Eva Morava
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Verena Peters *Editors*

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RESEARCH REPORT

LC-MS/MS Analysis of Cerebrospinal Fluid Metabolites in the Pterin Biosynthetic Pathway

Erland Arning • Teodoro Bottiglieri

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Abstract The analysis of (6R)-5,6,7,8-tetrahydrobiopterin (BH4) and neopterin in cerebrospinal fluid (CSF) is often used to identify defects in the pterin biosynthetic pathway affecting monoamine metabolism that can lead to pediatric neurotransmitter diseases. Low levels of BH4 and neopterin alone may not be sufficient to determine the defect, and further testing is often required. We have developed a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for determination of BH4, 7,8-dihydrobiopterin (BH2), neopterin, and sepiapterin in CSF, which provides a more comprehensive evaluation of the pterin pathway. The method utilizes labeled stable isotopes as internal standards and allows for a fast 10-minute analysis by LC/MS/MS over a linear working range of 3 to 200 nmol/L. Total analytical imprecision is less than 14.4% for all pterin metabolites. Accuracy for BH4 and neopterin was determined by comparing data obtained by an alternative method using HPLC with EC and fluorescence detection. Excellent correlation was demonstrated for BH4 ($r = 0.9646$, 1/slope = 0.9397; $n = 28$; concentration range 3 to 63 nmol/L) and neopterin ($r = 0.9919$, 1/slope = 0.9539; $n = 13$; concentration range 5 to 240 nmol/L). CSF specimens from patients diagnosed with inborn errors of sepiapterin reductase (SR), 6-pyruvoyl-tetrahydropterin synthase (PTPS), dihydropteridine reductase (DHPR), and guanosine triphosphate cyclohydrolase (GTPCH) have been analyzed, and distinct pterin metabolite patterns were consistent with the initial diagnosis. This method differentiates patients with DHPR and SR deficiency from other pterin defects (GTPCH and PTPS) and will be useful for the diagnosis of specific defects in the pterin biosynthetic pathway.

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Competing interests: None declared

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Introduction

A variety of rare inherited inborn errors related to (6R)-5,6,7,8-tetrahydrobiopterin (BH4) metabolism have been described, often referred to as pediatric neurotransmitter disease. These disorders may be associated with or without hyperphenylalaninemia (HPA) and can lead to low or decreased homovanillic acid and 5-hydroxyindoleacetic acid (Blau et al. 2001; Fig. 1). Some BH4 disorders are detected by neonatal newborn screening since they present with HPA and are caused by enzyme deficiencies of guanosine triphosphate cyclohydrolase (GTPCH, OMIM 600225; MIM 233910; EC 3.5.4.16)-autosomal recessive form (Niederwieser 1984), pterin-4-α-carbinolamine dehydratase (PCD, OMIM 126090; EC 4.2.1.96) (Thöny et al. 1998), 6-pyruvoyl-tetrahydropterin synthase (PTPS, OMIM 261640; EC 4.2.3.12), and dihydropteridine reductase (DHPR, OMIM 261630; EC 1.5.1.34) (Kaufman et al. 1975). The neurological symptoms associated with these disorders can vary and include mental disability, convulsions, dystonia, oculogyric crises, drowsiness, irritability, and swallowing difficulties. However, other inborn errors of pterin metabolism that do not present with HPA have been described and include GTPCH-autosomal dominant form (MIM 128230), initially described as Segawa disease (Segawa et al. 1976) and sepiapterin reductase (SR, OMIM 182125; EC 1.1.1.153) deficiency (Bonafe et al. 2001). Initial screening for these defects

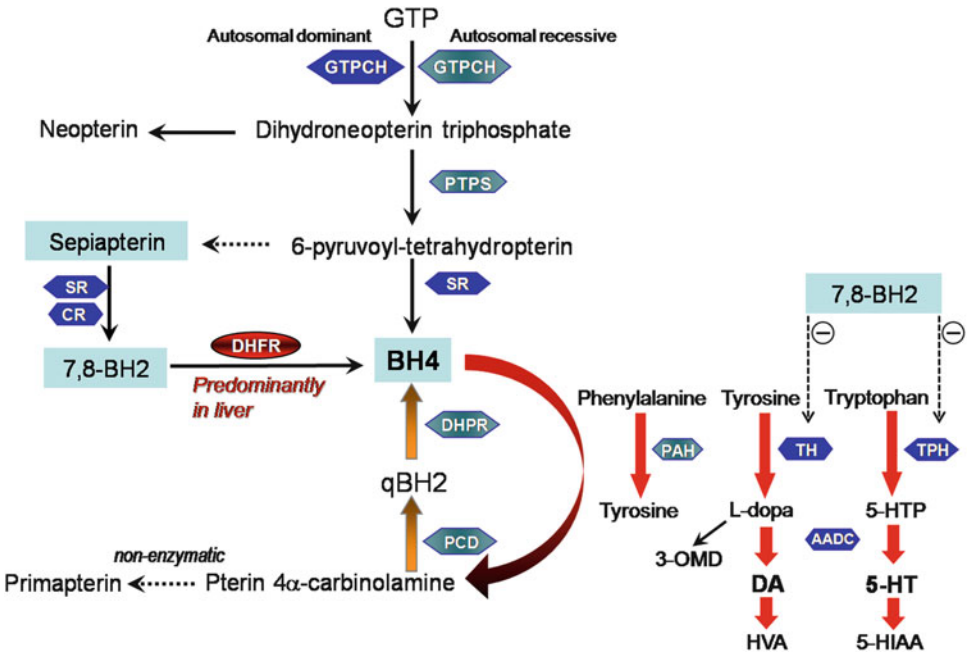

Fig. 1 Pterin and monoamine metabolism. Abbreviations: *GTP* guanosine triphosphate, *GTPCH* GTP cyclohydrolase, *PTPS* 6-pyruvoyl-tetrahydropterin synthase, *SR* sepiapterin reductase, *BH4* (6R,S)-5,6,7,8-tetrahydrobiopterin, *BH2* 7,8-dihydrobiopterin, *DHPR* dihydropteridine reductase, *PCD* pterin-4-α-carbinolamine dehydratase, *CR* carbonyl reductase, *DHFR* dihydrofolate reductase, *qBH2* quinine-dihydrobiopterin, *TH* tyrosine hydroxylase, *TPH* tryptophan hydroxylase, *PAH* phenylalanine hydroxylase, *AADC* aromatic amino acid decarboxylase, *L-DOPA* L-3,4-dihydroxyphenylalanine, *5-HTP* 5-hydroxytryptophan, *3-OMD* 3-o-methyldopa, *DA* dopamine, *5-HT* serotonin, *HVA* homovanillic acid, *5-HIAA* 5-hydroxyindoleacetic acid

relies on analysis of pterins and monoamine neurotransmitter metabolites in cerebrospinal fluid (CSF).

Numerous methodologies have been employed to determine pteridines, including radioenzymatic assay (Guroff et al. 1967), radioimmunoassay (Nagatsu et al. 1981), HPLC with fluorescence (Stea et al. 1979; Fukushima and Nixon 1980a), HPLC with electrochemistry (Lunte and Kissinger 1983; Bräutigam et al. 1982), and HPLC or UPLC with electrochemistry and fluorescence (Hyland 1985; Guibal et al. 2014). One of the first HPLC methods developed relied on the highly fluorescent characteristics of the fully oxidized pteridines and the differential oxidation of reduced biopterin states (Fukushima and Nixon 1980b; Fukushima et al 1978). This allowed an indirect method of detecting both BH4 and 7,8-dihydrobiopterin (BH2) through the measurement of total biopterin. This method has been applied to detect BH4 and BH2 in tissues, urine, plasma, and dried blood spots (Schmidt et al. 2006; Antonozzi et al. 1988).

We here describe a method using stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) to quantitate BH4, BH2, neopterin, and sepiapterin in CSF that can be applied as a screening method for the differentiation of inborn errors of pterin metabolism.

Materials and Methods

Standards and ^{15}N-labeled stable isotope internal standards, BH4, BH2, neopterin, sepiapterin, ^{15}N-BH4, ^{15}N-BH2, and ^{15}N-neopterin, were obtained from Schircks Laboratories (Switzerland). Formic acid, heptafluorobutyric acid, ammonium acetate, and dithiothreitol (DTT) were obtained from Fluka and Optima LC-MS grade methanol from Fisher Scientific. Diethylenetriaminepentaacetic acid (DETAPAC) was purchased from Sigma. Stock standards for each pterin metabolite and internal standard were prepared as 1 mmol/L solutions in water containing 0.2% DTT and stored at −80°C. Microtiter plates used for were purchased from NUNC.

Sample Collection

CSF samples were collected in CSF collection kits provided by the neuropharmacology laboratory at the Baylor Research Institute. Specimens used for accuracy validation of CSF BH4 and neopterin were performed on tube #3 of 5 which contained DTT and DETAPAC. After collection, samples were frozen and shipped by overnight courier on dry ice and are stored at −80°C until analysis.

Table 1 MS/MS parameters of pterins and internal standards (declustering potential (DP); collision energy (CE); and collision exit potential (CXP))

Analyte	Retention time (min)	MRM transition		Dwell time (ms)	DP (V)	CE (V)	CXP (V)
		Q1 (*m/z*)	Q3 (*m/z*)				
BH_4	5.9	242.2	166.0	80	66	25	16
^{15}N-BH_4	5.9	243.1	167.0	80	66	25	13
BH_2	5.7	240.1	164.9	80	51	29	14
^{15}N-BH_2	5.7	241.1	197.0	80	66	19	14
Neopterin	5.2	254.1	206.1	80	40	23	10
^{15}N-Neopterin	5.2	255.1	206.1	80	40	23	10
Sepiapterin	5.9	238.1	165.1	80	40	27	10

Blood-contaminated CSF samples are rejected, since hemoglobin from erythrocytes may cause auto-oxidation of pterins. This can be avoided by centrifuging prior to freezing, and the clear CSF transferred to another vial and frozen.

Analysis of Amine Neurotransmitters in CSF

CSF amine neurotransmitters were analyzed by HPLC with electrochemical detection by a previously published method (Hyland et al. 1986).

Analysis of BH4 and Neopterin in CSF by HPLC with Electrochemical and Fluorescence

CSF BH4 and neopterin were analyzed by HPLC with electrochemical and fluorescence detection by a previously published method (Hyland 1985).

Analysis of BH4, BH2, Sepiapterin, and Neopterin in CSF by LC-MS/MS

Sample Preparation

The calibration curve was prepared in water containing 0.2% DTT over a range of 3 to 200 nmol/L for each pterin analyte. Internal standard concentrations of ^{15}N-BH4, ^{15}N-BH2, and ^{15}N-neopterin were prepared in water containing 0.2% DTT at a final concentration of 100 nmol/L each. Sample preparation for CSF pterin analysis consisted of combining 210 µL of 100 nmol/L internal standard solution with 30 µL of blank, standard, control, or sample in 1.5 mL Eppendorf tubes, mixed by vortex and loaded in a microtiter plate for analysis.

LC-MS/MS

The LC-MS/MS analysis was performed on an AB Sciex 5500QTRAP mass spectrometer (Foster City, CA, USA) coupled with a Shimadzu ultrahigh pressure Nexera chromatograph system (Kyoto, Japan). Nitrogen gas was supplied by an AB-3G gas generator (Peak Scientific) and was used as the drying, nebulizing, curtain, and collision gas. The MS/MS experiments were performed under positive electrospray ionization (+ESI) with multiple-reaction monitoring (MRM) using a Turbolon Spray electrospray source operating at a voltage of 1.1 kV and desolvation temperature of 700°C. All pterin standards exhibited intense protonated molecular ions under + ESI conditions. The parameters for ion selection and collision-activated fragmentation of the molecular ions of pterin standards and ^{15}N-labeled isotopes were optimized by continuous infusion of pure compounds (1 µmol/L) at a flow rate of 10 µL/min. The selected precursor and fragment ions used for the measurement of unlabeled and labeled pterins are summarized in Table 1. The mass spectrometer was operated under unit resolution. The LC-MS/MS data was acquired and processed using Analyst 1.5.2 software (AB Sciex).

Liquid chromatography was performed on a Shimadzu Nexera system by reversed-phase HPLC (EZfaast 250 × 2 mm 4 µm AAA-MS column, with a 4 × 2 mm SecurityGuard column, Phenomenex, CA, USA) equilibrated at 40°C. The system consisted of a binary gradient: Eluent A (water containing 0.1% formic acid and 0.1% heptafluorobutyric acid) and Eluent B (methanol containing 0.1% formic acid). The flow rate was 0.20 mL/min, and 10 µL of processed sample was injected for analysis. The LC gradient was optimized to retain the pterin compounds

on the column while eluting ion-suppressing moieties. The initial composition of the gradient was 95% A and increased linearly to 25% A during the first 4 min. The concentration of Eluent B was increased to 100% at 4.1 min and held until 5.0 min. At 5.1 min eluents were returned to initial conditions for equilibration. Total analytical analysis, including column re-equilibration, was 10 minutes. Eluent flow from the column was diverted to waste at the beginning and end of each run and was only directed to the source for the period from 5.0 to 7.5 min.

Assay Validation (Precision, Accuracy, and Recovery)

The method was validated by investigation of linearity, method precision studies (intra- and inter-day), accuracy, detection limit, and recovery. CSF was spiked with external pterin standards to produce a bi-level quality control (QC) used for both intra- and inter-day precision studies. Intraday precision ($n = 10$) was evaluated by analysis of bi-level QC material acquired within a single analytical run. Inter-day precision ($n = 20$) was evaluated by repeated analysis of bi-level QC material analyzed in duplicate over a period of 20 different days. Precision was determined as the relative standard deviation (RSD %). Recovery of pterins was performed by spiking CSF with water, 50 or 200 nM of each analyte. Determination of accuracy was performed only for BH4 and neopterin by comparing results obtained by HPLC with electrochemical and fluorescence detection currently used in our laboratory for these metabolites only.

Results

The collision-induced dissociation spectra of the protonated molecular ions of each pterin standard are given in Fig. 2. Representative chromatograms for the analytes in CSF are also shown in Fig. 2. The precursor-product transitions for each analyte and internal standard are shown: *m/z* 242.2 → 166.0 (BH4), 240.1 → 164.9 (BH2), 254.1 → 206.1 (neopterin), 238.1 → 165.1 (sepiapterin), 243.1 → 167.0 (^{15}N-BH4), 241.1 → 197.0 (^{15}N-BH2), and 255.1 → 206.1 (^{15}N-neopterin) (Table 1). Monitoring of a second transition was not possible because there were no other unique fragments of significant intensity. In order to minimize ion suppression, this LC method was developed to retain analytes and internal standards later in chromatographic run. In preliminary ESI both positive mode and negative mode were tested. Positive ESI produced greater signal intensity for all analytes.

Assay Performance and Validation

Linear regression analysis showed linearity for all analytes over their calibration range (data not shown). The calibration curves for BH4, BH2, sepiapterin, and neopterin were linear over a concentration range of 3 to 200 nmol/L. Intra-assay precision ($n = 10$) was assessed in spiked bi-level CSF with coefficient of variation (CVs) for all analytes <6% (Table 2). The inter-assay precision ($n = 20$) was assessed by analysis of duplicates of bi-level quality controls over a period of 20 different days. The CVs for BH4, BH2, and neopterin were <10% (Table 2). The inter-assay precision for sepiapterin was higher than the other pterins since ^{15}N-BH2 was used as the internal standard since labeled sepiapterin is unavailable (Table 2). Recovery of 50 and 200 nM spikes of BH4, BH2, and neopterin ranged from 98 to 112%, whereas recovery for sepiapterin was 85 to 75% (data not shown).

Method Comparison (BH4 and Neopterin)

Accuracy of the LC-MS/MS method was determined by comparing BH4 and neopterin concentrations in CSF to values obtained by HPLC with electrochemical and fluorescence detection (Hyland 1985). CSF neopterin could not be directly compared between the two methods due to the electrochemical cell oxidizing reduced forms of neopterin completely to neopterin. The LC-MS/MS method only detects native oxidized neopterin and not any of the reduced forms. In order to obtain native oxidized neopterin on the HPLC with electrochemical and fluorescence method, the electrochemical cell was switched off, and this allowed only the native oxidized neopterin present in the CSF to be quantified by fluorescence. Method comparison data of BH4 (HPLC with electrochemical and LC-MS/MS) and neopterin (HPLC fluorescence and LC-MS/MS) is shown in Fig. 3.

Analysis of Pterins in Known Cases of BH4 Deficiencies

CSF specimens from subjects with pterin enzyme deficiencies, confirmed by genomic DNA analysis, having GTPCH-autosomal dominant PTPS, SR, and DHPR, were analyzed by LC-MS/MS. Pterin metabolite results for each specific enzyme deficiency, with data on monoamine neurotransmitter metabolites, are shown in Table 3. In some cases CSF samples were obtained either prior to treatment or following L-dopa therapy. As expected low BH4 levels were detected in subjects with GTPCH-autosomal dominant form and PTPS, whereas BH2 levels

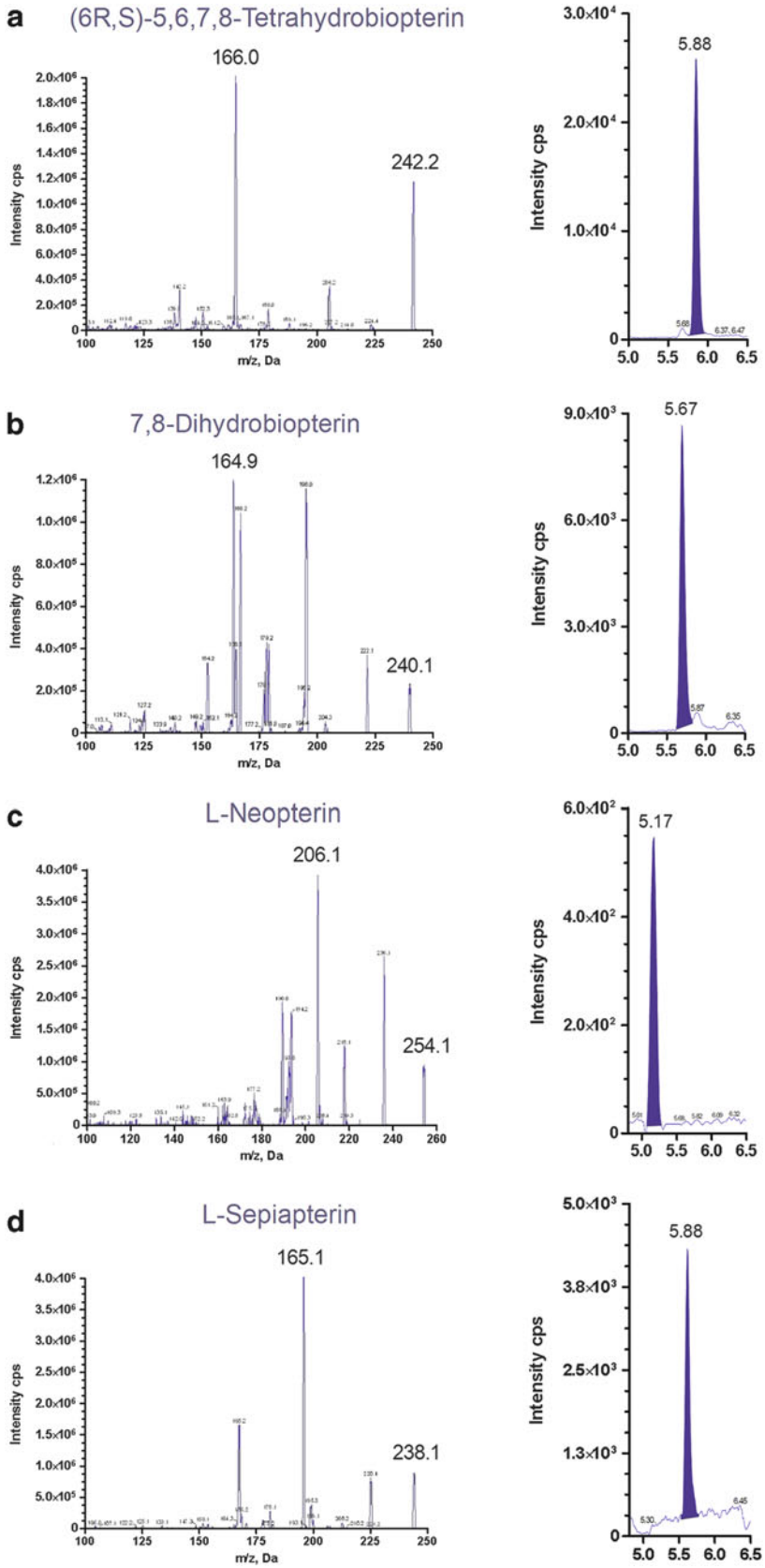

Fig. 2 Product ion spectra of each pterin and a representative chromatograph of each metabolite in CSF. (**a**, BH4 = 51 nM; **b**, BH2 = 39 nM; **c**, neopterin = 20 nM; and **d**, sepiapterin = 23 nM). Spectra were generated with + ESI-MS/MS by infusion (10 μL/min) of pure standards (1 μM/L each)

Table 2 Assay precision, linearity, and limit of detection (LOD)

		Intra-assay ($n = 10$)		Inter-assay ($n = 20$)			
Analyte (nmol/L)	Internal standard	Level 1 (CV%)	Level 2 (CV%)	Level 1 (CV%)	Level 2 (CV%)	Linearity	LOD
BH4	^{15}N-BH$_4$	55.1 ± 1.7 (3.0)	156.1 ± 7.2 (4.6)	50.2 ± 2.9 (5.9)	149.0 ± 6.2 (4.2)	3–200	1
BH2	^{15}N-BH$_2$	41.0 ± 1.8 (4.4)	170.9 ± 5.9 (3.5)	38.6 ± 3.2 (8.2)	152.7 ± 12.8 (8.4)	3–200	1
Neopterin	^{15}N-Neopterin	35.6 ± 2.0 (5.6)	160.2 ± 7.6 (4.7)	29.1 ± 2.8 (9.5)	141.3 ± 10.3 (7.3)	3–200	1
Sepiapterin	^{15}N-BH$_2$	CV% (5.3)	CV% (4.3)	CV% (14.4)	CV% (12.5)	3–200	1

Data expressed as nmol/L, mean ± standard deviation (coefficient of variation, %)

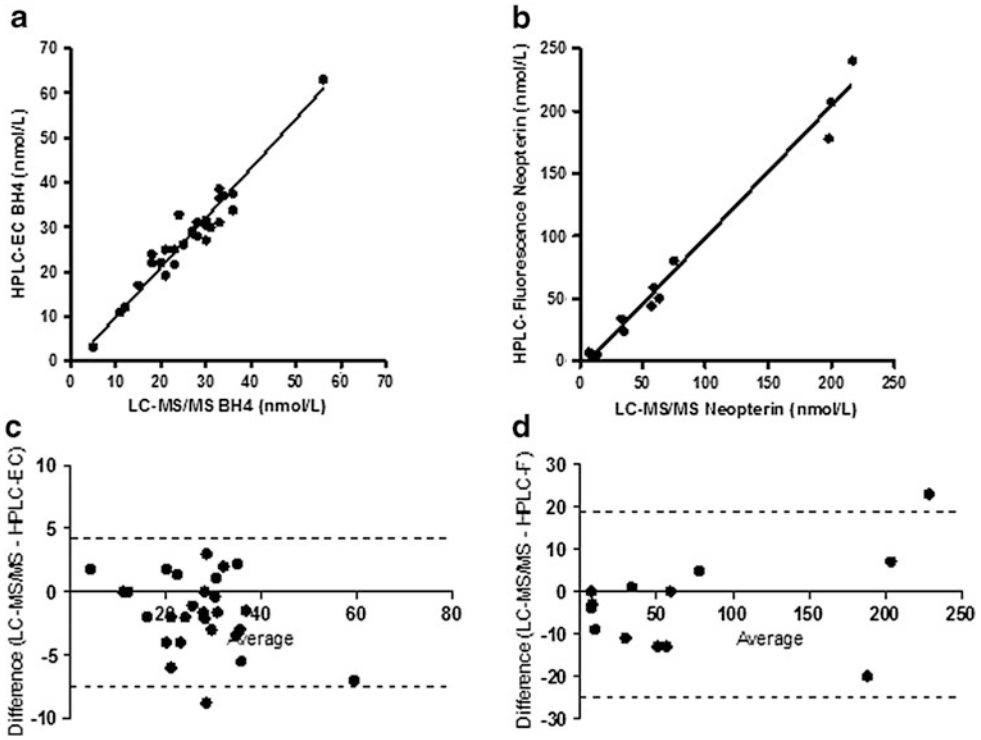

Fig. 3 Pearson correlation coefficients (95% CLs) for BH4 and neopterin (**a** and **b**) were $r = 0.9646$ (0.9240, 0.9837) and $r = 0.9919$ (0.972, 0.998)%, respectively. Deming regression analysis for BH4 (**a**) yielded an intercept (95% CLs) of 1.05 (−1.16, 1.65) nmol/L and a 1/slope of 0.9397; neopterin (**b**) yielded an intercept of (95% CLs) of −7.17 (−2.86, −11.48) nmol/L and a 1/slope of 0.9539. Bland–Altman proportional bias analyses (**c** and **d**) relative bias (95% CLs) for BH4 and neopterin were −1.6 (4.1, −7.4)% and −2.9 (18.8, −24.5)%, respectively. All data expressed as nmol/L

were elevated in patients with SR and DHPR deficiency. Elevated concentrations of sepiapterin were detected in two patients with SR deficiency and were undetectable (<1 nmol/L) in the other pterin disorders. Sepiapterin was undetectable in CSF from other subjects ($n = 20$) without any defects in pterin metabolism and having monoamine metabolite concentrations within the normal reference range (data not shown). The patient with PTPS deficiency presented with an increase in neopterin and with decreased BH4 and BH2 concentrations in CSF.

Discussion

An LC-MS/MS method was developed and validated for the simultaneous determination of four important pterin metabolites in CSF BH4, BH2, neopterin, and sepiapterin. The sample preparation procedure was optimized for increased accuracy and ease of use as a screening procedure for defects in pterin metabolism, which lead to pediatric neurotransmitter diseases. This method was further validated by analysis of CSF from subjects with known

Table 3 Differentiation of pterin defects through LC-MS/MS analysis

Subject ID	Age	BH_4	BH_2	Neopterin	Sepiapterin	5-HIAA	HVA	3-OMD
Sepiapterin reductase deficiency								
Subject 1	8 years	16	42.8 H	2	7 H	3 L	49 L	50
Subject 1	9 years	22	55.4 H	4	6 H	9 L	81 L	208* H
Subject 2	6 years	24	46.5 H	4	9 H	4 L	67 L	27
Subject 2	7 years	31	58.1 H	5	11 H	10 L	106 L	205* H
Dihydropteridine reductase deficiency								
Subject 3	4 months	27	54.0 H	7	<1	82 L	281 L	1835* H
Subject 3	11 months	22	48.0 H	17	<1	39 L	171 L	453* H
6-Pyruvoyl-tetrahydropterin synthase deficiency								
Subject 4	11 years	<1 L	1 L	29 H	<1	119	199	1533* H
Subject 4	11 years	2 L	3	27 H	<1	90	199	1870* H
G TP Cyclohydrolase deficiency – autosomal dominant								
Subject 5	36 years	3 L	3	<1	<1	NA	NA	NA
Reference range								
	2–6 months	23–98	3–18	0–18	<1	179–711	450–1,132	0–300
	6 months–2 years	20–58	3–18	0–18	<1	129–520	294–1,115	0–300
	2–5 years	20–58	3–18	0–18	<1	74–345	233–928	0–150
	5–10 years	20–58	3–18	0–18	<1	66–338	218–852	0–100
	10–15 years	9–40	3–18	0–18	<1	67–189	167–563	0–100
	>15 years	9–32	3–18	0–18	<1	67–140	145–852	0–100

Data expressed as nmol/L. (L = below reference range; H = above reference range; * denotes L-dopa therapy)
Subjects 1 and 2 are siblings with confirmed SR deficiency having a homozygous 587A>G (Y196G) mutation. Subject 3 has a confirmed DHPR deficiency with a homozygous IVS7-2 A>G mutation. Subject 4 is assumed PTPS deficiency based on biopterin profile and clinical symptoms. Subject 5 has a confirmed GTPCH-1 deficiency with a Q180R mutation
NA not available

defects in pterin metabolism. Results showed the expected pattern of changes in pterin metabolites in subjects (Table 3) with defects in GTPCH-autosomal dominant (low BH4 and low BH2), SR (increased sepiapterin, increased BH2, and normal BH4), PTPS (increased neopterin, low BH4, and low BH2), and DHPR (increased BH2). Detection of neopterin is limited to native oxidized neopterin, which may not be reliable to discriminate GTPCH-autosomal dominant cases. However, elevated levels can distinguish patients that have PTPS deficiency or in cases of macrophage activation due to infection and inflammation.

Previous reports in which metabolites were measured in subjects with suspected disorders of pterin metabolism have relied on either multiple analytical analyses or differential oxidation of BH4 and BH2 to obtain a more complete understanding of the state of pterin synthesis. For example, shortly after the discovery of SR deficiency resulting in decreased BH4 metabolism (Bonafe et al. 2001), a HPLC-fluorescence method was reported to measure sepiapterin alone in CSF of patients suspected of having this disorder (Zorzi et al. 2002). Two recently published methods demonstrated the utility of LC-MS/MS for the determination of pterins (Fismen et al 2012; Kim et al 2012). The study by Fismen et al. (2012) first described the use of ^{15}N-labeled stable isotopes to quantify different oxidative states of biopterin forms without differential oxidation. However, these pterins were only measured in human umbilical vein endothelial cells and did not include sepiapterin and neopterin. The study by Kim et al. (2012) described the quantitative analysis of BH4 and dopamine in rat brain extracts only, using LC-MS/MS and stable isotopes as internal standards.

The LC-MS/MS method described in this study is rapid and precise and requires minimal sample preparation for the analysis of CSF samples The high sensitivity provided by positive ESI LC-MS/MS eliminates the need for derivatization, and the HPLC separation is sufficient to avoid the analytes of interest co-eluting with matrix components that can cause ion suppression. The ability to quantitate BH4, BH2, sepiapterin, and neopterin in a single method will allow differentiation of pterin defects, particularly identifying those with DHPR and SR deficiency from other

pterin defects (GTPCH and PTPS), by detecting elevated BH_2 and the presence of sepiapterin in the case of SR deficiency. The pterin metabolite profile provided by this method will be beneficial in ascertaining the specific pterin defect involved and the correct course for follow-up testing. This method offers a significant improvement in screening patients and will lead to prompt diagnosis and management of inborn errors of pterin metabolism as well as therapeutic monitoring.

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Synopsis

Newly developed LC-MS/MS method for determination of CSF pterins will improve diagnostic differentiation of patients with inborn errors of pterin metabolism.

Compliance with Ethics Guidelines

Conflict of Interest

Erland Arning and Teodoro Bottiglieri declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Appropriate informed consents were obtained from all subjects or attending pediatric neurologists. All biochemical data was obtained through the routine analysis required for regular clinical care of each subject, as approved by the attending pediatric neurologist.

Animal Rights

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

Details of the Contributions of Individual Authors

Article contributions by Erland Arning: conception and design, analysis, interpretation of data, drafting, and revising manuscript. Article contributions by Teodoro Bottiglieri: conception and design, interpretation of data, and revising manuscript. Erland Arning is the guarantor for this article and accepts full responsibility for the work and conduct of the study, had access to the data, and controlled the decision to publish.

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RESEARCH REPORT

Renal Involvement in a French Paediatric Cohort of Patients with Lysinuric Protein Intolerance

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Abstract Lysinuric protein intolerance (LPI) is a rare autosomal recessive metabolic disorder, caused by defective transport of cationic amino acids at the basolateral membrane of epithelial cells, typically in intestines and kidneys. The *SLC7A7* gene, mutated in LPI patients, encodes the light subunit (y+LAT1) of a member of the heterodimeric amino acid transporter family.

The diagnosis of LPI is difficult due to unspecific clinical features: protein intolerance, failure to thrive and vomiting after weaning. Later on, patients may present delayed growth osteoporosis, hepatosplenomegaly, muscle hypotonia and life-threatening complications such as alveolar proteinosis, haemophagocytic lymphohistiocytosis and macrophage activation syndrome. Renal involvement is also a serious complication with tubular and more rarely, glomerular lesions that may lead to end-stage kidney disease (ESKD). We report six cases of LPI followed in three different French paediatric centres who presented LPI-related nephropathy during childhood. Four of them developed chronic kidney disease during follow-up, including one with ESKD. Five developed chronic tubulopathies and one a chronic glomerulonephritis. A histological pattern of membranoproliferative glomerulonephritis was first associated with a polyclonal immunoglobulin deposition, treated by immunosuppressive therapy. He then required a second kidney biopsy after a relapse of the nephrotic syndrome; the immunoglobulin deposition was then monoclonal (IgG1 kappa). This is the first observation of an evolution from a polyclonal to a monotypic immune glomerulonephritis. Immune dysfunction potentially attributable to nitric oxide overproduction secondary to arginine intracellular trapping is a debated complication in LPI. Our results suggest all LPI patients should be monitored for renal disease regularly.

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Introduction

Lysinuric protein intolerance (LPI) (MIM 222700) is a rare autosomal recessive metabolic disorder with an estimated incidence of 1.7 cases for every 100,000 live births in France. It is caused by defective sodium-independent

transport of cationic amino acids (CAA, i.e. L-arginine, L-lysine, L-ornithine) at the basolateral membrane of epithelial cells, mostly of the intestine and kidney (Camargo et al. 2008). The *SLC7A7* gene, mutated in LPI patients, encodes the light subunit (y+LAT1) of a member of the heterodimeric amino acid transporter (HAT) family. y+LAT-1 is associated with 4F2hc, the heavy subunit required for normal expression of the transporter at the basolateral membrane (Palacin et al. 2004).

The highest prevalence of LPI (1/60,000 live births) is found in Finland, due to a founder mutation (c.895-2A>T) (Thelle et al. 2006). There is also a high prevalence in Southern Italy and Japan (Sebastio et al. 2011). More than 50 mutations have been described to date (Ogier de Baulny et al. 2012). The mutations result in defective transport of CAA and subsequently low blood concentrations of CAA due to poor intestine absorption and high renal clearance. The reduced availability of arginine and ornithine for the urea cycle is responsible for hyperammonaemia after rich protein intakes (Ogier de Baulny et al. 2012).

The diagnosis of LPI is often difficult due to unspecific and sometimes subtle clinical features. Protein intolerance and failure to thrive are common findings in infants after weaning. Later on, patients may present growth delay, osteoporosis, hepatosplenomegaly, muscle hypotonia, pancreatitis (Parenti et al. 1995) and life-threatening complications such as alveolar proteinosis, haemophagocytic lymphohistiocytosis with macrophage activation syndrome and autoimmune disorders (Ogier de Baulny et al. 2012). Renal involvement is also a serious complication with tubular and more rarely, glomerular involvement that may lead to end-stage kidney disease (ESKD) (Tanner et al. 2007).

We report herein six LPI patients followed in three different French paediatric centres who presented LPI-related nephropathy during childhood: five chronic tubulopathies and one chronic glomerulonephritis. Four of them developed chronic kidney disease (CKD) during follow-up, including one with ESKD at last follow-up.

Materials and Methods

Patients

After reviewing the last 30 years of archives (1984–2013) of all the French paediatric metabolic disease departments and paediatric nephrology departments, we identified six LPI patients who developed a renal disease before 18 years of age in an estimated 25 patients diagnosed during this period. The mean follow-up duration was 13 years (6 to 17 years). Data were collected from clinical and biological files. Renal biopsy reports were all reviewed.

Written informed consent for genetic analysis was obtained from participants or their parents. Patient care and study conduct complied with good clinical practice and the Declaration of Helsinki Principles.

The cohort consisted of six patients (four boys) (Table 1). The age at LPI diagnosis ranged from few days of life to 11 years of age. Patient 1 and patient 2 were siblings (brother and sister) from a non consanguineous Moroccan family. Patient 3 and patient 4 were from Turkish families; they were not related, but both of them came from a consanguineous family. Patient 5 was Caucasian; his parents were not related. Patient 6 had consanguineous Moroccan parents; his older brother had LPI without renal impairment.

Biochemical and Molecular Analyses

The diagnosis of LPI was confirmed for all patients if they had low lysine (<71 μmol/L), arginine (<23 μmol/L) and ornithine (<27 μmol/L) blood concentrations associated with high urinary concentrations of these following cationic amino acids: lysine (>150 μmol/mmol creatinine), arginine (>5 μmol/mmol creatinine) and ornithine (>5 μmol/mmol creatinine). They all had high orotic aciduria (>1.2 μmol/mmol creatinine). LPI was confirmed by Sanger sequencing in patient 5, bearing the already-described homozygous c.106-108delGAG *SLC7A7* mutation (Sperandeo et al. 2005).

Results

General LPI Features

The main presenting symptom was significant growth retardation detected between 2 and 19 months of age (patients 2, 3, 4). Patient 1 presented with coma with metabolic acidosis at 3 months of age. Patient 5 was diagnosed after investigations for proteinuria discovered at 11 years of age, and patient 6, who had an affected sibling and neonatal hypotonia, was diagnosed soon after birth. All of the six patients had biochemical features characteristic of LPI.

They all received a protein-restricted diet and citrulline supplement, associated with arginine supplement in patient 5 or lysine supplement in patients 3 and 6, and three patients (patients 3, 4 and 6) received also a temporary L-carnitine supplement. All had hyperferritinaemia (ferritin >80 μg/L) (data not found for patient 2) and enlarged liver and spleen. Patient 1 had serious lung involvement with severe interstitial syndrome at 8 years of age. The lung biopsy showed extended fibrosis and fatty acid crystals. She died 2 years later from refractory hypoxemia. Two other patients had asymptomatic pulmonary impairment discovered on systematic tomodensitometry (patients 3 and 6) (Table 1).

Table 1 Characteristics and treatments of this study's patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Birth/gender	1976/F (sister of patient 2)	1977/M (brother of patient 1)	1991/F	1997/M	1997/M	2005/M
Ethnic background	Morocco	Morocco	Turkey	Turkey	Caucasian	Morocco
Age at the diagnosis of LPI	3 months old	10 months old	19 months old	2 months old	11 years old	Few days old
Presenting symptoms	Coma, metabolic acidosis	Failure to thrive	Vomiting, failure to thrive	Failure to thrive	Proteinuria	Neonatal hypotonia
Remarkable symptoms	Interstitial pulmonary syndrome, severe growth retardation		Local emphysema on pulmonary tomodensitometry			Interstitial pulmonary syndrome on tomodensitometry
Treatment	Citrulline (dose not known)	Citrulline (3 g/day)	Citrulline (3 g/day), lysine, L-carnitine	Citrulline (dose not known), L-carnitine	Citrulline (3 g/day), arginine (250 mg/kg/day)	Citrulline (1.6 g/day), L-carnitine, lysine
Age(yo) at renal impairment diagnosis	8	7	3	6	12	2
Renal dysfunction	Tubular	Tubular	Tubular	Tubular	Tubular, glomerular	Tubular
Kidney biopsy	Normal	No	No	No	Membranoproliferative glomerulopathy	No
Nephrocalcinosis	Yes	No	Yes	No	No	Yes
CKD at the end of study (clearance mL/min/ 1.73 m^2)	No	Yes (70)	ESKD	Yes (44)	No	Yes (72)
Supplementation	Bicarbonate, phosphate	Bicarbonate	Bicarbonate, phosphate	Bicarbonate, phosphate	Bicarbonate, phosphate	No
Age (years) at the end of study	Death at 10	36	21	16	16	8

M male, *F* female, *CKD* chronic kidney disease, *ESKD* end-stage kidney disease

Renal Involvement

All patients had renal tubular impairment with increased β2 microglobulinuria discovered between 2 and 12 years of age (Table 1). Four had phosphate leaks (patients 1, 3, 4 and 5), and five had renal tubular acidosis (patients 1, 2, 3, 4, 5) requiring bicarbonate supplements. Three patients developed nephrocalcinosis (patients 1, 3 and 6), secondary to hypercalciuria (ranging from 1.3 to 1.8 mmol ca/mmol creatinine), detected between 2 and 8 years of age. In two of them, nephrocalcinosis was associated with CKD that was diagnosed, respectively, at 7 and 14 years of age (patients 3 and 6); the third one (patient 1) died at 10 years of age with preserved renal function.

Four patients had CKD diagnosed between 6 and 14 years of age (Table 1). Three had CKD diagnosed before 10 years old (patients 2 and 6 had CKD stage 2 and patient 4 had CKD stage 3 at the end of the study). Patient 3 presented CKD at 14 years of age that eventually progressed to ESKD at 21 years of age. Concurrently, he developed a non-nephrotic range proteinuria (0.71 g/L) and hypoalbuminaemia (29 g/L) but did not undergo kidney biopsy.

Patient 3 had high blood pressure, revealing his renal impairment at three years old; patients 4, 5 and 6 had normal blood pressure (data not found for patients 1 and 2). Patient 5 had microscopic haematuria when the renal impairment was diagnosed (no data for the other patients).

Kidney biopsy was performed in two patients (Table 1). Patient 1 underwent kidney biopsy at 9 years of age because of nephrocalcinosis: kidney histology was normal. Patient 5 had presented hyperferritinaemia and hepatosplenomegaly since infancy, and a tubular proteinuria appeared at 11 years of age, etiological investigation of which finally led to the diagnosis of LPI. He developed a nephrotic syndrome two years later (hypoalbuminaemia 9 g/L, hypoproteinaemia 40 g/L, proteinuria> 3 g/L). Renal biopsy, performed at the onset of the nephrotic syndrome, showed a membranoproliferative glomerulonephritis with polyclonal immunoglobulin deposits associated with C3 and C1q deposits (Fig. 1). He received corticosteroids (60 mg/day) associated with mycophenolate mofetil (1,600 mg/day) and achieved partial remission within

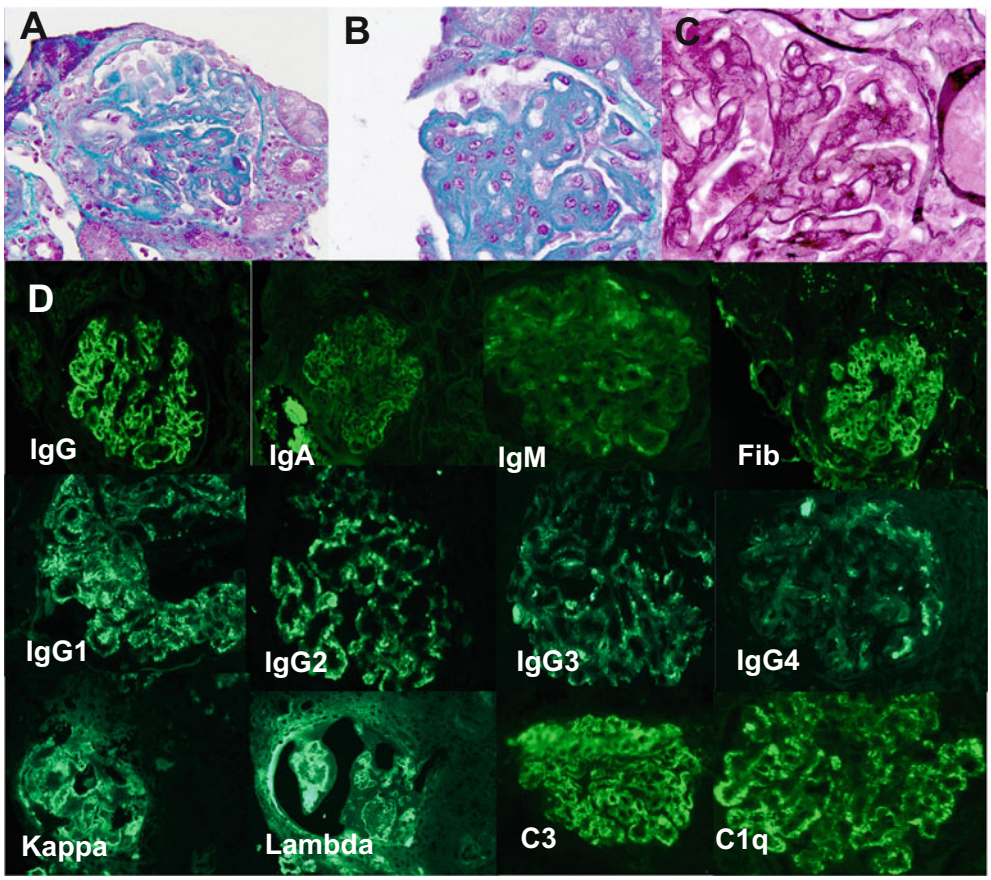

Fig. 1 Patient 5, first kidney biopsy: membranoproliferative glomerulonephritis: (**a**) mesangial proliferation, accumulation of mesangial matrix (trichrome Masson, ×100). (**b**) Intramembranous, extramembranous and subendothelial deposits (trichrome Masson, ×250). (**c**) Diffuse doubles contours (Marinozzi staining, ×250). (**d**) Immunofluorescence study (×100): Ig G, Ig A, Ig M, fibrin (Fib), IgG1, IgG2, IgG3, IgG4, C3 and C1q positivity: granular deposits along glomerular capillary walls, kappa and lambda light chains positivity

6 months (normalization of albuminaemia, but persistence of a non-nephrotic proteinuria (0.6 g/L)). Steroid was tapered off after 11 months, and the patient was treated with mycophenolate mofetil alone. The nephrotic syndrome relapsed 10 months after. A second kidney biopsy was performed and showed the same membranoproliferative glomerulonephritis pattern but with a surprising evolution of the immunoglobulin deposits that were monoclonal IgG1 kappa (Fig. 2). Corticosteroid was started again (30 mg/day) and resulted in a complete remission, but proteinuria reappeared after tapering corticosteroid doses. Immunologic investigations found antinuclear antibodies (1/400) in plasma with anti-SSA (Sjögren syndrome A) antibodies (49 mUA/L); C3 nephritic factor was inconsistently detectable. No monoclonal proliferation was detected after myelogram, plasma and urinary protein electrophoresis and immunoelectrophoresis.

Discussion

We report six patients with LPI and renal involvement, consisting of either tubular or glomerular features, which appeared before 18 years of age. In a Finnish series including mostly adults, 74% of the 39 patients were reported to have had proteinuria, 38% haematuria and 15% an elevated serum creatinine (Torrents et al. 1999). Few other cases of glomerulonephritis have been reported (Parto et al. 1994).

In our series, five out of six patients were diagnosed with LPI before 2 years of age because of characteristic symptoms such as failure to thrive, vomiting, hepatomegaly, hyperammonaemia. The renal impairment was detected during the follow-up between 2 and 8 years of age. In patient 5, however, the work-up of kidney disease led to the diagnosis of LPI at 11 years of age. It is well known that LPI can be diagnosed late in life (Dionisi-Vici et al. 1998), and complications may reveal the disease because LPI severity is rather dependent on complications (renal, pulmonary) than on moderate metabolic disorders (hyperammonaemia, low cationic amino acids in blood and high in urine) (Ogier de Baulny et al. 2012; Tanner et al. 2007).

The renal disease varies from tubular dysfunction to glomerulonephritis, with or without CKD (Tanner et al. 2007; Verzola et al. 2012). Tubular impairment is often the first manifestation of kidney involvement. All of our six patients had rather severe tubular dysfunction that ranged from isolated metabolic acidosis with normal anion gap (patient 2) to complete Fanconi syndrome (patient 3) and preceded glomerular features for patient 5. It was associated

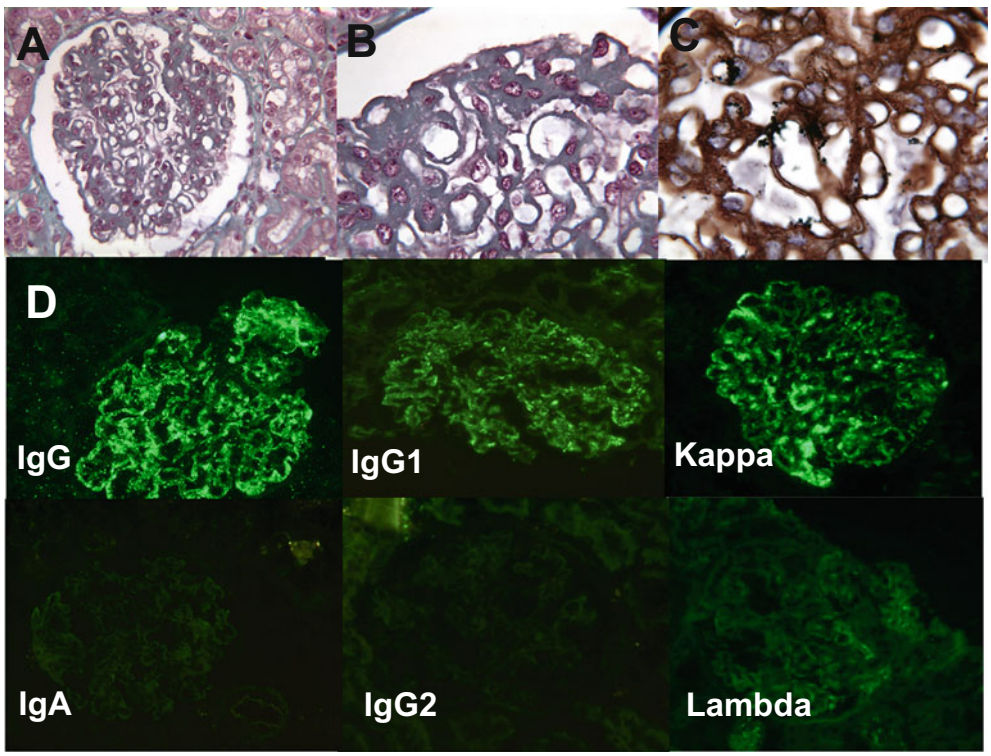

Fig. 2 Patient 5, second kidney biopsy, membranoproliferative glomerulonephritis: (**a**) mesangial proliferation, extramembranous deposits (trichrome Masson, ×100). (**b**) Intramembranous and subendothelial deposits (trichrome Masson, ×250). (**c**) Irregular thickening of basal membranes, double contours (Marinozzi staining, ×250). (**d**) Immunofluorescence study (×100): Ig G, IgG1 and kappa light chain: intramembranous and extramembranous deposits. IgA, IgG2 and lambda light chain: no deposit; IgM, Ig3, Ig4 and fibrinogen: no deposit (data not shown)

with hypercalciuria and nephrocalcinosis in three out of six patients probably responsible, at least in part, for CKD. Tanner et al. did not report any nephrocalcinosis in the Finnish LPI cohort (Tanner et al. 2007). We could not find any previous report of nephrocalcinosis in LPI in the literature. However, our results are not surprising since nephrocalcinosis is a classical complication of proximal or distal tubular dysfunction (Ogier de Baulny et al. 2012).

Four out of six patients developed CKD between the age of 6 years and adulthood (before 10 years of age for three of them) attributed to chronic tubular damage. However, since no kidney biopsy was performed in these patients, an associated glomerulopathy cannot be excluded. Patient 3 had severe renal impairment with ESKD at 20 years of age. The proteinuria and hypoalbuminuria attributed to nephron reduction may have been caused by other glomerular lesions. Patient 1 died early at 10 years of age from lung disease: her renal function was normal at that time. Patient 5 developed glomerulonephritis with normal renal function at the last follow-up at 15 years of age. Our results suggest that when LPI is complicated with renal disease during childhood, the evolution of renal impairment is severe.

Pathophysiological mechanisms of renal impairment in LPI are not fully elucidated, and several explanations have been suggested. The first is a direct toxicity of intracellular trapped lysine on proximal tubular cells (Thelle et al. 2006; Verzola et al. 2012). Indeed oral lysine supplementation in rats promotes proteinuria and inhibits albumin reabsorption by proximal tubular cells (Thelle et al. 2006). Lysine can also trigger apoptosis through an NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-dependent mechanism that increases ROS (reactive oxygen species) production (Verzola et al. 2012). And the renal oxidative stress plays a central role in hypertension, CKD and diabetic nephropathy (28). Moreover, patients with cystinuria (another amino acid transport defect) have a high urinary concentration of cysteine, a natural anti-oxidant, but do not develop any tubular leaks (they typically develop microcalculi). The anti-oxidative properties of cysteine may have a protective effect on the tubular cells (Verzola et al. 2012). Finally, one patient with LPI has been described with renal Fanconi syndrome, and the kidney biopsy showed a sloughing of brush border and vacuolization of proximal tubular cells. But the author did not consider the lysine accumulation as the toxic agent since patients with hyperlysinaemia do not develop renal Fanconi syndrome (Benninga et al. 2007).

The second hypothesis is a dysregulation of the immune system, in particular locally in renal tissue through the nitric oxide (NO) pathway. Arginine which accumulates in LPI cells due to its defective efflux (Mannucci et al. 2005) is the substrate of inducible nitric oxide synthase (iNOS), which synthesizes NO and citrulline (Nagasaka et al. 2009). Three LPI patients have been reported with increased plasma NO (Mannucci et al. 2005). *i*NOS is naturally present in epithelial tubular and renal mesangial cells and also plays a key role in fine regulation of vascular resistance; NO plays a role in inflammation and triggers mechanisms of immune defence (Nagasaka et al. 2009). *i*NOS synthesis is stimulated in LPI so the increased NO production may further contribute to the development of a multisystemic inflammation status (Mannucci et al. 2005). This overstimulation of the immune system could lead to autoimmune diseases. Patients with LPI often show disturbed immune functions and lupus erythematosus is the major autoimmune disease observed in patients with LPI (Aoki et al. 2001; Di Rocco et al. 1998; Kamoda et al. 1998; Parto et al. 1994). Several cases of lupus erythematosus diagnosed before LPI have been reported (Di Rocco et al. 1998; Parto et al. 1994). Patient 5 illustrates a new involvement of the immune system in LPI, through a very unique immune glomerulopathy. In this case, renal impairment revealed the diagnosis of LPI with a proximal tubular dysfunction (beta2 microglobulinuria) but unexpectedly evolved towards a glomerular disease with nephrotic syndrome. On initial kidney biopsy, a membranoproliferative pattern and immunoglobulin deposits associated with C3 and C1q deposits led to the diagnosis of lupus-like kidney disease. Plasma analyses found anti-SSA antibodies. Immunosuppressive treatment efficacy is an additional argument for the involvement of the immune system in LPI. For patient 5, an immunosuppressive therapy with corticosteroid and mycophenolate mofetil led to the remission of the nephrotic syndrome. Several other LPI cases have been treated successfully with various immunosuppressive therapies (steroid, polyvalent immunoglobulin or azathioprine) (Aoki et al. 2001; Dionisi-Vici et al. 1998; Di Rocco et al. 1998). These treatments were introduced because of a systemic disorder with fever, skin rash or erythroblasto-phagocytosis without macrophagic activation syndrome and showed an improvement of clinical symptoms. Patient 5's kidney biopsy immunofluorescence pattern also supports the hypothesis of the immune system involvement in LPI glomerular injuries, with a very unique evolution from polyclonal Ig deposition at onset to a monoclonal IgG1 kappa deposition 18 months later. The unsuccessful search for a systemic immune abnormality with monoclonal peak of Ig or plasmocyte abnormalities supports the hypothesis of a local dysregulation. Increased immunoglobulin concentration has already been reported but never as a monoclonal disease (Yoshida et al. 1995).

Conclusion

We report six paediatric cases of LPI with renal involvement. All of them had tubular disease, while three were also diagnosed with nephrocalcinosis and another had a glomerular disease. The renal evolution was severe in three cases

with CKD during childhood. The occurrence of glomerulopathy in LPI supports the role of an immune system dysregulation in LPI renal disease. All LPI patients should therefore be regularly monitored for renal disease.

Take-Home Message

Kidney involvement can complicate lysinuric protein intolerance, and it is therefore essential to monitor patients for the appearance of kidney diseases such as tubulopathy or glomerulopathy.

Compliance with Ethics Guidelines

Conflict of Interest

Camille Nicolas, Nathalie Bednarek, Vincent Vuiblet, Olivia Boyer, Anais Brassier, Pascale De Lonlay, Louise Galmiche, Pauline Krug, Véronique Baudouin, Samia Pichard, Manuel Schiff, Christine Pietrement declare that they have no conflict of interest.

All authors have nothing to declare.

The authors declare that the content of the article has not been influenced by the sponsors.

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

Patient care and study conduct complied with good clinical practice and the Declaration of Helsinki Principles.

Camille Nicolas wrote the paper and gathered patients' data. Vincent Vuiblet and Louise Galmiche interpreted the biopsy pictures, and all authors contributed to the interpretation and final manuscript preparation.

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RESEARCH REPORT

A Highly Diverse Portrait: Heterogeneity of Neuropsychological Profiles in cblC Defect

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Abstract Cobalamin C is a rare inborn disorder of metabolism that results in multisystemic abnormalities, including progressive visual deficits. Although the cellular pathophysiology of cblC is a field of active study, little attention has been dedicated to documenting the cognitive consequences of the defect. The neuropsychological assessment of nine individuals aged between 23 months and 24 years was conducted to establish cognitive profiles. Results reveal a marked heterogeneity, with intellectual functioning ranging from extremely low to average, and cognitive difficulties (e.g., attention) evidenced even in those who are not intellectually disabled. Central nervous system abnormalities and multisystem disease are likely to be major contributing factors to the observed cognitive impairments, with the presence of visual deficits constituting an additional impediment to normal cognitive development. This study underscores the importance of conducting in-depth neuropsychological assessments in individuals with cblC, the results of which may be particularly helpful for clinical management, guidance toward rehabilitation services, and educational/vocational planning.

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Introduction

Cobalamin C defect (cblC, OMIM 277400), a clinically distinct autosomal recessive inborn error of intracellular vitamin B_{12} metabolism, results in multiple metabolic, developmental, neurological, and ophthalmological abnormalities. The pathophysiological mechanisms of cblC are only partly understood. CblC is due to mutations in the *MMACHC* gene on chromosome 1p34.2 and results in impaired conversion of vitamin B_{12} into two active forms, adenosylcobalamin and methylcobalamin, leading respectively to increased plasma and urine methylmalonic acid and to increased plasma homocystine and low plasma methionine levels because of impaired remethylation of homocystine to methionine (e.g., Lerner-Ellis et al. 2006; Morel et al. 2006). Impaired remethylation and oxidative stress are felt to contribute to the neurological complications of cblC and increased homocystine concentration to arterial thrombosis (McGuire et al. 2009; Richard et al. 2009).

Clinically, cblC can be divided into early- and late-onset forms, depending on whether the clinical presentation is before or after one year of age, respectively (Martinelli et al. 2011). Characteristic clinical manifestations of the early-onset type include feeding difficulties, failure to

thrive, developmental delay, and muscular hypotonia (e.g., Fischer et al. 2014; Rosenblatt et al. 1997). Some early-onset patients develop a hemolytic-uremic-like syndrome, optic atrophy, and congenital or acquired cardiomyopathy (Martinelli et al. 2011), sometimes with noncompaction (Profitlich et al. 2009). Other clinical signs include protein-losing enteropathy, pulmonary hypertension (Iodice et al. 2013), acidosis, and hyperammonemia (Fischer et al. 2014; Martinelli et al. 2011). Although cblC is part of a group of conditions that are accompanied by an elevation of both homocystine and methylmalonic acid in blood and plasma, it is clinically unique because some features are reported only in this defect, such as the distinct maculopathy (Gizicki et al. 2014; Weisfeld-Adams et al. 2015) and hemolytic-uremic-like syndrome.

Neurological findings relative to the cblC defect include seizures, hydrocephalus, microcephaly, cerebral atrophy, and diffuse white matter edema and demyelination (Rossi et al. 2001; Weisfeld-Adams et al. 2013). More recent patient series emphasize the heterogeneous nature of magnetic resonance findings (Longo et al. 2005); common abnormalities involve white matter changes such as callosal thinning, an increased T2 FLAIR signal in the periventricular and periatrial white matter (Weisfeld-Adams et al. 2013) as well as supratentorial white matter abnormalities (Longo et al. 2005), and craniocaudal shortening of the pons (Weisfeld-Adams et al. 2013). Longo and colleagues (2005) also found that 30% of their patients had basal ganglia lesions. Concerning ophthalmological findings, progressive visual deficits such maculopathy, retinopathy, and nystagmus, in addition to abnormal vision, are extremely common in early-onset cblC and most children will have developed these deficits before school entry, despite early and aggressive biochemical treatment (Gizicki et al. 2014; Weisfeld-Adams et al. 2015). Strabismus and optic atrophy also affect individuals with the early-onset type relatively frequently (Weisfeld-Adams et al. 2015).

Developmental delay and intellectual deficits are also frequent in cblC (Biancheri et al. 2001), and although it is clinically obvious that cognitive difficulties are a major component of cblC defect, few studies have focused on this critical feature (Rossi et al. 2001). Shinnar and Singer (1984) provided evidence of low-average intellectual functioning (IQ = 84) in a 14-year-old female treated for cblC. Beauchamp et al. (2009) reported intellectual decline and attentional-executive deficits (e.g., difficulties concerning selective and sustained attention, inhibition, emotional control, initiation, and/or goal setting) in two female patients between preschool and 12 years of age. In a group of 13 patients aged 9 to 76 months, Weisfeld-Adams et al. (2013) highlight significant and variable developmental delays in a range of adaptive skills, with a relative sparing of socialization abilities (i.e., the skills that allow for adequate communication and interaction with others, such as the modulation of one's behavior according to contextual cues). Lastly, neuropsychiatric disturbances such as personality, emotional, and behavioral changes in the presence of severe executive dysfunction are documented in adult late-onset cblC (Boxer et al. 2005; Tsai et al. 2007).

Preliminary evidence of adverse cognitive outcome despite active clinical management and treatment suggests that there is a pressing need to better identify and understand the neuropsychological repercussions of cblC. Accordingly, the goal of the present study was to describe the neuropsychological profiles of a group of individuals with cblC.

Method

Patients

Nine cblC patients aged between 23 months and 24 years were recruited through the Division of Medical Genetics, CHU Sainte-Justine, Montreal, Quebec, Canada. Five patients (63%) were initially seen following referral for increased urinary methylmalonic acid by the Québec urinary screening network. This is a voluntary program offered for newborns at 21 days of age and also after international adoption. In three patients (33%), clinical signs were present before the screening results were available and led to the diagnosis. Finally, in one case (Patient 3), diagnosis was made by family screening. The cblC patients described received once- or twice-weekly intramuscular injections of hydroxocobalamin (1 mg/injection) and supplementation with betaine (see Table 1). Dietary protein intake is currently within the normal range and total levels of homocystine are followed serially. Most also received acetylsalicylic acid (~1 mg/kg/day). The practice in our institution is to provide sufficient protein intake to ensure normal levels of plasma methionine while maintaining plasma homocystine as low as possible by the use of betaine supplementation. Lipoproteins and carotid ultrasounds are obtained at least at baseline and at 14 years of age or more frequently, if judged necessary. Patients' medical and clinical characteristics, symptomatology, *MMACHC* mutations, pretreatment metabolite levels, treatment parameters, and visual deficits are presented in Tables 1, 2, and 3.

Table 1 Pre-, peri-, and neonatal medical and diagnostic information

Patient	Delivery	Birth weight (g)	Length (cm)	Head circumference (cm)	Apgar	Pre- and neonatal particularities
1	At term	2,863	49,5	Unknown	9-9-10	*Neonatal*: feeding difficulties
2	At term	2,125	49	34	9-9-10	*Prenatal*: fetal pericardial hyperechogenicity (resolved at birth)
3	At term	Unknown	Unknown	Unknown	Unknown	Normal
4	At term	2,360	48	32	9-9-10	*Prenatal*: placental abruption *and* oligohydramnios (14 weeks); delivery induced at 37 weeks
5	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
6	At term	2,590	Unknown	Unknown	7-8-8	*Prenatal*: small placental detachment. *Neonatal*: feeding difficulties, jaundice, hypotonia; rehospitalized at 10 days for one month of investigations
7	At term	3,190	52	31	7-9-9	*Prenatal*: insulin-treated gestational diabetes. *Neonatal*: cyanosis, weak cry, lethargy, feeding difficulties
8	At term	3,345	48	34,5	7-9-9	Normal
9	At term	2,750	45	33,5	9-10-10	*Pre- and neonatal*: intestinal duplication

Patient	Age at diagnosis (months/days)	Diagnostic method	Homocysteine (μmol/L)[a]	Methionine (μmol/L)[a]	Methylmalonic acid (plasma)[a]	Methylmalonic acid (urine)[a]	Allele mutations[b]	
1	1,6	Screen	135	18	23	3,690	c.271 dupA (p.Arg91Lysfs*14)	c.388_390delTAC (p.Tyr130del)
2	1,3	Screen	Unknown	13	Unknown	4,208	c.271dupA (p.Arg91Lysfs*14)	c.271dupA (p.Arg91 Lysfs* 14)
3	58,5	Family evaluation	26	18	6	216	c.271 dupA (p.Arg91 Lysfs* 14)	N/A[c]
4	2,5	Screen	44	27	15	785	c.271 dupA (p.Arg91 Lysfs*14)	N/A[c]
5	6,2	Screen	Unknown	Unknown	73	1290	N/A[c]	N/A[c]
6	0,5	Medical[d]	203	10	46	1597	c.271 dupA (p.Arg91 Lysfs* 14)	c.271dupA (p.Arg91 Lysfs* 14)
7	1,5	Screen	234	<5	50	803	c.271 dupA (p.Arg91Lysfs*14)	c.388_390delTAC (p.Tyr130del)
8	0,8	Medical[d]	166	14	Unknown	711	c.388_390delTAC (pTyr130del)	c.331>T (p.Arg111 Ter)
9	2,5	Medical[d]	Unknown	Unknown	Unknown	Unknown	c.271 dupA (p.Arg91Lysfs*14)	c.271dupA (p.Arg91 Lysfs* 14)

(continued)

Table 1 (continued)

Patient	Age treatment onset	Betaine (mg/kg/day)	B12 (mg/day)	Complementation	Neurological conditions	Neuroimaging abnormalities	Other medical conditions
1	10 weeks	263	1	1	Febrile seizures; attention-deficit disorder	Not performed	Genu valgum
2	Unknown	392	2	1	Seizures; epilepsy (controlled); migraine	Mild cerebral and cerebellar atrophy	Arterial hypertension, osteopenia, hemolytic-uremic-like syndrome at 21 months
3	61 months	0	1	0	None	Not performed	Patent foramen ovale with mild mitral insufficiency; normal ventricular function
4	2,5 months	0	1	1	None	Not performed	Clinically normal
5	6 months	180	1	1	None	Not performed	Hypertrophic cardiomyopathy with pericardial effusion at presentation (8.5 mos), resolved; von Willebrand disease
6	1 month	201	2	1	Hypotonia	Loss of periventricular white matter, bilaterally symmetric, thin corpus callosum, unilateral carotid thrombosis and occlusion present in neonatal period	None
7	21 days	299	1	1	Seizures	Loss of periventricular white matter, T2 hypersignal, irregular ventriculomegaly particularly the atria of the lateral ventricles	None
8	1 month	224	2	1	Afebrile convulsions with multifocal activity on EEG, hydrocephaly corrected surgically; transient CVA (brief facial hemiplegia and dysarthria); migraine	Hydrocephalus (surgically corrected); loss periventricular white matter volume; thin corpus callosum; delayed myelination	Recurrent pericardial effusion and Raynaud syndrome (suspected familial Mediterranean fever); scoliosis; hyperlactatemia
9	6 weeks	199	2	1	Tonic-clonic epileptic seizures (controlled); microcephaly	Loss of periventricular white matter; thin corpus callosum	Intestinal duplication (surgically corrected), mild segmentation abnormality of thoracic spine with scoliosis; interventricular septal defect; sleep disorder; megaloblastic anemia (resolved with treatment)

[a] Normal range for metabolite values: homocystine = 6.5–12.8 μmol/L; methionine = 13–25 μmol/L; methylmalonic acid (pl.) = <0.4 μmol/L; methylmalonic acid (ur.) = <10 μmol/L

[b] Reference sequence used: NM_015506

[c] *N/A* not available at the time of writing

[d] Clinical signs leading to medical attention prior to age of screening. Patient 6: at 2 weeks, presence of hypotonia and somnolence. Patient 8: at 8 weeks, presence of convulsions. Patient 9: 2 days after birth, presence of intestinal duplication diagnosed prenatally and corrected surgically. The child also experienced convulsions at 4 weeks.

Table 2 Psychological aspects, rehabilitation services, and educational/occupational status

Patient	Age	Psychological	Professional services related to development[a]	Medication related to neuropsychiatric function	Schooling or work environment
1	3 years 9/12	Developmental delay, sensory integration disorder (hypersensitivity), anxiety	Npsych, OT, psych ed, psych, special ed., ST. Attends a rehabilitation center specialized in visual deficits	Methylphenidate (unknown dosage)	Specialized class for children with autism spectrum disorder
2	24 years 4/12	Developmental delay, oro-linguo-facial and ideo-motor dyspraxia, dysdiadochokinesia, hyperreflexivity, anorexia	OT, physiatry, PT, psych, special ed, ST	Methylphenidate (ceased – headaches), amitriptyline (25 mg die)	Adult education, receptionist at family business 3 days/week
3	8 years 5/12	None	Remedial instruction	None	Regular curriculum with support from remedial teacher
4	6 years 5/12	None	None	None	Regular curriculum
5	16 years 9/12	None	None	None	Regular curriculum
6	1 year 11/12	Failure to thrive, developmental motor delay (gross and fine), dysphagia, hypotonia, difficulties in feeding (uncoordinated suction, swallowing, breathing), involuntary movements	OT, PT, psych, ST	None	At home with mother
7	9 years 1/12	Developmental delay, hypotonia	OT, PT, psych, special ed, ST	None	Special class for children with mild intellectual disability
8	12 years 11/12	Psychomotor developmental delay, bucco-lingual dyspraxia	Child psychiatry, OT, physiatry, PT, psych, ST. Attends a rehabilitation center specialized in visual deficits	Methylphenidate (unknown dosage), amitriptyline (25 mg die – ceased), clobazam (10 mg bid)	Elementary: school for children with speech delays/disorders High school: special education class
9	14 years 1/12	Failure to thrive, oral dysphagia, oral hypersensitivity, diffuse hypotonia with hyperreflexivity, dyskinesia and hyperkinesia	Child psychiatry, OT, physiatry, PT, psych ed, remedial instruction, ST, social work, low vision services	Melatonin (9 mg die), lorazepam (0.5 mg die)	Class for children with severe to profound intellectual disabilities (reduced teacher to student ratio)

[a] Abbreviations: *PT* physiotherapy, *OT* occupational therapy, *ST* speech therapy, *psych* psychology, *Npsych* neuropsychology, *psych ed* psychoeducation, *special ed* special education

Table 3 Ophthalmic findings in patients with cblC

Patient	Gender	Initial examination age	Follow-up (years)	Initial best corrected visual acuity (right eye to left eye)	Last best corrected visual acuity (right eye to left eye)	WHO category of visual impairment[a]	Abnormal looking retina	Electroretinogram	Nystagmus	Optic atrophy	Strabismus
1	M	6 months	2	Fx-Fx	20/150-20/150	Moderate	+	N	+	+	–
2	F	5 months	23	Fx-Fx	20/800-20/800	Blindness	+	AbN	+	–	+
3	M	6 years	0	20/20-20/20	20/20-20/20	Mild or absent	–	N/A	–	–	–
4	F	19 months	5	Fx-Fx	20/25-20/25	Mild or absent	–	N	–	–	–
5	F	9 months	13	FF-FF	20/25-20/25	Mild or absent	–	N	–	–	–
6	M	2 months	1	Fx-Fx	20/200-20/200	Moderate	+	AbN	+	–	–
7	M	2 months	7	FF-FF	FF-FF	N/A	+	AbN*	+	–	–
8	F	5 months	11	LP-LP	20/100-20/100	Moderate	–	N	+	+	–
9	M	5 years	12	LP	20/100-20/100	Moderate	+	N	+	+	+

F female, *M* male, *Fx* fixates, *LP* light perception, *FF* fixates and follows, + yes, – no, *AbN* abnormal, *N* normal, *AbN** abnormal with documented deterioration, *N/A* not available, *WHO* World Health Organization. [a]World Health Organization (2003)

Neuropsychological Assessment

For patients who were able to complete intellectual functioning assessments (Patients 1, 2, 3, 4, 5), a battery of age-appropriate standardized tasks was administered. Tests were chosen to minimize visual stimuli.

General Intellectual Functioning

Developmentally appropriate scales from the Wechsler batteries (Wechsler 1997, 2002, 2003; see Table 4) were used to test intellectual functioning including verbal comprehension and perceptual reasoning. The working memory (i.e., online capacity to retain and manipulate information) and information processing speed indices were calculated when visual acuity and cognitive capacities permitted. Intellectual functioning is presented with respect to age-referenced normative data (standard score, mean = 100, SD = 15; see Table 4 for IQ classifications). The term "intellectually disabled" is used here for patients with an IQ below 70.

Visual Attention

Patients completed a selective/divided visual attention task (d2, 16+ years of age, mean = 100, SD = 15, and NEPSY-I, 3 to 12 years of age, mean = 10, SD = 3; Brickenkamp and Zillmer 1998; Korkman et al. 1998) in which target items have to be crossed out while ignoring distractors.

Auditory Attention

Patients completed the auditory attention (NEPSY-II; 3 to 16 years of age, mean = 10, SD = 3; Korkman et al. 2007) or the elevator counting task (TEA; 18 + years of age, mean = 10, SD = 3; Robertson et al. 1994). In the former, children touch a designated shape when an auditory stimulus is presented and ignoring distractors. In the latter, individuals count elevator floors, with each level increase denoted by a "beep" sound amidst auditory distractors.

Visual Memory

Immediate and delayed visual memory was evaluated using memory for designs (NEPSY-II; 3 to 16 years of age; Korkman et al. 2007), in which individuals have to memorize a configuration of abstract designs on a grid over the course of four or five learning trials and complete a 20-min delayed recall (scaled score, mean = 10, SD = 3).

Verbal Memory

A word list (CMS, 5 to 16 years of age, and WMS-III, 16+ years of age; Cohen 1997; Wechsler 1997) is presented

Table 4 Neuropsychological results on standardized cognitive tasks

	Patient				
	1	2	3	4	5
Age	3 years 9/12	24 years	8 years 5/12	6 years 8/12	16 years 9/12
Intelligence [IQ (percentile)]					
Verbal comprehension IQ (VIQ)	71 (2.7) Borderline	61 (0.5) Extremely low	77 (6.3) Borderline	89 (23.2) Low average	93 (32) Average
Perceptual reasoning IQ (PIQ)	67 (1.4) Extremely low	49 (<0.1) Extremely low	80 (9.1) Low average	87 (19.3) Low average	104 (60.5) Average
Working memory index (WMI)	N/A**	N/A*	91 (27.4) Average	86 (17.5) Low average	86 (17.5) Low average
Processing speed index (PSI)	N/A**	50 (<0.1) Extremely low	85 (15.9) Low average	80 (9.1) Low average	80 (9.1) Low average
Global IQ	63 Extremely low	56 Extremely low	79 Borderline	82 Low average	84 Low average
Attention [SS (percentile)]					
Visual	N/A*	1(0.1) Extremely low	5 (5) Borderline	7 (15) Low average	7 (17.5) Low average
Auditory	N/A*	N/A*	7 (15) Low average	8 (25) Low average	1 (0.1) Extremely low
Memory [SS (percentile)]					
Immediate visual recall	N/A**	N/A*	7 (15) Low average	10 (50) Average	14 (90) Superior
Delayed visual recall	N/A**	N/A*	9 (37) Average	10 (50) Average	13 (84) High average
Immediate verbal recall	N/A**	5 (5) Borderline	10 (50) Average	11 (63) Average	7 (15) Low average
Delayed verbal recall	N/A**	7 (15) Low average	9 (37) Average	10 (50) Average	8 (25) Low average
Visual-motor integration [SS (percentile)]					
Visual perception	N/A*	<1 (0.02) Extremely low	5 (6) Borderline	7 (16) Low average	6 (10) Borderline
Motor coordination	N/A*	<1 (0.02) Extremely low	2 (0.7) Extremely low	7 (18) Low average	8 (25) Low average
Visual-motor integration	N/A*	<1 (0.02) Extremely low	4 (2) Extremely low	8 (25) Low average	7 (14) Low average

(continued)

Table 4 (continued)

	Patient				
	1	2	3	4	5
Executive functions [parental report; T score (percentile)]					
Behavioral regulation index	N/A***	46 (50) Typical	55 (72) Typical	46 (40) Typical	38 (11) Typical
Metacognition index	N/A***	70 (90) Clinical	53 (63) Typical	47 (41) Typical	46 (41) Typical
Global executive composite	N/A***	62 (89) Subclinical	54 (66) Typical	46 (38) Typical	42 (29) Typical
Inattention/hyperactivity [parental report; T score (percentile)]					
DSM-IV: inattention	63 (90) Subclinical	63 (90) Subclinical	64 (92) Subclinical	48 (42) Typical	47 (37) Typical
DSM-IV: hyperactivity/impulsivity	61 (86) Subclinical	47 (37) Typical	50 (50) Typical	59 (81) Typical	43 (23) Typical
DSM-IV: total	63 (90) Subclinical	58 (79) Typical	58 (79) Typical	53 (61) Typical	45 (30) Typical
Adaptive behavior [parental report; standard score (percentile)]					
Global adaptive composite	N/A***	N/A***	98 (45) Average	103 (58) Average	111 (77) High average

Shaded data indicate performance in the intellectually disabled or clinically significant range. N/A* = participant unable to complete the tasks due to visual and intellectual limitations; N/A** = tasks/indices do not apply given the age of the participant; N/A*** = data could not be obtained. Evaluation of intellectual functioning: (1) Wechsler Preschool and Primary Intelligence Scale-III (WPPSI-III; 2 years and 3 months to 7 years and 3 months; Wechsler, 2002) – verbal (VIQ), performance (PIQ), and full scale (FSIQ) intellectual quotient (IQ) scores. (2) Wechsler Intelligence Scale for Children-IV (WISC-IV; 6 to 16 years and 11 months of age; Wechsler, 2003) – verbal comprehension (VCI), perceptual reasoning (PRI), and full scale (FSIQ) IQ scores. Working memory (WMI) and processing speed indices (PSI) were calculated when possible. (3) Wechsler Adult Intelligence Scale-III (WAIS-III; 16+ years of age; Wechsler, 1997) – verbal (VIQ), performance (PIQ), and full scale (FSIQ) IQ scores

Legend IQ scores/composite scores: extremely low (≤69), borderline (70–79), low average (80–89), average (90–109), high average (110–119), superior (120–129), very superior (≥130)

Legend scaled scores (SS): extremely low (1–4), borderline (5–6), low average (7–8), average (9–11), high average (12–13), superior (14–15), very superior (16–19)

Legend *T* scores: normal range (<60), subclinical range (60–64), clinical range (≥65)

Table 5 Results on the Vineland Adaptive Behavior Scale for patients unable to complete standardized cognitive tasks

	Patient			
	6	7	8	9
Age	1 year 11/12	9 years 1/12	12 years 11/12	14 years 1/12
Adaptive behavior (percentile)	1	2	1	<1
Adaptive level	Mild deficit	Mild deficit	Mild deficit	Severe deficit
Communication (percentile)	3	1	1	<1
Daily living skills (percentile)	2	3	4	<1
Socialization (percentile)	4	5	<1	<1
Motor skills (percentile)	3	3	7	<1
Lowest age equivalency (subdomain)	4 months (coping skills)	3 years 2 months (expressive)	2 years 2 months (community)	10 months (expressive)
Highest age equivalency (subdomain)	1 year 2 months (receptive)	7 years 7 months (domestic)	10 years 6 months (personal)	3 years (gross)

Shaded area indicates performance in the deficient range. Domains (subdomains): communication (receptive, expressive, written), daily living skills (personal, domestic, community), socialization (interpersonal relationships, play and leisure, coping skills), and motor (gross, fine)

orally four times, and individuals must recall as many words as possible immediately after each reading and after a 30-min delay (scaled score, mean = 10, SD = 3).

Visual-Motor Integration

The Beery-Buktenica Developmental Test of Visual-Motor Integration (VMI; full form, 2 to 100 years of age; Beery et al. 2010) consists of the following: (1) visual-motor integration in which simple and integrated geometric forms must be reproduced, (2) visual perception in which a geometric figure must be identified among distractors, and (3) motor coordination in which stimulus forms are traced with a pencil (30 items each, scaled score, mean = 10, SD = 3).

Parent Questionnaires

The Behavior Rating Inventory of Executive Function (BRIEF; 5 to 18 years of age; Gioia et al. 2000) is an 86-item parent rating of executive behavior on a three-point scale. Three summary indices are reported: behavioral regulation index (scales: inhibit, shift, emotional control), metacognition index (scales: initiate, working memory, plan/organize, organization of materials), and global executive composite (*T* scores, mean = 50, SD = 10).

The Conners Performance Rating Scale (CPRS-R:L; 6 to 18 years of age version; Conners 2000) was used to evaluate attentional difficulties and consists of 80 items rated on a four-point frequency scale. Three criteria pertaining to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) are reported (*T* scores, mean = 50, SD = 10).

Adaptive behavior, defined as the everyday practical abilities necessary to respond adequately to environmental demands such as autonomy, social interactions, and communication skills, was assessed in Patients 1–5 using the Adaptive Behavior Assessment System (ABAS-II; Harrison and Oakland 2003) which includes 211 items that must be rated according to the frequency with which children demonstrate these behaviors, without help, when necessary (standard score; mean = 100, SD = 15). The 383-item Vineland Adaptive Behavior questionnaire (Vineland-II; birth to 90 years of age; Sparrow et al. 2005) was used for patients who were unable to complete the intellectual functioning assessment (Patients 6, 7, 8, 9; see Table 5).

Neuropsychological and Statistical Analyses

Cognitive profiles were established by comparing individual performances to age-referenced normative data (interpersonal comparisons). Relative strengths and weaknesses were determined by comparing individual performances in each cognitive function to overall intellectual functioning, which serves as a baseline of expected cognitive ability (intrapersonal comparisons). A strength or weakness was considered to be present when individuals performed 1.5 to 2 standard deviations above or below their intellectual level (Lezak et al. 2004, pp. 99, 148). Fisher's exact test was conducted to determine whether there were any differences between individuals with and without intellectual disability in terms of interventions received. Additionally, Pearson correlations were performed to examine the association between age at diagnosis and age at treatment initiation and cognitive outcomes (in terms of intellectual quotient or adaptive quotient for the severely disabled individuals).

Results

Patients 3, 4, and 5 (See Table 4)

Five patients (1, 2, 3, 4, 5) completed intellectual functioning scales. Three (3, 4, 5) had an IQ within the nonintellectually disabled range (($\geq$70); see supplementary Fig. 1). None presented with a *specific* deficit in terms of attentional abilities, but attention was nonetheless impaired for two of the three patients (3, 5). These latter two individuals also presented with visual-motor integration difficulties, which are the result of both poor visual perception and motor coordination. They had relative strengths in long-term verbal and visual memory, respectively. Memory was also slightly above expected levels for Patient 4, but not enough to warrant categorization as a strength.

BRIEF results were in the normal range for Patients 3, 4, and 5, suggesting intact attentional-executive abilities, as perceived by parents. This observation is supported by the results of the Conners questionnaire for Patients 4 and 5 whose parents reported no significant difficulties in terms of attentional control and hyperactivity/impulsivity. Parental reports for Patient 3 revealed clinically significant ratings for the cognitive problems/inattention and the hyperactivity dimensions, resulting in subclinical ratings on global attentional-executive measures including the Conners ADHD index and the DSM-IV inattentive index. The ABAS-II results revealed normal to slightly superior adaptive functioning compared to same-aged peers for Patients 3, 4, and 5. The intellectual and adaptive functioning scores are displayed in Table 4.

Patients 1 and 2 (See Table 4)

The other two patients (1, 2) who completed the Wechsler scales presented with severe intellectual limitations, as demonstrated by extremely low IQ scores (Table 4). Patient 2 completed all domain-specific measures except the auditory attention and the visual memory tasks, due to visual and cognitive limitations. The results reveal extremely low to borderline functioning in the areas of visual attention, short-term verbal memory, and fine motor skills. The only exception concerns a relative strength in long-term verbal memory. Both the BRIEF and Conners questionnaires reveal subclinical ratings for the global executive composite and the DSM-IV inattention index, as well as clinically significant ratings for the metacognitive index, suggesting the presence of attentional-executive difficulties. Additionally the questionnaire may underestimate difficulties in this 24-year-old patient, given it was validated only up to 18 years. Data concerning adaptive behavior (ABAS-II) could not be obtained for Patient 2.

Although Patient 1 was capable of completing the Wechsler IQ measure, severe visual and cognitive limitations precluded the completion of all domain-specific cognitive tasks. Subclinical ratings on all three indices of the Conners questionnaire suggest the presence of attentional-executive difficulties. Additional data concerning executive functions (BRIEF) and adaptive behavior (ABAS-II) could not be obtained.

Patients 6, 7, 8, and 9 (See Table 5)

An estimate of global functioning was obtained for Patients 6, 7, 8, and 9 using the Vineland-II questionnaire as they were unable to complete the Wechsler IQ scales. Adaptive deficits were reported for all of these patients (Table 5) with age equivalencies well below the individuals' chronological age.

Associated Medical and Clinical Factors

Clinical profiles and medical histories were explored in order to determine which factors may be common to those who have severe cognitive limitations. No correlations were found between age at diagnosis and age at treatment initiation and intellectual/adaptive functioning (as measured by global IQ for Patients 1, 2, 3, 4, 5 and by global adaptive behavior for Patients 6, 7, 8, 9; $r(9) = 0.29$, $p = 0.45$, $r(8) = 0.29$, $p = 0.49$).

As depicted in Tables 1, 2, and 3, all patients with intellectual disability and/or adaptive dysfunction (IQ < 70, Patients 1, 2, 6, 7, 8, 9) also had visual impairment (mainly retinopathy, maculopathy, and nystagmus). Many had a history of neurological complications (convulsions/epilepsy, hydrocephalus) and the involvement of other organ systems. Of note, all those who underwent clinical neuroimaging (i.e., Patients 2, 6, 7, 8, 9) had positive radiological findings, with four out of five (Patients 6, 7, 8, 9) presenting altered white matter integrity and corpus callosum atrophy, among other structural abnormalities. Globally, these individuals have extensive multisystemic involvement that requires follow-up by primary care and rehabilitation services and which has a significant impact on daily functioning, impeding them from leading independent lives. All of these patients (except Patient 6 who is too young to attend school) require specialized and adapted school curricula and services aimed at developing basic living skills and maximizing independence.

Conversely, none of the three individuals with intellectual functioning in the nondisabled range (IQ > 70) have visual and/or neurovisual deficits. While two of them (Patients 3 and 5) had nonneurological medical complications at a young age (e.g., mild mitral insufficiency, hypertrophic cardio-

myopathy, von Willebrand disease), these complications were not clearly associated with cblC and did not lead to chronic sequelae. All these patients are currently leading age-appropriate independent lives and follow the regular school curriculum. Only one of the three patients (Patient 3) requires remedial support. Of note, Patient 3 obtained the lowest IQ score (borderline) among those who did not have intellectual disability.

Lastly, the patients with an intellectual disability/adaptive dysfunction did not have recourse to rehabilitation services significantly more than those in the nondisabled intellectual range ($p = 0.08$).

Discussion

Cognitive Functioning and Associated Developmental, Neurobiological, and Medical Factors

IQ or adaptive skills for patients in this study ranged from extremely low to average, revealing marked cognitive differences among cblC individuals. Poorer cognitive outcome was observed in conjunction with multisystem disease, associated neurological and neuroradiological abnormalities, as well as visual deficits. In particular, most individuals with impaired functioning had corpus callosum atrophy and white matter abnormalities. Given that myelinated nerve fibers are involved in the efficient transmission of signals between neurons, facilitating speed of interhemispheric communication and information integration, white matter abnormalities may contribute to the severe cognitive limitations observed. Other potential correlates of outcome, such as metabolite levels and causal mutations, require larger cohorts to draw conclusions. To fully understand the complexity of neurocognitive profiles in cblC within a developmental context, findings must be interpreted as an interaction between child development and defect progression. When cognitive deficits are observed, genuine deterioration may be occurring, as in neurodegenerative diseases; however, it may also be that children are simply not progressing at the same rate as their peers or have reached a plateau in terms of their intellectual functioning, thereby widening the gap between their functioning and that of normally developing children, as is likely the case in cblC.

Neuropsychological Assessment in cblC Patients

Cognitive difficulties were observed in eight of the nine patients studied, regardless of their level of intellectual functioning and despite the fact that all patients received treatment including hydroxocobalamin injections. This demonstrates that current biomedical treatment of cblC does not prevent cognitive impairment, even if started at an early age in screened, asymptomatic newborns, and indicates the potential utility of timely neuropsychological assessment. The clinical heterogeneity revealed here extends the previously reported findings (Beauchamp et al. 2009; Longo et al. 2005; Varvogli et al. 2000; Weisfeld-Adams et al. 2013). Clearly, cblC does not cause a consistent pattern of cognitive functioning. This underscores the need for individualized evaluation to understand each patient's idiosyncratic patterns of cognitive functioning. Strengths identified through such assessments can be used to compensate for potential weaknesses, as well as to determine optimal learning conditions on an individual basis. A complete neuropsychological evaluation can also identify subtle, yet significant weaknesses. For example, difficulties in visual-motor integration were observed here in patients with age-appropriate intellectual functioning and in regular school curriculums. Additionally, Patient 3 had borderline intelligence and cognitive difficulties that were not detected until middle to late primary school.

Pathophysiology, Molecular Analyses, and Treatment in Relation to Neuropsychological Outcome

The most common mutation is c.271dupA (p.Arg91Lysfs*14), a frequent protein-truncating frameshift mutation associated with severe outcome (Lerner-Ellis et al. 2009). Studies are ongoing to establish the complete mutational profile of our population of cblC patients.

As previously mentioned, the pathophysiology of cblC defect is largely unknown. The characteristically elevated metabolites of cblC, homocystine and methylmalonic acid, cannot explain many of the most important manifestations of this condition. Conversely, neither hyperhomocysteinemia nor methylmalonic acidemia can be assumed to be benign in cblC. For instance, to our knowledge, no episodes of hemolytic-uremic-like syndrome have occurred in previously asymptomatic patients during hydroxocobalamin treatment, and a rapid albeit partial decrease in homocystine occurs within weeks in most patients following the initiation of treatment. The choice of hydroxocobalamin dose has previously been discussed in the literature (Dionisi-Vici et al. 2013); our patients receive 1 mg intramuscularly once or twice weekly. Marked protein restriction is avoided because of its established negative effects on growth and anabolism. Dietary methionine provides a source of methionine that may compensate in part for the defective intracellular synthesis of methionine that follows closely from the primary defect in cblC. Although patients with cblC were traditionally restricted in protein intake, in a recent survey, none of 88 European patients received protein restriction (Fischer et al. 2014). Furthermore, recent studies of treatment in cblC found no benefit in restricting protein access and a possibly deleterious effect of using low-methionine medical foods (Manoli et al. 2015). Hyperhomocystinuria is a known risk

factor for thrombosis. General measures of prevention of thrombosis include aspirin treatment and hydration, optimization of the dose of hydroxocobalamin and avoidance of excessive methionine intake, and reduction of other cardiovascular risk factors if present, such as hypercholesterolemia and hypertension. Methylmalonic acidemia is usually considered to be a clinically minor finding in cblC, and the ketoacidic decompensations seen in classical methylmalonic aciduria resulting from methylmalonyl-coenzyme A mutase deficiency are rarely, if ever, seen in cblC defect. However, the basal ganglia lesions seen in some cblC patients (Longo et al. 2005) are reminiscent of those seen in many diseases of acyl-CoA metabolism including methylmalonyl-CoA mutase deficiency (Mitchell et al. 2008), and some role for methylmalonic acid or methylmalonyl-CoA in the pathogenesis of cblC cannot be formally excluded. From the above, it is clear that no dietary regimen can normalize the concentrations of all the metabolites that are currently considered as potentially involved in aspects of neurodevelopmental function.

Similarly, the effect of newborn screening on clinical outcome in cblC defect is unknown. Some patients detected by urine screening and for whom treatment is initiated in the first weeks of life have early-onset defect and have manifestations at birth or prenatally like cardiomyopathy (De Bie et al. 2009). Therefore, newborn screening will not correct the defect in all patients, but arguably, early detection and treatment might influence neurodevelopment favorably in some individuals and will also detect other forms of methylmalonic aciduria.

Conclusions and Future Directions

The current findings constitute the most in-depth neuropsychological evaluation to date in a large group of cblC patients. Detailed studies of cognition were previously described in only three cblC patients (Beauchamp et al. 2009; Shinnar and Singer 1984). Links with documented medical and radiological findings, despite being qualitative in nature, suggest preliminary hypotheses as to the mechanisms underlying cognitive impairment. Although visual deficits can affect cognitive performance, the deficits observed in these patients are not reducible to visual problems. Central nervous system abnormalities detected by neuroimaging as well as extensive multisystem disease are probably the main factors contributing to severe cognitive limitations. The great clinical diversity in cblC patients invites future studies of larger numbers of patients, with longitudinal neuropsychological evaluation and detailed prospective studies of *MMACHC* genotype and of dietary and pharmacological treatments.

A multicentric study could be useful in increasing the patient base and in enabling age group comparisons. This would allow the scientific community to examine genotype and pathophysiological mechanisms in relation to clinical outcome, as well as allowing the exploration of the cognitive benefits of newborn screening. Additionally, longitudinal follow-up of patients with cblC is recommended to monitor the stability of the cognitive profile (or lack thereof) and further specify what factors promote favorable cognitive outcome. From a clinical perspective, we suggest that neuropsychological evaluation should be offered to children and adolescents with cblC defect. As well as being an essential element for accurate evaluation of the impact of treatments, neuropsychological evaluation has practical impacts for patient management. It allows for orientation to appropriate educational and remedial services, which may even benefit individuals whose difficulties are not obvious on standard pediatric evaluation. As such, early intervention, *even in children with intellectual functioning in the normal range*, may increase the chances of maintaining adequate functioning when children are faced with increasing cognitive and environmental demands at older ages.

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Synopsis

This article explores the heterogeneous nature of neuropsychological functioning in individuals with cobalamin C defect.

Compliance with Ethics Guidelines

Conflict of Interest

Jenny Bellerose, Mathilde Neugnot-Cerioli, Catherine Brunel-Guitton, Grant A. Mitchell, Luis H. Ospina, Karine Bédard, and Miriam H. Beauchamp declare that they have no conflict of interest.

Informed Consent

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

Details on the Contribution of Individual Authors

Jenny Bellerose: Development of study protocol and methodology, ethics submissions, data collection, analyses, and interpretation, writing of manuscript.

Mathilde Neugnot-Cerioli: Development of study protocol and methodology, data collection and analyses, interpretation of findings, manuscript editing and review.

Luis H. Ospina: Consultant for ophthalmological aspects, interpretation of opthalmological data, manuscript review.

Grant A. Mitchell: Development of study protocol, recruitment of patients, consultant for medical aspects, writing of manuscript.

Catherine Brunel-Guitton: Recruitment of patients, manuscript review.

Miriam H. Beauchamp (principal investigator): Development of study protocol and methodology, funding, supervision of J.B. and M.N.C., interpretation of data, writing of manuscript.

Karine Bédard: Performance and interpretation of molecular sequencing data.

Appendix: Abbreviations and Details of Neuropsychological Tests Administered

ABAS	**Adaptive Behavior Assessment System** Harrison PL, Oakland T. Adaptive Behavior Assessment System – Second Edition. The Psychological Corporation, San Antonio, TX, 2003
BRIEF	**Behavior Rating Inventory of Executive Function** Gioia GA, Isquith PK, Guy SC, Kenworth L. Behavior Rating Inventory of Executive Function. Psychological Assessment Resources, Odessa, FL, 2000
CMS	**Children's Memory Scale** Cohen M. Children's Memory Scale. The Psychological Corporation, San Antonio, TX, 1997
CPRS–R:L	**Conners Performance Rating Scale–Revised: Long version** Conners KC. Conners Performance Rating Scale - Revised: Long version. Multi-Health Systems Inc., North Tonawanda, NY, 2000
d2	**d2 Test of Attention** Brickenkamp R, Zillmer E. The d2 Test of Attention. Hogrefe & Huber Publishers, Seattle, WA, 1998
TEA	**Test of Everyday Attention** Robertson IH, Ward T, Ridgeway V, Nimmo-Smith I. The Test of Everyday Attention. The Psychological Corporation, San Antonio, TX, 1994
VMI	**Beery-Buktenica Developmental Test of Visual-Motor Integration.** Beery KE, Buktenica NA, Beery NA. The Beery-Buktenica Developmental Test of Visual-Motor Integration - Sixth Edition. The Psychological Corporation, San Antonio, TX, 2010
NEPSY I-II	**Neuropsychological Assessment** Korkman M, Kirk U, Kemp S. NEPSY-II: A Developmental Neuropsychological Assessment – Second Edition. The Psychological Corporation, San Antonio, TX, 1998 & 2007
WMS-III	**Wechsler Memory Scale** Wechsler D. Wechsler Memory Scale - Third Edition. The Psychological Corporation, San Antonio, TX, 1997
WPPSI-III	**Wechsler Preschool and Primary Scale of Intelligence** Wechsler D. Wechsler Preschool and Primary Scale of Intelligence – Third Edition. The Psychological Corporation, San Antonio, TX, 2002
WISC-IV	**Wechsler Intelligence Scale for Children** Wechsler D. Wechsler Intelligence Scale for Children – Fourth Edition. The Psychological Corporation, San Antonio, TX, 2003
WAIS-III	**Wechsler Adult Intelligence Scale** Wechsler D. Wechsler Intelligence Scale for Children – Fourth Edition. The Psychological Corporation, San Antonio, TX, 1997
Vineland-II	**Vineland Adaptive Behavior Scales** Sparrow SS, Cicchetti DV, Balla DA. Vineland Adaptive Behavior Scales – Second Edition. The Psychological Corporation, San Antonio, TX, 2005

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RESEARCH REPORT

Heterozygous Monocarboxylate Transporter 1 (MCT1, *SLC16A1*) Deficiency as a Cause of Recurrent Ketoacidosis

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Abstract We describe two half-siblings with monocarboxylate transporter 1 (MCT1, *SLC16A1*) deficiency, a defect on ketone body utilization, that has only recently been identified (van Hasselt et al., N Engl J Med, 371:1900–1907, 2014) as a cause for recurrent ketoacidoses. Our index patient is a boy with non-consanguineous parents who had presented acutely with impaired consciousness and severe metabolic ketoacidosis following a 3-day history of gastroenteritis at age 5 years. A 12.5-year-old half-brother who shared the proband's mother also had a previous history of recurrent ketoacidoses. Results of mutation and enzyme activity analyses in proband samples advocated against methylacetoacetyl-coenzyme A thiolase ("beta-ketothiolase") and succinyl-coenzyme A: 3-oxoacyl coenzyme A transferase (SCOT) deficiencies. A single heterozygous c.982C>T transition in the *SLC16A1* gene resulting in a stop mutation (p.Arg328Ter) was detected in both boys. It was shared by their healthy mother and by the proband's half-sister, but was absent in the proband's father. MCT1 deficiency may be more prevalent than is apparent, as clinical manifestations can occur both in individuals with bi- and monoallelic mutations. It may be an important differential diagnosis in recurrent ketoacidosis with or without hypoglycemia, particularly in the absence of any specific metabolic profiles in blood and urine. Early diagnosis may enable improved disease management. Careful identification of potential triggers of metabolic decompensations in individuals even with single heterozygous mutations in the *SLC16A1* gene is indicated.

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Introduction

The ketone bodies acetoacetate and D-3-hydroxy-*n*-butyric acid are derived from fatty acids and ketogenic amino acids such as leucine. They are important vectors of energy transport from the liver to extrahepatic tissues during prolonged fasting or in cases of enhanced energy requirements (Sass 2012; Fukao et al. 2014). Ketone bodies play a key role in glucose-sparing energy supply, particularly in the brain, which is unable to utilize fatty acids directly (Mitchell et al. 1995). The interconversion of acetoacetate with D-3-hydroxy-*n*-butyric acid is catalyzed by D-3-hydroxy-*n*-butyrate dehydrogenase. It reflects the oxidation status of the mitochondrial matrix.

Ketolysis (ketone body utilization) occurs in extrahepatic tissues. Its first and rate-limiting step requires the enzyme

succinyl-coenzyme A: 3-oxoacyl coenzyme A transferase (SCOT; EC 2.8.3.5) which activates acetoacetate to acetoacetyl-coenzyme A. A mitochondrial acetoacetyl-coenzyme A thiolase ("beta-ketothiolase"; EC 2.3.1.9) then catalyzes the formation of two acetyl-coenzyme A (acetyl-CoA) molecules per molecule acetoacetyl-CoA. Since this enzyme has also a role in isoleucine catabolism, where it catalyzes the thiolytic cleavage of methylacetoacetyl-coenzyme A, it is more unambiguously named methylacetoacetyl-CoA thiolase (MAT; EC 2.3.1.9) (Sass 2012).

Defects in the genes encoding for SCOT (*OXCT1*) and MAT (*ACAT1*) are the cause of the established inborn errors of ketolysis (OMIM 245050 and OMIM 203750) (Mitchell et al. 1995; Sass 2012; Fukao et al. 2014). Patients usually present with ketoacidotic episodes, which may be life-threatening. Due to accumulating isoleucine metabolites, the laboratory diagnosis of MAT deficiency can be rather straightforward by analyses of urinary organic acids and blood acylcarnitines, as long as a defect of the preceding step in the isoleucine pathway is considered in the differential diagnosis (Sass 2012). In contrast there is no specific metabolite marker for SCOT deficiency. This ketolysis defect is suspected in cases of unexplained pronounced or frequent ketoacidotic episodes and in some cases is associated with persistent ketonuria (Fukao et al. 2004). Enzyme activity testing in blood cells or cultivated fibroblasts may clarify whether SCOT deficiency is present or not. Sequence analysis of the *OXCT1* gene is another option.

Recently, van Hasselt et al. (2014) have revealed homozygous and heterozygous mutations in the *SLC16A1* gene, which encodes the monocarboxylate transporter 1 (MCT1), in ketoacidotic patients with a suspected defect in ketolysis, but normal enzyme activities of SCOT and MAT. Such a finding may have major impact on the diagnostics of ketoacidosis, but so far awaits confirmation in other patients. Here we report a family with two symptomatic boys and a pedigree which supports the opinion that even a single heterozygous mutation can result in clinically relevant symptoms and that biallelic mutations in the *SLC16A1* gene are not always required for clinical symptoms.

Case Reports

A 5-year-old boy born to non-consanguineous British parents presented acutely with impaired consciousness following a 3-day history of gastroenteritis while holidaying in Croatia. He was initially managed with oral rehydration solutions for the first 24 h; however, due to unrelenting vomiting, he presented to an emergency clinic. His capillary blood glucose was normal; however, no other blood or urine tests were done at that stage. After initiation of intravenous normal saline maintenance infusion, with sips of sweet drinks, his vomiting reduced. By the third day, he became extremely lethargic and was referred to a regional hospital. Shortly upon presentation to the hospital, he deteriorated rapidly and became encephalopathic. He was tachypnoeic and mildly dehydrated but afebrile with no localizing neurological signs. His arterial blood pH was 7.13, bicarbonate 7.3 mmol/L, BE −19.2 mmol/L, and anion gap 22.7 mmol/L. His plasma 3-hydroxy-*n*-butyrate was increased at 5.2 mmol/L, and significant urinary excretion of ketones was demonstrated. His plasma lactate, ammonia, glucose, and liver function remained normal. He was treated with a normal saline bolus, followed by continuous infusion of dextrose at 9 mg/kg/min with sodium and potassium supplements. His condition improved over the next few hours with fluid and caloric replacement, and he was discharged the next day. His urine metabolic screen showed massive excretions of 3-hydroxy-*n*-butyrate and acetoacetate and detectable amounts of 2-methyl-3-hydroxybutyrate (2M3HB), 2-methylacetoacetate (2MeAA), and tiglylglycine (TG), leading to an initial suspicion of mitochondrial MAT deficiency. A Guthrie card collected during the crisis was not analyzed then, but provided to the parents and studied later (see below). Back home in Perth, Western Australia, the proband was referred to the metabolic service. Upon further recollection, his parents reported a possible previous history of recurrent ketotic episodes associated with fruity odor on his breath and fast breathing during intercurrent infections or unwell periods. Clinical examination was unremarkable. He was a well-thrived child with age-appropriate developmental milestones. Interestingly, a similar history of recurrent ketoacidosis was elicited in an elder 11.5-year-old half-brother who shared the proband's mother. He was previously managed at a local hospital in the United Kingdom, having first presented at 18 months of age, following a day of profuse vomiting and diarrhea. He had significant metabolic acidosis with a pH of 7.24, bicarbonate of 5 mmol/L, and BE of −13 mmol/L. He was discharged home after a short stay, but readmitted 10 days later with gastroenteritis. His blood pH was 7.33, bicarbonate 15 mmol/L, and BE −7 mmol/L. His blood glucose, plasma ammonia, and urine organic acids were reported to be normal. He was reported to be ketotic; however, a documented level was not provided. At 2 years of age, while holidaying in Tenerife, he had another vomiting illness and became apathetic, but responded to antiemetics. At 3.5 years of age, an occipital skull fracture sustained from a fall off play equipment resulted in recurrent vomiting necessitating hospital admission. His blood pH was 7.20, bicarbonate 9.5 mmol/L, and BE −15.5 mmol/L. He responded well after the commence-

ment of an intravenous glucose infusion. Neuroimaging did not reveal any intracranial hemorrhages or cerebral concussions. He was discharged soon thereafter.

A conclusive diagnosis for the recurrent ketoacidosis had not been reached. It was suggested that he may outgrow the ketotic episodes and was advised to have sweetened drinks when unwell. He was subsequently discharged from the pediatric clinic, and the family migrated to Australia shortly thereafter.

Investigations performed on both well boys aged 5.5 and 12 years included plasma glucose, amino acids, free carnitine, acylcarnitines, 3-hydroxy-*n*-butyrate, free fatty acids, urate, blood gas, and liver function tests which were all normal. The crisis bloodspot collected from the index patient in Croatia was analyzed, but did not show any abnormalities; of specific relevance, the C5:1 was 0.02 μmol/L (normal range <0.04) and C5OH was 0.30 μmol/L (normal range <0.44). The levels of 2M3HB, 2MeAA, and TG were all undetectable in noncrisis urine samples from both boys.

The index patient has subsequently had a few mild episodes of ketonuria when unwell or if he has exerted himself during strenuous activities. He has, however, responded well with early initiation of increased caloric intake using glucose polymers, mild protein restriction during unwell periods, and early use of antiemetics when nonobstructive vomiting is a predominant feature.

Interestingly, the initially suspected resolution of ketotic episodes that the parents had reported in the elder half-brother was proven otherwise when suggested home monitoring displayed intermittent mild ketonuria when unwell. His mother had previously monitored his urine ketones when he was younger, but ceased over the last couple of years.

The other family members including the boys' biological fathers and their mother were all reported to be healthy. The proband's almost 15-year-old half-sister had poor weight gain in early childhood but caught up later and was otherwise well. The absence of relevant symptomology in the mother and half-sister was particularly interesting as they too carried the same mutation.

Material and Methods

Enzyme Activity Assays

Assays for acetoacetyl-CoA thiolases (with MAT being the only such enzyme activated by potassium ions) and for SCOT were performed as described, based on spectrophotometric monitoring of conversion of the substrate acetoacetyl-CoA spectrophotometrically at 303 nm (Fukao et al. 1996; Sakazaki et al. 1995).

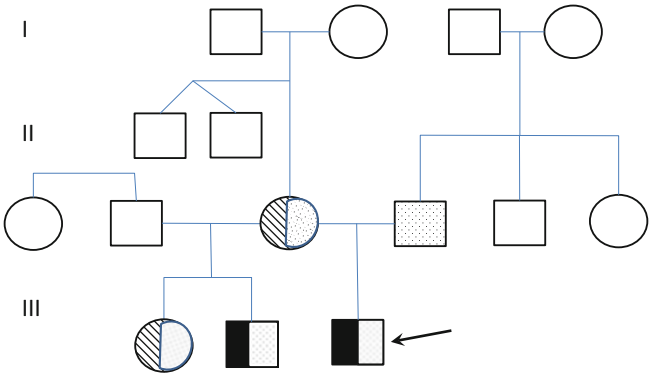

Fig. 1 Pedigree. The index patient is indicated with an *arrow*. *Black and hatched black-and-white fills* indicate a monoallelic c.982C>T (p.Arg328Ter) mutation in the *SLC16A1* gene of symptomatic (*full black*) and asymptomatic (*hatched*) family members, respectively (*Grey fills* indicate the investigated individuals)

Molecular Testing

Molecular genetic investigations were performed by PCR and sequence analysis of genomic DNA. We tested all exons of the *ACAT1* gene of the index patient plus flanking intronic regions. Reference sequence for the *ACAT1* gene was ENSG00000075239. Reference sequence for the *ACAT1* cDNA was NM_000019.3. We tested all four coding exons of the *SLC16A1* gene plus flanking noncoding sequences in the parents and children indicated in the pedigree (Fig. 1). Reference sequence for the *SLC16A1* gene was ENSG00000155380; reference sequence for the *SLC16A1* cDNA was NM_003051.3.

Immunoblot Analysis

In Western blot analysis, three fibroblast samples of the index patient (different passages) were analyzed on one or two gels each, and the mean value ± standard deviation of intensity ratios was calculated from the (mean) value obtained for each of the three samples. In parallel to the patient samples, 18 control samples were investigated. They comprised cells of individuals with various confirmed metabolic disorders. Samples of patients with a suspected defect in ketone body utilization were only included as controls if such a defect had been confirmed ($n = 1$ SCOT deficiency; $n = 2$ MAT deficiency), but not if any suspicion for an undiagnosed defect on ketone body utilization was still pending. One of the 18 controls was analyzed on all three gels; in that case, the mean values were used for the calculation.

Fibroblasts were homogenized in RIPA buffer (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma).

Table 1 Activities of enzymes involved in ketolysis were assessed in fibroblast homogenates in three different series and are given in nmol min^{-1} mg^{-1} protein

Proband and controls	Thiolase activity (substrate acetoacetyl-CoA) without K^+	Thiolase activity (substrate acetoacetyl-CoA) with K^+	Ratio of thiolase activities with/without K^+	Succinyl-CoA: transferase (SCOT) activity	Ratio of activities of SCOT and thiolase with K^+
Proband ($n = 3$)	2.10 ± 1.06	3.55 ± 2.44	1.59 ± 0.29	2.63 ± 0.42	0.96 ± 0.47
Positive control SCOT deficient ($n = 3$)	5.55 ± 0.05	12.3 ± 1.61	2.27 ± 0.22	Not detectable/n.d.	Not detectable/n.d.
Positive control MAT deficient ($n = 3$)	2.46 ± 1.27	2.38 ± 0.61	0.96 ± 0.13	3.04 ± 1.29	1.36 ± 0.70
Negative control A ($n = 3$)	3.31 ± 1.28	7.47 ± 2.78	2.26 ± 0.26	3.68 ± 0.60	0.58 ± 0.35
Negative control B ($n = 3$)	7.09 ± 1.11	13.3 ± 0.60	1.90 ± 0.29	6.13 ± 4.02	0.46 ± 0.29
Negative control C ($n = 2$)	3.46/3.51	8.53/5.01	2.47/1.43	6.01/5.15	0.70/1.03

Cell homogenates of the patient and of controls were analyzed in parallel. For $n = 3$, the results are displayed as mean value ± standard deviation; for one control ($n = 2$ only), individual values of both series are given

Aliquots of 30 µg of total protein were separated on 4–20% gradient Mini Protean TGX Gels (Biorad) and transferred onto PVDF membranes by semidry blotting using the Trans Blot Turbo system (Biorad). Membranes were blocked with 4% nonfat dry milk in TBS/0.05% Tween 20 (TTBS) for 1 h at room temperature and then incubated overnight at 4°C with affinity purified rabbit polyclonal antibody against MCT1 (Millipore, AB3538P) diluted 1:1,000 in 5% nonfat dry milk/TTBS. As a loading control, the membranes were stripped and reprobed with a polyclonal antibody against GAPDH (GeneTex, #GTX100118) diluted 1:10,000 in 5% nonfat dry milk/TTBS. After incubation with a horseradish-peroxidase-conjugated secondary antibody (GE Healthcare, NA934V) diluted 1:10,000 in 5% nonfat dry milk/TTBS for 1 h at room temperature, the antigen-antibody complexes were visualized by use of Super Signal West Femto Maximum Sensitivity Substrate (Pierce, #34094) for MCT1 detection and ECL (GE Healthcare, RPN2106) for GAPDH detection in an imaging apparatus (My ECL Imager, Fisher Scientific). Ratios of signal intensities of the MCT1 and GAPDH bands were determined with the program Image J (http://rsbweb.nih.goc/ij/download.html).

Results

MAT and SCOT enzyme activities were assessed in fibroblast homogenates (Table 1). MAT activity was considered normal, since thiolase activity was clearly increased if potassium ions were added, thus reflecting the results in negative control samples, while the positive control for MAT deficiency showed no such increase. SCOT activity was also normal. Since small signals of isoleucine metabolites had been noted in the urinary organic acids analyzed during that metabolic decompensation which prompted the metabolic work-up, a special effort was made to exclude MAT deficiency. Although MAT deficiency would be expected to be identified by fibroblast analysis, the focus on potential MAT deficiency was due to the fact that metabolite abnormalities may be subtle (Fukao et al. 2011). Therefore, mutation analysis was added by Sanger sequencing of the *ACAT1* gene of the patient, but also revealed no abnormality. Thus, it was concluded that neither MAT nor SCOT deficiency was the cause for the metabolic decompensations in the index patient. The small signals of urinary isoleucine metabolites which were not identified in any other urine samples of the patient and his brother and were not reflected by abnormalities in the acylcarnitine pattern during the crisis very likely represent nonspecific changes during a heavy metabolic crisis, as it is especially known for 2M3HB.

Sanger sequencing of the *SLC16A1* gene was performed in the index patient and his parents and half-siblings. The mother and all children were found to be heterozygous for the c.982C>T mutation which is predicted to result in a premature stop of protein synthesis (p.Arg328Ter) (Fig. 1, Table 1). This was not identified in the DNA of the father of the index case. In homozygous form, this mutation has already been reported by van Hasselt et al. (2014) in the ketoacidotic son of consanguineous Turkish parents. Since c.982C>T was identified both in symptomatic and asymptomatic individuals, we also studied the distribution of single nucleotide polymorphisms (SNPs) of the *SLC16A1*

Table 2 Sequence variations identified in the *SLC16A1* gene of the proband and his family members

Proband and relatives	c.982C>T, R328X (truncating mutation)	c.362-14_362-11delTATT (rs149491709); MAF = 0.1230	c.362-18_-11delTATTTATT (rs199817200); MAF = not available	c.362-21A>C (rs201021807); MAF = not available	c.1470T>A, D490E (rs1049434); MAF = 0.3233
Index	+/–	+/–	–/–	+/–	–/–
Mother	+/–	+/+	–/–	+/–	+/–
Father	–/–	+/–	–/–	+/–	+/–
Half-brother	+/–	+/–	+/–	+/–	+/–
Half-sister	+/–	+/+	–/–	+/+	+/+

"+" indicates the presence of the sequence variant on one allele, "–" its absence. Codes (rs. . .) and global minor allele frequency (MAF) refer to the database of single nucleotide polymorphisms (SNPs) and multiple small-scale variations available at http://www.ncbi.nlm.nih.gov/snp/

gene among the five individuals to reveal a possible contribution of one of these SNPs to disease manifestation (Table 2).

The immunoblot analysis of the three fibroblast homogenate samples of the index patient did not yield a relative decrease of the MCT1/GAPDH signal ratio compared with control fibroblasts ($n = 18$); mean values ± standard deviations were 0.70 ± 0.15 for the patient cells and 0.49 ± 0.19 for the controls.

Discussion

Our investigations on a family with affected half-siblings further support that even a single heterozygous mutation in the *SLC16A1* gene can lead to clinical symptoms of MCT1 deficiency (OMIM 616095). In their study, van Hasselt et al. (2014) had confirmed heterozygosity of mutations by ruling out exon-sized deletions and confirmed biallelic expression by cDNA sequencing. In our family, the identification of only the maternal mutation in both symptomatic boys with different fathers further supports that a monoallelic mutation in the *SLC16A1* gene can be the only relevant change in this gene. The presence of the mutation in the asymptomatic mother and the patients' (half) sister underlines that additional triggers are needed for the development of ketoacidotic episodes. The study of SNPs in the *SLC16A1* gene of the five individuals provided no strong evidence for an association of any of the analyzed SNPs with the clinical phenotype, although one SNP (c.362-14_362-11delTATT; rs149491709) is present in heterozygous form in the affected half-brothers, but in homozygous form in the mother and her daughter. However, this is not only a frequent SNP (MAF = 0.123) but also localized in an intron and unlikely to result in functional interference with the truncating mutation c.982C>T (p.Arg328Ter).

The relevance of polymorphisms in the *SLC16A1* gene has recently been highlighted by Fei et al. (2015) who have shown that in genomic DNA obtained from blood, the SNP rs1049434 was significantly associated with the death risk of colorectal cancer patients. However, in our family, there was no consistent difference regarding this SNP if the symptomatic boys were compared with their nonsymptomatic relatives.

It is noteworthy that our immunoblot analyses, for which we used the same polyclonal antibody against MCT1 protein as van Hasselt et al. (2014), did not yield a decreased amount of MCT1/GAPDH ratio in the index patient, who carries p.Arg328Ter mutation. Van Hasselt et al. (2014) reported a significantly reduced relative level of MCT1 protein in fibroblasts from patients with other heterozygous truncating mutations. However, they have studied fibroblasts with the p.Arg328Ter mutation in homozygous form only and showed a decreased ratio between MCT1 and their reference protein tubulin. The discrepancy is surprising, so far it is not clear whether it reflects a functional difference between heterozygosity for the p.Arg328Ter mutation and that for other truncating mutations in the *SLC16A1* gene, differences in the sensitivity of the immunoblots performed in different laboratories or has a yet undefined cause.

A variety of causes of ketoacidoses are known (for review, see Sass 2012). Thus far, however ketoacidosis in subjects without associated hypoglycemia, with normal blood lactate, and in the absence of any specific metabolic markers in blood or urine analysis has usually prompted diagnostic work-up for SCOT deficiency. MCT1 deficiency should be considered in the differential diagnosis. Awareness of those diseases is crucial for taking early preventive

measures and thus minimizing the damaging effects of ketoacidotic episodes. In case of MCT1 deficient patients with some residual activity, as expected in heterozygotes, one may speculate, whether treatment with methionine precursor DL-2-hydroxy-(4-methylthio)butanoic acid (HMTBA) might have a supporting effect. Further studies should be carried out to investigate whether HMTBA, which is widely used as a supplemental methionine source in animals, will enhance MCT1 expression in vivo, after this has successfully been demonstrated in vitro with immortalized human epithelial colorectal adenocarcinoma cells (Caco-2) (Martín-Venegas et al. 2012).

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One-Sentence Take-Home Message

MCT1 deficiency is an only recently described cause of recurrent ketoacidosis with clinical manifestation observed both in individuals with bi- or monoallelic mutations.

Compliance with Ethics Guidelines

Conflict of Interest

Shanti Balasubramaniam, Barry Lewis, Lawrence Greed, David Meili, Annegret Flier, Raina Yamamoto, Karmen Bilić, Claudia Till, and Jörn Oliver Sass declare have no conflict of interest.

Informed Consent and 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.

The investigated family has agreed to the publication and provided informed consent. This article does not contain any studies with animal subjects performed by the any of the authors.

Details of the Contributions of Individual Authors

SB was the physician in charge of the family. BL, LG, DM, AF, RY, KB, CT, and JOS performed/supervised/interpreted laboratory investigations. SB and JOS have drafted the manuscript. All authors have read/critically revised the manuscript.

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RESEARCH REPORT

Spectrum of Mutations in 60 Saudi Patients with Mut Methylmalonic Acidemia

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Abstract Defects in the human gene encoding methylmalonyl-CoA mutase enzyme (MCM) give rise to a rare autosomal recessive inherited disorder of propionate metabolism termed mut methylmalonic acidemia (MMA). Patients with mut MMA have been divided into two subgroups: mut^0 with complete loss of MCM activity and mut^- with residual activity in the presence of adenosylcobalamin (AdoCbl). The disease typically presents in the first weeks or months of life and is clinically characterized by recurrent vomiting, metabolic acidosis, hyperammonemia, lethargy, poor feeding, failure to thrive and neurological deficit. To better elucidate the spectrum of mutations causing mut MMA in Saudi patients, we screened a cohort of 60 Saudi patients affected by either forms of the disease for mutations in the *MUT* gene. A total of 13 different mutations, including seven previously reported missense changes and six novel mutations, were detected in a homozygous state except for two compound heterozygous cases. The six novel mutations identified herein consist of three nonsense, two missense and one frameshift, distributed throughout the whole protein. This study describes for the first time the clinical and mutational spectrum of mut MMA in Saudi Arabian patients.

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Introduction

Methylmalonic acidemia (MMA, OMIM 251000) is a common inborn error of organic acid metabolism occurring with a worldwide incidence rate ranging between 1:50,000 and 1:100,000 (Coulombe et al. 1981; Lemieux et al. 1988; Sniderman et al. 1999; Chace et al. 2001; Shigematsu et al. 2002; Sakamoto et al. 2007) and is inherited in an autosomal recessive manner (Matsui et al. 1983; Fenton 1995; Fenton et al. 2001). This disorder is caused by genetic defects in *MUT*, the gene encoding for L-methylmalonyl CoA mutase (MCM, EC 5.4.99.2), which catalyses the

conversion of L-methylmalonyl-CoA to succinyl-CoA utilizing adenosylcobalamin (AdoCbl) as a co-factor. Unconverted methylmalonyl-CoA is subsequently hydrolyzed to free coenzyme A (CoA) and methylmalonic acid (MMA), leading to accumulation of MMA in tissue and body fluids of affected individuals (Kovachy et al. 1983; Fenton et al. 2001). The exact incidence of MMA among live births in Saudi Arabia is not known; however, newborn screening results suggest that 1 in every 12,178 live newborns may be affected with this disease of which the majority are due to mutase deficiency (unpublished data). This is considered high when compared to the worldwide frequency, but it is not surprising due to the high rate of consanguineous marriages in Saudi Arabia.

Patients harbouring defects in the *MUT* gene have been distinguished by two biochemical criteria. Mutants with residual mutase activity in cell homogenates under saturating AdoCbl conditions, and whose ability to incorporate [1-14C] propionate is responsive to hydroxocobalamin supplementation of the culture medium, are designated as mut-, whereas those with no residual activity and no response of propionate incorporation to hydroxocobalamin are designated as mut^0 (Willard and Rosenberg 1980; Thoma and Leadlay 1996; Fowler et al. 2008). Mut^0 patients manifest as early as the neonatal period with poor feeding, vomiting, lethargy, hypotonia, altered level of consciousness, life-threatening metabolic ketoacidosis and moderate to severe hyperammonemia. If patients are not treated early and aggressively, the disease progresses to coma, neurological damage especially involving the basal ganglia and death in some cases (Lempp et al. 2007). Mut^- patients have a milder phenotype and present within the first 1–2 years of life (Martinez et al. 2005). Accumulation of MMA is associated with dysfunction of the mitochondrial respiratory chain reaction characterized by reduced ATP production and increased oxidative stress (Matsui et al. 1983; Fenton 1995). Evidence of respiratory chain impairment and/or oxidative stress was reported in MMA patients (Chandler et al. 2009; Ribas et al. 2012). Several complications have been described in long-term survival patients including neurodevelopmental delay, basal ganglia abnormalities, progressive renal failure, recurrent pancreatitis, recurrent bone marrow suppression and cardiomyopathy (Baumgarter and Viardot 1995; Nicolaides et al. 1998; Horster et al. 2007).

The human *MUT* gene was mapped to chromosome 6, consisting of 13 exons and spanning over 35 kb (Nham et al. 1990). The nuclear-encoded MUT is synthesized as a 750 amino acid long cytoplasmic precursor, bearing a 32 amino acid mitochondrial leader sequence cleavable upon transport into the mitochondria, where it homodimerizes with another cleaved precursor forming the mature enzyme. The human MUT primary structure has two major domains, as revealed by X-ray crystal structure and homology modelling studies, connected via a small interdomain linker: the N-terminal $(\beta/\alpha)_8$ barrel domain accommodating the substrate binding site and the C-terminal AdoCbl-binding domain, with the active site residing at the interface between these domains (Thoma and Leadlay 1996; Froese et al. 2010).

To date, 243 pathogenic mutations have been identified in the human *MUT* gene in various populations (HGMD®: http://www.hgmd.cf.ac.uk/ac/index.php). This study is the first to report a total of 14 mutations, 8 of which are novel in a cohort of 60 patients with mut MMA in Saudi Arabia. Three are believed to be founder mutations as all affected families originate from specific geographical locations in Syria and Saudi Arabia.

Materials and Methods

Patients and MMA Diagnosis

This study includes samples from 60 patients (from 56 different nuclear families) with mut MMA. Patients were ascertained through three sources: (1) index cases where the diagnosis was established based on the clinical presentation, abnormal acylcarnitine profile and urine organic acids, (2) siblings of index cases who were born and tested positive for MMA and (3) state-based newborn screening. Patients were recruited as part of an institutionally approved research project (RAC# 2020 011); informed consent was obtained, which adhered to the institutional guidelines and to the tenets of the Helsinki Declaration of 1975, as revised in 2000. Subsequently as diagnostic molecular testing was established for *MUT* gene locally, patients with clinical and biochemical diagnosis of MMA had routine genetic testing for mutation identification. Mutations in the *MUT* gene were identified in all patients.

Mutation Detection

Genomic DNA from all affected individuals was extracted from whole blood using the conventional salting-out method. Intronic primers were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/) to flank each of the 12 coding exons of *MUT* (primer sequences and conditions are available on request). PCR reactions for all patients and subsequently normal control samples were typically performed in a 25 μL reaction volume containing standard reagents and 10 ng of genomic DNA. Sequencing reactions were desalted and unincorporated nucleotides removed using ethanol precipitation and re-suspended in a formamide EDTA solution for injection on a MegaBACE 1000 DNA Analysis System (Molecular Dynamics; Sunnyvale, CA, USA). Purified PCR products covering the entire

coding region of *MUT* (accession no. ENSG00000146085) as identified on Ensembl (http://www.ensembl.org/index.html) were directly sequenced with the dideoxy chain-termination method using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. Sequence analysis was performed using the SeqMan 6.1 module of the Lasergene (DNA Star Inc. WI, USA) software package and then compared to the reference GenBank sequence (accession no. # NM_000255.3). Numbering commenced with the A of the ATG initiation codon as +1.

Results

The identified mutations and their associated phenotypes are summarized in Table 1. All patients except one came from consanguineous marriage. Where genomic DNA from parental and unaffected siblings was available, the molecular analysis was performed as described. All parents were heterozygous carriers and unaffected siblings were heterozygous carriers or wild-type normal. Briefly, the majority of index cases presented had an early neonatal presentation. Patients with Y110C mutation had a variable age of presentation ranging from neonatal to early childhood. Complications observed in childhood included growth delay, neurodevelopmental delay, chronic renal impairment and recurrent pancreatitis. Table 1 summarizes the identified mutations and their incidence.

In the current study we investigated the molecular background of mut MMA in a cohort of 60 patients, whereby the entire coding region and intron-exon boundaries of *MUT* were directly sequenced in both the forward and reverse direction using genomic DNA. In total, we have identified 13 different mutations in these 60 patients. Six of these mutations were novel, while the remaining seven have been previously described. Novel mutations included two missense mutations (c.329A>C resulting in p.Y110C and c.2075T>C resulting in p.L692P), three nonsense mutations (c.88C>T resulting in p.Q30*, c.109C>T resulting in p.Q37* and c.2200C>T resulting in p.Q734*) and one frameshift mutation (c.810_811delGGinsA p.A271LfsX11). None of the six novel mutations were reported in the locus-specific mutation databases such as the Human Gene Mutation Database Professional 2013 (http://www.hgmd.org) and the National Center for Biotechnology Information (NCBI) SNP database (http://www.ncbi.nlm.nih.gov/SNP), nor were they present in 300 chromosomes from ethnically matched normal controls suggesting that these variants are not population-based polymorphisms. Moreover, in silico analysis performed using a suite of bioinformatics tools including PolyPhen (http://genetics.bwh.harvard.edu/pph2/) and MutationTaster (http://www.mutationtaster.org/) revealed that all the novel missense mutations are predicted to probably be disease-causing further confirming pathogenicity. However, expression studies are required to validate this notion. Furthermore, alignment of orthologous protein sequences from human, monkey, mouse, dog and zebrafish obtained from Ensembl or UCSC Genome browsers (http://genome.ucsc.edu/cgi-bin/hgGateway) using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) demonstrated a strong cross-species conservation of the novel missense mutations (p.Y110C and p.L692P) (Fig. 1). Seven different previously reported missense mutations were also identified in this study (p.R93H, p.R108C, p.F174S, p.G215S, p.Y364S, p.T387I and p.R694W). All the mutations were homozygous with an exception of two compound heterozygote cases harbouring the known p.R108C in combination with the novel p.Q37* mutation.

Among the 60 patients studied, p.R93H was the most prevalent mutation accounting for 35% of the cases. Interestingly, the next most common mutation was the novel p.Y110C missense mutation present in 25% of the cases. Q37* and Q734* mutations were equally observed.

Discussion

This study describes the spectrum of mutations in the *MUT* gene among 60 patients diagnosed with mut MMA in Saudi Arabia based on abnormal acylcarnitine profile and urine organic acids. It is important to note that there was a further patient in whom the *MUT* gene was sequenced and clear from mutation upon analysis. A deleterious mutation was subsequently identified in the *MMAA* gene (NM_172250) for this single case. Patients with methylmalonic aciduria types MMAB and MMADHC have not been found. All of the mutations reported here occurred in a homozygous state with the exception of two cases being compound heterozygous for p.R108C in association with the novel p.Q37* mutation, reflecting the consanguineous nature of the Saudi population. Considering the wide spectrum of mutations (nonsense, missense and frameshift), their distribution is heterogeneous with the majority clustering in the substrate-binding $(\beta/\alpha)_8$ barrel and the AdoCbl-binding domains. Six different previously reported missense mutations (p.R93H, p.R108C, p.F174S, p.G215S, p.Y364S and p.R694W) have been detected in our cohort of patients. Four of which (p.R93H, p.R108C, p.F174S and p.G215S) were located within the substrate-binding $(\beta/\alpha)_8$ barrel domain and one (p.R694W) residing in the AdoCbl-binding domain. The R93H mutation, detected in 35% of the cases, was first identified in a homozygous state in a cell line derived from a Caucasian patient with mut^0 MMA (Raff et al. 1991). Studies have revealed that p.R93H-expressing cells when

Table 1 Identified *MUT* mutations and phenotypes of the *mut* MMA patients

Mutation	Genotype	Amino acid change	Domain	Number of patients with the genotype	Age of onset	Renal impairment	Growth delay	Pancreatitis	References
c.88C>T	Homozygous	**p.Q30***	ML	1	Neonatal	Not observed[a]	Yes	Not observed	This study
c.109C>T	Homozygous	**p.Q37***	NT	4	Neonatal	Present	Yes	Observed	This study
c.278 G>A	Homozygous	p.R93H	$(\beta/\alpha)_8$	21	Neonatal	Present	Yes	Observed	Raff et al. (1991)
c.322C>T	Homozygous	p.R108C	$(\beta/\alpha)_8$	1	Neonatal[b]	Unknown	Unknown	Unknown	Worgan et al. (2006)
c.329 A>G	Homozygous	**p.Y110C**	$(\beta/\alpha)_8$	15	Neonatal (12)/ infantile (3)	Present	Variable	Observed	This study
c.521 T>C	Homozygous	p.F174S	$(\beta/\alpha)_8$	1	Neonatal	Not observed[a]	Yes	Not observed[a]	Fuchshuber et al. (2000)
c.643 G>A	Homozygous	p.G215S	$(\beta/\alpha)_8$	1	Neonatal	Not observed[a]	Yes	Not observed[a]	Acquaviva et al. (2005)
c.810_811delGGinsA	Homozygous	**p.A271LfsX11**	$(\beta/\alpha)_8$	2	Neonatal	Present	Yes	Observed	This study
c.1091 A>C	Homozygous	p.Y364S	$(\beta/\alpha)_8$	1	Neonatal	Present	Yes	Observed	Gradinger et al. (2007)
c.1160C>T	Homozygous	p.T387I	$(\beta/\alpha)_8$	1	Neonatal	Not observed[c]	Yes	Not observed[c]	Dundar et al. (2012)
c.2075 T>C	Homozygous	**p.L692P**	AdoCbl	1	Neonatal	Yes	Yes	Not observed[a]	This study
c.2080C>T	Homozygous	p.R694W	AdoCbl	5	Neonatal	Not observed[a]	Yes	Observed	Lempp et al. (2007)
c.2200C>T	Homozygous	**p.Q734***	AdoCbl	4	Neonatal	Present	Yes	Observed	This study
c.88C>T/c.322C>T	Compound heterozygous	**p.Q37***/p.R108C	NT/$(\beta/\alpha)_8$	2	Neonatal	Present	Yes	Observed	This study/ Worgan et al. (2006)

Novel mutations are in bold

ML mitochondrial leader sequence, *NT* N-terminal extended segment, $(\beta/\alpha)_8$ substrate-binding $(\beta/\alpha)_8$ barrel, *AdoCbl* AdoCbl-binding domain

[a] Patients are still young (complications may develop later)

[b] Unable to obtain data as patient not followed at our institute

[c] Long follow-up over 19 years

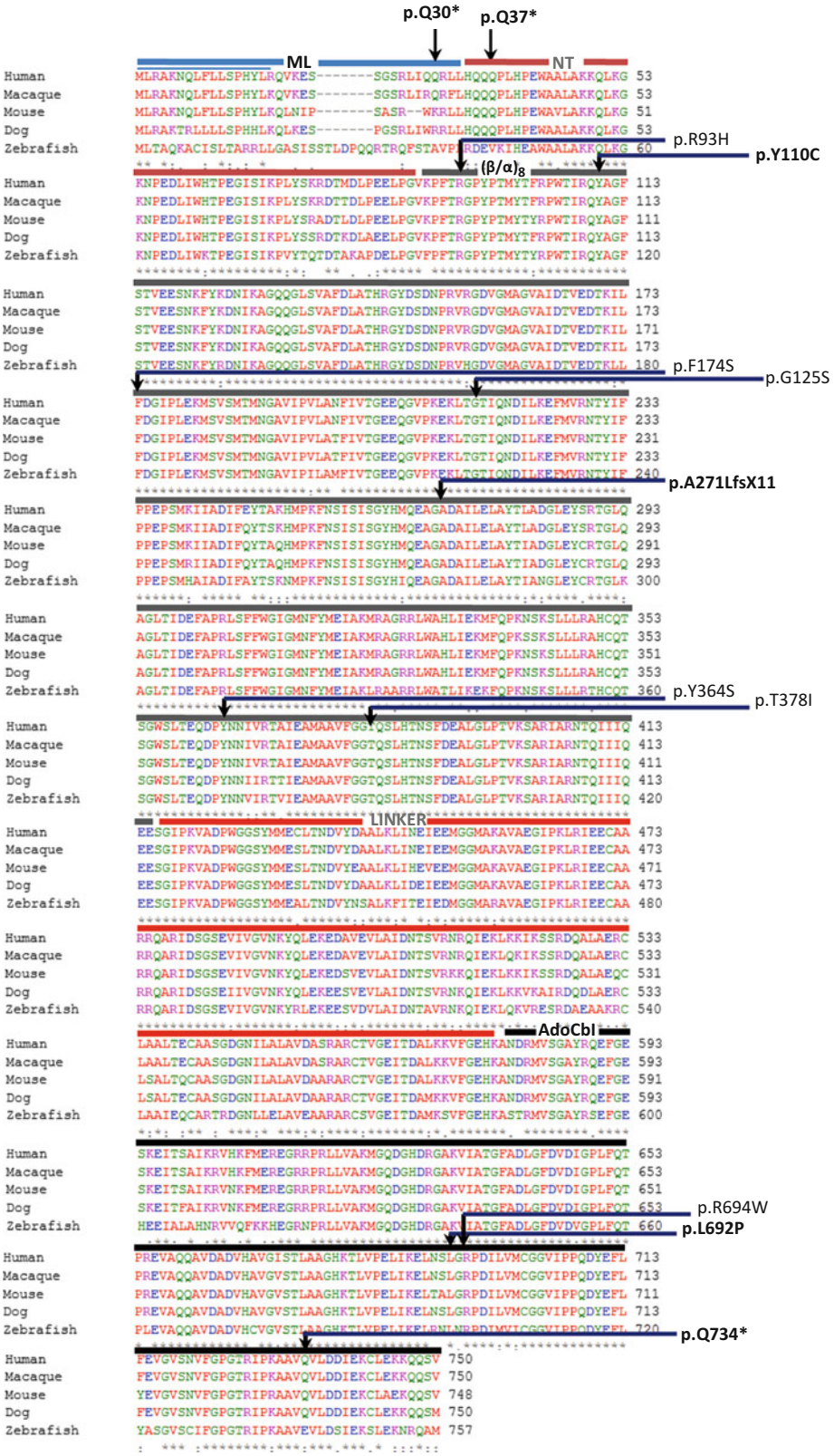

Fig. 1 Cross-species conservation of MUT protein between human (NP_000246.2), macaque (XP_005552835.1), mouse (AAH19175.1), dog (XP_532164.3) and zebrafish (AAI39861.1). ML, mitochondrial leader sequence; NT, N-terminal extended segment; $(\beta/\alpha)_8$, substrate-binding $(\beta/\alpha)_8$ barrel; Linker, interdomain linker region; AdoCbl, AdoCbl-binding domain. Novel mutations are in bold, (*) denotes amino acids identical in all sequences, (:) denotes conserved substitutions and (.) denotes semi-conserved substitutions. Alignment performed by ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/) using protein sequences from the National Centre of Biotechnology (NCBI)

co-transfected with clones bearing one of the other MUT mutations such as p.R694W, p.G648D and p.G626C or fused with cells expressing either of these mutations have the capacity to produce significant levels of enzyme activity as a result of interallelic complementation (Crane and Ledley 1994; Qureshi et al. 1994). Among Japanese patients, the p.R93H mutation was recurrent in compound heterozygous patients associated with other mutations (Kobayashi et al. 2006). Unlike Japanese patients, all Saudi patients harbouring the p.R93H in this study were homozygous for the mutation. It is likely that this is a founder mutation as all affected patients came from unrelated families from the southern region of Saudi Arabia. The mutation was observed to be associated with a severe early-onset phenotype. All patients uniformly had growth retardation, progressive renal disease, cognitive delay and recurrent pancreatitis. The next most common mutation was the novel p.Y110C missense mutation present in 25% of the cases. Again this mutation is expected to be a founder as all families affected with mutation had the same tribal orientation. Interestingly, this mutation has a more variable phenotype with age of onset ranging from neonatal to early childhood. Growth delay was also variable with some patients having normal growth and others with significant growth delay. In addition, cognitive function has also ranged from normal to moderate cognitive delay. Next were p.Q37* and p.Q734* mutations and both were equally observed. The first one affected unrelated families with roots back to a specific region in Syria near Damascus, and the second was identified in two siblings from a specific tribe in addition to two more patients. As predicted these two mutations result in a severe early-onset disease as p.Q37* introduces a termination codon at the start of the NT extended segment. The consequence of such mutations was first described by Ledley et al., whereby a nonsense mutation at position 17 terminated translation from the original AUG and reinitiated translation at an in-frame AUG codon internal to the mature protein sequence producing immunoreactive truncated protein (Ledley et al. 1990). The truncated protein lacking leader peptide and a portion of the amino terminus of the mature apoenzyme remains in the cytoplasm and undergoes degradation (Fenton et al. 1987). Recently, more termination mutations have been identified: One (p.Q7*) was found in a European patient occurring early in the sequence (Acquaviva et al. 2005) and the other (p.Q31*), adjacent to the one reported in our study (p.Q30*), was detected in a Thai patient (Vatanavicharn et al. 2012). Both mutations were predicted to result in the absence of functional gene product. p.Q734* was found in the AdoCbl-binding domain causing the loss of 16 amino acid residues in the C-terminus. Although the substrate-binding domain and most of the AdoCbl-binding domain are intact, patients with the p.Q734* mutation exhibited a phenotype reminiscent of many mut^0 patients suggesting that the last 16 amino acid residues are indispensable for the enzyme function. This observation is in agreement with previous reports on patients carrying another stop codon (p.Q727*), located 7 amino acids upstream of p.Q734*, which have been diagnosed with mut^0 form of MMA (Kobayashi et al. 2006; Worgan et al. 2006; Dundar et al. 2012) supporting the notion that such mutations can be detrimental to enzyme function.

The third most recurrent known mutation in our population is p.R694W, identified in four patients. Patients affected with this genotype range from 2 to 11 years of age. So far they do not show any signs of renal involvement and have growth delay, and pancreatitis was observed once only in one patient. Therefore, this genotype might be associated with a milder phenotype. This is consistent with previous reports of this mutation (Janata et al. 1997; Acquaviva et al. 2005). The rest of the mutations were seen in single cases and the associated clinical findings are summarized in Table 1. As predicted, p.Q30* which introduces an early termination codon at position 30 (p.Q30*) within the mitochondrial leader sequence is associated with a severe phenotype.

One of the two novel missense mutations identified in the present study (p.Y110C) was mapped to the substrate-binding $(\beta/\alpha)_8$ barrel domain and the other (p.L692P) was mapped to AdoCbl-binding domain, both affecting highly conserved amino acids. The preservation of these amino acids along with the absence of these missense changes in normal controls makes it very likely that these mutations are pathologically significant.

The p.Y110C mutation lies within the 1st β-strand of the substrate-binding domain. This domain is thought to be responsible for the binding of the CoA ester substrate (Thoma and Leadlay 1996); therefore, the substitution of the aromatic amino acid (tyrosine 110) which points directly to the active site with an uncharged polar residue (cysteine) may substantially affect activity. The second missense mutation resulted in a non-conservative amino acid substitution (p.L692P) within the AdoCbl-binding domain would most likely suffer from a breaking of the secondary structure (alpha-helix) of which the leucine is part of.

Finally, one frameshift mutation (p. Ala271LeufsX11) was found in the substrate-binding $(\beta/\alpha)_8$ barrel domain resulting in a premature stop codon. Predictably, such mutations would abolish the enzyme activity via mechanisms involving nonsense-mediated mRNA decay or elimination of truncated proteins.

In conclusion, we have detected a total of 13 different mutations, six of which were novel mutations, including three nonsense mutations, two missense mutations and one frameshift mutation, in 60 Saudi patients, in addition to seven previously reported mutations. The diversity of *MUT*

gene mutations detected in our patients suggests the pleomorphic nature of this condition in the Saudi population. Homoallelic mutations are almost universally observed in our cohort, due to the extensively consanguineous nature of the Saudi population, negating clinical heterogeneity resulting from interallelic complementation. Our study summarizes the spectrum of mutations in the *MUT* gene in Saudi Arabia. It provides useful genotype phenotype correlation that will help in predicting clinical outcome and genetic counselling of families affected with this disease. Using the presented information, rapid molecular diagnosis can be established and preventative reproductive counselling such as prenatal diagnosis, preimplantation genetic diagnosis and carrier testing can be implemented.

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Compliance with Ethics Guidelines

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

FI, BAM, AM, MH and RA performed molecular genetic studies, analysis and interpretation. ZH, MO, HZ, ZR, AQ, EF, AA, FM, MF, WE, MS and MAS provided patient information, clinical diagnosis and samples and were involved in data interpretation. All authors were all involved in drafting and revising the article.

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RESEARCH REPORT

CoQ_{10} Deficiency Is Not a Common Finding in GLUT1 Deficiency Syndrome

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Abstract CoQ_{10} deficiency has been recently described in tissues of a patient with GLUT1 deficiency syndrome. Here, we investigated patients and mice with GLUT1 deficiency in order to determine whether low CoQ is a recurrent biochemical feature of this disorder, to justify CoQ_{10} supplementation as therapeutic option.

CoQ_{10} levels were investigated in plasma, white blood cells, and skin fibroblasts of 16 patients and healthy controls and in the brain, cerebellum, liver, kidney, muscle, and plasma of 4-month-old GLUT1 mutant and control mice.

CoQ_{10} levels in plasma did not show any difference compared with controls. Since most of the patients studied were on a ketogenic diet, which can alter CoQ_{10} content in plasma, we also analyzed white blood cells and cultured skin fibroblasts. Again, we found no differences. In mice, we found slightly reduced CoQ in the cerebellum, likely an epiphenomenon, and activity of the mitochondrial respiratory chain enzymes was normal.

Our data from GLUT1 deficiency patients and from GLUT1 model mice fail to support CoQ_{10} deficiency as a common finding in GLUT1 deficiency, suggesting that CoQ deficiency is not a direct biochemical consequence of defective glucose transport caused by molecular defects in the *SLC2A1* gene.

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Introduction

GLUT1 deficiency syndrome (GLUT1DS) (OMIM 606777) is an autosomal dominant genetic trait due to mutations in the glucose transporter *SLC2A1* gene. GLUT1DS was first recognized in two children with seizures and persistently low glucose in the cerebrospinal fluid (CSF) (De Vivo et al. 1991; Seidner et al. 1998).

Since GLUT1 is the glucose transporter in the blood–brain barrier, the key hallmark is a low CSF glucose concentration in the presence of normoglycemia, with a CSF/blood glucose ratio commonly less than 0.4.

The clinical phenotype is variable; the most severe presentation is characterized by infantile epilepsy, developmental delay, cognitive impairment, spasticity, ataxia, and dystonia; some patients may present with paroxysmal head and eye movements, without epilepsy. A wide spectrum of mutations has been identified, but no obvious genotype–phenotype correlation has been detected (Pearson et al. 2013). The ketogenic diet is the standard of care, providing ketones in partial lieu of glucose as the alternative fuel for brain energy metabolism (Alter et al. 2015).

Recently, Yubero and colleagues reported a 15-year-old girl with GLUT1 deficiency and reduction of CoQ_{10} levels in muscle, plasma, and skin fibroblasts (Yubero et al. 2014).

CoQ supplementation in the patient leads to a striking improvement of neurological phenotype, suggesting that

adjunctive treatment with CoQ_{10} may be wise when treating this disorder.

To determine if low levels of CoQ_{10} are a common biochemical finding in GLUT1 deficiency, we measured CoQ levels in fibroblasts, plasma, and white blood cells of several patients with GLUT1 deficiency and in affected and unaffected tissues of a model mice with GLUT1 haploinsufficiency ($GLUT1^{+/-}$), as previously described (Wang et al. 2006).

Patients and Methods

Sixteen patients were diagnosed according to the following clinical and laboratory criteria: seizures, microcephaly, developmental delay, and low CSF/blood glucose ratio (~0.33 ± 0.01). Diagnosis was confirmed by molecular genetic analysis in 11. Informed consent was obtained from the parents of children who participated in this study. Plasma was collected from 14 patients; white blood cells were collected from eight patients, separated from plasma using Ficoll®-Paque, according to manufacturer instructions. Fibroblasts from five patients and six age-matched controls were cultured for CoQ_{10} assessment as previously described (Lopez et al. 2006). Briefly, cells were grown in 15-cm-diameter culture plates until confluent. CoQ_{10} was extracted in a hexane–ethanol mixture. The lipid component of the extract was separated by high-performance liquid chromatography (HPLC) on a reverse Symmetry® C18 3.5-μm, 4.6 × 150-mm column (Waters), using a mobile phase consisting of methanol, ethanol, 2-propanol, acetic acid (500:470:15:15), and 50 mM sodium acetate at a flow rate of 0.8 mL/min. The electrochemical detector consisted of an ESA Coulochem II with the following setting: guard cell (upstream of the injector) at +900 mV and conditioning cell at −650 mV (downstream of the column), followed by the analytical cell at +450 mV. CoQ_{10} concentration was estimated by comparison of the peak area with those of standard solutions of known concentration. Four $GLUT1^{+/-}$ heterozygous mice and four wild-type mice, aged 4 months, were euthanized; the brain, cerebellum, liver, kidney, muscle, and plasma were collected for biochemical analyses. CoQ_9 and CoQ_{10} were extracted by mixing tissue extracts with 1-propanol, and measurement was performed as above. To measure activities of mitochondrial respiratory complexes, 40–70 mg tissue was homogenized in CPT medium (0.5 M Tris–HCl, 0.15 M KCl, pH 7.5) and centrifuged at 2,500*g* for 20 min at 4°C to obtain 10% homogenates. The supernatant was used for protein determination and enzymatic assays. Complex I and III (CI + III) activity was measured by observation of the reduction of cytochrome *c* (cyt *c*) at 550 nm. In brief, samples were incubated at 30°C in a medium containing 100 mM KH2PO4 (pH 7.5), 10 mM KCN, 2 mM NADH, and 1 mM cyt *c*. The increase of absorbance was observed for 1 min and for an additional 2 min after addition of rotenone (10 μg/mL). The residual activity in the presence of rotenone was subtracted from total activity. The results were expressed in nmol of reduced cyt *c*/min/g tissue. Complex II and III activity was measured by observation of the reduction of cytochrome *c* at 550 nm. Samples were incubated at 30°C in medium containing 100 mM KH2PO4 (pH 7.5), 30 mM succinate, 10 mM KCN, 2.5 μM rotenone KCN, and 1 mM cyt *c*, and increase of absorbance was observed for 2 min. The results were expressed in nmol of reduced cyt *c*/min/g tissue. Citrate synthase (CS) activity was measured following the reduction of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 1 M Tris–HCl (pH 7.5) at 412 nm (30°C) in the presence of sample, 10 mM acetyl-CoA, and 10 mM oxalacetic acid. The results of CI + III, CII + III, and CIV were normalized to CS activity and to protein.

To determine the level of oxidative damage in the cerebellum, we performed Western blot analysis of carbonyl group content using the OxyBlot kit (Chemicon, Millipore Corp, Billerica, MA, USA) following the manufacturer's instructions. Briefly, 15 μg of proteins were incubated with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazine (DNP) derivates. 2,4-Dinitrophenyl-derivatized proteins were separated on a 12% polyacrylamide gel and transferred to polyvinyl prolidone membranes for 30 min at 50 V in Mini *Trans*-Blot cell apparatus (Bio-Rad, Hercules, CA, USA). The blots were hybridized with rabbit anti-DNP antibody and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Chemicon, Millipore Corp, Billerica, MA, USA) and visualized by autoradiography using ECL substrate (Pierce, Rockford, IL, USA).

All animal studies were performed in accordance with protocols approved by the Columbia University Institutional Animal Care and Use Committee.

Mann–Whitney test was used to measure statistical significance ($p < 0.05$). Results are expressed as mean ± standard deviation.

Results

At the time of sample collection, clinically all patients (4 males and 12 females, mean age 15.4 ± 7.2 years) were symptomatic, and all but two were on a ketogenic diet (Table 1).

CoQ_{10} levels measured in plasma and white blood cells of seven patients showed no difference compared with controls (0.49 ± 0.06 vs. 0.41 ± 0.05 μg/mL; and 62.53 ± 20.97 vs. 68.05 ± 22.3 μg/mg prot, respectively) (Fig. 1a, b). We also assessed CoQ_{10} levels in cultured skin

Table 1 Clinical features of GLUT1DS patients

Patient no.	Gender	Age of onset (month)	Age (year)	Disease duration (year)	Ketogenic diet	Clinical features	Genetic defect	Tissue studied
1	M	9	3	2	Y	S, M, D	Neg	P, WBC
2	F	12	4	3	Y	S, D	c.209 C>A	P, WBC
3	F	72	18	12	Y	S, D	c.438A>C	P, WBC
4	F	84	13	6	N	S, D	Neg	P
5	F	9	16	15	N	S, D	c.436G>A	P
6	M	19	30	28	Y	S, M, D	c.377G>A	P, WBC
7	F	4	20	20	Y	S, M, D	Microdeletion *3,336 bp* (*3,269 from intron 2 and the first 67 bp from exon 3*)	P, WBC
8	F	3	19	19	Y	S, M, D	Microdeletion (*start*, *43119150*; *end*, *43177300*)	P, WBC
9	F	12	15	14	N	S, M	c.940G>A	P
10	M	3	2,5	2	Y	S, D	c.388G>C	P
11	F	30	14	12	N	S, M, D	c.276-1G>A	P
12	F	3	20	20	na	S, M, D	c.907delG	F
13	F	3	19	19	na	S, M, D	c.562_563insC	F
14	M	2	22	22	na	S, M, D	c.1383_1384insATCG	F
15	F	6	17	17	na	S, M, D	c.911_912insAC	F
16	F	1	14	14	na	S, M, D	c.791delG	F

P plasma, *WBC* white blood cells, *F* fibroblasts, *na* not available, *S* seizures, *M* microcephaly, *D* developmental delay

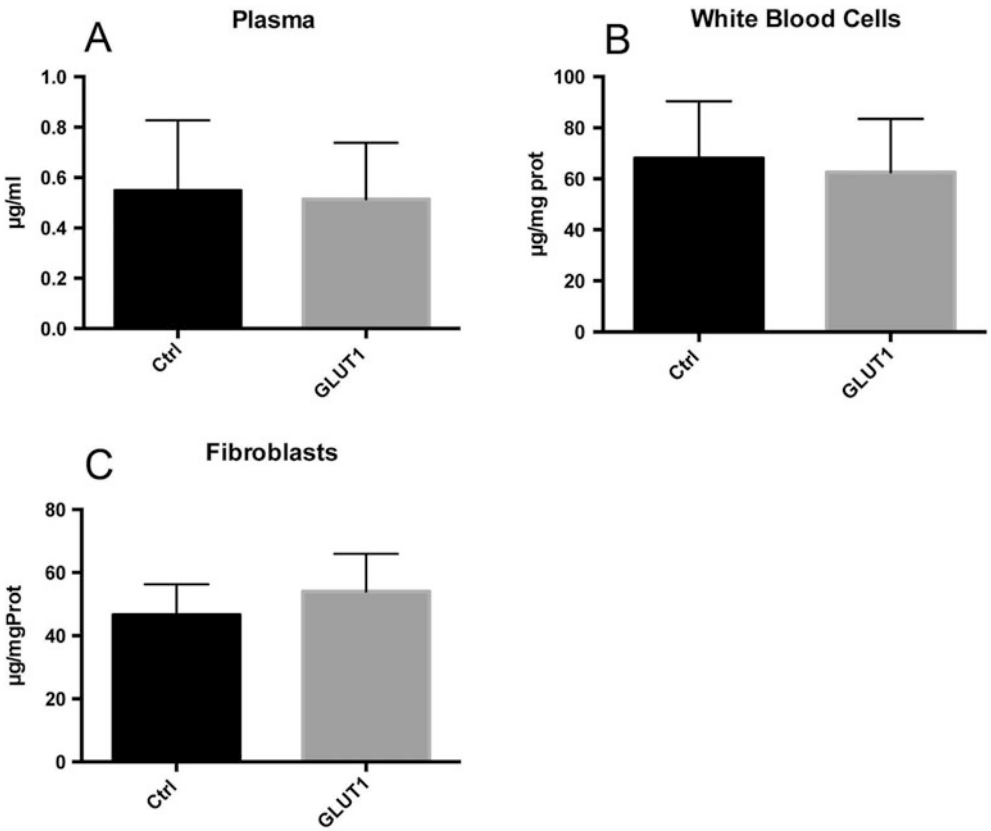

Fig. 1 CoQ_{10} measured in (**a**) plasma of controls (ctrl; $n = 9$) and patients (GLUT1; $n = 8$), (**b**) white blood cells of controls (ctrl; $n = 6$) and patients (GLUT1; $n = 6$), and (**c**) skin fibroblasts of controls (ctrl; $n = 6$) and patients (GLUT1; $n = 6$). Data are expressed as mean; *bars* represent standard deviation

fibroblasts from an additional six patients with GLUT1 deficiency and found no differences compared to 6 age-matched controls (54.05 ± 11.9 vs. 46.6 ± 9.7 μg/mg prot) (Fig. 1c).

CoQ_{10} deficiency has been shown to be tissue specific (Emmanuele et al. 2012). Thus, we measured CoQ levels in the brain, cerebellum, and skeletal muscle of GLUT1 mice. The liver and kidney were analyzed as non-affected tissues. CoQ_9, the most common ubiquinone in rodents, as well as CoQ_{10} levels, was measured in wild-type and heterozygous animals. CoQ_9 was normal in the brain (22.4 ± 2.6 vs. 22 ± 2.8 ng/mg tissue) (Fig. 2b), muscle (14.7 ± 4 vs. 17 ± 7 ng/mg tissue) (Fig. 2c), kidney (136.3 ± 3 vs. 136 ± 3 ng/mg tissue), and liver (85.7 ± 27.7 vs. 84 ± 26.5 ng/mg tissue). A very mild although statistically significant reduction of CoQ_9 and CoQ_{10} was present in the cerebellum (24.45 ± 0.6 vs. 23.65 ± 0.3 ng/mg tissue; $p < 0.05$) (Fig. 2a).

We did not observe any difference between $GLUT1^{+/-}$ and wild-type animals when CoQ levels were normalized to CS, an index of mitochondrial mass (data not shown).

CoQ transports electrons from NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) to cytochrome c reductase (complex III). Therefore, to assess the functional impact of CoQ levels on the mitochondrial electron transport, we measured the activity of complexes I + III and II + III in the cerebellum, normalized to proteins and CS, and we did not find any difference between $GLUT1^{+/-}$ and wild-type animals (data not shown). Quantitative analysis of the blots carried out using the ImageJ software did not show any difference in protein oxidation between mutant and control mouse cerebellums (77.09 ± 19.05 vs. 68.22 ± 15.53).

Discussion

GLUT1DS is a rare neurological disorder with about 400 patients now reported in the literature worldwide (Pearson et al. 2013). The treatment with a ketogenic diet is based on the fact that brain metabolic fuels are limited to glucose and ketones. Recently, Yubero and colleagues reported a patient with GLUT1 deficiency, who showed consistently increased levels of blood lactate and alanine (Yubero et al. 2014). Mitochondrial function was investigated, and CoQ_{10} was reduced in skeletal muscle, fibroblasts, and plasma.

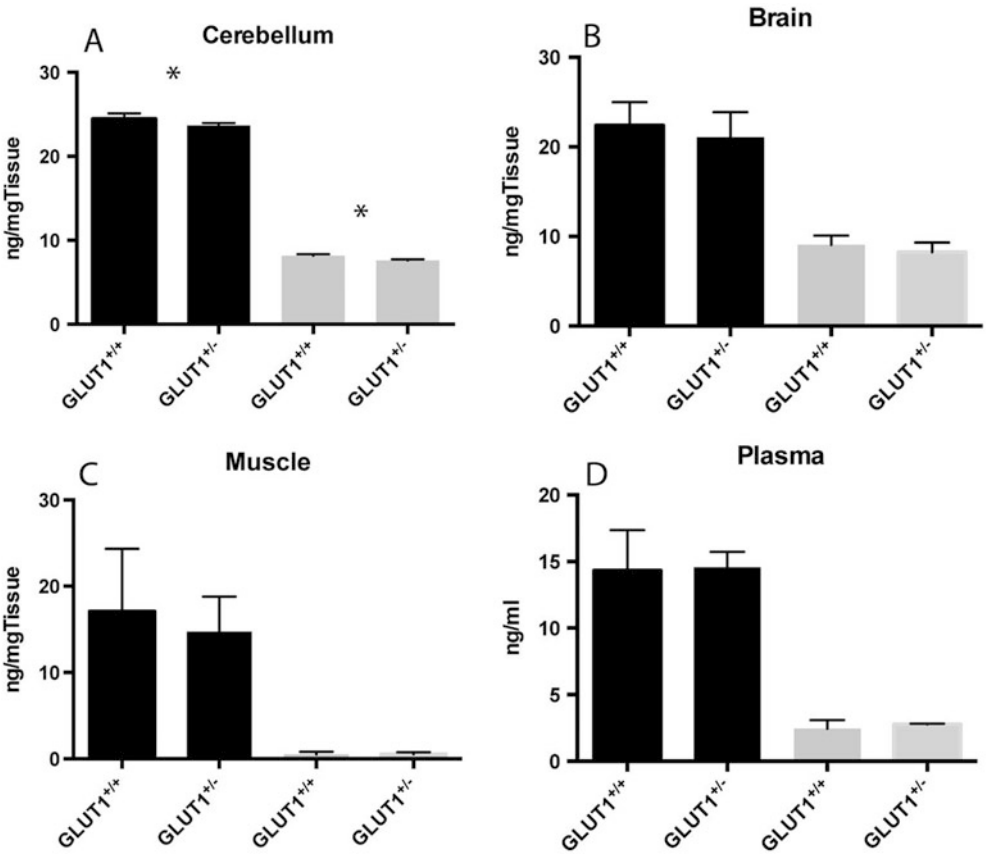

Fig. 2 CoQ_9 (*solid black*) and CoQ_{10} (*solid gray*) in mutant ($GLUT^{+/-}$; $n = 4$) and control ($GLUT^{+/+}$; $n = 4$) mice: (**a**) cerebellum, (**b**) brain, (**c**) muscle, (**d**) plasma. Data are expressed as mean; *error bars* represent standard deviation. *$p < 0.05$

Supplementation therapy with CoQ_{10} dramatically ameliorated some of the neurologic symptoms, especially cerebellar ataxia. Interestingly, CoQ_{10} deficiency has been observed in different conditions associated with cerebellar ataxia suggesting a specific susceptibility of this brain region to oxidative stress and/or mitochondrial bioenergetics impairment due to CoQ_{10} reduction (Emmanuele et al. 2012). GLUT1DS also is characterized clinically as an infantile-onset cerebellar phenotype, and the cerebellar region is unduly vulnerable as documented by brain imaging (Akman et al. 2015).

In order to assess if CoQ_{10} reduction is a common feature in GLUT1DS, we collected tissue samples from 16 patients with GLUT1 deficiency. Plasma CoQ_{10} levels, in seven patients, were comparable to controls. However, since plasma COQ_{10} levels can be influenced by diet (Emmanuele et al. 2012), we extended the studies to white blood cells (WBC) collected from peripheral blood. Again, the CoQ_{10} levels in WBC were not different from controls, congruent with the plasma data. We also studied 6 skin fibroblast cell lines derived from GLUT1 patients and found normal levels of CoQ_{10}.

As is often the case in mitochondrial diseases, including CoQ deficiency, plasma and skin fibroblast CoQ levels do not necessarily reflect the biochemical phenotype (Emmanuele et al. 2012). Therefore, we decided to study affected and unaffected organs in model mice with GLUT1 deficiency. In $GLUT1^{+/-}$ mice, CoQ levels were mildly reduced only in the cerebellum (~7%), while CoQ levels elsewhere in the brain, muscle, kidney, and liver were normal. The cerebellum, and other specific brain regions, is noticeably vulnerable to GLUT1 deficiency (Akman et al. 2015). Therefore, we think the slightly reduced CoQ levels in this brain region reflect the tissue pathology as an epiphenomenon. Reduced brain lipid synthesis has been shown in a different GLUT1 mouse model (Marin-Valencia et al. 2012) and also may account for reduced CoQ levels in the cerebellum. However, such mild deficiency can hardly alter mitochondrial energy metabolism, as confirmed by our observations of normal transport of electrons from complexes I and II to complex III.

Since CoQ_{10} is the predominant antioxidant of the inner mitochondrial membrane, we studied oxidative stress as a possible consequence of CoQ_{10} deficiency, and we did not find any evidence of oxidative stress in $GLUT1^{+/-}$ mouse cerebellum, compared with controls.

In conclusion, our aggregate data from patients and model mice fail to support CoQ_{10} deficiency as a common finding in GLUT1 deficiency, and CoQ deficiency cannot be viewed as a direct biochemical consequence of defective glucose transport associated with a disease-causing mutation in the *SLC2A1* gene. It is possible that the patient described by Yubero et al. had the double misfortune of inheriting GLUT1 deficiency and a second (yet undefined) genetic disorder causing CoQ_{10} deficiency.

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Synopsis

CoQ is not a recurrent biochemical feature of GLUT1DS; therefore, routine CoQ_{10} supplementation as adjuvant therapy with the ketogenic diet cannot be justified.

Compliance with Ethics Guidelines

Conflict of Interest

Emanuele Barca declares that he has no conflicts of interest.

Maoxue Tang declares that he has no conflicts of interest.

Kristin Engelstad declares that he has no conflicts of interest.

Giulio Kleiner declares that he has no conflicts of interest.

Salvatore DiMauro declares that he has no conflicts of interest.

Catarina M. Quinzii declares that he has no conflicts of interest.

Darryl C. De Vivo declares that he has no conflicts of interest.

Informed Consent

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

Animal Rights

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

Author Contribution

EB performed experiments and statistical analysis and wrote the manuscript, MT provided mouse samples and revised the manuscript, KE provided patients' data and revised the manuscript, GK performed experiments and wrote the manuscript, SD conceived and designed the project and revised the manuscript, CMQ conceived and designed the project and wrote the manuscript, and DCD diagnosed and takes care of the patients, conceived and designed the project, and wrote the manuscript.

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RESEARCH REPORT

Correlation Between Flexible Fiberoptic Laryngoscopic and Polysomnographic Findings in Patients with Mucopolysaccharidosis Type VI

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Abstract This study aimed to compare flexible fiberoptic laryngoscopy (FFL) and polysomnography (PSG) findings in patients with mucopolysaccharidosis (MPS) type VI and to describe upper airway anatomical findings and abnormal PSG results in these patients. In this cross-sectional study, all MPS VI patients followed up at the genetic division of a hospital in southern Brazil were included. Overnight PSG was performed, and the results were classified as normal or mildly, moderately, or severely abnormal. FFL was performed between 7 days before and 7 days after PSG. FFL findings were classified as (1) no obstruction, (2) mild obstruction, (3) moderate obstruction, or (4) severe obstruction of the airways, using the highest score obtained in all the regions.

Eleven patients with MPS VI were included. FFL detected severe airway obstruction in eight (72.7%) patients, moderate obstruction in two (18.2%), and mild obstruction in one (9.1%). PSG revealed obstructive sleep apnea syndrome (OSAS) in nine (81.8%) patients. Among these, mild OSAS was observed in five (45.5%) patients, moderate OSAS in three (27.2%), and severe OSAS in one (9.1%). Moderate to severe hypertrophy of the nasal turbinates was found in 81.8% of the patients, and 64% had severe infiltration in the supraglottic region. There was no association between FFL and PSG findings ($p = 0.454$; $\kappa = -0.09$; 95%CI $= -0.34$ to 0.17), indicating no agreement between the two methods. In the present study, all patients with MPS showed some degree of airway obstruction. We suggest performing PSG in MPS patients to determine disease severity.

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Introduction

Mucopolysaccharidoses (MPSs) consist of a rare group of inherited lysosomal storage diseases, specifically related to glycosaminoglycan (GAG) metabolism defects. Each type of MPS is caused by deficiency of a specific enzyme involved in the degradation of GAGs, causing accumulation of partially degraded products. MPS is estimated to affect one in every 20,000 live births (McKunsick and Neufeld 1983; Kakkis and Wynn 2015).

Head and neck are often affected by MPS, usually at an early stage (Wold et al. 2010). Obstructive sleep apnea, otitis media with effusion, sinusitis, frequent respiratory infections, adenotonsillar hyperplasia, and speech disorders are very common (Gönüldaş et al. 2014). Airway symptoms are the main cause of morbidity and mortality in these patients, airway obstruction is a common finding, and

pharyngeal hypotonia may cause obstructed breathing during sleep (Leighton et al. 2001; Santamaria et al. 2007).

Mucopolysaccharidosis type VI (MPS VI), also known as Maroteaux-Lamy syndrome, is caused by *N*-acetylgalactosamine-4-sulfatase deficiency. As there is no central nervous system involvement in this case, cognitive capacity is not impaired. However, airway infiltration by GAGs is a major concern in these patients, as it leads to the obstructive sleep apnea syndrome (OSAS) and, consequently, to *cor pulmonale*. Death occurs early on, often in the second or third decades of life (Wraith 1995; Kakkis and Wynn 2015).

Polysomnography (PSG) is the gold standard for the diagnosis of OSAS. While flexible fiberoptic laryngoscopy (FFL) is a widely used diagnostic tool in upper airway obstructions in the general population, there is not any information about the agreement of its results with PSG findings in MPS VI patients or about the importance of identifying the site of obstruction for therapeutic planning (Leighton et al. 2001). Actually, no studies to date have compared the findings of FFL and PSG for the assessment of MPS VI patients with airway obstruction symptoms.

The aim of the present study is to compare FFL and PSG findings in MPS VI patients.

Materials and Methods

All MPS VI patients followed up at the Hospital de Clínicas de Porto Alegre, southern Brazil, and whose parents or legal representatives signed a consent form after being fully informed about the study were allowed to participate.

Both clinical and epidemiological data were collected from the patients after interviews with their parents or legal representatives and after analysis of medical charts.

The patients were submitted to overnight PSG, including electroencephalogram, electrooculogram, and electromyography for the classification of sleep stages. Leg electromyography, electrocardiogram, transcutaneous hemoglobin saturation (oximetry), snoring intensity detected with a microphone attached to the neck, and body position were recorded continually. An oronasal thermal sensor was used to detect the absence of airflow. Apnea was defined as blocked airflow for >10 s. Obstructive sleep apnea was defined as absence of airflow for >10 s associated with the presence of continued or increased chest and/or abdominal movements throughout the period of airflow obstruction. Hypopnea was considered whenever nasal airflow decreased by at least 30%, compared to the previous two ventilations, and when arterial oxygenation dropped by at least 4% in the subsequent 30 s. The apnea/hypopnea index (AHI), i.e., the number of apneas and hypopneas per hour of sleep, was calculated. Arousals were regarded as a sudden increase in EEG frequency for 3 or more seconds. The arousal index, defined as the number of arousals divided by the number of hours of sleep, and sleep efficiency, defined as sleep time divided by the number of hours in bed, were also measured (Santamaria et al. 2007; Iber et al. 2007; Epstein et al. 2009).

PSG was classified as normal or mildly (AHI ≥5 and <15/h), moderately (AHI ≥15 and ≤30/h), or severely (AHI >30/h) abnormal, according to the criteria established for adults (Iber et al. 2007).

All patients were also examined in the outpatient clinic, using FFL without sedation 7 days before to 7 days after PSG. The examination was performed with the patient sitting and after administration of a local topical anesthetic inside the nose (xylocaine 2% gel). A flexible fiberoptic 2.2-mm laryngoscope (Olympus®, model ENF-XP, Olympus America, Melville, NY, USA), employed exclusively to this end and connected to a Storz® microcamera (Karl Storz, Tuttlingen, Germany) and to a Storz® light source, was used. The examination was recorded on DVD and later reviewed by a researcher blinded to the PSG findings and widely experienced in pediatric airways (D.M.), who classified airway obstructions according to the following parameters:

Nasal cavities (lower nasal turbinates): classified, according to the grade of hypertrophy, into normal turbinates or mildly, moderately, or severely hypertrophic turbinates or fully obstructed nasal cavities

Cavum (pharyngeal or adenoid tonsils): classified, according to Brodsky and Koch (1992), based on the percentage of cavum filled by the pharyngeal tonsils, absence of hyperplasia, mild hyperplasia (pharyngeal tonsils filling up to 25% of the cavum), moderate hyperplasia (26–50%), severe hyperplasia (51–75%), or total obstruction (76–100%) (Brodsky 1989; Brodsky and Koch 1992)

Retropalatal region (palate and posterior and lateral walls): classified, according to the level of infiltration, into normal, mild, moderate, or severe infiltration or total obstruction

Oropharynx (palatine tonsils): classified, according to Brodsky (1989), into absence of tonsils, grade 1 hypertrophy (up to 25% of the oropharynx filled by the tonsils), grade 2 hypertrophy (26–50%), grade 3 hypertrophy (51–75%), and grade 4 hypertrophy (76–100%) (Brodsky 1989; Brodsky and Koch 1992)

Oropharynx (base of the tongue and epiglottis): classified, according to the level of infiltration, into normal, mild, moderate, or severe infiltration or total obstruction

Supraglottis (arytenoids/aryepiglottic folds): classified, according to the level of infiltration, into normal, mild, moderate, or severe infiltration or total obstruction

Glottis (vocal folds, ventricles of Morgani): classified, according to the level of infiltration, into normal, mild, moderate, or severe infiltration or total obstruction

Hypopharynx (pyriform sinuses): classified, according to the level of infiltration, into normal, mild, moderate, or severe infiltration or total obstruction/effacement of pyriform sinuses

After the assessment, taking into account the high grade of obstruction observed in different regions, the patients were classified as having: (1) no airway obstruction, (2) mild obstruction, (3) moderate obstruction, and (4) severe obstruction.

The data were analyzed by the SPSS® software program, version 21.0. The quantitative variables were described as mean and standard deviation or median and interquartile range. The categorical variables were described by absolute and relative frequencies. Pearson's chi-square test was used to assess the association between the diagnostic methods. The kappa statistic was used to assess the amount of agreement. The significance level was set at 5%.

Results

Eleven MPS VI patients aged 17.8 ± 3.2 years, seven (63.6%) of whom were male were included in the study. Snoring was the most frequent clinical manifestation (72.7%), followed by nasal obstruction (54.5%). Secretion, rebound upper airway infection, swallowing difficulty, and dysphagia were also common (45.5%). Doctor-diagnosed sleep apnea was reported by 36.4% of the patients. No patient had a previous history of stridor or cyanosis. Most patients (72.7%) had a previous history of intubation and the use of CPAP was reported by 27.3% of them. Prior airway surgeries had been necessary in 45.5% of the individuals and consisted of adenoidectomy, tonsillectomy, and nasal turbinate cautery (Table 1).

FFL revealed moderate to severe hypertrophy of nasal turbinates in 81.8% of the patients, moderate to severe hypertrophy of pharyngeal tonsils in 27.2%, and grades 3 and 4 hypertrophy of palatine tonsils in 27.2%. Approximately 64% of the patients had moderate to severe obstruction in the supraglottic region, 36.4% revealed moderate to severe obstruction in the retropalatal region, and 54.5% had moderate to severe obstruction at the base of the tongue.

Taking into account the high incidence of obstruction in the regions, FFL detected severe obstruction in eight patients (72.7%), moderate obstruction in two patients (18.2%), and mild obstruction in one patient (9.1%).

PSG revealed OSAS in nine patients (81.8%). Among these, mild OSAS was observed in five (45.5%), moderate OSAS in three (27.2%), and severe OSAS in one (9.1%).

Sleep efficiency averaged 76.4% (±12.8%), and a median of 7.9 (0.4–16.9) apneas per hour and of 3.4 (0.3–11.8) hypopneas per hour was observed. Mean oxygen saturation was as high as 95.6% (±3.7) while minimum saturation was 80.2% (±8.6). A median of 12 (2–40) microarousals per hour was obtained.

As shown in Fig. 1 and in Tables 1 and 2, there was no association between FFL and PSG findings ($p = 0.454$), which shows lack of agreement between the diagnostic methods ($\kappa = -0.09$; 95%CI $= -0.34$ to 0.17).

Discussion

The present study was the first one to describe these findings exclusively in MPS VI patients, thereby providing a more homogeneous sample of individuals.

Airway obstruction is quite common in patients with this disease, which is progressive and may be fatal. Although nearly 50% of the individuals included in the study (45.5%) had already undergone surgical procedures to treat airway obstruction, 81.8% of them, according to the PSG findings, still had OSAS. This finding was highly frequent and corroborates the literature data, according to which the prevalence of this symptom ranges from 40 to 90% in patients with different types of MPS (Leighton et al. 2001; Santamaria et al. 2007).

Similarly to our study, Yeung et al. (2009) used FFL and PSG to assess some patients with different types of MPS. They found that 19 of their 27 patients presented with upper airway obstruction. Of these 19 patients, seven were submitted to PSG and had an AHI between 10 and 17. Only five of the patients were submitted to direct laryngoscopy, which revealed the presence of macroglossia and of redundant tissues in the oropharynx and in the supraglottis.

In our study, in which all individuals were submitted to FFL, major obstruction was observed in the nasal fossae, at the base of the tongue, and in the supraglottic region. Presumably, this is due to the accumulation of GAGs in these regions, causing airway narrowing.

Santamaria et al. (2007) assessed 11 patients (five children and six adults) with MPS. They also performed PSG and FFL in all of them, in addition to computerized tomography (CT) of the upper airways. They found OSAS in 100% of the children and in 17% of the adults. In the CT scan, they observed a smaller retropalatal and retroglossal space whereas nasal endoscopy revealed hypertrophic adenoids in all of the patients. However, they assessed only the size of pharyngeal tonsils and did not perform a thorough examination of the larynx and pharynx as we did in the present study.

Table 1 Flexible fiberoptic laryngoscopic and polysomnographic findings

Patient	Sex	Age	Height (m)	Prior airway surgeries	FFL	AHI	Number of central apneas	PSG – OSAS
1	M	22	1.29	No	Severe	44	0	Severe
2	M	24	1.12	Yes (adenoidectomy)	Severe	20.2	0	Moderate
3	M	19	0.89	Yes (adenoidectomy)	Severe	16.3	1	Moderate
4	M	15	1.02	No	Severe	12.2	0	Mild
5	M	14	1.21	Yes (adenoidectomy)	Severe	12.8	3	Mild
6	F	14	1.09	Yes (adenoidectomy + nasal turbinate cautery)	Mild	4.2	0	Without OSAS
7	F	17	1.08	No	Severe	13.1	0	Mild
8	M	16	1.12	Yes (adenoidectomy + tonsillectomy)	Severe	7.2	0	Mild
9	M	19	1.15	No	Moderate	19.3	0	Moderate
10	F	17	1.26	No	Severe	12.7	0	Mild
11	F	19	1.22	No	Severe	3.1	0	Without OSAS

FFL flexible fiberoptic laryngoscopy, *AHI* apnea/hypopnea index, *PSG* polysomnography, *OSAS* obstructive sleep apnea syndrome, *M* male, *F* female

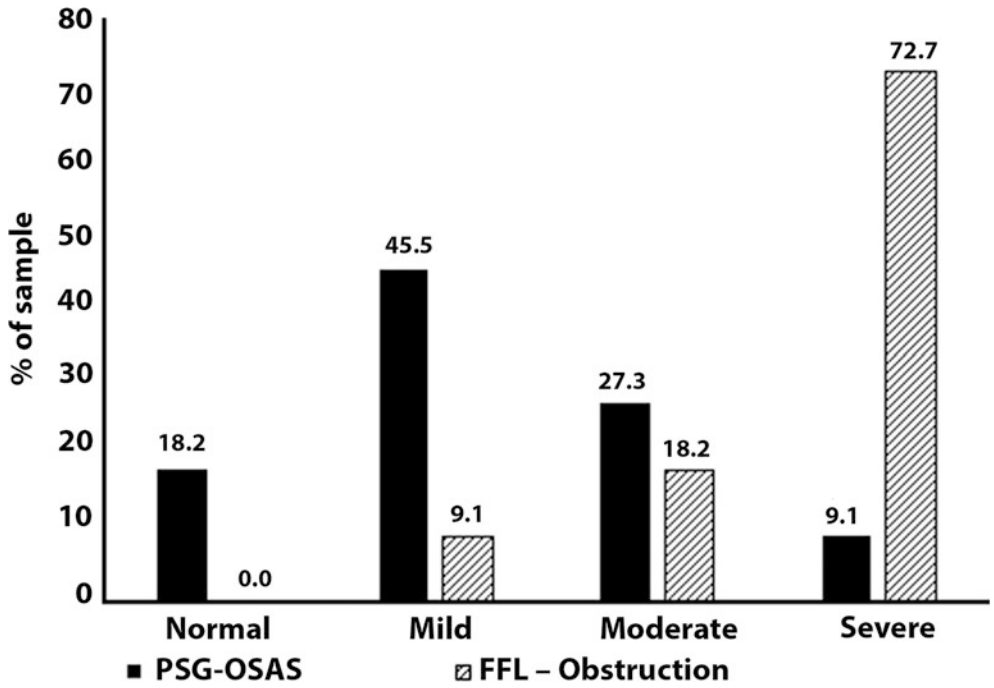

Fig. 1 Comparison of flexible fiberoptic laryngoscopic and polysomnographic findings. *FFL* flexible fiberoptic laryngoscopy, *OSAS* obstructive sleep apnea syndrome, *PSG* polysomnography. Normal: PGS without OSAS and FFL without obstruction

The FFL with sedation, with consequent loss of muscle tone, could be ideal for the assessment of upper airways during sleep. Nevertheless, the high anesthetic risk should only be taken in cases where the procedure has actual potential to change the clinical outcomes of these patients (Simmons et al. 2005). In our population, all the individuals tolerated the procedure without sedation, and the information could be obtained as expected.

Finally, by comparing FFL and PSG findings, we did not observe good agreement between them. Thus, not all those patients in which FFL showed airway obstruction had an abnormal PSG. These data suggest it is necessary to

Table 2 Comparison of flexible fiberoptic laryngoscopic and polysomnographic findings

	Polysomnography			
Fiberoptic laryngoscopy	Without OSAS *n* (%)	Mild OSAS *n* (%)	Moderate OSAS *n* (%)	Severe OSAS *n* (%)
Mild obstruction ($n = 1$)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Moderate obstruction ($n = 2$)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)
Severe obstruction ($n = 8$)	1 (12.5)	4 (50.0)	2 (25.0)	1 (12.5)

The data are shown as absolute and percentage numbers
OSAS obstructive sleep apnea syndrome, *n* number of patients

perform PSG in MPS patients to identify those at risk for OSAS.

Conclusions

MPS VI is a heterogeneous and progressive disease with major systemic involvement. All the assessed patients have some airway obstruction. We suggest performing PSG in all individuals in order to verify the severity of each case. The role of FFL in the identification of obstructive lesions in this population still needs clarification.

Take Home Message (Synopsis)

Obstructive sleep apnea syndrome is frequently found in patients with mucopolysaccharidosis type VI and should be investigated with polysomnography.

Compliance with Ethics Guidelines

Details of the Contributions of Individual Authors

D.R.R.P., C.S., and D.M. were responsible for the design of the study and for data collection. D.R.R.P., C.S., and S.F. carried out the tests. G.K. and P.J.C.M. participated in the design of the study and performed the statistical analysis. C. F.S. and R. G. participated in its design and helped to draft the manuscript. D.R.R.P. was responsible for data analysis and interpretation and helped to draft the manuscript. All authors read and approved the final manuscript.

Name of the Author Who Serves as Guarantor

Denise Rotta Ruttkay Pereira

Competing Interest Statement

Denise Rotta Ruttkay Pereira, Claudia Schweiger, Carolina Fischinger de Souza, Simone Fagondes, Denise Manica, Roberto Giugliani, Gabriel Kuhl, and Paulo José Cauduro Marostica declare that they have no conflict of interest.

Details of Funding

The authors have no financial relationships relevant to this article to disclose.

Details of Ethics Approval

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Patient Consent Statement

Patients' parents or legal representatives signed a consent form after being fully informed about the study.

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RESEARCH REPORT

Continual Low-Dose Infusion of Sulfamidase Is Superior to Intermittent High-Dose Delivery in Ameliorating Neuropathology in the MPS IIIA Mouse Brain

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Abstract Mucopolysaccharidosis IIIA (MPS IIIA) is a neurodegenerative lysosomal storage disorder characterised by progressive loss of learned skills, sleep disturbance and behavioural problems. Reduced activity of lysosomal sulfamidase results in accumulation of heparan sulfate and secondary storage of glycolipids in the brain. Intra-cisternal sulfamidase infusions reduce disease-related neuropathology; however, repeated injections may subject patients to the risk of infection and tissue damage so alternative approaches are required. We undertook a proof-of-principle study comparing the ability of slow/continual or repeat/bolus infusion to ameliorate neuropathology in MPS IIIA mouse brain. Six-week-old MPS IIIA mice were implanted with subcutaneously located mini-osmotic pumps filled with recombinant human sulfamidase (rhSGSH) or vehicle, connected to lateral ventricle-directed cannulae. Pumps were replaced at 8 weeks of age. Additional MPS IIIA mice received intra-cisternal bolus infusions of the same amount of rhSGSH (or vehicle), at 6 and 8 weeks of age. Unaffected mice received vehicle via each strategy. All mice were euthanised at 10 weeks of age and the brain was harvested to assess the effect of treatment on neuropathology. Mice receiving pump-delivered rhSGSH exhibited highly significant reductions in lysosomal storage markers (lysosomal integral membrane protein-2, G_{M3} ganglioside and filipin-positive lipids) and neuroinflammation (isolectin B4-positive microglia, glial fibrillary acidic protein-positive astroglia). MPS IIIA mice receiving rhSGSH via bolus infusion displayed reductions in these markers, but the effectiveness of the strategy was inferior to that seen with slow/pump-based delivery. Continual low-dose infusion may therefore be a more effective strategy for enzyme delivery in MPS IIIA.

Abbreviations

CNS	Central nervous system
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine
GFAP	Glial fibrillary acidic protein
LIMP-2	Lysosomal integral membrane protein
MPS	Mucopolysaccharidosis
OCT	Optimal cutting temperature compound
rhSGSH	Recombinant human sulfamidase

Introduction

Mucopolysaccharidosis type IIIA (MPS IIIA) is an inherited lysosomal storage disorder that results from the absence or defective function of lysosomal sulfamidase, which is involved in the stepwise degradation of heparan sulfate, resulting in the accumulation of heparan sulfate in lysosomes and subsequent clinical disease. The main feature of this disorder is central nervous system (CNS) pathology, with progressive neurodegeneration and subsequent mental decline resulting in a greatly shortened lifespan, often <20 years (Neufeld and Muenzer 2001).

We have identified a naturally occurring mouse model of MPS IIIA (Crawley et al. 2006), which exhibits similar neuropathological features to the human condition, and

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have used it to investigate therapy options for this condition. Whilst intravenous enzyme replacement therapy is useful for reducing lysosomal storage in non-CNS tissues, the blood–brain barrier prevents access of conventional doses of sulfamidase delivered intravenously (Gliddon and Hopwood 2004). Enzyme uptake into the brain parenchyma and subsequent reductions in lysosomal storage and related neurodegenerative changes, together with improvements in clinical function have, however, been observed in MPS IIIA mice receiving repeated injection of sulfamidase into the cisternal cerebrospinal fluid (CSF) (e.g. Hemsley et al. 2007, 2008, 2009). This treatment is also efficacious at ameliorating neuropathology in the larger brain of the MPS IIIA Huntaway dog (Crawley et al. 2011).

Similar observations have been made in MPS I dogs (Kakkis et al. 2004), Krabbe mice (Lee et al. 2007), late-infantile neuronal ceroid lipofuscinosis mice (LINCL; Chang et al. 2008), Niemann–Pick A mice (Dodge et al. 2009), Sandhoff disease mice (Tsuji et al. 2011) and fucosidosis dogs (Kondagari et al. 2011). Therefore, this approach appears to be a disease-spanning therapeutic strategy, and the movement towards application in MPS IIIA patients (www.clinicaltrials.gov #NCT01299727, #02060526) appears both rational and justified.

At present, application of enzyme to MPS IIIA patients occurs via bolus injection using an indwelling intrathecal drug-delivery cannula, with enzyme administered over a short period. Indeed, the majority of the preclinical studies described above have administered the respective enzyme over similar time frames (i.e. minutes). To explore the impact of varying the enzyme supply rate on the amelioration of neuropathology in the MPS IIIA mouse brain, we have compared the effectiveness of continually supplying low-concentration recombinant human sulfamidase (rhSGSH) via a subcutaneous mini-osmotic pump device connected to a cannula directed at the right lateral ventricle, with repeated high-concentration bolus delivery to the cisternal CSF. The total quantity of enzyme supplied to both groups was the same (200 μg over 1 month).

Materials and Methods

Enzyme

RhSGSH was provided by Shire at 25 μg/μL and stored at −70°C until used. The enzyme was diluted to 1.2 μg/μL in 10 mM sodium phosphate and 138 mM sodium chloride (pH 7) and injected into the pump (Alzet; pump rate 0.25 μL/h; Jomar Bioscience, Australia) >48 h prior to surgery. During this time, the pumps were stored at 37°C under sterile conditions. Vehicle was infused in the same manner. Enzyme activity was determined using a natural tritiated tetrasaccharide substrate (Hopwood and Elliott 1982).

Mice

Congenic C57BL/6 MPS IIIA mice (Crawley et al. 2006), or unaffected −/+, +/+ littermates (hereafter referred to as "Normal" mice), were bred, housed and maintained in the institutional Animal House, with all breeding and experimental procedures undertaken with the approval of the Women's and Children's Health Network Animal Ethics Committee, with regard to the guidelines of the National Health and Medical Research Council of Australia on the Use and Care of Experimental Animals. The mice were genotyped using previously established methods (Gliddon and Hopwood 2004).

Intra-cisternal CSF Injections

All methods have been previously described in full (Hemsley et al. 2009). Briefly, 6-week-old mice were anesthetised with ketamine (87 mg/kg; Parnell Laboratories, Australia)/xylazine (13 mg/kg; Troy Laboratories, Australia) (i.p.). Four microlitres of rhSGSH (25 μg/μL) or vehicle was injected into the cerebellomedullary cistern using a 27G dental needle attached to an infusion pump via plastic tubing. MPS IIIA mice received rhSGSH or vehicle injections according to the schedule shown in Fig. 1a ($n = 3$/group); unaffected mice received vehicle only ($n = 3$). All mice received butorphanol tartrate (2 mg/kg; Fort Dodge, NSW Australia) following surgery. Figure 1c shows the position of the injection site.

Cannula/Pump Implantation and Pump Replacement

A further nine mice were anaesthetised as above, secured in a stereotaxic frame (David Kopf Instruments, California, USA), and an incision was made in the scalp. A small hole was made above the right lateral ventricle with a 0.5 mm hand drill bit (Flintware, Adelaide, Australia). The coordinates in reference to bregma and the midline were 0.5 mm posterior and 1.0 mm lateral (Paxinos and Franklin 2001). The cannula (Alzet Brain Infusion Kit 3 fitted with two 0.5 mm spacers; Jomar Bioscience, Australia) was inserted into the brain and secured to the skull with glue. The enzyme/vehicle-filled pump was inserted through the scalp incision and positioned subcutaneously on the flank. The skin wound was sutured and all mice received butorphanol tartrate for analgesia (2 mg/kg; Fort Dodge, Baulkham

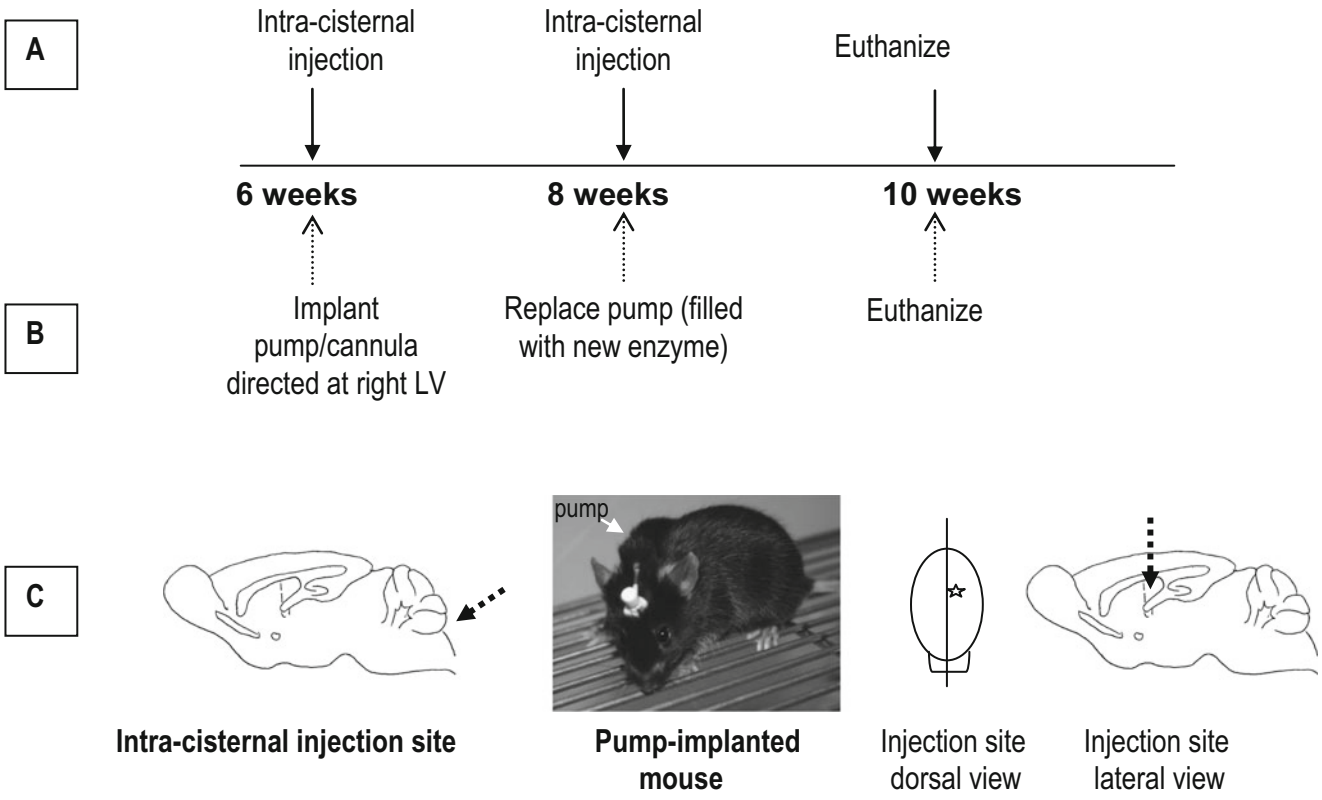

Fig. 1 Experimental plan. Mice received either (**a**) bolus injection of 100 μg recombinant human sulfamidase (rhSGSH) via the cisterna magna (CM) at each of 6 and 8 weeks of age, with euthanasia at 10 weeks of age, or (**b**) implantation of a mini-osmotic pump and cannula, directed at the right lateral ventricle (LV) at 6 weeks of age. The pump was removed and replaced at 8 weeks of age and mice were euthanised at 10 weeks of age. During the experimental period, the pump delivered rhSGSH (1.2 μg/μL) at 0.25 μL/h, delivering 100 μg over each 2-week period. (**c**) Illustrates the injection region in each treatment strategy and shows a photo of a pump-/cannula-implanted mouse

Hills, NSW, Australia), following surgery. Pump replacement was performed at 8 weeks of age (see experimental plan, Fig. 1b) and required a skin incision above the pump site (under anaesthesia as above), to remove the pump and replace it with a new one. The wound was sutured and analgesia administered as above. Figure 1c shows a photo of a mouse fitted with a pump/cannula and the location of the cannula in the right hemisphere of the brain.

Necropsy and Sample Collection

Mice were euthanised via CO_2 asphyxiation at 10 weeks of age, blood was sampled, and mice were fixation-perfused for light microscopy examination (4% paraformaldehyde in PBS, pH 7.4). The brain was removed and each hemisphere was sectioned in a sagittal plane at 1 mm from the midline (i.e. at the lateral coordinate of the cannula), producing a medial and lateral portion of each hemisphere. Medial portions of the left and right hemisphere were embedded in paraffin; lateral portions of the left and right hemispheres were infiltrated with 30% sucrose and snap-frozen in OCT.

Measurement of Anti-rhSGSH Antibodies in Sera

Previously published methods were used to determine the presence of anti-rhSGSH antibody titres in mouse sera (Hemsley et al. 2009).

Histochemistry, Immunohistochemistry and Quantification of Neuropathology Using Light Microscopy

Reagents for Staining

Rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP, #Z334) were purchased from Dako (Glostrup, Denmark). A monoclonal anti-lysosomal integral membrane protein (LIMP-2) antibody (Hemsley et al. 2008), a mouse monoclonal antibody to rhSGSH (Shire) and a mouse monoclonal antibody against G_{M3} (Seikagaku, Japan) were used. Peroxidase-conjugated lectin from *Bandeiraea* (*Griffonia*) *simplicifolia* (isolectin B_4, BSI-B_4, #L5391; Sigma, Missouri, USA), which recognises α-galactosyl groups, was used as a marker for activated microglia. Filipin complex from *Streptomyces filipinensis* (Sigma #F9765)

was resuspended in *N*,*N*-dimethylformamide. Biotinylated secondary antibodies, donkey anti-rabbit IgG and donkey anti-mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA), and goat anti-mouse IgM was obtained from Vector Laboratories (Burlingame, CA USA).

Immunohistochemistry

All procedures and post-staining image analyses were undertaken by an experimenter blinded to genotype/treatment status, and all methods have been previously described (Hemsley et al. 2009; Lau et al. 2010). Briefly, 6 micron thick sagittal sections of fixed paraffin-embedded brain tissue were deparaffinised and exposed to heat-induced epitope retrieval. Tissues were blocked and incubated with primary antibodies (anti-GFAP 1:12,000; anti-LIMP-2 1:800; anti-rhSGSH 1:50). Endogenous peroxidases were quenched prior to addition of a species-specific biotinylated secondary antibody (1:2,000). Sections were visualised using a Vectastain ABC Kit (PK-6100; Vector Laboratories, California, USA) and diaminobenzidine (DAB; #K3468; Dako). BSI-B_4 (1 mg/mL) was diluted 1:80 in TRIS buffer and reactions were visualised with DAB. Sections were batched for each stain. Histochemical detection of filipin- or G_{M3}-positive inclusions was carried out on frozen sections (6 μm), with anti-G_{M3} antibodies applied overnight (1:750) at 4°C. Visualisation was as for the paraffin sections. Filipin staining was carried out using previously published methods (Lau et al. 2010).

Sections were viewed on either an Olympus BX41 or BX61 microscope; digital images were collected using either an Olympus UC50 or Colorview III camera. Several brain regions were imaged and analysed, with all regions defined using a mouse brain atlas (Paxinos and Franklin 2001). All parameters of imaging and calibration remained constant for each comparative region and stain. Images were analysed using AnalySIS LifeScience software (version 2.8, Build 1235). Thresholding, based on the optical density of positive immunostaining for LIMP-2, GFAP and G_{M3}, was applied to the images in a consistent manner. Data are reported as percentage immunoreactivity. The number of BSI-B_4-stained activated microglia (per mm^2) was determined; rhSGSH immunostaining was qualitatively assessed, and the accumulation of filipin-positive inclusions was examined semi-quantitatively (i.e. number of cells with positively stained inclusions), using a +/++/+++ scale.

Statistics

Data were assessed using GraphPad Prism v6.05 software. Data were log transformed $Y = \log(Y + 1)$ and examined using the analysis of variance and post-hoc testing with Bonferroni correction. Mice were compared with their respective control groups (i.e. Normal v MPS cisternal vehicle v MPS cisternal enzyme and Normal v MPS pump vehicle v MPS pump enzyme). $P < 0.05$ was considered to be statistically significant. Data are shown as mean ± SEM.

Results

Anti-rhSGSH Antibodies in Sera

All MPS IIIA mice receiving pump-delivered rhSGSH developed serum anti-rhSGSH antibodies (titres of 1/3,200 to 1/51,200 in enzyme-treated mice cf. titres of ≤1/100 in vehicle-treated mice). The sera from the cisternal high-dose bolus group were unfortunately unable to be analysed; however, we have consistently observed anti-rhSGSH antibodies in the sera from mice treated in this manner (Hemsley et al. 2007, 2008, 2009). There was no apparent clinical effect of the humoral immune response on the mice.

Enzyme Distribution in the Brain Following Cisternal Bolus or Pump Infusion

Immunohistochemical detection of rhSGSH in 10-week-old pump-infused MPS IIIA mouse brain revealed very intense immunostaining in many regions of the brain (Fig. 2b–g). Immunopositive puncta were observed in the cerebral cortex overlying the lateral ventricle and the adjacent hippocampus and in the olfactory bulb. Diffusion from the ventricle was evident, with cells in the thalamus, retrosplenial cerebral cortex and cerebellum displaying rhSGSH-positive puncta. Morphologically, cells containing positive immunoreactivity appeared to include neurons, glia, endothelial cells and macrophages. In contrast, cisternal bolus enzyme-treated MPS IIIA mouse brain revealed low numbers of immunopositive cells in the outer layers of the posterior lobes of the cerebellum (Fig. 2h) and pontine nucleus. Some positive staining was also seen in the ventral meninges. All other areas of the brain, including the cerebral cortex, hippocampus, thalamus, and the colliculi, were negative.

Impact of rhSGSH Treatment on Storage-Related Neuropathology

Three brain regions were examined to determine the effect of rhSGSH delivery on MPS IIIA-related neuropathology. On the basis of previously described disease-related changes and proximity to each of the injection sites, we selected the rostral aspect of the cerebral cortex, thalamus or subiculum of the hippocampus (stain dependent) and the

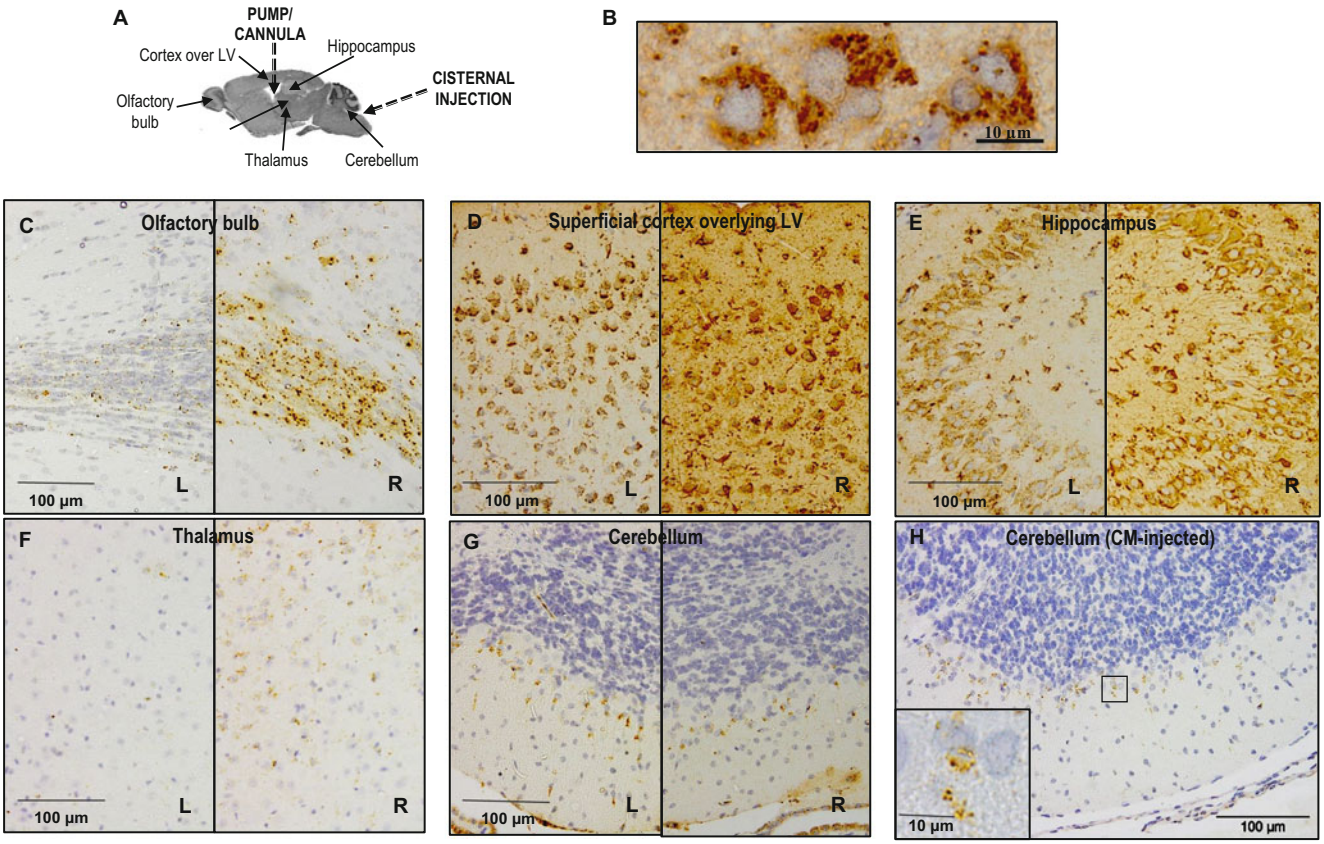

Fig. 2 Immunohistochemical localisation of rhSGSH in regions shown in (**a**) within the pump-/cannula-implanted (**b**–**g**) and the cisternally injected (**h**) MPS IIIA mouse brain at 10 weeks of age. Staining in both *left* (L) and *right* (R) hemispheres of brain is shown for the cannulae-implanted mice (**c**–**g**). The photo in (**b**) shows a high power view of the immunopositive puncta seen in the cerebral cortex of a pump-/cannula-implanted MPS IIIA mouse. The staining is presumptively in endo-/lysosomes

inferior colliculus. The regions and their relationship to the injection locations are shown in Fig. 3.

Sustained delivery of rhSGSH to the MPS IIIA mouse brain resulted in complete normalisation of two of the three disease markers (LIMP-2 and G_{M3}) in the rostral cerebral cortex (right hemisphere; Fig. 3a, d) after 4 weeks of treatment. Near-normalisation of filipin staining was also observed in the deeper pyramidal cell layers III/IV in this brain region at this time (Fig. 3g). The ventricular infusion site is proximal to the right rostral cortex; however, the impact of treatment on LIMP-2 and G_{M3} staining was bilateral, with both cortices exhibiting similar reductions in inclusion number at 10 weeks of age. Further, even in brain areas distant from the ventricle (e.g. inferior colliculus), pump-delivered rhSGSH mediated a significant and bilateral reduction in (LIMP-2) or near-complete amelioration of G_{M3} and filipin-stained inclusions (Fig. 3c, f, i). Reductions in these secondarily stored substrates were also observed in the subiculum of the hippocampus (bilaterally). This structure is located caudal but adjacent to the injection region.

In contrast, whilst reductions in LIMP-2 immunoreactivity and filipin-stained inclusion levels were noted in the inferior colliculus of bolus-injected mice at 10 weeks of age (i.e. 2 weeks after the last injection), LIMP-2 and G_{M3} staining in the thalamus and hippocampus (respectively) was not significantly different to that seen in bolus vehicle-infused MPS IIIA mice (Fig. 3b, e). The impact of bolus cisternal enzyme delivery on neuropathology in the rostral cerebral cortex was statistically negligible, although two of the three cisternally injected MPS IIIA mice exhibited reductions in G_{M3} and filipin-positive lipid accumulation in this region.

Impact of rhSGSH Treatment on Micro- and Astrogliosis

Microgliosis was completely ameliorated in the rostral cortex in both hemispheres (Fig. 4a) after 4 weeks of sustained delivery of low-dose rhSGSH. A small, non-statistically significant reduction in microgliosis was observed in this region after bolus delivery. In the thalamus, microglia appeared to be completely deactivated on the

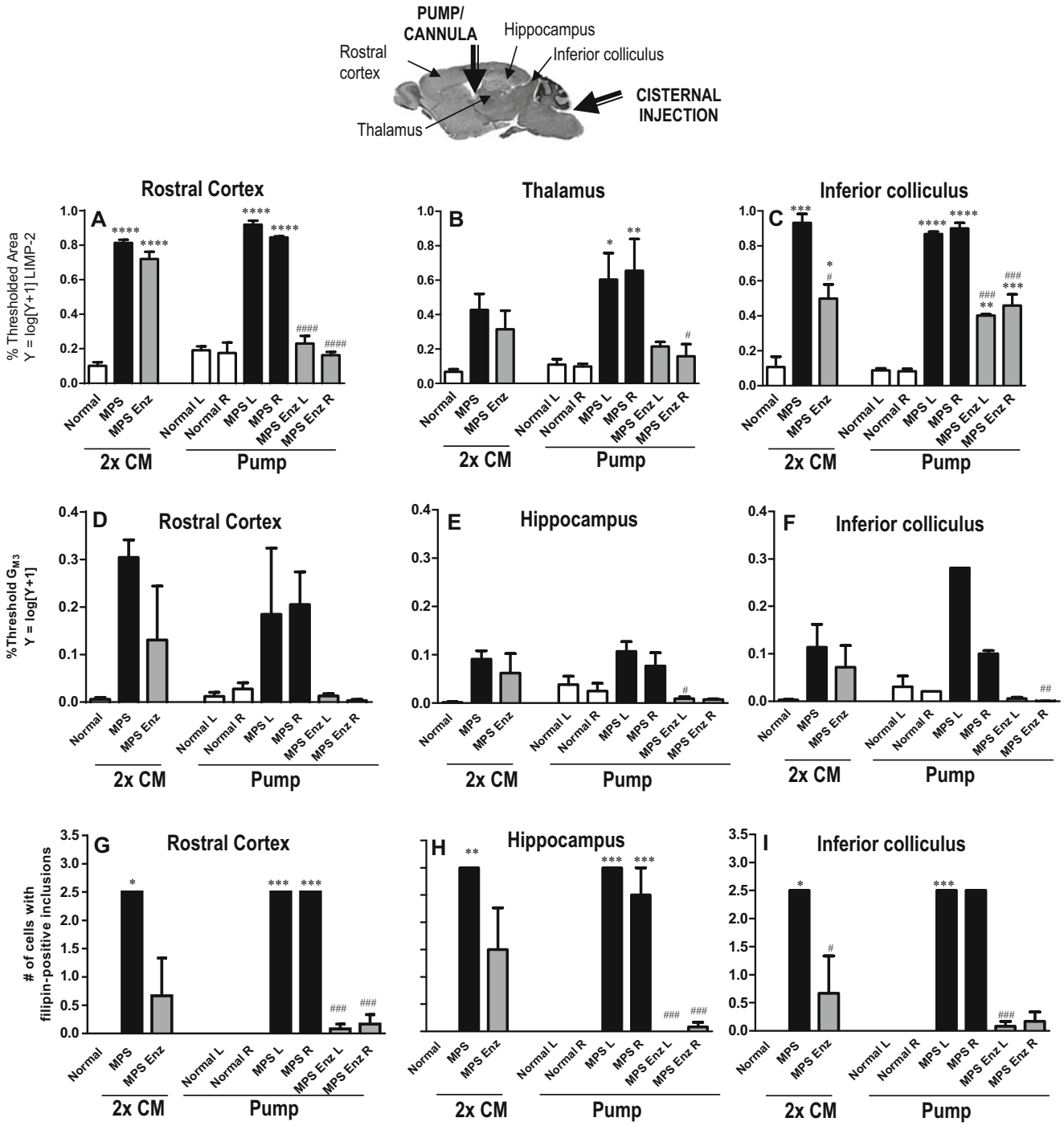

Fig. 3 Quantification of immunohistochemical staining of lysosomal integral membrane protein-2 (LIMP-2; **a**–**c**) and G_{M3} (**d**–**f**) and semi-quantification of filipin-stained inclusions (**g**–**i**) in 10-week-old MPS IIIA and unaffected mouse brain following cisternal bolus (CM) or pump-delivered enzyme treatment. The location of the brain regions analysed with respect to the injection sites is shown in the sagittal brain section ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ cf. Normal; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, $\#\#\#p < 0.0001$ cf. MPS vehicle)

injected side (right hemisphere; Fig. 4b), and a small (non-statistically significant) reduction in the number of activated microglia was observed in the non-injected (left) hemisphere of the brain in pump-treated MPS IIIA mice. The reason for the disparity between left and right hemispheres is unknown.

In contrast, there was no impact on microgliosis in this region when mice receiving bolus delivery of rhSGSH were

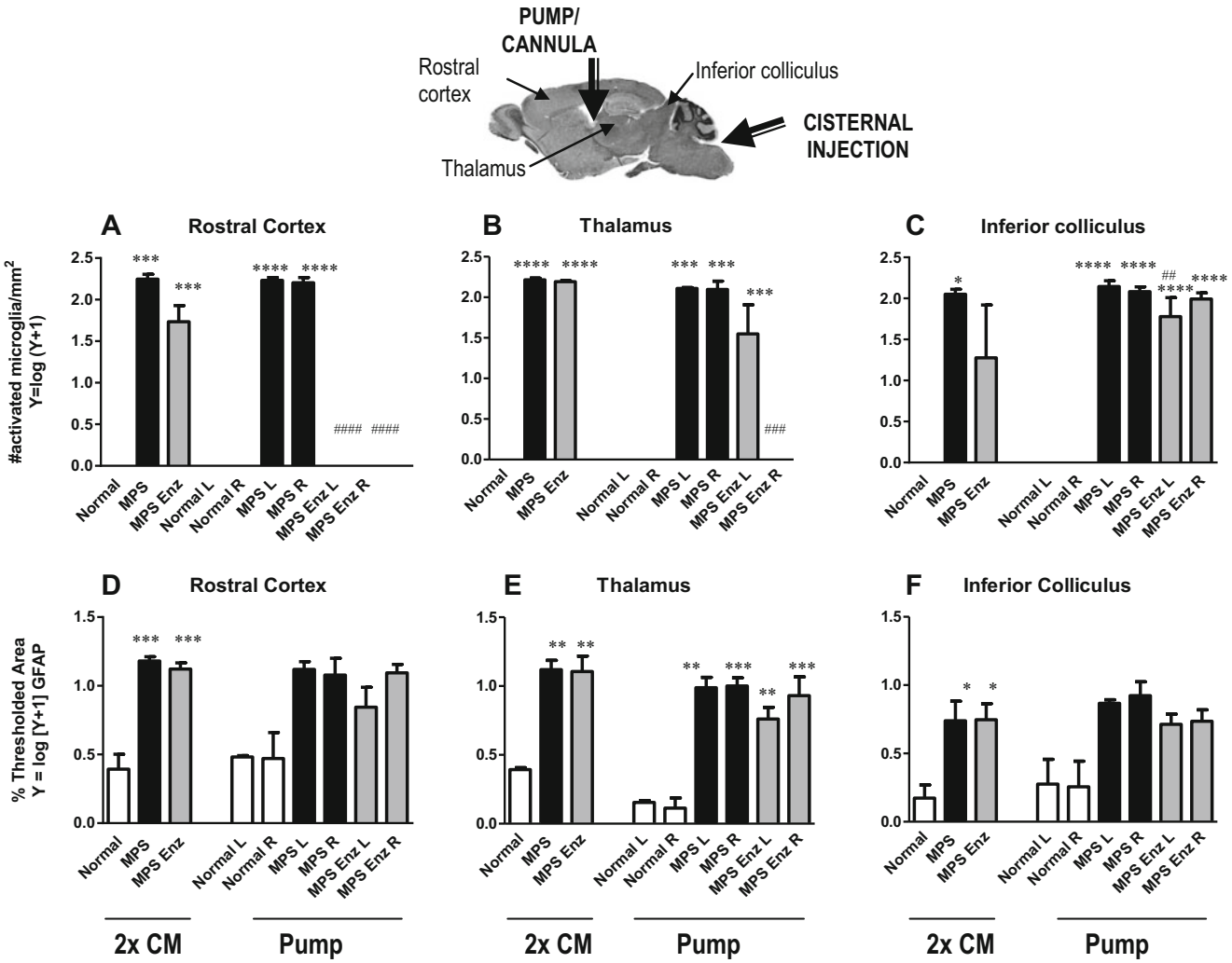

Fig. 4 Quantification of immunohistochemical staining of isolectin B4 (activated microglia; **a**–**c**) and glial fibrillary acidic protein (GFAP; activated astrocytes; **d**–**f**) in 10-week-old MPS IIIA and unaffected mouse brain. The location of the brain regions analysed, with respect to the injection sites, is shown in the sagittal section of brain (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ cf. Normal; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ###$p < 0.0001$ cf. MPS vehicle)

examined at 10 weeks of age (2 weeks after the last injection). Neither treatment strategy initiated large reductions in microgliosis in the inferior colliculus (Fig. 4c), and astrogliosis remained at vehicle-treated MPS IIIA mouse levels in all regions of the brain examined following both high-dose bolus and low-dose continual enzyme delivery (Fig. 4d–f).

Stability of rhSGSH

At the end of each 2-week period, the remaining enzyme was removed from the pump and its activity was determined against a natural substrate. The rhSGSH removed from pumps explanted at 8 weeks of age exhibited 95.0 ± 5.0% of its original, undiluted activity, and enzyme removed from pumps taken at the end of the experiment retained 78.7 ± 2.0% of its original activity.

Discussion

In this proof-of-principle study, we evaluated the efficacy of continuous low-dose rhSGSH delivery to the ventricular CSF of MPS IIIA mice. Outcomes have been compared with those from MPS IIIA mice receiving equivalent amounts of rhSGSH (200 μg) over the same time frame (1 month) via intra-cisternal bolus infusions. Until now, all injections into MPS IIIA animals have been performed using bolus injection over the course of several minutes, and this is the methodology presently used in MPS IIIA patients enrolled in a clinical trial of this therapy (www.clinicaltrials.gov #NCT 01155778 and 01299727).

Data presented here suggest the superiority of low-dose continual enzyme supply over high-dose bolus injection, in both achieving and maintaining a low pathology burden in the MPS IIIA mouse brain. We hypothesise that continual

delivery enables the maintenance of brain SGSH concentration at or above the effective concentration required for substrate catabolism. In contrast, bolus delivery may result in an initially high SGSH concentration, but this could be followed by a period of time during which the amount of SGSH falls below the effective concentration. As this is a proof-of principle study, this hypothesis is yet to be proven.

Once stability of the enzyme within the pump can be assured, increased duration experiments would need to be carried out to determine the long-term ability of this infusion method to both achieve and maintain low storage pathology burdens and to elucidate the impact of continuous delivery of rhSGSH on neurological function in MPS IIIA.

Two clinically feasible strategies accessing different CSF injection points were utilised in this study. To have compared ventricularly infused pump-delivered rhSGSH to a ventricular bolus strategy, we would have needed to have undertaken repeated intracerebral (lateral ventricle) injections. This is an invasive method and would have caused considerable damage to the overlying brain tissue. Similarly, we were unable to direct infusion cannulae (connected to the pumps) towards the cisterna magna, to enable comparison with cisternally infused bolus rhSGSH. The difference in CSF infusion points may/may not have biased the outcomes towards one infusion strategy or the other. Further investigation of the effectiveness of bolus versus slow delivery of rhSGSH via a single infusion site is now warranted, and these studies would be feasible in a larger animal model.

Pump-infused mice exhibited anti-rhSGSH antibodies in sera at post-mortem; however, we were unable to study the humoral response in the present cohort of cisternal bolus-infused mice. We have previously shown that repeat bolus rhSGSH-treated animals exhibit anti-rhSGSH antibodies in sera (Hemsley et al. 2007, 2008, 2009). In all instances (i.e. in both previously bolus-treated and the current pump-infused mice), there is no apparent clinical change in the mice. Thus, as anti-rhSGSH antibodies are found in the sera of rhSGSH-treated mice regardless of treatment method, we suggest that this is not a factor contributing to the superiority of one method of enzyme delivery over another.

To our knowledge, this is the first study to directly compare bolus and sustained-release enzyme delivery to the brain in a lysosomal storage disorder animal model. Other neurodegenerative lysosomal storage disorders that have been treated with slow, continuous infusion of enzyme into the CSF are late-infantile neuronal ceroid lipofuscinosis mice (Chang et al. 2008), metachromatic leukodystrophy mice (Stroobants et al. 2011) and MPS II mice (Sohn et al. 2013); Niemann–Pick A mice have received repeated slow (but not continuous) infusions of enzyme (Dodge et al. 2009). In the Chang et al. study (2008), 100 μg of recombinant human tripeptidyl peptidase (TPP1) was delivered over 14 days with no subsequent pump replacement. Euthanasia was carried out 45 days after treatment initiation, and post-mortem analyses revealed bilateral enzyme distribution, with 2- to 18-fold heterozygote levels of TPP1 detected in all ten 1 mm coronal slices of mouse brain at euthanasia, reduced cortical gliosis and autofluorescence, which was able to initiate an improved tremor phenotype. Whilst anti-TPP1 antibodies were recorded in treated mice, as also observed here, mouse health was not adversely affected. The influence of the antibodies on enzyme uptake and internalisation was not explored.

Mice with metachromatic leukodystrophy received recombinant human arylsulfatase A into the lateral ventricle over 4 weeks (Stroobants et al. 2011) using the same methodology employed here. The authors observed significant reductions in sulfatide storage and improved gait parameters. It was reported that various brain cell types responded differently to enzyme infusion, with macrophages most readily treated, as judged by catabolism of stored sulfatide. Intra-CSF enzyme-infused mice did not develop anti-arylsulfatase antibodies, in contrast to those animals enrolled in previous intravenous enzyme delivery studies (Matzner et al. 2005, 2008).

Finally, in the Dodge et al. study (2009), freely moving Niemann–Pick A mice were treated with recombinant human acid sphingomyelinase (rhASM) either once with one of three doses (7.5, 25, 250 μg) at 14 weeks of age or every 2 weeks from 6 to 30 weeks of age. Slow and repeated enzyme administration (25 μg) occurred over the course of 6 h. Treated mice exhibited intracellular (presumptively lysosomal) uptake of rhASM throughout the rostro-caudal axis, and dose-dependent reductions in sphingomyelin in the brain, liver and lung were noted. Cholesterol accumulation began to be ameliorated within 24 h post-infusion of rhASM, with levels subsequently re-accumulating to reach those of untreated affected mice by 3 weeks post-infusion. This is similar to the time course for pathology amelioration/re-accumulation in the MPS IIIA mouse (Hassiotis et al. 2014). Repeated treatment of Niemann–Pick A mice normalised forelimb and near-normalised hind-limb gait and improved performance in a foot-fault assay, but did not improve grip strength.

The results of the present study provide proof-of-principle support for continued evaluation of sustained delivery of lysosomal enzyme as a treatment for MPS IIIA. This strategy may significantly reduce the invasiveness of lifelong enzyme treatment and improve patient and family quality of life by reducing hospital/clinic visits.

Acknowledgements We acknowledge Hanan Hannouche for genotyping the mice, the WCHN Animal Care Facility staff for caring for our mice and Shire for the rhSGSH and anti-rhSGSH antibody used in the study. The Australian National Health and Medical Research Council (Grant #565074 to JJH and KMH) and Shire are acknowledged for provision of funds to support the study.

Conflict of Interest Statement

Role of Commercial Funding Source

Shire provided the rhSGSH and the anti-rhSGSH antibody. Shire did not have any role in study design, data collection, data analysis, interpretation of data, writing of the report or in the decision to submit the paper for publication. Shire was given the opportunity to review the manuscript for scientific accuracy and legal/IP compliance, but the views expressed remain those of the authors.

Disclosure Statement

An international patent is held by JJH and others for mammalian sulfamidase and genetic sequences encoding it, for use in the investigation, diagnosis and treatment of subjects suspected of suffering from sulfamidase deficiency (US Patent # 5,863,782).

Synopsis

Continual rhSGSH infusion is superior to repeat bolus CSF injection.

Compliance with Ethics Guidelines

Conflict of Interest

Helen Beard, Sofia Hassiotis, Amanda J. Luck and Tina Rozaklis declare that they have no conflict of interest.

John J. Hopwood has received research grant funding from Shire and the Australian National Health and Medical Research Council for this and other studies. He and others hold an international patent for mammalian sulfamidase and genetic sequences encoding it, for use in the investigation, diagnosis and treatment of subjects suspected of suffering from sulfamidase deficiency (US Patent # 5,863,782). Kim M. Hemsley has received research grant funding from Shire and the Australian National Health and Medical Research Council for this and other studies.

Animal Rights

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

Details of the Contributions of Individual Authors

Helen Beard and Sofia Hassiotis carried out tissue processing, undertook the immunohistochemical and histochemical studies outlined in the manuscript, analysed and interpreted data and edited the manuscript for accuracy. Amanda J. Luck carried out the mouse husbandry, assisted with the surgical procedures, monitored the mice post-surgery, carried out post-mortems/tissue collection, interpreted the data and edited the manuscript for accuracy. Tina Rozaklis carried out the enzyme activity and antibody titre assays analysed and interpreted the data and edited the manuscript for accuracy. John J. Hopwood conceived the study, obtained funding and edited the manuscript for accuracy. Kim M. Hemsley conceived and designed the study, obtained required consents, performed the surgery, analysed and interpreted data and wrote the manuscript.

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RESEARCH REPORT

A Short Synthetic Peptide Mimetic of Apolipoprotein A1 Mediates Cholesterol and Globotriaosylceramide Efflux from Fabry Fibroblasts

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Abstract Fabry disease is an X-linked sphingolipid storage disorder caused by a deficiency of the lysosomal enzyme α-galactosidase A (AGA, EC 3.2.1.22) resulting in the intracellular accumulation of globotriaosylceramide (Gb3). We found that Gb3 storage also correlates with accumulation of endosomal–lysosomal cholesterol in Fabry fibroblasts. This cholesterol accumulation may contribute to the phenotypic pathology of Fabry disease by slowing endosomal–lysosomal trafficking. We found that LDL receptor expression is not downregulated in Fabry fibroblasts resulting in accumulation of both cholesterol and Gb3. 5A-Palmitoyl oleoyl-phosphatidylcholine (5AP) is a phospholipid complex containing a short synthetic peptide that mimics apolipoprotein A1, the main protein component of high-density lipoprotein (HDL) that mediates the efflux of cholesterol from cells via the ATP-binding cassette transporter. We used 5AP and HDL to remove cholesterol from Fabry fibroblasts to examine the fate of accumulated cellular Gb3. Using immunostaining techniques, we found that 5AP is highly effective for depleting cholesterol and Gb3 in these cells. 5AP restores the ApoA-1-mediated cholesterol efflux leading to mobilization of cholesterol and reduction of Gb3 in Fabry fibroblasts.

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Introduction

Fabry disease is an X-linked glycosphingolipid storage disorder caused by a deficiency of the enzyme α-galactosidase A, which causes the accumulation of glycosphingolipids, particularly globotriaosylceramide (Gb3) in most cell types. Fabry disease is a progressive debilitating disorder affecting multiple organ systems with an incidence of ~1 in 40,000 males. Signs and symptoms include angiokeratoma, lymphedema, cornea verticillata, hypohidrosis, neuropathic pain, cardiac hypertrophy, proteinuria, progressive kidney failure, abdominal pain, diarrhea, fatigue, vertigo, and strokes. Fabry disease demonstrates significant morbidity even in childhood. Investigators analyzed dried blood spots collected from 34,736 newborns in an Austrian newborn screening program and found that 1 per 2,315 babies had a lysosomal storage disorder, much higher than the previous estimated incidence of 1 per 7,700 births. The most frequent mutations found were for Fabry's disease (Mechtler et al. 2012).

Enzyme replacement therapy (ERT) for Fabry disease is now the therapy of choice. It appears to show some beneficial effects, but the overall effects of ERT have been modest with regard to strokes, myocardial infarctions, and renal involvement.

A report exists about the postmortem findings of a male patient with Fabry disease who was on ERT for more than 2 years. He received α-galactosidase A infusions (agalsidase

beta; Genzyme Corporation, Cambridge, MA) at a dosage of 1 mg/kg every 2 weeks for the last 2.5 years of his life. The autopsy revealed that he had widespread atherosclerotic coronary artery disease that culminated in a massive acute myocardial infarction at age 47. Typical Fabry cardiomyopathy and glomerular nephropathy were found. With the exception of vascular endothelial cells, extensive glycolipid storage deposits were seen in all vascular and nonvascular cells and organ systems. In this patient, repeated infusions with α-galactosidase A over a prolonged period did not appreciably clear storage material in cells other than vascular endothelial cells. Also remarkable was the history of his blood cholesterol levels. His total blood cholesterol at age 36 was 201 mg/dL (5.21 nmol/L) and his HDL-cholesterol and triglyceride levels were 87 mg/dL (2.25 nmol/L) and 92 mg/dL (2.38 nmol/L), respectively. At age 43, total cholesterol was 241 mg/dL (6.24 nmol/L), LDL-cholesterol level was 161 mg/dL (4.16 nmol/L), and HDL-cholesterol level was 24 mg/dL (0.62 nmol/L). Normal ranges for total cholesterol should be below 200 mg/dL (5.21 nmol/L) for LDL cholesterol below 70 mg/dL (1.81 nmol/L) and for HDL cholesterol 40–60 mg/dL (1.04–1.55 nmol/L) (Schiffmann et al. 2006).

The presence of marked storage in cell types other than vascular endothelia cells such as smooth muscle cells and pericytes after more than 2 years of enzyme infusions suggests that the infused enzyme has limited access cells other than vascular endothelia cells. Thurberg analyzed pretreatment and posttreatment endomyocardial biopsies from 58 Fabry patients enrolled in a 5-month, phase 3, double-blind, randomized, placebo-controlled trial, followed by a 54-month open-label extension study of recombinant human α-galactosidase A. No clearance of GL-3 was observed in the cardiomyocytes during this trial (Thurberg et al. 2009).

Therefore, exploration of new therapies that may achieve more complete clearing of storage material is imperative.

It has been known for several decades that high-density lipoprotein (HDL) exerts a protective effect on atherogenesis. This effect is primarily mediated by the ATP-binding cassette (ABC) transporter ABCA1 that promotes the efflux of excess cholesterol from cells. ABCA1 resides on the plasma membrane, as well as in endocytic vesicles, that shuttle between late endocytic compartments and the cell surface. ABCA1 in late endocytic vesicles (late endosomes and lysosomes) can mobilize lipids for ApoA-1-mediated cellular efflux. A model for the pathway of ABCA1-mediated intracellular lipidation of ApoA-1 was postulated (Neufeld et al. 2004). ApoA-1 binds to the cell surface and is internalized along with ABCA1 into early endosomes. A portion of ABCA1 and ApoA-1 is delivered to late endocytic compartments. ABCA1 at the cell surface and in early endocytic compartments mediates the lipidation of ApoA-1. ApoA-1 traffics back to the cell surface where it is released as the nascent HDL particle.

The association of glycosphingolipid storage and cholesterol accumulation is well known (Puri et al. 2003). The physical interaction between GSLs and cholesterol is thought to cause intracellular cholesterol "trapping." Cholesterol together with other lipids accumulates as primary or secondary storage in several lysosomal storage disorders and has been proposed to impede trafficking in the endolysosomal system (Walkley and Vanier 2009). This "traffic jam" can impair lysosomal function such as delivery of nutrients through the endolysosomal system leading to a state of cellular starvation (Schulze and Sandhoff 2011). ApoA-1-mediated cholesterol efflux occurs via ATP-binding cassette transporter A1 (ABCA1) and is a key regulator of cellular cholesterol balance. The accumulation of cellular cholesterol that accompanies glycosphingolipid storage is associated with an impaired capacity to efflux cholesterol to ApoA-1. Fabry disease fibroblasts express increased ABCA1 mRNA levels while still exhibiting a suppressed capacity to efflux cholesterol to ApoA-1 (Glaros et al. 2005). An agent that is able to increase ApoA-1-mediated cholesterol efflux and reduce lysosomal Gb3 would be of enormous benefit for the treatment of Fabry disease.

It has been found that the variant of ApoA-1 in which there is an arginine to cysteine substitution at amino acid 173 (ApoA-1Milano) appears to have superior anti-atherogenic properties compared with conventional ApoA-1. This finding led to the first positive clinical trial of HDL replacement therapy (Remaley et al. 2008). However, the production of therapeutic quantities of recombinant HDL and the Milano variant is costly. Fabry disease fibroblasts show impaired apolipoprotein A1-mediated cholesterol efflux compared with normal control fibroblasts (Glaros et al. 2005). We showed that Gb3 storage in Fabry fibroblasts correlates with the accumulation of endosomal–lysosomal cholesterol. LDL receptor expression is not downregulated when fibroblasts accumulate both cholesterol and Gb3.

It was recently found that short synthetic peptides that mimic ApoA-1, the main component of HDL, can mediate the efflux of cholesterol from cells (Sethi et al. 2008) and may be a cost-effective alternative to a full-length ApoA-1 as a therapeutic agent. The 5A peptide is a bihelical amphipathic peptide that can be complexed with the

phospholipid palmitoyl oleoyl-phosphatidylcholine (5AP). 5AP specifically effluxes cholesterol from cells by ATP-binding cassette transporter 1 (ABCA1) (Amar et al. 2010). In our studies, we used 5AP and HDL to remove cholesterol from Fabry fibroblasts to examine the fate of accumulated cellular Gb3. Using immunostaining techniques, we could show that 5AP is highly effective for depleting cholesterol and Gb3. 5AP restores the ApoA-1-mediated cholesterol efflux which leads to mobilization of cholesterol and reduction of Gb3 in Fabry fibroblasts.

Materials and Methods

Materials

Fabry fibroblasts GM 00107 with 0.0% of normal control alpha-galactosidase-A enzyme activity were from the Coriell Institute of Medical Research (Camden NJ, USA). Control Fibroblasts were from a normal healthy subject. Fetal bovine serum was obtained from HyClone Laboratories, Inc., Logan, UT. Lipoprotein-deficient bovine serum (LPDS), human low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were prepared by Advanced Bioscience Laboratories, Rockville, MD. Dr. Alan Remaley, National Heart, Lung, and Blood Institute (NHLBI) at NIH, provided the peptide 5AP. Filipin was purchased from Polysciences, Warrington, PA; mouse monoclonal anti-Gb3 antibody was from Seikagaku, Falmouth, MA; and Alexa-568 tagged IgG goat anti-mouse antibody and DiI-LDL were from Life Technologies, Grand Island, NY. Glass and plastic microscope culture chamber slides were from Nunc, Inc., Naperville, IL.

Methods

Fabry fibroblasts were cultured in McCoy's medium supplemented with 5% FBS (HyClone) and 100 units of penicillin–streptomycin/mL in a humidified incubator with 95% air and 5% CO_2 at 37°C. Fibroblasts were seeded at a density of 20,000 cells/chamber in human fibronectin-coated two-chamber glass slides. Cells were cultured in McCoy's medium supplemented with 5% LPDS (lipoprotein-depleted serum) and 100 units of penicillin–streptomycin/mL (McCoy's/5% LPDS) medium for 5 days for cholesterol depletion and cell synchronization. Cells were then incubated in fresh medium containing LDL (low-density lipoprotein) (50 μg/mL) (Sethi et al. 2008) for 24 h and then HDL (50 μg/mL) or peptide 5AP (50 μg/mL) was added to different groups. One experimental group was washed after LDL exposure, with phosphate-buffered saline (PBS), and subsequently grown another 24 h in McCoy's/5% LPDS as a cholesterol washout control.

Immunocytochemical Analyses

Filipin Staining: Filipin Staining Was Used to Visualize Unesterified Cholesterol

Cells in chamber slides were PBS washed, fixed in 3% paraformaldehyde for 30 min at room temperature, washed with PBS, and then incubated overnight with 0.05% filipin in PBS at 4°C. After incubation, cells were washed with PBS and mounted in *p*-phenylenediamine glycerol. Cells were viewed with a Zeiss LSM 410 confocal fluorescence microscope using excitation filter BP-350-364 for filipin. Then images were taken using a Zeiss Axiovert 100 TV fluorescent microscope. Eight images per slide were taken of random fields. The images were then analyzed using the I-vision program and fluorescent pixels per cell were calculated.

Gb3 Staining

Cells in chamber slides were washed in PBS and fixed in 3% paraformaldehyde for 30 min. Cells were immunolabeled using an indirect procedure in which all incubations were performed in blocker solution containing goat IgG (10 mg/mL) and phosphate-buffered saline with saponin (0.2%). Slides were incubated with primary antibody mouse monoclonal antibody against human Gb3 at a 1:500 dilution and followed detection with an Alexa-568-labeled anti-mouse antibody at a 1:100 dilution. Fluorescence was viewed with a Zeiss LSM 410 laser scanning confocal microscope, using a krypton–argon (Milles-Griot) laser with an excitation wavelength of 568 nm. Imaging and image analysis were performed as described for filipin.

LDL Uptake and Gb3 Accumulation

Monitoring of LDL uptake in Fabry and control fibroblasts with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-LDL). DiI-LDL is a marker for receptor-mediated cellular endocytosis of LDL (Stephan and Yurachek 1993). Fabry and control fibroblasts were

seeded in 2-well chamber slides and 24-well plates at 20,000 cells per well and cholesterol depleted for 4 days. Cells were then incubated in fresh LPDS medium containing DiI-LDL (20 μg/mL). Twenty-four-well plates were analyzed using the Cytofluor 4000 plate reader at excitation of 540 nm and emission 620 nm at 0, 6, 12, and 24 h. After 24 h, wells were washed 2 times with PBS, the cells were dissolved with 0.05 N NaOH, and total protein was determined with the micro-BCA assay using bovine serum albumin as a standard. Images of the cells stained for Gb3 and cholesterol were taken at the same time using the Zeiss LSM 700.

Results

We monitored LDL uptake in Fabry and control fibroblasts with DiI-LDL as a marker for receptor-mediated cellular endocytosis of LDL. We found that DiI-LDL uptake was markedly increased in Fabry fibroblasts compared to controls, suggesting that LDL receptor expression is markedly upregulated in Fabry fibroblasts (Fig. 1).

The time course of DiI-LDL uptake and Gb3 increases in Fabry fibroblasts (Fig. 2).

A micrograph of Fabry Fibroblasts stained with filipin (blue) for cholesterol and immunostaining for Gb3 (red). The typical granular pattern of lysosomal staining is increased in fibroblast loaded with LDL and reduced in cells subsequently cultured with 5AP for 24 h (Fig. 3).

We treated cholesterol-depleted Fabry and control fibroblasts with LDL for 24 h to load them with cholesterol and then for 24 h with either LPDS, HDL, or 5AP for an additional 24 h. In Fabry fibroblasts Gb3 increases with LDL uptake and decreases with 5AP or HDL treatment. In control fibroblasts the Gb3 levels showed almost no fluctuation (Fig. 4).

T-Test Statistics

The difference between LPDS wash and 5AP is statistically significant, ($p < 0.05$).

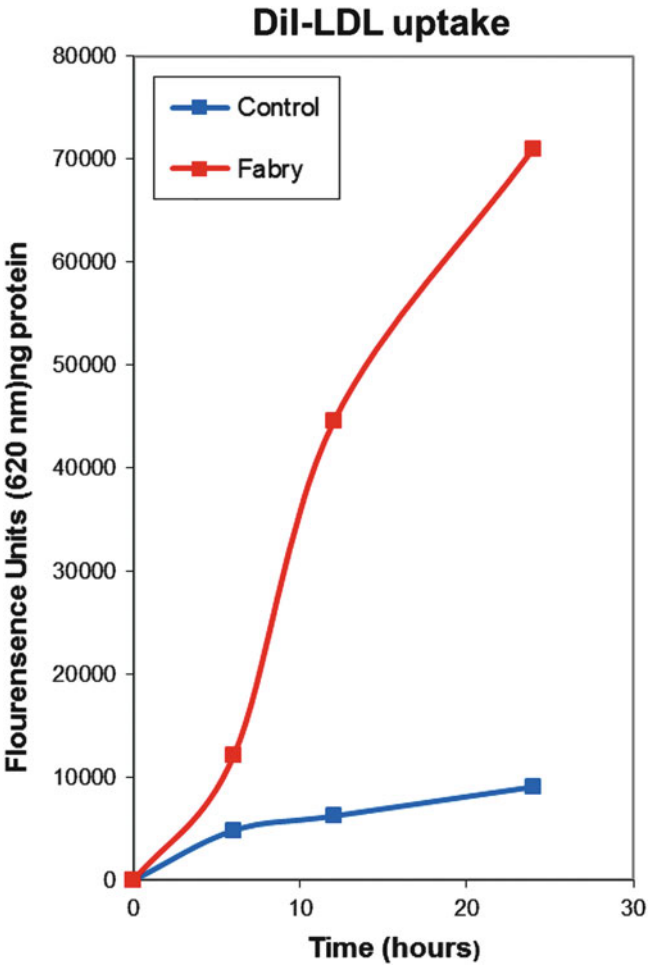

Fig. 1 DiI-LDL uptake in Fabry and control fibroblasts monitored over 6, 12, and 24 h measured with a fluorescence plate reader (Cytofluor 4000). Results are expressed as fluorescence units per mg of protein

The difference between LPDS wash and HDL is statistical very significant, ($p < 0.01$).

Our cell culture studies have shown that peptide 5AP is highly effective for depleting cholesterol and Gb3 from Fabry fibroblasts.

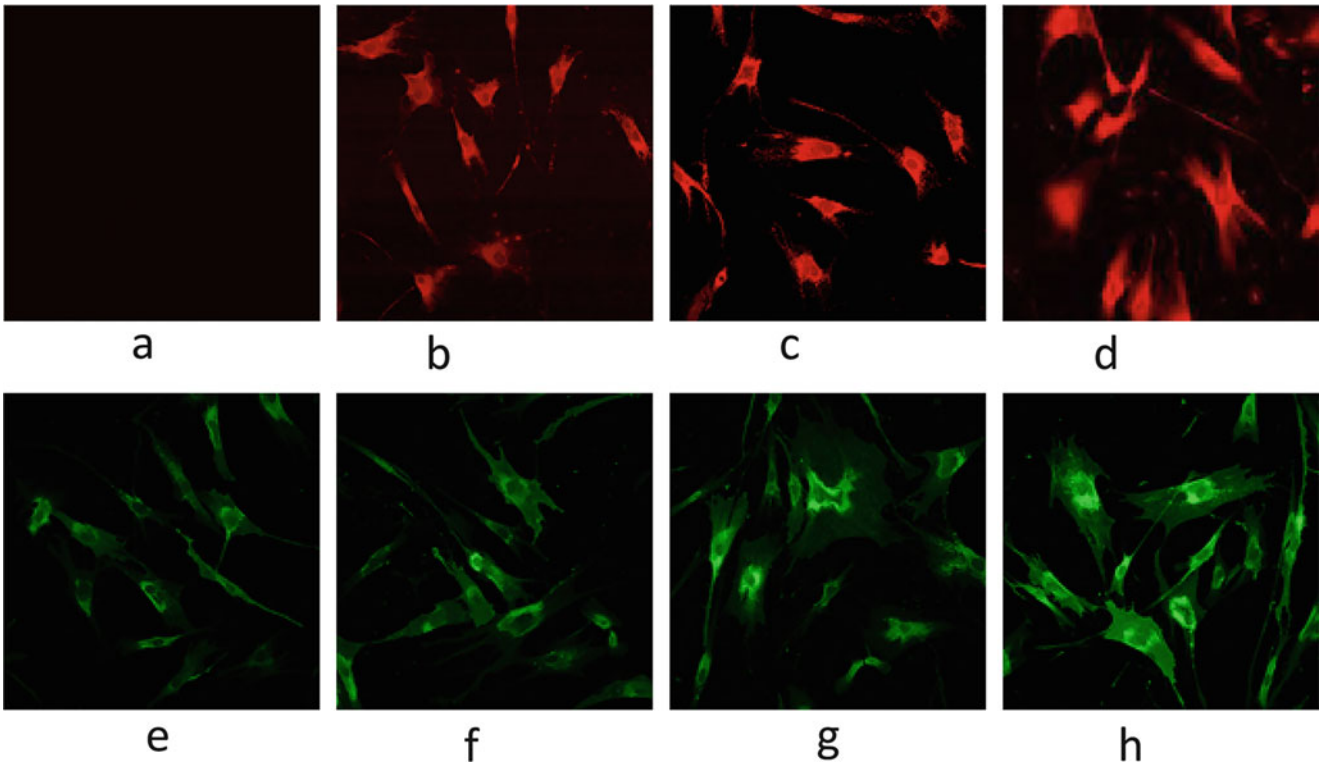

Fig. 2 Imaging of DiI-LDL uptake and staining for Gb3 in Fabry fibroblasts. *Upper row*: DiI-LDL visualized after 0 (**a**), 6 (**b**), 12 (**c**), and 24 (**d**) hours of incubation. *Lower row*: Gb3 increase visualized by Gb3 staining after 0 (**e**), 6 (**f**), 12 (**g**), and 24 (**h**) hours (original magnification 200×)

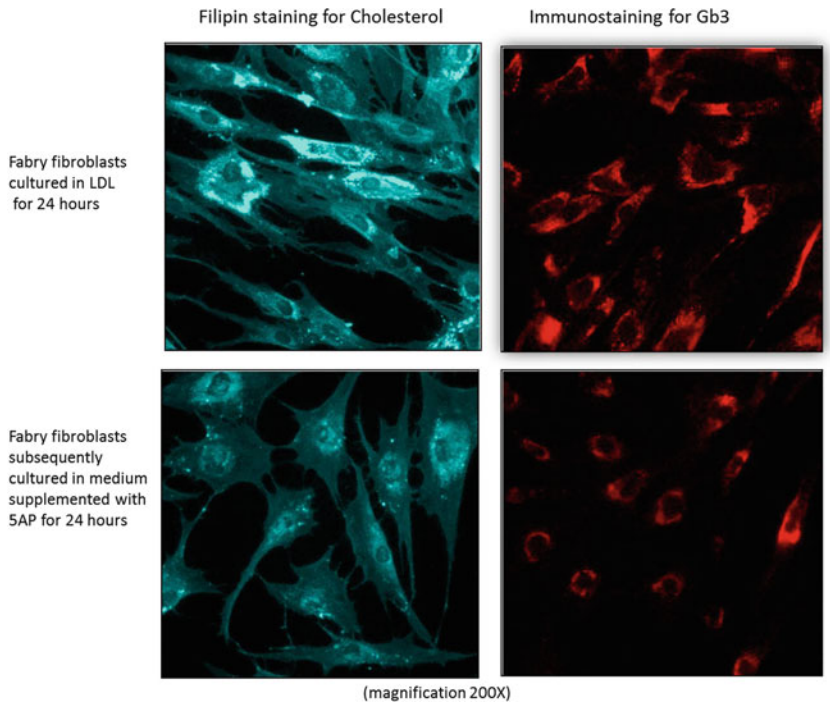

Fig. 3 Micrograph of Fabry fibroblasts stained with filipin for cholesterol, *left*, and Gb3, *right* shows a typical granular staining for lysosomes

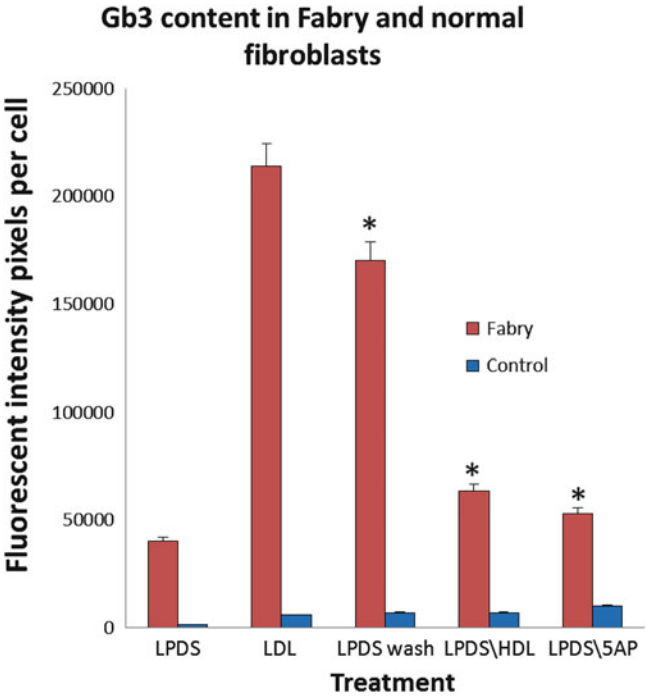

Fig. 4 Determination of Gb3 content in Fabry and control fibroblasts by image analysis. Cells were treated as described in methods, and the average intensity of pixels per cells was determined using I-vision software. The *error bars* indicate a 5% SE

Discussion

The postmortem findings of a male patient with Fabry disease, who was on ERT for more than 2 years, showed the presence of marked storage in cell types other than vascular endothelial cells, such as smooth muscle cells and pericytes. Accumulation of lipid after more than 2 years of enzyme infusions suggests that the infused enzyme has limited access to cells other than vascular endothelial cells (Schiffmann et al. 2006). Askari also found that substantial amounts of lysosomal and extralysosomal Gb3 immunoreactivity remains in cells and tissues even after years of enzyme replacement (Askari et al. 2007).

Our finding that cholesterol efflux induced by 5AP treatment reduces the accumulation Gb3 in Fabry fibroblasts offers promise for developing an improved treatment for patients with Fabry disease.

It is known that glycolipid storage promotes cholesterol accumulation and is often called cholesterol "trapping." Cholesterol can form a complex with glycolipids (GSL). For example, Fantani and coworkers (Fantini et al. 2013) found in the cholesterol–galactoceramide complex that the OH of cholesterol is a donor group that forms a hydrogen bond with the oxygen atom of the glycosidic linkage between galactose and ceramide. A similar hydrogen bond is formed between cholesterol and GM1. The other contacts between cholesterol and the GSL give rise to stabilizing van der Waals interactions between the apolar ceramide part of the GSL and cholesterol (Fantini et al. 2013). Cholesterol is thought to accumulate as a secondary storage product. This accumulation has been proposed to slow trafficking in the endosomal–lysosomal system, like a traffic jam in the lysosome. Blanchette-Mackie postulates that the late endosomal tubules that can be visualized by immunostaining or with a fluorescent tag become immobile and that depletion of cellular and lysosomal free cholesterol in Fabry fibroblasts correlates with reestablishment of the late endosomal tubular trafficking and results in mobilization of Gb3 from the lysosome (Joan Blanchette-Mackie, personal communication).

Glaros has shown that glycolipid accumulation inhibits the cholesterol efflux via the ABCA1/apolipoprotein A-1 pathway. The ApoA-1-mediated cholesterol efflux from fibroblasts derived from patients with genetic GSL storage diseases like Fabry disease was impaired compared with control cells (Glaros et al. 2005). We showed that the cholesterol uptake is also impaired in Fabry fibroblast. According to Brown and Goldstein, LDL receptor syntheses are shut down as a consequence of cellular cholesterol saturation (Brown and Goldstein 1975). Fabry fibroblasts do not slow down LDL-mediated cholesterol uptake and therefore cholesterol, as well as Gb3, accumulates in the lysosome. The effect of selective LDL apheresis in a Fabry patient was studied by Utsimi (Utsumi et al. 2006). They used this strategy to deplete the accumulated circulating Gb3.

It is known that circulating Gb3 is mostly transported by LDL particles in the plasma (Clarke et al. 1976; Chatterjee and Kwiterovich 1984). The patient that was treated by Utsimi suffered from severe Fabry-related pain and was able to remain without pain medication for 2–3 days after LDL apheresis. The markers for endothelial activation and inflammation were reduced as well.

We showed that the apolipoprotein A1 mimetic peptide 5AP mobilizes cholesterol and Gb3 from Fabry fibroblasts. We hypothesize that the apolipoprotein A1 mimetic peptide 5AP activates cholesterol efflux and effluxes Gb3 simultaneously. As a consequence of the lipid and cholesterol overload in Fabry fibroblasts, cholesterol and Gb3 form a complex in the lysosome as described by Fantani for other GSLs.

Whether the Gb3 can finally leave the body or is stored in a different compartment of the cell then the lysosome remains to be elucidated. We hope that Fabry mouse studies will help us to answer this question.

Synopsis

Cholesterol efflux facilitated by a mimetic apolipoprotein A1 peptide reduces globotriaosylceramide in lysosomes of Fabry fibroblasts.

Compliance with Ethics Guidelines

Conflict of Interests

Christine Kaneski, Alan Remaley, Stephen Demosky, Nancy Dwyer, Joan Blanchette-Mackie, John Hanover, and Roscoe Brady declare that they have no conflict of interest.

Ulrike Schueler has received a research grant from the Genzyme Corporation.

Informed Consent

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

Contribution

Ulrike Schueler designed, performed, and analyzed the experiments and wrote the manuscript.

Christine Kaneski grew the cells and provided technical support.

Alan Remaley provided the 5AP peptide and edited the manuscript.

Stephen Demosky provided technical support.

Nancy Dwyer provided imaging support.

Joan Blanchette-Mackie evaluated the experiments.

John Hanover evaluated the experiments and edited the paper.

Roscoe Brady evaluated the experiments, edited the paper, and mentored on the project.

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RESEARCH REPORT

Development of Metabolic Phenotype in Phenylketonuria: Evaluation of the Blaskovics Protein Loading Test at 5 Years of Age

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On behalf of the German Collaborative Study of Phenylketonuria (PKU)/Hyperphenylalaninaemia (HPA)

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Abstract *Background*: As part of the German Collaborative Study on Phenylketonuria (PKU)/Hyperphenylalaninaemia (HPA) Study Protocol, a Blaskovics protein loading test (180 mg phenylalanine (phe) protein equivalent per kg body weight and day for 72 h) had been applied to 145 children at the age of 6 months. For investigating possible age-related changes of metabolic phenotype, 51 of them received a 2nd loading test at 5 years of age.

Methods: Besides the analysis of blood phe levels, acidic phe transamination metabolites were quantified in urine.

Results: Compared to the 6-month data, the mean blood phe level 72 h after start of loading (Phe72) was found to be decreased by 7% ($P = 0.06$), whereas the mean urinary excretion (per 1.73 m^2 body surface and day) of 2-hydroxyphenylacetic acid was increased 1.9-fold ($P < 0.01$). Corresponding with these analytical data, the kinetic model constant k_{out} of metabolic plus renal phe disposal was found increased 1.3-fold in mean ($P < 0.01$).

In 3 of the 51 patients, Phe72 was very high at 6 months while in the medium range at 5 years, suggesting that catabolic states may mimic a more severe metabolic defect.

The blood phe level response of mild PKU (type II) was assigned identically at both ages in 7/9 patients. Diverging results were (i) response type III (mild hyperphenylalaninaemia) at 6 months and type II at 5 years and (ii) type II at 6 months and type III at age 5.

Conclusion: Renal elimination of OHPAA and phe tolerance increase significantly between the age of 6 months and 5 years, suggesting that, at least in childhood, formation and/or renal disposal of phe transamination metabolites may be major distal determinants of phe tolerance.

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Abbreviations

AV	Assigned value of PKU phenotype (Guldberg et al. 1998)
bs	Body surface
HPA	Hyperphenylalaninaemia
k_{out}	First-order kinetic constant of metabolic plus renal loss/disposal
OHPAA	2-Hydroxyphenylacetic acid
phe	Phenylalanine
PAH	Phenylalanine hydroxylase (E.C. 1.14.16.1)
pAH	*Para*-aminohippuric acid
Phe72	Blood phenylalanine 72 h after start of the loading test (morning of day 4)
PKU	Phenylketonuria (OMIM +261600)
PLA	Phenyllactic acid
PPA	Phenylpyruvic acid

$t_{1/2}$	Phenylalanine half-life (50% elimination time)
6ML	Loading test at age of 6 months
5YL	Loading test at age of 5 years

Introduction

Phenylketonuria (PKU) due to deficient activity of phenylalanine hydroxylase (PAH) is characterized by a very high degree of molecular heterogeneity, with 872 variants of PAH presently known (Blau et al. 2015). Among the first hints at genetic heterogeneity of this disease was the observation that children with measurable PAH activity in their liver had a higher phe tolerance than children with undetectable PAH activity (Bartholomé et al. 1975). These results of microenzyme assay on liver needle biopsy specimens are at the root of the German Collaborative Study on Phenylketonuria (PKU)/Hyperphenylalaninaemia (HPA) (Collaborative study of children treated for phenylketonuria (PKU) in the Federal Republic of Germany 1990) which was designed in 1976. On the whole, it consisted of studies in three fields: (i) differential diagnosis of subtypes of HPA by analysis of the clinical, metabolic and molecular phenotype as an aid to rational treatment; (ii) therapeutic outcome in terms of intellectual, behavioural and neurological development; and (iii) decisions on suspending or continuing dietary treatment at given ages (Lutz et al. 1990).

For defining their metabolic phenotype, at the age of 6 months of life (6ML), patients with PKU/HPA were loaded with natural protein, corresponding to 180 mg phe/kg bw/day over a period of three consecutive days (72 h). The analysis of acidic transamination metabolites in urine was made part of this test because possible benefits of such data for investigating heterogeneity and pathogenesis of PKU were still contemplated at that time (Chalmers and Watts 1974). Building on a preceding study (Lutz et al. 1982), the blood phe level 72 h after start of loading (Phe72) was identified in these studies as a reliable surrogate parameter of residual PAH enzyme activity as estimated by in vitro analysis of PKU cell lines of defined genotype. This finding strongly supported the hypothesis of a molecular basis of phenotypic heterogeneity of PKU/HPA and opened the way for predicting metabolic and clinical phenotypes from molecular data (Okano et al. 1991).

To investigate the stability of metabolic phenotype beyond 6 months of age, e.g. excluding possible cases of delayed PAH maturation, the same type of loading was repeated at the age of 5 years of life (5YL) in a subset of the 6ML patients. After description of the 6ML data (Lutz et al. 1990; Mönch et al. 1990; Langenbeck et al. 2009) and of preliminary results of 5YL (Schmidt et al. 1989), we here present for all 5YL study patients the analytical results of phe disposal in blood and of urinary excretion of acidic phe transamination metabolites. Synopsis of the 5YL blood and urine data suggests maturation of phe transamination and/or renal metabolite transport between 6 months and 5 years as the reason for both the predictability of phe tolerance from 2 years on (van Spronsen et al. 2009) and reliable classification of PKU/HPA through age 5 phe tolerance data (Güttler and Guldberg 1996).

Patients and Methods

Selection of Patients

For the 6ML study, 165 patients with PKU/HPA had been enrolled, and 155 received a protein loading test in the years 1978–1984. Because of incomplete sampling, only 145 loads were analysed, with 14 classified as response type II (Lutz et al. 1990). The detailed analysis of complete test samples included 134 study cases, and 40 more patients were tested during routine diagnostic workup in the years 1996–1998 and 2007 (Langenbeck et al. 2009). The protein loading of the study patients was repeated between 1983 and 1989 at age 5 years in 51 of the original patients and is described in the present communication.

Analytical Methods

Blood phe levels were determined by an amino acid analyser (Lutz et al. 1982). Urinary organic acids were quantified as trimethylsilyl derivatives with gas chromatography in a sample of the 24 h urine collection of day 3 (Mönch et al. 1990). The *PAH* gene mutations were determined as previously described (Zschocke and Hoffmann 1999).

Classification and Kinetic Analysis of Phenylalanine Blood Level Response

The protein loading test elicits three main types of phe blood level response (O'Flynn et al. 1980; Lutz et al. 1990) which in typical cases can be recognized from the data pattern over the 72 h observation period: response type I (classical PKU) shows a continuous increase beyond 1,200 μmol/L, and type II (mild PKU) starts with an increase of blood phe concentration up to around 1,200 μmol/L followed by a spontaneous decrease well below 1,200 μmol/L starting at 36–48 h of the loading, i.e. when intake of high doses of phe is still continued. Type III (mild hyperphenylalaninaemia) shows a data pattern fluctuating around 600 μmol/L. Therefrom, classification of HPA is done on the basis of the Phe72 values as

>1,200 μmol/L (type I), 600–1,200 μmol/L (type II) and <600 μmol/L (type III), respectively.

For kinetic analysis of blood phe level response during dietary treatment and protein loading tests, a model was built with the Windows™-based simulation and model analysis package ModelMaker 4 of Cherwell Scientific Ltd. (2000, now available at modelkinetix.com and exetersoftware.com). It comprises a single compartment with a single input, i.e. alimentary phe on 3 meals per day, and dual output, i.e. zero-order net protein synthesis and first-order, time-dependent metabolic plus renal phe disposal (dphe/dt = phe × k_{out}). In the following, this original model (Langenbeck et al. 2001) without activation is called kinetic model type 1. It fits reaction types I and III. Kinetic model type 2 with time-dependent, exponentially saturated activation of phe disposal (dphe/dt = phe × $A[1 - e^{B \times t}]$) (see Keen and Spain 1992) and maximal $k_{out} = A \times [1 - e^{B \times 3}]$ 72 h after start of loading fits reaction type II (Langenbeck et al. 2009).

For the present analysis, model parameters were set as follows: (i) mean body weight of girls and boys combined (bw), 7.63 and 18.94 kg at 6 months and 5 years, respectively (Neuhauser et al. 2013); (ii) net protein synthesis, 35 and 21 mg phe/kg bw/day at 6 months and 5 years, respectively (van Spronsen et al. (2009); (iii) predicted total body water, i.e. volume of distribution of phe, 0.588 and 0.602 L/kg bw at 6 months and 5 years, respectively (Wells et al. 2005); and (IV) rate of intestinal phe uptake k_{in} (d^{-1}): 2.1. At this latter value, the model fit for the mean time courses of types I, II and III was found closest, with the metabolic plus renal rate reaching a plateau.

To statistically distinguish kinetic types 1 and 2, the variable parameters of both models were estimated in parallel with the loading data by initial Simplex and final Marquardt optimization. For model 1, the initial phe blood level (mg/dL) and rate of metabolic plus renal disposal/loss k_{out} (d^{-1}) were optimized together. For model 2, A was empirically set to 5, and B was optimized together with initial phe blood level.

Urinary Excretion of Acidic Phenylalanine Metabolites

Spontaneous morning urine was collected on days 1, 2 and 5. A 24 h urine was collected from morning of day 3 till morning of day 4. Data of the latter sample are presented here.

Urinary excretion data are normalized to 1.73 m^2 body surface (bs) because this format has been shown in the Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study to be an age-independent parameter of renal net organic acid excretion in healthy subjects (Manz and Wentz 2000; Berkemeyer and Remer 2006), thus allowing identification of disease-related changes depending on age. Mean bs at 6 months and 5 years is taken as 0.3904 and 0.7809 m^2, respectively, by applying the height and body weight data of the German Health Survey for Children and Adolescents (KiGGS) Study (Neuhauser et al. 2013) and the mean from five different equations for estimating bs (www.halls.md/body-surface-area/refs.htm).

Data Analysis

After parallel optimization of the type 1 and 2 kinetic models of phe disposal, the kinetic type was assigned by comparing with Z statistics the R^2 values of model fit (Langenbeck et al. 2009). Data with statistically assigned kinetic type 2 are classified as response type II, else as response type I or III, respectively. With response type III data, because of their flat profile over time, it is not possible, as a rule, to detect statistically possible cases of activation.

The 6ML and 5YL analytical and model data were tested for normality by the non-parametric one-sample Kolmogorov-Smirnov test. If normality is not excluded, the data are compared by linear least squares regression with and without intercept (Phe 72, urinary OHPAA). The significance of observed quantitative changes is computed with the non-parametric Wilcoxon matched-pairs signed-rank test (Forthofer et al. 2007).

The SYSTAT 11 program package (2004) of Systat Software GmbH, D-40699 Erkrath, Germany, was used for statistical analysis and graphics.

Results

The study of the 51 5YL patients yielded 48 Phe72, 30 urinary OHPAA and 51 model constant k_{out} values. From these same patients are available at 6ML 48 Phe72, 44 urinary OHPAA and 49 model constant k_{out} data. Due to missing values in either series and exclusion of three 6ML patients because of their excessive Phe72 levels (see below), 43 Phe72, 24 urinary OHPAA and 46 k_{out} values remained for the final 6ML versus 5YL comparison.

Analysis of Phenylalanine Disposal

Two parameters characterize the time course of blood phe during protein loading tests, the observed Phe72 (Lutz et al. 1982; Okano et al. 1991) and the estimated first-order rate constant k_{out} of metabolic plus renal phe disposal. Both are closely correlated, with lower values of Phe72 corresponding to higher ones of k_{out} (Langenbeck et al. 2009).

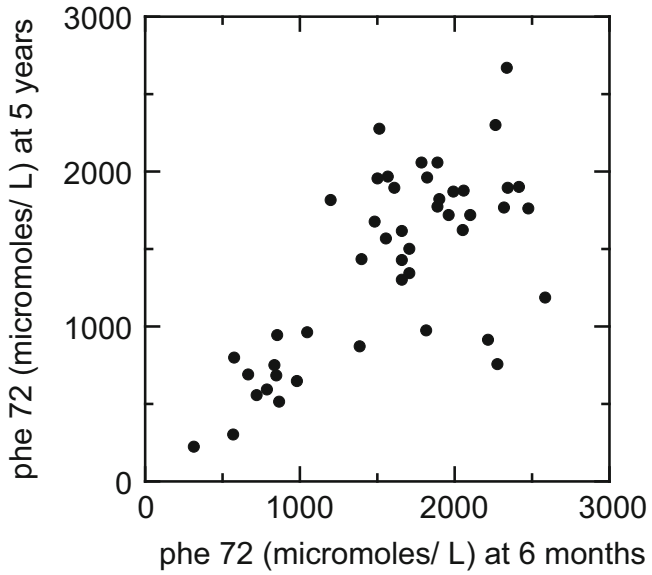

Fig. 1 Relation of the 72 h phe blood level (Phe72) at ages 6 months and 5 years of life. Linear least squares regression without intercept, the 3 outliers ignored: Slope = 0.9312 ± 0.0310 s.e., mult. $R = 0.98$, $N = 43$, $P < 0.01$

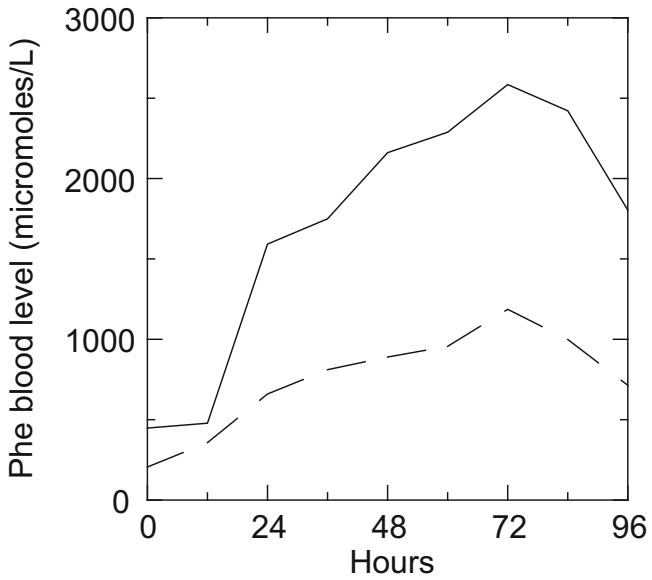

Fig. 2 Phe blood level response during protein loading tests of a patient with the genotype p.R261Q/c.1066-11G>A at 6 months (*line*) and 5 years (*dashed*). The Phe72 values are 2.585 and 1.186 μmol/L, respectively

The 43 6ML and 5YL Phe72 values are found in the same range. Linear least square regression (Fig. 1) indicates a 7% decrease of Phe72 at 5YL (Wilcoxon $Z = 1.89$, $P_{2\text{-tail}} = 0.06$). In contrast, the Phe0 values (μmol/L; mean ± s.d.) at the beginning of the test are almost identical in 6ML and 5YL: 448.4 ± 276.6 vs. 550.3 ± 245.2, respectively.

Consistent with the lower Phe72 values at 5 years of age, there is a 1.30-fold increase of k_{out} at this age (mean ± s.d.): 0.8946 ± 0.6111 vs. 1.1609 ± 0.7872, $N = 46$. In 38 of the 46 cases, k_{out} was found higher in 5YL, a highly significant difference (Wilcoxon $Z = 4.09$, $P_{2\text{-tailed}} < 0.01$).

Not included in this statistical analysis are three patients with Phe72 >1.200 μmol/L higher at 6ML than at 5YL. For one of them (Fig. 2), the genotype p.R261Q/c.1066-11G>A, with an AV of 3 (Guldberg et al. 1998), and the Phe72 at age 5 indicate a moderate to mild PKU. The genotypes of the two other patients are not known.

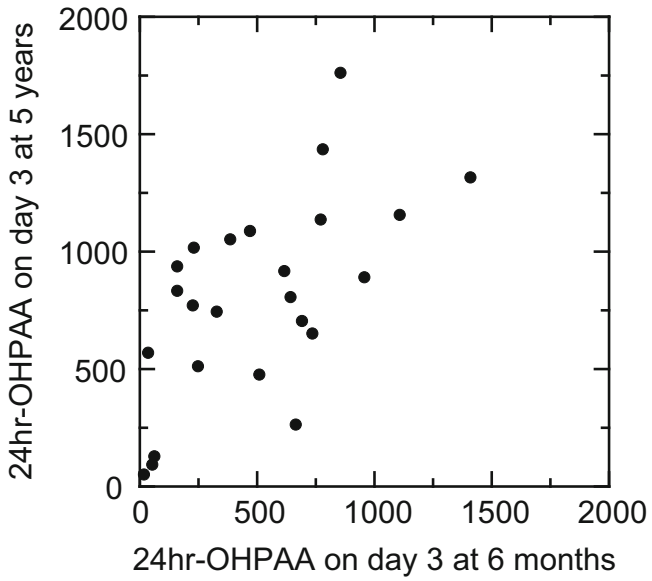

Fig. 3 Relation between urinary excretions of 2-hydroxyphenylacetic acid (μmol per 1.73 m^2 body surface area) on day 3 at 6 months and 5 years. Linear least squares regression with intercept, the 3 Phe72 outliers ignored: (OHPAA at 5 years) = (450.0 ± 119.8 s.e.) + (0.7025 ± 0.1927 s.e.) × [OHPAA at 6 months]; $N = 24$, mult. $R = 0.61$, $P < 0.01$

Catabolic states at the time of the first test (instead of delayed PAH maturation) most probably explain these observations.

Urinary Phenylalanine Metabolites

The urinary levels of the phe transamination metabolites phenylpyruvic acid (PPA), phenyllactic acid (PLA) and 2-hydoxyphenylacetic acid (OHPAA) are linearly related in older children to the logarithm of their corresponding plasma levels (Langenbeck et al. 1992). Analytically valid quantitative urine data of these metabolites may therefore be taken as proxy of the endogenous phe transamination capacity.

Besides PLA, which attains urinary concentrations in the range of PPA only after prolonged phe loads (Langenbeck et al. 1992), OHPAA is established as the analytically most stable phe transamination metabolite in urine (Dhondt et al. 1974) when gas chromatography with traditional derivatives is applied (Langenbeck et al. 1980). Therefore, urinary OHPAA is taken in the present communication as an indicator of phe transamination during the protein loading tests. Complete 6ML findings were reported by Mönch et al. (1990).

Compared to the 6ML findings, the 24 h urinary excretion of OHPAA on day 3 is increased 1.9-fold at 5YL, comprising 427 (18–1,409; 42) and 806 (51–1,761; 27) μmol per 1.73 m^2 bs area and day (mean; range; N), respectively; see Fig. 3. This positive difference is highly significant (Wilcoxon $Z = 3.11$, $N = 24$, $P_{2\text{-tailed}} < 0.01$) and consistent with the apparent age-dependent rise of phe disposal, as indicated by the changes of Phe72 and k_{out} shown above.

Recognition of the Response Type II

The blood phe level response type II was assigned statistically to 6 and visually to 2 of the 51 5YL data sets. One patient with response type II at 5YL was type III at 6ML, and one case with response type II at 6ML was response type III at 5YL. Two more patients were assigned response type III (mild hyperphenylalaninaemia) at both 6ML and 5YL. In summary, the blood phe level response type II was assigned identically to both ages in 7/9 patients, and no case of delayed PAH maturation was detected.

As shown in Table 1, the response type II patients carry genotypes characteristic of mild PKU, i.e. with AVs of 5–7 (Guldberg et al. 1998) and residual PAH activities around 60% (Blau et al. 2015). Of the 51 5YL patients, 9 (17.6%) manifested response type II at one or both ages which is close to the 6ML study data (19/125 = 15.2%), implying absence of a significant sampling bias in recruitment of the 5YL patients.

Discussion

As documented above, renal elimination of OHPAA and phe tolerance increase significantly between the age of 6 months and 5 years. Mechanistically, this could be

Table 1 Assignment of phenylalanine blood level response types II and III at 6 months (6ML) and 5 years (5YL) protein loading test by kinetic analysis (*) and visual classification (**)

PAT	6ML	5YL	Genotype	AV	% ACT
1	II*	II*	[p.Y414C] + [p.R408W]	5	59
2	II*	II*	[p.Y414C] + [p.R251Q]	5	57
3	II*	II**	[p.Y414C] + [p.P281L]	5	59
4	II*	II*	[p.Y414C] + [c.1315 +1G>A]	5	57
5	II*	II*	[p.R68S] + [c.165delT]	5	57
6	III**	II*	[p.L48S] + [p.R241H]	5(7)	62
7	II*	II*	n.d.	–	–
8	II*	II**	n.d.	–	–
9	II*	III**	[p.Y414C] + [p.R408W]	5	59
10	III**	III**	[p.E390G] + [c.842 +1G>A]	9	62
11	III**	III**	n.d.	–	–

AV: assigned value of PKU phenotype (Guldberg et al. 1998); % ACT: % PAH residual activity in vitro (Blau et al. 2015); *n.d.* not determined

explained with maturation of either phe transamination or renal organic acid transport. Also concomitant evolution of both systems may take place.

Phenylpyruvate is cleared in the kidney through proximal tubular secretion, mediated by the para-aminohippuric acid (pAH) transporter (Vink and Kroes 1961). The activity for its pAH substrate is low in newborns and reaches the normal values of older children only at the end of the first year (Bertram et al. 1970). In wild-type mice, the pAH clearance increases till 10 weeks of age. In adult mice, OAT1 is the principal pAH transporter (Sweeney et al. 2011).

If all phe transamination metabolites shared this transport system, their excretion in early life is expected to be low. Rey et al. (1974) first described the influence of age on excretion of OHPAA in children with PKU and found adult threshold values only after 2 years. Accordingly, metabolic data of PKU children older than 2 years conform with respective findings in juvenile patients (Langenbeck et al. 1980, 1992), and phe tolerance at 10 years of age can be predicted reliably from the respective data at 2 years (van Spronsen et al. 2009).

Whether delayed maturation of phe transaminase, as suggested by Rey et al. (1974), and/or delayed maturation of the pAH transporter explains the data could be decided by knowledge of phe metabolite blood levels. However, we only know of three such observations: Partington and Vickery (1974), using the enol-borate method for quantifying PPA, found in a 3-week-old infant plasma PPA levels in the range seen in older children. The urinary PPA levels, however, were very low. In contrast, using the trimethylsilyl-quinoxalinol method, Bebehani and Langenbeck (1980, unpublished) found in two 11-day-old infants very low plasma PPA levels in relation to plasma phe (1,501 vs. 11 and 2,700 vs. 50 μmol/L) but more 'normal' PPA excretion in relation to plasma PPA (39 and 291 μmol/mmol creatinine, respectively). Only detailed clearance studies in PKU infants and young children could resolve the joint effects of these mechanisms.

The intricate interplay of factors determining phe tolerance is documented in a study by Treacy et al. (1996) of two siblings with mild PKU (p.R408W/p.I65T). The child with the higher phe tolerance at 42 months (600 vs. 350 mg phe/day) excreted higher amounts of phe transamination metabolites into urine (10.9 vs. 4.9 μmol/kg × h), whereas the sibling with much lower excretion had plasma metabolite levels in the toxic range (220 vs. 134 μmol/L; cf. Langenbeck et al. 1992).

There are few reports only on the further development of juvenile, transitional and adult patients. Further studies would therefore be welcomed on therapeutically relevant aspects like the possible influence of obesity on phe tolerance (MacLeod et al. 2009), the degree of oxidative stress at elevated blood phe levels (Okano and Nagasaka 2013), the value of self-management in improving the patients' individual responsibility and adherence (ten Hoedt et al. 2011), the development and production of more palatable and satiating low-phe food products (van Calcar and Ney 2012) and the practicality of home monitoring (Wendel and Langenbeck 1996). Progress in these fields would contribute to converting a cumbersome disease into an aspect of self-determined life, like diabetes mellitus.

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Compliance with Ethics Guidelines

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975 (at the time of data collection) as revised in 2000 (at the time of data analysis). Prior to study

enrolment, all families were informed in written and oral form that the clinical-therapeutic study was in technical and formal accordance with legal data protection regulations, and informed proxy consent for participation was obtained from all of them. All data obtained were stored in pseudonomized form in a central database.

Conflict of Interest

Peter Burgard has received speaker honoraria from Vitaflo Ltd., Merck Serono GmbH and Swedish Orphan Biovitrum GmbH. Eberhard Mönch has received speaker honoraria from Vitaflo Pharma GmbH., metaX Institut für Diätetik GmbH, Cytonet GmbH & Co. KG and Swedish Orphan Biovitrum GmbH. Johannes Zschocke has received financial support for educational, research and diagnostic activities from Nutricia (Milupa) and Merck Serono. Udo Wendel and Ulrich Langenbeck declare that they have no conflict of interest.

Contributions of Individual Authors

P.B. curates the collaborative study's database and designed and edited the present communication, E.M. supervised the collection and analysis of urine samples, J.Z. analysed and verified the DNA data and U.W. helped interpret the clinical data and test results. U.L. developed and performed the model calculations. U.L. and P.B. drafted the report, and all other authors critically reviewed the report. All authors saw and approved the final submitted version. As the corresponding author, U.L. confirms that he had full access to the data and had final responsibility for the decision to submit for publication.

Animal Rights

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

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RESEARCH REPORT

The Lactose and Galactose Content of Cheese Suitable for Galactosaemia: New Analysis

P.A. Portnoi • A. MacDonald

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Abstract *Introduction*: The UK Medical Advisory Panel of the Galactosaemia Support Group report the lactose and galactose content of 5 brands of mature Cheddar cheese, Comte and Emmi Emmental fondue mix from 32 cheese samples. The Medical Advisory Panel define suitable cheese in galactosaemia to have a lactose and galactose content consistently below 10 mg/100 g.

Methods: A total of 32 samples (5 types of mature Cheddar cheese, Comte and "Emmi Swiss Fondue", an emmental fondue mix) were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) technology used to perform lactose and galactose analysis.

Results: Cheddar cheese types: Valley Spire West Country, Parkham, Lye Cross Vintage, Lye Cross Mature, Tesco West Country Farmhouse Extra Mature and Sainsbury's TTD West Country Farmhouse Extra Mature had a lactose and galactose content consistently below 10 mg/100 g (range <0.05 to 12.65 mg). All Comte samples had a lactose content below the lower limit of detection (<0.05 mg) with galactose content from <0.05 to 1.86 mg/100 g; all samples of Emmi Swiss Fondue had lactose below the lower limit of detection (<0.05 mg) and galactose between 2.19 and 3.04 mg/100 g.

Conclusions: All of these cheese types were suitable for inclusion in a low galactose diet for galactosaemia. It is possible that the galactose content of cheese may change over time depending on its processing, fermentation time and packaging techniques.

Communicated by: Alberto B Burlina, MD

Competing interests: None declared

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Introduction

In the last 15 years, the UK Galactosaemia Support Group (GSG) Medical Advisory Panel have reported eight separate lactose and galactose analyses on 134 samples of 15 cheese types (Portnoi and MacDonald 2009, 2013). They identified that seven types were suitable in a low galactosaemia diet: West Country Farmhouse Cheddar, Emmental, Italian Parmesan, Grana Padano, Gruyere and Jarlsberg. The UK GSG Medical Advisory Panel recommends cheese should have a lactose and galactose consistently below 10 mg/100 g for its inclusion in a low galactose diet. In this short paper, we report the lactose and galactose content of five brands of mature Cheddar cheese, Comte and Emmi Emmental fondue mix from 32 cheese samples. We do not report any cheese analyses that had a lactose/galactose content consistently above 10 mg/100 g.

Methods

Five samples of each cheese (exception Valley Spire West Country Cheddar Parkham) listed in Table 1 (five types of mature Cheddar cheese, Comte and "Emmi Swiss Fondue") were purchased from retail outlets or supplied by cheese makers from 2013 to 2014. They were prepared and

Table 1 The lactose and galactose content of cheese

Cheese	Information about manufacturer	Number of analyses	Lactose content (mg/100 g of cheese samples)	Galactose content (mg/100 g of cheese samples)
Valley Spire West Country Cheddar Parkham	Parkham farm: formerly part of the West Country Farmhouse Cheesemakers Group	2014: $n = 2$	<0.05 mg/100 g: $n = 2$ *Historical analysis of 10 other samples of West Country Farmhouse Cheese makers Cheddar were < lower limit of detection*	<0.05 mg/100 g: $n = 2$
Lye Cross Vintage Cheddar	Lye Cross Vintage Cheddar Maturation: aged >15 months	2013: $n = 1$ 2014: $n = 4$	<0.05 mg/100 g: $n = 4$ 1.3 mg/100 g: $n = 1$	<0.05 mg/100 g: $n = 4$ 12.65 mg/100 g: $n = 1$
Lye Cross Mature Cheddar	Lye Cross Mature Cheddar Maturation: aged >9 months	2013: $n = 1$ 2014: $n = 4$	<0.05 mg/100 g: $n = 5$	2.20 mg/100 g: $n = 1$ 2.44 mg/100 g: $n = 1$ 2.43 mg/100 g: $n = 1$ <0.05 mg/100 g: $n = 2$
Tesco West country Farmhouse Extra Mature Cheddar	Tesco West Country Farmhouse Extra Mature Cheddar	2013: $n = 2$	<0.05 mg/100 g : $n = 5$	2.48 mg /100 g: $n = 1$
	Made on Ford Farm on Ashley Chase Estate	2014: $n = 3$		2.55 mg/100 g: $n = 1$ 2.55 mg/100 g: $n = 1$ 2.79 mg/100 g: $n = 1$ 2.87 mg/100 g: $n = 1$
Sainsbury's TTD West Country Farmhouse Extra Mature Cheddar	Made on Barbers farm Made traditionally but packed in block form Maturation: aged >9 months	2013: $n = 1$ 2014: $n = 4$	6.82 mg/100 g: $n = 1$ <0.05 mg/100 g: $n = 4$	5.51 mg/100 g: $n = 1$ 10.15 mg/100 g: $n = 1$ 11.78 mg/100 g: $n = 1$ <0.05 mg/100 g: $n = 2$
Comte	Mild French mountain cheese using rennet to coagulate Maturation: aged >12 months	2013: $n = 5$	<0.05 mg/100 g: $n = 5$	<0.05 mg/100 g: $n = 2$ 0.43 mg/100 g: $n = 1$ 1.86 mg/100 g: $n = 1$ 1.48 mg/100 g: $n = 1$
Emmi Swiss Fondue	Packet mix of Emmental cheese, wine and potato starch to make Swiss cheese fondue	2013: $n = 1$ 2014: $n = 4$	<0.05 mg/100 g: $n = 5$	2.32 mg/100 g: $n = 1$ 2.19 mg/100 g: $n = 1$ 2.21 mg/100 g: $n = 1$ 2.22 mg/100 g: $n = 1$ 2.31 mg/100 g: $n = 1$

analysed by Leatherhead Food Research. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) technology was used to perform lactose and galactose analysis. The HPAEC-PAD had a limit of detection for lactose and galactose of <0.05 mg/100 g. Valley Spire West Country Cheddar Parkham was only analysed twice as this cheese was formerly part of the West Country Farmhouse Cheese makers group. Their Cheddar cheese had a consistently low lactose and galactose content from previous analysis (Portnoi and MacDonald 2009, 2013).

Results (Table 1)

Cheddar Cheeses All Cheddar cheeses (Valley Spire West Country, Parkham, Lye Cross Vintage, Lye Cross Mature, Tesco West Country Farmhouse Extra Mature, Sainsbury's

TTD West Country Farmhouse Extra Mature) had a median lactose and galactose content consistently below 10 mg/100 g (range <0.05 to 12.65 mg).

Comte All Comte samples had a lactose content below the lower limit of detection (<0.05 mg) and galactose content ranging from <0.05 to 1.86 mg/100 g.

Emmi Swiss Fondue All samples had a lactose content below the lower limit of detection (<0.05 mg) and a galactose content ranging from 2.19 to 3.04 mg/100 g.

Discussion

All of the cheeses reported (five specific brands of mature Cheddar cheese, Comte and Emmi Swiss Fondue) are suitable for inclusion in a galactosaemia diet. The testing of additional cheese types to examine their suitability for patients with galactosaemia is beneficial. This patient group is at risk of osteoporosis, and intake of calcium and vitamin D from a low galactose diet may be suboptimal. In the UK, the introduction of low lactose/galactose cheese has gained wide acceptance by patients (Ford et al. 2012).

In this paper, we excluded two brands of mature Cheddar cheese as the lactose/galactose content was consistently over 10 mg/100 g. Not all mature Cheddar cheese is processed in the same way. In the traditional manufacture of Cheddar cheese, it dries in large barrel shapes called truckles. The lactose content decreases as cheese dries over many months, which may be protected by a cloth only. The cheese may also be dried in blocks or covered in a rind, with extra lactose lost at this stage as the cheese dries naturally. However, in less traditional large-scale manufacture, the cheese may be packed in a plastic wrapper soon after production, with maturation occurring within the package. Consequently lactose is not lost within increasing maturity within the package.

In conclusion, this lactose and galactose analysis has expanded the range of the cheese types allowed in a low galactose diet. Using systematic and reproducible analysis, with a technique with a very low level of lactose and galactose detection, has enabled the inclusion of an expanded range of cheeses. It is important that professionals are fully aware of the suitable cheese types and understand the differences in cheese production so they can accurately advise and support their families with galactosaemia. The suitability of cheese may change over time depending on their processing and packaging techniques. More traditional manufacturing processes will continue to provide the lowest levels of lactose and galactose.

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Conflicts of Interest

Pat Portnoi has no conflicts of interest.

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Compliance with Ethics Guidelines

This study did not involve humans. It was a food analysis study only, so informed consent was unnecessary.

Details of the Contributions of Individual Authors

Pat Portnoi helped with study design, organised food analysis and interpretation of the data.

Anita MacDonald helped with study design, interpretation of the data and writing of the paper.

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CASE REPORT

Atypical Clinical Presentations of *TAZ* Mutations: An Underdiagnosed Cause of Growth Retardation?

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Abstract Barth syndrome is known as a highly recognizable X-linked disorder typically presenting with the three hallmarks: (left ventricular non-compaction) cardiomyopathy, neutropenia, and 3-methylglutaconic aciduria. Furthermore, growth retardation, mild skeletal myopathy, and specific facial features as well as mitochondrial dysfunction in muscle are frequently seen. Underlying mutations are found in *TAZ* and lead to defective cardiolipin remodeling.

Here, we report atypical clinical manifestations of *TAZ* mutations in two male patients initially presenting with growth retardation and very mild skeletal myopathy. As other phenotypic hallmarks were missing, Barth syndrome had not been suspected in these patients. One of them has been incidentally diagnosed in the frame of an in-depth cardiolipin research analysis, while the underlying genetic defect was unexpectedly identified in the second one by exome sequencing.

Conclusion: These cases underline that *TAZ* mutations might well be an underdiagnosed cause of skeletal myopathy and growth retardation and do not necessarily manifest with the full clinical picture of Barth syndrome.

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Abbreviations

BTHS	Barth syndrome
CL	Cardiolipin
UOAA	Urinary organic acid analysis

Introduction

Barth syndrome (BTHS, MIM #302060, for review Roberts et al. 2012; Clarke et al. 2013) is known as a distinctive clinical syndrome characterized by (cardio)myopathy, neutropenia, and 3-methylglutaconic aciduria (3-MGA-uria). Most prominent and often the key to diagnosis are the

cardiac features including left ventricular dilation, hypertrophy, and non-compaction, with varying degrees of congestive heart failure and endocardial fibroelastosis. Sudden cardiac death and ventricular arrhythmia have been reported. Cardiomyopathy can be apparent at birth, or even in utero, but mostly develops within the first year of life (Cardonick et al. 1997; Spencer et al. 2006). Exercise intolerance due to cardiac problems in combination with skeletal myopathy is common. Most patients have delayed milestones and muscular hypotonia but are ambulatory and do not show progression of these symptoms. Prepubertal growth delay is another frequent finding. Neutropenia varies between mild depression of neutrophils and complete absence and can be cyclic. Patients are at risk for overwhelming bacterial infections, especially in the newborn period and chronic aphthous stomatitis due to candida infections. More recently, a cognitive phenotype and typical facial features have been added to the phenotypic description of BTHS. Mutations in X-chromosomal *TAZ* encoding tafazzin underlie BTHS. Tafazzin is a transacylase located in the mitochondrial membrane and involved in cardiolipin (CL) remodeling. CL is a phospholipid in the mitochondrial membrane and as such holds a key position in mitochondrial energy metabolism and apoptosis. Heterozygous females are asymptomatic.

We describe two atypical patients with mutations in *TAZ* who initially presented with growth retardation and mild myopathy without any other cardinal features of BTHS such as cardiomyopathy, 3-MGA-uria, or neutropenia.

Patients and Methods

Patient 1

The male patient was born to healthy unrelated German parents after a normal pregnancy at 35 weeks of gestation with a birth weight on p10. Postnatal adaption was uneventful. A slight delay in motor development was already apparent during the first year. At the age of nearly 4 years, he was referred for evaluation of growth retardation (Fig. 1). Physical examination revealed chubby cheeks and a mild myopathy with positive Gowers' sign. Creatine kinase, leukocyte count, and serum lactate were normal;

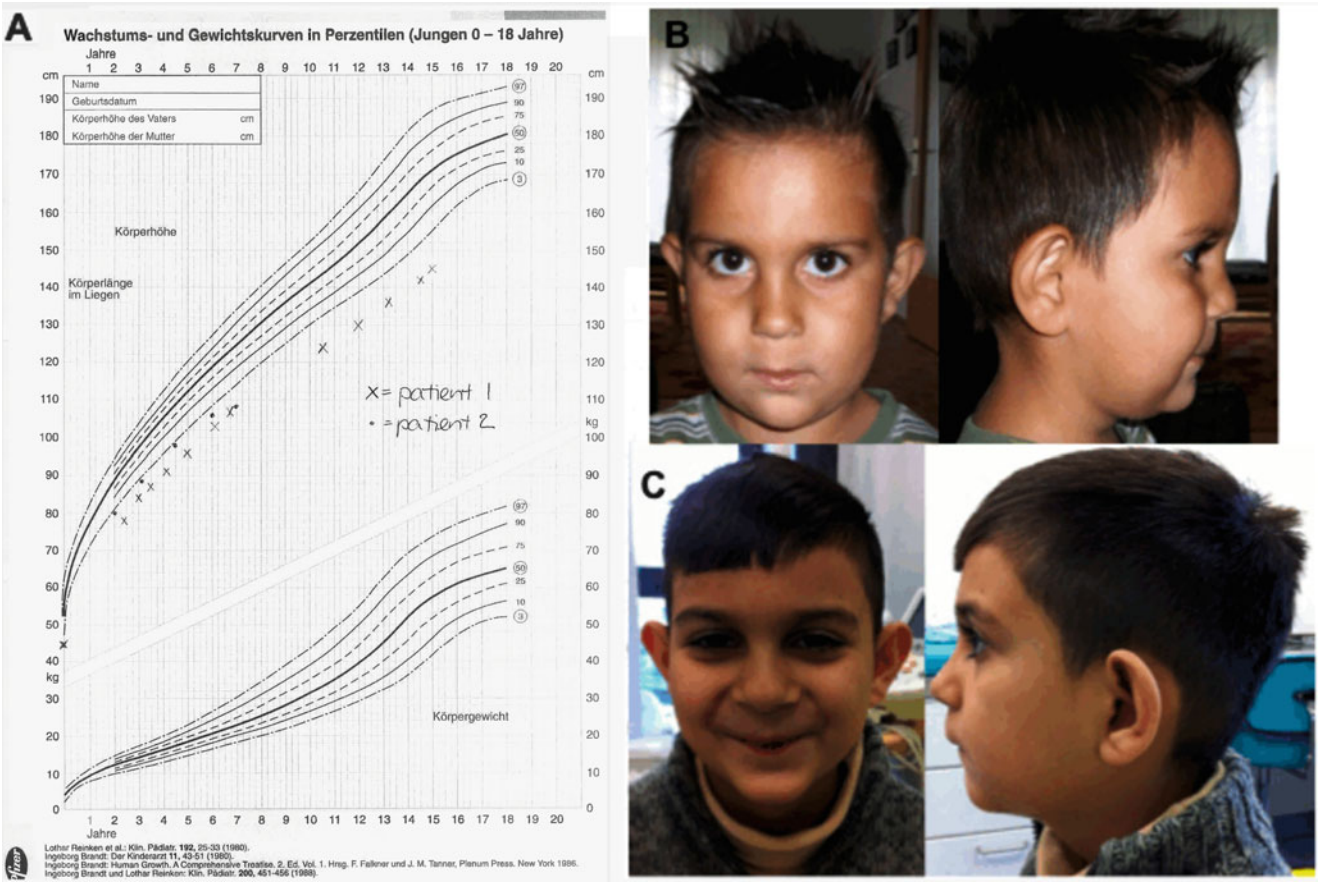

Fig. 1 Clinical findings in our patients. (**a**) Growth charts of patient 1 (X) and patient 2 (o). (**b**) Patient 2 at age six and (**c**) 10 years; note the chubby cheeks and large ears

urinary organic acid analysis (UOAA) was not performed. Over time myopathy became more apparent, as did the short stature. At the age of 5.5 years, serum lactate was elevated (4.9 mmol/l, normally <2 mmol/l); serum cholesterol, leukocyte count, and UOAA were normal. Based on the involvement of two organ systems (growth retardation and myopathy) and the elevated serum lactate, an oxidative phosphorylation system (OXPHOS) disorder was suspected, and a fresh muscle biopsy was performed. It showed strongly reduced mitochondrial energy producing capacity (ATP + CrP production from pyruvate 12.7 nmol/h/mUCS, reference range 42.1–81.2) with deficiencies of the mitochondrial complexes I (74%), III (27%), and IV (22%). Western blot showed a reduced amount of complex I (Fig. 1). Analysis of the OXPHOS in fibroblasts was normal. Fibroblasts were sent as control cells for validation of the method of CL analysis in muscle, without clinical suspicion of this diagnosis (the biochemical, but no genetic or clinical data are described in Houtkooper et al. 2009). CL analysis of muscle tissue showed the typical pattern of TAZ deficiency with a decrease in tetra-linoleoyl species of CL and an accumulation of monolyso-CL. Sanger sequencing of *TAZ* revealed a hemizygous frameshift mutation in exon 9 of *TAZ* (c.655_656insAAGT, p.(Asp219Glufs*6); RefSeq NM_000116.4) (Houtkooper et al. 2009). At the age of 7 years, 3-methylglutaconic acid (3-MGA) was found elevated for the first time (after more than five unremarkable UOAA), being 50 μmol/mmol creatinine (normally <20 μmol/mmol creatinine); leukocyte count was normal. Echocardiography was repeatedly normal until the age of 9 years when a diastolic dysfunction of the left ventricle was detected. Shortly afterward, the patient developed heart failure with a shortening fraction <20% and highly elevated NT-pro-BNP (2,002 ng/l). The end-diastolic diameter of the left ventricle was still within limits (37 mm, p75) as was the myocard. Cardiac function improved significantly under treatment with ACE inhibitors (NT-pro-BNP 1,136 ng/l).

Currently, the patient is 12 years old; his height is 129 cm (5 cm < p3). The proximal myopathy is stable; he is fully ambulatory, but has difficulties climbing stairs. He successfully attends regular school, joins sports classes, and never experienced frequent or serious infections. Recently, diuretics had to be added to cardiac treatment due to worsening shortening fraction (18%) and increasing NT-pro-BNP (2,346 ng/l). Still the dimension of the left ventricle is within limits (45 mm, p90), but end-diastolic diameter is increased.

Patient 2

A 6-year-old boy of Roma decent was primarily referred because of growth retardation. Length, weight, and head circumference had been normal at birth and crossed the p3 from age 12 months onward, remaining stable from then (Fig. 1). Upon physical examination, mild generalized muscular hypotonia without functional impairment was present. He had chubby cheeks and large ears (Fig. 1). His motor and intellectual development was age appropriate.

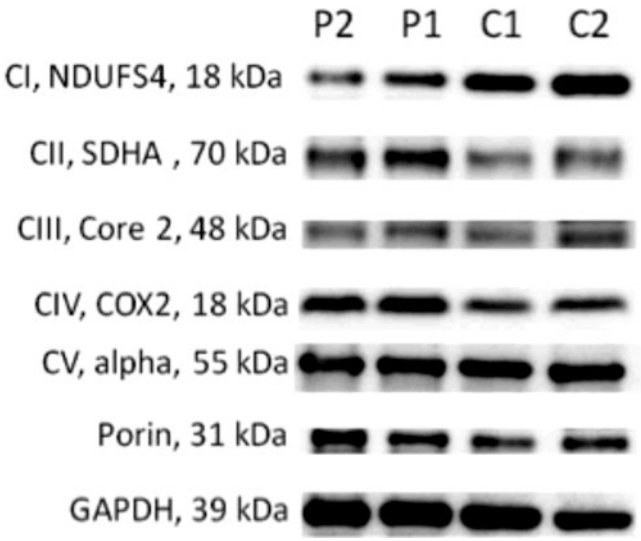

Fig. 2 Western blot analysis of oxidative phosphorylation enzymes in muscle. Western blot analysis (SDS-PAGE) of oxidative phosphorylation enzymes was performed by blotting 10 μg of protein loaded on an SDS-polyacrylamide gel and probing with the respective antibodies as previously reported (Feichtinger et al. 2014). A significant decrease was found in complex I in both patients

Gastrointestinal, endocrinological, and nutritional workup revealed no diagnostic evidence, and serum lactate, serum amino acids, creatine kinase, and UOAA were normal. MRI/MRS of the brain was unremarkable. At the age of nearly 8 years, triggered by febrile infection, the patient presented with acute episodes of painless muscular weakness and significant exercise intolerance. Clinical examination revealed muscular hypotonia and positive Gowers' sign in the absence of muscular atrophy. Metabolic tests as described above were repetitively normal as was myosonography.

The combination of unexplained muscular symptoms and persistent growth retardation prompted mitochondrial workup. A muscle biopsy showed reduced activity of complexes I (50%), III (50%), and IV (88%) and the oligomycin-sensitive ATPase (71%) in relation to citrate synthase (related to the lowest level of normal). The ratio of CCCP versus ADP-stimulated respiration of pyruvate + malate was increased to 1.43 (normally 1.00 ± 0.09) and therefore indicative for a deficiency in ATP synthesis, including the F_1F_0 ATP synthase, the adenine nucleotide translocator, and mitochondrial phosphate carrier. Western blot analysis showed a reduction especially of complex I (Fig. 2). In parallel, exome sequencing (performed essen-

Table 1 Clinical signs and symptoms seen in our patients compared with patients reported in literature

Clinical sign or symptom	Estimated frequency in Barth syndrome	P1 at initial presentation	P1 during the disease course	P2 at initial presentation	P2 during the disease course
Cardiomyopathy (any type)	70% in first year (n = NA (Clarke et al. 2013)), 91% (n = 16 (Rigaud et al. 2013)), 94% (n = 73, (Roberts et al. 2012))	–	+	–	–
Delayed motor milestones	61–72% (n = 73 (Roberts et al. 2012))	+	+	+	+
Neutropenia	69% (n = 73 (Roberts et al. 2012)), 73% (n = 16 (Rigaud et al. 2013)), 90% (n = NA (Clarke et al. 2013))	–	–	–	–
3-Methylglutaconic aciduria	50% (n = 16, (Rigaud et al. 2013)), 87% (n = 56 (Wortmann et al. 2013)), 100% (n = 6 (Ferri et al. 2013))	–	+	–	–
Growth delay	50% (n = 16 (Rigaud et al. 2013)), 58% (n = 34 (Spencer et al. 2006))	+	+	+	+

tially as described in Haack et al. 2014) revealed a known pathogenic hemizygous mutation c.281G>A, p.(Arg94His) in *TAZ* (RefSeq NM_000116). CL analysis in leucocytes was consistent with TAZ deficiency (CL 13 pmol/mg protein, normally >130, in Barth patients <30; monolyso-CL/CL ratio 3.43, normally <0.007, in Barth patients >2.52, performed as described in Bowron et al. 2013). Currently, at age 10 years, the patient shows persistent growth retardation (Fig. 1) and very mild exercise intolerance. The boy is able to participate in normal school activities, can run and play but complains that longer walks or hikes cause muscle fatigue and pain. Cardiac evaluation, leukocyte count, and UOAA were repeatedly normal.

Discussion

The two patients presented here did not exhibit the classical hallmarks of BTHS at initial presentation and also did not develop the full clinical phenotype during the course of disease (Table 1). Therefore, it would be more precise to describe them as being TAZ-deficient or to refer to a BTHS spectrum where these patients would be at the mild end.

Both males were referred for growth retardation. The additionally discovered muscular signs and symptoms were not perceived as limiting in daily life; however, one of the patients had a positive Gowers' sign at presentation, and the other developed it during the course. In none of them BTHS was suspected on clinical grounds. One of them has been incidentally diagnosed in the frame of an in-depth cardiolipin research analysis, while the underlying genetic defect was unexpectedly identified in the second one by exome sequencing.

In the first patient, a previously unreported predicted loss-of-function mutation was found, and its pathogenicity was proven by CL analysis (Houtkooper et al. 2009). Interestingly, the mutation found in the second patient, with growth retardation and very mild myopathy at age 10 years, has been described earlier in a severely affected patient presenting with fetal cardiomegaly from 32 weeks of gestation who died on day 12 of life (Brady et al. 2006). Of note, also this patient had never displayed 3-MGA-uria or leukopenia.

Muscle biopsies have been performed in both patients due to suspicion of a defect in mitochondrial energy metabolism. Interestingly, biochemical investigations revealed a clear combined defect of oxidative phosphorylation enzymes in both patients with a predominantly decreased amount of complex I. Investigation of mitochondrial energy metabolism in muscle tissue has only been reported from a very limited number of BTHS patients. The predominant decrease of complex I is in line with previously published data and can be explained by impaired remodeling of CL in these patients, which directly affects the respiratory chain structure and function (Dudek et al. 2013).

The presented cases extend the mutational and phenotypic spectrum of BTHS. More important, they underline that TAZ mutations do not have to manifest with the full phenotypic spectrum of BTHS. Additionally, these cases illustrate that unexplained growth retardation with involvement of a second organ system (e.g., myopathy) should prompt workup for a mitochondrial disorder. To avoid invasive procedures such as a muscle biopsy and given its increasing availability, exome sequencing should be considered an early diagnostic step in this clinical setting. Specific studies of CL metabolism may be valuable concerning the evaluation of unclear variants.

Compliance with Ethics Guidelines

Conflict of Interest

Charlotte Thiels, Martin Fleger, Martina Huemer, Richard J. Rodenburg, Frederic M. Vaz, Riekelt H. Houtkooper, Tobias B. Haack, Holger Prokisch, René G. Feichtinger, Thomas Lücke, Johannes A. Mayr, and Saskia B. Wortmann declare that they have no conflict of interest.

Informed Consent

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

Authors' Contributions

Saskia Wortmann designed the study, collected the clinical info, and wrote the paper. Charlotte Thiels, Martin Fleger, Martina Huemer, and Thomas Lücke were the attending clinicians and collected the clinical info. Tobias Haack and Holger Prokisch performed the exome sequencing on patient 2. Richard Rodenburg, Frederic Vaz, Riekelt Houtkooper, René G. Feichtinger, and Johannes Mayr performed the biochemical experiments. All authors revised the manuscript.

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RESEARCH REPORT

Long-Term Cognitive and Functional Outcomes in Children with Mucopolysaccharidosis (MPS)-IH (Hurler Syndrome) Treated with Hematopoietic Cell Transplantation

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Abstract The long-term cognitive and functional outcomes of children with mucopolysaccharidosis type I (MPS-IH) post-hematopoietic cell transplant (HCT) are not well documented, and the role of genetic and treatment factors in these outcomes has yet to be defined. In this multi-site, international study, we (1) characterize the cognitive and functional status of 47 individuals (ages 2–25, mean of 10.6 years) with MPS-IH who are 1–24 years post HCT (mean = 9 years) and (2) examine contributions of genotype, transplant characteristics, and sociodemographic factors to cognitive ability, adaptive behavior, and quality of life. The overall cognitive ability of our sample was mildly impaired, more than two standard deviations below general population norms. Parent reported adaptive behaviors (i.e., communication, daily living, and motor skills) were similarly impaired with a relative strength in socialization. Quality of life, as reported by parents, fell more than two standard deviations below population norms for physical functioning; however, psychosocial quality of life (emotional well-being) approximated population norms. In linear regression analysis, adjusted for demographic and treatment factors, mutation severity was associated with lower cognitive ability ($p = 0.005$) and adaptive functioning ($p = 0.004$), but not parent ratings of children's quality of life. Older age at HCT was associated with poorer physical quality of life ($p = 0.002$); lower socioeconomic status ($p = 0.028$) and unrelated bone marrow HCT ($p = 0.010$) were associated with poorer psychosocial quality of life. Implications for screening and early intervention for children at risk for poorer cognitive and functional outcomes are described.

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Introduction

Mucopolysaccharidosis type I (MPS I) is an inherited metabolic disorder caused by the absence or deficiency of lysosomal enzymes needed to degrade glycosaminoglycan (GAG). Progressive accumulation of GAG within the cells of various organs ultimately compromises their function. The severe form of MPS I (Hurler syndrome MPS-IH) usually presents in infancy. If left untreated, worsening neurological, cardiovascular, and respiratory problems result in death in early childhood. The standard of care for MPS-IH is treatment with hematopoietic cell transplantation (HCT) (Muenzer et al. 2009; Tolar et al. 2007).

While survival rates and short-term outcome are known to be improved by HCT, the long-term cognitive, adaptive, and quality of life outcomes of these children post transplant are not well understood. Additionally, genetic, demographic, and treatment-related factors that may influence these important functional outcomes have yet to be defined.

Prior reports on the cognitive and adaptive outcomes of children with Hurler syndrome have shown that most children continue to acquire cognitive skills after HCT, albeit at a slower rate compared to unaffected children. These studies suggest that, when performed early enough in the disease process, HCT improved or stabilized neurocognitive development and adaptive skills in most children, particularly in combination with enzyme replacement therapy, preventing progressive deterioration (Malm et al. 2008; Bjoraker et al. 2006; Eisengart et al. 2013). Nonetheless, the neurocognitive and adaptive outcomes after HCT for Hurler syndrome are highly variable (Aldenhoven et al. 2008). Several factors are suspected to play a role in this heterogeneity, including genotype, age at transplantation, graft source, and demographic factors, but their relative contributions have been incompletely studied (Grewal et al. 2003; Prasad and Kurtzberg 2010; Peters et al. 1998). A recent longitudinal multisite study (Aldenhoven et al. 2015) found that the neurodevelopmental outcome of patients with MPS-IH after HCT was determined in large part by the degree of central nervous system damage prior to transplant, with those showing higher baseline cognitive functioning and receiving early HCT showing better long-term outcomes.

The presence of a nonsense mutation on both IDUA (lysosomal enzyme alpha-L-iduronidase) alleles is known to cause severe Hurler syndrome (Matte et al. 2003). These nonsense mutations are believed to result in a complete lack of residual enzyme activity, causing rapid accumulation of lysosomal GAG storage, early onset of clinical signs, and rapid disease progression. Two of these mutations, W402X and Q70X, are particularly common, accounting for up to 70% of MPS I disease alleles, although there is considerable variation in frequency across racial/ethnic groups and countries (Terlato and Cox 2003). While the role of homozygous nonsense mutations in the clinical severity of Hurler syndrome is well established, their potential role in long-term cognitive and adaptive outcomes following transplantation has not been examined. Additionally, the role of other mutation types presumed to be severe (i.e., nonsense-deletion and deletion-deletion) in cognitive and functional outcomes is not known.

In this collaborative study between investigators in the United Kingdom and the University of Minnesota in the United States, we examined the cognitive status, adaptive functioning, and quality of life of 47 individuals with MPS-IH who were between 1 and 24 years status-post HCT. Additionally, we evaluated the relative influence of demographic, genetic, and transplant-related factors on these key outcomes.

Materials and Methods

Participants

The United Kingdom

Forty-nine participants with MPS-IH who were members of the Society for MPS and Related Diseases in the United Kingdom and previously transplanted at either the Royal Manchester Children's Hospital or Great Ormond Street Hospital were invited and agreed to participate in this study. The ethics board of these two participating medical centers reviewed and approved the protocol. A home visit was scheduled for interested families at which time informed consent was obtained and a psychologist administered the test battery described below. Medical background data related to transplant and treatments were obtained from the two participating medical centers. Two families were excluded because of incomplete data. One family with two siblings affected consented for only one child to participate. Two children were ultimately not able to participate due to significant health issues at the time of the study. Seven children were excluded from this analysis, as they were less than 1 year from HCT at the time of the study. Three additional children were excluded from analyses due to missing genetic mutation information. Thus, a total of 34 participants and their families from the United Kingdom were included in this research. Data were collected between 2004 and 2005.

University of Minnesota (UMN), the United States

Seventeen patients diagnosed with MPS-IH who were at least 1 year from having completed HCT and at least partially engrafted were selected from the pool of patients who were followed in our clinics after transplant. Written informed consent was obtained from parents at the time of the patient's assessment visit at the University of Minnesota Medical Center. Data were collected between 2004 and 2012. Cognitive and adaptive behaviors were collected as part of their clinical assessment, and quality of life assessments was collected as an additional research measure. Four participants were excluded due to lacking genetic mutation information, central for our study question.

Combined data from the United Kingdom and University of Minnesota yielded a total of 47 participants with MPS-IH that were at least 1-year post-HCT. Diagnosis of MPS-IH was made in accordance with the clinical guide-

lines at the time (Muenzer et al. 2009; Pastores et al. 2007), and all patients included in this study manifested early onset of severe symptoms prior to 2 years of age. All children were at least partially engrafted (i.e., 70% were > 90% engrafted; the remainder were 50–90% engrafted) and were transplanted between 1985 and 2007.

Measures

Cognitive Functioning

Children in the United Kingdom were administered the Griffiths Mental Development Scales (GMDS) (Griffiths 1996) for subjects from birth to 8 years of age, the Wechsler Intelligence Scale for Children Third Edition (WISC-III) (Wechsler 1991) for subjects 8 through 16 years of age, and the Wechsler Adult Intelligence Scale Third Edition (WAIS-III) for those ages 17 and over. The GMDS measures gross motor skills, personal-social development, hand and eye coordination, and performance, resulting in a general developmental quotient (GDQ) comparable to a Wechsler IQ (Luiz et al. 2001). Normative information for the GMDS is based on a national standardization sample representative of children between their second and eighth years of age in the United Kingdom (England, Wales, Scotland, and Northern Ireland) and Eire (Republic of Ireland). At the University of Minnesota, children under the age of 6 were given the Mullen Scales of Early Learning (Mullen 1995) ($n = 6$). Children older than 6 years of age were administered one of the Wechsler tests (WISC-IV or Wechsler Abbreviated Scale of Intelligence; $n = 9$) (Wechsler 1999) or were administered the Stanford-Binet Intelligence Scales Fifth Edition (Roid 2003) ($n = 8$; children who were generally more developmentally delayed). Full-scale IQ scores were collapsed across instruments to index cognitive ability with a mean of 100 and a standard deviation of 15 representing the average range of functioning and scores more than two standard deviations below the mean (i.e., <70) representing impairment.

Adaptive Behavior

The Vineland Adaptive Behavior Scales (VABS) (Sparrow et al. 1984) were used to measure children's daily functioning. The VABS is a widely used norm-referenced parent-report measure of personal and social sufficiency in the areas of communication, daily living skills, socialization, and motor function. These domains are combined to form an adaptive behavior composite (ABC) score. Similar to IQ scores, domain areas and the composite score have a mean of 100 and a standard deviation of 15 with two standard deviations below the mean representing clinical impairment. Two editions of the VABS are available, with a second edition published in 2005 (Sparrow et al. 2005) offering a normative update and revisions to scale items. Due to differences in the availability of these instruments across centers and the timing of data collection, all UK participants received the first edition of the test and the majority of participants in Minnesota received the second edition.

Quality of Life

The Child Health Questionnaire (CHQ) (Landgraf et al. 1996) PF-50 was administered to measure participant's physical and psychosocial well-being, as reported by parents. This 50-item survey has been widely used in studies of children with chronic illness and has established reliability and validity. While the CHQ is normed for parents of children 5–18 years of age, parents of all participants in the study completed this survey, given that none of our young adult participants ($n = 8$) were living independently and self-report ratings would have been questionable given estimates of their cognitive functioning. The CHQ assesses 14 physical and psychosocial domains: general health perceptions, physical functioning, role/social physical functioning, bodily pain, role/social-emotional functioning, role/social behavioral functioning, parent impact-time, parent impact-emotional, self-esteem, mental health, behavior, family activities, family cohesion, and change in health. Scales are transformed to a 0–100 scale, where 0 = the worst possible health state and 100 = the best possible health state. The individual scale scores are aggregated to derive two summary component scores: the physical functioning and psychosocial health summary scores. These scores are converted into norm-referenced *T*-scores with a mean of 50 and a standard deviation of 10. Poor quality of life has been defined as two standard deviations below the mean of the normative sample or a physical functioning or psychosocial health summary score <30.

Socioeconomic Status

The Hollingshead and Redlich (2-factor) classification of socioeconomic status (SES) was used (Hollingshead, unpublished manuscript 1975; Hollingshead 1957). Two raters (E.S. and E.B.) independently classified both the UK and the US families based on parental occupation and education with 94% agreement. A consensus was reached on the two disagreements. SES was categorized as ≥ 4 ("lower middle to lower socioeconomic class") versus <4 ("middle to upper socioeconomic class").

Genotype

Genotype was obtained from medical records and classified as known severe (homozygous for nonsense or deletion mutation or heterozygous for a combination of these) and

other (homozygous or heterozygous for missense mutation).

Statistical Analysis

Descriptive characteristics were examined by mutation type and in aggregate. Unadjusted comparisons were based on a *t*-test with unequal variance and Welch degrees of freedom. Separate multiple linear regression models were used to examine the adjusted influence of demographic (SES, sex of the child), treatment factors (age at transplant, time since transplant, radiation treatment (yes/no), type of transplant (related versus unrelated marrow versus cord blood), number of transplants (one versus more than one) and genetic factors (severe versus not severe mutation type)) on cognitive (IQ), adaptive functioning composite (VABS ABC), physical quality of life, and psychosocial quality of life. All analyses were adjusted for treatment center (i.e., UK center or UMN) and regression analysis of adaptive behavior functioning was further adjusted for version type (Vineland I or II). Robust variance estimation was used for confidence intervals and *p*-values. All statistical analyses were performed using R v3.0.3 (Pinheiro et al. 2014).

Results

Participants

Descriptive statistics for the 47 patients with available information regarding mutation type (severe versus other) can be found in Table 1. Participants were between 2.2 and 25.4 years of age at the time of evaluation and were transplanted at a mean age of 18.6 months (range of 6–44 months). Given the time at which diagnosis and treatment took place, only two participants (both at the University of Minnesota) received enzyme replacement therapy (ERT) as part of their conditioning regimen. Twenty-three percent of the total sample received total body irradiation as part of their conditioning regimen (6 at the UMN and 5 in the United Kingdom). The majority of participating patients were found to have known severe mutations (66%), and the remainder (34%) had other types of mutations. The specific mutation types that comprise these groups are reported in Table 2. Children with severe mutations were older at the time of assessment and further from transplant than those with other known mutations. Overall cognitive ability for participants fell in the mildly impaired range, with similar level of impairment reported in their adaptive skills, consistent with prior reports in the literature. Children's physical quality of life was rated as poor by parents, with the sample mean falling more than two standard deviations below the general population mean. In contrast, parent ratings of children's psychosocial quality of life fell within the average range and were not discrepant from general population norms, suggesting that parents perceive their children to be functioning well from a social-emotional-behavioral perspective.

Predictors of Cognitive and Adaptive Functioning

In multiple linear regression analyses, adjusted for demographic and treatment factors described above, overall cognitive ability was significantly associated with genotype, showing that those who had a known severe genotype scored more than one standard deviation lower (−16.76 points, 95% CI, −25.58 to −4.94, $p = 0.005$) on average for measures of IQ compared to those with a known other mutation. Specific transplant-related variables (i.e., type of transplant, number of transplants, age at transplant, time since transplant, total body irradiation treatment) and demographic factors (sex, SES) were not significant predictors in the multivariate model. Similarly, parent ratings of patient's adaptive functioning were also significantly associated with genotype, but not with demographic and treatment factors in multivariate analysis. Specifically, parents of patients with a severe mutation reported significantly lower adaptive functioning on average (−9.27 points, 95% CI, −18.33 to −0.20, $p = 0.045$).

Predictors of Quality of Life

Age at transplant was significantly associated with physical quality of life, with older age at transplant associated with poorer physical functioning (−8.10, 95% CI −13.16 to −3.05, $p = 0.002$). Parents of patients who received total body irradiation as part of their transplant regimen reported better physical quality of life (e.g., better physical functioning, fewer limitations due to physical difficulties) than children who did not have total body irradiation (13.88, 95% CI 5.12–22.64, $p = 0.002$). Within the psychosocial domain of quality of life, children from lower SES families (−5.35, 95% CI −10.15 to −0.57, $p = 0.028$) and those who received unrelated bone marrow HCT (−7.73, 95% CI −13.62 to −1.83, $p = 0.010$) were reported to have poorer psychosocial functioning.

Discussion

This study utilized a unique multi-institutional, international cross-sectional cohort of children with MPS-IH who were between 1 and 24 years post transplant to characterize the cognitive, adaptive, and quality of life outcomes of these patients. We investigated the role of mutation type, treatment factors, and demographic characteristics in these clinically important outcomes.

Table 1 Participant characteristics by mutation type

Covariate	Overall $N = 47$ *n* (%)	Severe mutation $N = 31$ *n* (%)	Other mutation $N = 16$ *n* (%)
United Kingdom	34 (72.3)	21 (67.7)	13 (81.2)
UMN	13 (27.7)	10 (32.3)	3 (18.8)
Female	18 (38.3)	12 (38.7)	6 (37.5)
Male	29 (61.7)	19 (61.3)	10 (62.5)
Transplant type			
HCT-related marrow	26 (55.3)	20 (64.5)	6 (37.5)
HCT-unrelated marrow	14 (29.8)	8 (25.8)	6 (37.5)
Cord blood	7 (14.9)	3 (9.7)	4 (25.0)
Total body irradiation	11 (23.4)	7 (22.6)	4 (25.0)
No GVHD	15 (31.9)	9 (29.0)	6 (37.5)
Known GVHD	18 (38.3)	13 (41.9)	5 (31.2)
Missing GVHD	14 (29.8)	9 (29.0)	5 (31.2)
Engraftment			
<=90%	9 (19.1)	8 (25.8)	1 (6.2)
>90%	33 (70.2)	20 (64.5)	13 (81.2)
Missing	5 (10.6%)	3 (9.7%)	2 (12.5%)
Single transplant	32 (68.1)	20 (64.5)	12 (75.0)
Two transplants	15 (31.9)	11 (35.5)	4 (25.0)
Vineland version I	35 (74.5)	22 (71.0)	13 (81.2)
Vineland version II	12 (25.5)	9 (29.0)	3 (18.8)
SES (Hollingshead)			
SES < 4 (middle to upper)	23 (48.9)	17 (54.8)	6 (37.5)
SES >=4 (low-mid to low)	24 (51.1%)	14 (45.2)	10 (62.5)
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
Age at time of evaluation (year)	10.5 (6.8)	11.4 (7.1)	8.9 (6.1)
Age at transplant (month)	18.5 (8.2)	18.8 (8.2)	17.9 (8.2)
Time since transplant (year)	9.0 (6.7)	9.8 (7.0)	7.4 (5.9)
IQ	67.1 (22.3)	61.1 (19.8)	78.9 (22.9)
Vineland adaptive behavior			
Communication	70.6 (23.9)	64.4 (23.2)	82.7 (21.0)
Daily living skills	63.1 (24.4)	61.3 (25.6)	66.6 (22.2)
Socialization	75.9 (20.7)	72.9 (22.8)	81.6 (14.8)
Adaptive composite	64.8 (21.6)	61.4 (21.8)	71.3 (20.4)
CHQ: PHS	24.1 (15.3)	25.1 (15.5)	22.3 (15.1)
CHQ: PSS	45.0 (11.2)	45.4 (11.6)	44.1 (10.7)

Note: *HCT* hematopoietic cell transplant, *GVHD* graft versus host disease, *SES* socioeconomic status (Hollingshead 2-factor), *IQ* intelligence quotient (from age-appropriate cognitive test), *CHQ* child health questionnaire, *PHS* physical summary scale, *PSS* psychosocial summary scale

Consistent with prior reports (Bjoraker et al. 2006; Souillet et al. 2003; Shapiro et al. 1995), the overall cognitive ability of our sample of children and young adults with MPS-IH was in the mildly impaired range, with a similar level of impairment reported in their adaptive skills. Mutation type was significantly and independently associated with both cognitive ability and adaptive skills, with those who had a known severe genotype scoring much lower on average than those with a known other mutation. Unlike prior reports (Aldenhoven et al. 2015; Peters et al. 1996; Guffon et al. 2009; Vellodi et al. 1997), age at transplant was not significantly associated with either IQ or adaptive functioning in this cohort. This may be due to restricted range of age at transplant as the majority of the

Table 2 Distribution of specific genotypes representing severe and other non-severe mutations

%	Severe mutation $n = 31$ Nonsense mutations or deletions	%	Other mutation $n = 16$ Missense mutations or one unknown
45.2	W402X/W402X	25.0	W402X/A327P
10.0	Q70X/Q70X	6.2	W402X/P533R
23.0	W402X/Q70X	6.2	W402X/S633L
3.1	W402X/R619X	6.2	W402X/T387R
3.1	W402X/R621X	6.2	A75T/A75T
3.1	W402X/Q561X	6.2	L490P/L490P
3.1	W402X/153delC	6.2	P533R/P533R
3.1	W402X/C49delC	6.2	Q70X/G208D
3.1	c.783delC/c.783delC	6.2	Q70X/A75T
3.1	Q70X/35del12	6.2	T388R/W402X
		6.2	Int9 2850g>t/A75T
		13.0	W402X/unknown

sample was transplanted at less than 2 years old, consistent with the modern standard of care. Surprisingly, this study did not replicate prior reports that have documented TBI as a risk factor for poor neurocognitive functioning. However, the absence of a relationship between this well-known risk factor and neurocognitive outcome is likely due to the relatively small size of the current sample and limited power for detecting such influences after adjusting for mutation type. Additionally, within this small sample of individuals who received TBI, some patients received brain-sparing techniques ($n = 2$), which may have further attenuated these relationships.

In examination of quality of life, individuals with MPS-IH appear to be doing well from a psychosocial standpoint, but are greatly affected physically. Mean physical quality of life fell more than two standard deviations below the general population mean, reflecting poor physical functioning and limitations in daily activities due to physical disability. In contrast, parent ratings of their children's psychosocial quality of life were average and not discrepant from the general population, suggesting that parents perceive their children to be functioning well from a social-emotional-behavioral perspective. Mutation type was not significantly associated with psychosocial or physical quality of life. Rather, specific demographic and treatment factors appeared to play a more prominent role. Even within the limited range of age at HCT in the current cohort, we found that every year older a child was at time of HCT was associated (on average) with almost one standard deviation poorer physical functioning. This is consistent with previous studies that have examined the relationship between age and health-related quality of life after HCT in children more broadly and may reflect developmental differences in the way in which both MPS and HCT are experienced and expressed by children as they age. Parents of children who had received total body irradiation as part of their transplant regimen were more likely to report better physical functioning than those who had not received this treatment, which may be secondary to better engraftment associated with use of total body irradiation conditioning in this cohort and consequently better disease and symptom management in the context of the available treatments and conditioning regimens during this time period. In more recent years, optimization of chemotherapy protocols has led to sustained donor-derived engraftment, reducing the need for total body irradiation for most children treated on modern treatment protocols, reducing the need for total body irradiation which has been consistently associated with poor neurodevelopmental outcomes.

Risk factors for poor psychosocial quality of life included lower socioeconomic status and having undergone an unrelated donor bone marrow HCT (as opposed to receiving bone marrow HCT from a donor relative). It should be noted that the significant contribution of socioeconomic status to psychosocial QOL remained even when adjusting for study center (UMN versus the United Kingdom) suggesting that SES is an important contributor to QOL in these children even after consideration of other contextual differences that exist between these samples (i.e., access to healthcare). The relationship between lower SES and parent/patient-reported poor quality of life has been well documented in other populations (Chen et al. 2006; Von Rueden et al. 2006; Kunin-Batson et al. 2014), but has not been examined in children with MPS disorders. Future prospective studies should examine the socioeconomic impact of MPS-IH on families to better understand whether socioeconomic disparities develop over the course of treatment and represent the economic toll of MPS-IH on quality of life. Such information may be useful for guiding intervention development and timing/delivery of interventions for at-risk families. Our findings regarding the role of transplant type (i.e., graft source) in quality of life are similar to some previous studies of children with hematologic malignancies (Phipps et al. 2002; Clarke et al. 2008), which have reported relatively poorer quality of life after unrelated donor bone marrow transplants when compared with recipients of transplants from matched sibling donors. While analyses were adjusted for GVHD, we did not have information about other potential complications that may differ between transplant types and may account for differences in quality of life after transplant.

Our results must be understood in the context of limitations. As this was a cross-sectional cohort including individuals from the United Kingdom, no information was available about cognitive functioning prior to transplant. A prospective, longitudinal design (including a pre-HCT assessment) would

have been needed to allow for a precise examination of genetic, treatment, and demographic factors influences changes over time in cognitive and adaptive functioning, as well as quality of life. We also lacked information on current health status (e.g., physical symptoms or neurological symptom severity) and physical mobility at the time of assessment and were thus not able to explore the role of such factors that are likely relevant to adaptive functioning quality of life after transplant. Such information would be important to include in future investigations of quality of life. While the CHQ is a validated and well-established measure for examining quality of life in children with chronic health conditions, use of an MPS-specific measure or health assessment tool (e.g., MPS Health Assessment Questionnaire) would have been beneficial to more precisely cover the diverse features and sequelae of MPS-IH. This is an important area for further study, and our team is actively working to develop and validate these tools at the University of Minnesota. It should also be noted that the majority of this cohort received bone marrow donor HCT, and few had received cord blood, consistent with the time frame during which transplants took place (i.e., 1985 and 2007). Given that cord blood transplants represent the current standard of care for children with MPS-IH, and there have been a number of advances in unrelated donor bone marrow transplant procedures since the time during which transplants took place for current study participants (e.g., advances in tissue type matching, enzyme delivery, and supportive care), our findings may not represent the long-term outcomes of children treated on these more modern treatment protocols. Nonetheless, our study has important strengths including our use of a multi-institution, international cohort from whom we have detailed information about treatment histories and mutation type, as well as performance-based cognitive testing. We also included caregiver ratings of functional adaptive skills and quality of life, important aspects of functioning that have been historically under reported in this population, and our sample includes a longer time frame for follow-up from transplant than most prior studies.

In summary, mutation type (i.e., homozygous for nonsense or deletion mutations or heterozygous for a combination of these) is significantly associated with both cognitive and functional adaptive outcomes post transplant, and thus mutation analysis may have relevance for early identification of children at risk for long-term severe neurocognitive impairment despite treatment. While recommendations for cognitive screening are beneficial for all children with MPS-IH, early assessment and routine monitoring appear particularly important for those with known severe mutations. Physical manifestations of MPS-IH appear to be a major hurdle in attaining good quality of life post transplant, whereas psychosocial functioning was consistent with population norms. Early intervention and adaptations for children's physical functioning are important, and research efforts aimed at developing efficacious interventions to improve the physical function of children with MPS-IH are needed. Further attention should also be paid to the assessment of socioeconomic risk factors to help at-risk families overcome barriers and access to resources needed for optimizing their child's psychosocial well-being.

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Synopsis

Mutation type (i.e., homozygous for nonsense or deletion mutations or heterozygous for a combination of these) is significantly associated with both cognitive and functional adaptive outcomes post transplant and may have relevance for early identification of children at risk for severe long-term neurocognitive impairment despite treatment.

Compliance with Ethics Guidelines

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed Consent

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

A.S. Kunin-Batson, led manuscript writing and scientific conceptualization.

E.G. Shapiro assisted in writing paper and scientific conceptualization and assisted in obtaining Minnesota data.

K.D. Rudser statistical analysis, writing and editing.

C. Lavery scientific conceptualization; IQ, data collection in the United Kingdom, manuscript review and editing.

K.J. Bjoraker scientific conceptualization; data collection in Minnesota, manuscript review and editing.

S. Jones provided medical data and collaborated with Ms. Lavery in getting study accomplished, manuscript review and editing.

R. Wynn provided medical data regarding transplant and participated in manuscript review and editing.

A. Vellodi provided medical data regarding transplant, manuscript review and editing.

J. Tolar provided medical data regarding transplant, manuscript review and editing.

P. Orchard provided medical data regarding transplant, manuscript review and editing.

J.E. Wraith (deceased) collaborated with Ms. Lavery in getting study accomplished.

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RESEARCH REPORT

Treatment with Mefolinate (5-Methyltetrahydrofolate), but Not Folic Acid or Folinic Acid, Leads to Measurable 5-Methyltetrahydrofolate in Cerebrospinal Fluid in Methylenetetrahydrofolate Reductase Deficiency

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Abstract *S*-adenosyl methionine, which is formed from methionine, is an essential methyl donor within the central nervous system. Methionine is formed by the enzyme methionine synthase for which 5-methyltetrahydrofolate (5-MTHF) and homocysteine are substrates. Patients with severe methylenetetrahydrofolate reductase (MTHFR) deficiency cannot make 5-MTHF and have extremely low levels in the CSF. As a consequence, methylation reactions in the CNS are compromised, and this is likely to play an important role in the neurological abnormalities that occur in MTHFR deficiency. Although treatment with oral betaine can remethylate homocysteine to methionine in the liver, betaine crosses the blood-brain barrier poorly, and CSF levels of methionine remain low. We report three patients with severe MTHFR deficiency (enzyme activity $\leq$1% of controls) who had undetectable levels of CSF 5-MTHF at diagnosis and while on treatment with either folic acid or calcium folinate. Only treatment with oral 5-MTHF given as calcium mefolinate at doses of 15–60 mg/kg/day resulted in an increase in CSF 5-MTHF.

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Background

Severe methylenetetrahydrofolate reductase (MTHFR) deficiency is a rare autosomal recessive condition leading to a wide spectrum of neurological symptoms, mainly encephalopathy, hypotonia, microcephaly, seizures, developmental delay and episodes of apnoea (Burda et al. 2015; Huemer et al. 2015). Hydrocephalus is an additional rare but recognised complication. The condition generally presents in infancy and is associated with a high morbidity and mortality.

MTHFR is an enzyme required for the formation of 5-methyltetrahydrofolate (5-MTHF), a form of folate able to cross the blood-brain barrier and which is necessary as a substrate for the remethylation of homocysteine to methionine by methionine synthase (Fig. 1). A deficiency of this enzyme results in a low methionine and hence a reduction in *S*-adenosylmethionine, with elevated homocysteine levels (Watkins et al. 2012). Although the pathology in MTHFR deficiency is not fully understood, *S*-adenosylmethionine, an important methyl donor, is required for the formation and maintenance of myelin in the brain; a defect in methylation is likely to contribute to the neurological sequelae seen in the condition (Jadavji et al. 2012). Additionally, raised levels of homocysteine may result in an increased risk of thrombosis.

If treatment is started early, it can improve the outcome for children with MTHFR deficiency, although the outlook is still often rather poor. The treatment consists of betaine, hydroxocobalamin and folate (in various forms) which work in combination to reduce homocysteine, increase methionine, and increase CSF folate levels (Diekman et al. 2014). Betaine lowers homocysteine and increases methionine levels by acting as a methyl donor for the remethy-

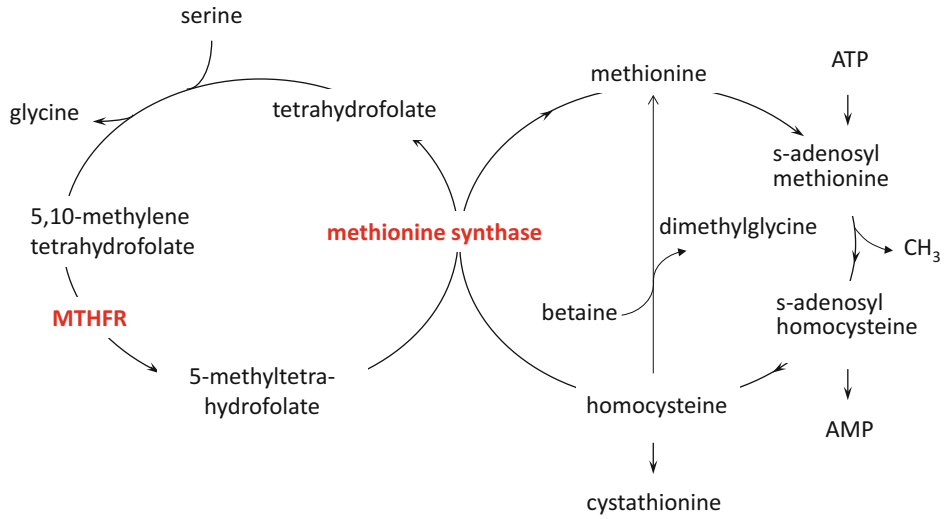

Fig. 1 Folate metabolism and related enzymes. *MTHFR* methylenetetrahydrofolate reductase

lation of homocysteine to methionine, although the homocysteine levels remain elevated above normal. Methylcobalamin is derived from hydroxycobalamin and, along with 5-MTHF, is a cofactor in methionine production. Folate is given in an attempt to increase 5-MTHF levels.

This report will focus on the various forms in which folate can be given in practice and their effects in MTHFR deficiency. In a mouse model of severe MTHFR deficiency, 5-MTHF given to mothers during pregnancy and lactation led to reduced mortality in the pups, but this effect was not seen with folic acid treatment (Li et al. 2008). A previous single case (reported in abstract form only) showed better clinical effects when treated with 5-MTHF compared with folinic acid but no change in the CSF 5-MTHF levels, which remained undetectable (El-Gharbawy et al. 2011). In this report we will present three unrelated patients with severe MTHFR deficiency whose CSF 5-MTHF remained undetectable when treated with folate or folinic acid but improved when this was changed to relatively high dosages of 5-MTHF, commercially available as calcium mefolinate.

Patient Cases

Patient 1

Patient 1 is a female born by normal vaginal delivery at term with a birth weight of 3.04 kg. She is the second child of healthy, non-consanguineous parents. She presented to her local hospital at 5 weeks of age with lethargy, reduced feeding and low temperatures. Treatment for sepsis with intravenous fluids and antibiotics was started but despite this she deteriorated further. A CT head showed areas of low attenuation but no acute changes. Blood tests revealed a raised homocysteine of 176 μmol/L (normal <18 μmol/L) and low methionine of <2 μmol/L (normal range 18–62 μmol/L). Urine organic acid analysis showed no increase in methylmalonic acid and CSF 5-MTHF was undetectable. A skin biopsy was undertaken which confirmed the diagnosis of MTHFR deficiency, with clear deficiency of fibroblast MTHFR activity of around 1 % of the mean control activity with no increase in the presence of added cofactor, flavin adenine dinucleotide. Molecular genetic investigations showed her to be homozygous for c.1781G>A in *MTHFR*.

Following diagnosis patient 1 was commenced on betaine (200 mg/kg/day), hydroxycobalamin (10 mg/day po) and folic acid (5 mg/day). She made a satisfactory clinical recovery from her acute presentation, and the plasma homocysteine and methionine improved being 110 μmol/L and 31 μmol/L, respectively, after 2 weeks. However, at 2 months of age, the CSF 5-MTHF was undetectable and folic acid was changed to calcium folinate. At 11 months of age, the CSF 5-MTHF was still undetectable; calcium folinate was then changed to mefolinate 15 mg/day. At 15 months of age on this treatment, CSF 5-MTHF had increased to 17 nmol/L. However, at 42 months of age, CSF 5-MTHF was again undetectable, but it transpired that due to a pharmacy error at 18 months of age, calcium folinate had been given instead of mefolinate for this period of time. Mefolinate was then restarted at 30 mg twice daily; repeat CSF 5-MTHF levels at 55 months of age had increased to 26 nmol/L (Table 1). At 5 years of age, she has normal speech and is fully mobile, attends a normal school and has only mild learning difficulties.

Table 1 CSF 5-MTHF concentrations and medication in our three patients

Patient	Age	Medication	Dose (mg/day)	CSF 5-MTHF (nmol/L)[a] (reference range 52–178 nmol/L)
1	2 months	Folic acid	5	0
	10 months	Calcium folinate	10	0
	1 year 3 months	Mefolinate	15	17
	3 years 10 months	Calcium folinate	60	0
	4 years 8 months	Mefolinate	60	26
2	3 months	Folic acid	5	0
	1 year 7 months	Mefolinate	15	18
	5 years 11 months	Mefolinate	45	33
3	5 years 8 months	Calcium folinate	15	0
	6 years 3 month	Mefolinate	15	18

[a] All CSF samples were collected at least 4–6 h after the last oral dose of medication. CSF 5-MTHF measurements were made by HPLC in the laboratory of Prof Simon Heales, London

Patient 2

Patient 2 is a male born by normal vaginal delivery at term. He is the first child of distantly consanguineous parents. He first presented at 20 days of age with a bronchiolitic illness and at this point was noted to be below the 0.4th centile for both weight and head circumference. At 8 weeks of age, he was readmitted with poor feeding and mild respiratory distress associated with an abnormal, jerky breathing pattern. He was also found to have some developmental delay – he had nystagmus and was unable to fix and follow and also had central hypotonia. Urine tests during admission revealed homocystinuria. Blood tests showed a raised homocysteine level of 204 μmol/L and low methionine level of 14 μmol/L. There was no increase in methylmalonic acid on urine organic acid analysis.

A diagnosis of 5-MTHFR deficiency was suspected and treatment with betaine (100 mg/kg/day), hydroxycobalamin (10 mg/day po) and folic acid (5 mg/day) was started. The plasma homocysteine and methionine levels responded quickly to treatment, with levels of 109 μmol/L and 64 μmol/L, respectively, within 3 days. However, the patient clinically deteriorated with intermittent sunsetting of the eyes, a bulging fontanelle and increasing head circumference. Cranial ultrasound showed dilatation of the lateral ventricles, and CT scan showed dilatation of all four ventricles with periventricular oedema. An MRI scan revealed further abnormalities with marked hydrocephalus, atrophy of forebrain, pons and medulla and severe hypoplasia of the cerebellum with opening of the inferior aspect of the fourth ventricle into the foramen magnum, indicating a Dandy-Walker malformation.

At 11 weeks of age, he underwent surgery for insertion of a ventriculoperitoneal shunt. CSF analysis at this time showed no sign of infection, but CSF 5-MTHF was undetectable. The diagnosis of 5-MTHFR deficiency was subsequently confirmed by skin biopsy with severely reduced MTHFR activity (0.7% of the mean control value) in extracts of cultured skin fibroblasts. Molecular genetic investigations showed the patient to be homozygous for c.1530G>A in exon 9 of *MTHFR*. This variant is not predicted to result in an amino acid change in the MTHFR protein, but because it changes the last nucleotide of exon 9, there was suspicion that it may affect splicing; this was confirmed by cDNA sequencing from cultured skin fibroblasts (Burda et al. 2015)

The patient continued on betaine treatment and the vitamin B12 was changed to oral administration (previously intramuscular). The folate treatment was changed to mefolinate 15 mg/day and a repeat CSF 5-MTHF level at 23 months of age was 18 nmol/L. The mefolinate has since been increased to 45 mg/day and the CSF 5-MTHF level has risen to 33 nmol/L.

Following insertion of the VP shunt, his breathing pattern, responsiveness and feeding improved. There was also an improvement on imaging, with reduction in size of the lateral and third ventricles and resolution of periventricular oedema. More clinical improvement was seen 1 month following surgery as he was able to fix and follow with no strabismus or nystagmus. Although there was some progression of development, his head circumference remained below the 0.4th centile and developmental milestones continue to be delayed. He sat without support at 18 months of age, crawled at 2 years and walked at 3.5 years. He acquired his first words at approaching 5 years of age. He has cortical visual impairment and has now developed an intermittent divergent squint. He also requires feeding via a gastrostomy.

Patient 3

Patient 3 is a female born by normal vaginal delivery at term with a birth weight of 2.8 kg. She is the third child of consanguineous (first cousin) parents. Her older siblings and parents are all healthy. There were concerns regarding microcephaly and development from an early age, as she could only sit unsupported at 1 year of age, stood without support at 18 months and walked with support at 5 years. By 5 years of age, she had not developed any expressive language. There were also reports of possible seizures at 8 months of age so she was commenced on sodium valproate.

At 5.5 years of age, she presented with a 6-week history of lethargy and poor feeding and required intubation due to deterioration with hypothermia and respiratory depression. Initial investigations were performed, and an MRI brain showed diffuse generalised atrophy of cerebral hemispheres, cerebellum, and brainstem, with reduced volume of both grey and white matter. Blood tests revealed a raised total homocysteine of 256 μmol/L and a methionine of 0 μmol/L. Urine organic acid analysis was normal and CSF 5-MTHF was undetectable. Subsequent studies on cultured skin fibroblasts confirmed showed MTHFR activity to be approximately 1 % of controls.

She was commenced on treatment with betaine (200 mg/kg/day), hydroxycobalamin (10 mg/day po) and folic acid (5 mg/day). Her level of consciousness improved over the next few weeks, but she required a tracheostomy and was ventilator dependent for 4 months. CSF 5-MTHF remained undetectable on folic acid and after being changed to calcium folinate 15 mg/day. She was then switched to mefolinate 15 mg/day, and a repeat CSF 5-MTHF was measurable with a level of 18 nmol/L, 7 months after presentation. The mefolinate has since been gradually increased to 45 mg/day.

On review 9 months after presentation, she was able to vocalise, use her hands and mobilise on her knees. Her muscle tone and reflexes were mildly increased in her arms and markedly increased in her legs. Her vision and hearing were normal. The sodium valproate was stopped at this time and there have been no concerns regarding seizures. On review at 7 years, she was able to take a few steps with the assistance of a walking frame. She was also able to say her name and demonstrated some understanding of language.

Discussion

The main treatment used to treat severe MTHFR deficiency is betaine, with the aim to increase CSF methionine levels and reduce blood homocysteine levels. However, despite betaine working to increase the plasma methionine levels, it crosses the blood-brain barrier poorly (Kempson et al. 2014). It is suggested that an increase in blood methionine and *S*-adenosylmethionine, as a result of betaine treatment, may provide a sufficient supply of methionine and *S*-adenosylmethionine to the CNS for methylation reactions and that providing this is the case the developing brain may be tolerant to low 5-MTHF (Strauss et al. 2007). Indeed, some patients with severe MTHFR deficiency diagnosed and treated early with betaine may do reasonably well (Diekman et al. 2014). Unfortunately long-term outcome in most other patients is poor, as demonstrated in our report by patient 2, who developed hydrocephalus, and by patient 3 who was diagnosed late. Diekman (Diekman et al. 2014) reviewed the published literature on the outcome of patients with MTHFR deficiency treated with betaine. They found that of the 36 patient included in their study, only those five patients treated early with betaine had satisfactory outcome both in terms of growth and cognitive development. However, the number of early treated patients reported by Diekman is small, and the period of their follow-up was only 1–3 years. Clayton et al. (1986) concluded that defective methyl folate metabolism is the key to neurological damage in this condition and that treatment should be directed towards maintaining methionine turnover as well as a supply of folate to the CNS. If folate levels within the brain remain very low, there may still be a risk of demyelination/subacute degeneration occurring later despite treatment with betaine. Consequently, it would seem appropriate to provide treatment with a form of folic acid that will both increase brain 5-MTHF in addition to using betaine to increase blood levels of methionine and *S*-adenosylmethionine that may cross the blood-brain barrier.

As demonstrated in our case reports, folic acid can be given in various forms and these have varying effects on CSF 5-MTHF levels. Commercial synthetic folic acid is slowly converted to tetrahydrofolate by dihydrofolate reductase and may inhibit the uptake of 5-MTHF into the brain and cause further clinical deterioration (Clayton et al. 1986). Calcium folinate is rapidly converted to tetrahydrofolate, but it can only cross the blood-brain barrier following further metabolism to 5-MTHF, a reaction that requires MTHFR activity (Levitt et al. 1971).

Schiff et al. reported six children with MTHFR deficiency (Schiff et al. 2011) One patient who previously had mild cognitive delay presented at the age of 11 years with an abrupt neurological deterioration. In this child CSF 5-MTHF increased to 15.5 and 10.4 nmol/L with oral 5-MTHF at 45 mg/day but subsequently on folinic acid at 20 mg/day still managed to maintain a level of 19 nmol/L. Since this patient had relatively late-onset disease, it is likely that he had some residual MTHFR activity that was able to covert some THF to 5-MTHF, hence the limited response to folinic acid treatment.

Although we were only able to achieve CSF 5-MTHF levels between 18 and 33 nmol/L with oral 5-MTHF in our patients, this may be sufficient. A patient diagnosed at 4 years with congenital folate malabsorption, a disorder in which there is folate deficiency and impaired transport of folate into the CNS, had at the time of diagnosis undetectable levels of CSF 5-MTHF (Torres et al. 2015). With twice daily intramuscular folinic acid, CSF levels increased to between 18 and 46 nmol/L. At 7 years of age, she had reached all developmental milestones and had a normal MR brain scan. The authors concluded that these levels of CSF 5-MTHF may be enough to eradicate CNS disease.

In conclusion, folic acid and calcium folinate both appear to be ineffective in increasing CSF levels of 5-MTHF in severe early-onset MTHFR deficiency. Treatment should include oral 5-MTHF as calcium mefolinate, rather than other forms of folic acid, and relatively high doses are required.

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Take-Home Message

In severe methylenetetrahydrofolate reductase deficiency, measurable 5-methyltetrahydrofolate in cerebrospinal fluid is only achieved with mefolinate (5-methyltetrahydrofolate) supplements and not with either folic acid or folinic acid**.**

Compliance with Ethics Guidelines

Conflict of Interest

Dr. Linzi Knowles, Dr. Andrew Morris and Prof. John Walter declare that they have no conflict of interest.

Informed Consent

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

This manuscript details the results of investigations and treatment that were undertaken as part of routine clinical care and not as part of a research study. Consent for treatment and routine investigations was obtained from the parents of all patients reported in this article.

Details of the Contributions of Individual Authors

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

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CASE REPORT

Abnormal Glycosylation Profile and High Alpha-Fetoprotein in a Patient with *Twinkle* Variants

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Abstract The *C10orf2* gene encodes Twinkle, a protein involved in mitochondrial DNA (mtDNA) replication. *Twinkle* mutations cause mtDNA deletion or depletion and are associated with a large spectrum of clinical symptoms including dominant progressive external ophthalmoplegia (adPEO), infantile-onset spinocerebellar ataxia (IOSCA), and early-onset encephalopathy. The diagnosis remains difficult because of the wide range of symptoms and lack of association with specific metabolic changes. We report herein a child with early-onset encephalopathy, unusual abnormal movements, deafness, and axonal neuropathy. All laboratory investigations were normal with the exceptions of high alpha-fetoprotein levels and an abnormal glycosylation profile. These abnormal parameters resulted in misdiagnosis as a previously unidentified congenital disorder of glycosylation (CDG) type I syndrome. Whole exome sequencing revealed two point mutations in *C10orf2* that were confirmed by Sanger sequencing; neither had been previously reported. This report enlarges the clinical phenotype of *Twinkle* mutations and suggests that an abnormal glycosylation profile suggestive of CDG type I associated with high blood alpha-fetoprotein levels without obvious cause should prompt *Twinkle* sequencing.

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Introduction

Mitochondrial DNA (mtDNA) depletion syndromes (MDS) are disorders with varied genetic origins and clinical symptoms, characterized by a severe reduction in mtDNA content. Genes associated with MDS encode proteins involved in maintenance of the mitochondrial nucleotide pool or in mtDNA replication (Copeland 2012; El-Hattab and Scaglia 2013), as observed for chromosome 10 open reading frame 2 (*C10Orf2*), also called *Twinkle*.

Twinkle mutations were initially described in patients who exhibited dominant progressive external ophthalmoplegia (adPEO) (Spelbrink et al. 2001) and infantile-onset spinocerebellar ataxia (IOSCA) (Nikali et al. 2005). The clinical symptoms of IOSCA are progressive; symptoms initially include ataxia, athetosis, and muscle hypotonia with loss of deep tendon reflexes, then ophthalmoplegia

and hearing loss, and finally sensory neuropathy and epilepsy. The onset of the disease is between 1 and 2 years of age (Koskinen et al. 1994). Recently, case studies of two children with early-onset encephalopathy and liver failure associated with *Twinkle* mutations were reported (Hakonen et al. 2007). The phenotype was close to Alpers hepatocerebral syndrome (Alpers 1931), which is caused by mutations in *POLG*. Thus, *Twinkle* mutations result in a highly nonspecific clinical spectrum, and no suggestive biochemical changes have thus far been identified, explaining in part why the diagnosis remains difficult.

Here we report on a patient who presented with early-onset encephalopathy, abnormal eye and limb movements, deafness, and axonal neuropathy very likely related to *Twinkle* mutations. Interestingly, an abnormal glycosylation profile of serum glycoproteins was observed, evoking a congenital disorder of glycosylation (CDG).

Case Description

This male infant was born from non-consanguineous parents at 38 weeks of gestation, after an uneventful delivery with a birth weight of 3,560 g (P50), a height of 49 cm (P50), and a head circumference of 35 cm (P50). The mother presented with a dengue infection during the first trimester of pregnancy. There was no significant family history, except for an early miscarriage. The first months of the infant's life were marked by regular vomiting. The child was hospitalized at 20 days of life for malaise ascribed to a severe gastroesophageal reflux. Concerns about neurological development were noted first at the age of 5 months, when the child presented with progressive hypotonia and psychomotor regression. The first assessment was carried out at the age of 7 months. At that time, generalized hypotonia, persistence of archaic reflexes, and motor incoordination were observed. Episodes of abnormal eye movements, sometimes with tongue protrusion, were also noted. Feeding difficulties became progressively more prominent with recurrent vomiting episodes. At the age of 11 months, the child had intermittent chorea, especially in the upper limbs and face, non-systematized eye movements with normal eye tracking and ophthalmoscopy, and absence of deep tendon reflexes. The head circumference growth was regular on −1 standard deviation, whereas growth of weight and height faltered from 7 months of age. There was no dysmorphic feature. The patient exhibited a progressive loss of contact, recurrent episodes of abnormal movements, and died at the age of 21 months.

At 1 year of age, brain and spinal cord MRI were normal. An MIBG scan (metaiodobenzylguanidine scintigraphy) was also performed to rule out neuroblastoma and did not reveal abnormalities. Echocardiography and abdominal ultrasounds were normal. Electroencephalogram showed a poorly organized spontaneous electric activity, without physiological background rhythm, but no focal or generalized discharges. Auditory evoked potentials showed a deep and bilateral sensorineural hearing loss.

Electromyography confirmed sensorimotor neuropathy of the four limbs, with neuropathy more pronounced on the lower limbs. A neuromuscular biopsy disclosed neurogenic muscular atrophy in conjunction with a moderate but progressive axonal neuropathy.

Extensive laboratory investigations (including urine and blood amino acids, urine organic acids, very long chain fatty acids, ceruloplasmin and copper, amino acids, glucose, and neurotransmitters in the cerebrospinal fluid [CSF]) were normal. Repeated plasma lactate measurements were normal. CSF lactate was normal (1.62 mmol/L) as was the CSF lactate to pyruvate ratio.

Alpha-fetoprotein (AFP) was measured as early-onset ataxia-telangiectasia was suspected. AFP levels were consistently high (>4,000 ng/mL, normal value: <100 ng/mL) in the absence of any liver failure or liver tumor. Molecular analyses of the *ataxia-telangiectasia* (*ATM*) and the aprataxin (*APTX*) genes were negative. Western blots of serum glycoproteins (Seta et al. 1996), were performed to rule out a CDG syndrome, and showed an abnormal glycosylation profile suggestive of type I CDG (Fig. 1). Both the *O*-glycosylation pattern and the apoC3 pattern were normal. PMM2-CDG, the most frequent type of CDG, was excluded because of a normal fibroblast activity of phosphomannomutase, and metabolic labeling on fibroblasts did not show accumulation of intermediate sugars. Sequencing of 16 genes known or possibly involved in CDG I (*MPI*, *ALG6*, *DPM1*, *ALG2*, *ALG7*, *ALG9*, *DK1*, *RFT1*, *ALG11*, *SRD5A3*, *ALG13*, *ALG14*, *ALG5*, *ALG10*, *DOLPP1*, and *DHDDS*) did not reveal any mutations, and there were no gene rearrangements in 94 genes involved in glycosylation on a custom CGH array. Taken together these results led to the diagnosis of CDG Ix. Unfortunately, transferrin isoelectric focusing was not performed at that time and remaining serum is not sufficient to perform this analysis.

Finally, a whole exome sequencing (WES) was performed, which revealed two heterozygous mutations in the gene *c10orf2*, encoding the mtDNA helicase Twinkle. These mutations were confirmed by Sanger sequencing. Each mutation was inherited from one parent. These two missense mutations in exon 1 (c.316G>A (p.Lys106Glu) and c.1181G>A (p.Arg394His)) were predicted to be deleterious by three of four functional effect prediction algorithms (PolyPhen-2, SIFT, Align GVGD, Mutation Taster). Neither mutation had been previously reported.

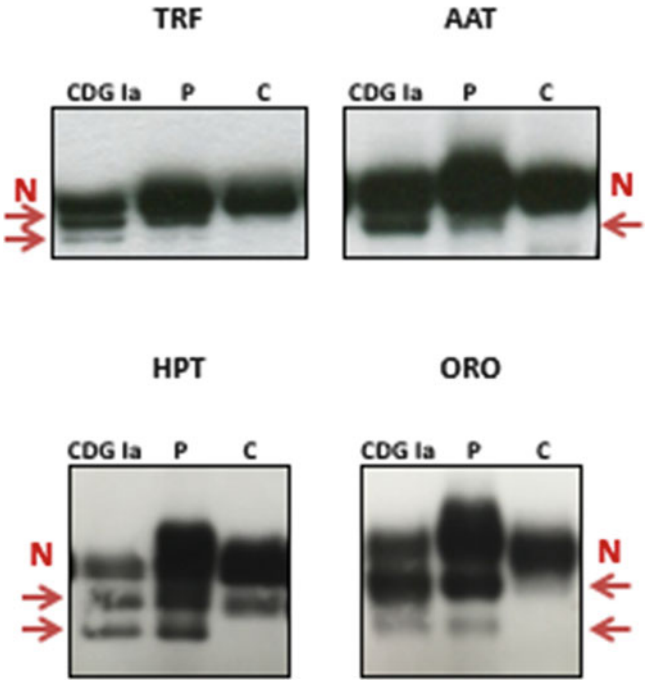

Fig. 1 For CDG I screening, an SDS-PAGE electrophoresis followed by western blotting for four serum *N*-glycan proteins (transferrin, TRF; alpha-1 antitrypsin, AAT; haptoglobin, HPT; orosomucoid, ORO) was performed. The profile from a CDG I patient (lane CDG Ia, the previous name for PMM2-CDG) revealed several bands corresponding to glycoforms missing one or more *N*-glycans (*arrows*). Proteins from the patient sample (lane P) presented abnormal profiles with a main normal band (N) and additional bands with lower molecular weight in comparison to the non-CDG I control (lane C)

Retrospectively, severe mtDNA depletion in skeletal muscle (90% depletion) was found. Respiratory chain activities measurements showed inconclusive results in the muscle (due to a long period of storage) and were normal in cultured skin fibroblasts.

Discussion

We report for the first time a patient with secondary glycosylation disorder and elevated AFP caused by previously unreported missense mutations in *Twinkle*. Despite this is a single case with newly reported mutations, the pathogenicity of these latter is highly probable, as (1) they were predicted as pathogenic by three different algorithms, (2) the clinical phenotype is very similar to early-onset *Twinkle*-related disorders, and (3) severe mtDNA depletion in skeletal muscle was found. In addition, we looked for mutations in all genes involved in CDG but did not find any. Given the delay since the patient's death, further functional validation was not possible.

The first patients described with *Twinkle* mutations were from a Finnish cohort with IOSCA also called Perrault syndrome (Koskinen et al. 1994; Lönnqvist et al. 1998). The described phenotype is that of a slow loss of acquisitions, starting with hypotonia, ataxia and athetosis, subsequent ophthalmoplegia, deafness and sensory neuropathy in adolescence, and very late epilepsy.

Hakonen et al. described a more severe disease associated with compound heterozygous *Twinkle* mutations in two brothers, who had very early-onset encephalopathy at around 6 months of age with abnormal eye, face, and limb movements, sensory neuropathy, and hypotonia (Hakonen et al. 2007). The evolution was marked by the emergence of intractable epilepsy and liver failure, mimicking Alpers syndrome described in patients with *POLG* mutations. The clinical story of our patient resembles that described by Hakonen et al. No seizures were reported for our patient, perhaps because of his death at 21 months of age; the reported patients presented their first seizures at 25 months and 3.5 years of age. AdPEO patients and IOSCA patients with *Twinkle* mutations were described with mtDNA deletions in skeletal muscle, but Hakonen's patients had mtDNA depletion instead of deletion, mainly in the brain and liver samples, but also in the muscle. Similarly, our patient exhibited severe mtDNA depletion in the muscle.

High AFP was the only liver abnormality in our patient. In other patients with *Twinkle* mutations reported to have liver failure, AFP levels were not mentioned. AFP may be elevated in non-mitochondrial disorders such as hepatocarcinoma or any cause of liver regeneration, ataxia-telangiectasia, and ataxia with oculomotor apraxia. All these

possibilities were investigated and eventually ruled out. There are no reported links between mitochondrial disease and elevated AFP, apart from situations of liver failure and/or liver regeneration, which was not the case for our patient. This elevated AFP could be an early sign of liver disease, before liver failure with elevated transaminases and protein synthesis decrease.

Secondary abnormalities of protein glycosylation have been reported in patients with liver disease, alcohol intake, hereditary fructose intolerance, galactosemia, and hemolytic uremic syndrome (Helander et al. 2001; Jaeken 2011). In addition, there are some nonspecific CDG presentations that could mimic mitochondrial disease. In contrast, this is the first description of abnormal CDG profile in mitochondrial disease. The lack of specificity of the clinical manifestations presented by our patient initially led to extensive diagnostic investigations. These investigations revealed no abnormalities suggestive of mitochondrial disease, specifically no lactate accumulation. The only biochemical abnormality was an atypical glycosylation defect, suggestive of CDG type I. The absence of mutations or micro-rearrangements in known CDG genes led us to perform WES. It has been shown that mitochondria play an important role in glycosylation, and *O*- and *N*-glycan chain sialyltransferases and glycosyltransferases are located in the outer mitochondrial membrane (Gasnier et al. 1991a, b). We speculate that mtDNA depletion could disorganize mitochondrial membranes and therefore disrupt glycosylation processes. Conversely, glycosylation abnormalities are known to affect mitochondrial function (Tan et al. 2014). Further experiments are necessary to study the interplay among *Twinkle* mutations, mtDNA depletion, and glycosylation abnormalities. A systematic investigation (even retrospectively) of glycosylation defects in *Twinkle* patients might reveal other patients with similar abnormal glycosylation profile.

In conclusion, these data further expand the phenotype of *Twinkle* mutations and suggest that high AFP along with abnormal CDG type I profile in absence of other cause should prompt *Twinkle* sequencing.

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Take-Home Message

Twinkle mutations can be associated with elevated alpha-fetoprotein and an abnormal CDG profile.

Compliance with Ethics Guidelines

Conflict of Interest

Juliette Bouchereau, Sandrine Vuillaumier Barrot, Thierry Dupré, Stuart E. H. Moore, Ruxandra Cardas, Yline Capri, Pauline Gaignard, Abdelhamid Slama, Hélène Ogier de Baulny, Nathalie Seta, Manuel Schiff, and Laurent Servais declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

Juliette Bouchereau analyzed the results and wrote the paper.
Sandrine Vuillaumier Barrot performed the experiments.
Thierry Dupré performed the experiments.
Stuart E. H. Moore performed the experiments.
Ruxandra Cardas performed the experiments.
Yline Capri performed the experiments.
Pauline Gaignard performed the experiments.
Abdelhamid Slama performed the experiments.
Hélène Ogier de Baulny performed the experiments.
Nathalie Seta performed the experiments.
Manuel Schiff wrote the paper.
Laurent Servais wrote the paper.
Manuel Schiff and Laurent Servais contributed equally to the manuscript.

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RESEARCH REPORT

Erratum to: LC-MS/MS Analysis of Cerebrospinal Fluid Metabolites in the Pterin Biosynthetic Pathway

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The last row in Table 2 was incorrectly typeset in the original publication; the correct values are provided in the table below:

Table 2 Assay precision, linearity, and limit of detection (LOD)

		Intra-assay ($n = 10$)		Inter-assay ($n = 20$)			
Analyte (nmol/L)	Internal standard	Level 1 (CV%)	Level 2 (CV%)	Level 1 (CV%)	Level 2 (CV%)	Linearity	LOD
BH4	^{15}N-BH$_4$	55.1 ± 1.7 (3.0)	156.1 ± 7.2 (4.6)	50.2 ± 2.9 (5.9)	149.0 ± 6.2 (4.2)	3–200	1
BH2	^{15}N-BH$_2$	41.0 ± 1.8 (4.4)	170.9 ± 5.9 (3.5)	38.6 ± 3.2 (8.2)	152.7 ± 12.8 (8.4)	3–200	1
Neopterin	^{15}N-Neopterin	35.6 ± 2.0 (5.6)	160.2 ± 7.6 (4.7)	29.1 ± 2.8 (9.5)	141.3 ± 10.3 (7.3)	3–200	1
Sepiapterin	^{15}N-BH$_2$	17.5 ± 0.9 (5.3)	101.3 ± 4.4 (4.3)	23.1 ± 3.3 (14.4)	119.7 ± 15.0 (12.5)	3–200	1

Data expressed as nmol/L, mean ± standard deviation (coefficient of variation, %)

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RESEARCH REPORT

Erratum to: Treatment with Mefolinate (5-Methyltetrahydrofolate), but Not Folic Acid or Folinic Acid, Leads to Measurable 5-Methyltetrahydrofolate in Cerebrospinal Fluid in Methylenetetrahydrofolate Reductase Deficiency

L. Knowles · A.A.M. Morris · J.H. Walter

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The final sentence of the abstract should read 'Only treatment with oral 5-MTHF given as calcium mefolinate at doses of 15–60 mg/day resulted in an increase in CSF 5-MTHF'.

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