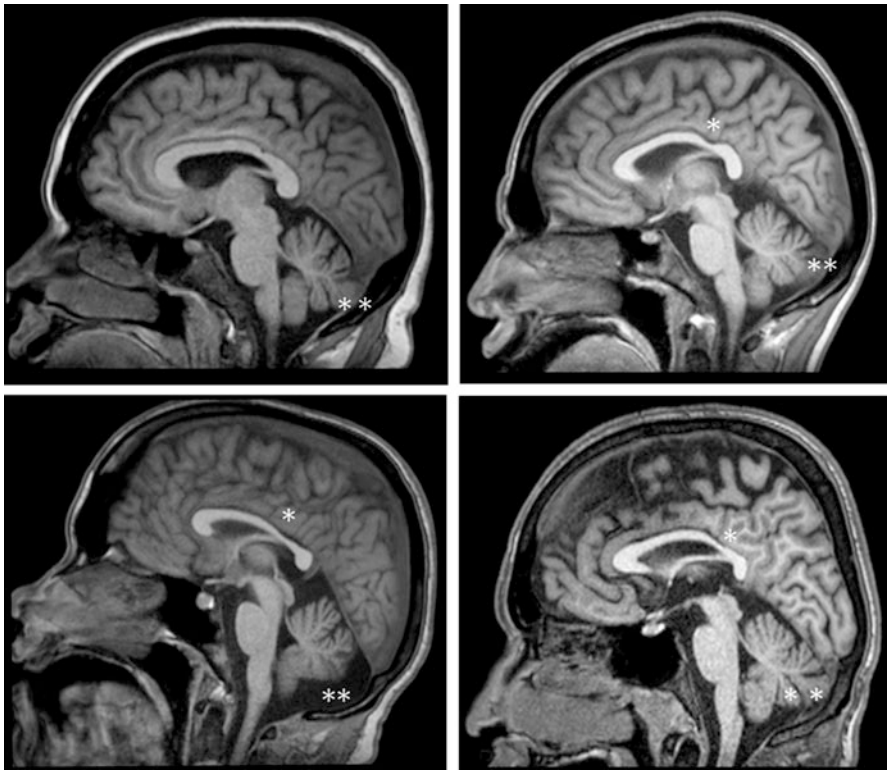

Fig. 1 Magnetic resonance imaging showing thinning of corpus callosum (*white star*) and mild cerebellum atrophy in four affected patients (*two white stars*)

we found three nonsynonymous variants in *ZNF646*, *ZNF629* and *GPT2*. The three genetic changes segregated with the disease in the family. Nevertheless, the genetic variations in *ZNF646* and *ZNF629* had been previously reported in dbSNP database with an allele frequency of 1:2,000 and therefore were considered unlikely as disease causing mutations.

After validation through Sanger sequencing, we found that genes *SMPD3* (Sphingomyelin Phosphodiesterase 3), *SETD1A* (Set domain-containing protein 1A), *TAOK2* (TAO kinase 2) y *KIAA0556* (mRNA not yet characterized) that had heterozygote genetic changes and were in the region of interest were false positives. We also sequenced exon 4 of *SBK1* (*Homo sapiens* SH3-binding domain kinase 1) that was not covered by the NGS and had been previously related to neurodevelopment but did not find any genetic changes.

We found a mutation in exon 5 of *GPT2* (OMIM #616281) that changed a guanine for an adenine (c. 286G>A), which resulted in an amino acid change in position 96 of the protein (p. 96G>R) (Fig. 2). This mutation was detected only once in 121,290 alleles, with a frequency of 0.0000008245. According to the latest version of Exome Aggregation Consortium (ExAC) this single allele was not found in 11,574 Latinos (Lek et al. 2016). The crystal structure of an inhibited GPT2 shows that G96 is in the immediate vicinity of the substrate-binding site and makes contacts with the substrate (Fig. 1a, b). In the G96R variant, the arginine sidechain protrudes into the ligand-binding site and would result in steric clashes with a bound substrate (Fig. 3c) thereby affecting catalytic activity.

Bioinformatics prediction programs (SIFT, Phylop, PolyPhen, and Mutation Taster) determined that this position is evolutionarily highly conserved and that the variant is predicted to be pathogenic. We did not find any pathogenic CNVs or other chromosomal structural or numerical abnormalities associated with the disease.

Discussion

We found a novel mutation, c. 286G>A p. 96G>R, in *GPT2* associated with autosomal recessive severe nonsyndromic intellectual disability in four children of a Costa Rican family of consanguineous marriage. *GPT2* encodes for a pyridoxal dependent enzyme involved in a reversible reaction that transfers an alpha amino group from oxoglutarate to L-alanine and the formation of pyruvate and L-glutamate. Therefore, *GPT2* has an important role in gluconeogenesis, amino acid metabolism and neurotransmitter production (Yang et al. 2002). We suggest that this mutation is the cause of ID in this family.

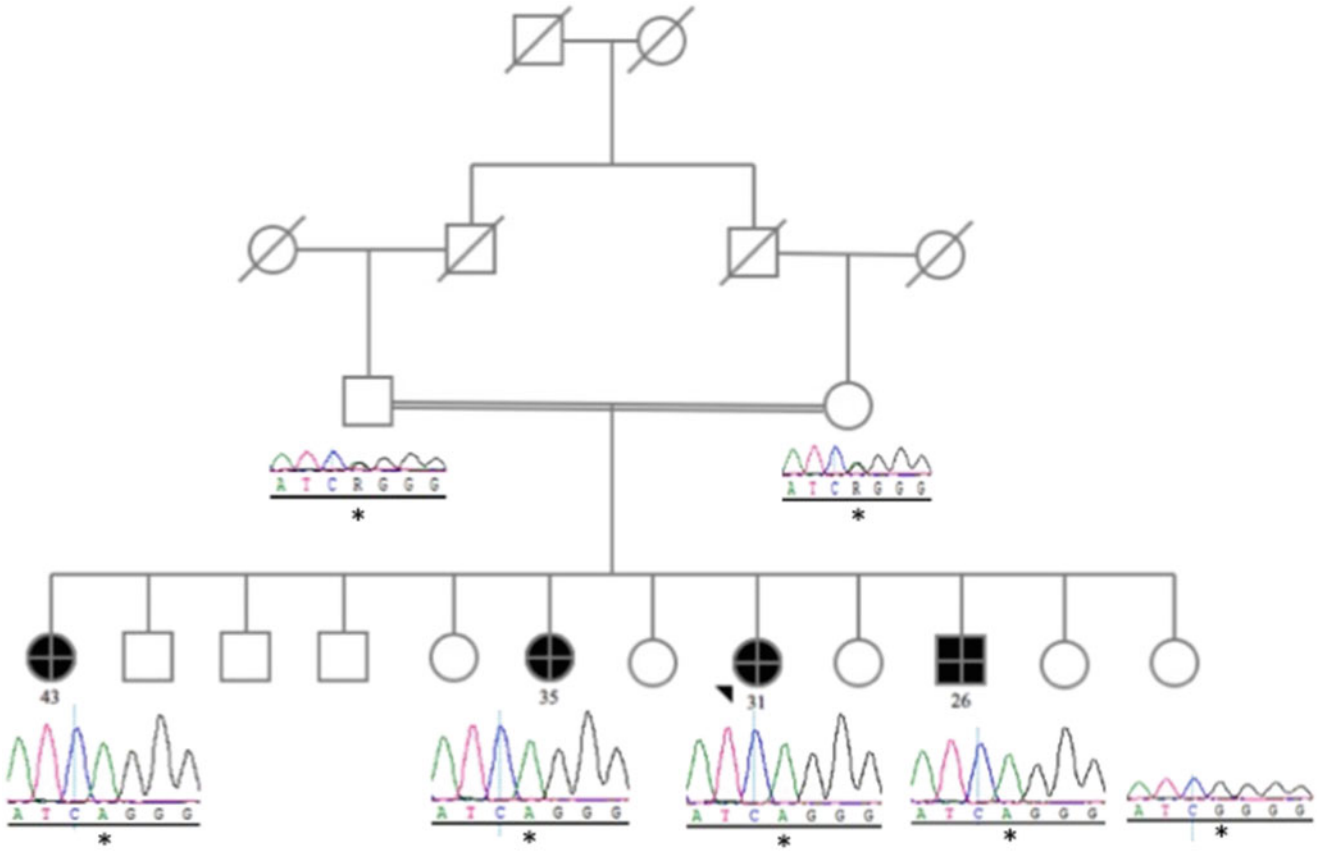

Fig. 2 Family pedigree and location of homozygous recessive mutation c. 286G>A in *GPT2* in four affected patients (*black*), heterozygous in both parents and dominant homozygous in a healthy sibling

GPT2 also known as ALT2 is highly expressed in muscle, adipose tissue, kidney and brain and lower quantities have been found in liver and breast (Yang et al. 2002). The other isoform, GPT1 or ALT1 is expressed in most of the same organs except brain and it is known as an indicator of liver damage. Deregulations in either GPT isoforms have been associated with liver damage (Sherman 1991), obesity (Strauss et al. 2000) and muscle disease (Rutledge et al. 1985).

Recently, a missense homozygous mutation in *GPT2* was found to be associated with severe developmental encephalopathy in a Jewish origin family with three affected siblings (Celis et al. 2015). They present a *GPT2* nonsynonymous homozygous mutation, c. 459C>G, p. 153S>R, associated with static encephalopathy related to severe ID. Celis et al. (2015) reported that their mutation was near a catalytic residue highly conserved in evolution and, after functional assays that mutation resulted in a severe loss of enzymatic function. Inspection of the GPT three-dimensional structure shows that p. 153S>R is located in a loop of one subunit that is placed in close proximity to the active site of the second subunit (Fig. 3a). In the p. 153S>R variant, the longer sidechain of the arginine causes steric clashes with S372 (Fig. 3e) that are not present in the wildtype due to the shorter S153 sidechain (Fig. 3d). The clashes formed in the p. 153S>R variant will cause misfolding of the loop thus offering a structural explanation for the experimentally observed reduced catalytic activity.

In the case of the Costa Rican family, the mutation was also found close to the substrate binding site (Fig. 3a) suggesting that both mutations affect catalytic activity. In contrast to the p. 153G>R variant, the p. 96G>R variant has a direct structural effect on substrate binding by protruding into the active site (Fig. 1c). This might indicate that both mutations affect catalytic activity to a different extent.

When comparing the Celis et al. patients with ours, some different clinical signs could be noticed. Both affected families share clinical signs such as a severe developmental delay, non-verbal language (except for two of the Costa Rican patients) and some clinical findings suggesting pyramidal dysfunction (e.g., brisk reflexes and bilateral extensor plantar responses, high arched palate, and sialorrhea). Nevertheless, when compared to the Costa Rican, Celis et al. family had characteristic facial dysmorphic features (e.g., bifrontal narrowing, hypotelorism, low or rotated set ears and epicanthal folds), microcephaly or

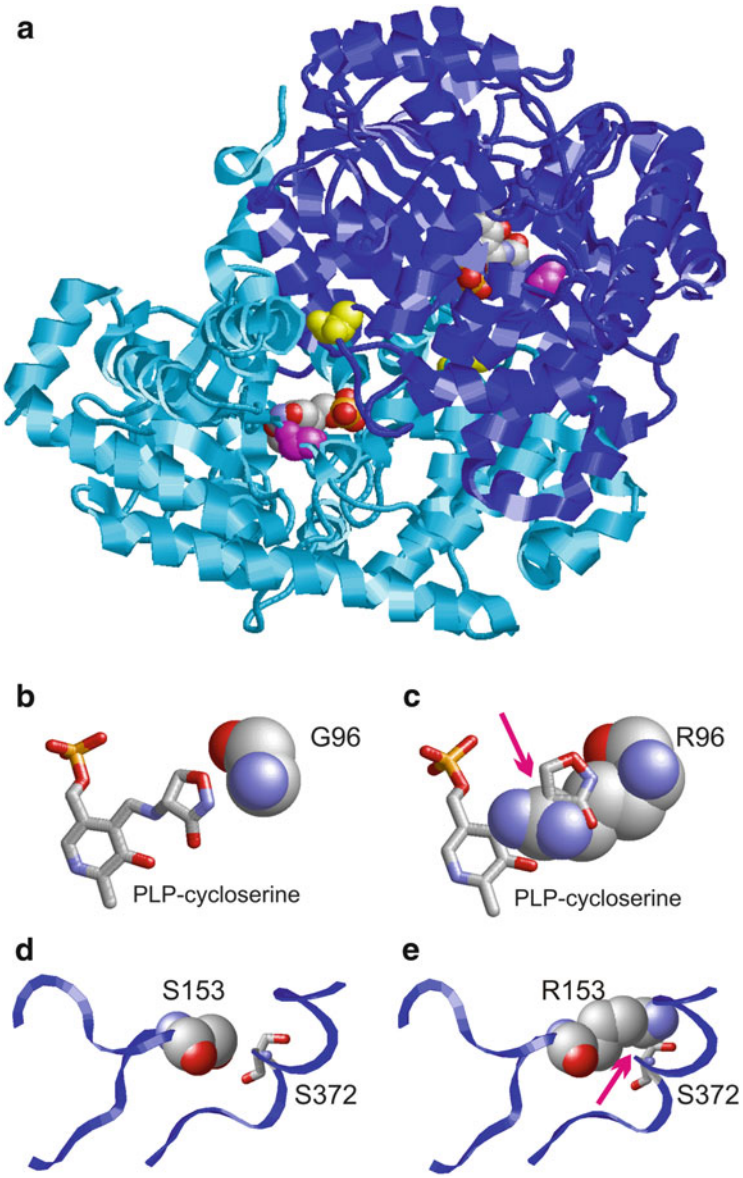

Fig. 3 (**a**) Structure of dimeric GPT2 showing the subunits as *blue* and *cyan* ribbons, respectively. The sites of the p. 96G>R (Costa Rican family) and p. 153S>R (Mizrahi Jewish origin family) exchange are highlighted in *magenta* and *yellow*, respectively. A PLP-cycloserine inhibitor present in the active site is shown in *space-filled* presentation and *colored* according to the atom types. (**b**, **c**) Effect of p. 96G>R variant. (**b**) p. 96G>R is located in close proximity to the substrate binding site and forms interactions with the substrate-analogous inhibitor PLP-L-cycloserine. (**c**) The larger side-chain of arginine causes steric clashes with the substrate which will block substrate access to the active site. (**d**, **e**) Effect of a p. 153S>R variant. (**d**) p. 153S>R is located in a loop in close proximity to S372. (**e**) The larger sidechain of arginine causes steric clashes with S372, which will cause a rearrangement of the loop. Clashes are indicated by *magenta arrows*

dolichocephaly, developmental encephalopathy, seizures, axial hypertonia or hypotonia, and an incapacity to walk and talk. In our family, after and exhaustive clinical evaluation, we found some specific clinical features such as summarized in Table 2. We also found that the severity of the disease progresses with maternal age. The wide spectrum of clinical features in both families suggests that *GPT2* is highly important in brain functioning but much is still to be learnt about the interactions of *GPT2* with other proteins and its role in brain development and physiology.

Glutamate – an excitatory neurotransmitter – is highly abundant in brain and it has been proven to be implicated with different processes involved in complex adaptive functioning: cognition, memory, and learning (McEntee and Crook 1993). Evidence has shown the importance of glutamate availability in brain homeostasis. Numerous neurodegenerative diseases

such as Parkinson, Alzheimer's, epilepsy, Huntington's, multiple sclerosis, and nonsyndromic ID have been associated with modifications in glutamate receptors or free glutamate levels. In our family, we suspect that c. 286G>A is probably affecting *GPT2* enzymatic activity by diminishing the available glutamate in the synaptic cleft and, therefore, causing severe ID.

Our findings of a second family carrying an independent missense mutation in *GPT2* support the hypothesis that homozygous missense mutations near catalytic residues of GPT2 are associated with severe ID. The mutation c. 286G>A p. 96G>R found in our patients probably affects substrate binding to the active site, altering the enzymatic activity, therefore, glutamate metabolism. However, these findings must be interpreted with caution, however, since functional analysis is lacking. Functional studies are required to further confirm the importance of GPT2 in neural development. In addition, regardless of high accuracy of exome sequencing coverage, there are coding regions that are not fully covered by this method. In our study the coverage was 80%, leaving some exons where possible mutations could be queried. Moreover, with the increasing understanding of the importance of noncoding regions in disease pathogenesis, we cannot rule out other mutations might be contributing to the phenotype seen in our family.

In conclusion, this study fortifies the importance of GPT2 in the pathogenesis of ID. Given the severity of this disease, future studies into the molecular pathways in which this protein is involved are of crucial importance for a better understanding of the condition and to find potential targets to improve care for these patients.

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Conflict of Interest

T L-P, H S, S B-L, A E, S U, A R, A L declare that we have no conflict of interest. We declare that this article has not been published previously and all the authors approve its publication.

Authors' Contribution

Tanya Lobo-Prada was involved in the conception design analysis interpretation and drafting of the article. PhD. Alejandro Leal and Prof. André Reis contributed to the conception and design and revised the manuscript critically for important intellectual content. Heinrich Sticht contributed with in silico modeling of the enzyme and contributed critically to the main article draft. Sixto Bogantes did valuable to contributions to the clinical characterization of the patients and revised the manuscript critically. Arif Ekici and Steffen Uebe contributed to analysis and interpretation of the data and revised the article critically.

Guarantor and Corresponding Author

Alejandro Leal accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Details of Ethical Approval

The study was performed as per protocol of the University of Costa Rica's Institutional Review Board.

Patient Consent Statement

All authors declare that informed consent was obtained from all the subjects on this research in accordance with the Code of Ethics of the World Medical Association. The privacy rights of the human subjects were also protected.

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RESEARCH REPORT

Impact of Dietary Intake on Bone Turnover in Patients with Phenylalanine Hydroxylase Deficiency

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Abstract Phenylalanine hydroxylase (PAH) deficiency is a genetic disorder characterized by deficiency of the PAH enzyme. Patients follow a phenylalanine-restricted diet low in intact protein, and must consume synthetic medical food (MF) to supply phenylalanine-free protein. We assessed relationships between dietary intake and nutrient source (food or MF) on bone mineral density (BMD) and bone turnover markers (BTM) in PAH deficiency. Blood from 44 fasted females 11–52 years of age was analyzed for plasma phenylalanine, serum BTM [CTx (resorption), P1NP (formation)], vitamin D, and parathyroid hormone (PTH). BTM ratios were calculated to assess resorption relative to formation (CTx/P1NP). Dual energy X-ray absorptiometry measured total BMD and age-matched Z-scores. Three-day food records were analyzed for total nutrient intake, nutrients by source (food, MF), and compliance with MF prescription. Spearman's partial coefficients (adjusted for age, BMI, energy intake, blood phenylalanine) assessed correlations. All had normal BMD for age (Z-score >-2). Sixty-four percent had high resorption and normal formation indicating uncoupled bone turnover. CTx/P1NP was positively associated with food phenylalanine ($r^2 = 0.39$; p-value $= 0.017$), energy ($r^2 = 0.41$; p-value $= 0.011$) and zinc ($r^2 = 0.41$; p-value $= 0.014$). CTx/P1NP was negatively associated with MF fat ($r^2 = -0.44$; p-value $= 0.008$), MF compliance ($r^2 = -0.34$; p-value $= 0.056$), and positively with food sodium ($r^2 = 0.43$; p-value $= 0.014$). CTx/P1NP decreased significantly with age (p-value $= 0.002$) and higher PTH (p-value $= 0.0002$). Phenylalanine was not correlated with any bone indicator. Females with PAH deficiency had normal BMD but elevated BTM, particularly resorption. More favorable ratios were associated with nutrients from MF and compliance. Younger females had less favorable BTM ratios. Promoting micronutrient intake through compliance with MF may impact bone metabolism in patients with PAH deficiency.

Synopsis: Bone mineral density was normal in 44 females with PAH deficiency; however, bone turnover markers suggested uncoupling of bone resorption and formation, particularly in younger patients. Adequate nutrient intake from medical food and overall medical food compliance may positively impact bone turnover.

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Introduction

Phenylketonuria, also known as phenylalanine hydroxylase (PAH) deficiency (OMIM #261600), is an autosomal recessive inborn error of metabolism (IEM) diagnosed in infants through newborn screening (NBS) (Williams et al. 2008). Patients with PAH deficiency lack sufficient activity of the PAH enzyme (OMIM 612349) due to mutations in the PAH gene. Under normal physiologic circumstances, PAH metabolizes the amino acid phenylalanine to tyrosine. In patients with PAH deficiency, blood phenylalanine can increase to high concentrations and cross the blood-brain barrier, affecting cognitive development in addition to other sequelae (Blau et al. 2010). In addition, deficiency of tyrosine and products of tyrosine metabolism can occur in patients with PAH deficiency.

To prevent the severe effects of high blood phenylalanine and low tyrosine in patients with PAH deficiency, dietary treatment should be implemented immediately. Patients follow a phenylalanine-restricted diet to limit natural (intact) protein. Since protein is required for normal growth and development, patients must consume a synthetic medical formula (medical food) which contains all amino acids in elemental form except phenylalanine. Medical food can supply up to 85–90% of total protein intake in patients with classical PAH deficiency (Camp et al. 2012). Medical food is prescribed to meet 120–140% of patients' total protein needs defined by the Recommended Dietary Allowance (RDA) for age and sex (IOM 2005). Complete medical food also contains other nutrients (carbohydrates, fat, calcium, vitamin D, B12, etc.) that are difficult to consume in adequate amounts with dietary protein restriction (Lammardo et al. 2013). Previous studies have shown that the PAH deficiency diet generally meets and even exceeds requirements for most nutrients (Evans et al. 2014), but there are no reports of nutrient intake by source: food versus medical food. Phenylalanine restriction and medical food are recommended for life in all patients with PAH deficiency (Singh et al. 2014).

Early and continuous dietary treatment of patients with PAH deficiency results in near normal cognitive outcomes (Burgard 2000; Enns et al. 2010). A recent systematic review, however, showed suboptimal outcomes in 140 of 150 studies including at least one primary indicator of health in early treated patients, including low bone mineral density (BMD) (Enns et al. 2010). Since the first report in 1962 of abnormal bone structure in neonates and infants with phenylketonuria, low BMD has been described in male and female patients of all age groups (Feinberg and Fisch 1962). Though inconsistent criteria have been used to define low BMD, osteopenia, and osteoporosis, low BMD may be present in nearly 50% of individuals with PAH deficiency (Modan-Moses et al. 2007). The most recent meta-analysis, however, suggests a much lower prevalence of low BMD of 10% (Demirdas et al. 2015). Although the cause and extent of low BMD is uncertain, low BMD is the precursor to osteoporosis and an increased risk of fractures (Kanis 2002).

Bone mineral density is a good indicator of bone status, but bone turnover markers (BTM) are valuable indicators of dynamic bone remodeling (Lee and Vasikaran 2012). The by-products of bone turnover may provide insight into acute bone metabolism (Wheater et al. 2013) including resorption by osteoclasts and formation by osteoblasts (Naylor and Eastell 2012; Burch et al. 2014). Gold-standard markers of bone resorption (CTx) and formation (P1NP) (Lee and Vasikaran 2012) have not been measured in patients with PAH deficiency, or with factors such as dietary intake, compliance with dietary treatment (phenylalanine restriction and medical food), and blood phenylalanine concentrations. The primary aim of this study was to assess the impact of dietary intake and nutrient source (food versus medical food) on BMD and gold-standard BTM (Lee and Vasikaran 2012) in patients with PAH deficiency. Secondary aims were to assess differences across age categories and correlations among bone biomarkers.

Subjects and Methods

Subjects

Females 11 years of age and older attending a research-based camp for girls and women with PAH deficiency from 2013 to 2015 were eligible for this study. Subjects also had the option to attend camp but not participate in the study. Exclusion criteria included those not participating in camp research, those with a diagnosis of maple syrup urine disease (MSUD), those who were pregnant, and those who the PI did not feel comfortable enrolling (i.e., could not understand the study, abnormalities in pre-camp physical). The study was approved by the Emory University Institutional Review Board (IRB) and data were collected in compliance with Health Insurance Portability and Accountability (HIPAA) guidelines. Phone consents (adults) and assents (<18 years) were performed with a study coordinator to explain all components of the study, including blood collection to dual energy X-ray absorptiometry (DXA) scans. Subjects also had time to ask questions about the study.

Study Design

All subjects attended study visits on the first day of camp at either the Clinical Research Network (>18 years of age) at Emory University Hospital or Children's Healthcare of Atlanta – Egleston Pediatric Research Center (<18 years). Seasonality was consistent for all participants since all visits occurred during the summer in Atlanta, Georgia. All subjects

were fasting for at least two and a half hours before study visits. A history and physical was performed and research nursing staff assessed anthropometrics, vitals and collected urine and blood samples. Urine pregnancy tests were completed and documented as negative prior to performing DXA scans. A trained phlebotomist drew venous blood for all blood samples and a research laboratory technician performed preliminary processing and aliquoting of the samples.

Dietary Intake

Three-day diet records were submitted by all subjects and reviewed individually with a metabolic Registered Dietitian (RD) to ensure completeness and accuracy. If subjects did not bring a completed 3-day food record to camp, the RD performed a 24-h recall. All food records and recalls were analyzed by a single RD using Nutrition Data System for Research (NDSR) (NCC 2011). All foods and medical foods that were missing from the database were requested through the NDSR administrators at the University of Minnesota, Nutrition Coordinating Center. The 3 days of dietary intake were averaged to calculate median daily intake of each nutrient. Median nutrient intake from medical food (not including low-protein foods) over the 3-day record was also calculated. Median nutrient intake from medical food was subtracted from median total nutrient intake to calculate median nutrient intake from food sources.

The following nutrients were investigated based on known or proposed relationship with bone health: energy, fat, carbohydrates, protein, vitamin A, vitamin D, vitamin B12, calcium, phosphorus, magnesium, iron, zinc, selenium, sodium, and cholesterol. Nutrient intakes were compared to age- and gender-specific Dietary Reference Intakes (DRI) (Institute of Medicine (U.S.) Subcommittee on Interpretation and Uses of Dietary Reference Intakes et al. 2000) to assess adequacy of reported dietary intake. For nutrients with an established RDA, total nutrient intake was compared to RDA as percent RDA. For nutrients without an established RDA, nutrient intake was compared to the Adequate Intake (AI) as percent AI. For each nutrient, the percent of RDA met from food sources and the percent of RDA met from medical food was also calculated.

DXA Scans

All study subjects had a dual energy X-ray absorptiometry (DXA) scan at the Winship Cancer Institute by the same technician on a GE Lunar Prodigy machine. DXA measured bone mineral content (BMC, g), bone mineral density (BMD, g/cm^2), and body composition [fat mass (g), % fat mass, lean mass (g), % lean mass]. BMD Z-scores were calculated by DXA software to compare BMD to the average for a healthy person of the same age and sex. International Society for Clinical Densitometry (ISCD) standards recommend using Z-scores to assess BMD in pediatric subjects, pre-menopausal women, and men under 50 years of age (ISCD 2013a, b). Using ISCD recommendations, subjects were categorized as having normal BMD (Z-score >-2) or low BMD (Z-score <-2) (ISCD 2013a, b).

Laboratory Samples

Plasma amino acids were analyzed at Emory Genetics Laboratory via ion exchange chromatography. Aliquots of serum were sent on dry ice to Maine Medical Center Research Institute to measure bone turnover markers [bone-specific alkaline phosphatase (BALP), C-terminal telopeptide of type 1 collagen (CTx), pro-peptide of type 1 collagen (P1NP), vitamin D (25-hydroxyvitamin D) and intact parathyroid hormone (PTH)] using Immunodiagnostic System's (IDS) multi-discipline automated system (IDS-iSYS). The system, one of only two validated methods to accurately measure CTx and P1NP, uses a single sample of serum to measure all five markers using chemiluminiscence techniques (Cavalier et al. 2012a, b; Morovat et al. 2013). CTx and P1NP were selected as the two best markers of resorption and formation, respectively, based on a literature review by the International Osteoporosis Foundation and the International Federation for Clinical Chemistry in 2013 (Johansson et al. 2014). CTx and P1NP have diurnal variation with concentrations peaking in the morning, thus all blood samples were collected between 8:30 a.m. and 12:00 p.m. to minimize variation (Qvist et al. 2002).

Other Variables

Subjects also completed a bone health questionnaire to assess previous fractures, medications, concurrent conditions that could affect bone, vitamin D and multivitamin supplement use, and pubertal status [pre-menstrual, menstrual (regular or irregular), or post-menopausal]. All participants also completed surveys on demographics, medical information, current dietary and medical food prescriptions, and health insurance.

Statistical Analysis

All statistics were performed using Statistical Analysis Software (SAS) version 9.4 (SAS 2013). Since the study was a cross-sectional pilot analysis, a power calculation was not performed. The primary dependent variables included: CTx, P1NP, BMD and BMD Z-score and the

primary independent variables included: median nutrient intake by source (total, food, and medical food) and plasma phenylalanine. A BTM ratio (CTx/P1NP) was calculated to assess the relative degree of bone resorption to formation (Fardellone et al. 2014) with a higher ratio indicating higher resorption compared to formation. Normality of each variable was assessed using the Shapiro-Wilk test. If outliers in any primary dependent variables were identified during exploratory analyses, statistical tests were performed including and excluding the outlier and compared. If results were similar, outliers were retained in final analyses.

Descriptive statistics including prevalence and 95% confidence intervals (CI) were calculated for the following categories: age based on DRI-defined groups [11–13, 14–18, >19 years (Ryan-Harshman and Aldoori 2006)], BMI [underweight <18.5, normal weight 18.5–24.9, overweight 25–29.9, and obese >30 (kg/m^2)], menstrual status (premenstrual, regular menstrual cycle, and irregular menstrual cycle), pharmaceutical use [no medications, treatment with Kuvan, treatment with pegylated ammonia-lyase (PEG-PAL)], reported fracture or break (yes or no), and BMD Z-score category [normal (>−2) or low (<−2)]. Continuous variables including dietary intake by source and biomarkers (plasma phenylalanine, vitamin D, PTH, BALP, CTx, and P1NP) were described by mean and standard deviation (or median and interquartile range for non-normally distributed variables). Biomarkers were also compared to clinical reference ranges for females by age group and determined to be low, normal, or high. For biomarkers found to be higher or lower than the reference range, mean difference and mean percent difference from the reference range were calculated using the upper or lower limit as appropriate.

Paired t-tests (or Wilcoxon signed rank tests for non-parametric data) were performed to determine if nutrient intake from food differed from nutrient intake from medical food for each nutrient. Nutrients with greater than 50% of daily intake from medical food were noted. Pearson's (or Spearman's for non-parametric data) partial correlation coefficients were calculated to examine associations between nutrient intake by source and BTM and BMD, adjusting for age, BMI, total energy intake, and plasma phenylalanine. Pearson's or Spearman's partial correlation coefficients were also calculated to examine associations between medical food prescription and medical food compliance (% of prescription consumed) and dietary phenylalanine prescription and dietary phenylalanine compliance (% of prescription consumed), and BTM and BMD. A correlation matrix was also constructed to examine relationships between all bone-related parameters included in this study (CTx, P1NP, CTx/P1NP ratio, BALP, PTH, vitamin D, BMD, and BMD Z-score), adjusting for age and BMI.

To compare independent variables (CTx, P1NP, CTx/P1NP, BMD, BMD Z-score) by age category, each non-normal variable was first log-transformed. Linear regression was then used to control for BMI and blood phenylalanine. Tukey's adjustment for multiple comparisons was applied (criterion p-value <0.01) to calculate significant differences by age category.

Results

Study Subjects

Over 3 years of Metabolic Camp, 97 females were eligible for the study. The following participants were excluded: 9 (9%) had MSUD; 20 (21%) did not choose to participate in research, and 24 (25%) had already enrolled in the study in 2013 or 2014. Forty-four females with PAH deficiency were included in the final study, ranging from 11 to 54 years of age (Table 1). Fifteen (34%) were overweight or obese. Ninety-three percent had reached menarche, though 46% of these women reported an irregular cycle. The majority of subjects (n = 34, 77%) had phenylalanine concentrations above the therapeutic range, defined as 120–360 μmol/L (2–6 mg%) (Singh et al. 2014), even though 16 (36%) were taking phenylalanine-lowering pharmaceuticals. Nearly half (n = 20) reported at least one previous fracture.

All subjects had normal BMD for age (Z-score >−2). Nine (20%) had a BMD Z-score between −1 and −2. CTx, marker of bone resorption, was above normal (Cavalier et al. 2012a, b) in 81% (n = 34) of the sample while P1NP, marker of bone formation, was normal in 83% (n = 35) (Cavalier et al. 2012a, b). Normal reference ranges have not been established for CTx or P1NP by assay manufacturers, thus comparisons were made to two studies calculating mean CTx and P1NP by age groups and 95% confidence intervals to designate "high" (above the upper limit), "normal" (within 95% CI) and "low" (below the lower limit). On average, CTx was 0.79 ng/mL (342%) above the upper limit of the 95% confidence interval. The majority of the sample (n = 27; 64%) had high CTx and normal P1NP indicating a possible uncoupling of normally coupled bone turnover.

Dietary Intake

Table 2 shows dietary intake of individual nutrients including source (food or medical food). Total intakes of protein, vitamin D, calcium, and zinc were predominantly from medical food (>50% of total intake). Patients reported a median medical food prescription of 45.0 g of protein

Table 1 Characteristics of females with PAH deficiency ($n = 44$)

	Mean (SD)	*N*	Percent	95% CI
Demographics				
Age (years)	20.1 (10.0)			
11–13		7	15.9	4.7, 27.2
14–18		25	56.8	41.6, 72.1
>19		12	27.3	13.6, 41.0
BMI (kg/m^2)	25.5 (8.9)			
Underweight (<18.5)		2	4.5	0, 11.0
Normal (18.5–24.9)		27	61.4	46.4, 76.3
Overweight (25–29.9)		8	18.2	6.3, 30.0
Obese (>30)		7	15.9	4.7, 27.2
BMD Z-score Category	0.045 (0.141)			
Normal (>−1)		35	79.5	67.1, 92.0
Between −1 and −2		9	20.5	8.0, 32.9
Low for chronological age (<−2)		0	0	–
Laboratory measures				
Plasma phenylalanine (μmol/L)	777.3 (424.7)			
Below treatment range (<120)		2	4.5	0, 11.0
Normal (120–360)		8	18.2	6.3, 30.0
Above treatment range (>360)		34	77.3	64.4, 90.2
Serum 25-hydroxyvitamin D (ng/mL)	37.6 (14.3)			
Normal (>30)		28	66.7	51.8, 81.5
Insufficient (20–30)		12	28.6	14.3, 42.8
Deficient (<20)		2	4.8	0.0, 11.5
Carboxyterminal telopeptide of type I collagen (CTx) (ng/mL)[a]	0.947 (0.68)			
Low for age		0	0	–
Normal for age		8	19.0	6.7, 31.4
High for age		34	81.0	68.6, 93.3
Aminoterminal propeptide of type I collagen (P1NP) (ng/mL)[b]	171.5 (200.5)			
Low for age		1	2.4	0, 7.2
Normal for age		35	83.3	71.6, 95.1
High for age		6	14.3	3.2, 25.3

[a] CTx indicates active bone resorption; 95% confidence intervals of mean CTx concentrations (ng/mL) in females (Cavalier et al. 2012a, b): 10–<12 years (0.388–1.094), 12–<14 years (0.082–0.650), 14–<16 years (0.538–0.300), 16–<18 years (0.0251–0.148), 18–<20 years (0.0241–0.118), premenopausal women (0.03–0.66)

[b] P1NP indicate active bone formation; 95% confidence intervals of median P1NP concentrations (ng/mL) in females (Morovat et al. 2013): 9–12 years (386–1,070), 13–16 years (59.3–672), 17–20 years (25.2–160), premenopausal women (13.7–71.1)

(IQR 28.4–56.6) per day and a median phenylalanine allotment of 450 mg per day (IQR 75–825). Median intake of most nutrients exceeded the RDA/AI by an average of 147% (excluding cholesterol intake). Only median vitamin D and cholesterol intake fell below the RDA/AI (75% of RDA and 5% of AI, respectively). Thirty-six percent ($n = 16$) of subjects reported taking a multivitamin and/or vitamin D supplement, in addition to their prescribed medical food.

Differences in source of nutrients were compared between those under age 18 and adults. A higher percent of vitamin D came from medical food in the younger age group compared to the older age group (76% versus 52%; p-value $= 0.04$); all other nutrients were comparable. Though median vitamin D intake was 75% of the RDA, 80% of subjects' vitamin D intake fell below the RDA of 600 IU/day, two-thirds of whom were in the youngest age group (Ross 2011).

Table 2 Intake of individual bone-related nutrients and source from 3-day food records

	Total intake		Food intake		Medical food intake		
	Median (IQR)	% RDA/AI	Median (IQR)	% RDA/AI	Median (IQR)	% RDA/AI	*p*-value[a]
Macronutrients							
Total energy (kcal)	1,532 (531)	–	1,139 (554)	–	277 (423)	–	<0.01
Fat (g)	50.7 (35.9)	–	35.6 (24.3)	–	4.9 (19.6)	–	<0.01
Carbohydrates (g)	218.7 (70.9)	168 (55)	187.5 (60.7)	141 (41)	21.0 (44.2)	27 (34)	<0.01
Protein (g)[b]	56.8 (34.0)	126 (61)	17.6 (20.0)	54 (50)	30.4 (34.8)	92 (101)	0.13
Micronutrients							
Vitamin A (IU)	4,810 (4,350)	219 (183)	2,685 (2,597)	120 (158)	1,452 (2,513)	91 (115)	<0.01
Vitamin D (mcg)[b,c]	11.2 (10.4)	75 (69)	0.6 (3.4)	5 (27)	6.4 (9.8)	61 (72)	<0.01
Vitamin B12 (mcg)[b]	5.1 (5.7)	213 (238)	0.8 (2.2)	27 (49)	2.9 (6.1)	153 (254)	<0.01
Calcium (mg)[b]	1,235 (984)	102 (82)	255 (424)	24 (36)	655 (1,103)	79 (88)	<0.01
Phosphorus (mg)	1,240 (801)	110 (80)	443 (283)	43 (50)	581 (930)	77 (82)	0.16
Magnesium (mg)	391 (252)	110 (82)	134 (83)	39 (29)	164 (300)	77 (81)	0.11
Iron (mg)	20.9 (15.6)	142 (111)	6.7 (5.9)	53 (52)	8.6 (16.8)	87 (111)	0.21
Zinc (mg)[b]	16.3 (16.3)	191 (195)	3.4 (3.4)	41 (45)	8.4 (17.3)	136 (197)	<0.01
Selenium (mcg)	70.1 (51.0)	128 (114)	27.8 (35.0)	61 (89)	27.4 (43.5)	78 (84)	0.74
Sodium (mg)	2,800 (1,244)	187 (83)	1,868 (1,081)	121 (52)	425 (1,125)	43 (73)	<0.01
Cholesterol (mg)[d]	14.4 (37.6)	5 (13)	14.4 (37.7)	5 (13)	0 (0)	0 (0)	<0.01

[a] Wilcoxon signed rank test, unadjusted differences between mean intake from food and medical food
[b] >50% of intake from medical food
[c] 5 mcg of dietary vitamin D is equivalent to 200 International Units (IU)
[d] Cholesterol based on recommended intake of <300 mg/day (no defined AI/RDA) (IOM 2005)

Correlation Between Nutrient Intake and Bone Markers

Table 3 shows correlations between nutrient intake by source and bone indicators adjusted for age, BMI, plasma phenylalanine, and total energy intake. P1NP (bone formation) was correlated positively with food energy ($r^2 = 0.44$), and negatively with total magnesium intake ($r^2 = -0.43$) and medical food compliance ($r^2 = -0.53$). CTx/P1NP was positively correlated ($p < 0.05$) with energy ($r^2 = 0.41$), zinc ($r^2 = 0.41$), and phenylalanine ($r^2 = 0.39$) from food sources. CTx/P1NP was negatively correlated ($p < 0.01$) with medical food fat intake ($r^2 = -0.44$) and positively with sodium intake from food ($r^2 = 0.43$). BMD was negatively correlated with cholesterol from food ($r^2 = -0.42$), despite the low reported median. BMD Z-score was marginally positively correlated with sodium intake from food ($r^2 = 0.39$, p-value $= 0.014$), but no other nutrient. Dietary phenylalanine intake was not associated ($p > 0.01$) with BTM, BMD, or Z-score.

Differences Across Age Categories

As a secondary aim, we examined variables by age group given the wide range of age in the sample (Table 4). BMI significantly increased with age (p-value <0.01), by nearly 10 kg/m^2 from the youngest group to the oldest group. As expected, BMD was higher in the oldest group compared to the youngest group (mean = 1.150 g/cm^2 vs 1.008 g/cm^2, p-value = 0.016).

All BTM (BALP, CTx, and P1NP) decreased with age (p-value <0.0001), as expected based on the physiological decrease in bone turnover with aging (Adami et al. 2008). CTx/P1NP ratio also decreased significantly with age (p-value = 0.0014) suggesting younger patients have a higher degree of uncoupling in the direction of higher resorption, that is opposite to normal bone metabolism in adolescent females (Levine 2012). CTx decreased by 77% from the youngest to the oldest age group while P1NP decreased by 90%, indicating P1NP decreases more over time than CTx in patients with PAH deficiency. Blood phenylalanine was also significantly different over age categories with those in the middle age group (14–18 years), having higher concentrations than both their younger and older peers.

Associations Between Bone Biomarkers

As a final aim, we examined associations between biomarkers, adjusting for age, BMI, and plasma phenylalanine. CTx and P1NP had a strong negative association with age ($r^2 = -0.72$ and -0.84, p-value <0.001) and a marginal

Table 3 Source of nutrient intake and spearman's partial[a] correlation coefficients with bone turnover markers, BMD and BMD Z-score

	CTx (ng/mL)		P1NP (ng/mL)		CTx/P1NP		BMD		BMD Z-score	
	r^2	*p*-value	r^2	*p*-value	r^2	*p*-value	r^2	*p*-value	r^2	*p*-value
Total energy (kcal)	0.14	0.39	*0.35*	*0.03*	0.16	0.34	−0.22	0.17	−0.09	0.57
Medical food	−0.19	0.36	−0.13	0.45	−0.28	0.08	0.11	0.49	−0.05	0.75
Food	*0.33*	*0.047*	**0.44**	**0.006**	*0.41*	*0.011*	*−0.39*	*0.015*	0.01	0.94
Total fat (g)	−0.15	0.37	−0.13	0.45	−0.24	0.16	−0.05	0.76	0.24	0.15
Medical food	−0.26	0.13	−0.28	0.10	**−0.44**	**0.008**	0.24	0.14	0.04	0.81
Food	0.02	0.89	−0.04	0.80	0.09	0.60	−0.31	0.056	0.22	0.18
Total carbohydrates (g)	0.24	0.16	0.24	0.15	0.14	0.42	−0.14	0.41	0.08	0.64
Medical food	−0.26	0.12	−0.32	0.057	*−0.37*	*0.03*	0.26	0.12	−0.02	0.93
Food	*0.38*	*0.02*	*0.35*	*0.03*	*0.36*	*0.03*	−0.25	0.14	0.12	0.47
Total protein (g)	−0.14	0.42	*−0.39*	*0.019*	−0.19	0.28	0.10	0.55	−0.23	0.17
Medical food	−0.25	0.14	*−0.39*	*0.017*	*−0.33*	*0.047*	0.20	0.23	−0.06	0.70
Food	0.19	0.26	0.03	0.87	0.25	0.14	−0.15	0.36	−0.06	0.71
Vitamin A (IU)	0.03	0.84	−0.09	0.58	−0.02	0.92	0.28	0.09	−0.20	0.24
Medical food	−0.21	0.21	−0.30	0.08	*−0.37*	*0.03*	0.29	0.09	0.04	0.81
Food	0.13	0.46	0.02	0.91	0.13	0.46	0.11	0.50	−0.17	0.31
Total vitamin D (mcg)	0.07	0.69	−0.12	0.49	−0.10	0.57	0.17	0.31	0.02	0.90
Medical food	−0.10	0.56	−0.25	0.14	−0.24	0.15	0.30	0.07	0.01	0.94
Food	0.18	0.28	0.05	0.77	0.29	0.09	−0.04	0.82	0.00	0.98
Total vitamin B12 (mcg)	−0.07	0.69	−0.20	0.25	−0.27	0.12	0.25	0.14	0.11	0.52
Medical food	−0.22	0.20	−0.27	0.12	*−0.37*	*0.02*	0.28	0.09	0.06	0.73
Food	0.07	0.68	−0.06	0.71	0.11	0.52	−0.24	0.14	0.19	0.25
Calcium (mg)	−0.15	0.39	*−0.34*	*0.04*	*−0.35*	*0.04*	0.15	0.36	−0.10	0.54
Medical food	−0.22	0.21	*−0.34*	*0.04*	*−0.36*	*0.03*	0.28	0.08	0.01	0.96
Food	0.15	0.38	−0.06	0.71	0.04	0.83	−0.24	0.14	−0.10	0.53
Iron (mg)	−0.04	0.81	−0.28	0.10	−0.21	0.22	0.26	0.12	0.08	0.64
Medical food	−0.21	0.22	−0.32	0.06	*−0.34*	*0.045*	0.28	0.08	0.02	0.90
Food	0.29	0.09	0.10	0.55	0.26	0.12	−0.14	0.39	0.17	0.32
Magnesium (mg)	−0.14	0.42	**−0.43**	**0.008**	−0.27	0.10	0.21	0.20	−0.12	0.48
Medical food	−0.21	0.22	*−0.36*	*0.03*	*−0.34*	*0.04*	0.24	0.15	0.01	0.97
Food	0.21	0.22	−0.08	0.63	0.26	0.13	−0.03	0.89	−0.11	0.52
Phosphorus (mg)	−0.15	0.38	*−0.35*	*0.03*	−0.28	0.10	0.24	0.15	−0.12	0.47
Medical food	−0.22	0.20	−0.35	0.04	*−0.35*	*0.03*	0.30	0.07	0.00	0.99
Food	0.23	0.17	0.09	0.61	0.27	0.12	−0.24	0.15	0.01	0.94
Selenium (mcg)	0.05	0.78	−0.09	0.60	−0.06	0.73	0.11	0.51	0.08	0.63
Medical food	−0.19	0.26	*−0.34*	*0.04*	*−0.33*	*0.049*	0.26	0.11	0.04	0.83
Food	0.32	0.06	0.16	0.34	*0.35*	*0.03*	−0.16	0.34	0.09	0.59
Zinc (mg)	−0.05	0.78	−0.21	0.23	−0.23	0.17	0.27	0.11	0.01	0.97
Medical food	−0.23	0.18	*−0.33*	*0.05*	*−0.34*	*0.04*	0.28	0.08	0.02	0.91
Food	*0.40*	*0.016*	0.24	0.16	*0.41*	*0.014*	−0.18	0.28	0.05	0.75
Sodium (mg)	0.13	0.46	0.15	0.38	0.07	0.71	−0.03	0.86	*0.32*	*0.048*
Medical food	−0.23	0.18	−0.30	0.07	*−0.38*	*0.02*	0.26	0.11	0.04	0.80
Food	*0.34*	*0.046*	0.28	0.10	**0.43**	**0.008**	*−0.33*	*0.04*	*0.39*	*0.014*
Cholesterol (mg)	0.21	0.21	0.31	0.059	0.21	0.23	*−0.41*	*0.011*	−0.03	0.86
Medical Food	–	–	–	–	–	–	–	–	−0.00	0.98
Food	0.21	0.21	*0.32*	*0.046*	0.20	0.24	**−0.42**	**0.008**	−0.02	0.91

(continued)

Table 3 (continued)

	CTx (ng/mL)		P1NP (ng/mL)		CTx/P1NP		BMD		BMD Z-score	
	r^2	p-value	r^2	p-value	r^2	p-value	r^2	p-value	r^2	p-value
Phenylalanine (mg)	0.31	0.07	0.29	0.08	*0.39*	*0.02*	−0.20	0.22	0.02	0.91
Medical food	–	–	–	–	–	–	–	–	–	–
Food	0.31	0.06	0.30	0.08	*0.39*	*0.017*	−0.21	0.21	0.02	0.90
Medical food prescription (g)	0.02	0.91	0.10	0.56	0.22	0.21	0.13	0.48	−0.06	0.75
Medical food compliance (%)	−0.25	0.16	**−0.53**	**0.001**	−0.34	0.056	0.16	0.38	−0.10	0.60
Phe prescription (mg)	0.16	0.37	−0.05	0.77	0.06	0.72	−0.12	0.51	0.18	0.32
Phe compliance (%)	0.06	0.75	0.22	0.23	0.16	0.38	−0.18	0.32	−0.00	0.99

Correlations significant at $p < 0.05$ in *italics*; Correlations significant at $p < 0.001$ *bolded*
[a] Correlation coefficients adjusted for age, BMI, total calorie intake, and blood phenylalanine

Table 4 Differences in key variables by age group in females with PAH deficiency

	Age 11–13 ($n = 7$) Mean (SD)	Age 14–18 ($n = 25$) Mean (SD)	Age >19 ($n = 12$) Mean (SD)	p-value[d]
Demographics				
Body mass index (kg/m^2)	22.8 (5.1)[a]	23.1 (5.7)[a]	32.2 (12.4)[b]	0.0054
Bone mineral density (g/cm^2)	1.008 (0.111)[a]	1.090 (0.094)	1.150 (0.096)[b]	0.0920
BMD Z-score	−0.428 (1.163)	−0.276 (1.009)	0.400 (1.065)	0.5705
Percent with fracture or break	57.1%	36.0%	58.3%	0.28
Biomarkers				
Phenylalanine (μmol/L)	470 (252)[a]	871 (415)[c]	762 (464)[b]	0.1875
25-hydroxyvitamin D (ng/mL)	28.2 (9.2)	40.3 (14.6)	36.4 (14.8)	0.1934
Parathyroid hormone (pg/mL)	29.6 (15.5)	25.7 (8.6)	32.1 (14.9)	0.7746
Bone-specific alkaline phosphatase (μg/L)	84.1 (59.4)[b]	29.4 (18.9)[a]	20.6 (7.6)[a]	<0.0001
C-terminal cross-linking telopeptide of type I collagen (ng/mL)	1.88 (0.86)[c]	0.95 (0.52)[b]	0.43 (0.23)[a]	<0.0001
Procollagen type I N propeptide (ng/mL)	546.4 (288.9)[c]	133.8 (89.2)[b]	52.7 (24.3)[a]	<0.0001
CTx/P1NP ratio	0.075 (0.036)[b]	0.042 (0.035)	0.017 (0.012)[a]	0.0014

[a] significantly less than b
[a] significantly less than c
[b] significantly less than c
[d] Mantel-Haenszel Chi-Square test with Tukey's post-hoc p-value adjustment, controlling for BMI and plasma phenylalanine

association with BMI ($r^2 = -0.31$ and -0.32, p-value <0.05). Blood 25-hydroxyvitamin D negatively correlated with PTH concentration ($r^2 = -0.40$, p-value = 0.01) as expected, but no other biomarkers. Bone turnover markers CTx, P1NP and BALP had strong positive correlations; the strongest correlation was between CTx and P1NP ($r^2 = 0.74$, p-value <0.0001). The ratio of CTx/P1NP had a strong negative association with age ($r^2 = -0.55$, p-value = 0.002) and PTH ($r^2 = -0.59$, p-value = <0.0001), adjusting for BMI and blood phenylalanine. Blood phenylalanine and BMD Z-score had no significant correlation with any marker of bone metabolism.

Discussion

In this sample of females with PAH deficiency, dietary intake of most nutrients exceeded RDA/AIs, but nutrients related to bone health (protein, vitamin D, calcium, zinc and vitamin B12) were consumed predominantly through medical food. Nearly two in three had normal or low P1NP (formation) but high CTx (resorption) indicating an uncoupling of bone turnover. Younger patients had a less favorable turnover ratio, with higher resorption compared to formation, than their older counterparts. BTM was impacted by dietary intake, but not blood phenylalanine, and nutrients from medical food correlated

to more favorable BTM ratio than nutrients from food sources. Higher intake of food phenylalanine correlated to a BTM ratio favoring resorption (p-value = 0.017) while better compliance with medical food correlated to a BTM ratio favoring formation (p-value = 0.056) suggesting following diet is critical.

DXA showed normal BMD for age in all subjects and a mean total BMD Z-score of 0.045 (range −1.9–2.3) contradicting low BMD found in previous studies (Carson et al. 1990; Zeman et al. 1999; Perez-Duenas et al. 2002; Koura et al. 2011) but in line with more recent reports (Demirdas et al. 2015; Geiger et al. 2016). We defined low BMD by ISCD criteria (Z-score <−2) instead of alternative definitions used in previous studies (Demirdas et al. 2015). Prevalence of previous fractures, though cause was not assessed, was higher compared to population estimates for adolescent females (44% vs an estimated 19.4–33.9%) (Chevalley et al. 2012; Faje et al. 2014) and adult females (47.4% vs 25.3%) (Young et al. 2011), and much higher than the 20% fracture rate reported in a systematic review of males and females with PAH deficiency (Hansen and Ney 2014). Though BMD was normal in all subjects, uncoupled bone turnover, particularly in younger patients, and high prevalence of vitamin D insufficiency, still suggests potential abnormalities in bone health and vitamin D status in females with PAH deficiency.

We found higher bone turnover was associated with nutrients from food, and lower bone turnover was associated with nutrients from medical food and compliance. Since nutrients are likely co-correlated, we emphasize significant findings indicate an overall correlation between multiple nutrients in medical food and bone turnover. We hypothesize medical food compliance is associated with a more favorable BTM ratio because its high content of nutrients improves P1NP suppression. P1NP is a fragment of collagen released by osteoblastic cells as collagen is incorporated into the bone matrix, and an indicator of bone formation (Wheater et al. 2013). Increasing medical food compliance may increase P1NP more than CTx, decreasing CTx/P1NP ratio, promoting bone formation and the attainment of peak bone mass in adolescents or maintaining BMD in adults.

BMD and BMD Z-scores were not associated with any nutrient except for BMD's negative relationship with cholesterol intake from food. Other studies also find no correlations between dietary calcium, protein, and energy intake and BMD, BMD Z-score or BTM in patients of any age (Carson et al. 1990; McMurry et al. 1992; Hillman et al. 1996; Modan-Moses et al. 2007; Lage et al. 2010). Positive correlations have been reported, however, between total calcium and phosphorus intake and BMD Z-score (Perez-Duenas et al. 2002) and calcium and protein from medical food and spine BMD (Geiger et al. 2016). None of these investigations controlled for total energy intake (Willett et al. 1997).

The ratio of bone resorption to formation decreased with age and PTH concentration suggesting uncoupling is more pronounced in younger patients and those with lower PTH. Our findings are opposite of the expected relationship between CTx/P1NP and aging. Bone formation should exceed bone resorption prior to attaining peak bone mass in late teenage years. A small sample size could limit the interpretation of this finding, but we believe the higher degree of uncoupling in younger patients warrants further investigation.

The inverse relationship between PTH and CTx/P1NP ratio, even after adjustment for age, BMI, and plasma phenylalanine, is difficult to interpret. PTH was positively correlated to P1NP in subjects under age 18 but not in adults. Thus, different sets of factors could influence bone turnover in adults compared to children with PAH deficiency including vitamin D concentrations. Eighty-eight percent of adolescents in this study did not meet the RDA for vitamin D and 83% of those with insufficient blood vitamin D were children or adolescents. Given the propensity for younger girls to have higher bone resorption to formation ratios and lower vitamin D concentrations than adults, this may be a particularly high-risk group for developing low BMD and even osteoporosis later in life.

Blood phenylalanine was not significantly correlated with any blood biomarker, but moderately positively correlated with CTx (r^2 = 0.27, p-value = 0.09). A single in vitro study found higher phenylalanine concentrations were associated with higher circulating osteoclast precursor cells (Roato et al. 2010) and that T cells, indicators of immune activation, were activating the precursor cells into mature osteoclasts (Teitelbaum 2000). Assessing immune activation with BTM could provide further insight into relationships with blood phenylalanine in future studies.

Our sample of patients with PAH deficiency included a cross-section of females only. Results are not generalizable to males with PAH deficiency, or females under 11 years of age. Subjects were attending a camp for Emory Genetics Clinic patients or were recommended by clinics familiar with Metabolic Camp and may have been more invested in managing their disorder compared to other patients. The nature of this sample may have produced better compliance rates with diet and potentially better bone health. Bias could also exist in 3-day food records as subjects may have changed their usual diet to appear more compliant or recorded false food items and false compliance with medical food (median compliance rate with medical food prescription was 99%). Only 28% of subjects reported a medical food intake of less than 50% of their prescription, but we believe a higher percent were non-compliant. Cause of previous fractures was not assessed and future studies must examine if fractures are caused by typical mechanisms including falls, collisions, and trauma (Hedstrom et al. 2010) or result from low BMD in females with PAH deficiency. Finally, a control group was not

included, thus population-specific reference values were used for comparisons when available.

In future studies, we recommend investigating BTM with other more accessible measures of bone status such as a quantitative heel ultrasound (QUS), in addition to DXA (Faulkner et al. 1994). We also recommend assessing menstrual status and hormone levels in association with bone health. Finally, prospective cohort studies are needed to assess the relationship between bone turnover markers and long-term risk of developing low BMD, fractures and/or osteoporosis. Finally, since key nutrients are coming predominantly from synthetic medical food, not whole foods in most patients with PAH deficiency, bioavailability of nutrients from medical food must be examined.

Adhering to the Genetic Metabolic Dietitians International (GMDI) recommendation to monitor BMD by DXA regularly in PAH deficiency is suggested (Singh et al. 2014). Twenty percent of subjects had a BMD Z-score below −1, of which long-term clinical implications are unknown. Assessing CTx and P1NP in regular clinical practice could be useful if longitudinal studies show BTM can predict fracture risk. Patients who are not compliant with medical food may be at-risk for nutrient deficiencies and less favorable bone metabolism. Patients should be encouraged to consume medical food as prescribed to promote normal bone turnover and minimize uncoupling and excess resorption.

In conclusion, patients with PAH deficiency may have uncoupled bone turnover. Younger patients and those with low PTH appear to have BTM ratios favoring resorption. Optimal dietary intake, particularly medical food compliance and adequate micronutrient intake, likely impacts bone turnover more than metabolic control in patients with PAH deficiency.

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KEC and RHS designed research; KEC, EIF, and RHS conducted research; KEC, VT, and PWW analyzed data; KEC wrote the paper; KEC and RHS had primary responsibility for final content. All authors read and approved the final manuscript.

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Conflict of Interest Statement

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RESEARCH REPORT

Hypogonadotropic Hypogonadism in Males with Glycogen Storage Disease Type 1

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Abstract *Background*: Glycogen storage disease type 1 is an autosomal recessive disorder with an incidence of 1 in 100,000. Long-term complications include chronic blood glucose lability, lactic academia, short stature, osteoporosis, delayed puberty, gout, progressive renal insufficiency, systemic or pulmonary hypertension, hepatic adenomas at risk for malignant transformation, anemia, vitamin D deficiency, hyperuricemic nephrocalcinosis, inflammatory bowel syndrome (type 1b), hypertriglyceridemia, and irregular menstrual cycles. We describe hypogonadotropic hypogonadism as a novel complication in glycogen storage disease (GSD) type 1.

Case Studies and Methods: Four unrelated patients with GSD 1a ($N = 1$) and 1b ($N = 3$) were found to have hypogonadotropic hypogonadism diagnosed at different ages. Institutional Research Ethics Board approval was obtained as appropriate. Participant consent was obtained. A retrospective chart review was performed and clinical symptoms and results of investigations summarized as a case series.

Results: All patients were confirmed biochemically to have low luteinizing hormone (LH) and follicular stimulating hormone (FSH), and correspondingly low total testosterone. Clinical symptoms of hypogonadism varied widely. Investigations for other causes of hypogonadotropic hypogonadism were unremarkable. In addition, all patients were found to have disproportionately low bone mineral density at the lumbar spine compared to the hip. Common to all patients was erratic metabolic control, including recurrent hypoglycemia and elevated lactate levels.

Discussion: Recurrent elevations in cortisol in response to hypoglycemia may be the underlying pathology leading to suppression of gonadotropin-releasing hormone (GnRH) release. Incorporating clinical and/or biochemical screening of the hypothalamic–pituitary–gonadal axis may be important in the management of this disease. Testosterone therapy however needs to be carefully considered because of the risk of hepatic adenomas.

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Introduction

Glycogen storage disease type 1 (GSD1) is a rare autosomal recessive disorder (Bali et al. 2013) classified into two major subtypes: 1a caused by deficiency in glucose-6-phosphatase

activity and 1b caused by a defect in glucose-6-phosphate (G6P) translocase. These defects result in decreased conversion of G6P to glucose, leading to hypoglycemia, lactic acidemia, organomegaly, and dyslipidemia. Long-term complications include short stature, osteoporosis, delayed puberty, gout, progressive renal insufficiency, systemic or pulmonary hypertension, hepatic adenomas at risk for malignant transformation, anemia, vitamin D deficiency, hyperuricemic nephrocalcinosis, and irregular menstruation (Bali et al. 2013). Patients with GSD1b may develop neutropenia, inflammatory bowel disease, and autoimmune thyroid disease (Bali et al. 2013).

Case 1

A 46-year-old male with a diagnosis of GSD1a prior to age 5 had a history of recurrent hypoglycemia and hyperlactatemia with hypoglycemic unawareness and poor metabolic control which was thought to be related in part to noncompliance with dietary recommendations including duration of fasting.

His clinical course was complicated by biopsy-proven hepatic adenomas, first diagnosed at age 23, measuring 2.5 and 2.8 cm with slow progressive growth. Most recently at age 45, CT imaging showed a 2.3 cm and several subcentimeter lesions consistent with adenomas which were unchanged for 4–5 years.

His first bone mineral density (BMD) at age 29 showed low BMD at the lumbar spine (LS) (Z-score −3.54, BMD 0.73 g/cm^2) relative to hip (Z-score −0.97, BMD 0.89 g/cm^2). With this disproportionate pattern in bone loss, hypogonadism was considered. Investigations revealed low-normal total testosterone at 12.1 nmol/L (10–30) with normal LH 3 IU/L (<10.0) and FSH 3.4 IU/L (<10.0). The patient denied erectile dysfunction and declined testosterone supplement at this point.

Repeat BMD at age 31 was unchanged but because his bone mass placed him at moderate to high risk of fracture, etidronate was discussed and started at age 32. After 1 year of etidronate, LS BMD increased (0.964 g/cm^2, Z-score −2.3), and hip BMD was unchanged (0.912 g/cm^2, Z-score −1.1). He continued etidronate and repeat BMD every 3–4 years was stable. Most recently at age 45, L1–L4 BMD was 0.992 g/cm^2, Z-score −2.0, and hip BMD was 0.941 g/cm^2, Z-score −1.1. The disproportionate pattern in bone density is still present.

Around age 43, he developed erectile dysfunction but declined testosterone therapy. Repeat investigations showed gradual testosterone decrease: at age 43, 44 and 45, total testosterone was 7.6, 5.3 and 5.1 nmol/L, respectively, with inappropriately normal FSH and LH (Table 1). Pituitary MRI at age 44 was normal and evaluation of his other pituitary hormones revealed no other deficiencies (Table 1). Ferritin and iron saturation were normal. Hemoglobin was low at 123 g/L (131–169) with an mean corpuscular volume (MCV) of 88 fL (80–95).

Case 2

An 18-year-old male was diagnosed with GSD1b at 3 weeks of age. He had erratic metabolic control, with recurrent

Table 1 Laboratory results

Test	Case 1	Case 2		Case 3	Case 4	Normal
		Age 12	Age 18			
Total testosterone	3.4	1.6	4.4	5.0	2.1	8.4–28.8 nmol/L
FSH	4.0	2.7	6.6	4.4	2.4	<10.0 IU/L
LH	7.0	0.3	7.8	6.1	0.7	<10.0 IU/L
AM cortisol	700	527	636	–	252	>500 nmol/L
Cortisol post-ACTH stimulation	–	–	–	–	637	>500–550 nmol/L
Prolactin	17.0	10.3	10.3	6.1	–	4.0–15.0 μg/L
TSH	2.0	1.74	1.72	2.1	–	0.30–5.50 mU/L
Free T4	12.0	9.6	12.0	11.9	12.0	11–22 pmol/L
Ferritin	134	171	171	134	58	15–300 μg/L
IGF-1	–	4.3 nmol/L (N 24–68)	–	–	14 μg/L (N 82–807)	–
GH post-stimulation	–	6.6 μg/L (N >5.6)	–	–	13.0 μg/L (N >8)	–
Iron saturation	0.29	–		0.29	–	0.20–0.55

N normal; *AM* morning; *ACTH* Adrenocorticotropic hormone

hypoglycemic symptoms, including a hypoglycemic seizure. He is currently managed with Glycosade® at night but compliance with other recommended aspects of dietary therapy and home blood glucose monitoring is incomplete and he has symptoms suggestive of hypoglycemia, which respond to eating, and persistent hyperlactatemia.

At age 12 years, growth hormone (GH) stimulation test with arginine and clonidine was normal although his IGF-1 was low for his age. LH, FSH, and testosterone were consistent with his pre-pubertal clinical status (Table 1). Other pituitary hormones were normal. At age 12 years 11 months, growth velocity was 4.2 cm/year but by 13 years and 6 months, fell to 1.7 cm/year. He did undergo spontaneous puberty and by age 13 years 11 months was noted to be Tanner stage 3 pubic hair and stage 2 genitalia, with a growth velocity of 7.0 cm/year. However, at age 14 years and 6 months, growth velocity decreased to 0.6 cm/year and pubertal progression was stalled so GH therapy was initiated given his low IGF-1 with improvement in linear growth (9.5 cm/year) and pubertal progression to Tanner stage 5 by age 17. Of note, he did have a longstanding history of low bone mass. At age 9, L1-L4 BMD was 0.475 g/cm^2 (Z-score −1.7) and total body less head (TBLH) BMD 0.745 g/cm^2 (Z-score −1.0). He suffered a T8 compression fracture at age 7 and had received IV pamidronate between age 9 and 14. At age 11, BMD at all sites was >−2.0. At the conclusion of his linear growth, L1–L4 BMD was 0.844 g/cm^2 (Z-score −1.5), TBLH BMD 1.130 g/cm^2 (Z-score −0.9), and TH BMD was 1.130 g/cm^2 (Z-score +0.2). GH was discontinued at age 18 years, as his growth rate had plateaued for 3 years. Most recently at age 17 LS BMD was 0.844 g/cm^2 (Z-score −1.5) and TH BMD 1.130 g/cm^2 (Z-score 0.2).

At age 18, biochemical testing confirmed hypogonadotropic hypogonadism; other pituitary hormones and ferritin were normal (Table 1). Hemoglobin was low at 87 g/(MCV of 81 fL). MRI revealed a normal pituitary gland. At age 19, testosterone cypionate was started at 50 mg IM every 2 weeks IM with improvement in energy. A hepatic lesion was first seen at age 15 on CT, measuring 1.4 × 1.5 × 1.7 cm but not seen on follow-up MRI at age 18.

Case 3

A 24-year-old male diagnosed with GSD1b in infancy had a history of recurrent hypoglycemia and inflammatory bowel disease (presumed to be GSD1b-related), requiring recurrent hospitalization. His current GSD management includes nocturnal elemental feeds. He is known to have multiple hepatic adenomas stable on serial imaging. At age 19, a 6 mm hypodensity, and a 2.1 × 2.1 cm focus were seen by CT. MRI at age 23 showed multiple liver lesions with the largest measuring 2.4 × 1.6 cm, a second lesion of 1.2 cm, and at least 7 subcentimeter lesions, consistent with adenomas which were unchanged at follow-up imaging 1 year later.

BMD at age 23 showed low bone mass, with lower BMD at the lumbar spine (L1–L4 BMD 0.748 g/cm^2, Z-score −2.8) relative to hip (BMD 0.739 g/cm^2, Z-score −2.1). In addition, he had short stature, measuring 151 cm (<1st percentile), decreased body and facial hair, and he reported low energy. At the same time, investigations revealed a low total testosterone and inappropriately normal FSH and LH. Prolactin, TSH, ferritin, and iron saturation were normal (Table 1). Testosterone cypionate therapy was initiated at 100 mg IM every 2 weeks with improvement in energy and well-being.

Case 4

A 30-year-old male with GSD1b diagnosed in infancy has had a history of erratic metabolic control with persistent hyperlactatemia. He is currently managed with frequent meals. Over the course of his life, his GSD has been complicated by unilateral renomegaly (stable over time), hepatomegaly, and hepatic adenomas.

When he was investigated at the age of 12 years for poor growth, IGF-1 and IGF-binding protein 3 (IGF-BP3) were low, but GH stimulation testing was appropriate. LH and FSH were low with correspondingly low total testosterone, at pre-pubertal levels. ACTH stimulation test was adequate and free T4 was normal (Table 1). Ferritin was normal, with a hemoglobin 114 (130–170 g/L) with an MCV of 94.7 fL (80–100).

Ongoing monitoring for clinical signs of puberty continued until age 16 and 17, but he remained pre-pubertal so at 17 years, testosterone was initiated with testosterone cypionate 50 mg monthly. After 1 year, height velocity was 10 cm/year with progression of puberty (Tanner stage 3 pubic hair and genitalia, coarsening facial hair). Testosterone was increased by 50 mg every year to 200 mg monthly and he reached Tanner stage 5 at age 22. Liver enzymes remained stable. Hepatic imaging at age 27 and 28 showed multiple focal lesions which remained stable in size in both lobes of liver (measuring 2.1 × 1.5 cm, and 1.3 × 0.9 cm, and several subcentimeter lesions), consistent with adenomas.

Low bone mass was also noted. At age 19, he had a low LS BMD (LS 0.39 g/cm^2, Z-score −4.1) and TBLH (0.761 g/cm^2, Z-score −3.4, 71st percentile). At age 21 he had a disproportionately low LS BMD at 0.845 g/cm^2 (Z-score −3.3), in comparison to his TH BMD of 0.856 g/cm^2 (Z-score −1.8). Over the years, this gap closed with his most recent LS BMD at LS 0.945 g/cm^2 (Z-score −2.3) and TH BMD 0.789 g/cm^2 (Z-score −2.3) at age 27.

Discussion

We present four patients with suboptimal metabolic control of GSD and hypogonadotropic hypogonadism. Within minutes of onset of hypoglycemia, there is a decrease in insulin secretion and an increase in glucagon and epinephrine secretion in a homeostatic effort to increase serum glucose levels by promoting delivery of substrates to the liver for gluconeogenesis, increasing hepatic glycogenolysis, and inhibiting peripheral utilization of glucose. Following this immediate response, cortisol and growth hormone are secreted to further promote gluconeogenesis and reduce rates of glucose utilization (Santiago et al. 1980).

Cortisol is known to suppress both the GH/IGF-1 axis and the hypothalamic–pituitary–gonadal axis (Gaillard and Wehrenberg 1996). Specifically in GSD1, poor linear growth has been documented caused by GH deficiency which improves with GH therapy. This abnormality may be related to the counterregulatory response, specifically recurrent surges in cortisol, in response to hypoglycemia (Noto et al. 2003; Wolfsdorf et al. 1999; Dunger et al. 1982). In untreated GSD1 patients, serum cortisol levels are elevated, up to 810 nmol/L, and subsequently decrease with a glucose load (Dunger et al. 1982). It is known that hypercortisolemia can lead to hypogonadism. Patients with Cushing syndrome have decreased LH and FSH levels (Luton et al. 1977) and even short-term hydrocortisone therapy in normally menstruating women results in suppression of gonadotropin levels (Saketos et al. 1993).

These physiological processes provide a basis for our hypothesis that recurrent hypoglycemia in GSD1 patients could potentially contribute to hypogonadotropic hypogonadism. In GSD1 patients with suboptimal metabolic control, hypogonadism may be secondary to chronic recurrent elevations of cortisol in response to hypoglycemia, with resulting suppression of GnRH, LH, and FSH release (Gore et al. 2006; Breen and Karsch 2006).

Clinical symptoms of hypogonadism in males include delayed puberty, decreased libido and spontaneous erections, gynecomastia, small testes, infertility, hot flushes, and low BMD (Bhasin et al. 2010).

As seen in our patients, hypogonadism leads to a preferential loss of trabecular bone. Two types of bone exist: cortical and trabecular. Cortical bone (such as the hip or pelvis) is dense and compact, forming the outer layer of most bones. Trabecular bone (such as the vertebrae and femoral neck) forms the inner layer and is honey-comb like, thereby providing flexibility. Importantly, trabecular bone has a higher surface area to mass ratio and is the site of frequent remodeling. Therefore, in hypogonadism, greater loss of BMD occurs at sites with predominantly trabecular bone than with predominantly cortical bone (Khosla et al. 2008). Acute losses in testosterone and therefore estradiol, as seen in patients with prostate cancer treated with androgen deprivation therapy, leads to greater losses in trabecular bone than cortical bone (Greenspan et al. 2005). Bone loss in male hypogonadism is primarily due to loss of estradiol (produced by aromatization of testosterone) which accounts for about 70% of the effects on bone metabolism in men and testosterone itself is 30% of the effect (Clarke and Khosla 2010). Estradiol suppresses production of bone-resorptive mediators such as receptor activation of nuclear factor kB ligand (RANKL), thereby promoting the formation of resorption pits on bone surfaces (Clarke and Khosla 2010).

Other factors also contribute to bone loss, both cortical and trabecular, to varying degrees. Elevated cortisol such as glucocorticoid therapy will also preferentially affect trabecular bone. Aging also leads to trabecular bone loss, although later in life, cortical bone loss also occurs due to increased cortical porosity (Bousson et al. 2001). Parathyroid (PTH) excess leads to cortical bone loss and may be anabolic for trabecular bone (Duan et al. 1999). Primary hyperparathyroidism has been shown to predominantly affect cortical bone loss with preservation of trabecular bone (Silverberg et al. 1989). Secondary hyperparathyroidism such as renal disease, vitamin D or calcium deficiency, will affect both cortical and trabecular bone as will malnutrition, such as protein (Weaver and Heaney 2013; Giannini et al. 1997).

Less specific findings of hypogonadism include fatigue, depression, anemia, reduced muscle mass, and increased body fat. Androgen deficiency is defined as a persistently low morning total testosterone level (Bhasin et al. 2010). Once confirmed, measuring LH and FSH will distinguish between a primary cause (high LH and FSH) and a secondary cause (low or inappropriately normal LH and FSH). If secondary hypogonadism, investigations should include iron studies to rule out hemochromatosis, prolactin, TSH, and free T4 to assess for pituitary dysfunction, and pituitary imaging to exclude a pituitary adenoma (Bhasin et al. 2010). Other less common etiologies include tuberculosis and sarcoidosis. If primary hypogonadism is suspected, then karyotype should be performed to rule out Klinefelter syndrome or other underlying genetic or chromosomal abnormality (Bhasin et al. 2010).

Hypogonadism itself can potentially exacerbate complications of GSD, such as osteoporosis, and therefore it is important to consider treating this. Treatment with testosterone is directed at restoring levels to normal.

Replacement results in development or maintenance of secondary sexual characteristics, increases in libido, lean body mass, muscle strength, and BMD. Therapeutic options include intramuscular injections, oral and transdermal preparations. Overall, rates of venous thromboembolism and cardiovascular events are not significantly elevated compared to placebo (Baillargeon et al. 2015; Corona et al. 2014); however, when

choosing amongst the different routes of administration, there is suggestion that oral testosterone, but not intramuscular or transdermal testosterone, may increase cardiovascular risk (relative risk = 2.20, 95% CI: 1.45–3.55, $p = 0.015$) (Borst et al. 2014). Most recent studies indicate that testosterone therapy does not increase the risk of prostate cancer, but long-term follow-up data are not yet available and testosterone therapy is considered contraindicated in men with advanced prostate cancer (Fernandez-Balsells et al. 2010). Given that GSD1 patients are at risk for hepatic adenomas with the potential for malignant transformation, testosterone therapy should be carefully monitored. Androgen receptors are expressed in the liver, and androgens can stimulate hepatocyte proliferation. Although rare, androgens have been shown in observational studies to induce adenoma development (Giannitrapani et al. 2006). Testosterone levels should be assessed for response 2–3 months after therapy is initiated.

To our knowledge, this is the first case series describing hypogonadotropic hypogonadism in patients with glycogen storage disease. Based on our observations ongoing assessment of the hypothalamic–pituitary–testicular axis function is important in managing such patients as treatment is available and may impact long-term outcome.

Take-Home Message

Hypogonadotropic hypogonadism is an important complication in glycogen storage disease (GSD) type 1 and incorporating clinical and/or biochemical screening of the hypothalamic–pituitary–gonadal axis may be important in the management of this disease.

Details of the Contributions of Individual Authors

All authors have been involved in the conception and design, analysis and interpretation of data, and drafting and revision of the article for important intellectual content.

Name of One Author Who Serves as Guarantor for the Article

SS accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

Competing Interests

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The patients have consented to the publication of their medical information.

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RESEARCH REPORT

Widespread Expression of a Membrane-Tethered Version of the Soluble Lysosomal Enzyme Palmitoyl Protein Thioesterase-1

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Abstract "Cross-correction," the transfer of soluble lysosomal enzymes between neighboring cells, forms the foundation for therapeutics of lysosomal storage disorders (LSDs). However, "cross-correction" poses a significant barrier to studying the role of specific cell types in LSD pathogenesis. By expressing the native enzyme in only one cell type, neighboring cell types are invariably corrected. In this study, we present a strategy to limit "cross-correction" of palmitoyl-protein thioesterase-1(PPT1), a lysosomal hydrolase deficient in Infantile Neuronal Ceroid Lipofuscinosis (INCL, Infantile Batten disease) to the lysosomal membrane via the C-terminus of lysosomal associated membrane protein-1 (LAMP1). Tethering PPT1 to the lysosomal membrane prevented "cross-correction" while allowing PPT1 to retain its enzymatic function and localization in vitro. A transgenic line harboring the lysosomal membrane-tethered PPT1 was then generated. We show that expression of lysosome-restricted PPT1 in vivo largely rescues the INCL biochemical, histological, and functional phenotype. These data suggest that lysosomal tethering of PPT1 via the C-terminus of LAMP1 is a viable strategy and that this general approach can be used to study the role of specific cell types in INCL pathogenesis, as well as other LSDs. Ultimately, understanding the role of specific cell types in the disease progression of LSDs will help guide the development of more targeted therapeutics.
One Sentence Synopsis: Tethering PPT1 to the lysosomal membrane is a viable strategy to prevent "cross-correction" and will allow for the study of specific cellular contributions in INCL pathogenesis.

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Introduction

Lysosomal storage diseases are monogenic diseases that affect many cell types and present with a complex clinical phenotype. Understanding the role of individual cell types in the disease process will allow for the rational development of effective therapies. However, "cross-correction" interferes with the ability to address basic biological questions in a complex setting.

Inter-cellular trafficking of lysosomal enzymes, "cross-correction," forms the foundation of therapeutic approaches to treat lysosomal storage disorders (Neufeld and Fratantoni 1970; Sands and Davidson 2006). Soluble lysosomal enzymes are glycosylated and phosphorylated, then transported to the lysosome via the mannose-6-phophate receptor (M6P/IGFIIr) (Neufeld et al. 1977; Dahms et al. 1989). A small proportion of lysosomal enzymes escapes the canonical mannose-6-phosphate pathway and is secreted into the intercellular space. The secreted enzymes can be endocytosed by adjacent cells via the plasma membrane-

localized M6P/IGFIIr and trafficked to the lysosome (Neufeld 1980; Dahms et al. 1989).

Deficiency of the lysosomal hydrolase palmitoyl-protein thiosesterase-1 (PPT1) results in infantile neuronal ceroid lipofuscinosis (INCL) (Vesa et al. 1995; Gupta et al. 2001; Verkruyse and Hofmann 1996). Human INCL and PPT1-deficient mice have progressive deficits in vision, motor function, and cognition (Macauley et al., 2009; Griffey et al., 2005; Santavuori et al. 1973; Dearborn et al. 2015). INCL histology reveals autofluorescent storage material (AFSM), neuronal death, glial activation, and neuroinflammation, implicating a variety of cell lineages in the pathogenesis (Haltia et al. 1973; Bible et al. 2004; Kielar et al. 2007; Macauley et al. 2009, 2011). Proteomic and metabolomic studies in INCL mice also demonstrated that PPT1 deficiency affects a variety of cell types (Woloszynek et al. 2007; Khaibullina et al. 2012; Tikka et al. 2016). To understand the relative contribution of each cell type to the disease process, it is necessary to prevent "cross-correction."

In this study, we tethered PPT1 to the C-terminus of lysosome-associated membrane protein-1 (LAMP1). The C-terminus of LAMP1 is necessary and sufficient for lysosomal targeting (Guarnieri et al. 1993; Rohrer et al. 1996). To target specific lineages, we employed the Cre-lox system. We report that membrane-tethered PPT1 retains enzyme activity in vitro and prevents the biochemical, histological, and behavioral phenotype of murine INCL. Unfortunately, cell specificity was lost due to rearrangement of the transgene. Nonetheless, this study demonstrates a proof-of-principle that this general methodology is a viable approach to address questions regarding the role of specific cell types in the progression of INCL.

Materials and Methods

PPT1-LAMP1 Transgenic Mouse and Husbandry

Transgenic animals were generated by microinjection of the transgene directly into C57Bl/6 embryos (Transgenic Knockout Microinjection Core, Washington University). Transgenic animals were crossed onto the PPT1−/− background (also on the congenic C57Bl/6 background) until PPT1−/− homozygosity. Congenic C57BL/6 wild-type and PPT1−/− mice were used as controls (Griffey et al. 2004). All procedures were carried out in accordance with an approved IACUC protocol from Washington University School of Medicine.

Chimeric PPT1-LAMP1 and Lentiviral Expression

The chimeric PPT1-LAMP1 expression plasmid includes the promoter and first intron of the chicken β-actin gene, the human PPT1 cDNA, a six-glycine linker, the 120 bp sequence encoding the transmembrane domain and lysosomal localization sequence of LAMP1 followed by a rabbit β-globin polyadenylation signal (Fig. 1d). The transgene is identical to the expression plasmid except that it contains a loxP-STOP-loxP sequence (L-S-L) from pBS302 (Addgene, Cambridge, MA).

Third-generation SIN lentiviral vectors were created for in vitro experiments (Dull et al. 1998; Zufferey et al. 1998). Briefly, the lentivirus contained the phosphoglycerate kinase (PGK) promoter, the target gene, an SV40 polyadenylation site, and a puromycin resistant cassette. The target gene contained wild type hPPT1 (LV-WT) or hPPT1-LAMP1 (LV-PPTLAMP). The empty lentiviral vector did not contain a target gene (LV-Empty).

Cross-Correction Experiments

PPT1−/− murine fibroblasts were plated, transduced, and then selected with puromycin. Untransduced PPT1−/− murine cells were plated on transwell inserts (Corning, Corning, NY) in a separate dish prior to transfer into the experimental plate. Three days post co-incubation, PPT1 enzyme activity was measured. "Cross-correction" experiments using primary dermal fibroblasts from F42 mice were performed similarly.

Genomic DNA Isolation and Sequencing

Genomic DNA from brain and liver was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The recombined transgene was sequenced using primers flanking the loxP-STOP-loxP sequence (PNACL, Washington University). Copy number was determined by qPCR on genomic DNA using primers flanking the human PPT1 and LAMP1 cDNA junction and calculated as previously described (Joshi et al. 2008).

SDS-PAGE/Western Blot

Western blots were performed on homogenates from transduced cells or primary fibroblast as previously described (Benitez et al. 2015). Membranes were probed for human PPT1 (Abcam 89022; 1:1,000) or the cytoplasmic domain of LAMP1 (Sigma L1418; 1:1,000).

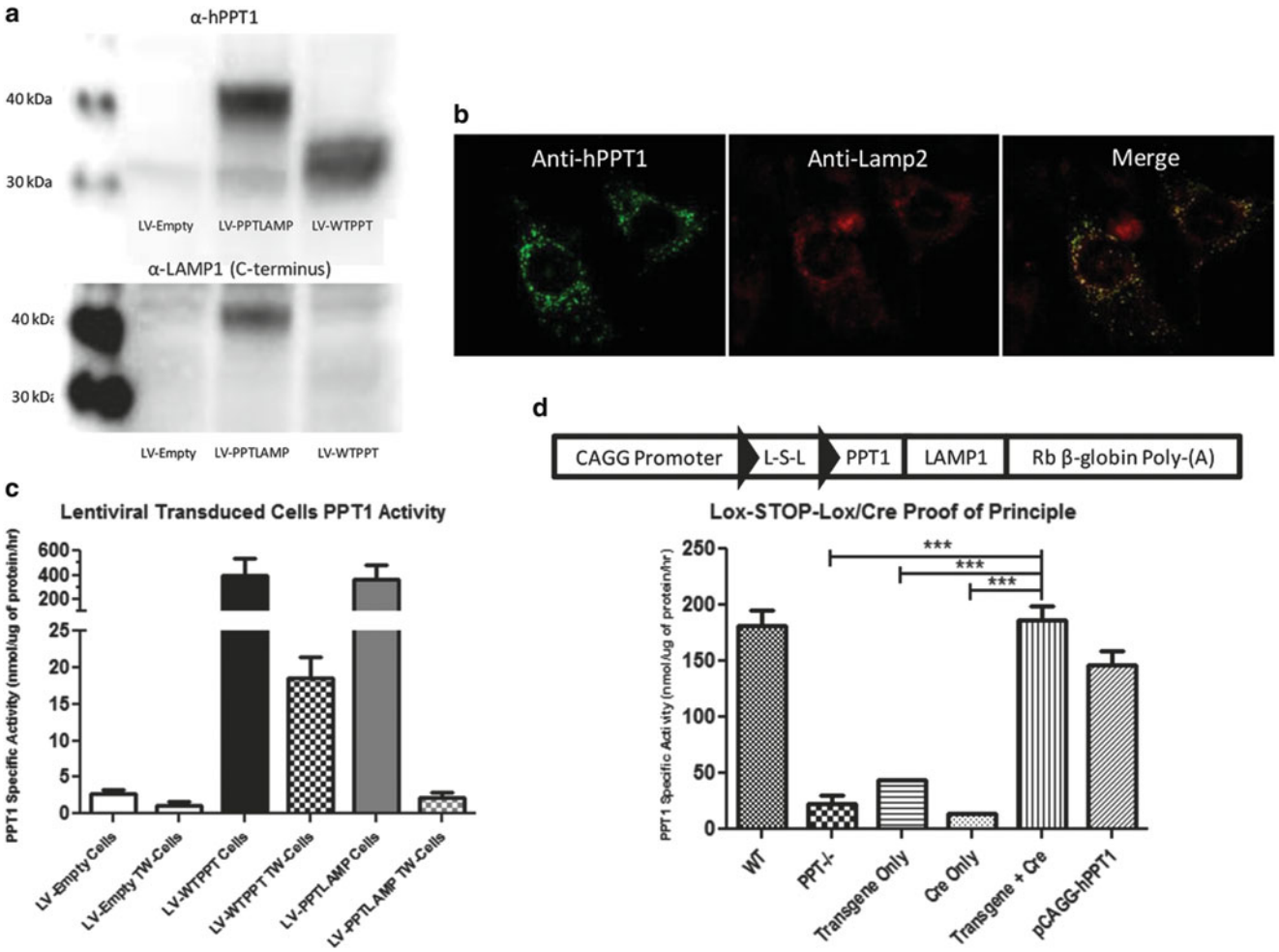

Fig. 1 In vitro characterization of PPT1-LAMP1 and generation of a Cre responsive transgene. (**a**) Expression of PPT1-LAMP1 in vitro. PPT1−/− cells were transduced with LV-Empty, LV-PPTLAMP, or LV-WTPPT. The top panel is probed for anti-hPPT1. hPPT1 has a predicted molecular weight of ~32 and 36 kDa, and hPPT1-LAMP1 is predicted at ~40 kDa. The bottom panel was probed for the C-terminus of LAMP-1. The left lanes show the markers for 30 and 40 kDa. A ~32 and ~36 kDa PPT1 signal was detected in the LV-WTPPT lane. PPT1 signal was detected at ~40 kDa in the LV-PPTLAMP lane. LAMP-1 signal was detected in the LV-PPTLAMP lane. No PPT1 or LAMP1 signal was detected in LV-Empty lane. (**b**) Immunofluorescence of PPT1 (*green*) and LAMP-2 (*red*). (**c**) Transwell experiment for "cross-correction." Cells were transduced with LV-Empty, LV-PPTLAMP, or LV-WTPPT. There was a significant increase in enzyme activity in the LV-WTPPT cells (394.7 nmol/μg/h) compared to LV-Empty (2.7 nmol/μg/h). LV-WTPPT cells and LV-PPTLAMP cells (367.9 nmol/μg/h) had similar levels of activity. There was a significant ($p < 0.05$) increase in PPT1 activity in the transwell inserts (TW-cells) in the LV-WTPPT TW cells (18.5 nmol/μg/h) compared to LV-Empty TW cells (1.1 nmol/μg/h) and LV-PPTLAMP TW cells (2.2 nmol/μg/h). (**d**) In vitro testing of cell-specific transgene. Schematic of PPT1LAMP1 transgene is above the graph. The transgene consists of the CAGG Promoter (Chicken β-actin first exon and intron), human palmitoyl-protein thioesterase-1 (PPT1) cDNA, lysosomal-associated membrane protein (LAMP-1) cDNA, and the rabbit β-globin polyadenylation terminator (Rb β-globin Poly-(A)). A loxp-STOP-loxp (L-S-L) cassette was added to the transgene (loxp sites: *black triangles*). There was not a significant increase in PPT1 activity in the PPT1−/− cells transfected with the transgene alone (43.9 nmol/μg/h) or the Cre-containing plasmid alone (14.0 nmol/μg/h) compared to PPT1−/− cells (22.4 nmol/μg/h). There was an increase in PPT1 activity when the transgene and the Cre-containing plasmid were transfected together (186.4 nmol/μg/h) to WT levels (181.5 nmol/μg/h). pCAGG-hPPT1 plasmid was used as a transfection control

PPT1 Enzyme Activity and Secondary Enzyme Elevations

PPT1 assays were performed using the 4-MU-palmitate fluorometric assay and normalized to total protein (Griffey et al. 2004). Whole blood was collected slowly by cardiac puncture through a 23G needle in an attempt to avoid cell lysis. The blood was allowed to clot then spun at 3,000 rpm. Serum was collected and flash frozen for subsequent PPT1 assays. β-glucuronidase assays were performed using 4-MU-β-D-glucuronide fluorometric assay and normalized to total protein as previously described (Roberts et al. 2012). Significance was determined using a two-way ANOVA followed by a Bonferroni post-hoc analysis.

Autofluorescent Storage Material

Autofluorescent storage material (AFSM) was imaged and quantified as previously described (Griffey et al. 2004). Twelve-micrometer sections were imaged using confocal microscopy.

Lifespan, Rotarod, and Electroretinography

Lifespan was determined by death or euthanasia out to 1 year ($n = 10–11$ mice/group). A Kaplan-Meier lifespan curve was used to measure survival and significant differences were determined using a log-rank analysis ($p < 0.05$).

Mice ($n = 10$/group) were tested on a constant-speed rotarod (3 rpm) at 7 months as previously described (Dearborn et al. 2015). Statistical significance was determined using one-way ANOVA followed by a Bonferroni post hoc test.

Electroretinography (ERG) was performed under dark or light conditioning as previously described (Vision Core, Washington University) (Griffey et al. 2005). b-wave amplitudes (microvolts) were recorded (UTAS-E-3000 LKC system, LKC Technologies, Gaithersburg, MD).

Results

PPT1-LAMP1 Function In Vitro

PPT1−/− fibroblasts were transduced in vitro with LV-Empty, LV-PPTLAMP, or LV-WT. Western blot analysis showed an increased molecular weight of the hPPT1-LAMP1 (~40 kDa) compared to the wild type human PPT1(~32–36 kDa). This was consistent with the addition of the LAMP1 transmembrane domain and six-glycine linker (~5 kDa). Endogenous LAMP1 (~120 kDa) and a signal at ~40 kDa were detected when the same membrane was probed with an anti-LAMP1 antibody (Fig. 1a). Immunofluorescent staining for LAMP2 and PPT1 in PPT1−/− fibroblasts transduced with LV-PPTLAMP demonstrates that PPT1 enzyme is localized to the lysosome (Fig. 1b).

Transwell experiments confirmed that PPT1-LAMP1 retained enzyme activity and was not secreted (Fig. 1c). Following LV-WT, PPT1 activity (~400 nmol/μg/h) was detected in the transduced cells and in the transwell cells (~20 nmol/μg/h). Nearly identical PPT1 activity to LV-WT was detected following LV-PPTLAMP transduction but no activity was detected in the transwell cells.

To achieve cell specificity for the expression of the transgene, a lox-STOP-lox sequence was included within the transgene (Fig. 1d). In vitro transfection of PPT1−/− cells with a Cre-recombinase plasmid or the transgene-containing plasmid alone resulted in no increase in PPT1 activity. Upon co-transfection of the plasmids containing Cre recombinase and the lox-STOP-lox PPTLAMP transgene, a significant increase in PPT1 activity to near WT-PPT1 activity levels was observed (Fig. 1d).

Generation of a PPT1-LAMP1 Transgenic Mouse

To study the role of specific cell types in INCL in vivo, transgenic animals were generated harboring the PPT1-LAMP1 transgene. Eight founders were identified and five demonstrated germ-line transmission. Only two founders (F38 and F42) were able to generate colonies. F38 did not express PPT1-LAMP1 in the presence or absence of Cre-recombinase. Surprisingly, a PPT1 assay of F42 detected supraphysiological levels of PPT1 activity in the absence of Cre recombinase.

Founder #42 Analyses

Genomic transgene characterization of F42 mice identified a ~1.5 Kb band consistent with the intact transgene and a ~260 bp band consistent with recombination of the loxP sites (Fig. 2a). Sequencing confirmed Cre-independent recombination. Approximately 11 transgene copies per genome were calculated using quantitative PCR.

Western blot analysis was performed on dermal fibroblasts from F42. A PPT1-specific signal was detected in human fibroblasts corresponding to differentially glycosylated PPT1 but was not detected in PPT1−/− or WT murine fibroblasts (Fig. 2b). A protein of ~40 kDa was detected in homogenates from F42 fibroblasts probed with an anti-hPPT1 antibody. Similarly, a protein of ~40 kDa was detected with an anti-LAMP1 antibody along with endogenous LAMP1 (~120 kDa). A transwell experiment showed that PPT1-LAMP1 from F42 fibroblasts does not "cross-correct" (Fig. 2c). Transwell insert cells exposed to WT cell media had increased PPT1 activity of ~10% of WT levels. In contrast, little to no PPT1 activity was detected in the transwell cells exposed to F42 cell media even though F42 fibroblasts had ~6-fold greater PPT1 activity compared to WT cells.

PPT1-LAMP1 Expression In Vivo Is Ubiquitous and Prevents AFSM Accumulation

Supraphysiological levels of PPT1 activity were detected in the brain and heart, and near normal levels in the kidney and spleen of F42 mice compared to wild type (Fig. 2d). PPT1 activity was not significantly increased in the serum of F42 mice compared to PPT1−/− mice (Fig. 2e). Surprisingly, very low PPT1 activity was detected in the

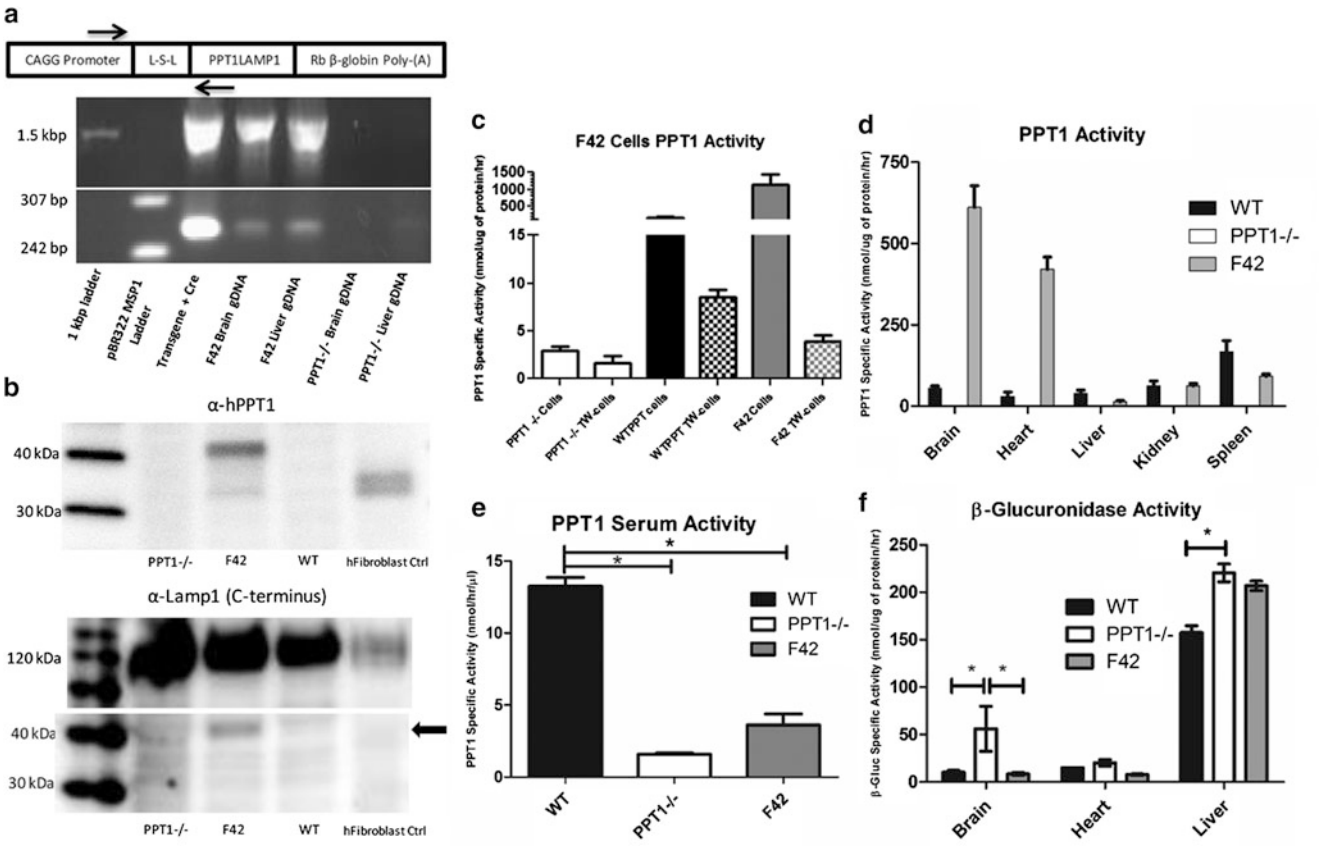

Fig. 2 Biochemical and molecular characterization of F42 mice. (**a**) Schematic of the transgene with the position of PCR and sequencing primers. PCR gel identifying spontaneous recombination in Founder #42. Intact loxp-STOP-loxp cassette is ~1.5 kbp and the rearranged cassette is ~260 bp. Lane 1: 1 kbp ladder, Lane 2: pBR322-MSPI digest ladder, Lane 3: in vitro Cre recombinase and transgene plasmid, Lane 4: genomic DNA isolated from F42 brain, Lane 5: genomic DNA isolated from F42 Liver, Lane 6: genomic DNA isolated from PPT1−/− brain, Lane 7: genomic DNA isolated from PPT1−/− liver, Lane 8: negative control. (**b**) Western blot for PPT1-LAMP1 expression. Expression of PPT1-LAMP1 was confirmed in primary dermal fibroblasts from F42. The top panel was probed for anti-human PPT1. A strong signal was detected at ~40 kDa in the F42 lane, and at the predicted ~32 (unglycosylated) and ~36 kDA (glycosylated) in the human control fibroblasts. The bottom panel was probed for the C-terminus of LAMP-1. There was a signal at ~120 kDa (endogenous LAMP1) in all lanes and ~40 kDa (PPT1-LAMP1) signal in the F42 lane (*black arrow*). (**c**) Transwell experiment for "cross-correction." Primary dermal fibroblast cells were used. There was a significant increase in enzyme activity in the WT-PPT1 (184.0 nmol/μg/h) compared to PPT1−/− cells (2.9 nmol/μg/h). F42 cells (1135.1 nmol/μg/h) have ~6-fold greater levels of PPT1 activity compared to WT cells. There was an increase in PPT1 activity in the transwell inserts in the WT-PPT1 transwell (8.5 nmol/μg/h) compared to PPT1−/− (1.5 nmol/μg/h) and F42 transwells (3.9 nmol/μg/h). (**d**) PPT1 enzyme activity tissue survey. Supraphysiological levels of PPT1 activity were detected in the brain and heart. Near normal levels were detected in the kidney, and ~50% activity in the spleen. The liver had ~5% normal levels of PPT1 activity. (**e**) PPT1 enzyme activity in the serum. There is a significant ($p < 0.05$) decrease in PPT1 activity in PPT1−/− serum compared to WT serum (1.6 vs 13.3 nmol/μg/h). PPT1 activity was significantly decreased in serum from F42 mice (3.6 nmol/μg/h, $p < 0.05$) compared to WT mice. There is no significant difference in PPT1 activity between F42 and PPT1−/− serum. (**f**) β-glucuronidase activity in the brain, heart, and liver from F42 and controls. There was a significant ($p < 0.05$) increase in β-glucuronidase activity in the brain (56.2 vs 10.2 nmol/μg/h) and liver (220.4 vs 157.4 nmol/μg/h) of PPT1−/− animals compared to WT ($p < 0.05$). β-glucuronidase activity was normalized to WT levels in the F42 brain (8.5 nmol/μg/h), however, the β-glucuronidase activity was not reduced in F42 liver (206.7 nmol/μg/h). The heart showed a small increase in β-glucuronidase activity in PPT1−/− animals compared to WT animals (20.2 vs 15.0 nmol/μg/h) but this increase was not statistically significant. There appeared to be decreased β-glucuronidase activity in the F42 heart (7.8 nmol/μg/h)

liver of F42 mice. There is an increase in secondary lysosomal enzyme activity in the murine model of INCL (Fig. 2f) (Griffey et al. 2004). The β-glucuronidase activity levels in the brain and heart of F42 animals were restored to WT levels. However, β-glucuronidase activity in the F42 liver was not significantly reduced.

PPT1-LAMP1 Prevents the Pathological and Clinical Defects Associated with Murine INCL

There was widespread AFSM throughout the PPT1−/− brain and liver and none detected in WT animals (Fig. 3a). Accumulation of AFSM was not detected in the cortex,

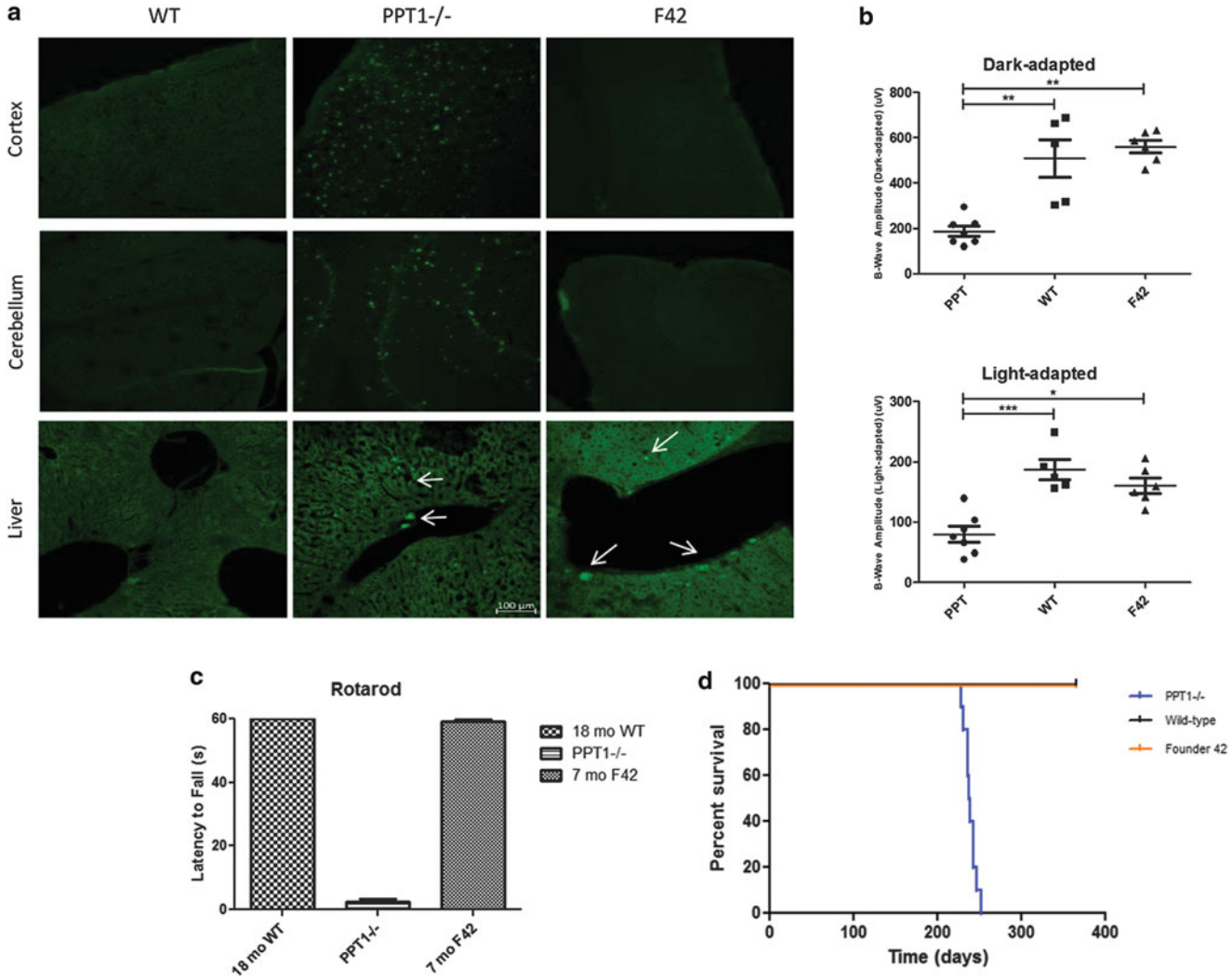

Fig. 3 Histological and clinical parameters of F42. (**a**) Representative images show AFSM accumulation in PPT1−/− brains (cortex and cerebellum). In contrast, AFSM was not detected in the cortex or cerebellum of WT or F42 animals. Representative images show similar levels and distribution of AFSM in the livers of PPT1−/− and F42 animals. No AFSM was detected in WT livers. All images were taken at the same magnification of 20x (100 µm). (**b**) Electroretinography of F42 and control eyes at 7 months. There was a significant decrease in b-wave amplitudes in the light-adapted (WT $p < 0.0001$, F42 $p < 0.01$) and dark-adapted (WT $p < 0.001$, F42 $p < 0.001$) PPT1−/− mice compared to WT and F42 mice. There was no significant difference between F42 and WT b-wave amplitudes in light-adapted or dark-adapted conditions. (**c**) Rotarod analysis of F42 mice showed no deficit in motor function at 7 months of age. PPT1−/− animals could not stay on the rotarod at 7 months of age. However, 7-month-old F42 mice were able to stay on the rotarod for 60 s. Similarly, the WT mice were also able to stay on for the duration of the study. (**d**) The lifespan of F42 mice (*orange*) was not reduced compared to WT animals (*black*) out to 1 year. In contrast, PPT1−/− animals had a median lifespan of 238 days (*blue*)

cerebellum or any other brain region in F42 mice. There was abundant AFSM throughout the liver of F42 mice (Fig. 3a).

PPT1−/− mice showed a significant decline in ERG amplitudes and motor function by 7 months of age, and are terminal by ~8.5 months. F42 mice showed no significant decline in either dark-adapted or light-adapted ERG amplitudes (Fig. 3b). At 7 months, F42 mice remained on the rotarod for 60 s and were indistinguishable from wild type mice (Fig. 3c). Lastly, the F42 mice lived to at least 1 year without any obvious clinical signs of disease (Fig. 3d).

Discussion

The first goal of this study was to generate PPT1 that was sequestered within the lysosome. We successfully generated a lysosomal membrane-tethered form of human PPT1 that was properly trafficked, enzymatically active, and retained within the lysosomal compartment in vitro. This demonstrates proof-of-principle that PPT1-LAMP1 can be used as a tool to limit enzyme expression to select cells. The second goal was to utilize the Cre-lox system and generate a mouse model where PPT1-LAMP1 was limited to specific cell

types, thereby creating novel models of cell-specific enzyme deficiencies. We succeeded in generating a mouse model of PPT1-LAMP1 using a loxP-STOP-loxP cassette. However, a spontaneous rearrangement resulted in near-ubiquitous expression of PPT1-LAMP1 throughout the mouse. Therefore, we characterized the mouse model with widespread expression of the transgene to determine whether PPT1-LAMP1 could correct INCL pathology. Since native PPT1 is a soluble protein, it was critical to determine if membrane-tethered PPT1 could access its substrates.

We demonstrated that tethering PPT1 to the lysosomal membrane eliminates "cross-correction" of neighboring cells in vitro and in vivo. Near-ubiquitous expression of PPT1-LAMP1 in vivo was sufficient to prevent the hallmark INCL pathology of AFSM accumulation in most tissues indicating its retention in the lysosome. AFSM did accumulate in the liver, which is consistent with the low level of PPT1-LAMP1 expression, possibly resulting from transgene integration into a closed genomic domain within hepatic cells. These data, combined with the very low level of PPT1 activity in the serum and high level of expression in other tissues, strongly support our conclusion that PPT1-LAMP1 is not secreted nor capable of "cross-correction." The apparent small increase in serum PPT1 activity in the F42 mice was not significantly greater than that measured in PPT1−/− mice but could be due to cell lysis during blood collection. Finally, we found that restricting PPT1 expression to the lysosome is sufficient to prevent several functional deficits observed in INCL mice, including loss of vision and motor deficits.

A previous study limited the secretion of sphingomyelinase (SMase), another soluble lysosomal hydrolase, by tethering SMase to the lysosomal membrane using the C-terminus of LAMP-1 (Marathe et al. 2000). The original goal of their study was to eliminate the secreted form ubiquitously. The transgenic/knockout hybrid model of Neimann-Pick A/B had modest lysosomal SMase activity in the brain (~20% WT) with little or no neurological disease. However, there was lower activity in systemic tissues (~1–14% WT) with widespread visceral disease. The data in the INCL mouse are similar with rescue of CNS pathology while liver pathology persisted. Although these represent two different disease models, taken together, the data suggest that secreted enzymes might be of greater importance to visceral organs compared to the CNS. Alternatively, these data suggest that secreted PPT1 serves no vital function since there is essentially no disease pathology in any tissue examined with the exception of the liver which has very low activity.

Our original model utilized a loxP-STOP-loxP system that was to be combined with cell-specific Cre-driver lines. However, a spontaneous rearrangement of the loxP sites led to loss of Cre-responsiveness. We detected ~11 transgene copies/genome in F42 mice which is consistent with previous studies demonstrating multiple integrants using a traditional transgenic approach (Haruyama et al. 2009). It is possible that the direct repeat sequences in the concatamers made the transgene prone to recombination and led to deletion of the loxP-STOP-loxP sequence (Bill and Nickoloff 2001; Hendricks et al. 2003; Wurtele et al. 2005).

While the current transgenic model may not allow for the study of cell autonomous expression of PPT1-LAMP1 in vivo, the model provides evidence that this basic approach is sound. In addition, expressing PPT1-LAMP1 in cultured cells will allow for in vitro experiments without the confounding issues of "cross-correction."

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Contributions

C.S. and M.S.S. wrote the paper. C.S., S.L.M., and M.S.S. designed research. C.S., S.L.M. and J.T.D. performed research. C.S. and J.T.D. analyzed the data. M.S.S. will serve as guarantor.

Compliance with Ethical Standards

Conflict of Interests: No conflicts of interest to report.
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RESEARCH REPORT

Gamma-Hydroxybutyrate (GHB) Content in Hair Samples Correlates Negatively with Age in Succinic Semialdehyde Dehydrogenase Deficiency

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Abstract Gamma-hydroxybutyrate (GHB) is a drug of abuse, an approved therapeutic for narcolepsy, an agent employed for facilitation of sexual assault, as well as a biomarker of succinic semialdehyde dehydrogenase deficiency (SSADHD). Our laboratory seeks to identify surrogate biomarkers in SSADHD that can shed light on the developmental course of this neurometabolic disease. Since GHB may be quantified in hair as a potential surrogate to identify victims of drug-related assault, we have opted to examine its level in SSADHD. We quantified GHB in hair derived from ten patients with SSADHD, and documented a significant negative age correlation. These findings are consistent with recent results in patient biological fluids, including plasma and red blood cells. These findings may provide additional insight into the developmental course of SSADHD (Jansen et al., J Inherit Metab Dis 39:795–800, 2016).

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Introduction

Succinic semialdehyde dehydrogenase (SSADH) deficiency (SSADHD), a rare disorder of GABA metabolism, manifests with accumulation of both gamma-aminobutyrate (GABA) and gamma-hydroxybutyrate (GHB) in patient biological fluids (Malaspina et al. 2016). While the neurochemistry of GABA is well known (Hillmer et al. 2015), the pharmacology of GHB remains unclear. GHB, an endogenous intermediate in the central nervous system present at ~1% of parent GABA, is neuromodulatory in its effects on dopamine release and uptake (Maitre et al. 2016). Additionally, GHB is used therapeutically for the treatment of narcolepsy, and employed illicitly as a drug of abuse and agent in the facilitation of sexual assault (Malaspina et al. 2016). Because of its short terminal plasma half-life (~0.5 h) (Brenneisen et al. 2004), forensic methods have been developed to quantify GHB in non-physiological fluids, including hair (Jagerdeo et al. 2015).

The natural history of SSADHD remains undefined, and a systematic developmental analysis of patients with age is lacking. In an effort to explore metabolic developmental characteristics, we recently quantified GABA and GHB in plasma and red blood cell (RBC) lysates obtained from a cohort of 18 patients (age range 5–41 years; median 8 years) and found that both compounds negatively correlated with age (Jansen et al. 2016). In that study, plasma and RBC GHB levels reached a nadir and

approximate steady-state by 10 years of age, whereas plasma GABA achieved an approximate steady state level at 30–40 years of age. These biomarker interactions shed light on additional GABA- and GHB-ergic neurotransmission imbalances potentially correlated with the onset of adolescent/adulthood neuropsychiatric morbidity and epilepsy. Here, we extend these studies by examining the levels of GHB in hair derived from a cohort of SSADHD patients, with the goal of investigating the potential of this method for long-term monitoring of GHB levels in SSADHD patients.

Patient Samples and Methodological Approach

Testing for drugs in hair is an established methodology complementing other methodologies in clinical and forensic toxicology (Cooper et al. 2012). Hair represents a durable sample, which is less affected by other contaminants (as in the case of urine and blood). Moreover, hair can provide an overview of drug exposure (e.g., narcotics, benzodiazepines) over extended periods depending upon the length of the sample analyzed. Hair samples contain keratinized cells composed of three distinct layers, including the medulla (core, or soft keratin), the cortex (composed of thick and hard keratin), and the cuticle (surface, composed also of hard keratin which is thinner than the cortex). Drugs of abuse (including GHB) are believed to disperse throughout all these regions, deposited from blood capillaries located at the root bulb (Cooper et al. 2012). Our methodology closely follows the guidelines of the Society of Hair Testing (SoHT) established for evaluation of drugs of abuse in hair. They include appropriate washing of collected samples with both aqueous and organic solvents, pulverization of samples prior to extraction, and analysis of an extract of each segment and washed fraction (Cooper et al. 2012). For GHB quantitation, the method of Wang et al. (2016) was used with multiple reaction monitoring (MRM) and quantification of the m/*z* 85 fragment from parent ion m/*z* 103, using $^{2}H_{6}$-GHB as internal standard. Hair samples were pulverized into a fine powder in extraction media containing solvent and buffer (Wang et al. 2016) at 37°C for 1.5 h. The limit of quantification (LOQ) for GHB was 0.32 ng/mg hair, with linearity of the assay to 50 ng/mg. Extraction recoveries were 62–92%, and the accuracy 90–108%. Relative standard deviations (%) obtained from daily controls for GHB were 9.1–11.3%. Quantitation of the glucuronide of GHB followed the same methodology (Wang et al. 2016).

Hair specimens from patients with genetically confirmed SSADHD were obtained with informed consent (WSU IRB #14100; Gibson, PI). Sample collection kits were sent to all participants following consent. Patient demographics included 10 patients, age range 3–36 years (median age, 13 years; 9 males), representing approximately 5% of published patients (Malaspina et al. 2016). Race as well as hair color can influence the concentration of GHB in hair (Goullé et al. 2003). Seven patients resided in the USA, presumed to be of N. European descent. The three remaining patients were from Germany, Greece, and Uruguay, respectively. All had brown hair, with the exception of one patient with blond hair. Hair was clipped from the back upper area of the skull (vertex posterior region), where hair growth is considered most regular. Hair shafts of 1 cm length were gathered by the individual collecting the specimen (with gloves; approximately 60 hairs), and clipped as close to the scalp as possible. Three samples were collected, placed in individual foil, and the foil crimped. Samples were shipped and stored at room temperature until analysis. Hair samples were processed into either 0.5 or 1 cm segments and washed with isopropanol and water several times before analysis.

Control samples were obtained from 10 controls (8 Caucasian, 2 Chinese), and hair color ranging from blond to black with ages from 9 to 44 years (median age 29 y.o.; 9 females). The GHB range was <0.32–1.00 ng/mg hair in controls, with 0.32 ng/mg representing LOQ. Other investigators have reported higher values, including Goullé et al. (2003), 0.32–1.86 ng/mg, $n = 61$; Bertol et al. (2015), 0–5.09 ng/mg, $n = 30$; and Shi et al. (2016), 0.28–4.91 ng/mg, $n = 66$. Statistical analysis of GHB concentrations in hair employed the Spearman rank order correlation for non-parametric data, with a post-hoc Bonferroni analysis employing a standard two-way *t*-test. Significance was set at the 95th centile. Data analysis employed the GraphPad 6.0 program (San Diego, CA).

Results

GHB levels in hair samples of patients are shown in Fig. 1, and a representative ion chromatogram of GHB analysis in Fig. 2. The left side of Fig. 1 depicts all data for patients with all hair segments (either in 0.5 or 1 cm segments). The four graphs of Fig. 1 on the right depict the same data stratified for cm 1–4 of hair segments as a function of patient age. The concentrations of GHB in hair reached a level within the control range (<0.32–1.00 ng/mg) at approximately age 12–13 years, with elevated levels observed in patients below this age (3–7 years). When stratified for age with the first cm of hair, there was a highly significant negative correlation as a function of age. This correlation was maintained in cm 2, 3 and 4 of hair samples. It was not as strong but still significant. For cm 5, 6 and 7, correlations failed to achieve significance but the sample number was low due to short hair for several

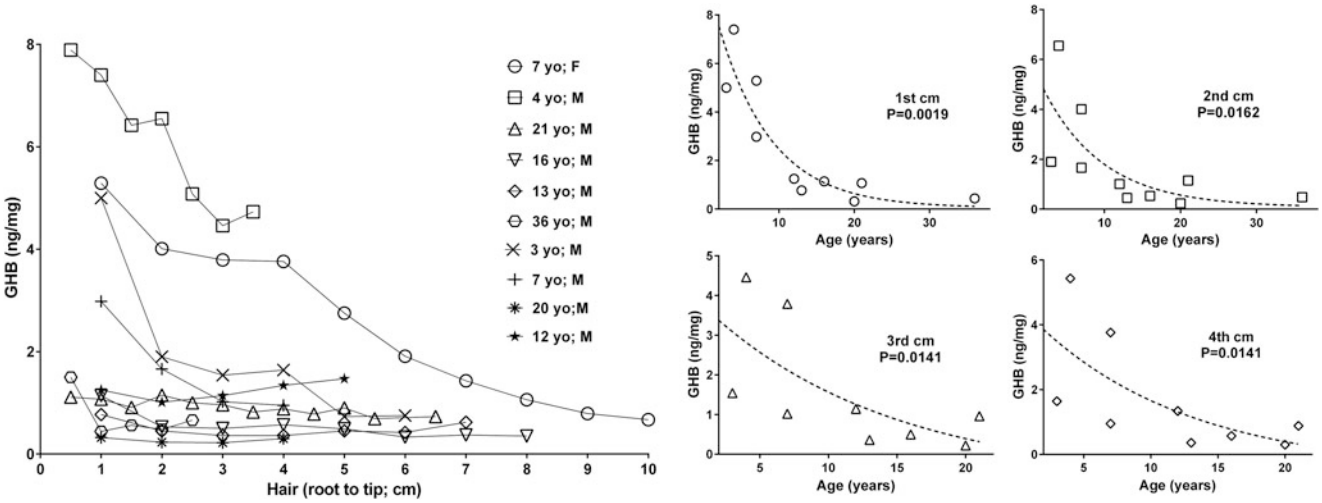

Fig. 1 (*Left*) GHB concentrations in all hair segments measured from ten patients with SSADHD. Age and gender of patients are shown in the legend. (*Right*) Stratification of GHB content in hair segments in cm 1, 2, 3, and 4 for all patients from whom samples were available. Each data point for a patient hair sample represents a unique measurement of GHB content within that single 0.5–1.0 cm hair segment derived from the entire length of the hair sample (depicted as analysis from root to tip). Statistical analyses employed the Spearman rank order correlation, with significance set at the 95th centile

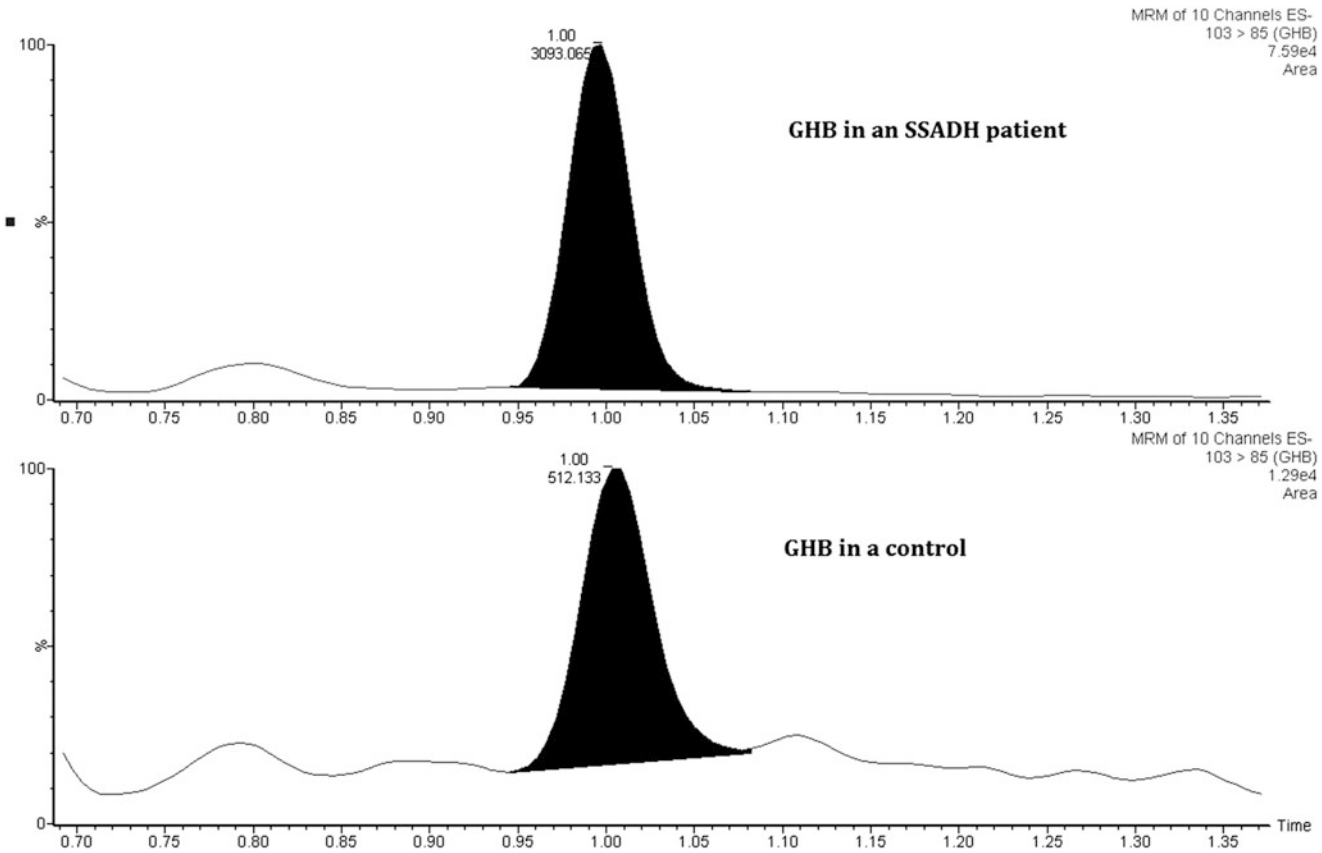

Fig. 2 Multiple reaction monitoring (MRM) of GHB in a representative hair sample from a patient with SSADHD and a control. The transition from m/*z* 103 > 85 was monitored with $^{2}H_{6}$-GHB as internal standard. This obviated interference from other potential contaminants, including other butyric acid compounds, which also elute at different retention times than GHB (Wang et al. 2016). The GHB level in the patient's hair sample was approximately 5.9-fold increased in comparison to control based upon peak area

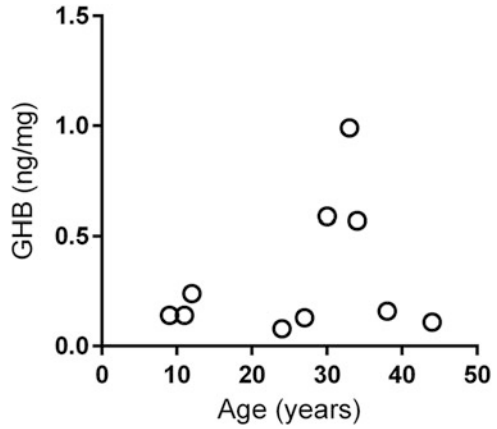

Fig. 3 Total hair GHB content for controls. The concentrations were calculated by standard addition method for each individual (age-dependent correlation was non-significant)

patients. Additionally, the levels of GHB in the most distant samples measured from the hair root (cm 5–7) might reflect wash out due to personal hygiene. Only younger patients showed hair GHB concentrations significantly above the control range; conversely, older patients demonstrated hair GHB levels within the control range of up to 1.0 ng/mg in all hair segments. There was no significant GHB vs age correlation in the control group (Fig. 3).

An explanation for the longitudinal decrease of GHB with length of hair is not readily available, although this implies that transport of GHB from blood to hair is not reversible. Several other factors could contribute to this phenomena, including diet and drug interactions, but direct studies to address this have not been presented. The analysis of the glucuronide of GHB in hair was also measured for the first seven patients, since the glucuronide of GHB represents its main metabolite (Ainslie et al. 2016). For all segments in these patients, the concentrations of GHB-glucuronide were below the LOQ or within the levels observed in controls (not shown) (Wang et al. 2016).

Discussion

These are the first data on GHB concentrations in the hair of patients with SSADHD. These findings are consistent with our recent study of GHB in plasma and red-blood cell lysates derived from patients with SSADHD. They showed similar age-dependent negative correlations that reached a steady-state at approximately 10 years of age. In the current study, patients in the median age-range of the cohort, ~12–13 years, showed hair GHB values within the control range (<1 ng/mg hair). Whether hair represents a better surrogate of GHB content in the brain than blood or urine remains to be determined (Staeheli et al. 2016). To answer this question, a longitudinal analysis of brain GHB in patients (either employing cerebrospinal fluid, or methods to quantify GHB in vivo using edited magnetic resonance spectroscopy) will be needed. Nonetheless, surrogate studies to date (hair, plasma, red blood cell GHB content) all point to an age-dependent lowering of GHB in brain in SSADHD (Jansen et al. 2016). It is important to point out that no negative correlation of GHB vs. age was observed in unaffected controls. Future studies will thus be needed to confirm the diagnostic value of GHB concentration in hair from SSADHD patients, as well as to explain the significance of elevated hair GHB concentration in younger SSADHD patients.

Direct comparisons of the level of GHB and GABA in cerebrospinal fluid (CSF) of SSADHD patients with hair GHB content are somewhat challenging based upon the matrices involved. CSF GHB in four patients with SSADHD were 116–1,110 nmol/ml (control range, 0–2.6, $n = 10$) and the values for total GABA in these same patients were 13.6–22.4 nmol/ml (control range, 4.7–11.8, $n = 10$) (Gibson et al. 1995). The corresponding highest GHB concentrations measured in the hair of the 4 youngest patients with SSADHD in our study (Fig. 1) was approximately 3–8 ng/mg (0.03–0.08 nmol/mg). Nonetheless, blood or CSF levels of GHB cannot be directly correlated to hair concentration in the present study. To achieve this, we will need controlled studies of GHB intake and GHB hair measurement (Busardò et al. 2016).

As mentioned previously, the content of GHB in hair can be dependent upon race and hair color. All patients in our cohort were Caucasian and the hair color for 9 of 10 patients was brown, but for one 3 years old patient the hair color was blond. This may provide an explanation for the observation that the GHB hair content of this 3-year-old patient was less elevated than we predicted (Fig. 1) (Goullé et al. 2003). The phenotype of SSADHD routinely includes neuropsychiatric morbidity from adolescence to adulthood, including attention deficit-hyperactivity, oppositional defiant and obsessive compulsive disorders (Malaspina et al. 2016), representing significant disease morbidity. It is tempting to speculate that the nadir of hair GHB levels we observed (~12–13 years of age) may correlate with altered GABA levels and the onset of neuropsychiatric morbidity in SSADHD (Parviz et al. 2014). Disruption of GABA-ergic neurotransmission has been confirmed in SSADHD (Pearl et al. 2009; Reis et al. 2012; Buzzi et al. 2006), using both mouse models and patients. High levels of GHB and GABA during ontogeny almost certainly offsets the balance of GABA-ergic/glutamatergic neurotransmission (Jansen et al. 2008; Vogel et al. 2016; Talaei et al. 2016). High levels of GHB may impact this balance both through GHB-ergic and $GABA_B$-ergic and $GABA_A$-ergic effects (Absalom et al. 2012).

Decreasing GHB levels with age will likely reset the homeostatic balance between inhibitory and excitatory neurotransmission, a process we plan to address in a more comprehensive longitudinal evaluation of SSADHD. A major goal in such a study will be to identify biomarkers that reflect chronic exposure to high GHB levels, and correlate with the clinical evolution of the disease. Our data suggest that hair GHB concentration which declines with age like plasma and RBC GHB levels, and is not subject to fast metabolic clearance, may be a suitable biomarker in a SSADHD natural history study. The data also suggest that hair GHB measurement will be useful as a SSADHD diagnostic tool. However, the present findings need to be confirmed in studies with SSADHD patients against strictly age-matched controls.

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Author Contributions

- JSS, WX, SPD: acquisition and analysis of data
- PLP, JBR, KRV, GRA: conception and design of study; drafting of manuscript
- KMG: conception and design of study; data analysis; manuscript drafting

Conflicts of Interest

The authors cumulatively declare they have no direct or perceived conflicts of interest.

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RESEARCH REPORT

An Audit of the Use of Gonadorelin Analogues to Prevent Recurrent Acute Symptoms in Patients with Acute Porphyria in the United Kingdom

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Abstract Severe recurrent acute attacks of porphyria have traditionally been treated with either prophylactic human haemin or gonadorelin analogues (GnA) in females. Evidence on the most effective treatment for this patient subgroup is lacking. This audit surveyed the use of prophylactic GnA in the UK.

Twenty female patients (who experienced between 2 and 45 acute attacks of porphyria requiring hospitalisation and treatment with human haemin prior to GnA prophylaxis) were included in the audit. Data was retrospectively collected based on patient history and case review.

Twenty three treatment courses were given lasting a median period of 12 months. Monthly subcutaneous Goserelin was most commonly used. In three patients in whom timing with the menstrual cycle was not considered, an acute attack occurred after initiation of the first dose. The majority of patients experienced oestrogen deficiency symptoms during treatment. Fifty percent of the prescribed courses of GnA resulted in a degree of clinical benefit. This successfully treated group experienced between 3 and 20 acute attacks prior to and between 0 and 6 acute attacks during GnA treatment.

The audit revealed large variation in practice in the United Kingdom regarding indication, duration of treatment, specific drug used and management of side effects. In view of the limited treatment options available for this cohort and the mixed outcome successes reported, we believe it is reasonable for porphyria specialists to continue offering GnA treatment to women with severe recurrent debilitating acute attacks of porphyria associated with the menstrual cycle, and we propose best practice guidelines to standardise management.

Introduction

The porphyrias are a group of mainly inherited conditions that result from a partial deficiency of one of the enzymes in the haem production pathway. The eight different porphyrias are characterised by either acute neurological and/or cutaneous symptoms and signs, depending on which enzyme is affected and the resultant pattern of toxic haem precursor build-up proximal to the metabolic block. Three autosomal dominant porphyrias cause acute neurological symptoms: acute intermittent porphyria (AIP), variegate porphyria (VP) and hereditary coproporphyria (HCP).

During an acute attack the majority of patients experience severe abdominal, back or thigh pain, other features of autonomic neuropathy such as vomiting, constipation, hypertension and tachycardia, behavioural disturbances (Peters and Sarkany 2005) and an increase in urine porphobilinogen (PBG) excretion. Treatment includes providing symptomatic relief including carbohydrate and

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administering intravenous human haemin (haem arginate Normosang®, Recordati, Paris) (Stein et al. 2013). Haem, the final product of the pathway, suppresses the rate limiting first enzyme, 5-aminolaevulinic acid synthase 1 (ALAS1). Provision of exogenous haem down-regulates the pathway with a decrease in toxic precursor build-up and resolution of symptoms.

A small proportion of patients, particularly those with AIP, develop recurrent debilitating attacks (Elder et al. 2013). Frequent hospital admissions have a devastating impact on their quality of life. In women these recurrent attacks are often associated with the luteal phase of the menstrual cycle; symptomatic disease usually resolves following the menopause.

Published evidence on the most effective way to manage this challenging patient subgroup is lacking. Prophylactic haem has been used for the last two decades to prevent or decrease the frequency of acute attacks (Stein and Cox 2011; Marsden et al. 2015). Liver transplant has been effective in a few cases (Soonawalla et al. 2004), but is used as a last resort (Dowman et al. 2012). For repeated pre-menstrual acute attacks, an alternative option is pituitary ovarian suppression using gonadorelin analogues (GnA) (Anderson et al. 1984), which down-regulate ovarian oestrogen and progesterone production, the likely triggers of acute attacks. Previously hormonal manipulation with oral contraceptive agents had been used successfully (Perlroth et al. 1965). However in view of the risks associated with exogenous sex hormone therapy (Andersson et al. 2003) this is no longer pursued as a treatment option.

The aim of this audit was to review the historic and varying practices in the use of GnA in females with severe recurrent acute attacks of porphyria in the UK, and where possible, to use this data together with existing evidence to develop a preliminary guide to aid patient management. These standards could subsequently be used for future prospective audit.

Method

Patients were identified through the UK National Acute Porphyria Service (NAPS) database, which was established in 2012 to manage patients with active acute porphyria and currently includes two expert and two outreach centres. Of the 135 patients followed up by NAPS, 62 were defined as having current or previously active recurrent porphyria. Amongst these patients, 20 were identified for inclusion in the audit who had been treated with GnA between 2000 (prior to the establishment of NAPS) and 2015. An audit proforma (Table 1) was created to enable the collection of data on the particulars of GnA administration and distributed to all centres. The questionnaire was retrospectively completed by the clinicians and/or specialist nurses with clinical responsibility for the patient based on patient history and case review and returned between September 2014 and May 2015.

An acute attack was generally defined as an acute worsening of porphyria symptoms which local clinicians had decided required admission to hospital. The historic, retrospective nature of the data collected precluded the use of a more specific definition.

The questionnaire included a question to both the patient and the responsible clinician to state whether in their view GnA was successful, partially successful or unsuccessful in reducing acute attack frequency without predefined quantitative outcome measures.

Results

Pre-GnA Treatment

Twenty female patients with repeated acute attacks of porphyria received GnA prophylaxis (Table 2), 18 of whom had AIP, 1 with VP and 1 with HCP. The age of diagnosis (genetic confirmation in 18 and biochemical diagnosis in 2 patients) ranged between 17 and 41 years and the age of the first acute attack was between 19 and 41 years.

First acute attack precipitants were documented as unsafe drugs in eight patients, menstrual cycle related in two patients, dieting in two patients, pregnancy in one patient, stress in one patient, the menstrual cycle and a drug in one patient, alcohol and drugs in one patient and in four patients a precipitant was not identified.

The number of hospital admissions and courses of symptomatic haem arginate treatments ranged between 2 and 45 and the number of Intensive Treatment Unit (ITU) admissions between 0 and 1. Only one patient had received prophylactic haem arginate prior to GnA which was ineffective in preventing recurrent attacks.

GnA Treatment

Twenty three courses of prophylactic GnA were administered to 20 patients (Table 2); patient 1, 14 and 15 received a second course. The median duration of a treatment course was 12 months and ranged between 1 and 54 months. The drug most commonly used was Goserelin (17/23 courses), 4 patients received Leuprorelin and 1 Triptorelin. For patients in whom this information was available most received the drug administered monthly subcutaneously. Three patients received either Leuprorelin or Goserelin every 3 months. No patients received intranasal preparations.

In only 6 of the 17 (35%) treatment courses for which this information is known, was the GnA treatment started in

Table 1 Audit proforma

Demographics Diagnosis	
Pre-GnA treatment	Age of first acute symptoms, precipitants, total number of acute attacks, hospital/ITU admissions prior to GnA, evidence of neuropathy, regular analgesia requirement, previous prophylactic humin haemin, co-morbidities, QOL assessment
During GnA treatment	Analogue, route, frequency and duration of treatment, timing of the dose in terms of menstrual cycle, adverse event with first dose, side effects with continued use, monitoring of bone density, oestrogen replacement, total number of hospital/ITU admissions for acute symptoms, evidence of neuropathy, regular analgesia need, QOL assessment, judgment of whether GnA was deemed successful or not
Post-GnA treatment	Occurrence of further attacks, number of hospital/ITU admissions, need for human haemin, evidence of neuropathy, QOL assessment

GnA gonadorelin analogue, *ITU* intensive treatment unit, *QOL* quality of life

the recommended first few days of the follicular phase of the menstrual cycle (Anderson 1989; BNF 2013). In none of these 6 treatment courses did initiation of GnA precipitate an acute attack. In total three acute attacks occurred after initiation of the first drug dose and in all three cases GnAs were not given during the first week of their cycle. In patient 1 in whom a first dose acute attack was precipitated, the same drug was subsequently reintroduced during the first week of the cycle without adverse effect.

Patients experienced oestrogen deficiency side effects in 16 of the 19 treatment courses for which this information is known. The majority reported hot flushes but a minority also described hair thinning (two patients), mood changes (three patients) and reduced libido (one patient). Seven patients received hormone replacement therapy which included oestrogen only, tibolone or combined oestrogen and progesterone preparations to treat symptoms. Bone mineral density was monitored in 9 of the 23 treatment courses ranging between 4 and 54 months. The treatment courses in which bone mineral density was not monitored ranged from 1 to 25 months. Information on bone mineral density measurements and whether hormone replacement therapy precipitated any acute attacks or not was insufficiently reported in the majority of patients on the returned audit forms.

The patient receiving Triptorelin had only recently been initiated on treatment, and outcome data was therefore not available. Of the remaining treatment courses, 8/22 (36%) were deemed by the patient and responsible clinician as successful with regard to reducing acute attack frequency, 3/22 (14%) as partially successful, 8/22 (36%) as unsuccessful and for 4/22 (18%) this information was not stated (Table 3). A quantitative definition of treatment success was unfortunately not predefined, but both the patient and clinician were asked their opinion on whether a clinical improvement in terms of reduction in acute attack frequency and therefore quality of life was achieved or not.

In the 11/22 (50%) successful or partially successful courses, patients were treated with either Goserelin or Leuprorelin. Of the eight patients in whom treatment was deemed a success, the initial precipitant was drugs in four, menstrual cycle one, menstrual cycle and a drug one, fasting/dieting one and unknown in one patient. Eight patients experienced between 3 and 20 attacks pre GnA and between none and 6 acute attacks whilst on GnA with no ITU admissions documented. Six patients completed their treatment course which was stated as lasting between 16 and 54 months. One patient discontinued treatment after 3 months due to unacceptable oestrogen deficiency side effects but deemed it as successful as she had no acute attacks during the 3 months; and one patient discontinued treatment at 12 months as she had the desire to conceive.

In the partially successful group (3/22), drugs or drugs and alcohol was the initial precipitant of symptomatic disease in all three cases. In this group one patient had no further attacks for 3 months (vs. 6 admissions pre-treatment) after which GnA was stopped when she underwent a hysterectomy and oophorectomy, one patient had 2 further attacks (baseline number of attacks unknown) and GnA was stopped after 9 months and 1 had treatment for 10 months during which she had only 1 attack (vs. 5 attacks pre-treatment) which was stopped due to unacceptable side effects.

Of the eight patients in whom the treatment was deemed unsuccessful, only one patient's symptomatic disease was assessed as associated with the menstrual cycle. She had 10 attacks pre-treatment, was treated for 24 months during which she had a further 4 acute attacks and suffered unacceptable side effects.

In patient number 1 an acute attack occurred after the first dose of Goserelin, as mentioned this was reintroduced and continued for 16 months and deemed as successful. Patient number 14 had a first course lasting 4 months which was deemed unsuccessful as she had a further 5 attacks, then had a second course over 36 months, the success of

Table 2 Patient demographics prior to and during GnA treatment

Demographics				Pre-GnA			GnA treatment					
Patient	1st attack age (years)	Diagnosis	Precipitant[a]	Acute attack hospital admissions[b]	Nr of haem courses for acute attacks	Prophylactic haem	Analogue	Frequency/route	Duration[c]	Timing[d]	First dose adverse event	E deficiency side effects
1	23	AIP	Drug	>6	U	N	Goserelin	Monthly SC	1	N	Acute attack	N
1							Goserelin	Monthly SC	16	Y	N	Y
2	21	HCP	Drug	6	3	N	Leuprorelin	Monthly SC	3	N	U	N
3	25	AIP	Drug	3	3	N	Leuprorelin	3 monthly SC	24	Y	N	Y
4	27	AIP	Drug	U	U	NS	Goserelin	Monthly SC	9	Y	N	Y
5	19	AIP	Drug	>3	2	N	Goserelin	Monthly SC	24	Y	N	Y
6	41	AIP	Dieting	9	7	N	Goserelin	Monthly SC	U	N	N	NS
7	26	AIP	U	28	28	N	Goserelin	Monthly SC	U	U	U	NS
8	27	AIP	U	2	2	N	Goserelin	Monthly SC	4	Y	N	Y
9	19	AIP	Drug	45	45	N	Goserelin	Monthly SC	12	NS	N	Y
10	19	AIP	U	13	6	N	Triptorelin	NS	Ongoing	N	Acute attack	Y
11	19	AIP	Drug	10	10	Y	Leuprorelin	Monthly SC	10	Y	N	N
12	18	VP	Menstrual cycle/drug	4	3	N	Leuprorelin	Monthly SC, later switched to 3 monthly	25	U	N	Y
13	Mid teens	AIP	Menstrual cycle	10	10	N	Goserelin	Monthly SC	24	N	Flushing, nausea, hypotension	Y
14	20	AIP	Second pregnancy	>5	U	N	Goserelin	Monthly SC	4	N	N	Y
14							NS	NS	36	U	N	NS
15	22	AIP	Drug	NS	1	N	Goserelin	Monthly SC	36	U	U	NS
15							Goserelin	NS	54	U	N	Y
16	22	AIP	Alcohol/drug	5	5	N	Goserelin	Monthly SC	10	N	N	Y
17	23	AIP	U	15–20	15–20	N	Goserelin	Monthly SC	12	N	N	Y
18	28	AIP	Dieting	5	5	N	Goserelin	Monthly SC	3	N	N	Y
19	22	AIP	Menstrual cycle	5–10	5–10	N	Goserelin	Monthly SC, later switched to 3 monthly	42	N	N	Y
20	21	AIP	Stress	4	4	N	Goserelin	Monthly SC	5	N	Acute attack	Y
Median[e] (range)	22 (19–41)			(2–45)	(2–45)				12 (1–54)			

NS not stated on returned audit questionnaire, *U* unknown, *N* no, *Y* yes, *SC* subcutaneous, *Nr* number, *E* oestrogen

[a] Precipitant of first acute attack

[b] Total number of acute attacks only; time intervals not documented

[c] Duration in months

[d] Whether administered in first week of menstrual cycle

[e] Calculated from the known data only

Table 3 Outcome

Deemed as successful	Patient	Number of pre-GnRH acute attack admissions	Precipitant of symptomatic porphyria	Analogue used	Duration in months	Number of acute attack admissions on GnRH	Reason for withdrawal
Y	1	>6	Drug	Goserelin	16 (unsure)	NS	Completed 16 months
Y	3	3	Drug	Leuprorelin	24	1	Completed 24 months
Y	5	>3	Drug	Goserelin	24	1	Completed 24 months
Y	12	4	Menstrual cycle/drug	Goserelin	25	0	Completed 25 months
Y	15	U	Drugs	Goserelin	54	1	Completed 54 months
Y	17	15–20	U	Goserelin	12	1	Wanted to conceive
Y	18	5	Dieting	Goserelin	3	0	Side effects
Y	19	5–10	Menstrual cycle	Goserelin	42	3–6	Completed 42 months
PS	2	6	Drug	Leuprorelin	3	Y[a]	Hysterectomy with oophorectomy
PS	4	U	Drug	Goserelin	9	2	Further attacks
PS	16	5	Alcohol/drugs	Goserelin	10	1	Side effects
N	20	4	Stress	Goserelin	5	1	Further attacks
N	1 (a second treatment course)	>6	Drug	Goserelin	1	NA[b]	Triggered attack
N	6	9	Dieting/fasting	Goserelin	U	8	Further attacks
N	8	2	U	Goserelin	4	1	Further attacks Side effects
N	9	45	Drug	Goserelin	12	12	Further attacks
N	11	10	Drug	Goserelin	10	3	Further attacks
N	13	10	Menstrual cycle	Goserelin	24	4	Side effects
N	14	>5	Second pregnancy	Goserelin	4	5	Further attacks
NS[c]	7	28	U	Goserelin	U	Y[a]	NS
NS[c]	14 (a second treatment course)	>5	Second pregnancy	NS	36	NS	NS
NS[c]	15 (a second treatment course)	U	Drugs	Goserelin	36	NS	Completed 36 months
NA[c]	10	13	U	Triptorelin	Ongoing	1	NA
	Median of known data (range)	(2–45)			12 (1–54)		

U unknown, *NA* not applicable, *NS* not specified, *Y* yes, *PS* partly successful, *N* no

[a] Number not specified on returned proforma

[b] Was in hospital for an acute attack when GnA was initiated and experienced a further attack whilst still in hospital

[c] For patient 7, 14 and 15 (one of the two treatment cycles they received) information on whether the treatment was deemed successful or not was not documented on the returned proforma. As patient 10 had not completed the treatment course an opinion on whether it was successful or not was not given on the returned proforma

this or number of attacks was not specified. Patient number 15 had a first course of treatment lasting 36 months which was not well documented and a second course lasting 54 months, deemed as successful during which she only had 1 acute attack.

Information on analgesia requirements and quality of life prior to and during GnA prophylaxis was not sufficiently complete to report.

Following completion of prophylactic GnA treatment 17 patients have subsequently experienced further acute attacks, 5 of whom had had >4 acute attacks at the time the audit was conducted.

Discussion

Acute attacks of porphyria in AIP, HCP and VP are clinically similar and potentially life threatening. There are a variety of known physiological and environmental triggers that precipitate acute attacks. Drugs, steroid hormones, alcohol, fasting and infection directly or indirectly via cortisol and other hormones induce nuclear receptor activation resulting in either hepatic ALAS1 induction or cytochrome P-450 (haem containing) enzyme production needed for drug metabolism or hormone synthesis (Badminton et al. 2012). The influence of steroid hormones such as oestrogen and progesterone and differences in gender control of pituitary axis regulation explains why symptomatic acute porphyria is more common in women than men and why in women recurrent attacks may be associated with the menstrual cycle (Badminton et al. 2012; Thunell 2006), particularly in AIP (Hift and Meissner 2005).

GnA prophylaxis for prevention of menstrual cycle associated acute attacks was first described in 1984 (Anderson et al. 1984). Prior to this, hormonal manipulation to suppress ovulation with oral contraceptives was used with limited success (Perlroth et al. 1965). GnA initially acts as an agonist, but when administered continuously, desensitises the pituitary to GnA action with decreased pituitary LH and FSH release and suppression of ovulation and ovarian hormone production.

This retrospective audit demonstrates a large variation in practice in the United Kingdom with regard to prophylactic GnA treatment for females with recurrent acute porphyria attacks. Between 2000 and 2015, 23 treatment courses of GnA were given to 20 females with recurrent acute attacks over a period ranging between 1 and 54 months with a median treatment period of 12 months. Monthly subcutaneous Goserelin was most commonly used. In only 3 of the 20 patients treated with GnA was the initial precipitant of the first acute attack thought to be hormonal changes associated with the menstrual cycle. However 50% of the prescribed courses of GnA were viewed as being of clinical benefit in reducing acute attack frequency. The successfully treated group experienced between 3 and 20 acute attacks prior to and between 0 and 6 acute attacks during GnA treatment.

Published case studies and series (Anderson et al. 1984; Herrick et al. 1990; Innala et al. 2010) demonstrated that menstrual cycle associated acute attacks usually occur in the luteal phase, prior to menstruation when mid cycle oestrogen surges and corpus luteum progesterone production occurs, with symptoms subsiding with the onset of menstruation as hormone levels fall. As a result it has been recommended that prophylactic GnA should be initiated in the first week of the menstrual cycle (Anderson 1989; Herrick et al. 1990), when the risk of precipitating an acute attack is the lowest, although the evidence to support this is anecdotal. The efficacy of GnA is believed to be due to the down-regulating and resistance of pituitary receptors to hypothalamic LH releasing hormone (Fraser and Baird 1987). In the six treatment courses in which this timing was adhered to, no acute attacks were precipitated. In 3 of the 17 courses for which the timing of the dose was not considered, an acute attack occurred after administration of the first dose, suggesting that it may be the safest to initiate treatment in the first week of the menstrual cycle.

GnA renders females post-menopausal and predictable side effects include oestrogen deficiency symptoms such as hot flushes, bone demineralisation (Studd and Leather 1996) and reduced libido. Pregnancy is contraindicated during treatment, and patients should be advised about alternative contraceptive methods. Intra-nasal, subcutaneous or intramuscular preparations are available. Patients in this survey were all treated with long acting subcutaneous preparations administered monthly apart from three patients who received it 3 monthly. Both of these approaches were successful in reducing acute attack frequency. Eleven of the 16 patients with documented oestrogen deficiency side effects reported hot flushes and a minority also described low mood, hair thinning and loss of libido. Hormone replacement therapy using oestrogen or progesterone or both has been used previously with some patients experiencing reactivation of their porphyria (Innala et al. 2010). Reluctance to prescribe hormone replacement therapy to alleviate these symptoms exists due to both the fear of precipitating an acute attack and the requirement for endometrial monitoring, as evident by only seven patients receiving hormone replacement for symptomatic relief. This demonstrates that post menopausal symptoms are very common using prophylactic GnA and patients need to be fully informed prior to treatment which may improve successful completion of treatment courses.

Other methods of ovulation prevention using oral contraceptives (combined or progesterone alone) have not

been successful in the past as acute attacks have been precipitated in a subgroup of symptomatic patients (Anderson et al 2003; Innala et al. 2010; Lamon et al. 1979; Levit et al. 1957; Edwards and Elliot 1975; Kappas et al. 1968).

The retrospective nature of this audit has significant limitations. Data quality is subject to recall bias as it relied on the accurate recording of clinical information and patient recollection. We have chosen to not report information where there were doubts over the accuracy. Data comparing symptoms, neuropathy, need for analgesia between attacks, quality of life measures prior to and during GnA prophylaxis and detailed information on bone mineral density monitoring and hormone replacement regimes were too incomplete to report. The quantitative criteria for what would be considered a successful treatment outcome were not predefined and relied on patient and clinician opinion on whether clinical improvement on GnA with regard to decreasing acute attack frequency occurred. In the absence of complete remission of acute attacks, many courses were reported as unsuccessful or partially successful despite a clear improvement in patient symptomatology. Without an exact successful outcome definition, the occurrence of side effects could also have influenced the outcome opinion.

Despite these shortcomings, this is the first attempt at auditing the use of prophylactic GnA for recurrent acute attacks of porphyria in females in the United Kingdom, the majority of treatment having occurred prior to the establishment of a nationally commissioned and governed service. The audit reveals large variation in practice with regard to indications for and duration of GnA treatment as well as monitoring and treatment of side effects necessitating a more unified approach and identifying the need for treatment guidelines despite the existing limited evidence.

Three previous case series using GnA in 27 patients in total have been reported with similar partial success rates (Anderson et al. 1990; Herrick et al. 1990; Innala et al. 2010). Including our audit, a degree of clinical benefit with regard to reducing acute attack frequency has been reported in 31 of the 47 patients described. In a prospective study of 7 women between the age of 22 and 35 (Herrick et al. 1990), buserelin 4 times a day intranasally was given for 12 months. Reduction in acute attack frequency was reported in 5 females and the authors concluded that clinical benefit was dependent on the strength of the association between attack onset and stage of the menstrual cycle. In another cohort of 14 females (Innala et al. 2010) treated with either intranasal, subcutaneous or intramuscular buserelin or triptorelin for between 5 months and 9 years, 11 reported the treatment as beneficial with 4 having almost no further attacks. The third cohort (Anderson et al. 1990) treated 6 females for up to 26 months and benefit was observed in 5. All three cohorts reported oestrogen deficiency side effects, predominantly flushing and vaginal dryness in the majority of patients. In the one case series (Innala et al. 2010), hormone replacement therapy induced recurrence of attacks in 7 of 11 patients receiving oestrogen alone or in combination with progesterone. This group monitored the endometrium with 6-monthly uterine ultrasound and endometrial sampling. In agreement with our audit there was a lack of systematic consideration of the effect of this treatment on bone health in all three series.

Conclusion

Randomised control trial data on the efficacy of GnA and comparison of GnA versus prophylactic human haemin in preventing recurrent acute attacks of porphyria in females are lacking. Similarly to the case series' previously published, this audit demonstrates mixed results with regard to acute attack prevention in females with acute porphyria. Approximately 50% of the prescribed courses of GnA in our audit resulted in some degree of subjective clinical benefit although clear outcome criteria were not defined which makes judging success rates difficult. In view of the limited treatment options available for this challenging patient cohort and the mixed outcome successes reported, we believe it is reasonable to continue offering GnA treatment to women with recurrent debilitating attacks of acute porphyria, particularly if the attacks are clearly associated with the menstrual cycle, and the first dose is given in the first week of the cycle. GnA treatment is generally less complicated to deliver than regular haem arginate, which requires insertion of a semi-permanent venous access device and repeated, usually weekly, intravenous administration with the accompanying risks of extravasation, infection, malfunction and potential for iron overload.

The audit also demonstrates large variation in practice in the United Kingdom with regard to the indication for GnA use, duration of treatment, specific drug used and degree of monitoring and treatment of side effects. Based on the experience of this patient cohort and review of the limited literature, we suggest the following best practice guidelines to standardise the management of this subgroup of severely affected acute porphyria patients. These can also be used as standards for future NAPS prospective audit.

Indication for Prophylactic GnA Treatment

1. Confirmed recurrent acute attacks ($\geq$4 per year) of sufficient severity to require hospitalisation and treatment

with human haemin and which are associated with increased urine PBG excretion.
2. Females in whom acute attacks are associated with the menstrual cycle. A decision to embark on preventative treatment with GnA should always be reached in partnership with the patient after thorough, candid discussion of the likely benefits and risks.

Treatment

3. A multidisciplinary approach should be adopted between a clinician with expertise in porphyria and a gynaecologist with experience in the use of GnA. In the United Kingdom this should be through the NAPS service.
4. The first dose should be given during day 1–5 of the menstrual cycle.
5. The risk of precipitating an acute attack appears low and therefore it is reasonable for prophylaxis to be initiated on an outpatient basis.
6. Both intranasal and subcutaneous (monthly or 3 monthly) depot preparations appear to be successful in reducing acute attack frequency. Depot preparations may be preferred by patients compared to nasal preparations that require several doses daily.
7. To date, our largest experience has been with monthly subcutaneous Goserelin injections.
8. Patients should be monitored monthly for efficacy and side effects during the early phase of treatment, and then 6-monthly if treatment is continued
9. In view of the natural history and possibility of disease remission, treatment duration should be for a maximum of 2 uninterrupted years.

Side Effects and Monitoring

10. Oestrogen deficiency side effects occur in the majority of women and patients should be informed regarding this prior to initiating treatment.
11. Contraceptive advice should be provided as anovulation is not guaranteed and unwanted pregnancies whilst on GnA prophylaxis have been described (Innala et al. 2010).
12. Bone mineral density should be determined pre-treatment and then annually whilst GnA treatment is continued (Herrick et al. 1990).
13. Ensure all patients are Vitamin D replete prior to commencement of GnA prophylaxis; adequate dietary calcium intake and Vitamin D supplementation is recommended.

Withdrawal of Treatment

14. Case studies and case series demonstrated that the benefit is not always immediate so withdrawal due to treatment failure should only be considered after 3 months of no apparent benefit.
15. After completing 2 years of prophylactic GnA. If acute attacks recur, then a further treatment course can be considered, subject to confirmation of satisfactory bone mineral density.

Take Home Message/Synopsis

Although no trial data exists, prophylactic GnA may be a reasonable alternative to prophylactic human haemin in women with severe recurrent attacks of acute porphyria, particularly where attacks are associated with the menstrual cycle.

Compliance with Ethics Guidelines

Conflict of Interest

Danja Schulenburg-Brand, Tricia Gardiner, Simon Guppy, David C. Rees, Penelope Stein, Julian Barth, M. Felicity Stewart and Michael Badminton declare that they have no conflict of interest.

Informed Consent

Not applicable as this was an audit.

Animal Rights

Not applicable.

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

Author Contributions

Danja Schulenburg-Brand: data analysis and authored the paper.

Tricia Gardiner: audit design, data collection and review of paper.

Simon Guppy, David Rees, Penelope Stein, Julian Barth, Felicity Stewart: review of audit design, data collection and review of paper.

Mike Badminton: audit design, data collection and co-authored the paper.

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RESEARCH REPORT

Altered Cellular Homeostasis in Murine MPS I Fibroblasts: Evidence of Cell-Specific Physiopathology

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Abstract Mucopolysaccharidosis type I (MPS I), a rare autosomal recessive disease, is caused by a deficiency of the lysosomal enzyme alfa-L-iduronidase. Impaired enzyme activity promotes glycosaminoglycans accumulation in several tissues and organs, leading to complex multisystemic complications. Several studies using animal models indicated different intracellular pathways involving MPS I physiopathology; however, the exact mechanisms underlying this syndrome are still not understood. Previous results from our group showed alterations in ionic homeostasis and cell viability of splenocytes and macrophages in *Idua*−/− mice. In the present study, we found altered intracellular ionic homeostasis in a different cell type (fibroblasts) from the same murine model. *Idua*−/− fibroblasts from 3-month-old mice presented higher cytoplasmatic and endoplasmic reticulum Ca^{2+} concentration, lower levels of mitochondrial Ca^{2+} and mitochondrial membrane potential and higher cytoplasmatic pH when compared to *Idua*+/+ animals. Also, Idua−/− fibroblasts were more resistant to the apoptotic induction with staurosporine, indicating a possible resistance to apoptotic induction in those cells. In addition, despite the intracellular ionic imbalance, no significant alterations were found in apoptosis and autophagy in *Idua*−/− fibroblasts, which implies that the ionic alterations did not activate those pathways. The investigation of mechanisms underlying the cellular physiopathology of lysosomal diseases is crucial for a better understanding about the progression of these diseases. Since splenocytes, macrophages, and fibroblasts have different embryonic origins and distinct structural and functional features, potentially altered signaling pathways found in a cell-specific manner in an alfa-L-iduronidase-deficient environment provide additional understanding of the clinical multisystemic presentation of this disease and provide new basis for improved therapeutic approaches.

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Introduction

Mucopolysaccharidosis type I (MPS I) is a progressive lysosomal storage disease caused by deficient α-L-iduronidase, responsible for GAG (heparan and dermatan sulfate) catabolism. These non-degraded substrates accumulates in several organs and tissues, leading to multisystemic complications (Neufeld and Meunzer 2001). The exact mechanisms underlying MPS physiopathology are still not well understood; however, several studies using cellular and animal models indicated alterations in different intracellular signaling cascades, not related to the enzyme deficiency (and subsequent GAG accumulation) itself (Clarke 2008). Dysfunctions in intracellular pathways such as Ca^{2+} signaling, apoptosis/autophagy, bone turnover, and inflammation were also observed in different cell types derived

from MPS animals (Kiselyov et al. 2007; Baldo et al. 2012; Lieberman et al. 2012). An impairment of the autophagic pathway was observed in mouse embryonic fibroblasts from MPS IIIA mice, leading to accumulation of dysfunctional organelles and toxic substrates and consequently cell death (Settembre et al. 2008). Mitochondrial defect and altered autophagy were related to neuronal death in MPS IIIC brains (Pshezhetsky 2016). Elevated levels of gangliosides (GM2 and GM3) and cholesterol were also found in neurons from MPS IIIA animals, indicating a secondary accumulation of storage material in MPS brains leading to a neuronal dysfunction (McGlynn et al. 2004). Decreased autophagy could also lead to an imbalance of reactive oxygen species production. Changes in redox state balancing have been described in MPS models. In a study using MPS VI rats, it was described a higher expression of cytochrome b5588, involved in oxidative burst of phagocytes (Simonaro et al. 2008). Our group have also found evidences of oxidative stress in blood cells from MPS I patients, where increased toxic products from lipid peroxidation, as malondialdehyde, led to lysosomal destabilization and consequently permeabilization (Pereira et al. 2008). Evidences of multi-organellar Ca^{2+} storage, intracellular pH alterations, and lysosomal permeability with higher cysteine proteases activity and apoptotic rate in murine *Idua*−/− splenocytes were also shown (Pereira et al. 2010). In a recent paper, we also demonstrated that changes in Ca^{2+} signaling itself were not sufficient to induce apoptosis in *Idua*−/− macrophages (in contrast to splenocytes); however, these cells appeared to be more sensitive to apoptotic induction and presented impaired phagocytosis ability (Viana et al. 2016). Thus, the present work shows altered homeostasis (ionic imbalance, apoptosis/autophagy induction, and GAG storage) of another cell type (fibroblasts) from 3-month-old *Idua*−/− mice.

Methods

Mice

C57BL/6 (*Idua*+/+) and α-L-iduronidase-deficient mice (*Idua*−/−), both 3-month-old, were used. MPS I mice (Ohmi et al. 2003) were kindly donated by Dr. Elizabeth Neufeld (UCLA, USA) and Dr. Nance B. Nardi (UFRGS, Brazil). The colony was established at Universidade Federal de São Paulo (UNIFESP) after heterozygous (*Idua* +/−) mating breeding and maintained on a light-dark 12:12 cycle under controlled temperature conditions (20 ± 2°C), with free access to food and water. Euthanasia was performed using cervical dislocation. All animal procedures were in accordance to the guidelines for care and use of animals and were approved by the Animal Care Ethics Committee of UNIFESP (#0594/10). After weaning, mice were identified according to their genotypes, by polymerase chain reaction, using specific oligonucleotides that amplify a fragment of *Idua* gene. After genotyping, 3-month old *Idua*+/+ and *Idua*−/− animals were selected for all experiments.

Peritoneal Fibroblast Culture

After euthanasia, the peritoneal membranes from both *Idua* +/+ and *Idua*−/− were removed and sliced into several small pieces of approximately 10 mm × 10 mm square sections. Under a laminar flow cabinet, pieces were then placed in a 35-mm Petri dish with R10 (RPMI-1640 medium supplemented with 10% of fetal bovine serum) to allow cell migration from tissue to the plate. After 5–10 days, cells were harvested, centrifuged at 200 × *g* for 6 min at 4°C, and seeded at a density of 10^6 cells/mL.

Ca^{2+} Signaling and Mitochondrial Membrane Potential

Ca^{2+} measurements were assessed using fluorescence microscopy (DM6000 microscope; Leica Microsystems Inc., Wetzlar, Hesse, Germany). The following fluorophores were used for cytoplasmic Ca^{2+} (Ca^{2+}_{cit}), mitochondrial Ca^{2+} (Ca^{2+}_{mit}), and mitochondrial membrane potential (ψ_{mit}) quantifications: Fluo-4AM (4 μM), Rhod-2AM (1 μM), and TMRE (250 nM), respectively. All fluorophores were purchased from Life Technologies (Carlsbad, CA, USA). Fluorescence was acquired with the following excitation/emission filter cubes, respectively: I3 (BP 470/40 nm and BP 525/50 nm) for Fluo-4AM and N2.1 (BP 515–560 nm and LP 590 nm) for Rhod-2AM and TMRE.

Intacellular pH

Lysosomal pH was quantified using Acridine Orange (AO; Molecular Probes, Invitrogen, USA). AO is a metachromatic fluorophore, which is accumulated in acidic organelles, especially in lysosomes. When excited by a blue light (BP 470/40 nm), AO emits green fluorescence (BP 505–530) related to cytosolic pH, or red fluorescence (LP 560 nm), related to lysosomic pH. Cells were plated in Petri dishes (35 mm; Mat Tek Corp, USA) at a density of 5×10^6. After adhesion, cells were incubated with AO (0.5 μM, 15 min), washed twice with HBSS, and visualized under fluorescence microscopy. Fluorescence intensities

were then quantified and expressed as relative fluorescence units (RFU) for cytoplasmic pH (alkaline) and lysosomal pH (acidic), as well the ratio alkaline/acidic.

Cellular Viability Assay

For cell viability evaluation, peritoneal fibroblasts from *Idua*+/+ and *Idua*−/− (2×10^5 cells/mL) were treated with 5 μM staurosporine (STS; Sigma–Aldrich, USA) for 24 h. After this period, cells were harvested and incubated for additional 15 min with Annexin-V-FITC (Life Technologies, USA) and Propidium Iodide (PI; Life Technologies, USA), indicators of apoptosis and necrosis, respectively. Assessment of cell viability and cell death (apoptosis and necrosis) was performed comparing values between both *Idua*+/+ and *Idua*−/− groups and between stimulated and non-stimulated cells (basal). The fluorescence readings were acquired in a Tali® ImageBased Cytometer (Life Technologies, USA).

Western Blotting: Apoptosis and Autophagy

Fibroblast protein extracts were obtained using NP-40 buffer supplemented with 10% of protease inhibitor cocktail (Sigma–Aldrich, USA). After quantification, proteins were then denatured in sample buffer (2% SDS, 62.5 mM Tris–HCl pH 6.8, 10% glycerol, 0.04 mg/mL bromophenol blue and 350 mM beta-mercaptoethanol), heated at 95°C for 5 min, and loaded (50 μg) into 15% (LC3-II, Bcl-2, Bax and GAPDH) or 10% (Beclin-1 and p62) polyacrylamide gels. Electrophoresis were done at constant voltage of 100 V. Proteins were then transferred to the nitrocellulose membranes at constant current (230 mA) for 90–120 min. After transfer, the nitrocellulose membranes were blocked in blocking solution (5% non-fat milk) for 1 h and incubated with primary antibodies overnight. All primary antibodies (produced in rabbit) were purchased from Novus Biologicals (Novus Biologicals Inc., USA), Sigma (Sigma–Aldrich, USA), or CST (Cell Signaling Technology, USA), with specific reactivity to mice. After primary antibody incubation, membranes were incubated with Alexa Fluor® 680-conjugated anti-rabbit IgG (Life Technologies, USA) for 45 min. The normalization of the results was performed with the GAPDH protein (glyceraldehyde 3-phosphate dehydrogenase) and the visualization of the results and band intensities quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences, USA).

Caspases 3, 8, and 9 Activities

The analysis of caspases activities was assessed using ImageiT™ LIVE Green Poly Caspases Detection Kit (Life Technologies, USA), with a fluorescent caspase inhibitor (FLICA) and a fluorescent nuclear marker (Hoeschst 33342). Approximately 5×10^5 cells/well were plated in a 96-wells black plate with a light bottom (Nunc, Germany). After adhesion, cells were treated with STS (5 μM; 4 h) or R10 (non-stimulated control); washed and incubated with FLICA and Hoeschst for 1 h. Cells were washed twice with a specific kit buffer and fluorescent emissions were read using SpectraMax M2 – MDS (Molecular Devices, USA). Green fluorescence indicated caspases activities (ex/em: 488/520 nm) and blue fluorescence indicated nuclear staining (ex/em: 350/461 nm). Results were expressed as a ratio of green and blue fluorescences to normalize the emission according to the cell density.

Statistics

All data were expressed as mean ± SEM. Comparisons were performed with unpaired Student's t test using Statistica 8.0 software (Stat Soft Inc., USA). The level of significance was set at $P < 0.05$.

Results

Ca2+ Signaling and Mitochondrial Membrane Potential

Measurements of Ca^{2+} homeostasis showed that basal cytosolic Ca^{2+} was significantly decreased in *Idua*−/− mice compared to *Idua*+/+ (Fig. 1a, d). No statistical difference was observed in basal mitochondrial Ca^{2+} (Fig. 1b, e). However, an increase in mitochondrial potential was found in *Idua*−/− group compared to control (Fig. 1c). Inhibition of Ca^{2+}-ATPase pump from endoplasmatic reticulum (ER) by thapsigargin (THG) produced a lower Ca^{2+} release in *Idua*−/− group (Fig. 1d). Similarly, mitochondrial Ca^{2+} and mitochondrial potential were also decreased after THG stimulus (Fig. 1e, f).

Lysosomal Homeostasis

Acridine orange has been known for many years as a convenient metachromatic dye to stain acidic vesicles, with fluorescence wavelength emission strongly dependent on concentration. The mechanism of AO accumulation in those vesicles is related to the protonation of AO molecules in a low pH environment, creating an electric charge that consequently hinders the ability of the dye molecules to cross the vesicular membrane (e.g., lysosomes) and escape back into the surrounding cytoplasm (Traganos and Darzynkiewicz 1994; Dobrucki et al. 2007). After incubation with AO, *Idua*−/− fibroblasts showed a higher cytosolic pH (Fig. 2a) and the ratio of lysosomal and

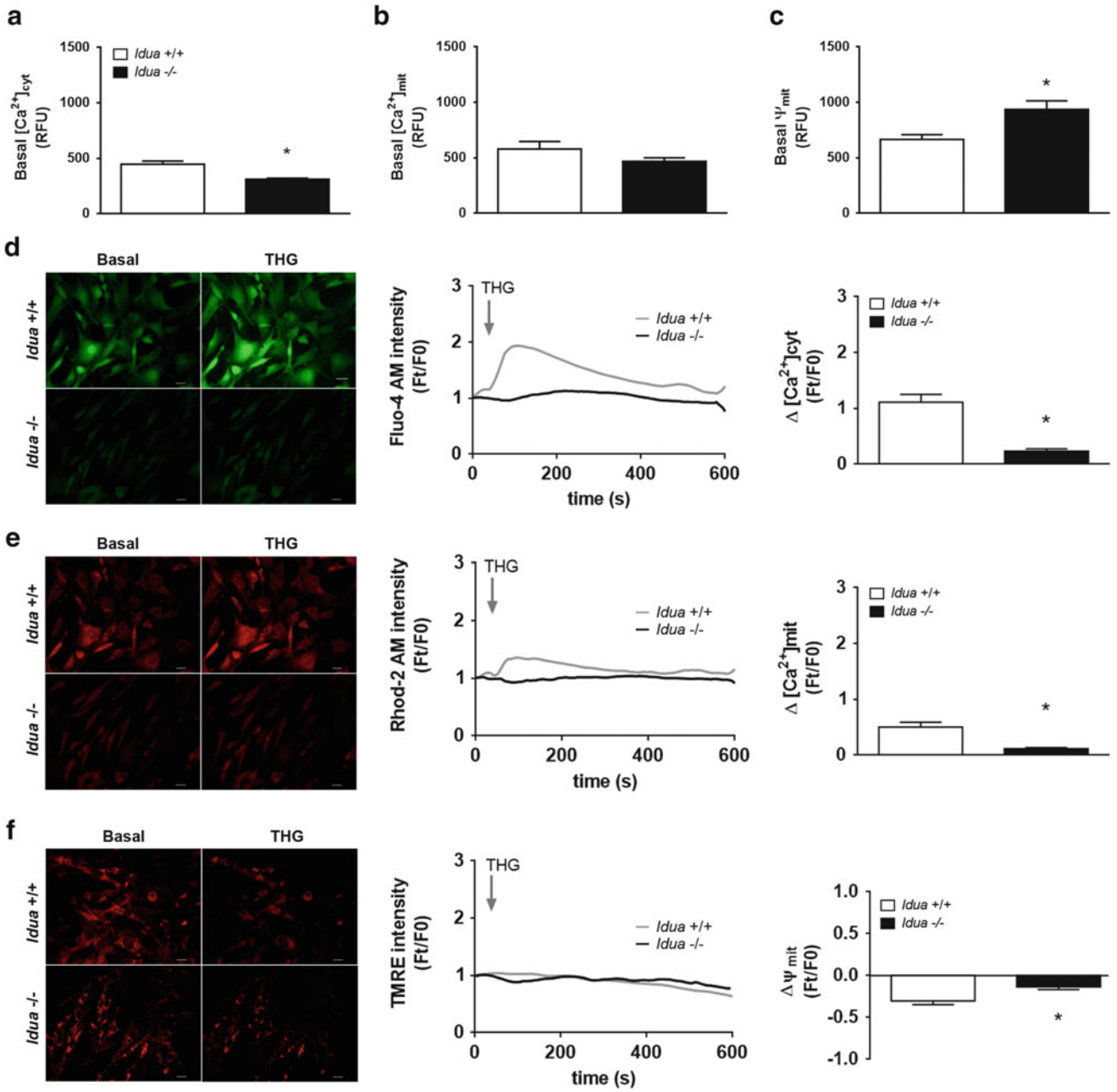

Fig. 1 Alterations in intracellular Ca^{2+} and mitochondrial homeostasis in *Idua* −/− fibroblasts. Peritoneal fibroblasts from Idua +/+ and Idua −/− mice were incubated with Fluo-4AM (4 μM) for cytoplasmatic Ca^{2+}, Rhod-2AM (1 μM) for mitochondrial Ca^{2+}, and TMRE (250 nM) for mitochondrial membrane potential measurements. Fluorescence microscopy was used to capture images, which were pseudocolored green (for cytoplasmatic Ca^{2+}) and red (mitochondrial Ca^{2+}) or mitochondrial membrane potential). As shown in (**a**), Idua −/− fibroblasts showed lower basal cytoplasmatic Ca^{2+} and higher mitochondrial membrane potential (**c**), despite no alterations in basal mitochondrial Ca^{2+} (**b**). To evaluate possible alterations in intracellular Ca^{2+} mobilization, cells were also incubated with THG (10 mM), a specific ER $Ca2^{+}$/ATPase inhibitor (**d**–**f**). The fluorescence intensity as a function of time corresponds to the images shown in the middle and the ratio between fluorescence readings before and after THG addition (Ft/F0) indicates the relative concentration of Ca^{2+} at ER (*right*). A reduced relative concentration ER Ca^{2+} was found in *Idua* −/− fibroblasts after THG addition (**d**). Lower mitochondrial Ca^{2+} variations were also found in these cells (**e**), indicating a reduced ability of Ca^{2+} buffering from this organelle, despite normal mitochondrial membrane potential (**f**). $Ca^{2+}{}_{cit}$: cytoplasmatic Ca^{2+}; $Ca^{2+}{}_{mit}$: mitochondrial Ca^{2+}; ψ_{mit}: mitochondrial membrane potential. Data are expressed as the mean ± S.E.M. of three independent experiments. $P < 0.05$ (Student's *t* test). $n = 4–6$

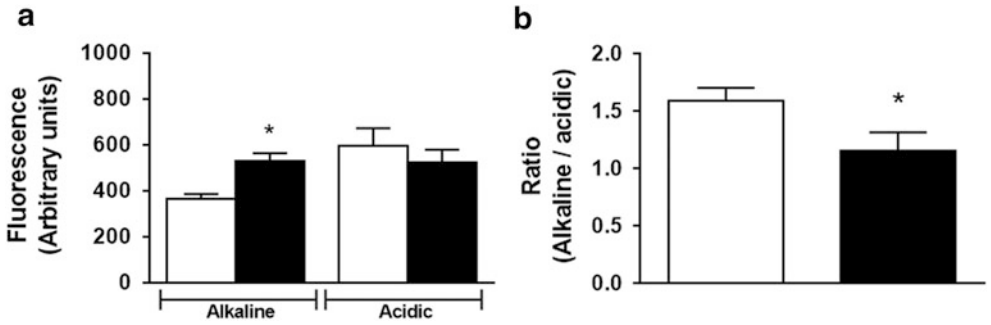

Fig. 2 Evidence of cytoplasmic alkalinization in *Idua* −/− fibroblasts. *Idua* −/− fibroblasts (5×10^5) were seeded in glass-bottom 35-mm Petri dishes and incubated with AO (0.5 μM) for 15 min and visualized under fluorescence microscopy. *Idua* −/− fibroblasts showed elevated cytoplasmic pH (alkaline) but normal lysosomal pH (acidic), when compared to *Idua* +/+ cells (**a**). In addition, alkaline/acidic ratio was lower in Idua −/−, indicating a disruption of intracellular H^+ homeostasis (**b**). Data are expressed as the mean ± S.E.M. of three independent experiments. $P < 0.05$ (Student's *t* test). $n = 4–6$

cytosolic pH decreased in the same group, compared to the control (Fig. 2b).

Apoptosis and Cellular Viability

There were no differences in the relative expression of apoptotic proteins (Bax and Bcl-2) and autophagic proteins (p62, beclin and LC3 II) between *Idua*+/+ and *Idua*−/− groups (Fig. 3a–d and Fig. 3e–h, respectively). Also, basal cellular viability appeared similar between both groups (Fig. 3i). However, after STS treatment, fibroblasts from *Idua*−/− group appeared more resistant to cell death, since we observed a higher percentage of viable fibroblasts and a lower number of apoptotic cells in *Idua*−/− mice (Fig. 3j). We also examined the activity of caspases 3, 6, and 9, but no statistical difference was observed between groups (Fig. 3k).

Discussion

Ionic intracellular homeostasis, autophagy, and apoptosis are some of the most affected mechanisms reported in studies related to animal models of lysosomal storage diseases (Kiselyov et al. 2010; Settembre et al. 2013). It is a consensus that MPS I is a progressive and multisystemic disease; however, it is still unclear whether the same mechanisms are similarly altered in different cell types. We decided to describe some physiological cellular parameters in fibroblasts of *Idua*−/− mice to implement the characterization of this important animal model and to compare previous data observed in splenocytes (Pereira et al. 2010) and macrophages (Viana et al. 2016). Although they share the same microenvironment, peritoneal macrophages and fibroblasts exert distinct functions in vivo. Macrophages act in immunological response against pathogenic agents, while fibroblasts maintain extracellular matrix and participate of tissue repairing and remodeling.

Ca^{2+} homeostasis is essential to cellular functions such as hormonal secretion, neurotransmitter release, muscle contraction, induction of cell death by apoptosis, necrosis, and autophagy (Harr and Distelhorst 2010). In fibroblasts, we observed a decrease in ER Ca^{2+}, as we have previously observed in macrophages of *Idua*−/− mice (Viana et al. 2016). Otherwise, our group observed a higher stock of ER Ca^{2+} in splenocytes of 6-month-old *Idua*−/− mice (Pereira et al. 2010). This suggests a failure of ER Ca^{2+}/ATPase, since Ca^{2+} maintenance depends on a coordinated action among calcium Ca^{2+} channels and Ca^{2+}/ATPase activity. Failure of Ca^{2+} homeostasis may also functionally affect lysosomes and mitochondria, such as the maintenance of cellular viability upon the cell death pathways control (Kiselyov and Muallem 2008). Pereira et al. (2010) encountered a higher level of cysteine proteases in the cytosol and an increased number of apoptotic splenocytes of *Idua*−/−. Furthermore, we had also previously described diminished ER Ca^{2+} in *Idua*−/− macrophages and an increase in mitochondrial Ca^{2+} basal levels (Viana et al. 2016).

Besides, after THG treatment, the Ca^{2+} mitochondrial uptake was lower, probably by low Ca^{2+} stocks in the ER, which reinforces the hypothesis of Ca^{2+}/ATPase impairment in this model. Although ER Ca^{2+} levels were lower in fibroblasts of *Idua*−/−, we have not found any difference in basal mitochondrial Ca^{2+}. However, the basal mitochondrial potential was increased in the same group. This result may be related to a protective cellular mechanism, as it has been described in cancer cells (Fulda et al. 2010; Ubah and Wallace 2014). On the other hand, an exaggerated increase of mitochondrial Ca^{2+} may also outcome in a metabolic overload, deficiency of ATP synthesis, excess of reactive oxygen species production,

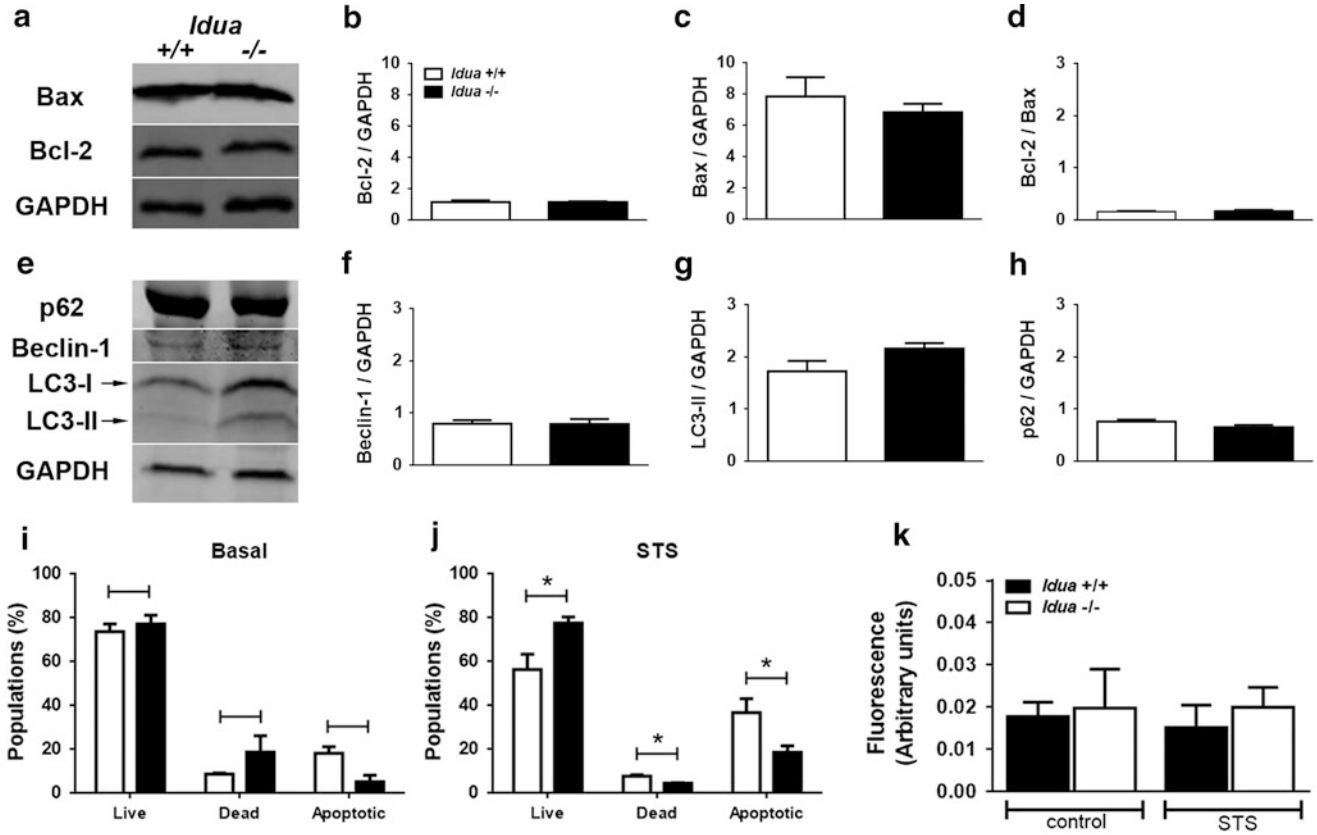

Fig. 3 *Idua* −/− fibroblasts are more resistant to apoptotic induction. Total protein extracts of Idua +/+ and Idua −/− fibroblasts (30–50 mg) were loaded into 10–15% SDS-polyacrylamide gels for evaluation of basal apoptotic (Bcl-2 and Bax) and autophagic (p62, beclin-1 and LC3-II) markers. No differences were observed in Bcl-2 and Bax relative expression (**a**–**d**), as well as in autophagy markers such p62, beclin-1, and LC3-II (**e**–**h**). Moreover, no changes in basal viability of peritoneal *Idua* −/− fibroblasts were observed (**i**). However, incubation with STS (5 μM) for 4 h resulted in an increase of viable (live) and a decrease of dead and apoptotic cells (**j**). In addition, no differences were observed in caspase activity of control (no STS) and STS-treated cells (**k**). Data are expressed as the mean ± S.E.M. of three independent experiments. $P < 0.05$ (Student's *t* test). $n = 4–6$

decrease in membrane potential, and consequently loss of mitochondrial functions and cell death (Patron et al. 2013). Ca^{2+} fluctuations may interfere in lysosomal homeostasis, which interfere in a variety of mechanisms.

Non-degraded substrates inside lysosomes change the membrane permeability and interfere in vesicle trafficking, altering the balance among cell proliferation and cell death (Lagadic-Gossmann et al. 2004). Activation of apoptosis has been described in different cell types from MPS animal models (Simonaro et al. 2008; Pereira et al. 2010). However, despite higher cytoplasmic pH and altered alkaline/acidic ratio found in cytoplasm of *Idua*−/− fibroblasts, no differences in cell viability were observed, as observed by reduced caspase activity and number of *Idua*−/− apoptotic fibroblasts after STS. Also, we have found no significant difference in Bcl-2 relative expression, neither in Bax expression in *Idua*−/− fibroblasts, indicating no mitochondrial damage.

Although some authors have indicated signs of autophagic deregulation in different tissues from MPS models (Ballabio 2009; Tessitore et al. 2009), we have not detected significant differences in relative expression of autophagic proteins in fibroblasts of *Idua*−/− mice. This data indicated that lysosomal dysfunction found in fibroblasts from MPS I mice could not impair endosomal vesicular traffic and cellular recycling, which is crucial to maintenance of cell viability.

However, it is important to stress that this lack of differences in autophagy markers in those cells is probably due to the absence of paracrine modulation between adjacent cells/tissues and possible differences in GAG deposition profile in several biological microenvironments in vivo. In fact, some studies on evaluation of intracellular signaling cascades can be only performed in isolated cultured cells from animal models, so general extrapolation of these results to humans should be carefully addressed.

In summary, the present study indicated significant differences of ionic homeostasis of ER, mitochondria, and lysosomes in *Idua*−/− fibroblasts. Despite the intracellular ionic imbalance in *Idua*−/− cells, no significant alterations were found in the relative expression of apoptosis and autophagy proteins in both wild type and Idua−/− fibroblasts, implying that such alterations did not activate these pathways. These evidences indicate that MPS I cellular physiopathology can be highly heterogeneous in a cell-specific manner and brings new perspectives to understand how multisystemic complications are possible related to changes in cellular physiology, tissue/cell type, storage substrate, and disease progression.

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Synopsis

Evidence of cell-specificity in murine MPS I physiopathology.

Compliance with Ethics Guideline

Conflict of Interest

Gustavo Viana, Cinthia Nascimento, Edgar Paredes Gamero e Vânia D'Almeida declare that they have no conflict of interest.

Animal Rights

All institutional and national guidelines for the care and use of laboratory animals were followed.

Details of the Contributions of Individual Authors

Gustavo Viana performed the experiments. Gustavo Viana, Cinthia Nascimento, Edgar Paredes Gamero, and Vânia D'Almeida analyzed data. Gustavo Viana, Cinthia Nascimento, and Vânia D'Almeida wrote the manuscript.

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RESEARCH REPORT

Irreversibility of Symptoms with Biotin Therapy in an Adult with Profound Biotinidase Deficiency

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Abstract We report a 36-year-old woman who exhibited progressive optic atrophy at 13 years old, then stroke-like episodes and spastic diplegia in her 20s. Biotinidase deficiency was not readily considered in the differential diagnosis, and the definitive diagnosis was not made until pathological variants of the biotinidase gene (*BTD*) were found by exome sequencing. Profound biotinidase deficiency was confirmed by enzyme analysis. Unfortunately, her symptoms did not resolve or improve with biotin treatment. Biotin therapy is essential for all individuals with profound biotinidase deficiency and for preventing further damage in those who already exhibit irreversible neurological damage. Newborn screening for the disorder would have avoided years of clinical symptoms that now appear to be irreversible with biotin treatment.

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Introduction

Biotinidase (EC 3.5.1.12) is the enzyme responsible for cleaving and recycling the vitamin biotin from biocytin and from dietary protein-bound sources (Pispa 1965; Wolf 2001). Profound biotinidase deficiency (<10% of mean normal serum activity) (OMIM #253260) is an autosomal recessively inherited metabolic disorder (Wolf 2012). Individuals with profound biotinidase deficiency, if untreated, usually exhibit seizures, hypotonia, skin rash, alopecia, vision problems, hearing loss, and developmental delay with accompanying ketolactic acidosis, and organic aciduria, usually in infancy or early childhood (Wolf 2001, 2012). Symptoms of the disorder can be successfully improved or prevented with pharmacological doses of oral biotin. However, if vision and hearing problems and developmental delay occur in an untreated individual, they are usually irreversible (Wolf 2012). Delayed-onset biotinidase deficiency presents with myelopathy with or without vision problems in adolescence or adulthood (Wolf et al. 1998; Wolf 2015a). All states in the United States and many countries screen their newborns for the disorder, although some countries do not (Wolf 2015b).

We report the case of a 36-year-old woman who had late, atypical clinical, and metabolic features, despite having profound biotinidase deficiency, and which resulted in a greatly delayed diagnosis and irreversible disability. It exemplifies the clinical variability of this potentially treatable disorder and the need to consider it in the differential diagnosis of diverse, even adult clinical presentations.

Case Report

A 27-year-old woman was referred to the inherited metabolic disease service at the Alberta Children's Hospital for further investigation of progressive optic atrophy and paraplegia. She had been healthy with normal development in infancy and childhood, except for a febrile seizure. At 13 years of age, her vision, which had previously been documented at 20/20, started to deteriorate, and she was diagnosed with optic atrophy. Her vision was adequate for reasonably normal functioning until about 19 years of age, when she experienced deterioration in her mid-20s when she became legally blind. From about 18 years of age, she experienced progressive leg weakness, balance difficulties, and severe pain in her abdomen, back, and legs but remained ambulatory until age 24 when she developed acute worsening associated with situational emotional stress. Since that time, she has not been able to walk. Significant pain in her legs has been her most concerning symptom, likely due to her spastic diplegia. Hearing was reported normal. She completed a Bachelor of Arts degree. Past surgeries included cholecystectomy, tonsillectomy, and a Caesarian section. Previous investigations did not reveal a cause for her problems, including a normal MRI of the brain and spinal cord, an EMG and nerve conduction, a muscle biopsy, an audiological evaluation and normal molecular testing for Leber hereditary optic neuropathy, spinal cerebellar ataxia, and Friedreich ataxia. Her parents are of Native American, Cree ancestry, but are not known to be consanguineous. She has two healthy siblings. She had three pregnancies, a son with type 1 diabetes diagnosed at 4 years, a daughter with multiple anomalies consistent with VACTERL association, and an early miscarriage. There is no other known family history of neurologic or neuromuscular disorders, blindness, or deafness.

Significant features on physical examination were obesity, left esotropia, spasticity, muscle weakness, brisk deep tendon reflexes, up-going plantar responses, and clonus in the legs and normal power, tone, and reflexes in her arms.

Further investigations included normal CBC, coagulation profile, urinalysis, electrolytes, glucose, liver function tests, lactate, plasma amino acids, plasma methylmalonic acid, plasma and urinary 3-methylglutaconic and 3-methylglutaric acids, and oxypurines. Plasma-free and total carnitine, and an acylcarnitine profile were normal (3-hydroxyisovaleryl carnitine was 0.09 μmol/L; normal <2 μmol/L); organic acids were normal except for a borderline elevated 3-hydroxyisovalerate at 2.01 mmol/mol creatinine (normal <2 mmol/mol creatinine). Molecular testing for the common mitochondrial DNA deletion for MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) and Charcot-Marie-Tooth, types 1A and 1B were all negative.

Plasma homocysteine concentration was elevated at 57.7 μmol/L (normal = 4.7–10.8 μmol/L). She was found to be a homozygote for the *MTHFR* 677C>T mutation. Further investigations revealed normal vitamin B12 and folate concentrations. Molecular analysis of the CBS gene for cystathionine beta-synthase was negative. Because this was not an adequate explanation for the degree of hyperhomocysteinemia, further detailed studies were performed on cultured fibroblasts (courtesy of Dr. David Rosenblatt, McGill University). [^{14}C]-propionate incorporation was slightly low, but higher than that seen in the methylmalonic acidurias, and [^{14}C]-methyltetrahydrofolate was normal. The uptake of [^{57}Co]-cyanocobalamin was low, but there was adequate synthesis of adenosylcobalamin and methylcobalamin. However, the specific activity of methylenetetrahydrofolate reductase was low at 5.4 compared to 16.5 nmol CHO/mg protein/h in extracts from a control cell line. Subsequent complete sequencing of the *MTHFR* gene did not reveal any other deleterious mutations.

She was prescribed betaine (3 g t.i.d), folate (5 mg/d), and vitamin B12 (0.5 μg b.i.d). Her other medications included baclofen, Elavil, ranitidine, ASA, and nitrazepam. Plasma homocysteine concentration decreased to 15.5 μmol/L, but compliance was inconsistent with later values in the 30–40 μmol/L range. There was no obvious clinical benefit from this regimen; in fact, there was some deterioration over about 8 years of subsequent follow-up evaluations. She had several sudden episodes of muscular spasm or stroke-like episodes, associated with vomiting and diarrhea, and accompanied by hemiparesis on one occasion and sensory loss in her lower extremity with facial palsy. Five months prior to her diagnosis, she woke up with right-sided facial weakness. This was followed by a severe headache in the right temporal and mastoid region and weakness of her right leg and arm. She also had an intermittent skin rash with the appearance of folliculitis pilaris and fragile nails, but her hair was normal with no alopecia. On two occasions, she had mildly abnormal organic acid analyses with moderate elevations of isovaleric acid, 3-methylglutaconic acid, and traces of methylcrotonylglycine and tiglylglycine and plasma acylcarnitines with a mild elevation of isovaleryl carnitine (1.48 and 1.19 μmol/L).

After appropriate counseling and informed consent, she was enrolled in the "Care4Rare" exome sequencing research program, through which she was discovered to have biallelic pathogenic mutations in the biotinidase (*BTD*) gene: c.511G>A;1330G>C; p.Ala171Thr;Asp444His and c.98_104delinsTCC; p.Cys33Phefs*36. This combination is known to cause profound biotinidase deficiency (Wolf et al. 1985) and was subsequently confirmed with serum biotinidase activity of <1.0 IU/L (normal 5.8–14.6 IU/L).

Biotin treatment, 16 mg for 2 weeks and then increased to 24 mg, over 3 months failed to improve any of the symptoms. Because there were no clinical improvements, she stopped her biotin after 3 months. However, biotin was

restarted after she developed significant pain in her posterior left thigh. She has had no further stoke-like episodes to date.

Discussion

This individual had an unusual, late, and confusing presentation of biotinidase deficiency. There apparently were no significant problems in childhood, and optic atrophy was the first issue developing in adolescence, followed by progressive muscle weakness, balance difficulties, and spastic paraplegia in early adult life and then by subsequent stroke-like episodes. In retrospect, her clinical picture is compatible with the diagnosis of biotinidase deficiency. Hyperhomocysteinemia at first appeared to be a diagnostic clue, but retrospectively turned out to be irrelevant, and diverted attention away from other possible diagnoses. Metabolic abnormalities in acylcarnitines and organic acids were unfortunately only borderline abnormal and disregarded because they were not consistent or not considered particularly significant in her clinical context. As a result, she underwent an unfruitful extensive diagnostic odyssey, only finally to be resolved by exome sequencing.

The phenotype of adolescents and adults with profound biotinidase deficiency is different from that of younger children (Wolf et al. 1998). These older untreated individuals with delayed-onset biotinidase deficiency exhibit diplegia/myelopathy with or without vision abnormalities (Wolf 2015a). There are multiple reports of older symptomatic individuals who have been diagnosed with various other disorders before the correct diagnosis was made and biotin therapy begun (Bottin et al. 2015; Wolf 2015a) However, when identified relatively rapidly, the symptoms can be ameliorated with therapy. The individual reported here is the oldest reported symptomatic individual to have been diagnosed with profound biotinidase deficiency and fails to demonstrate any improvement of symptoms with pharmacological doses of biotin. If diagnosis and treatment are delayed, the symptoms in adults may be irreversible, as they can be in older enzyme-deficient children. There has always been some improvement in symptoms, even complete resolution, in affected adolescents and other adults with biotin treatment, although not in the individual reported here (Wolf 2015a).

Based on previous experience, we expected to see some or significant clinical improvement after 3 months of biotin therapy. However, no improvement was observed. There is always the question of compliance, although she reported that she took the biotin during this initial 3-month period. If the clinical features are allowed to continue without a definitive diagnosis and the institution of biotin therapy, as in this case, they may become irreversible. Although subsequent biotin therapy will likely prevent new neurological problems from developing, many or most symptoms may have become permanent if sufficient time has passed before the diagnosis and biotin therapy has begun.

This individual appears to have some abnormality of homocysteine metabolism with an increased plasma homocysteine concentration and decreased MTHFR activity. Although the homozygous 677C>T alteration in *MFTHR* is known to be benign, in the presence of an elevated plasma homocysteine, we cannot entirely exclude that this individual is at increased risk for a thromboembolic or "stroke-like" event from her hyperhomocysteinemia. However, the major neurological symptoms of visual abnormalities and spastic diplegia seen in this individual are consistent with those seen in other symptomatic adults with profound biotinidase deficiency (Bottin et al. 2015; Wolf 2015a).

This case report emphasizes the following important points:

1. If a symptomatic adult with biotinidase deficiency is unrecognized for a sufficient length of time before being diagnosed and treated, the clinical symptoms may be irreversible with biotin therapy.
2. Biotin therapy is essential for all individuals with profound biotinidase deficiency and for preventing further damage in those who already exhibit irreversible neurological damage.
3. Exome or whole-genome sequencing was important in definitively establishing a diagnosis.
4. The importance of paying attention to abnormal results, even if seemingly minor and inconsistent, and at the same time not to be distracted by abnormal results that may lead to blind alleys.
5. Neonatal screening for biotinidase deficiency is important. At the date of her birth, biotinidase newborn screening had not yet been introduced in the Province of Alberta. If it had, early diagnosis and treatment would have significantly changed the patient's medical course.

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Contributions of the Authors

- Dr. Ferreira is a treating physician and helped write the manuscript.
- Dr. Chan is a treating physician and helped write the manuscript.
- Dr. Wolf planned, organized and wrote the manuscript.

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Statement About Competing Interest

No to all questions for all authors.

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Details of Ethics Approval

Informed consent, but not required for this case report.

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