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RESEARCH REPORT

Establishing New Cut-Off Limits for Galactose 1-Phosphate-Uridyltransferase Deficiency for the Dutch Newborn Screening Programme

E.A. Kemper • A. Boelen • A.M. Bosch • M. van Veen-Sijne • C.N. van Rijswijk • M.J. Bouva • R. Fingerhut • P.C.J.I. Schielen

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Abstract Newborn screening for classical galactosemia in the Netherlands is performed by five laboratories and is based on the measurement of galactose 1-phosphate-uridyltransferase (GALT) activity and total galactose (TGAL) in heel prick blood spots. Unexpected problems with the GALT assay posed a challenge to switch to a new assay. The aim of this study was to make an analytical and clinical evaluation of GALT assays to replace the current assay and to establish new cut-off values (COVs).

First, the manual assay from PerkinElmer (NG-1100) and the GSP assay were compared by analyzing 626 anonymous heel prick samples in parallel. Secondly, a manual GSP method was evaluated and 2,052 samples were compared with the automated GSP assay. Finally, a clinical evaluation was performed by collecting data from 93 referred newborns.

No satisfactory correlation was observed between GALT activity measured with the manual NG-1100 assay and the automated GSP assay. An acceptable correlation was found between the manual and automated GSP assay. Intra- and inter-assay variation of the automated GSP were 1.8–10.0% and 3.1–13.9%, respectively. Evaluation of clinical data demonstrated that adjusting the COVs for GALT to 2.0 U/dl and TGAL to 1,100 μmol/l improved specificity of screening for classical galactosemia.

An assay designed for automated processing to measure GALT activity in heel prick samples works equally well when processed manually. We therefore adopted both methods in the Dutch screening laboratories. As a result of this evaluation new COVs for GALT and TGAL have been introduced and are valid from July 2015.

Communicated by: Piero Rinaldo, MD, PhD

E.A. Kemper and A. Boelen contributed equally to the preparation of this manuscript.

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Introduction

Classical galactosemia (OMIM 230400) is an autosomal recessively inherited disorder of galactose metabolism caused by a severe deficiency of galactose 1-phosphate-uridyltransferase (GALT) enzyme (GALT; EC 2.7.7.12), (Isselbacher et al. 1956). Its incidence in Western Europe has been estimated to be between 1:23,000 and 1:44,000 (Bosch 2006). Affected children develop a severe illness with feeding difficulties, liver failure, *Escherichia coli* sepsis, and cataracts after ingestion of galactose from breast milk or infant formula in the first weeks of life. Timely treatment, consisting of a strict restriction of dietary galactose, is lifesaving, rendering this disease well suited for newborn screening.

The Dutch newborn screening for classical galactosemia, which was implemented in 2007, is primarily based on the measurement of GALT activity in filter paper blood spots. In heel prick samples with an activity level at or below 20% of average GALT activities of daily experimental batches, total galactose (TGAL) concentration was determined in the same blood sample (cut-off value (COV) for positive screening result and clinical referral was 700 μmol/l blood). The five Dutch screening laboratories aim to work with the same laboratory equipment and assays if possible to use the same, nationwide, COVs. For the galactosemia screening, three laboratories have experience using an automated GALT assay (PerkinElmer, 3303-0010-assay using the GSP analyzer) while two laboratories use a manual (Bio-Rad) assay.

Based on the data from the yearly reports of the newborn screening laboratories, in 2010–2014 (not publically available) about 0.4% of the newborn heel prick samples were considered abnormal and children were referred to a metabolic center; roughly 5% of referred children were diagnosed with classical galactosemia and roughly 6% were Duarte variants demonstrating GALT activity of 15–30%. So far, no cases of uridine diphosphate galactose 4-epimerase deficiency were found in the Netherlands.

Rather sudden delivery problems with the manual GALT assay in 2013 posed an immediate challenge for two laboratories to switch to a new assay that would fit as seamlessly as possible to the logistics and COVs of the current screening programme.

The aim of this study was to evaluate new GALT assays in order to fit these assays in the Dutch screening programme, maintaining uniformly applied COVs for GALT. Here we report the analytical and clinical evaluation of the new GALT assay in the context of the Dutch screening setting. The consequences for the Dutch newborn screening programme, including the COV for galactose, are discussed.

Methods

Study Population and Samples

Samples were selected from the routine screening programme in the laboratories immediately after performing the routine newborn screening. Approval to use anonymized samples for quality assurance properties and to improve test quality was obtained from the parents through the heel prick application form (in case parents notified informed dissent, cards were not used).

Assays

Three laboratories already had experience using the automated GALT assay (3303-0010-assay, PerkinElmer, Turku, Finland) and using the Genetic Screening Processor (GSP analyzer, PerkinElmer). The first choice for evaluation by the remaining two other laboratories was the manual GALT assay of PerkinElmer (NG-1100). An alternative method was to test whether the GSP assay could be used without a GSP (further referred to as manual GSP assay). To this end, the incubation steps carried out by the GSP analyzer were performed manually and the plates were read using a Fluorometer (Victor, PerkinElmer). This idea was originally suggested by Fingerhut and Torresani (2013).

Analytical Procedures

The experimental procedures for the manual and automated assays are comparable and were performed according to the manufacturer's protocol. Using a DBS puncher (Perkin Elmer), single 3.2 mm punches were collected in 96 well plates and incubated in a substrate reagent. The method applied in both GALT assays is an adaptation of the semiquantitative enzymatic assay of Beutler and Baluda (1966). GALT activity is determined using a coupled enzyme reaction, in which the end product (fluorescent NADPH) is measured by excitation with a laser source at 355 nm and detecting emitted light at 460 nm. Measurement of fluorescence was done using a Victor Fluorometer (PerkinElmer, 1420 Multilabel Counter) or with the GSP. Using this test, patients with GALT deficiency are identified, but also deficiencies of glucose-6-phosphate dehydrogenase and phosphoglucomutase (Tegtmeyer et al. 2014) can be picked up, since these enzymes are required for the test to function. The NG-1100 Newborn GALT kit for manual processing is provided with calibrators to express GALT activity in units per gram hemoglobin. The 3303-0010 GSP Newborn GALT kit was processed with the GSP automated analyzer and is provided with calibrators to express GALT activity in units per dl blood. Thus, both methods differ compared to the method routinely used, where GALT was expressed as percentage of the average of the daily batch.

For the comparative assays, experimental runs of 100–200 samples were analyzed in the routine daily screening in the laboratory of Bilthoven using the automated GSP method. Duplicate punches were taken to fill duplicate plates that were transported for parallel analysis at the laboratory of the Academic Medical Center in Amsterdam, where the NG-1100 assay was performed the next

day. For the comparison between the automated GSP and NG-1100 assay a total of 626 daily routine samples was used. For the comparison of both GSP assays (automated GSP versus manual GSP) a total of 2,052 daily routine samples and a selected group of 68 samples with low GALT activities were measured. In addition, intra- and inter-assay variation was determined for the automated and manual GSP assay.

Finally, data were collected from 93 newborns with low GALT activities (performed by the automated and manual GSP method) who were referred to the hospital for further analysis. Based on these data optimal COVs for GALT were assessed.

Statistical Analysis

Comparison was established using Passing–Bablok statistics and Pearson correlation (Analyse-it software, which is integrated in Microsoft Excel 2010). Intra- and inter-assay variation (as % coefficient of variance (%CV) where %CV is the quotient of SD and average × 100) were also established.

Results

Firstly, the manual assay (NG-1100) and the automated GSP assay were compared by analyzing 626 anonymous routine heel prick samples in parallel. Figure 1 shows the GALT activity measured by both assays. We did not observe a satisfactory correlation between both assays (Pearson correlation: $r = 0.37$ (95% confidence interval: 0.30–0.43) and Passing–Bablok analysis: slope = 0.37 and an intercept of 3.24).

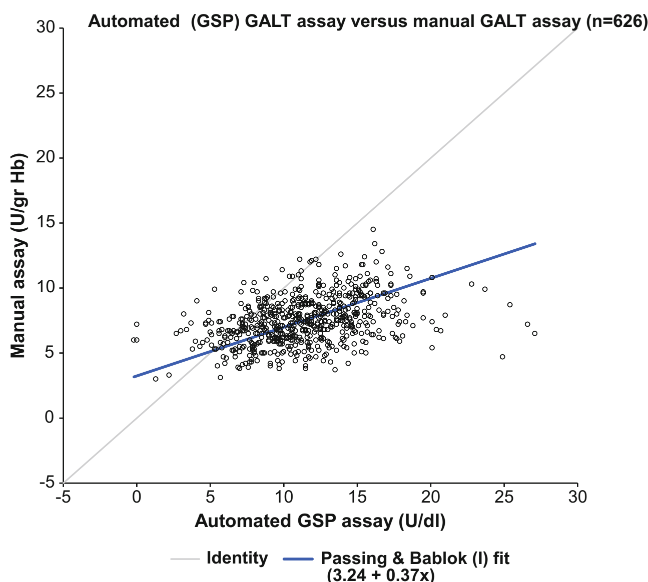

Fig. 1 GALT activity measured in 626 daily heel prick samples comparing the manual assay with the automated GSP assay

Secondly, the manual GSP method was carried out. Comparative results of 2,052 measurements using the 3303-0010-assay, performed either automated or manually, are given in Fig. 2a. With a slope of 0.86, an intercept of −0.8 and a correlation coefficient of 0.86 (0.85–0.87) between the manual and automated GSP method were deemed sufficiently comparable. In addition, data of a selected group of heel prick samples with low GALT values (<6.0 U/dl) were measured using both assays (Fig. 2b). Here, it is shown that the results were comparable to those shown in Fig. 2a, although GALT results of the manual assay were slightly lower compared to the automated GSP assay (with a slope of 0.74, intercept of 0.31, and correlation 0.64 (0.47–0.76)).

Intra- and inter-assay variation coefficients of the automated and manual GSP assay are listed in Table 1. Both assays showed acceptable variation coefficients (ranging from 1.8 to 13.9%) where the automated GSP assay performed better than the manual GSP assay.

Figure 3 reports the data collected from all newborns that were referred by all Dutch screening laboratories between July 2014 and June 2015 based on provisionally applied COVs (GALT activity ≤ 2.7 U/dl and TGAL ≥ 900 μmol/l). Two newborns were excluded from this evaluation, since referral data were incomplete. Ninety-three newborns were referred (0.05% of all newborns screened in this period). In this group, three patients were confirmed to have classical galactosemia, and 41 newborns had GALT activities that are indicative of a Duarte variant. All patients with classical galactosemia showed GALT activities ≤ 0.8 U/dl combined with TGAL concentrations ≥ 3,000 μmol/l. The positive predictive value (PPV) was 3.3% (Duarte variants being considered as false positive results).

Discussion

The present study describes the adoption of a manual assay for GALT used in the newborn screening for classical galactosemia in order to replace the assay of which the production was terminated. Choices were limited, and of those limited choices we showed that the manual GALT assay from Perkin Elmer (NG-1100 assay) did not produce results that were comparable with those measured using the automated GALT assay (3303-0010-assay, processed with the GSP analyzer). In additional experiments we demonstrated that adaptation of the automated GSP assay for manual processing did produce satisfactory results. Intra- and inter-assay variation were acceptable and comparable in both assays and, more importantly, a good correlation between the measurements of the automated and manual

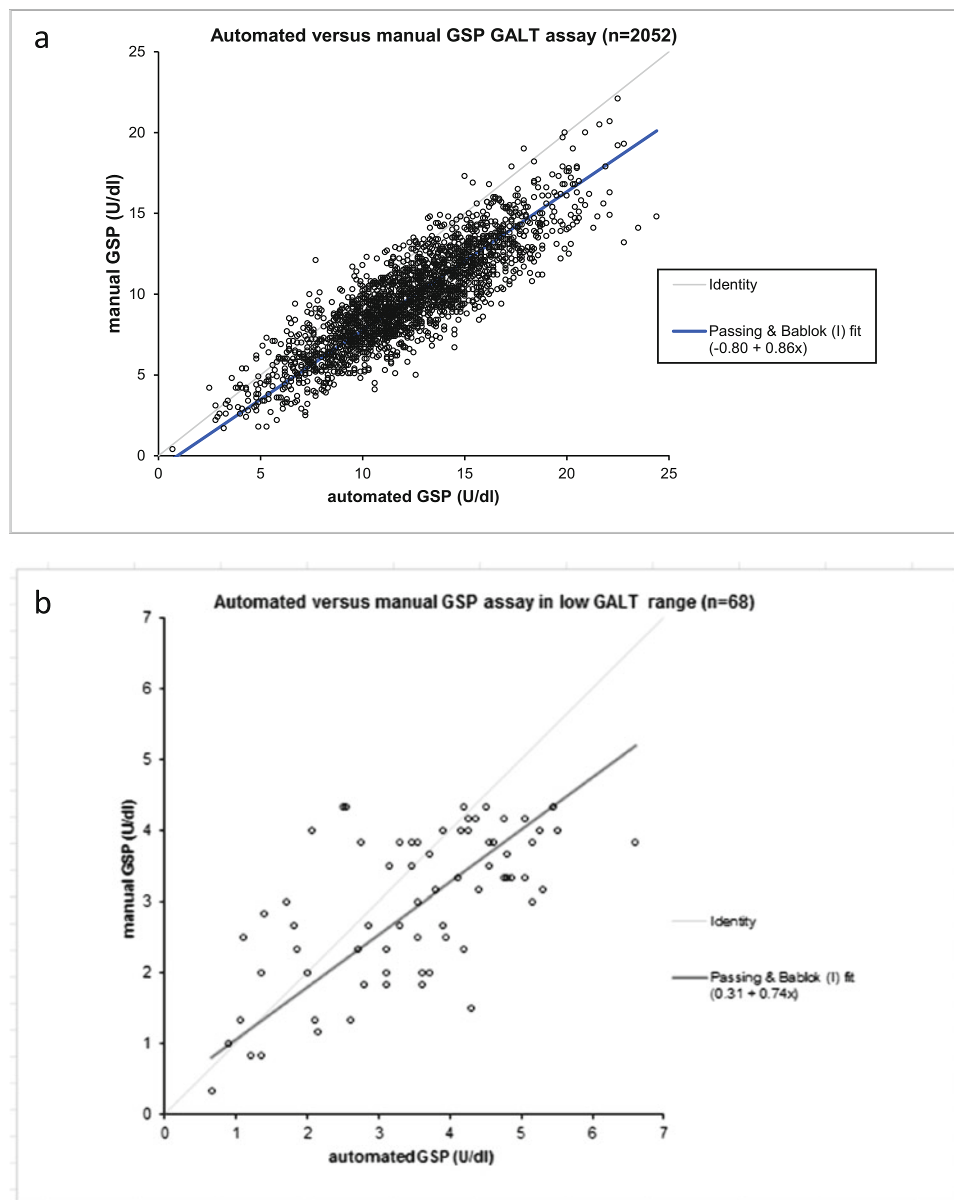

Fig. 2 GALT activity measured in 2,052 random heel prick samples (**a**) and in a selection of 68 heel prick samples (**b**) with GALT activity ranging from 0 to 6 U/dl using the automated assay and manual GSP assay. Slope and intercept of the Passing–Bablok analysis (*grey line*) are given in the diagram

Table 1 Intra- and inter-assay variation of GALT activity measured by the manual and automated GSP assay of low and medium control samples

	Low control (3.7 U/dl)		Medium control (13.8 U/dl)	
	Automated GSP	Manual GSP	Automated GSP	Manual GSP
Intra-assay				
Average	3.7	3.7	14.5	12.5
CV (%)	7.0	10.0	1.8	4.2
Inter-assay				
Average	3.7	3.8	13.8	12.5
CV (%)	3.1	13.9	5.0	3.4

Intra-assay variation determined in one experiment with $n = 6$ analyses, inter-assay variation in ten experiments with 4–6 analyses

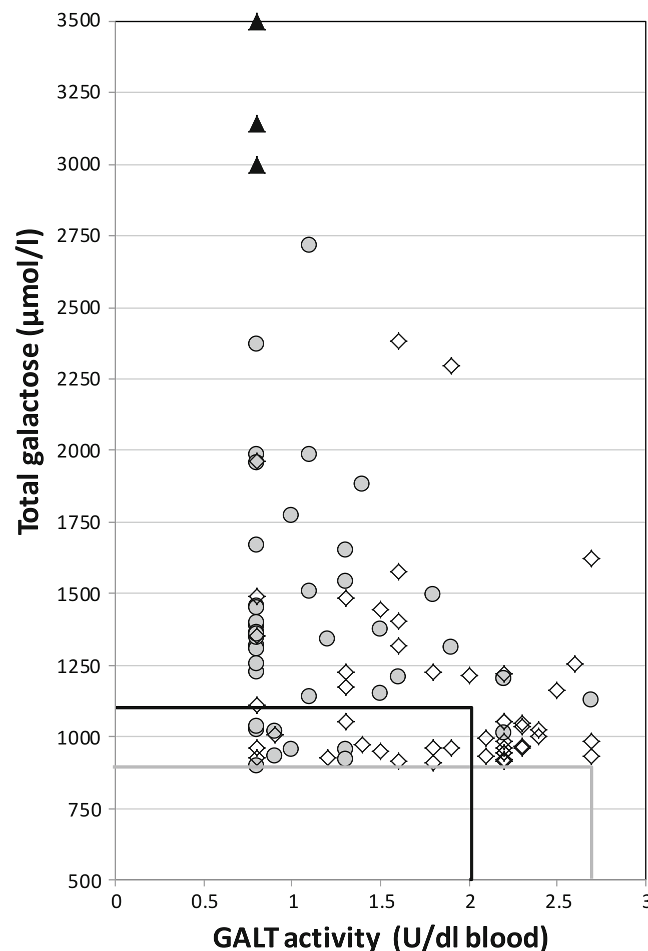

Fig. 3 Scattergram of results of all 93 newborns that were referred in a one year period (July 2014–June 2015) by all Dutch screenings laboratories based on GALT activity ≤ 2.7 U/dl and TGAL concentration ≥ 900 µmol/l (*gray line*). The *black line* indicates new adjusted COVs (see the section "Discussion"). Referred neonates that appeared normal after clinical evaluation are indicated by *open diamonds*, cases with the Duarte variant are indicated with *gray circles*, and classical galactosemia cases with *black triangles*. *Note*: analytical sensitivity for GALT was determined at 0.8 U/dl (based on our own validation studies), therefore results of ≤ 0.8 U/dl were all reported as 0.8 U/dl

GSP assay was shown. These results enabled implementation of this method in two of the five screening laboratories for measuring GALT activity in heel prick blood and set one nationwide-defined COV. The manual procedure was implemented on July 1, 2014. Concomitantly, a new COV for GALT, expressed in U/dl blood, had to be implemented.

The approach was to start with a relatively save, provisional COV, followed by a critical evaluation period, to allow fine tuning of the COV. In close collaboration with both the programme coordinator of the Dutch screening programme and the Dutch advisory board for newborn screening for metabolic disorders as of July 1, 2014, the initial COV for GALT activity in the Netherlands was set at GALT ≤ 2.7 U/dl blood (combined with TGAL ≥ 900 µmol/l). In a recent paper on GALT screening in the USA an overview was given of the applied COV for GALT screening (Pyhtila et al. 2014). Four out of nineteen laboratories used the PerkinElmer GSP assay, with COVs of 2.5, 2.7, 3.1, and 4.3 U/dl blood, respectively. Thus, 2.7 U/dl as a COV for the Dutch screening programme, compared to all comparative reference data together, seemed to be a reasonable choice.

After one year of experience, we have no indication of false negative results of the screening using the current COVs. GALT activities in cases of classical galactosemia were ≤ 0.8 U/dl (Fig. 3; three patients) and in case of the possible Duarte variants 0.8–2.7 U/dl blood. TGAL concentrations of the classical galactosemia cases were ≥ 3,000 µmol/l and of the Duarte variants between 900 and 2,717 µmol/l. Duarte variants are considered to be physiological variants and do not receive treatment.

To optimize COVs for GALT after the planned evaluation period we combined the limited available clinical and screening data of the current study and combined clinical and screening data (produced by using the exact same automated GSP method as the current study) from the Swiss newborn screening programme (kindly provided by

Dr. Fingerhut). In the Swiss study it was shown that GALT activity of five classical galactosemia cases was 0–1.4 U/dl and of 10 Duarte cases GALT varied between 0.7 and 3.3 U/dl. TGAL concentrations of the classical galactosemia cases ranged from 965 to 7,148 µmol/l blood and of the Duarte casus from 19 to 2,368 µmol/l blood. GALT activity results together led us to believe that a new COV for GALT activity to detect neonates with classical galactosemia only could safely be set at 2.0 U/dl.

To set a new COV for TGAL we sought additional information on the lowest TGAL concentration (965 µmol/l) in this dataset measured in a patient with classical galactosemia. It turned out that this patient was already on a galactose-free diet, because of the presence of a sibling with classical galactosemia.

It was therefore decided to increase the TGAL COV from 900 to 1,100 µmol/l. These new COVs (GALT $\leq$ 2.0 U/dl and TGAL $\geq$ 1,100 µmol/l) were approved by the National Screening Programme Committee and are valid as of July 1, 2015. Adjustment of the COVs will result in a significant decrease of the referral rate (48 instead of 93, projected on the July 2014–June 2015 data) and thereby reducing the amount of false positive referrals (45 instead of 90) and a subsequent increase in the PPV (6.5% instead of 3.3%). Duarte variants are included in the number of false positives. Adjustment of the COVs results in increased specificity (99.98%) compared to the formerly used COVs (specificity 99.95%) with unchanged sensitivity (100%).

The finding of one neonate with classical galactosemia that presented in 2011 with a very low GALT activity but a TGAL concentration of 959 hampered the decision on a COV of 1,100 µmol/l blood for some time. The fact that this particular neonate at the time of the heel prick was already on a partly galactose-free diet not only paved the way to elevate the COV for TGAL, but also stressed the importance of the dietary status of the neonate with respect to galactosemia screening. A question on the Dutch heel prick card might be a future consideration, allowing for an even higher TGAL COV, and thus a lower referral rate.

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Take-Home Message

A new method for measuring GALT activity for newborn screening was introduced in the Netherlands and has led to new COVs in newborn screening for classical galactosemia and may lower referral rates.

Contributions of Individual Authors

Boelen A: Conception and design, data analysis and interpretation of data, drafting and revising article.

Kemper EA: Conception and design, data analysis and interpretation of data, drafting and revising article.

Bosch AM: Interpretation of data, professional consultation, revising article.

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Guarantor for the Article

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Compliance with Ethics Guidelines

Conflict of Interest

Anita Boelen, Evelien Kemper, Annet Bosch, Marja van Veen, Carolien van Rijswijk, Marelle Bouva, Ralph Fingerhut, and Peter Schielen declare that they have no conflict of interest. With regard to the mandatory submission of the Conflict of Interest Disclosure Form, please see the following section on “Competing Interests”.

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RESEARCH REPORT

Four Years of Diagnostic Challenges with Tetrahydrobiopterin Deficiencies in Iranian Patients

Shohreh Khatami • Soghra Rouhi Dehnabeh • Sirous Zeinali • Beat Thöny • Mohammadreza Alaei • Shadab Salehpour • Aria Setoodeh • Farzaneh Rohani • Fatemeh Hajivalizadeh • Ashraf Samavat

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Abstract Hyperphenylalaninemia (HPA) is a condition caused by tetrahydrobiopterin (BH_4) and phenylalanine hydroxylase (PAH) deficiencies. It is essential that differential diagnosis be conducted to distinguish these two causes of HPA, because BH_4 deficiency is a more severe disease involving progressive neurologic deterioration. Based on the biological findings, HPA is defined as a plasma phenylalanine level of >2.0 mg/dl (>120 μmol/l). The National Biochemistry Reference Laboratory at the Pasteur Institute of Iran initiated BH_4 deficiency screening tests for the first time during the implementation of a nationwide phenylketonuria (PKU) screening program. Measurement of blood phenylalanine and urinary neopterin and biopterin was conducted by high-performance liquid chromatography in 617 patients with HPA. Dihydropteridine reductase (DHPR) activity was measured in all patients by kinetic spectrophotometry. Differential diagnosis was conducted for PKU, transient HPA, and BH_4 deficiencies.

Our results indicated that out of 76 cases involving BH_4 deficiencies, 37 had 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, 35 had DHPR deficiency, 1 case had pterin-4a-carbinolamine dehydratase (PCD) deficiency, and 3 cases had GTP cyclohydrolase I (GTPCH) deficiency. In this study, 1 novel deletion mutation and 18 novel missense mutations were reported in addition to mutations that had previously been identified and registered in BIOMDB. At present, the screening program for PKU in Iran includes tests that detect different forms of BH_4 deficiency presenting with HPA. Newborns that are BH_4-deficient benefit from the availability of the tests because they can receive necessary care before being clinically affected.

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Introduction

The fully functional phenylalanine hydroxylase (PAH) system for phenylalanine (Phe) metabolism consists of the PAH (EC 1.14.16.1) enzyme, the cofactor tetrahydrobiopterin (BH_4), and two regenerating enzymes: pterin-4a-

carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) (Blau et al. 2010). PAH deficiency and BH_4 deficiency result in hyperphenylalaninemia (HPA). Elevated Phe is a marker of this disorder; thus, a commonly used plasma Phe cutoff level for HPA diagnosis is >120 μmol/l (>2 mg/dl) (Blau et al. 2010).

BH_4 is an essential cofactor for aromatic amino acid hydroxylases, including phenylalanine, tyrosine, and tryptophan hydroxylases (Arai et al. 1982). Defects in the enzymatic conversion of phenylalanine to tyrosine, tyrosine to L-Dopa, and tryptophan to 5-hydroxytryptophan cause HPA and reduce dopamine and serotonin levels in the central nervous system (Arai et al. 1982).

The classical pathway for the de novo biosynthesis of BH_4 from guanosine triphosphate (GTP) requires three enzymes: GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS; EC 4.6.1.10), and sepiapterin reductase (SR; EC 1.1.1.153) (Blau et al. 2010). In addition, dihydropteridine reductase (DHPR; EC 1.6.99.7) and pterin-4a-carbinolamine dehydratase (PCD; EC 4.2.1.96) are critical for the regeneration of BH_4.

BH_4 deficiency is a severe disease involving progressive neurologic deterioration despite adequate dietary control of blood phenylalanine levels (Arai et al. 1982). Therefore, differential diagnosis to distinguish BH_4 deficiencies from PAH gene deficiencies is very important.

In Iran, screening for phenylketonuria (PKU) began as a pilot program in three provinces in 2006 and was expanded nationwide in 2011 under the direction of the Genetics Office of the Ministry of Health.

For a complete study, DHPR enzyme activity measurement and urinary neopterin and biopterin analyses are required to rule out BH_4 deficiencies. For this purpose, the National Biochemistry Reference Laboratory (NBRL) at the Pasteur Institute of Iran began to administer tests and serve patients in 2010. This program allows for the early identification and timely intervention to reduce morbidity and mortality rates, thus increasing the chances for healthy patient outcomes.

At present, NBRL is the only referral center in Iran to diagnose these disorders by urinary neopterin and biopterin analyses and DHPR enzyme activity determination. The purpose of this paper is to report on the implementation of these tests in Iran and present results from 4 years (2010–2014) of diagnosing autosomal-recessive BH_4 deficiencies at our laboratory.

Materials and Methods

Patients

463 patients diagnosed with HPA (blood phenylalanine >120 μmol/l) and 154 patients with HPA and neurological signs referred by physicians nationwide between 2010 and 2014 were investigated for possible BH_4 deficiencies at NBRL.

Selective tests conducted for all patients included blood phenylalanine measurement by high-performance liquid chromatography (HPLC), the DHPR activity test, and urinary neopterin and biopterin analyses by HPLC (Atherton 1989; Ponzone et al. 2004; Blau et al. 2008). Patients were on unrestricted diets for 3 days before sampling. Medications such as multivitamins, BH4, ferrous sulfate, and folic acid were not taken prior to before sampling. Dried blood samples (DBS) on filter paper cards were prepared by blood sampling, as the DHPR enzyme in DBS is stable enough for the sample to be mailed to a central laboratory (Ponzone et al. 2004).

For a complete evaluation to establish the exact nature of the genotype, blood samples from BH_4 deficient patients and their parents were sent to the genetic laboratory for molecular analysis. Out of 76 patients, gene mutation analysis was only performed for 44 cases because some patients did not consent to the molecular study.

Neopterin and Biopterin Measurement

Fresh clean-catch urine specimens were used to determine pterin profile levels. All urine samples were diluted 100-fold in normal saline (Ribeiro de Castro et al. 2004). Acid oxidation of reduced pterin was conducted according to the protocol of Blau et al. (2008). A Knauer HPLC system and Waters fluorimeter, equipped with a primary filter exciting at 350 nm and a secondary filter emitting wavelengths at 450 nm, and Tracer Excel 120 reversed-phase columns ODSB 5 μm 25 × 0.46 cm, part number: TR-016345, serial number: NF-31639, (Teknokroma) were used for pterin analysis.

Quality assurance of the neopterin and biopterin measurements was obtained by testing standard solutions and monitoring the inter-assay variation of urine precision control prepared in our lab.

DHPR Activity Assay

Enzyme activity was assayed based on spectrophotometric monitoring of the formation of ferrocytochrome C in a coupled reaction, according to the protocols of Arai et al. (1982) and Blau et al. (2008). Quality assurance of the DHPR assay was obtained by testing and monitoring the inter-assay variation of blood spot elutes from a normal adult and a DHPR-deficient patient.

Genetics

DNA was extracted from 5 ml peripheral blood collected from each patient and their parents. Direct sequencing of

PCR products was accomplished using a BigDye Terminator kit (Thermo Fisher Scientific, Life Technologies, USA) according to the manufacturer's protocol, using an ABI3130XL Genetic Analyzer for mutation detection in the Dr. Zeinali Human Genetics Laboratory, Tehran, Iran. Mutations in genomic DNA in GCH1 (OMIM: 600225), PCBD1 (OMIM: 126090), PTS (OMIM: 612719), and QDPR (OMIM: 612676) genes were studied (http://www.dnalc.org).

The bioinformatics analysis of these genes was performed using Gene Runner version 3.05, and mismatches were compared using an in-house MS Word file containing full details of exons (6 in GCH1, 4 in PCBD1, 6 in PTS, and 7 in QDPR genes), intron numbers, codon numbers, and details of known mutations.

Statistical Analysis

Statistical analysis of the results was performed in Microsoft Excel 2010. Median, minimum, and maximum values were extracted for all variables.

Results

In this cross-sectional study, 617 hyperphenylalaninemic cases were tested for DHPR activity and urinary neopterin and biopterin profiles. According to the data, of 617 HPA patients, 76 cases exhibited BH_4 deficiency.

The results, including the median, minimum, and maximum values of the 541 HPA cases, were shown in Table 1. PAH gene mutations were searched in some patients but are not reported in this article.

In the remaining cases, 76 individuals had BH_4 deficiencies with the following subtypes: DHPR deficiency (35 patients), PTPS deficiency (37 patients), PCD deficiency (1 patient), and GTPCH deficiency (3 patients). The results, including the median, minimum, and maximum values for laboratory data in these groups are shown in Table 2.

Out of 76 patients, 4 patients were Afghan and 6 patients had Arab ethnic origins. All 10 of these patients suffered from PTPS deficiency. For 11 patients with DHPR deficiency and 16 patients with PTPS deficiency, diagnosis

Table 1 Biochemical data according to age and phenylalanine level for 541 hyperphenylalaninemic cases

Phenylalanine 120–600 µmol/L				
	<1 Year	1–4 Years	4–10 Years	>10 Years
Phe (B) (µmol/L)	276 (127; 589)	262 (126; 583)	398 (177; 586)	223 (121; 570)
Neo (U) (mmol/mol Creat.)	3.54 (0.39; 10.10)	2.42 (0.40; 15.13)	0.99 (0.76; 2.51)	0.62 (0.46; 1.15)
Bio (U) (mmol/mol Creat.)	1.65 (0.30; 8.83)	3.04 (0.63; 9.06)	1.43 (0.76; 3.77)	1.68 (0.83; 2.49)
DHPR activity (mU/mg Hb)	2.80 (1.40; 4.70)	2.80 (1.40; 4.70)	2.20 (1.80; 4.60)	2.00 (1.40; 2.60)
No. of cases	149	35	12	4
Phenylalanine 600–1,200 µmol/L				
	<1 Year	1–4 Years	4–10 Years	>10 Years
Phe (B) (µmol/L)	863 (600; 1,199)	1,020 (600; 1,193)	1,055 (631; 1,182)	986 (606; 1,185)
Neo (U) (mmol/mol Creat.)	4.68 (1.25; 9.69)	2.66 (1.18; 5.43)	1.77 (0.74; 4.36)	1.23 (0.64; 2.61)
Bio (U) (mmol/mol Creat.)	3.84 (0.43; 8.55)	3.52 (0.80; 6.78)	2.66 (1.43; 5.20)	2.43 (1.17; 5.19)
DHPR activity (mU/mg Hb)	2.60 (1.60; 4.10)	2.40 (1.30; 3.50)	2.00 (1.40; 3.80)	2.10 (1.60; 2.80)
No. of cases	56	30	25	14
Phenylalanine >1,200 µmol/L				
	<1 Year	1–4 Years	4–10 Years	>10 Years
Phe (B) (µmol/L)	1,778 (1,204; 3,039)	1,600 (1,206; 2,707)	1,500 (1,212; 2,100)	1,551 (1,220; 1,961)
Neo (U) (mmol/mol Creat.)	4.43 (1.47; 21.05)	3.52 (1.36; 9.54)	1.95 (0.19; 8.13)	1.10 (0.41; 2.84)
Bio (U) (mmol/mol Creat.)	4.29 (0.81; 13.50)	4.06 (0.97; 15.54)	3.67 (1.00; 10.56)	1.76 (0.44; 4.80)
DHPR activity (mU/mg Hb)	2.60 (1.40; 4.40)	2.30 (1.40; 4.90)	2.00 (1.30; 3.60)	1.85 (1.30; 3.10)
No. of cases	88	45	55	28

Phe (B) phenylalanine in blood, *Neo (U)* neopterin in urine, *Bio (U)* biopterin in urine, *Creat.* urine creatinine, *Hb* hemoglobin

Table 2 Laboratory data of patients with BH_4 deficiency at the time of diagnosis

BH_4 Deficiencies Groups	PTPS Deficiency Group Median (Min; Max)	DHPR Deficiency Group Median (Min; Max)	PCD Deficiency Group Median	GTPCH Deficiency Group Median (Min; Max)	Reference range in normal group
Phenylalanine (B) (µmol/L)	864 (60; 2,064)	264 (49; 1,702)	1,327	1,248 (827; 1,747)	<1 month: 0–124 <16 years: 26–86 ≥16 years: 41–68
Neo (U) (mmol/mol Creat.)	24.08 (4.09; 57.15)	2.49 (0.74; 21.82)	1.52	<0.2	1 day to 10 years: 1.1–4.0 >11 years: 0.2–1.7
Bio (U) (mmol/mol Creat.)	0.11 (0.02; 0.64)	8.54 (2.87; 23.03)	–	<0.2	1 day to 10 years: 0.5–3 >11 years: 0.5–2.7
% Biopterin	0.49 (0.06–4.82)	77.50 (31.46-91.46)	–	–	
DHPR activity (mU/mg Hb)	3.8 (1.70; 4.60)	0.0 (0.0; 0.0)	–	4.20 (3.40–4.50)	1.8–3.8
No. of female cases	18	12	1	2	–
No. of male cases	19	23	0	1	–
No. of total (Percentage; %)	37 (49%)	35 (46%)	1 (1%)	3 (4%)	–
Ages	1 month to 12 years	1 month to 20 years	13 years	5 month to 4 years	–

Phe (B) phenylalanine in blood, *Neo (U)* neopterin in urine, *Bio (U)* biopterin in urine, *%Biopterin* calculated by ((biopterin/(biopterin + neopterin))*100); *Creat.* urine creatinine, *Hb* hemoglobin

occurred prior to one year of age. Consanguineous marriage existed in 64 (84.2%) families. In terms of gender, there was an early equal distribution of PTPS deficiency between females (49%) and males (51%), but in the DHPR deficiency group, the number of males (66%) was twice that of females (34%). Four of the reported cases of DHPR-deficient patients were siblings.

Also, decreases or absences of both neopterin and biopterin were observed in GTPCH deficiency group profile.

In PTPS-deficient patients, neopterin concentrations were significantly increased while biopterin levels were low. The data from this group imply that 37 PTPS-deficient patients (100%) had neopterin >4.0 mmol/mol creatinine, while 35 patients (94.6%) had biopterin ≤0.5 mmol/mol creatinine.

In PCD-deficient patients, elevated neopterin and primapterin levels were observed.

According to the past research, DHPR-deficient subjects showed slightly elevated amounts of biopterin combined with minor increases in neopterin (Blau et al. 2008). In our study, the data showed that 32 DHPR-deficient patients (91.4%) had biopterin >3.0 mmol/mol creatinine. In this group, only 2 cases (5.7%) of 35 DHPR-deficient patients exhibited normal levels of both neopterin and biopterin simultaneously.

Statistical analysis of the phenylalanine results determined that 29 DHPR-deficient patients (82.9%) and 13 PTPS-deficient patients (35.1%) had phenylalanine <600 µmol/l, which demonstrated that most of the BH_4-deficient patients have mild HPA.

The genomic structures of identified mutations for 44 cases in *GCH1*, *PCBD1*, *PTS*, and *QDPR* genes, their inheritance conditions, and other information including biochemical data are shown in detail in Table 3.

In our study, we found that mutations more frequently involved DHPR (46%) and PTPS (49%) than GTPCH (4%) and PCD (1%) (Table 3). All BH_4-deficient patients showed homozygous mutations, except one patient in the PTPS deficiency group, who had c.297C>A and c.84-3C>G in a compound heterozygous form.

Most detected mutations had been previously reported (http://www.biopku.org/BioPKU_DatabasesBIOMDB.asp), but 18 missense mutations (16 in exons and 2 in introns) and 1 deletion mutation were novel and had not been registered in the BioPKU_Databases BIOMDB yet. They are demonstrated by asterisks in Table 3 and included: c.163 + 2 T>C, c.217del(c.217delA), c.265G>A, c.266G>A, c.267A>G, c.673G>A, c.710C>T, c.49G>T, c.68G>A, c.488G>C, and c.344C>T in *QDPR* gene; c.281A>T,c.164.36A>G,c.331G>A,c.400G>A,

Table 3 Gene mutation analysis results and laboratory data for patients with BH_4 deficiencies

Row	Status	ID number	Date of birth	Age at diagnosis	Gender	Consanguinity	Ethnic origin	Phe (B) at birth µmol/l	Phe (B) at diagnosis µmol/l	Coding DNA reference Sequence	NCBI reference sequence for genes	Exon	Inheritance condition	Neo (U) mmol / mol Creat.	Bio (U) mmol / mol Creat.	% Biopterin	DHPR activity
1	GCTPH Def.	P.1030	13.06.2011	3.5 years	M	Yes	Fars	972	1,747	c.551G>A*	NM_000161.2	5	Homozygote	<0.2	<0.2	nd	4.2
2	GCTPH Def.	P.2137	16.12.2014	1.5 months	F	Yes	Fars	900	1,248	c.551G>A*	NM_000161.2	5	Homozygote	<0.2	<0.2	nd	3.4
3	GCTPH Def.	P.2149	12.08.2014	5 months	F	Yes	Azari	1,500	827	nd	–	–	–	<0.2	<0.2	nd	4.5
4	PCD Def.	P.1017	11.08.2000	12.5 years	M	Yes	Fars	–	1,116	c.313 T>C*	NM_000281.3	4	Homozygote	1.52	–	nd	nd
5	DHPR Def.	P.432	28.12.2003	7 years	F	No	Azari	–	220	c.53G>A	NM_000320.2	1	Homozygote	1.54	9.05	85.46	0
6	DHPR Def.	P.223	25.05.2000	10 years	M	Yes	Fars	–	–	c.49G>T*	NM_000320.2	1	Homozygote	1.7	5.28	75.64	nd
7	DHPR Def.	P.1477	25.05.2013	1.5 months	M	Yes	Fars	690	184	c.68G>A*	NM_000320.2	1	Homozygote	3.83	3.89	50.39	0
8	DHPR Def.	P.2104	09.07.2014	6 months	M	Yes	Azari	635	244	c.190 G>A	NM_000320.2	2	Homozygote	1.56	6.88	81.52	0
9	DHPR Def.	P.800	13.07.2011	3.5 years	F	Yes	Fars	360	48.6	c.265G>A*	NM_000320.2	3	Homozygote	1.79	5.08	73.94	0
10	DHPR Def.	P.2195	25.11.2008	6 years	M	Yes	Talesh	–	245	c.266G>A*	NM_000320.2	3	Homozygote	1.98	6.1	75.50	0
11	DHPR Def.	P.1172	06.05.2010	2.5 years	F	Yes	Fars	257	143	c.267A>G*	NM_000320.2	3	Homozygote	3.15	11.28	78.17	0
12	DHPR Def.	P.232	03.04.2009	1.5 years	M	Yes	Lor	–	228	c.341C>T*	NM_000320.2	4	Homozygote	8.47	21.53	71.77	0
13	DHPR Def.	P.935	12.10.2009	2.5 years	M	Yes	Fars	282	714	c.344C>T*	NM_000320.2	4	Homozygote	1.76	10.13	85.20	0
14	DHPR Def.	P.386	29.07.2010	4 months	F	Yes	Fars	–	240	c.449A>G	NM_000320.2	5	Homozygote	6.14	8.03	56.67	0
15	DHPR Def.	P.892	29.01.2004	8 years	M	Yes	Fars	–	506	c.449A>G	NM_000320.2	5	Homozygote	0.74	6.16	89.28	0
16	DHPR Def.	P.1019	22.01.2008	5 years	M	No	Azari	900	300	c.449A>G	NM_000320.2	5	Homozygote	4.72	11.16	70.28	0
17	DHPR Def.	P.1027	23.04.1999	13 years	M	No	Azari	–	228	c.449A>G	NM_000320.2	5	Homozygote	1.05	5	82.64	0
18	DHPR Def.	P.94-89	25.12.2005	9.5 years	F	Yes	Fars	440	593	c.470 T>G	NM_000320.2	5	Homozygote	4.08	22.25	84.50	0
19	DHPR Def.	P.94-124	03.08.2014	1 year	M	Yes	Azari	600	142	c.472 C>T	NM_000320.2	5	Homozygote	1.86	4.25	69.56	0
20	DHPR Def.	P.1524	16.06.2013	3 months	F	Yes	Fars	360	551	c.488G>C*	NM_000320.2	5	Homozygote	4.53	5.26	53.73	0
21	DHPR Def.	P.586	03.11.2008	2.5 years	M	Yes	Azari	–	389	c.217del (c.217delA)*	NM_000320.2	7	Homozygote	2.64	13.1	83.23	0
22	DHPR Def.	P.1117	23.12.2011	6 months	M	Yes	Fars	660	174	c.661C>T	NM_000320.2	7	Homozygote	4.75	6.58	58.08	0
23	DHPR Def.	P.366	26.02.2002	9 years	M	Yes	Azari	–	525.61	c.673G>A*	NM_000320.2	7	Homozygote	2.3	18.34	88.86	0
24	DHPR Def.	P.1274	31.12.1996	16 years	F	Yes	Azari	–	400.88	c.673G>A*	NM_000320.2	7	Homozygote	nd	nd	nd	0
25	DHPR Def.	P.1289	28.11.2012	8 months	M	No	Kord	312	175	c.710C>T*	NM_000320.2	7	Homozygote	1.13	2.88	71.82	0
26	DHPR Def.	P.94-17	29.03.2015	1 month	F	Yes	Azari	600	1,702	c.295 + 1 G>A	NM_000320.2	–	Homozygote	10.59	4.86	31.46	0
27	DHPR Def.	P.925	01.12.2008	3 years	F	Yes	Fars	540	534	nd	–	–	–	3.33	13.9	80.67	0
28	DHPR Def.	P.504	05.06.2008	3 years	F	Yes	Fars	–	626	nd	–	–	–	3.73	12.37	76.83	0
29	DHPR Def.	P.1571	10.11.2012	1 year	M	Yes	Azari	312	840	nd	–	–	–	2.87	15.98	84.77	0
30	DHPR Def.	P.582	05.08.2008	6 years	M	Yes	Azari	–	241	nd	–	–	–	2.34	10.83	82.23	0
31	DHPR Def.	P.211	27.03.2001	11 years	M	Yes	Fars	–	199	nd	–	–	–	1.34	6.95	83.84	0
32	DHPR Def.	P.1738	14.08.2013	6 months	M	Yes	Azari	–	161	nd	––	–	–	1.13	2.87	71.75	0
33	DHPR Def.	P.500	09.01.2010	15 months	M	No	Baloch	–	405	nd	–	–	–	4.72	23.03	82.99	0
34	DHPR Def.	P.1377	18.07.2009	3.5 years	M	Yes	Lor	–	240	nd	–	–	–	1.19	4.86	80.33	0
35	DHPR Def.	P.1383	18.05.2008	5 years	M	Yes	Bakhtiari	–	120	nd	–	–	–	1.77	4.36	71.13	0
36	DHPR Def.	P.1885	07.12.2013	9 months	M	Yes	Lor	432	387	nd	–	–	–	2.7	11.21	80.59	0
37	DHPR Def.	P.1948	08.09.1994	20 years	M	Yes	Tork	–	283	nd	–	–	–	1.59	17.02	91.46	0
38	DHPR Def.	P.94.145	14.06.2014	2 months	F	Yes	Fars	240	1,503	nd	–	–	–	11.04	10.87	49.61	0

(continued)

Table 3 (continued)

Row	Status	ID number	Date of birth	Age at diagnosis	Gender	Consanguinity	Ethnic origin	Phe (B) at birth µmol/l	Phe (B) at diagnosis µmol/l	Coding DNA reference Sequence	NCBI reference sequence for genes	Exon	Inheritance condition	Neo (U) mmol / mol Creat.	Bio (U) mmol / mol Creat.	% Biopterin	DHPR activity
39	DHPR Def.	P.1917	22.05.2014	1.5 months	F	Yes	Fars	570	1,020	nd	–	–	–	21.82	11.38	34.28	0
40	PTPS Def.	P.1160	14.04.2011	1.5 years	M	No	Afghan	–	940	c.155A>G	NM_000317.2	2	Homozygote	48.18	0.35	0.72	nd
41	PTPS Def.	P.247	21.06.2008	2.5 years	F	Yes	Fars	–	672	c.155A>G	NM_000317.2	2	Homozygote	42.9	0.55	1.27	nd
42	PTPS Def.	P.246	21.01.2008	2.5 years	F	Yes	Fars	–	766.02	c.155A>G	NM_000317.2	2	Homozygote	51.49	0.44	0.85	nd
43	PTPS Def.	P.2030	27.08.2014	4.5 months	F	Yes	Azari	630	807	c.199 C>T	NM_000317.2	2	Homozygote	36.57	0.23	0.63	4.3
44	PTPS Def.	P.1816	18.04.2011	3 years	M	Yes	Fars	180	124	c.259 C>T	NM_000317.2	5	Homozygote	7.65	0.03	0.39	1.9
45	PTPS Def.	P.227	30.01.2009	6 months	F	Yes	Kord	–	947	c.281A>T*	NM_000317.2	5	Homozygote	54.23	0.25	0.46	nd
46	PTPS Def.	P.238	18.08.2009	11.5 months	M	Yes	Kord	–	800.55	c.281A>T*	NM_000317.2	5	Homozygote	13.31	0.02	0.15	nd
47	PTPS Def.	P.1157	26.04.2012	4.5 months	F	No	Fars	794	1,353	c.297C>A & c.84-3C>G	NM_000317.2	5	Compound Heterozygote	35.5	0.25	0.70	nd
48	PTPS Def.	P.2001	04.03.2015	7 months	F	Yes	Fars	1,440	1,852	c.317 C>T	NM_000317.2	6	Homozygote	34.9	0.08	0.23	2.6
49	PTPS Def.	P.2194	17.01.2015	1.5 months	F	No	Azari	1,560	1,305	c.317 C>T	NM_000317.2	6	Homozygote	35.99	0.15	0.42	4.6
50	PTPS Def.	P.207	05.03.2001	12 years	F	Yes	Azari	–	2,064	c.331G>A*	NM_000317.2	6	Homozygote	11.87	0.04	0.34	nd
51	PTPS Def.	P.1551	04.08.2013	2 months	M	Yes	Afghan	841	596	c.351C>A*	NM_000317.2	6	Homozygote	12.57	0.16	1.26	nd
52	PTPS Def.	P.236	10.05.2005	5 years	M	Yes	Arab	–	480	c.373A>T	NM_000317.2	6	Homozygote	31.72	0.04	0.13	nd
53	PTPS Def.	P.94-26	19.12.2014	6 months	F	Yes	Arab	1,230	1,616	c.373A>T	NM_000317.2	6	Homozygote	57.15	0.19	0.33	3.8
54	PTPS Def.	P.1124	06.11.2008	4 years	M	Yes	Arab	–	420	c.373A>T	NM_000317.2	6	Homozygote	24.08	0.07	0.29	nd
55	PTPS Def.	P.1344	15.09.2011	1.5 years	M	Yes	Fars	660	1,331	c.373A>T	NM_000317.2	6	Homozygote	22.06	0.11	0.50	nd
56	PTPS Def.	P.1268	08.12.2012	1 month	F	Yes	Azari	1,020	1,241	c.400G>A*	NM_000317.2	6	Homozygote	33.13	0.02	0.06	nd
57	PTPS Def.	P.229	03.07.2009	1 year	M	Yes	Kord	480	90	c.84-3C>G	NM_000317.2	–	Homozygote	6.31	0.32	4.83	nd
58	PTPS Carrier	P.1918	05.06.2014	1 month	F	Yes	Fars	270	244	c.163 + 2 T>C*	NM_000317.2	–	Heterozygote	10.01	3.14	23.88	2.7
59	PTPS Def.	P.1122	19.02.2012	6.5 months	M	Yes	Arab	1,920	1,200	c.164.36A>G*	NM_000317.2	–	Homozygote	48.85	0.04	0.08	nd
60	Probably PTPS Def.	P.878	06.12.2010	1 year	M	Yes	Kord	–	60	nd	–	–	–	11.1	0.12	1.07	nd
61	Probabely PTPS Def.	P.1105	12.04.2012	4 months	F	Yes	Fars	864	894	nd	–	–	–	25.83	0.08	0.31	nd
62	Probabely PTPS Def.	P.622	01.10.2010	9 months	M	No	Fars	–	–	nd	–	–	–	20.4	0.06	0.29	nd
63	Probabely PTPS Def.	P.1213	23.04.2012	1.5 years	M	Yes	Fars	–	1,410	nd	–	–	–	29.48	0.15	0.51	4.1
64	Probabely PTPS Def.	P.260	02.12.2007	5 years	F	No	Fars	–	791	nd	–	–	–	16.27	0.1	0.61	nd
65	Probabely PTPS Def.	P.563	26.08.2007	3 years	M	Yes	Fars	–	1,129	nd	–	–	–	18.06	0.1	0.55	nd
66	Probabely PTPS Def.	P.596	31.10.2005	5 years	M	Yes	Azari	–	426	nd	–	–	–	4.09	0.08	1.92	nd
67	Probabely PTPS Def.	P.1141	16.08.2006	7 years	M	Yes	Kord	–	1,156	nd	–	–	–	22.82	0.11	0.48	nd
68	Probabely PTPS Def.	P.1375	23.08.2006	6.5 years	F	Yes	Arab	–	540	nd	–	–	–	13.81	0.09	0.65	nd
69	Probabely PTPS Def.	P.357	07.06.2010	4.5 months	F	Yes	Azari	–	1,890	nd	–	–	–	55.08	0.64	1.15	nd
70	Probabely PTPS Def.	P.1591	20.09.2012	13 months	F	Yes	Azari	510	1,080	nd	–	–	–	16.05	0.07	0.43	1.8
71	Probabely PTPS Def.	P.1717	15.08.2013	5 months	F	No	Afghan	–	1,573	nd	–	–	–	30.23	0.21	0.69	1.7
72	Probabely PTPS Def.	P.1736	23.03.2009	5 years	M	Yes	Azari	1,320	834	nd	–	–	–	16.62	0.2	1.19	1.8
73	Probabely PTPS Def.	P.1813	27.11.2011	2 years	F	Yes	Fars	–	1,251	nd	–	–	–	19.09	0.16	0.83	nd
74	Probabely PTPS Def.	P.94-128	23.08.2014	1 year	M	Yes	Afghan	–	585	nd	–	–	–	22.43	0.1	0.44	4.3
75	Probabely PTPS Def.	P.94-48	30.01.2014	1.5 years	M	Yes	Arab	–	579	nd	–	–	–	24.63	0.11	0.44	3.9
76	Probabely PTPS Carrier	P.94-95	11.06.2015	1 month	M	No	Azari	360	488	nd	–	–	–	45.62	2.03	4.26	4

Phe (B) phenylalanine in blood, *Neo (U)* neopterin in urine, *Bio (U)* biopterin in urine, *%Biopterin* calculated by ((biopterin/(biopterin + neopterin))*100), *nd* not detected

c.351C>A, and c.163 + 2 T>C in *PTS* gene; c.313 T>C in *PCBD1* gene; c.551G>A in *GCH1* gene.

Moreover, the diagnosis for one of the DHPR-deficient patients with DHPR activity equal to zero was performed by the University Children's Hospital of Zürich, Switzerland. All exons of the *QDPR* gene plus flanking intronic regions, and the complete coding sequence of the cDNA, were tested in this case. The new homozygous alteration c.267A>G, which seems to be synonymous for coding on the protein level (p. Gly89Gly) was found. The program used was "Human Splicing Finder," version 2.4.1 (http://www.umd.be/HSF/). Mutations in c.265G>A which coded (p.Gly89Arg) on the protein level and c.266G>A, coding (p.Gly89Gln) on the protein level, were observed in two other DHPR-deficient patients. All three mutations are novel on Cd89 *DHPR* gene. Phenylalanine levels were <250 μmol/: 49 μmol/l for patient with mutation in c.265G>A, 245 μmol/l for patient with mutation in c.266G>A, and 143 μmol/l for patient with mutation in c.267G>G.

Discussion

BH_4 deficiencies are a very heterogeneous group of diseases (Blau et al. 2001). Every newborn with even slight but persistent HPA should be tested for BH_4 deficiency. Such tests have been introduced in many developed countries, but even today, older children are more commonly diagnosed after the appearance of clinical symptoms, such as hypotonia of the trunk, hypertonia of the extremities, and often, myoclonic seizures unresponsive to a low-phenylalanine diet.

Because classical BH_4 deficiencies are a group of diseases that can be detected but not identified through neonatal mass screening for HPA, selective screening for a BH_4 deficiency is essential for every newborn with slightly elevated phenylalanine levels (Dhondt 1991).

Our findings show that there is no predominant mutation and that the majority of these mutations are isolated in families and scattered throughout the genes of the Iranian population. This finding aligns with a prior Iranian study about the *QDPR* gene (Foroozani et al. 2015). Furthermore, eight of the eleven new mutations reported in this article on the *QDPR* gene were previously reported by Foroozani et al. (2015).

It is interesting that in the DHPR deficiency group, the new homozygous alteration c.267A>G in the *QDPR* gene reported in this paper as a novel mutation, which seems to be synonymous coding on the protein level (p.Gly89Gly), is not an SNP. This mutation generates a new 9G8 (or SR-protein SFRS7) exon-splice-enhancer, which might alter pre-mRNA splicing.

The observation of two other new mutation on codon 89 (c.265G>A and c.266G>A) is a more interesting note that should be reported about Iranian DHPR-deficient patients. All mutations on this codon were novel, and all three patients had mild HPA. Therefore it will be interesting if a phenotype–genotype relation study were carried out.

In the PTPS deficiency group, there were no genetic study data or enzyme activity to confirm disease existence for 17 (46%) patients. According to biochemical findings and clinical signs, they were suspected of having PTPS deficiency. Despite the lack of confirmation for these patients, according to physicians' comments, they were treated with BH_4 tablets.

For a more complete diagnosis it is necessary to measure PTPS activity and assess 5-hydroxyindoleacetic acid, homovanillic acid, and 5-methyl tetrahydrofolic acid in the cerebrospinal fluid. The results in this article show gaps in the prevention and control programs for the diseases. These gaps can often be eliminated by using existing capacities in a standardized and systematic approach. If all newborns with HPA are screened to rule out BH_4 deficiencies within the first month of life, we would be able to report a precise incidence of BH_4 deficiencies per 100,000 live births in Iran in the future.

Under the present circumstances, BH_4-deficient newborns are benefiting from the availability of these tests in the context of a screening program in Iran, because they have the chance to be diagnosed and receive necessary care before clinical damage occurs.

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Compliance with Ethics Guidelines

Consortium

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Conflict of Interest

Shohreh Khatami, Soghra Rouhi Dehnabeh, Sirous Zeinali, Beat Thöny, Mohammadreza Alaei, Shadab Salehpour, Aria

Setoodeh, Farzaneh Rohani, Fatemeh Hajivalizadeh, and Ashraf Samavat declare that they have no conflict of interest.

Informed Consent

The project was supported financially with research grants for implemented programs by the Ministry of Health of Iran. Informed consent was obtained from all patients for inclusion in the study.

Animal Rights

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

Details of the Contributions of Individual Authors

Shohreh Khatami, as member of the PKU national scientific committee, contributed to the planning, conducting, and reporting of the BH_4 screening tests described in the article.

Soghra Rouhi contributed to the conception and design of the DHPR test and peterin analysis setup and to drafting the article.

Mohammadreza Alaei, Shadab Salehpour, Aria Sotoudeh, and Farzaneh Rohani contributed as members of the PKU national scientific committee and introduced patients for sample gathering.

Sirous Zeinali, Sarah Azadmeh, Tina Shirzad, Leyli Rejali, Mahbobeh Masoodifarand, and Beat Thöny contributed to the genetics study.

Soghra Khani, Rogiyeh Mirzazadeh, Sedigheh Sadeghi, Somayeh Mahmoudi Baram, Elham Farhangara, Arezu Asgari, Ghazaleh Dadashizadeh, Rayhaneh Hasanzaeh, Mina Barzegari, Parinaz Saeedi, Parastoo Bayat, Robabeh Ahadi, Hamid Mohammadaliha, and Saeedeh Saeedi contributed to the setup and performing of the tests.

Ashraf Samavat (Head of the Genetics Office, Ministry of Health of Iran) and Fatemeh Hajivalizadeh contributed to the design of the PKU screening program in Iran and to revising the article critically for important intellectual content.

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Competing Interests Statement

None

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RESEARCH REPORT

Endurance Exercise Training in Young Adults with Barth Syndrome: A Pilot Study

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Abstract *Background*: Barth syndrome (BTHS) is a rare X-linked disorder that is characterized by mitochondrial abnormalities, cardio-skeletal myopathy, exercise intolerance, and premature mortality. The effect on endurance exercise training on exercise tolerance, cardio-skeletal function, and quality of life in BTHS is unknown.

Methods: Four young adults (23 ± 5 years, $n = 4$) with BTHS participated in a 12-week, supervised, individualized endurance exercise training program. Exercise training was performed on a cycle ergometer for 30–45′ three times per week at a moderate intensity level. Exercise tolerance was measured by graded exercise testing and peak oxygen consumption, heart function via two-dimensional and M-mode echocardiography, skeletal muscle function by near-infrared spectroscopy, and quality of life through the Minnesota Living with Heart Failure questionnaire.

Results: There were no adverse events during exercise testing or training for any participant. Peak oxygen consumption modestly (~5%) improved in three or four participants. Mean quality of life questions regarding dyspnea and side effects from medications significantly improved following exercise training. Mean resting heart function or skeletal muscle oxygen extraction during exercise did not improve after exercise training.

Conclusion: Endurance exercise training is safe and appears to modestly improve peak exercise tolerance and certain measures of quality of life in young adults with BTHS. However, compared to improvements resulting from endurance exercise training seen in other non-BTHS mitochondrial myopathies and heart failure, these improvements appear blunted. Further research into the most beneficial mode, intensity and frequency of exercise training in BTHS is warranted.

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Barth syndrome (BTHS) is an X-linked disorder that results in cardio-skeletal myopathy, heart failure, fatigue, chronic/cyclic neutropenia, growth delay, and premature mortality (Barth et al. 1983). The full scope of the pathological and clinical manifestations of BTHS is not fully understood, but involves a tafazzin gene defect that results in cardiolipin deficiency and severe mitochondrial dysfunction. BTHS-associated mitochondrial dysfunction is assumed to mediate the cardio-skeletal myopathy seen in the majority of those with BTHS (Spencer et al. 2006).

Our group recently found that whole-body peak oxygen consumption (VO_{2peak}) during acute exercise was significantly impaired in BTHS when compared to healthy peers (Spencer et al. 2011). Further, deficits in VO_{2peak} in those with BTHS were mediated by impairments in both cardiac and skeletal muscle function (Spencer et al. 2011). This is important because peak oxygen consumption (i.e., exercise tolerance) is the single best predictor of cardiovascular and all-cause mortality in individuals with cardiovascular disease (Myers et al. 2002). In individuals with non-BTHS heart failure, endurance (aerobic) exercise training increases peak whole-body oxygen consumption, left ventricular function, skeletal muscle blood flow and oxidative function, plasma lactate concentration during exercise, and improves serum markers associated with the severity of the cardiac impairment (e.g., tumor-necrosis factor-α (TNF-α) and pro-brain natriuretic peptide(pro-BNP)) (Sullivan et al. 1988; Minotti et al. 1990; Coats et al. 1992; Hambrecht et al. 1997, 1998; Adamopoulos et al. 2002; Delagardelle et al. 2002; Giannuzzi et al. 2003; Conraads et al. 2004; Bartlo 2007). Most importantly, endurance exercise training in patients with non-BTHS heart failure was safe, improved survival (Belardinelli et al. 1999), decreased hospitalization (Belardinelli et al. 1999), and increased quality of life (Coats et al. 1992; Tyni-Lenne et al. 1996; Belardinelli et al. 1999). Similarly, endurance exercise training has been found to be safe, improve exercise capacity, increase skeletal muscle oxygen extraction, and improve quality of life in individuals with other non-BTHS mitochondrial myopathies (Taivassalo et al. 2001; Cejudo et al. 2005; Jeppesen et al. 2006, 2009). However, the effect of endurance exercise training on these measures in BTHS is unknown. Because there is no specific therapies for BTHS to date, identification of an intervention to improve cardiovascular and metabolic health and to improve quality of life in this population is of high clinical importance.

BTHS has many similar clinical characteristics with patients with non-BTHS-related heart failure and those with non-BTHS mitochondrial myopathies. Therefore we hypothesized that endurance exercise training would improve exercise tolerance (i.e., peak oxygen consumption), skeletal muscle oxygen extraction, cardiac function, and quality of life in young adults with BTHS. In this pilot study, we collected preliminary data on the effect of a 12-week endurance exercise training program in four young adults with BTHS.

Methods

Participants

Males with BTHS ages 15–35 years were identified through the Barth Syndrome Foundation Registry (BSFR). Inclusion criteria included sedentary lifestyle (exercises $<1\times$/week), willing to exercise, and with unchanged medications ≥ 3 months (Table 1). Participants were excluded if they had unstable heart disease or cardiac transplantation. Once identified, each potential participant was contacted by the principal investigator (WTC) to evaluate interest in participating in the study. If the participants were interested, a medical release for participation in the study's exercise program was obtained from the participant's personal cardiologist. Participant demographics are presented in Table 1. Informed written and verbal consent was obtained from all participants and the study was approved by the Human Research Protection Office at Washington University. 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.

Baseline Testing

Baseline testing was performed at the 2010 Barth Syndrome International Family Scientific, Medical and Family Conference in Clearwater, FL. Upon screening, all participants received a physical examination, including a medical history. Participants also received fasting complete blood chemistry including plasma pro-brain natriuretic peptide as a marker of left ventricular (LV) dysfunction (Nir et al. 2004). All post-exercise measurements were performed at the Washington University Institute for Clinical and Translational Sciences Clinical Research Unit.

Exercise Testing

Each participant performed a graded exercise test on an electronically braked cycle ergometer (SensorMedics/ VIASYS Healthcare, Yorba Linda, CA). The exercise test began with participants pedaling at a rate of 60 revolutions/ min with no load for 1 min (warm up). After the warm-up period, the work rate (WR) on the cycle ergometer started at 10 Watts (W) and increased 10 W/min until volitional exhaustion. Twelve-lead cardiography (ECG), blood pressure, O_2 consumption (VO_2), CO_2 production (VCO_2), and ventilation (VMax, SensorMedics/VIASYS Healthcare) were continuously measured. Exercise tests were considered to be maximal if the peak heart rate (HR) was $\geq$85% of that predicted for age (220-age) and/or the peak respiratory exchange ratio (RER; VCO_2/VO_2) was $\geq$1.15 (ACSM 1998).

Echocardiography

All participants underwent echocardiography using a standardized protocol designed to evaluate LV size, LV

Table 1 Demographics of participants

Variable	Participant #1	Participant #2	Participant #3	Participant #4
Age (yrs)	21	22	29	18
ICD (yes/no)	Yes	Yes	Yes	No
Pre-intervention endurance (city blocks)	2–3	2	2–3	1–2
Employment/student status	Graduate student	Undergraduate student	Part-time employed	Unemployed
Hobbies	Following sports, cooking	Following sports, gaming	Computers, gaming, photography	Gaming
Medications	Carvedilol 25 mg BID	Metoprolol 50 mg BID	Carvedilol 12.5 mg BID	Losartan 25 mg QD
	Furosemide 10 mg QD	Digoxin 0.125 mg QD	Captopril 25 mg QD	Atenolol 25 mg QD
	Digoxin 0.125 mg QD		Sertraline 50 mg QD	Carnitine 5 mL TID
	Losartan 50 mg QD		Metformin 50 mg BID	CEQ-10 200 mg TID
	Spironolactone 25 mg QD			Sulfamethoxazole TMP
	Warfarin 2.5 mg QD			DS tablet QD
	KCl 20 mg BID			
	Arginine 7 g QD			
	Cysteine 7 g QD			
	Methionine 7 g			
	Calcium/vitamin D 160 mg QD			

yrs years, *ICD* intra-cardiac defibrillator, *CEQ* co-enzyme Q, *BID* twice a day, *QD* once a day, *TID* three times a day

morphology, and systolic and diastolic function as previously described (Spencer et al. 2011). All echocardiograms were recorded digitally and each study participant and visit was interpreted and measured by the same cardiologist (CLT) who was blinded to subject identification. Echocardiograms were obtained using Phillips 7500 echocardiography machines (Phillips Medical Systems, Bothell, WA).

Near-Infrared Spectroscopy

Near-infrared spectroscopy (NIRS) is a well-described non-invasive technique that measures changes in oxy-hemoglobin and deoxy-hemoglobin which closely reflects muscular fractional oxygen extraction during exercise (DeLorey et al. 2003; Grassi et al. 2003). Relative concentration changes in oxy-(HbO_2), deoxy-(DeoxyHb), and total (TotalHb) hemoglobin of the vastus lateralis muscle and brain were measured using a four-wavelength continuous wave NIRS system (FORE-SIGHT®, CAS Medical Systems Inc., Branford, CT) as previously described (Spencer et al. 2011).

Assessment of Heath-Related Quality of Life

Subjective quality of life was measured by the Minnesota Living with Heart Failure Questionnaire (MLWHFQ). This tool examines the effects of heart failure and treatments for heart failure on the individual's quality of life and has been validated in individuals with heart failure (Rector et al. 1993) and a valid tool to evaluate therapies in this population (Rector et al. 1996). This tool contains 21 questions in response to the overall question "Did your heart failure prevent you from living as you wanted during the past month by?" in various categories (Supplementary Table 1) where the participants answer through a Likert scale from No (0) to Very Little (1) to Very Much (5).

Exercise Training

Volunteers participated in a 12-week, individualized, progressive, and supervised moderate-intensity endurance exercise training program performed at a hospital-based physical therapy clinic/cardiac rehabilitation program near the participants' homes. The ultimate goal of the training program was for the participants to perform a total of 45 min of continuous or intermittent moderate-intensity exercise (Borg scale 14–16 or "Somewhat Hard to Hard") on a cycle ergometer 3×/week or a total of 36 visits over 12 weeks. Cycle ergometer intensity (resistance in Watts) was increased to maintain moderate intensity as each participant's exercise tolerance increased. The principal investigator (WTC) personally traveled to the training site to initiate and train the local physical therapist/exercise physiologist on the intervention protocol, based on the

exercise tolerance of the participant. Weekly communication by the PI and site physical therapist/exercise physiologist was maintained throughout the training period. Total training time, heart rate, blood pressure, and perceived exertion (i.e., Borg Scale) were recorded for each training visit. In general, participants would exercise at a moderate intensity as long as they could and then rest before attempting another exercise bout. This was performed as many times within the 45 min time frame as the participant could tolerate with encouragement from the physical therapist/exercise physiologist.

Post-Exercise Testing

All post-exercise training testing was performed at Washington University School of Medicine as per previously described. Exercise testing and echocardiography equipment at Washington University were the same makes and models, and the NIRS equipment was identical as used in pre-testing at the 2010 Barth Syndrome Foundation Conference. Post-training NIRS was not performed in Participant #4 due to technical difficulties and thus data for this participant is not presented.

Statistics

The overall objective of this pilot study was to obtain preliminary data on the effect of endurance exercise training in BTHS. However, based on our preliminary data with peak exercise testing in BTHS (Spencer et al. 2011), for a statistically significant 20% improvement in peak oxygen consumption seen in populations with similar characteristics as BTHS, a sample size of 12 is needed. Pre–post data were analyzed through paired *t*-testing (IBM SPSS, Chicago, IL).

Results

Exercise Training

Participant #1 completed 100% of the exercise sessions plus three extra visits (39 visits) for a total of 1,920 min of exercise. Participant #2 completed 100% of the sessions (36 visits) for a total of 1,101 min of exercise. Participant #3 completed 100% of the exercise training plus eight visits extra (44 visits) due to personal reasons, there was a 10-day gap in the training where no exercise was performed and the extra visits were added to compensate for this. Participant #3 completed a total of 866 min of exercise. Participant #4 completed only 81% (29 sessions) of the exercise sessions for a total of 428 min of exercise (Table 2). Missed exercise sessions by Participant #4 were not due to medical but personal reasons. Also, compliance and motivation during the exercise sessions were reported to be variable in Participant #4 which might have affected the outcomes in this participant. Means ± SDs for heart rate, blood pressure, exercise duration, work and perceived exertion for each third of the training sessions (first third, second third, and final third) are presented in Table 3. Importantly, there were no adverse events for any participant during the training or testing sessions.

Demographics and Plasma Markers

As a group, there was no mean effect of exercise training on body weight; however, body weight slightly increased in three of the four participants and decreased in one participant. Mean hemoglobin/hematocrit, white cell count, absolute neutrophil count and percentage, pre-albumin, and pro-BNP were unchanged as a group following exercise training (Table 3).

Exercise Tolerance

As a group, mean peak oxygen consumption expressed absolutely and per kilogram body weight increased ~5% following exercise training; however, this was not significant (Table 3, Fig. 1a). Peak work, exercise time, and heart rate were unchanged following exercise training as a group. Peak respiratory exchange ratio (RER) tended ($p = 0.10$) to decrease following exercise training (Table 3). Three of four participants were able to tolerate more exercise volume (time exercising and intensity (watts)) from early in training to late in training (Fig. 1c).

Muscle Oxygen Extraction

The mean slope of deoxy-hemoglobin and time (i.e., Δdeoxy-hemoglobin) during exercise did not change following exercise training (Table 3). The deoxy-hemoglobin slope improved in one participant but mildly declined in two participants (Table 3).

Cardiac Function

Mean resting heart rate, blood pressure, and systolic and diastolic function were unchanged as a group as a result of exercise training (Table 3).

Subjective Quality of Life

As a group, mean total score from the MLWHFQ did not improve following exercise training. However, the effect of the participants' heart failure on the specific questions regarding dyspnea and side effects of medications signifi-

Table 2 Endurance exercise training data

Variable	Participant #1			Participant #2			Participant #3			Participant #4		
Mean +/−SD (session)	1/3	2/3	3/3	1/3	2/3	3/3	1/3	2/3	3/3	1/3	2/3	3/3
Exs duration (min)	52 ± 9	49 ± 3	47 ± 3	14 ± 4	21 ± 3	41 ± 10	17 ± 1	22 ± 2	22 ± 3	16 ± 5	17 ± 9	12 ± 3
HR (bpm)	123 ± 7	123 ± 6	126 ± 2	136 ± 6	139 ± 6	145 ± 4	130 ± 2	131 ± 4	128 ± 4	153 ± 19	136 ± 8	145 ± 6
SBP (mmHg)	103 ± 6	107 ± 8	108 ± 7	114 ± 20	109 ± 12	98 ± 20	112 ± 10	109 ± 5	103 ± 9	102 ± 10	106 ± 9	109 ± 14
DBP (mmHg)	61 ± 6	60 ± 3	59 ± 4	66 ± 7	64 ± 7	77 ± 18	64 ± 6	68 ± 7	62 ± 4	60 ± 8	62 ± 5	61 ± 8
Work (W)	42 ± 10	42 ± 8	49 ± 4	15 ± 1	18 ± 2	21 ± 3	15 ± 0	15 ± 0	15 ± 1	25 ± 0	25 ± 0	100 ± 0
RPE	12 ± 1	14 ± 0	14 ± 1	13 ± 1	13 ± 0	13 ± 1	15 ± 1	16 ± 1	15 ± 1	15 ± 2	16 ± 2	16 ± 1

1/3 mean ± SD of first third of exercise sessions, *2/3* mean ± SD of second third of exercise sessions, *3/3* mean ± SD of last third of exercise sessions, *Exs* exercise, *HR* heart rate, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *RPE* rating of perceived exertion, *bpm* beats per minute

Table 3 Metabolic and exercise responses to endurance exercise training

Variable Mean +/−SD	Participant #1		Participant #2		Participant #3		Participant #4		Mean		
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	*P* value
Weight (kg)	53.1	55.3	53.9	56.2	87.5	90.3	83.3	73.6	69.5 ± 18.5	68.9 ± 16.6	0.86
Hb (g/dL)	14.1	13.3	15.8	15.5	15.5	16.1	14.7	14.6	15.0 ± 0.8	14.9 ± 1.2	0.64
Hct (%)	40.6	37.1	44.3	44.3	44.0	46.4	43.1	41.5	43.0 ± 1.7	42.3 ± 4.0	0.62
WBC (K/cumm)	4.4	3.4	3.3	2.9	3.5	2.4	2.6	2.7	3.5 ± 0.7	2.9 ± 0.4	0.12
ANC (K/cumm)	1.6	1.1	0.5	0.6	1.7	0.7	0.7	0.4	1.1 ± 0.6	0.7 ± 0.3	0.16
Neutrophil (%)	37	33	13	22	49	31	25	13	31.0 ± 15.5	24.8 ± 9.2	0.36
Pre-albumin (mg/dL)	15.0	12.4	19.0	17.1	21.0	22.1	14.7	11.2	17.4 ± 3.1	15.7 ± 5.0	0.18
BNP (pg/mL)	196	121	26	43	15	18	10	15	61.8 ± 89.9	49.3 ± 49.5	0.60
Peak VO_2 (L/min)	0.55	0.58	0.63	0.68	0.97	1.16	0.76	0.66	0.73 ± 0.18	0.77 ± 0.26	0.52
Peak Work (W)	50	60	50	60	80	70	70	60	62.5 ± 15.0	62.5 ± 5.0	1.00
Peak Time (s)	306	370	327	380	477	468	370	360	62.5 ± 15.0	62.5 ± 5.0	0.30
Peak HR (bpm)	144	151	142	169	171	169	171	136	157.0 ± 16.2	156.3 ± 16.0	0.96
Peak RER	1.9	1.7	1.7	1.7	1.4	1.3	1.7	1.4	1.7 ± 0.2	1.5 ± 0.2	0.10
Peak Ve	75	41	60	50	56	52	**	30	63.7 ± 10.0	43.3 ± 10.0	0.22
ΔDeoxy-Hb (μM/s)	0.4	1.0	2.1	1.0	1.7	1.3	**	**	1.4 ± 0.9	1.1 ± 0.2	0.60
SBP (mmHg)	100	98	96	102	130	98	120	93	112 ± 16	113 ± 15	0.60
DBP (mmHg)	70	70	62	70	60	72	66	50	65 ± 4	66 ± 10	0.88
LV mass index	26.7	29.7	20.0	26.3	31.2	26.3	37.3	37.3	28.8 ± 7.3	29.9 ± 5.2	0.68
LVEDV (mL)	194.3	176.5	137.2	125.5	151.0	179.3	168.8	173.4	162.8 ± 24.6	162.5 ± 25.1	0.98
LVESV (mL)	157.0	125.6	72.4	69.1	79.5	100.7	79.6	78.7	97.1 ± 40.1	93.5 ± 25.1	0.76
EF (%)	19	29	47	45	47	44	53	55	42 ± 15	43 ± 11	0.67
FS (%)	9.0	8.9	19.4	19.1	20.3	19.8	26.9	26.6	18.9 ± 7.4	18.6 ± 7.3	0.96
MPI	0.9	0.7	**	**	0.44	0.54	0.51	0.68	0.62 ± 0.2	0.64 ± 0.1	0.86
E/A	2.7	1.8	1.6	1.5	1.4	1.4	1.5	1.4	1.8 ± 0.6	1.5 ± 0.2	0.28

Hb hemoglobin, *Hct* hematocrit, *WBC* white blood cell, *ANC* absolute neutrophil count, *BNP* brain natriuretic peptide, VO_2 volume of oxygen consumption, *Exs* exercise, *HR* heart rate, *RER* respiratory exchange ratio, *Ve* ventilation, *ΔDeoxy-Hb (μM/s)* slope of deoxy-hemoglobin and time in micromoles per second, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *LV* left ventricle, *LVEDV* left ventricular end diastolic volume, *LVESV* left ventricular end systolic volume, *FS* fractional shortening, *EF* ejection fraction, *MPI* myocardial performance index, *E/A* early to late diastolic filling ratio
** Missing data

cantly improved following exercise training (Fig. 1b, Supplementary Table 1). Of note, subjective fatigue improved in two participants and was unchanged in two participants after exercise training and the ability to perform house/yard work improved in two participants following training. However, as a group mean, neither specific question was significantly different following exercise training.

Discussion

This is the first study examine the effects of endurance exercise training on peak exercise tolerance, peak skeletal muscle oxygen extraction, cardiac function, and quality of life in individuals with BTHS. Our primary findings were that in four young adults with BTHS, 12 weeks of endurance exercise training: (1) was safe, (2) induced modest (~5%) improvement in peak exercise tolerance (i.e., oxygen consumption) in three of four participants, and (3) improved the participants' subjective impact of heart failure on dyspnea and the side effects from treatment. In addition, the majority of participants were able to tolerate longer and more intense exercise as the training sessions increased. Surprisingly, endurance exercise training did not appear to improve skeletal muscle oxygen extraction or cardiac function to a significant extent these participants.

Overall, in four individuals with BTHS, endurance exercise training was safe as there were no adverse events or known occurrences of life-threatening arrhythmias. Of note, three of the four participants with BTHS had a previous history of life-threatening arrhythmias as evidenced by implantation of ICD's but were able to safely undergo exercise training. These data suggest that individ-

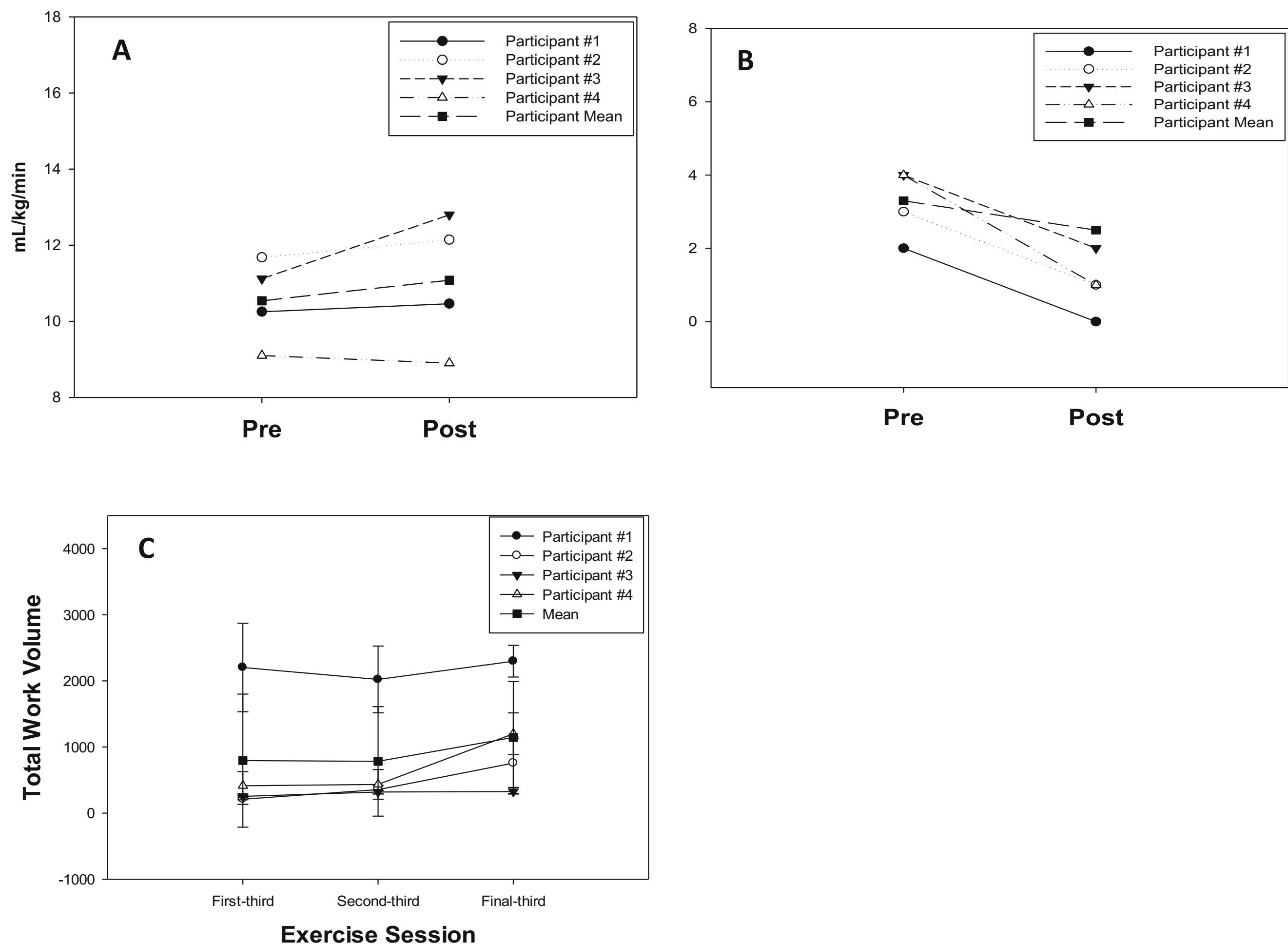

Fig. 1 (**a**) Peak oxygen consumption before and following 12 weeks of endurance exercise. (**b**) Dyspnea scores from the Minnesota Living with Heart Failure Quality of Life Questionnaire before and following 12 weeks of endurance exercise. (**c**) Total exercise work volume (time × resistance) during over the first, second, and last third of exercise sessions

uals with BTHS with stable disease can participate in endurance-type exercise without risk of life-threatening arrhythmias. These data agree with the documented safety of aerobic exercise training in patients with non-BTHS congestive heart failure (Belardinelli et al. 1999). Additional benefits of endurance exercise training in non-BTHS heart failure include decreased hospitalization and improved survival rates (Belardinelli et al. 1999) however; additional and longer term studies are needed to confirm these benefits in BTHS.

Chronic endurance (i.e., aerobic) exercise training is a well-established intervention that improves exercise tolerance and increases cardiac function and skeletal muscle mitochondrial function in healthy individuals (ACSM 1998). Endurance exercise training has also been found to be safe and improve peak exercise tolerance and cardiac and skeletal muscle function in conditions that share similar characteristics as BTHS: mitochondrial myopathies (Cejudo et al. 2005; Jeppesen et al. 2006, 2009) and congestive heart failure (Coats et al. 1992; Delagardelle et al. 2002; Giannuzzi et al. 2003; Bartlo 2007). In patients with other mitochondrial myopathies, an endurance exercise program (3–5×/week, 12 weeks, 70% VO_{2peak}) increased peak oxygen consumption 23–29% and peak work rate 16–100% (Cejudo et al. 2005; Jeppesen et al. 2006, 2009). Also, 14 weeks of endurance exercise training increased peak oxygen consumption (~25%), peak skeletal muscle oxygen extraction (20%), and mitochondrial function, enzyme activity and volume (~50%) without an improvement in cardiac function in individuals with mitochondrial myopathies. These findings demonstrate that endurance exercise training in non-BTHS mitochondrial myopathies specifically increases the ability of skeletal muscle to extract and utilize oxygen for energy production during exercise (Taivassalo et al. 2001). In addition, endurance exercise training increased peak oxygen consumption (23%) (Sullivan et al. 1988) and improved skeletal muscle oxidative function in individuals with

non-BTHS heart failure (Sullivan et al. 1988; Minotti et al. 1990; Hambrecht et al. 1997). In the current study, young men with BTHS were able to tolerate greater amounts (i.e., time and resistance) of submaximal exercise as the training period progressed; however, we observed only a modest increase in peak oxygen consumption (~5%) compared to larger increases seen in other mitochondrial myopathies (20–30%). This might suggest an improved ability for Cori cycling/gluconeogenesis in BTHS with exercise training (Bongaerts and Wagener 2007); however, this is speculative as we did not measure this. We also did not find an improvement in skeletal muscle oxygen extraction during peak exercise as seen in studies in patients with non-BTHS mitochondrial myopathies. One potential reason for these differences might include slight differences in exercise intensity: we used perceived exertion (i.e., Borg scale) to guide exercise intensity rather than %VO_{2peak} as used in other studies and thus we might have exercised our participants at a lower intensity level. Also, with the heterogeneity of mitochondrial DNA mutations, it is possible that exercise training increases the density of non-mutated mitochondria, resulting in better improvements in peak oxygen consumption. In contrast, the mitochondrial pathophysiology of BTHS is of nuclear origin and therefore endurance exercise training might only increase the density of defective mitochondria, thus blunting the response to exercise training. However, this is speculative as we did not obtain muscle biopsies. Lastly, it is possible that the training period (i.e., 12 weeks) was not long enough to elicit skeletal muscle and cardiac adaptations in the participants with BTHS. In healthy individuals, 12 weeks of endurance exercise is associated with a ~8–11% increase in peak oxygen consumption; however, improvements increase to 10–14%, 13–17%, and 16–17% with 24, 36, and 52 weeks of training, respectively (Iwasaki et al. 2003; Scharhag-Rosenberger et al. 2009). Thus, it is possible that cardiorespiratory adaptations might have further increased with a longer duration of exercise training and warrants further study.

Moderate-intensity endurance exercise training has been shown to improve left ventricular function in individuals with non-BTHS heart failure as well as improve levels of plasma markers known to be associated with the severity of cardiac impairment (e.g., TNF-α and pro-BNP) (Adamopoulos et al. 2002; Conraads et al. 2004; Chen et al. 2012). However, in the current study we did not observe improvements in resting nor exercise-stimulated left ventricular function, plasma pro-BNP, or plasma TNF-α following endurance exercise training in four participants with BTHS. It is possible that the training period was not long enough to demonstrate improvements in cardiac function as the majority of studies in non-BTHS heart failure that demonstrated cardiac improvements had training period ≥6 months (Chen et al. 2012). Like skeletal muscle, it is also possible that endurance exercise training in BTHS increased more genetically impaired cardiac mitochondria that did not result in improvement of cardiac energetics and function; however, this is also speculative as cardiac biopsies were not performed.

Endurance exercise training has been shown to improve subjective quality of life in non-BTHS heart failure (Tyni-Lenne et al. 1996; Belardinelli et al. 1999; Taylor et al. 2014) and mitochondrial myopathy (Cejudo et al. 2005). In the current study, endurance exercise training did not significantly improve overall (i.e., total score of the Minnesota Living with Heart Failure Questionnaire) subjective quality of life but did significantly improve scores on specific questions related to dyspnea and side effects of heart failure treatment. Two of four participants also reported an improvement in fatigue and rest during the day questions however as a group, these were not significantly improved following exercise training. Larger and longer studies are needed to fully determine the beneficial effects of endurance exercise training on subjective quality of life in BTHS.

There are limitations associated with this small pilot study. First, we were not adequately powered to demonstrate an effect of endurance exercise training on exercise tolerance. We were also not powered to detect differences in cardiac function or quality of life. However, we were able to show a trend towards improvement of exercise tolerance in the three of four participants with BTHS. In addition, during the training, exercise intensity was guided by perceived exertion rather than heart rate/VO_2. This was performed as all participants were on beta-blocker medication which blunts exercise-stimulated heart rate thus making heart rate guided exercise prescription unreliable. Lastly, the endurance exercise intervention was applied by four different physical therapists/exercise physiologists that could have led to varying motivational techniques for each participant. However, the PI (WTC) provided the same instructions and general communication to all participating therapists/physiologists.

In conclusion, in four young adults with BTHS, 12 weeks of moderate-intensity endurance exercise training was safe, well-tolerated, and modestly improved exercise tolerance and specific areas of subjective quality of life. Training improvements in BTHS however were not as great as those seen in other conditions that share characteristics as BTHS. Endurance exercise induced adaptation might be blunted in BTHS due to the more severe cardiac involvement and homogenous mitochondrial alterations in BTHS compared to other mitochondrial myopathies (Spencer et al. 2006; Finsterer and Kothari 2014). Randomized clinical trials that examine higher intensity and longer duration interventions of endurance exercise training are needed to

determine if endurance exercise training is clearly beneficial in BTHS. It is also possible that an exercise training mode that targets less oxidative muscle fiber types (i.e., Type II) such as resistance training (or combination of resistance and endurance) might be more effective in improving exercise tolerance in BTHS however; this needs to be tested in future studies.

Compliance with Ethics Guidelines

Synopsis

Conflict of Interest

Endurance exercise training is safe and appears to modestly improve exercise tolerance and certain measures of quality of life in young adults with BTHS.

W. Todd Cade, Dominic Reeds, Linda Peterson, Kathryn Bohnert, Rachel Tinius, Paul Benni, Barry Byrne, and Carolyn Taylor declare that they have no conflict of interest.

Author Contributions

WTC planned the study, performed the study, analyzed the data, wrote the manuscript.

DNR performed the studies, wrote the manuscript.

LRP performed the studies, wrote the manuscript.

KLB performed the studies, wrote the manuscript.

RAT performed the studies, wrote the manuscript.

PBB analyzed the data, wrote the manuscript.

BJB planned the study, wrote the manuscript.

CLT planned the study, analyzed the data, wrote the manuscript.

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RESEARCH REPORT

Hydroxysteroid 17-Beta Dehydrogenase Type 10 Disease in Siblings

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Abstract Hydroxysteroid 17-beta dehydrogenase type 10 (HSD10) deficiency (HSD10 disease) is a rare X-linked neurodegenerative condition caused by abnormalities in the *HSD17B10* gene. A total of 10 mutations have been reported in the literature since 2000. Described phenotypes include a severe neonatal or progressive infantile form with hypotonia, choreoathetosis, seizures, cardiomyopathy, neurodegeneration, and death, as well as an attenuated form with variable regression. Here we present the second report of a c.194T>C (p.V65A) mutation in two half-brothers with a clinical phenotype characterized by neurodevelopmental delay, choreoathetosis, visual loss, cardiac findings, and behavioral abnormalities, with regressions now noted in the older sibling. Neither has experienced a metabolic crisis. Both of the siblings had normal tandem mass spectroscopy analysis of their newborn screening samples. The older brother's phenotype may be complicated by the presence of a 3q29 microduplication. Diagnosis requires a high index of suspicion, as the characteristic urine organic acid pattern may escape detection. The exact pathogenic mechanism of disease remains to be elucidated, but may involve the non-dehydrogenase functionalities of the HSD10 protein. Our report highlights clinical features of two patients with the less fulminant phenotype associated with a V65A mutation, compares the reported phenotypes to date, and reviews recent findings regarding the potential pathophysiology of this condition.

Summary Sentence Hydroxysteroid 17-beta dehydrogenase type 10 (HSD10) disease (HSD10 disease) is a rare X-linked neurodegenerative condition with a variable clinical phenotype; diagnosis requires a high index of suspicion.

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Introduction

Hydroxysteroid 17-beta dehydrogenase type 10 (HSD10) is a multifunctional NAD^+-dependent mitochondrial enzyme involved in the degradation of isoleucine, metabolism of neuroactive steroids, and processing of mitochondrial tRNA transcripts (Yang et al. 2011; Zschocke 2012). It is encoded by the 3.11 kb *HSD17B10* gene, located on chromosome Xp11.2. The encoded protein, HSD10, is a 261 amino acid, 27 kDa protein which combines to form a 108 kDa homotetramer. It is the only 17-beta-hydroxysteroid dehydrogenase located in the mitochondria, and has a versatile active site that acts on a variety of substrates, including 17-beta-estradiol, allopregnanolone, isoleucine, fatty acids, bile acids, and xenobiotics (Holzmann et al. 2008; Yang et al. 2011).

HSD10 has been implicated in both childhood and adult-onset neurodegenerative conditions, such as HSD10 disease, MRXS10 disease, Alzheimer disease, and multiple sclerosis (Lenski et al. 2007; Yang et al. 2014). Missense mutations in the *HSD17B10* gene lead to HSD10 disease, a rare X-linked condition characterized by neurodegeneration, progressive visual impairment, choreoathetosis, and cardiomyopathy (Zschocke 2012). While primarily affecting males, variable X-inactivation has been described, leading to broad phenotypic expression in carrier females

(Yang et al. 2013a). The first case of HSD10 disease was reported in 2000 by Zschocke et al. who described a 14-month-old boy with progressive cognitive and motor regression, choreoathetoid movements, loss of vision, and death at 3.5 years of age after developing intractable epilepsy (Ofman et al. 2003; Zschocke et al. 2000).

Disease pathogenicity was initially thought to be related to HSD10's function in isoleucine metabolism, as this enzyme is known to catalyze the 2-methyl-3-hydroxybutyryl-CoA dehydrogenation (MHBD) reaction in isoleucine metabolism. Those with HSD10 disease have elevated urine levels of 2-methyl-3-hydroxybutyrate and tiglylglycine, without increase in 2-methylacetoacetate as would be seen in beta-ketothiolase deficiency (Korman 2006). Unlike beta-ketothiolase deficiency, which is characterized by intermittent episodes of ketoacidosis often associated with illness, HSD10 disease follows a pattern of progressive neurodegeneration that is unresponsive to dietary or other intervention (Ofman et al. 2003; Yang et al. 2014). Pathogenicity is thought to be related either to an impaired role in metabolizing neuroactive steroids, such as 17-beta estradiol and allopregnanolone (Yang et al. 2007, 2009), and/or impaired processing of mitochondrial tRNA and mitochondrial dysfunction (Rauschenberger et al. 2010), as HSD10 is an essential component of mitochondrial ribonuclease (RNase) P (Holzmann et al. 2008).

Subsequent case reports identified another four patients with similar clinical features and metabolic profiles (Ensenauer et al. 2002; Sutton et al. 2003; Poll-The et al. 2004; Sass et al. 2004). Genetic analysis confirmed pathogenic missense mutations in the *HSD17B10* gene in these patients (Ofman et al. 2003). Over the past decade, a total of 10 missense mutations have been identified in about 20 cases of HSD10 disease (Yang et al. 2013b; Zschocke 2012). Whereas approximately 50% of these cases are associated with one common missense mutation, c.388C>T (p.R130C), other mutations have been reported as well. Less fulminant courses have been described, associated with the E249Q, L122V, Q165H, A145T, and V65A mutations (Olpin et al. 2002; Poll-The et al. 2004; Rauschenberger et al. 2010; Fukao et al. 2014; Seaver et al. 2011). In particular, the V65A mutation was reported by Seaver in 2011 in a case of a 10-year-old male with refractory epilepsy, choreoathetosis, ataxia, visual loss, and developmental regression with onset at approximately 2–3 years of life (Seaver et al. 2011). Here we present the second report of a V65A mutation in *HSD17B10* occurring in two half-brothers with neurodevelopmental delay, unusual movements, autistic features, and progressive visual loss.

Case History

A now 7-year-old male was referred at 19 months of age due to a family history of intellectual disability, visual impairment, and microduplication (dup 3q29). He was born at term without complications. Birthmother admitted to consuming alcohol during early pregnancy. Urine toxicology screen was negative. Hearing screen via otoacoustic emission was normal. Newborn screen by tandem mass spectroscopy (which included assessment for beta-ketothiolase deficiency and may detect deficiencies in 2-methyl-3-hydroxybutyryl-CoA dehydrogenase) was normal. He had two hospitalizations for bronchiolitis prior to the age of 7 months, the latter of which was complicated by Klebsiella urosepsis and probable endocarditis with Wolff-Parkinson-White (WPW) syndrome and mild left ventricular hypertrophy. After treatment with gentamicin, a hearing test identified sensorineural hearing loss. At 19 months of age he was noted to have delays in speech, fine motor, and social skills with some regression in social functioning. Chromosome microarray was normal, excluding the familial microdeletion, and Fragile X testing was normal. By 5 years of age he was having staring spells, had low truncal tone, and had been diagnosed with autism spectrum disorder with delays in speech, social skills, and toilet training. EEG revealed benign Rolandic epilepsy. Brain MRI was normal. Eye examination revealed pale optic nerves with foveal changes, suggestive of incomplete stationary night blindness. At about 7 years of age, he began to experience exertion-related spells of pallor, collapse, and near loss-of-consciousness felt to be related to WPW. Repeat echocardiogram showed increased left ventricular hyperplasia with mild diastolic dysfunction. In one of two urine organic acid analysis samples there was a characteristic pattern of elevated 2-methyl-3-hydroxybutyric acid and tiglylglycine without elevation of 2-methylacetoacetic acid, typical of HSD10 disease. A hemizygous c.194T>C (p.V65A) mutation in exon 3 of the *HSD17B10* gene was identified, confirming a diagnosis of HSD10 disease (Table 1). Given the lack of known treatment, and the clinical similarity to disorders of mitochondrial function (Rauschenberger et al. 2010), he was prescribed a "mitochondrial cocktail," including coenzyme Q, Vitamins B1, B2, C, and E, acetylcysteine, and selenium, as well as digestive enzymes and probiotics. There has been subjective behavioral improvement noted by his family and teachers, but quantitative evidence for treatment effect has not been obtained. No further regressions have been observed.

Table 1 Reported pathogenic mutations in the *HSD17B10* gene

Patient	Genotype	Age of onset	Neurological findings	Developmental Findings	Regression (deceased)	Growth retardation	Visual loss	Hearing loss	Abnormal UOA	Reference
I-1 (F, adult)	c.194T>C (obligate), (p.V65A)	Unknown	Unknown	Learning difficulties in college	No	No	Unknown	Unknown	Unknown	N/A
II-1 (M, 7 years)	c.194T>C (p. V65A)	18 months	Hypotonia; benign Rolandic epilepsy; autism spectrum	Speech delay; behavioral issues	Progressive visual loss	No	Yes	Yes[a]	Yes	N/A
II-2 (M, 20 years)	c.194T>C, (p.V65A)	18 months	Decreased white matter volume of occipital lobes; cognitive impairment; choreoathetosis	Global developmental delays	Progressive visual loss	No	Yes	No	Yes	N/A
Number of reported cases					**Regression**					
One case	c.194T>C; p. V65A	2–3 years	Ataxia; choreoathetosis; dysarthria; seizures; hypotonia	Moderate cognitive and mild social impairments	Yes	No	Yes	Unknown	Yes	Seaver et al. (2011)
One case	c.745G>C; p. E249Q	6 years	Gait abnormality; dysarthria; hyperreflexia; dystonic posturing with upper extremity tremor; behavioral difficulties	Regression of verbal and motor skills	Yes	No	No	No	Yes	Olpin et al. (2002) (mutation info by Yang et al. (2009))
One Case	c.257A>G; p. D86G	Neonatal	Absent neurological development	N/A	No	No	Unknown	Unknown	Unknown	Rauschenberger et al. (2010)
One case	c.364C>G; p. L122V	Prior to 13 months	Broad-based gait; spastic paraparesis; increased lateral ventricle size; increased white matter perivascular spaces	Verbal and motor delays	No	No	Unknown	No	Yes	Poll-The et al. (2004) (mutation info: Ofman et al. (2003))
Ten cases	c.388C>T, p. R130C	Birth to 15 months	Motor regression; choreoathetosis; epilepsy; slight frontotemporal atrophy	Relatively normal development followed by progressive loss of milestones	Yes for males (death w/in several years of onset); no for females	No	Yes	No	Yes	Zschocke et al. (2000), Ensenauer et al. (2002) (mutation info: Ofman et al. (2003)), Sutton et al. (2003), Sass et al. (2004) (mutation info: Zschocke (2012)), Carzola et al. (2007), Garcia-Villoria et al. (2005, 2009), Zschocke (2012)

(continued)

Table 1 (continued)

Patient	Genotype	Age of onset	Neurological findings	Developmental Findings	Regression (deceased)	Growth retardation	Visual loss	Hearing loss	Abnormal UOA	Reference
One case	c.460G>A; p. A154T	6 years	Slightly below normal neurological development; normal MRI brain	Mildly delayed gross motor skills; difficulty with fine motor skills	No	No	No	No	Yes	Fukao et al. (2014)
One case	c.495A>C; p. Q165H	Not reported	Microcephaly	Growth retardation	No	Yes	Unknown	Unknown	Unknown	Rauschenberger et al. (2010)
Two cases	c.628C>T; p. P210S	3 months	Hypotonia	Developmental regression	Yes	No	Yes	Unknown	Unknown	Garcia-Villoria et al. (2009)
One case	c.677G>A; p. Q226R	1 day old	Myoclonus; seizures	Developmental regression	Yes (deceased at 7 months)	No	Unknown	Unknown	Unknown	Garcia-Villoria et al. (2009)
Three cases	c.740A>G; p. N247S	0–4 months	Ataxia, hyperkinetic behavior, myoclonus during stress	Psychomotor and speech delay	Yes for males (both deceased in infancy); no for female	No	Unknown	Yes	Yes	Garcia-Villoria et al. (2005), Chatfield et al. (2015)
Two cases	Silent mutation: c.574C>A; p.R192R (MRXS10)	1 month	Axial hypotonia; choreoathetosis; dysarthria; wide-based gait; lumbar lordosis	Motor delays	Unknown	No	No	Unknown	Unknown	Reyniers et al. (1999), Lenski et al. (2007)
Eight cases	Duplication Xp11.22	Unknown	hypotonic mouth; dysarthria; moderate intellectual disability; short attention span	Cognitive and speech delays	Unknown	No	Unknown	Unknown	Unknown	Froyen et al. (2008)

[a] Noted after exposure to gentamicin

His older maternal half-brother, now 20 years old, had onset of global developmental delays, visual impairment, and behavioral disturbances with autistic features at a similar age as our proband. His history is also significant for cryptorchidism, left inguinal hernia, and mild dysmorphisms (coarse facial features, inverted nipples). His cognitive functioning is at the approximate level of a 2–3 year old. His visual difficulties were progressive and he is now legally blind. Full ophthalmological assessment is significant for myopia and progressive macular degeneration, with prolonged VEP responses consistent with optic nerve atrophy. He achieved walking at 2 years of age, but began to experience choreoathetoid movements during his childhood. These movements were more pronounced with ambulation and most prominent in his upper extremities, but also involved his face and lower extremities. With time, these movements have become nearly constant and are accompanied by intermittent dystonic posturing. By 19 years of age his gait had become slow, spastic, and shuffling with lower extremity hyperreflexia and sustained ankle clonus. More recently, he has experienced increasing difficulties with dystonia, thought due to a combination of side effects of psychiatric medication for aggression and his underlying HSD10 disease. An extensive work-up over the years, including plasma amino acids, urine amino acids, mitochondrial encephalopathy profile (including MELAS, MERRF, and NARP), 2-methylglutaconic acid, and testing for Leber Hereditary Optic Neuropathy were all normal. Free and total carnitine were mildly depressed. Chromosome microarray analysis identified a 3q29 microduplication, a finding known to be associated with mild to moderate cognitive impairment and mild dysmorphic features. EEG was normal. Brain MRI obtained at 8 years of age showed no structural abnormality, but decreased white matter volume of occipital lobes. Repeat MRI at 20 years of age showed progression of cerebral volume loss with increased ventricular size. Echocardiogram was normal at 17 years of age, but by 20 years of age showed decreased left ventricular size with moderate to severe concentric thickening. The characteristic HSD10 pattern was detectable on urine organic acid analysis; however, the findings were subtle and could have been consistent with recent ketosis. Analysis of the *HSD17B10* gene revealed the same hemizygous c.194T>C (p.V65A) mutation as seen in his younger half-brother.

The older brother's full sister shares his 3q29 microduplication, but not his *HSD17B10* gene mutation. She is a high school graduate with only mild cognitive disability. Their birthmother, a presumed carrier of the 3q29 microduplication and the HSD10 mutation, graduated from high school, but was diagnosed with learning problems and is reported to have “autism.”

Discussion

HSD10 disease is a rare X-linked condition characterized by neurodevelopmental regression, choreoathetoid movements, hypotonia, progressive visual loss, hearing impairment, and seizures (Zschocke 2012). Despite few case reports (Table 1), a broad phenotypic spectrum has been described, ranging from a fatal neonatal form, to a rapidly progressive neurodegenerative infantile form, to an attenuated form lacking regression (Zschocke 2012). The severe R130C mutation accounts for approximately 50% of reported cases of HSD10 disease, possibly due to the presence of a hypermutable 5-methylcytosine prone to deamination (Yang et al. 2013b).

Here we describe the second case(s) of HSD10 disease associated with the V65A mutation first described by Seaver et al. (2011). The c.194T>C (p.V65A) mutation in *HSD17B10* results in the substitution in amino acid of an alanine for a valine in the HSD active site, which weakens interaction with NAD+. Our cases contribute important information regarding the V65A mutation phenotype, which is associated with a less fulminant clinical course than the more prevalent c.388C>T (p.R130C) mutation. Whereas R130C mutations generally result in severe neonatal or progressive infantile neurodegeneration with hypotonia, choreoathetosis, seizures, and death within a few years of onset, the V65A mutation causes a more prolonged course. The V65A phenotypes described here and by Seaver et al. (2011) share the features of abnormal choreoathetoid movements, dystonia, neurocognitive abnormalities, and progressive visual impairment, but lack the rapid deterioration described in individuals with the R130C mutation. Cardiomyopathy is a feature of the severe form of HSD10 disease (Zschocke 2012), and cardiac involvement, found in both of our patients, may be part of the expanded phenotype in attenuated cases. The neurodevelopmental regression in HSD10 disease appears variable, although regressions have become apparent in our older patient. Visual loss with myopia and bilateral optic nerve pallor is a common feature among all three V65A cases, as are social delays and abnormal choreoathetoid movements. The presence of hearing loss, autistic features and seizures are variably present (Table 1).

Interestingly, there appears to be a similar but distinct disorder, MRXS10 syndrome (OMIM #300220), caused by the silent c.574C>A (p.R192R) mutation. This mutation is associated with cognitive difficulties and choreoathetosis with normal vision. This abnormal HSD10 protein has essentially normal activity in catalyzing the MHBD reaction, but the amount of protein expression is reduced to approximately 60–70% (Lenski et al. 2007; Reyniers et al. 1999). Urine organic acid analysis was not reported.

Whereas HSD10 protein amount is reduced in MRXS10 syndrome, microduplication of the *HSD17B10* gene leads to higher protein levels and mild to moderate cognitive difficulties, dysarthria and shortened attention spans, without regression, choreoathetosis, or reported visual impairment (Froyen et al. 2008).

Researchers have tried to correlate clinical phenotype with residual HSD10 activity in the MHBD reaction of isoleucine metabolism, but the data appear variable and inconclusive (Yang et al. 2013b); R130C with 0–14% MHBD enzymatic residual activity, L122V with 25.4% residual activity, V65A with 50.5% residual activity, and R192R with 60–70% residual activity. Other mutations do not follow this trend: Rauschenberger describes a boy with a Q165H mutation and complete loss of MHBD enzymatic activity (<3% residual activity), who has normal neurologic development in the context of failure to thrive and microcephaly, and contrasts this with a case of a D86G mutation having 30% residual MHBD activity and a severe neonatal presentation that is fatal (Rauschenberger et al. 2010). Thus, impairment of the MHBD function does not appear to consistently correlate with the clinical phenotype of HSD10 disease.

Although a specific mechanism for the pathogenicity of HSD10 abnormalities remains elusive, researchers have been examining its function in mitochondrial ribonuclease (RNase) P (Deutschmann et al. 2014; Rauschenberger et al. 2010), and its role in the metabolism of neuroactive steroids (Yang et al. 2014). The *HSD17B10* gene encodes one of the three protein components of RNase P, an essential component of post-translational mitochondrial RNA processing (Holzmann et al. 2008). Variable disruption of mitochondrial morphology in cultured fibroblasts containing the Q165H, D86G, and R130C mutations has been demonstrated (Rauschenberger et al. 2010), with the amount of disruption being significantly higher in the more severe versus attenuated clinical phenotypes (Rauschenberger et al. 2010). A recent study of post-mortem tissue confirmed disrupted mitochondrial architecture and showed evidence of abnormal mitochondrial RNA processing, with markedly increased levels of unprocessed mitochondrial RNA in affected tissues (Chatfield et al. 2015). Such findings support the developing hypothesis that HSD10 disease is primarily related to an impairment of mitochondrial function.

The HSD10 protein also plays a vital role in the metabolism of neuroactive steroids, including allopregnanolone, an important modulator in gamma-aminobutyric acid type A ($GABA_A$) receptor functioning (Yang et al. 2009, 2014). Elevated levels of HSD10 negatively affect $GABA_A$ receptor functionality. Interestingly, HSD10 amount is increased in Alzheimer disease, Down syndrome, and multiple sclerosis (Yang et al. 2014), all of which are associated with neurocognitive abnormalities.

The clinical characterization of our proband's older brother and their mother is complicated by the presence of a microduplication at chromosome 3q29 not present in the proband. The sister serves as a case control. 3q29 duplication syndrome is typically characterized by mild to moderate cognitive difficulties and microcephaly (Lisi et al. 2008). In the family presented here, the sister has the 3q29 microduplication and not the HSD10 mutation, and has minimal cognitive disabilities. Although we have considered the possible contribution of this microduplication in the older brother's neurocognitive disabilities, we believe that this is not the primary etiology underlying his significant global developmental delays, progressive visual disability, cardiac findings, abnormal neurologic exam and movements, and recently progressive course.

The diagnosis of HSD10 disease can be facilitated by urine organic acid analysis, which typically reveals elevated levels of intermediate metabolites of isoleucine metabolism (2-methyl-3-hydroxybutyrate and tiglylglycine). Unfortunately, the findings may be subtle, absent, or present only during times of metabolic stress. The pattern may mimic that of beta-ketothiolase deficiency (the next step in isoleucine metabolism), however, the absence of elevations in 2-methylacetoacetate excludes that diagnosis. Addition of an isoleucine load 6 h prior to measurement of urine organic acids can improve detection of HDS10 disease, particularly in carrier females, in whom diagnosis may require assessment of an additional metabolite, 2-ethylhydracrylic acid, to make the diagnosis (Garcia-Villoria et al. 2009). Acylcarnitine profile may also be abnormal, with possible elevations of C5:1 or C5-OH species (Fukao et al. 2014; Garcia-Villoria et al. 2005; Sass et al. 2004; Zschocke et al. 2000). Diagnosis is confirmed by identification of a disease-causing mutation in the *HSD17B10* gene. Though some states' newborn screening includes beta-ketothiolase deficiency in their panel of assessed conditions, it is important to note that tandem mass spectroscopy assessment of newborn screen samples in our patients was normal on initial and/or post-diagnosis reanalysis.

No standard or proven treatments options for HSD10 disease have been identified to date. As the disease pathogenesis appears independent of the enzyme's role in isoleucine metabolism, it is not surprising that dietary restriction does not ameliorate symptom progression (Ofman et al. 2003).

Conclusion

We present the second reported instance(s) of a c.194T>C (p.V65A) mutation in the *HSD17B10* gene causing HSD10 disease in two maternally related half-brothers. This

mutation is associated with an attenuated phenotype compared to the classic infantile form of HSD10 disease. Our report highlights the ophthalmologic, neurologic, cardiac, and behavioral aspects of this specific mutation as well as the subtleties of detection and diagnosis of this rare disease, which has characteristic but variably present metabolic features. Current newborn screening protocols are unlikely to detect this condition, and diagnosis requires a high index of suspicion. Pharmacologic or dietary interventions to reverse or slow the progressive nature of this complex multisystemic disease will require a better understanding of the enzymatic and cellular roles of this multifunctional protein.

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Compliance with Ethical Guidelines

Neither this work nor any similar work has been submitted for previous or simultaneous publication. The above authors have substantially contributed to the analysis of these cases and the writing or revision of the included manuscript and agree to its submission for publication.

Annely Richardson – primary author, consolidated the case material for presentation, performed thorough literature review, wrote and edited the final manuscript for publication

Gerard T. Berry – provided metabolic genetics clinical consultation for these patients as well as expert analysis and critique of manuscript

Cheryl Garganta – provided expert analysis of clinical and laboratory data, and critique of the manuscript

Mary-Alice Abbott – primary geneticist of the patients described, initiated and supervised all aspects of the cases, data analysis, literature review, manuscript preparation, and final editing

Informed Consent

The patients and their guardians discussed provided their informed consent and permission to have their medical information discussed in this manuscript. Written documentation of verbal consent is available on request.

Conflict of Interest

Annely Richardson, Gerard T. Berry, Cheryl Garganta, and Mary-Alice Abbott declare that they have no conflict of interest.

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RESEARCH REPORT

Reliable Diagnosis of Carnitine Palmitoyltransferase Type IA Deficiency by Analysis of Plasma Acylcarnitine Profiles

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Abstract *Background*: Carnitine palmitoyltransferase IA (CPT-IA) deficiency is an inherited disorder of the carnitine cycle (MIM #255120). Patients affected by this deficiency might be missed easily because of lack of specific and sensitive biochemical markers. In this study, sensitivity and specificity of plasma free carnitine (C0) and long-chain acylcarnitines (lc-ac: C16:0-, C16:1-, C18:0-, C18:1- and C18:2-ac) was evaluated, including the sum of lc-ac (∑lc-ac) and the molar ratios C0/(C16:0-ac+C18:0-ac) and C0/∑lc-ac.

Methods: Nine plasma acylcarnitine profiles of 4 CPT-IA deficient patients were compared with profiles of 2,190 subjects suspected of or diagnosed with an inherited disorder of metabolism. Age-dependent reference values were calculated based on the patient population without a definite diagnosis of an inborn error of metabolism ($n = 1{,}600$). Sensitivity, specificity, and Receiver Operating Characteristic (ROC) curves were calculated based on samples of the whole patient population.

Results: Concentrations of C0 in plasma were normal in all CPT-IA deficient patient samples. ROC analyses showed highest diagnostic values for C18:0-ac, C18:1-ac, and ∑lc-ac (AUC 1.000) and lowest for C0 (AUC 0.738). Combining two markers, i.e., a plasma C18:1-ac concentration <0.05 μmol/L and a molar ratio of C0/(C16:0-ac+C18:0-ac) >587, specificity to diagnose CPT-IA deficiency increased to 99.3% compared with either C18:1-ac (97.4%) or C0/(C16:0-ac+C18:0-ac) (96.9%) alone, all at a sensitivity of 100%.

Conclusions: Combination of a low concentration of C18:1-ac with a high molar ratio of C0/(C16:0-ac+C18:0-ac) ratio in plasma has high diagnostic value for CPT-IA deficiency. Patients with a clinical suspicion of CPT-IA deficiency can be diagnosed with this test combination.

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Background

Carnitine palmitoyltransferase IA (CPTIA, EC# 2.3.1.21) deficiency is a rare autosomal recessively inherited disorder of the carnitine cycle (MIM #255120) (Bonnefont et al. 2004). This enzyme is essential for transport of long-chain fatty acyl-CoA esters into mitochondria for subsequent beta-oxidation. It is located in the outer mitochondrial membrane and catalyzes the conversion of cytosolic long-chain acyl-CoA esters into their respective acylcarnitine

esters. Acylcarnitines are subsequently transported across the inner mitochondrial membrane and reconverted to acyl-CoA thioesters by the actions of carnitine/acylcarnitine translocase (CACT, SLC25A20) and carnitine palmitoyltransferase II (CPTII, EC# 2.3.1.21). Loss of CPT-IA activity diminishes the intra-mitochondrial substrate levels for fatty acid beta-oxidation, thereby impairing energy generation (Bonnefont et al. 2004). Clinical symptoms often appear during periods of prolonged fasting or illness. Life-threatening hypoketotic hypoglycemia, hepatic encephalopathy, hepatomegaly with or without acute liver failure, seizures, and coma are prominent presenting clinical features. Mild cardiomegaly and heart beat disorders may be part of the clinical spectrum, as well as renal tubular acidosis (Bonnefont et al. 2004).

Classically, high plasma concentrations of free carnitine (C0) were considered diagnostic (Bonnefont et al. 2004; Stanley et al. 1992). It became, however, increasingly clear that this diagnostic parameter is problematic and that the diagnosis could be easily overlooked since quite often the concentration of C0 in plasma was found to be normal (Bergman 1994; Bonnefont et al. 2004; Innes et al. 2000; Primassin and Spiekerkoetter 2010; de Sain-van der Velden et al. 2013). It has been suggested that a high concentration of C0 in blood spot is more sensitive than its concentration in plasma (Primassin and Spiekerkoetter 2010; de Sain-van der Velden et al. 2013; Sim et al. 2001). This could be debated. Fingerhut et al. (2001) investigated the concentrations of C0 and acylcarnitines in 6 blood spot samples of 3 patients diagnosed with CPT-IA deficiency (2 days to 12 months old) and compared the results with those of 177,842 samples from the central Bavarian newborn-screening program. Blood spot C0 concentrations were normal in two of three patients. Diagnostic accuracy improved considerably in these patients when the molar ratio of C0/(C16:0-ac+C18:0-ac) was used (Fingerhut et al. 2001). In the most commonly used matrix, i.e., plasma, this ratio might have high sensitivity as well (de Sain-van der Velden et al. 2013). Unfortunately, the specificity of this marker is currently unknown.

To gain insight in the sensitivity ánd specificity of biochemical markers of CPT-IA deficiency, acylcarnitines in plasma of patients with proven CPT-IA deficiency were compared with those of patients in our historical dataset.

Methods

(Acyl-) carnitines in nine plasma samples of four patients with enzymatically and genetically confirmed CPT-IA deficiency were analyzed in three hospital laboratories, using comparable analytical methods. Blood spot acylcarnitines were available for three patients. The plasma results were compared with the historical dataset of 2,190 subjects of whom samples were analyzed in one of our laboratories between 2006 and 2012, (2,194 including the samples of 4 CPT-IA deficient patients). Only analyses of the first sample presented to our laboratories were included. The need for formal ethical review was waived by the local ethics committee. The study design was in accordance with the current revision of the Helsinki Declaration.

Samples were prepared according to local standard operating procedures. In short, plasma was deproteinized with acetonitrile. Subsequently, 6 deuterium-labeled internal standards in methanol/H_2O were added and samples were analyzed by flow-injection tandem-mass spectrometry (API-3000, AB Sciex, Framingham, USA). Concentrations of C0 and the individual acylcarnitines were quantitated by multiple reaction monitoring. C0 by an m/z transition of 162->103 and acylcarnitines by selected precursor ions with m/z 85 as the common product ion, as described earlier by Derks et al. (2008). For free carnitine, a daughter-mass with an m/z 103 was chosen because it had a higher intensity than the m/z 85 fragment. Data evaluation was performed with Chemoview® 3.0 software (AB Sciex, Framingham, USA).

Reference values were calculated using a reference population, consisting of 1,600 subjects out of the total population of 2,194 subjects. The other 594 samples derived from subjects diagnosed with an inborn error of metabolism, mainly fatty acid oxidation disorders, organic acidurias, phenylketonuria, glycogen storage diseases, and urea cycle defect, or originated from subjects who underwent a function test, i.e., fasting, glucose tolerance, or exercise tests. Concentrations of C18:1-ac and C16:0-ac are relatively high compared to the other long-chain acylcarnitines (lc-ac) (see, e.g., Table 1). It was decided to study C0, lc-ac (C16:0-, C16:1-, C18:0-, C18:1-, and C18:2-ac), the sum of lc-ac ($\sum$lc-ac), and the molar ratios C0/(C16:0-ac +C18:0-ac) and C0/$\sum$lc-ac. Age-dependency of these biochemical markers was investigated in the reference population by Spearman rank tests (significant $p < 0.05$). For each established age category, 95% reference intervals were based on 2.5 and 97.5% percentiles (non-Gaussian distributed metabolites). Normality was assessed using Shapiro−Wilk tests (significant $p < 0.05$). Receiver Operating Characteristic (ROC) curves were generated from results of patients with CPT-IA deficiency versus all (age-matched) subjects in the total cohort. The values of the biochemical markers to diagnose CPT-IA deficiency were compared using the areas under the curve (AUC). Sensitivity and specificity were calculated at 95% reference limits; sensitivity = true positives (TP)/(TP+false negatives (FN)) and specificity = true negatives (TN)/(TN+false positives (FP)). Analyses were done using Excel 2007 and Analyse-it 2008 (Analyse-it software, Ltd).

Table 1 Reference values and ROC analyses for biochemical markers of CPT-IA deficiency

Reference values							ROC analysis			
	0–7 days ($n = 169$)		8–30 days ($n = 106$)		31 days and older ($n = 1,325$)		31 days and older ($n = 1,803$ *subjects + patients*)			
	P2.5	P97.5	P2.5	P97.5	P2.5	P97.5	AUC (95% Cl)	*Cut-off*	*Se*	*Sp*
Free carnitine (C0)	6.0	33.0	7.3	41.8	13.2	55.0	0.738 (0.513–0.964)	>55.0	25.0%	96.1%
C16:0-ac	0.04	0.40	0.03	0.24	0.04	0.22	0.992 (0.977–1.000)	<0.04	100.0%	96.7%
C16:1-ac	0.01	0.15	0.01	0.05	0.01	0.10	0.919 (0.827–1.000)	<0.01	50.0%	97.0%
C18:0-ac	0.01	0.10	0.01	0.10	0.02	0.08	1.000 (0.999–1.000)	<0.01	100.0%	97.2%
C18:1-ac	0.03	0.33	0.04	0.38	0.05	0.43	1.000 (1.000–1.000)	<0.05	100.0%	97.4%
C18:2-ac	0.01	0.17	0.01	0.16	0.02	0.19	0.994 (0.983–1.000)	<0.02	88.9%	97.1%
Σlc-ac	0.11	1.06	0.12	0.96	0.16	0.99	1.000 (0.999–1.000)	<0.16	100.0%	96.9%
C0/(C16:0-ac+C18:0-ac)	15	306	63	578	63	587	0.997 (0.990–1.000)	>587	100.0%	96.9%
C0/Σlc-ac	8	138	21	229	22	216	0.999 (0.998–1.000)	>216	100.0%	96.7%

Reference and corresponding cut-off values were based on the historical subject population, see text. Concentrations of C0, individual long-chain acylcarnitines (lc-ac), and the sum of lc-ac (∑lc-ac) are in µmol/L. Ratios are in mol/mol. All biochemical markers, except C18:1-ac, were significantly related to age (see text). The areas under the curve (AUC) for Receiver Operating Characteristic (ROC) curves and the sensitivity and specificity at 95% cut-off values are presented for subjects and patients ≥31 days of age only (see text)

Results

Reference Values and ROC Analysis of Biochemical Markers

Except for C18:1-ac, all markers were significantly age-dependent (Spearman rank $P < 0.001$), with correlation coefficients (R) between (−) 0.05 for ∑lc-ac and (+) 0.34 for C0. This relation was most significant in the first 30 days of life. Even though age-dependency remained to be significant after 30 days for C0 and C18:0-ac, their relation with age was considered too small to have a relevant effect on calculated reference values (+0.07 µmol/L per year for C0, and +0.0001 µmol/L per year for C18:0-ac (linear regression analyses)). Reference values were calculated for three age-categories: 0–6 days ($n = 169$), 7–30 days ($n = 106$), and ≥31 days ($n = 1,325$) (Table 1). All biomarkers in these subgroups had non-Gaussian distributions (Shapiro–Wilk $p < 0.05$).

Plasma and blood spot acylcarnitines concentrations and ratios of patients with CPT-IA deficiency are presented in Table 2. All nine plasma samples had normal concentrations of C0, whereas all had low C18:0-ac, C18:1-ac, and ∑lc-ac concentrations, and high molar ratios. The ROC curves, and the sensitivity and specificity using 95% reference limits are also presented in Table 1. Because all samples of CPT-IA deficient patients were obtained after 30 days of age, only subjects and patients ≥31 days of age were included ($n = 1,803/2,194$). AUC were highest for C18:1-ac, C18:0-ac, and ∑lc-ac, closely followed by C0/∑lc-ac. Using 95% reference limits, C18:1-ac <0.05 µmol/L had the highest diagnostic value with a sensitivity of 100% and a specificity of 97.4%. Values were slightly less for other lc-ac and ratios, see Table 1. The ratio C0/(C16:0-ac+C18:0-ac) had slightly better specificity at these cut-off values compared to C0/∑lc-ac.

Using a combination of two markers, i.e., a concentration in plasma of C18:1-ac <0.05 µmol/L and a molar ratio of C0/(C16:0-ac+C18:0-ac) >587, specificity to diagnose CPT-IA deficiency increased to 99.3% compared with either C18:1-ac (97.4%) or C0/(C16:0-ac+C18:0-ac) (96.9%) alone, all at a sensitivity of 100%.

Characteristics of Subjects with False-Positives Test Results

Figure 1 shows the values of the 2 biochemical markers: C18:1-ac and C0/(C16:0-ac+C18:0-ac) for all subjects and patients ≥31 days of age, including 5 follow-up samples of CPT-IA deficient patients. Forty-eight subjects had false-positive test results for C18:1-ac. Ten of them were diagnosed with an inborn error of metabolism other than CPT-IA deficiency, i.e., phenylketonuria ($n = 4$), medium chain acyl-CoA dehydrogenase (MCAD) deficiency ($n = 4$), biotinidase deficiency ($n = 1$), and molybdenum cofactor deficiency ($n = 1$). Two out of four patients with MCAD deficiency and the patient with biotinidase deficiency had low concentrations of the sum of all carnitine-containing species in plasma (total carnitine). Six patients without a diagnosis also had low total carnitine concentrations. None of the 48 subjects had a high total carnitine concentration.

Fifty-six subjects out of the total population had a false-positive test results for the molar ratio C0/(C16:0-ac

Table 2 Biochemical marker concentrations of 4 CPT-IA deficient patients

Case	1	1	1	1	1	2	3	3	4
Gender	m					v	m		v
Age (years, days)	1y, 206d	1y, 266d	1y, 268d	1y, 271d	1y, 278d	6y, 22d	16y, 273d	19y, 21d	13y, 53d
Plasma (μmol/L)									
C0	55.6	51.6	33.5	15.9	49.1	48.2	34.7	52.4	31.6
C16:0-ac	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.02	0.01
C16:1-ac	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00
C18:0-ac	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
C18:1-ac	0.01	0.00	0.00	0.01	0.01	0.02	0.02	0.02	0.02
C18:2-ac	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.02
Σlc-ac	0.04	0.01	0.01	0.02	0.02	0.05	0.09	0.06	0.05
C0/(C16:0-ac+C18:0-ac)	3,087	5,160	3,350	1,590	4,910	4,818	694	2,618	3,160
C0/(Σlc-ac)	1,592	5,160	3,350	795	2,455	964	385	873	632
Blood spot (μmol/L)									
C0	>100					197	270		228
C16:0-ac						0.00	0.10		0.10
C16:1-ac						0.00	0.00		0.00
C18:0-ac						0.00	0.00		0.00
C18:1-ac						0.00	0.00		0.00
C18:2-ac						0.00	0.10		0.00
C0/(C16:0-ac+C18:0-ac)						>5,000	1,350		2,280
C0/(Σlc-ac)						>5,000	1,350		2,280
Enzyme Activity (nmol min^{-1} mg^{-1} protein)[a]	0.08					0.08	0.15		0.15
DNA mutations	c.1241C>T (A414V)					c.1318G>A (A440T)	c.1318G>A (A440T)		c.1318G>A (A440T)

This table shows concentrations of C0, individual long-chain acylcarnitines (lc-ac), the sum of lc-ac (∑lc-ac), and ratios for 9 samples of 4 CPT-IA deficient patients in plasma and blood spots together with lymphocyte CPT-IA enzyme activities and results of DNA mutation analysis. Two plasma samples of case 1 had non-detectable concentrations of C18:1-ac. In Fig. 1, these samples were presented at a C18:1-ac concentration of 0.005 μmol/L. The blood spot C0 concentration of case 1 was obtained from the Dutch neonatal screening program (see text). Patients were homozygous for mentioned DNA mutations

[a] Reference values lymphocyte CPT-IA activity: 0.2–0.8 nmol min^{-1} mg^{-1} protein

+C18:0-ac). Nineteen of them were diagnosed with an inborn error of metabolism other than CPT-IA deficiency, i. e., phenylketonuria ($n = 11$), glycogen storage disease 1A ($n = 2$), multiple acyl-CoA dehydrogenase deficiency ($n = 1$), MCAD deficiency ($n = 1$), molybdenum cofactor deficiency ($n = 1$), Smith–Lemli–Opitz syndrome ($n = 1$), pyruvate dehydrogenase deficiency ($n = 1$), and OCTN2 deficiency ($n = 1$). The MCAD deficient patient and ten subjects without a diagnosis had plasma total carnitine concentrations above the 95% percentile of their reference age group. None of the 56 subjects had a low total carnitine concentration in plasma.

Twelve patients had a false-positive test result when using the combination of a low C18:1-ac and a high molar ratio of C0/(C16:0-ac+C18:0-ac), of whom three had phenylketonuria and one had molybdenum cofactor deficiency. Of the other eight subjects, one was clinically suspected to suffer from CPT-IA deficiency. This was ruled out by CPT-IA enzyme activity measurements. All patients and subjects had a normal concentration of total carnitine.

Biochemical Markers of CPT-IA Deficiency in Neonatal Blood Spot

Acylcarnitine profiles were analyzed in blood spots obtained at the time of presentation in three of our patients with CPT-IA deficiency (cases 2–4). In these patients, C0 concentrations were above 197 μmol/L, whereas all lc-ac concentrations were below 0.02 μmol/L (Table 2). For case 1 the neonatal blood spot was requested at the Dutch

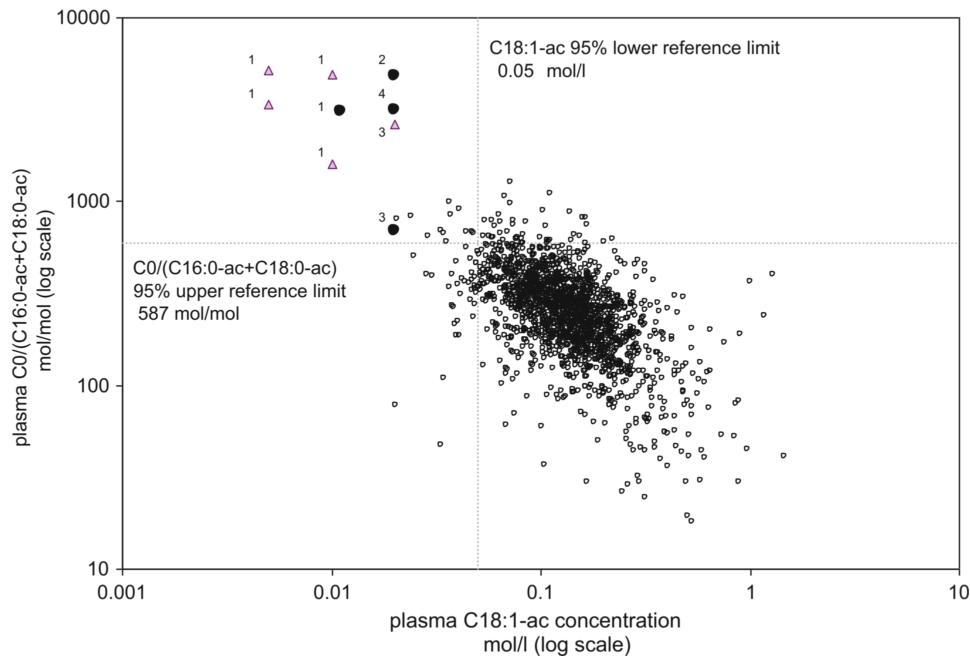

Fig. 1 Presents the results of C18:1-ac versus C0/(C16:0-ac+C18:0-ac) of all subjects and patients ≥31 days of age, including the first (*circles*) and follow-up (*triangles*) samples of four patients with CPT-IA deficiency [n = 1,808 (1,803 + 5 follow-up samples)]. The numbers correspond to the case numbers presented in Table 2. The *dashed lines* indicate the 95% cut-off limits for C18:1-ac (0.05 μmol/L) and C0/(C16:0-ac+C18:0-ac) (587). The quadrant limited by C18:1-ac <0.05 μmol/L and C0/(C16:0-ac+C18:0-ac) >587 had a sensitivity of 100% and a specificity of 99.3% for diagnosis of CPT-IA deficiency in our study population

neonatal screening authority. In this bloodspot, the concentration of C0 was found to be >100 μmol/L. In controls, C0 concentrations are lower and lc-ac concentrations are higher in blood spots compared to plasma (de Sain-van der Velden et al. 2013). This implies that these values are highly abnormal, even when applying plasma reference values.

Discussion

The results clearly show that CPT-IA deficiency can easily be overlooked when using the classical biomarker, i.e., high concentrations of C0 in plasma, as the sole criterion for diagnosis, as previously reported (Bergman 1994; Bonnefont et al. 2004; Innes et al. 2000; Primassin and Spiekerkoetter 2010; de Sain-van der Velden et al. 2013). Blood spot acylcarnitines, in particular the ratio of C0/(C16:0-ac+C18:0-ac), seem to have high diagnostic value for CPT-IA deficiency (Fingerhut et al. 2001; McHugh et al. 2011; Primassin and Spiekerkoetter 2010; de Sain-van der Velden et al. 2013; Sim et al. 2001). Even though our data support this suggestion, the present study also demonstrates that plasma, the more commonly used matrix, can be used for this purpose as well. The current study confirmed the high sensitivity of the molar ratio of C0/(C16:0-ac+C18:0-ac) in plasma, as proposed earlier (de Sain-van der Velden et al. 2013). Sensitivity of low plasma lc-ac concentrations was, however, equally high. When taking specificity under consideration, the diagnostic value became much better when using the combination of a high molar ratio of C0/(C16:0-ac+C18:0-ac) >587 mol/mol ánd a low C18:1-ac <0.05 μmol/L (cut-offs for patients ≥31 days of age). For low prevalent diseases such as CPT-IA deficiency, the increase of specificity from 97 to 99% has a dramatic effect on the positive predictive value of a test. In our selected population with a prevalence of 0.0018 (4/2,194), the positive predicted value increases from 7 to 25%.

ROC curve analysis is a well-known tool to compare diagnostic values of biomarkers. Using this analysis (Table 1), plasma C18:0-ac, C18:1-ac, and ∑lc-ac had highest and similar diagnostic values (AUC 1.000), closely followed by C0/∑lc-ac (AUC 0.999). To reduce the chance of missing a CPT-IA deficient patient, fixed cut-off values based on 95% reference values were evaluated. Using these cut-offs, the molar ratio C0/∑lc-ac was equally sensitive, but slightly less specific than the originally proposed molar ratio, i.e., C0/(C16:0-ac+C18:0-ac). False-positive results for C0/(C16:0-ac + C18:0-ac) were explained by high total carnitine concentrations in 11 of 56 patients and subjects. On the other hand, low C18:1-ac concentrations were associated with low total carnitine concentrations in 9 of 48

patients. Combining both markers significantly diminished the number of false positives, partly due to low or high carnitine concentrations. False-positive results were not clearly associated with other (known) inborn errors of metabolism. Three patients with phenylketonuria (3/100 in this cohort) had low C18:1-ac concentrations in combination with high C0/(C16:0-ac+C18:0-ac) ratios. After reviewing other acylcarnitine profiles of the three patients, only one sample, i.e., the first sample, had this remarkable result. The reason for this is unknown.

An important limitation of this study is the relatively low number of patients with CPT-IA deficiency. Plasma acylcarnitines have high intra-individual biological variation, depending on nutritional status (fasting), exercise, and clinical condition. The large biological variation may impair the diagnostic value of the markers. This is illustrated by the results of case 3. The analysis of the first sample gave near-normal values of the molar ratios of C0/(C16:0-ac+C18:0-ac) and C0/∑lc-ac, whereas the analysis of the second sample gave clearly abnormal values, see Table 2. When reviewing all nine plasma samples of our CPT-IA deficient patients (see Table 2 and Fig. 1), it is clear that the suggested cut-off values are highly sensitive and that the diagnostic value of the (combination of) markers is not dependent on the clinical condition and treatment regimens of our patients. When reviewing six plasma C0 concentrations and C0/(C16:0-ac+C18:0-ac) ratios of the 2 CPT-IA deficient patients presented in the study of de Sain-van der Velden et al. (2013), all the samples of these patients would probably also have been identified using the proposed marker combination. It is nevertheless important to investigate these markers in a larger cohort of CPT-IA deficient patients.

Conclusion

CPT-IA deficiency is rare and can easily be overlooked by relying on the classical marker, i.e., high plasma C0 concentrations. It has therefore been suggested to use blood spots acylcarnitines for diagnosis of CPT-IA deficiency. This study, however, demonstrates that CPT-IA deficiency can be diagnosed reliably with high sensitivity and specificity in plasma by a combination of a low C18:1-ac concentration and a high molar ratio of C0/(C16:0-ac+C18:0-ac). It is advised to further validate these markers in a larger cohort of patients with CPT-IA deficiency.

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Authors' Contributions

MR Heiner-Fokkema was responsible for the study design, statistical analyses, and writing of this manuscript. FJ van Spronsen and D-J Reijngoud made significant contributions to the conception and design of this study. Data from CPT-I deficient patients were supplied by MR Heiner-Fokkema, FM Vaz, R Maatman, and LAJ Kluijtmans. All authors critically read the manuscript and gave final approval for this version.

Conflicts of Interest

MR Heiner-Fokkema, FM Vaz, R Maatman, LAJ Kluijtmans, FJ van Spronsen, and D-J Reijngoud declare that they have no conflicts of interest regarding the topic in this manuscript.

Take-Home Message

Carnitine palmitoyltransferase IA deficiency can be diagnosed reliably by plasma acylcarnitine analysis.

Compliance with Ethics Guidelines

All procedures followed were in accordance with the ethical standards of the medical ethical committee on human experimentation (institutional and national) and with the Helsinki.

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RESEARCH REPORT

Relationships Between Childhood Experiences and Adulthood Outcomes in Women with PKU: A Qualitative Analysis

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Abstract *Background*: The enduring impact of the childhood experiences of people with phenylketonuria (PKU) on their adulthood outcomes is equivocal. As the effect of childhood experiences on adulthood is well documented amongst the general population, the aim of this study was to explore childhood experiences considered significant by women with PKU as they relate to adult experiences and management of PKU, and psychological wellbeing.

Method: Eight women with PKU in South Australia underwent semi-structured interviews. The audio-recorded interviews were transcribed verbatim and analyzed using thematic analysis.

Results: Interviews revealed that feeling different to peers as a child, challenges with management of the condition during adolescence, parental and extended family support, and the perception of PKU as a burden during childhood were associated with adulthood experiences.

Conclusions: Thus, it is proposed that these childhood factors have a combined, long-term impact. These findings have significant clinical implications, suggesting that early psychosocial intervention relating to these identified childhood experiences has the potential to enhance positive outcomes for adults with PKU.

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Successful management of phenylketonuria (PKU) can prove onerous across the lifespan (van Spronsen and Burgard 2008). Several factors influencing the experience of PKU during childhood have been identified. Family cohesion, comprising parental support (Shulman et al. 1991; MacDonald et al. 2010) and extended family support (MacDonald et al. 2010) are associated with dietary control. Conversely, children with separated or divorced parents (Olsson et al. 2007; Alaei et al. 2011), an unemployed parent (Alaei et al. 2011), or low socioeconomic status (Weglage et al. 1992) had reduced dietary control and elevated phenylalanine (Phe) levels (MacLeod and Ney 2010). Anguish and feelings of isolation have also been found to result from awareness of the differences in eating behavior between young people with PKU and their peers (Vegni et al. 2009). Adolescence can be particularly challenging. Adolescents experience less life satisfaction, restricted social experiences, and lower frustration tolerance than peers without PKU (Weglage et al. 1992). Additionally, adolescence has been associated with reduced dietary compliance (MacLeod and Ney 2010).

Despite the lifelong nature of PKU, there is little research on the influence of these early experiences on the adulthood experiences of the condition. To our knowledge, only MacDonald et al. (2010) have highlighted the important role of parental encouragement on children's

independence and increasing responsibility in relation to dietary control and long-term adherence to the PKU diet. This is because overreliance on parents for PKU treatment may negatively impact adjustment to full responsibility in adulthood (Bosch et al. 2007).

Whilst research specific to people with PKU is limited in this area, the long-term impact of childhood experiences is well documented within the general population. For example, childhood adversity has been associated with poor health and psychological distress in adulthood (Kestilä et al. 2005), whilst dietary patterns established in childhood extend into adulthood (Mikkilä et al. 2005). As such, it is proposed that the experiences of children with PKU would impact on their adult experience of the condition, psychological wellbeing, and management of the condition.

Irrespective of childhood experiences, and despite quality of life among adults with PKU being comparable with the general population (Mütze et al. 2011), adults with PKU experience greater psychological morbidity than the general population (Ris et al. 1997), including anxiety and depression (Pietz et al. 1997). The risk of mental health problems is further increased when one is non-adherent with the PKU diet (Koch et al. 2002). Accordingly, emphasizing childhood experiences that may impact adult outcomes is of significant clinical interest – early intervention may optimize outcomes for individuals with PKU not only during childhood but also across the lifespan.

This qualitative study aimed to explore the experiences of women with PKU, gaining an account of factors considered significant during childhood from the women's own perspectives. Subsequently, tentative links between childhood experiences and adult outcomes in terms of overall experience of PKU, psychological wellbeing, and PKU management are explored.

Method

Participants

All South Australian women known to be diagnosed with PKU were invited to participate ($n = 20$). Eight women participated (40% response), aged 21–42 years ($M = 29.9$, SD = 7.9). Reasons given for non-participation were primarily related to insufficient time. All participants were diagnosed and treated for PKU from soon after birth and all had tested intellectual functioning in the normal range. All but one of the participants ($n = 7$) were maintaining the PKU diet at the time of the interview, and were taking a supplement (medical formula). Six women were having regular blood tests although the frequency of testing ranged from weekly to every four to six weeks. Lifetime Phe levels were estimated based on a review of case notes by a Metabolic Physician (DB). For six participants, Phe levels were within acceptable ranges for the majority of the time, one for approximately 80% of the time, and one for less than 10% of the time (see Table 1). Five women were married/de facto and three were single. Seven women were employed, four had completed Year 11–12, two had vocational qualifications, and two had university degrees. Three participants were undertaking tertiary education. Three participants (37.5%) were assessed on the M.I.N.I. as experiencing significant psychological disorders in their lifetime (two participants had a history of major depressive disorder; one of whom also was experiencing recurrent bulimia nervosa; one participant had a history of bipolar affective disorder, with a current depressive episode).

Table 1 Participant characteristics

Participant number	Age range	Education	Diet status	Phe levels in target range (approximate % over lifetime)
1	20s to 30s	High school	Off diet	<10%
2	20s to 30s	Post high school	Strict	100%
3	20s to 30s	Post high school	Strict	100%
4	20s to 30s	High school	Strict	100%
5	20s to 30s	Post high school	Strict	100%
6	30s to 40s	Post high school	Relaxed	100%
7	30s to 40s	High school	Relaxed	100%
8	30s to 40s	Post high school	Relaxed	25%

Procedure

This study, approved by the Human Research Ethics Committee of the Children, Youth, and Women's Health Service, forms part of a study to explore women's experiences of PKU in relation to pregnancy (Roberts et al. 2014). As such, participants were all female. Experiences of PKU across the lifespan were explored within the initial study and childhood experiences were noted to be of importance in relation to outcomes in adulthood, resulting in the current research.

Semi-structured interviews (which took 30–90 min, depending on the length of participant's responses) were audio-recorded, then transcribed. Interviews began with women being asked "Can you tell me about the impact that PKU has had on you throughout your life?" (see Appendix for full list of interview questions). Participants' experiences of PKU across their lifetime were explored (reported here), followed by their perceptions and/or experiences of pregnancy (reported in Roberts et al. 2014). Interviews were analyzed using thematic analysis (Braun and Clarke 2006). Initial interviews, data coding and sorting into

themes was conducted by TR, a graduate clinical psychology student with basic training in PKU management. Reliability of coding and themes was verified through review with two psychologists/researchers with extensive experience working with people with PKU (RR, AG).

In addition, The M.I.N.I. (English Version 6.0.0) was administered. The M.I.N.I. is a brief, verbally administered questionnaire assessing lifetime experience of psychological disorders (Sheehan et al. 1998).

Results

Participants are identified by age group, education, and self-reported dietary adherence – strictly on diet, relaxed on diet or off diet (Table 1). Thematic analysis identified five themes relating to childhood experiences of PKU (Table 2) and three themes relating to adulthood experiences of PKU (Table 3). We also describe the association between childhood experiences and adulthood outcomes reported by the women.

Table 2 Childhood themes

Theme	Examples
1. Comparisons to and feeling different from peers	. . .you see all your friends eating this and then you're like, well I can't have that and they're all buying their lunch and you can't buy nothing cos you know, it's pies and pasties and everything else. (Participant 1) Back then it's, oh my god what's wrong with you! You know, you can't eat that, why, what's wrong with you, is something wrong with you, you know that sort of attitude. . . It was hard, very difficult. (Participant 4) So I was sort of, almost felt an oddball, being a child growing up with PKU, or a teenager. (Participant 7) . . .there's just me with like, my you know, different dish and you know it makes it a bit hard, hard to do, but it never bothered me. (Participant 3)
2. Management of PKU during adolescence	. . . It [management of PKU] was ok probably to my teens. . . I sort of strayed there and just had whatever. . . just the chips and you know, a pasty or. . . not counting anything. (Participant 8) . . . high school was worse. . . you eventually stop eating at school. . . and you know, you just think, I'm not eating at school, and you slowly drift from the diet. (Participant 1) . . . I remember at 14, and for that, for like nearly a year it was really, really hard to get me to take my formula. (Participant 6) Girls are trying to fit in, they're going through puberty and there's boys and this and, so it's really tough. And then trying to stick to the diet. You're not always gonna do it, you're gonna fail eventually. You think, what's the point? (Participant 1) . . .back then you think you can go on without doing the right thing for your body (Participant 8) . . .a teenager's brain would not care at all about what happens in the future. (Participant 6)
3. Parental support	. . .she [Participant's Mother] went out of her way to prepare stuff for me so I did have stuff there and stuff that was, I suppose special to me, and so it didn't make me feel like I was the oddball as much. . . She'd make me fairy bread out of my own bread and so I'd be able to eat the fairy bread when the other kids were eating fairy bread. . . (Participant 7) . . .mum's always been a great cook and she kind of always adapted recipes to suit me and I think that helped a lot because you know, there's, I guess I would still be having like normal food, but with the low protein food. (Participant 3) . . .mum never experimented with me, like with the drinks and food and stuff. (Participant 1) Mum would get me in the kitchen and we'd bake, make all the low protein stuff. . . (Participant 5) . . .I guess for many, my, my mum did pretty much everything for me. (Participant 8) . . . extremely thankful to my parents for sticking to the diet so umm, so greatly and so accurately, because you know, here I am, I'm a, you know, I'm a graduate student, I've got a profession, and if it weren't for their compliance and their understanding of PKU and, and what my needs were as a child and as a baby, I wouldn't be here today. So I have them to thank for that and I'm very grateful. (Participant 6)
4. Extended family support	. . . if there was a big party or something, they'd ring mum and say, you know, what can she have. Or we've made her this, so she can have that as well. . . always made sure I had something so yeah, it was good. (Participant 5) . . . yeah they were supportive, they always said, if we went over there for tea, they always said, what can [participant] have, what can she eat, and all that type of thing, so yeah. They were all supportive. (Participant 7)
5. Perception of PKU as a burden	I wouldn't really call it more an impact, it's just, it's just I've grown up this way, it's all I know. (Participant 5) So, it's sort of like, ok, how it is, that's how it is and you don't know any different. (Participant 4) Having to eat all my low protein foods and do all that type of thing and have my supplements, take them to friends' places. Or even something as simple as going on camps and stuff like that. . . That would probably be the hardest thing. (Participant 7)

Table 3 Adulthood themes

Themes	Examples
1. Management of PKU	I like the liberty of having a normal cookie or biscuit every now and again. (Participant 6)
2. The unique experience of having PKU	
2a. Eating in a social context	. . .when they [restaurants] make salads, some of the salads, like Caesar salads or something, has cheese in it and say could you not have cheese, and they look at you like you're weird. . . I just feel different. . . it always brings your mood down. (Participant 1) . . .But it's just so hard, like even when you're happy, you go out for tea and, you could just imagine if you were on the diet, then what would you eat? You know, places like that don't exactly have menus for PKU people, do they? (Participant 1) . . .so really socially, it's probably been an impact and going to parties and things like that. Having functions on where umm. . . I almost have to eat before I go, I can't eat there. (Participant 7) It's normally fine. . . I try not to eat tons of protein during the day, so I can have like a bowl of pasta or things like that. . . I really like Asian food and Indian food which is great because it's just easy to get a pure vegetable dish. (Participant 3)
2b. Effects of dietary compliance	. . .when my levels get higher umm. . . obviously the mood swings umm you feel, you get like, you feel sort of a little bit lethargic and you don't really want to do anything and you, it takes a while to umm to process stuff and just, my thinking's just probably a little bit slower. . . (Participant 5) I can feel it. I just go, oh I feel like my brain's all fuzzy and I can't think straight. (Participant 6). . . . having depression as well and not being on diet really is a bad combination. (Participant 1) So everything was just so much better. And mood swings were probably better too. . . huge change just in how I handled day-to-day things with stress and stuff like that. (Participant 7)
2c. Transition from childhood to adulthood management of the condition	. . .like counting out properly and that type of stuff, yeah. . . she [her mother] used to make a lot of food for me, so she did it all, so umm. . . yeah I didn't really do it myself, kind of thing. (Participant 8)
2d. Attitudes and perceptions	I don't really worry about it, I don't think about it. . .I've had it my whole life, it's who I am, you know. (Participant 2) You know, it's just kind of been part of my life so I've just dealt with it and I just know exactly what I can eat and I can't eat and how much of, you know, something I can eat and that kind of thing. (Participant 3) . . .oh my god, this is so hard, I've gotta weigh everything, I can't go out, I can't do this, I have to take my own food with me, so it is a big, it is a big stress. . . (Participant 7) . . . your life revolves around your diet. . . you always think about what you're going to be eating. (Participant 6)
3. Psychological wellbeing	. . .my eating was umm. . . a problem for me. . . so I think it [PKU] might have been a part of it. Like it was all, like cuz I wasn't allowed. I didn't have a choice, I wasn't allowed to. . . (Participant with previous eating disorder) . . .you need to be working on both things at the same time. So getting back on diet and look after yourself, after your mental health, is two things that need to happen together. (Participant 1)

Childhood Themes

Comparisons to and Feeling Different from Peers

A key theme noted within discussions of childhood was feelings of difference between the women and their peers, predominantly related to eating within a social context, such as at school or parties. Whilst discussed by the majority of women, the experience of such differences varied. Some women found it to be a negative experience, associated with feelings of isolation, whilst others were unconcerned and did not feel excluded or distressed. The women who found such differences to be a negative experience described mealtime away from home as challenging as they received questions regarding their restricted diet, heightening feelings of difference and exclusion. In contrast, other women noted that they were aware of differences between themselves and peers, but this did not result in distress.

Management of PKU During Adolescence

Women noted that management of PKU was challenging during adolescence, resulting in reduced dietary compliance. The reasons noted for reduced compliance were similar. Two women attributed it to the general challenges of adolescence, with Participant 6 reporting that she lived her ". . .frustration as a teenager through my diet." Reduced compliance was also attributed to the perception of adolescence being a period associated with limited consideration of the future consequences of present actions.

Parental Support

All women spoke of the support received from their parents in childhood, highlighting the ways in which their parents assisted in the management and experience of PKU. Whilst all women emphasized the importance of this care, the nature and level of the support received varied. Several women reported receiving high levels of parental support which centered on reducing their feelings of exclusion and difference. For example, for social events with peers, parents routinely prepared and brought low-Phe food for the children similar to those foods eaten by others. These women reported that this assisted them to feel included, minimizing feelings of difference. In contrast, others indicated that the support received was more limited. Whilst Participant 1 stressed how adapting "normal" meals to be low-Phe helped to "...deal with the differences..." as a child, she reported minimal experimentation with dishes within her family. Of note, these women reported more feelings of difference from their peers.

Additionally, differences in the nature of parental support were identified. Some women reported that their parents included them in meal preparation, which assisted the development of their skills in the management of PKU in addition to providing an enjoyable experience. In contrast, one woman reported that her mother took responsibility for her diet and that she was rarely involved. She felt that this resulted in a lack of development in skills and contributed to difficulties in her adjustment to independent management of the condition as an adult. Regardless of the nature, parental support was appreciated and perceived as important.

Extended Family Support

The involvement of extended family members in the management of PKU was discussed, specifically with regard to catering at family events. When extended family members were not involved in the preparation of "PKU-friendly" meals or lacked knowledge of the condition, women felt excluded, for example, Participant 1 claimed that her extended family saw her as an outcast due to her condition.

Perception of PKU as a Burden

All women commented on the overall impact of PKU on their childhood and adolescence. Women who did not indicate that their condition was a significant burden instead appeared to have accepted the requirements for management, perceiving PKU as a way of life. In contrast, women who found PKU to be a burden as a child made reference to the difficulties associated with PKU and the sense of feeling "different" to peers.

Adulthood Themes

Themes were identified regarding PKU as it related to adulthood and current circumstances (Table 3). As noted within childhood themes, both similarities and differences were apparent in their experiences.

Management of PKU

Self-reported management of PKU formed a continuum from complete non-compliance to vigilant dietary control. Participant 1 was off diet, did not take the supplement, nor had blood tests to measure Phe levels. Participants 2, 3, and 5 were strictly on diet, took the supplement, and had regular blood tests. Participant 4 was also on diet and took the supplement but did not undertake blood tests. Participants 6, 7, and 8's management was situated in the middle of the spectrum, all being effectively on diet and consuming the supplement, but having a somewhat relaxed approach towards dietary adherence. Participant 6 deviated from the diet occasionally and Participants 7 and 8 did not measure their Phe intake. As such, some women had elevated Phe levels.

The Unique Experience of Having PKU

Women referred to several specific aspects of having PKU as adults.

Eating in a Social Context

Food in social contexts was problematic for some women. Similar to during childhood, Participants 1, 7, and 8 were conscious of differences, still find this upsetting. Eating out socially generally continued to be perceived as difficult due to the lack of suitable meals available. Participant 8 avoided going out to dinner altogether as it "...was just too hard..." In contrast, Participant 3 did not report eating out as an adult problematic as she was able to adapt sufficiently in social eating situations.

Effects of Dietary Compliance

Several women mentioned the negative effects of being off diet, or having high Phe levels. Women reported tiredness, inattentiveness, poor memory, mood swings, and anxiety. In cases where a psychological issue was present, symptoms were reported to be exacerbated. Maintenance of Phe levels within, or close to, the recommended range was associated with improved psychological and physical wellbeing. Women reported enhanced concentration, improved coping ability, more stable mood, decreased anxiety, and higher

energy levels. These benefits of dietary compliance were a motivation for adherence.

Transition from Childhood to Adulthood Management of the Condition

Some women reported difficulty in adjusting to full responsibility for management of their condition as an adult. Contributing factors included timely access to adult services and the lack of development of appropriate knowledge and skills to achieve independent management. Participant 8, for example, noted that she had to acquire several skills required for dietary compliance in adulthood as she was not very involved in PKU management as a child. Participant 2 reported cessation of dietary compliance for a year following delays in access to adult services.

Attitudes and Perceptions

Overall, the women's attitudes towards, and perceptions of, their condition varied along a continuum. Women had either developed acceptance of, or spoke negatively about PKU. Acceptance of PKU and the requirements for its management was related to expression of relatively positive views about the condition. Women who expressed such views accepted PKU as a part of their life and experienced minimal distress related to PKU as they had adapted their lifestyle to accommodate the condition from an early age. In contrast, those with less positive attitudes towards PKU highlighted the difficulties associated with management of the condition. Additionally, poor psychological wellbeing appeared to heighten negative attitudes. One woman with depression reported, "I just really can't be bothered with my life. I just, there's no point anymore, so what's the point with the diet?"

Psychological Wellbeing

Significant differences in psychological wellbeing were noted between the women. Three women reported significant historical mental health concerns with one also experiencing current symptoms. The women who had experienced mental health concerns all perceived PKU as contributing to, or exacerbating, their mental health condition. In reference to her depressive episodes, one woman said "It's [PKU] got everything to do with it", with another questioning whether "...PKU people are fragile or predisposed to it [depression]..." In addition, a woman with bulimia nervosa suggested that the food restriction required in PKU contributed to its onset. Hence, PKU was perceived as a risk factor for poor psychological wellbeing. One woman also stressed the negative effect of poor psychological wellbeing on dietary compliance and the overall PKU experience, highlighting the importance of positive psychological wellbeing for successful dietary management.

Connection Between Childhood Experiences and Adult Outcomes

Connections were noted between the women's childhood experiences and their outcomes as adults, including management of the condition, experiences of PKU, and their psychological wellbeing. Positive adult outcomes, such as acceptance of PKU, continued appropriate management of PKU, positive psychological wellbeing, perception of minimal difficulty eating in social contexts, and ease of transition to independent management of the condition may be related to childhood experiences such as high levels of parental and extended family support, parental support to develop skills and knowledge to manage PKU, increased feelings of inclusion, and minimal perception of PKU as a burden. For example, Participant 1 who reported poor mental health and who was not on diet discussed feeling socially excluded as a child, and reported less support than many other participants from her parents and extended family, as well as perceiving PKU as a burden. This contracts with Participant 5 who reported good mental health, was on diet (strict) and who described extensive parental and extended family support as a child, who did not report feeling socially excluded as a child and who did not describe perceiving PKU as a burden. Table 4 highlights adulthood outcomes and potential related factors in childhood.

Table 4 Adulthood outcomes and associated childhood experiences

Adulthood outcomes		Associated childhood experiences
Eating out socially	→	Feelings of difference to peers
Transition to full responsibility of management		Management of PKU during adolescence
Attitude towards PKU		Parental support
Psychological wellbeing		Extended family support
Management of PKU		Perception of PKU as a burden

Discussion

This study explored childhood experiences of women with PKU as they relate to adulthood management of PKU, experiences of the condition, and psychological wellbeing. Consistent with previous literature, feelings of difference to peers due to eating behavior (Zwiesele et al. 2015; Vegni et al. 2009), increased difficulty with dietary compliance during adolescence (Weglage et al. 1992; MacLeod and Ney 2010), parental support (Shulman et al. 1991), and extended family support (MacDonald et al. 2010) were identified as key childhood experiences for women with PKU, shaping the perception of PKU during childhood. Consistent with Kestilä et al.'s (2005) and Mikkilä et al.'s (2005) findings regarding the long-term impact of early experiences, connections were noted between these childhood themes and adulthood outcomes. Adulthood outcomes ranged along a continuum dependent upon childhood experiences. Current findings suggest that the identified childhood themes collectively shape the overall experience of PKU as a child, in turn, having a combined effect on adulthood management of PKU, experiences of the condition, and psychological wellbeing.

More specifically, the experience of minimal emotional distress regarding differences with peers due to eating behavior, minimal difficulty with dietary compliance as an adolescent, and high levels of parental and extended family support together may reduce the perceived burden of PKU as a child. These factors appear to build resilience in the individual, facilitating adult outcomes characterized by effective management of PKU, minimal perception of difficulties eating in social contexts, reduced difficulty in transition to self-management, an attitude of acceptance towards PKU, and positive psychological wellbeing. This is consistent with Finkelson et al. (2001) who reported that social support, positive attitudes towards the PKU diet, and perceptions that the diet is manageable in one's daily life predicted whether adults with PKU successfully returned to being on diet.

The enduring significance of childhood experiences for individuals with PKU makes sense given the well-documented impact of early life experiences on long-term physical and psychological wellbeing amongst the general population (Kestilä et al. 2005). Distress during childhood over eating differences could lead in adulthood to deviation from the PKU diet to avoid feeling excluded, as well as negative views of the condition and poor psychological wellbeing. Difficulty with dietary compliance as an adolescent may result in relaxed or total non-adherence as an adult, with reestablishment of the diet being difficult (MacLeod and Ney 2010). Accordingly, women either off diet or with lenient dietary compliance in this study reported increased difficulty adhering during adolescence. Parental support is important in modeling management of PKU and establishing the child's dietary patterns, potentially assisting adjustment to adulthood in terms of attitude, diet, and psychological wellbeing. Consistent with Bosch et al.'s (2007) notion that children who are overly dependent on their parents in regard to PKU management may be susceptible to difficulties in adjustment to full responsibility in adulthood, the current study revealed that parental encouragement of growing self-management is important for the ease of transition. A sense of "teamwork" provided by supportive extended family members during childhood may also influence how accepting the individual is of PKU as an adult. Accordingly, women who reported high levels of extended family support during childhood were less overwhelmed by the demands of PKU in adulthood.

The self-report, qualitative nature of this study has revealed the perspective of women with PKU regarding their childhood and adult experiences, extending the current literature, much of which has previously explored the perspective of parents when consideration is made of childhood. Although appropriate for a study of this type, the sample was limited by the number of women with PKU in South Australia who were interested in participating. Participants were open to and had sufficient time to discuss their condition and experiences whereas those who were not as willing to discuss such matters and those who had more time pressures were likely underrepresented. It is also possible that those who chose not to participate were more likely to be those who perceived fewer psychological impacts of having PKU; future work should address this. However, the authors were satisfied that saturation had been reached for the key themes described in this paper. Given these findings reflect women's experiences only, future research with both genders would provide a broader perspective and validation of the themes identified here. Further, the results provide an illustration of the experiences of women treated within one hospital. Given that treatment within other health facilities may vary, multi-site research would be useful. Finally, future work that compares the experiences of adults with PKU with adults with other conditions, particularly those that require dietary management, may be useful.

The findings of this study have significant clinical implications. Given the difficult circumstances adults with PKU can experience regardless of their childhood experiences (Ris et al. 1997; Pietz et al. 1997; Koch et al. 2002; Gassió et al. 2003), intervention as a child can prove beneficial over the life span. In conjunction with diet and Phe level monitoring, treatment strategies should also encompass a psychosocial approach. Encouragement of parental support, specifically the delivery of advice regarding best care for children with PKU (as also described by Zwiesele et al. 2015), involvement of extended family

members, and provision of PKU knowledge to school peers should be included. Additional support services for adolescents with PKU may also assist in preventing or reducing potential issues with dietary adherence as a teenager, such as hands-on meal preparation “fun” days.

Conclusion

Adult outcomes for women with PKU may be influenced by certain childhood experiences relating to the condition, in particular the feeling of difference in comparison to peers, increased difficulty with dietary compliance during adolescence, parental support, extended family support, and the overall perceived burden of PKU. Results suggest that the unique experience of these may have a combined effect on adulthood management of PKU, experiences of the condition, and psychological wellbeing. Regardless of childhood experiences, lifelong management of PKU can prove onerous for some individuals and adults with PKU are at increased risk of mental health difficulties, despite good quality of life. Adoption of a broader approach to clinical management of PKU during childhood may facilitate positive adult outcomes.

Synopsis

Childhood experiences of women with PKU include feeling different to peers, challenges during adolescence, parental and extended family support, and the perception of PKU as a burden; these have a combined effect on adulthood management of the condition and psychological wellbeing.

Compliance with Ethics Guidelines

Conflict of Interest

R. Roberts, T. Muller, A. Sweeney, D. Bratkovic, A. Gannoni, and B. Morante declare that they have no conflict of interest.

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.

All authors contributed to the planning and reporting of the work described in the article. T Muller conducted the interviews and the thematic analysis.

Interview Questions

Can you tell me about the impact that PKU has had on you throughout your life?

As you were growing up, what were your plans about having children, knowing that you had PKU?

Have your plans changed now you are an adult?

What was your understanding about how pregnancy might be different for women with PKU?

Have you had any pregnancies, how many, were they planned or unplanned?

What did (would) you consider when deciding to become pregnant (if it was a planned pregnancy)?

Questions for Women Who Have Had a Pregnancy

I’d like to hear about your experience(s) of pregnancy with PKU.

Can you tell me about what you found particularly difficult during your pregnancy?

What did you find was helpful in coping with these difficulties?

Did you find that you used different ways of coping at different times in your pregnancy (for example, 1st trimester vs 3rd trimester)?

Can you tell me about what you found particularly positive during your pregnancy?

In general, how did you feel during your pregnancy?

How did you feel during the difficult times of your pregnancy?

Can you tell me about the supports you felt were available to you during your pregnancy, for example, people in your life, medical staff, or groups you were involved in?

Which supports did you find particularly helpful during your pregnancy and why was this?

Can you tell me about some supports that you would have liked to have had during your pregnancy?

Questions for Women Who Have Had Multiple Pregnancies

Can you tell me about the differences you noticed between your pregnancies?

How do you think your previous pregnancies affected your later pregnancies experiences?

Can you tell me about any knowledge that you feel that you would’ve found helpful during your first pregnancy?

Questions for Women Who Have Not Had a Pregnancy, but Are Planning to in the Future

I'd like to hear about the impact you believe PKU will have on your pregnancy.

Can you tell me about the difficulties you believe you may face?

Can you tell me about what you believe may be helpful in coping with these difficulties?

What supports do you believe may be helpful during your pregnancy, for example, people in your life, medical staff, or groups?

What supports would you like to have available to you during your pregnancy?

Questions for Women Who Have Not Had a Pregnancy, and Are Not Planning to in the Future

Can you share with me about the reasons why you have chosen not to become pregnant?

What impact did PKU have on this decision?

Can you tell me about the difficulties you experienced in making this decision?

Can you tell me about what you found helpful in coping with these difficulties?

Can you tell me about the supports you felt were available to you when you were making this decision, for example, people in your life, medical staff, or groups you were involved in?

Which supports did you find particularly helpful during this time and why was this?

Can you tell me about some supports that you would have liked to have had during this time?

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CASE REPORT

The Effect of S-Adenosylmethionine on Self-Mutilation in a Patient with Lesch–Nyhan Disease

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Abstract *Background*: Lesch–Nyhan disease (LND) is an X-chromosomal disorder of purine metabolism characterized by hyperuricemia, dystonia, and self-mutilation, leading to an extremely high burden of disease in affected patients and families. Although allopurinol therapy can control hyperuricemia, it has no effect on self-mutilation and neurological symptoms. Single reports describe a beneficial effect of S-adenosylmethionine (SAM) on the neurological symptoms, which motivated us to evaluate this alternative treatment.

Methods: We performed a double-blind placebo-controlled trial to analyze the effects of SAM on self-mutilation attempts in a male patient affected by LND. The trial lasted for 282 days and comprised three alternating verum and placebo periods of 50 days each. The mother of the patient recorded attempts of self-mutilation during the entire trial.

Results: While verum and placebo were both well tolerated, a total of 1,762 events of self-mutilation were recorded, of which 1,281 events were in the placebo period and 481 in the verum period. The daily mean of events was 8.6 with placebo and 4.5 with SAM corresponding to a 50 % decrease in self-mutilation events under SAM treatment ($p < 0.05$).

Conclusion: The results of this double-blind placebo-controlled single-case trial suggest that SAM can have a beneficial effect on self-mutilation in patients with LND, possibly by replenishing the purine pool in affected brain cells.

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Introduction

Lesch–Nyhan disease (LND, OMIM #308000) is a severe metabolic disorder due to a lack of hypoxanthine-guanine phosphoribosyltransferase (HPRT E.C. 2.4.2.8). The disease is characterized by hyperuricemia and neurological symptoms including dystonia, psychomotor retardation, and self-mutilation. In humans, the main role of HPRT is to salvage hypoxanthine and guanine and, hereby, to replenish the intracellular purine pool (Fig. 1). In LND, hypoxanthine is degraded to uric acid by xanthine oxidase, thus resulting in hyperuricemia. The disease is X-linked with an incidence of 1 in 380,000 (Bertelli et al. 2004). The defective enzyme is encoded by the *HPRT1* gene, and more than 615 mutations are known (Fu et al. 2014; Jinnah et al. 2004). There are several variants of the disease with different clinical severity (Jinnah et al. 2000, 2010; Puig et al. 2001; Schretlen et al. 2005). HPRT is expressed in all tissues with high levels found in many regions of the brain.

The clinical phenotype manifests mainly in males, but there are also female patients with LND (Chen et al. 2014;

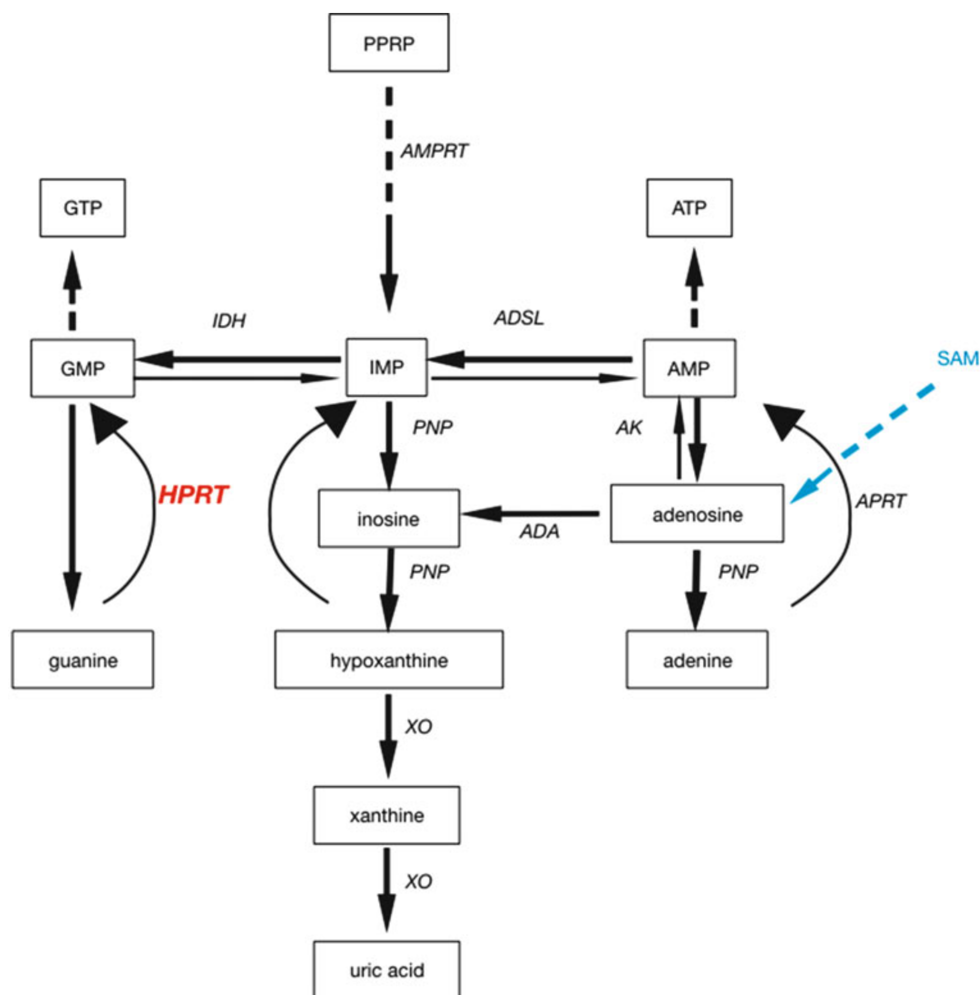

Fig. 1 Scheme of the metabolic pathway of purines and their relation to hypoxanthine-guanine-phosphoribosyltransferase (HPRT). In Lesch–Nyhan disease (LND), the lack of HPRT activity leads to a failure in recycling of both guanine and hypoxanthine, thus creating a deficiency of both inosine monophosphate (IMP) and guanosine monophosphate (GMP). Hypoxanthine (and guanine) concentrations are elevated in LND and will be metabolized to uric acid by xanthine oxidase (XO). With S-adenosylmethionine (SAM, depicted in *blue*), an adenosine donor, the lack of purines in LND can be recovered by turning adenosine to adenosine monophosphate (AMP) by adenine phosphoribosyltransferase (APRT) and then to IMP. *ADA* adenosine deaminase, *ADSL* adenylosuccinate lyase, *AK* adenosine kinase, *AMPRT* amido phosphoribosyltransferase, *ATP* adenosine triphosphate, *GTP* guanosine triphosphate, *IDH* isocitrate dehydrogenase, *PNP* purine nucleoside phosphorylase, *PPRP* phosphoribosylpyrophosphate

De Gregorio et al. 2000). Manifestation of LND is often by developmental delay in infancy, followed by dystonia and spasticity, which may provoke misdiagnosis as cerebral palsy. The presence of orange crystals in the diapers may raise suspicion of hyperuricemia.

LND usually leads to several severe complications along each of the clinical presentations, and all have a huge impact on the (social) life of patients and their families. The movement disorder is often severe and may necessitate the use of a wheelchair from early childhood. Self-mutilation and autoaggressive behavior are almost pathognomonic and may require wearing of gloves and/or dental removal to prevent bite wounds to fingers/hands and lips/cheeks. Overproduction of uric acid causes nephrolithiasis leading to renal insufficiency, which is a main cause of death. Furthermore, there are several sudden death cases related to LND (Neychev and Jinnah 2006).

The pathophysiology of LND is not fully understood, but the general hypothesis includes depletion of adenosine (Criswell et al. 1988; Green et al. 1982; Kopin 1981; Stone 1981). Lack of purines due to HPRT deficiency in LND may be rescued by salvaging purines via adenosine → inosine monophosphate (IMP) → guanosine monophosphate (GMP). S-adenosylmethionine (SAM) is thought to replenish the purine pool by serving as an adenosine donor (Fig. 1).

SAM, previously used in another purine deficiency disease, Arts syndrome (de Brouwer et al. 2007; Duley

et al. 2011), has been beneficial for treatment of self-mutilation in a single LND patient who initially received SAM for liver detoxification and elevated transaminases (Bottiglieri 2002; Glick 2006) and was tested in 14 patients from the Italian LND cohort with unequivocally opposite effects on patients (Dolcetta et al. 2013). A recent study in four additional patients (Chen et al. 2014) demonstrated a positive effect of SAM on the neurological symptoms, mainly self-mutilation.

Here, we prospectively investigated the effect of SAM in a double-blind placebo-controlled trial in a single patient with LND. From the findings of this trial, we suggest that SAM therapy could be beneficial for control of self-mutilation in LND.

Methods

Patient Presentation and Clinical Course

We describe a firstborn male child from unrelated healthy Swiss parents with normal postnatal adaptation. After normal development during his first 6 months of life, parents noticed first signs of developmental delay and muscular hypotonia. Evolution of dystonia and spasticity as well as orange crystals in the diapers and elevations of uric acid in blood and urine led to the suspicion of LND. This was confirmed by enzymatic testing in leukocytes (Laboratory of Genetic Metabolic Disease, Erasmus Medical Center, Rotterdam) at an age of 18 months. Mutation analysis of the *HPRT1* gene showed a c.319-1G>A *de novo* mutation in the patient, but not in the mother, probably leading to a splice defect of the gene. Allopurinol therapy was started at a low dose (as the only additional pharmacological treatment at the time of the SAM trial) and adapted in relation to the uric acid levels in serum and urine (Fig. 2). The patient showed first self-mutilation attempts at age 2.5 years by biting his fingers, and this symptom was progressive leading to severe destruction of lips and repeated deep oral wounds. Physiotherapy led to some improvement of hypotonia and spasticity. The patient became wheelchair bound at age four years. To prevent malnutrition, supportive gastric tube feeding was initiated at age 3.7 years. For treatment of sleep disorders and anxiety, risperidone seemed beneficial.

Planning of Therapeutic Trial and Study Design

Self-mutilation became the most stressful symptom for patient and parents and led to restraining of hands and legs for most of the time during days and nights. In an attempt to quantify self-mutilation attempts, recording by the parents was started as a pre-study period (Fig. 2). Self-mutilation attempts were defined as abrupt arm movements of the patient aimed at biting his fingers or hands. This pre-study period comprised 165 days, during which 212 self-mutilation attempts were documented confirming that recording of these events was feasible and tolerated by the parents. Later analysis of these recordings showed a possible association of self-mutilation attempts with pain and/or infections. In order to evaluate a possible effect of SAM on the number of self-mutilation events, a double-blind placebo-controlled trial was performed over eight months in which the parents continued to record self-mutilation events. Informed consent of parents was obtained. The trial consisted of alternating periods of 50 days on either placebo or SAM (verum) (Figs. 2 and 3). The patient was given capsules with 100 mg SAM twice daily (corresponding to 17 mg/kg/day) or placebo via his nasogastric tube. SAM (Abbot AG, Baar, Switzerland) and placebo were prepared as capsules indistinguishable by appearance and smell/taste. As this was a therapeutic trial with a licensed drug in a single patient, there was no need to obtain formal approval by the local ethical board.

Statistical Analysis

To analyze results, an unpaired *t*-student test was used with average of recorded events $\pm$ standard deviation. Results were considered significant in case of $p < 0.05$.

Results

The study was tolerated without adverse events, and total study duration was 9 months. All periods of the study (placebo and verum) could be completed as planned with 50 days each. As the only exception, capsules taken in the last study period changed appearance and smell probably because of the high temperature and/or humidity in the air during summer months. Therefore, the study was finished slightly ahead of schedule. Before unblinding of the study, the parents mentioned their suspicion of having lastly received verum based on the recent smell of the compound and their impression of a clinical improvement during every second period.

During the entire study period, a total of 1,762 self-mutilation events were recorded. Of these, 1,281 events occurred during the placebo periods and 481 during the verum periods of the study. This corresponds to a significant reduction of self-mutilation events from 8.6 ± 4.75 mean daily events in the placebo periods to 4.5 ± 2.57 mean daily events when SAM was taken ($p < 0.001$). Based on this almost 50 % reduction of self-mutilation events (Fig. 3) and based on the parental impression of a clear therapeutic benefit of SAM when

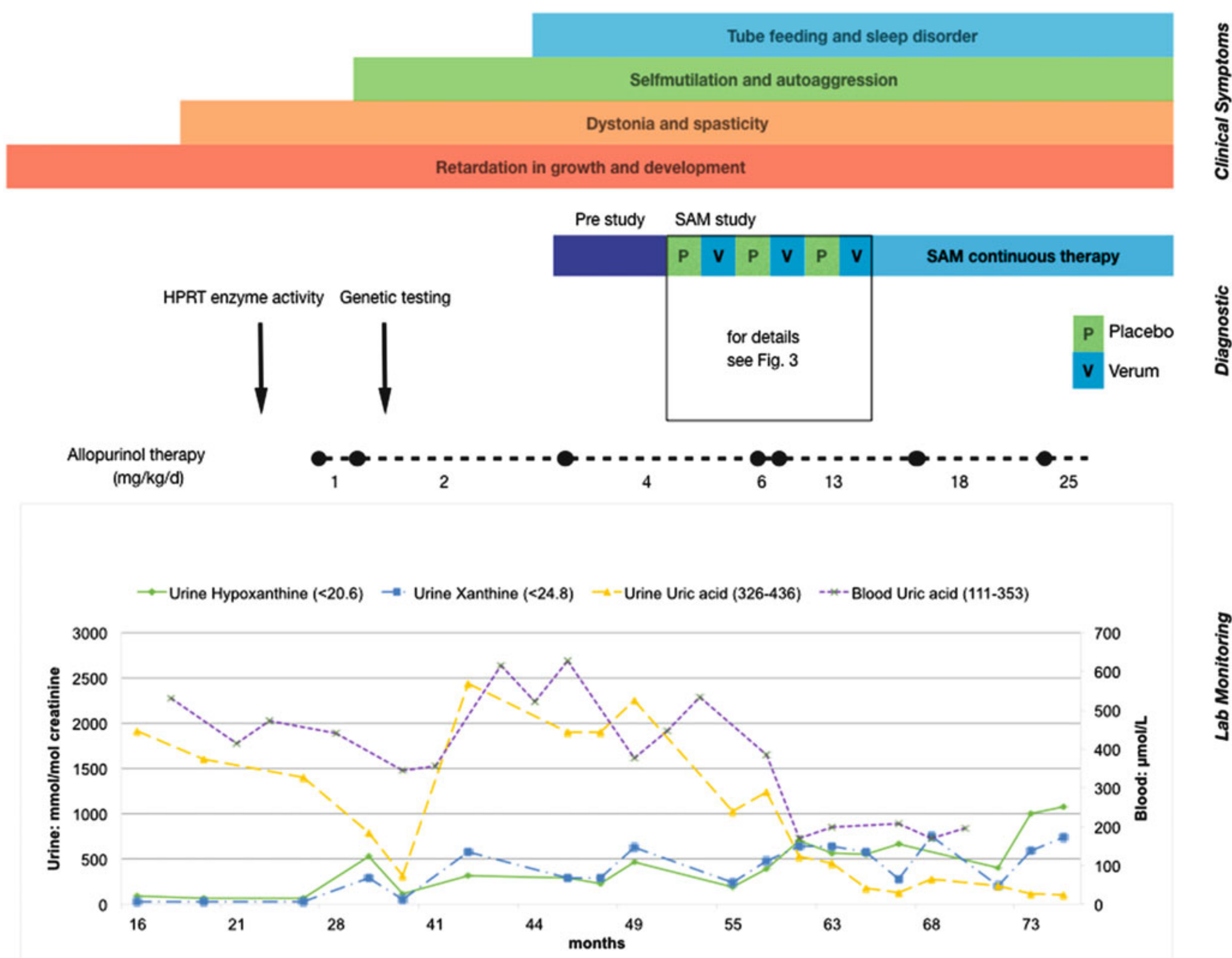

Fig. 2 Details of the patient divided into three main groups: clinical symptoms, diagnostic interventions, and lab monitoring. In the top in differently colored bars, the clinical manifestations of the disease in the patient are depicted. Below another bar shows the pre-study period followed by the SAM trial. The *dotted black line* indicates the adjustments of allopurinol therapy. A small drop in uric acid concentrations can be seen after start of allopurinol therapy, but the dose had to be adapted several times until urine and blood uric acid decreased to therapeutic ranges, while urinary xanthine and hypoxanthine increased

study periods were unblinded, SAM was continued at the same dose (100 mg twice daily) as long-term therapy.

Along increasing allopurinol dosages, levels of uric acid in blood and urine decreased, while concentrations of xanthine and hypoxanthine, the latter the best soluble of the three, increased (Fig. 2).

Discussion

Treatment of LND is symptomatic and often of only limited efficacy. The result from our placebo-controlled trial in a single patient showed a clear reduction in self-mutilation attempts. Self-mutilation is a key symptom in LND and most often includes biting lips or fingers (Robey et al. 2003). Most patients experience this as involuntary and request help or try to protect themselves by wearing gloves (Nyhan 1976). Our patient started self-mutilation with about 2.5 years, which is in line with other reports (Anderson and Ernst 1994).

Although we only studied one patient, we tried to compensate this with a careful pre-study period and a strict study protocol. In the pre-study period, we ensured quantification of self-mutilation events as good as this can be achieved with this partly subjective symptom that seemed to be associated with tiredness and pain, which is typical for LND patients. The study itself was double blind and placebo controlled, lasted 282 days, and was thus the optimum we could achieve in this $n = 1$ situation. Another limitation of our study is the fact that self-injury varies over

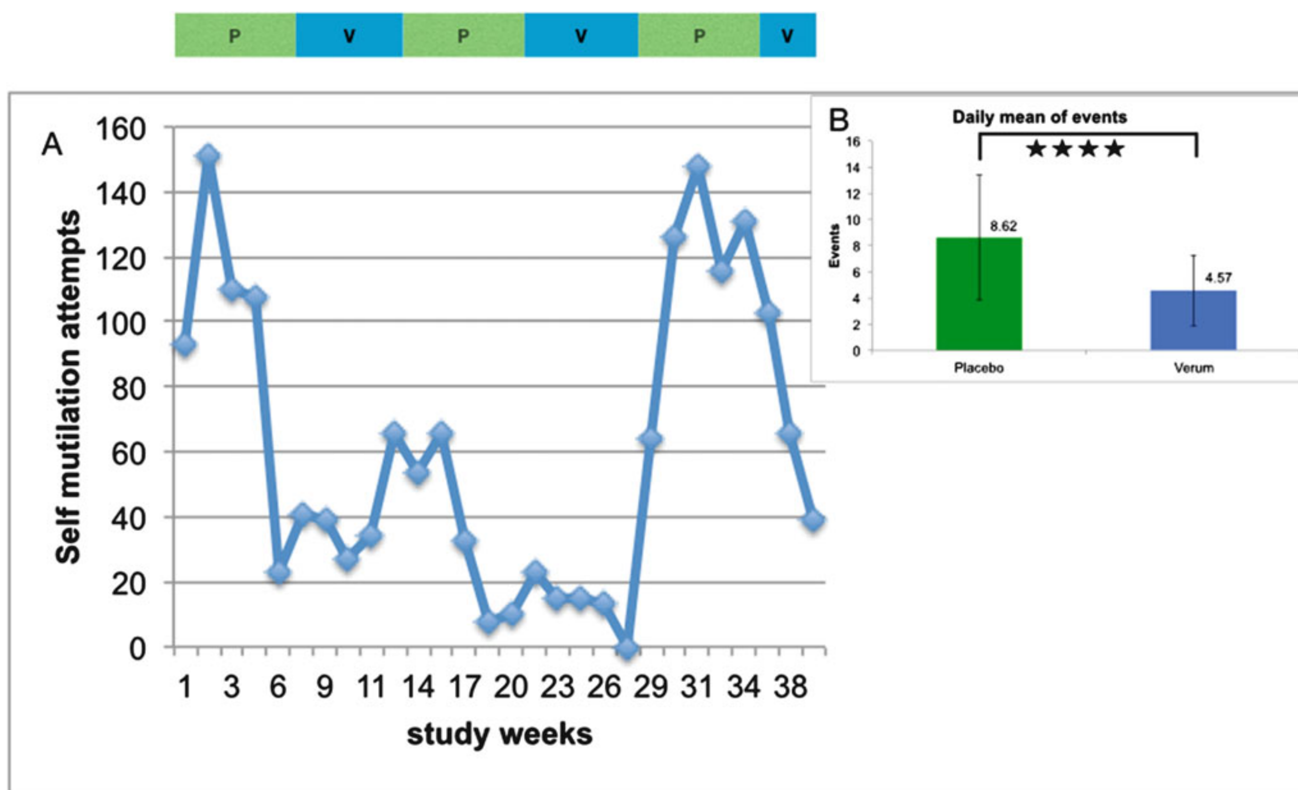

Fig. 3 Graph illustrating the number of self-mutilation events during the SAM trial. Panel **A**: Events during trial periods (a bar above the graph shows placebo periods in *green* and SAM periods in *blue*). For clarity of presentation, each 50-day period is calculated as 5 parts of 10 days each. Panel **B**: Daily mean ± SD of self-mutilation events; *green column* represents placebo periods (total days, 154), and *blue column* represents verum period (total days, 128). The difference between the two periods was significant ($p < 0.001$)

time and some of the changes may not be related to SAM. Nevertheless, while we are aware of these limitations, we still consider the improvement of the clinical situation under SAM therapy, as demonstrated by the significant decrease in self-mutilating events, as very encouraging because it relieved the physical and psychological distress for the patient and his family. The result may be further valuable for the understanding of the pathophysiology of LND and may open the way for further improvement of treatment.

SAM treatment has previously been reported in only few patients (Chen et al. 2014; Dolcetta et al. 2013; Glick 2006); in some of these, supplementation with SAM led to a drastic reduction in self-mutilation events and very good clinical outcome. This was investigated with (Dolcetta et al. 2013) or without (Chen et al. 2014; Glick 2006) formal assessment tools, and we decided here to base effect on parental judgment and documentation.

Action of LND is not fully understood, but SAM may replenish adenosine in cells, which can then be formed into adenosine monophosphate by adenosine kinase, and adenosine triphosphate. Furthermore, IMP can be formed by adenylosuccinate lyase, which can then be formed to GMP by isocitrate dehydrogenase, thus replenishing guanosine triphosphate purines. Purine depletion in tissue culture models and selective vulnerability of basal ganglia in HPRT-deficient mice was shown in several studies (Dunnett et al. 1989; Finger et al. 1988; Jinnah et al. 1992, 1994, 1999), and SAM could act on this by replenishing adenosyl stores in LND. Other authors discuss a role of SAM in neurotransmitter synthesis and metabolism (Dolcetta et al. 2013; Miller 2008), but this clearly needs more studies.

Although self-mutilation events cause most stress in LND, many patients die from renal failure due to nephrolithiasis. Prevention or treatment of nephrolithiasis should therefore be a priority, and a lesson can be learned from management in our patient. Lowering urinary uric acid concentration, the main therapeutic goal in LND, is coupled to an increase of renal xanthine and hypoxanthine excretion, which are a risk of urolithiasis. However, the solubility of these three metabolites should guide dosing of allopurinol: uric acid has by far the lowest solubility in water (10.5 mmol/L, HMBD, http://www.hmdb.ca/), while hypoxanthine is much better soluble (95.5 mmol/L, HMBD; this concentration will even with high-dose allopurinol hardly ever be reached). We thus suggest that

a high dose of allopurinol should be administered from the beginning of treatment in LND.

In conclusion, our $n = 1$ trial could show that SAM significantly reduces self-mutilating behavior in LND and may offer an additional therapeutic means adding to current symptomatic therapy. Furthermore, the course of purine metabolites in blood and urine underline the recommendation for an early start of high-dose allopurinol to prevent development of kidney stones. Finally, the findings of our study point toward adenosine depletion as a factor in the still dubious pathophysiology of LND.

Acknowledgments We appreciate the help of the family of our patient in conducting this trial and specifically the excellent recording of self-mutilation events. We also thank Mrs. Ursina Spörri, Zurich, for her help in data analysis.

Compliance with Ethics Guidelines

Conflict of Interest

Matthias Lauber, Barbara Plecko, Miriam Pfiffner, Jean-Marc Nuoffer, and Johannes Häberle declare that they have no conflict of interest.

This article does contain studies with a human subject. The patient received a medication as compassionate use after approval was obtained from the SwissMedic/Schweizerisches Heilmittelinstitut. As this was a therapeutic trial with a licensed drug in a single patient, there was no need to obtain formal approval by the local ethical board.

Jean-Marc Nuoffer and Johannes Häberle have together planned the study that was performed under the supervision of Johannes Häberle. Analysis and interpretation of data, drafting of the article, and revision were performed by Matthias Lauber, Barbara Plecko, Miriam Pfiffner, Jean-Marc Nuoffer, and Johannes Häberle.

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RESEARCH REPORT

Low Protein Formula: Consequences of Quantitative Effects of Pre-analytical Factors on Amino Acid Concentrations in Plasma of Healthy Infants

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Abstract *Objective*: Quantifying pre-analytical effects of postprandial sampling delay and daily protein intake on plasma amino acid concentrations in healthy infants fed formula with low protein content (1.8–1.9 g/100 kcal). Intake of formula with higher protein content bears a risk for later obesity (Kirchberg, J Clin Endocrinol Metab 100 (1):149–158, 2015). Formulas containing less than 1.8 g protein might be adequate but not safe (Fomon, J Pediatr Gastroenterol Nutr 28:495–501, 1999). With on-demand feeding reproducible controls of indispensible amino acid concentration cannot be made at trough level.

Methods: Data of 102 healthy infants aged 1 month and 79 aged 4 months fed formula with low protein content were obtained from a previous study (Haschke-Becher, J Inherit Metab Dis 39(1):25–37, 2016). They were analysed by multiple regression. Independent variables were the postprandial sampling delay from 2.25 to 4.5 h and the daily protein intake. Dependant variables were the amino acid concentrations. The combined effect was calculated with the natural logarithm of the amino acid concentration.

Results: Most amino acids fitted a significant exponential decrease due to the sampling delay, except of aspartate, citrulline, glutamine, glutamate, histidine, tryptophan and tyrosine at 1 month; and at 4 months except of citrulline, glutamine, glutamate, glycine and ornithine. Significant effects of protein intake were found for lysine and serine at 1 month and for glutamate at 4 months of age. Lowest limits of significant amino acid concentrations were calculated by extrapolation of sampling delay to 5 h and using the 10th percentile after back-transformation to μmol/L. A procedure to avoid the pitfall of overestimating amino acid concentration is presented.

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Introduction

When infants cannot be breast-fed formulas with low protein content (1.8–1.9 g/100 kcal) have been recommended in order to avoid an excessive protein supply or amino acid imbalance during organ development in infants (Koletzko et al. 2005). Furthermore, infant formulas with higher protein content are risk factors for later obesity (Kirchberg et al. 2015; Ong et al. 2009; Toschke et al. 2004). Formulas containing less than 1.8 g protein might be adequate but not safe as shown by Fomon et al. (1999). Frequent controls of amino acid concentrations in plasma of infants are thus needed when low protein formulas are exclusively fed in order to exclude that very low amino acid concentrations limit protein synthesis of infants during this phase of change of growth rate. For the interpretation of amino acid results the effect of pre-analytical factors must be taken into account; however, most often pre-analytical factors are not documented and/or communicated.

Relevant pre-analytical factors which modify the plasma levels are (1) the time elapsed between the end of the last

feed and the sampling of blood and (2) the actual daily intake of protein and its composition. For following the course of a patient or for excluding protein malnutrition blood sampling should be done at trough levels, i.e. between 4.5 and 5.5 h postprandial (p.p.) except of citrulline (Haschke-Becher et al. 2016; Windmueller and Spaeth 1981). However this is not always done or feasible. If blood is sampled during food absorption (i.e. until 2 h p.p.) the results are useless since no reference data of healthy infants exist. In practice samples are most often taken during the exponential decrease of essential and of some non-essential amino acids before trough level is reached, e.g. if the infants are fed on demand. Such results might, however, lead to pitfalls of interpretation: Most essential amino acids and some non-essential ones decrease exponentially after 2.25 postprandial (p.p.) hours until the trough level (>4.5 h) is reached; when blood is sampled during the exponential drop, the actual amino acid concentration determined will be higher than at trough level. If this is not taken into account in infants fed low protein formulas there is a risk of missing amino acid deficiencies and to interpret the amino acid concentration as safe level.

To our knowledge the effect of time of blood sampling and of the actual daily intake of formulas with low protein content on plasma amino acids has not been quantitatively estimated in healthy infants of 1 and 4 months of age. In this period of rapid growth the concentrations of indispensible amino acids in plasma should be safe, adapted to the needs and neither rate limiting for protein synthesis nor in excess.

We show quantitative data of plasma amino acid concentrations of healthy infants aged 1 and 4 months fed low protein formula on demand. We focus on the combined effect of the p.p. sampling delay from 2.25 to 4.5 h and of the individual daily protein intake on the amino acid concentrations in plasma. Furthermore we present low limits (the 10th percentile) of amino acid concentrations in plasma of healthy infants aged 1 and 4 months after extrapolating the time of sampling to trough levels (5 h p.p.). By comparison of patient results with these data the risk of amino acid deficiency might be evaluated in patients fed amino acid mixtures of low protein content.

Study Population and Methods

In order to quantify and estimate the influence of postprandial sampling delay after the end of last feed and of daily protein intake on the plasma amino acids we used a population of healthy infants of 1 and 4 months of age fed exclusively formulas with low protein content (1.8–1.9 g protein/100 kcal). The population and the analytical methods used for the amino acid analysis including total plasma tryptophan have been published elsewhere (Haschke-Becher et al. 2016). For this study we included only those infants who had information on actual protein intake per day, on postprandial sampling delay (independent variables) and plasma amino acid concentrations (dependent variables). 102 samples were of infants aged 1 month and 79 of infants aged 4 months. We do not report data of the following amino acids: taurine (added to the protein mixture of the formulas), hydroxyproline (posttranslational product of proline), cystine (pre-analytical bias) and 1- and 3-methylhistidine (low precision of low values); as shown in Haschke-Becher et al. (2016) on Fig 1 and in its electronic supplemental material. We focussed on the kinetics of plasma amino acids in blood samples obtained between 2.25 and 4.5 h postprandial. We used the natural logarithm of amino acid concentrations and determined intercept and parameter estimates of both independent variables. Furthermore we used standardized Beta coefficients of the multiple regressions, i.e. the coefficients of the dependent variables transformed to a standard deviation of 1 for weighting the impact of the two independent variables.

Statistics

Sample distribution of amino acids was analysed with Shapiro-Wilk, Kolmogorov Smirnov and Cramer von Mises tests and quantile regression with SAS 9.4 TS1M2 software for Windows (SAS Institute Inc., Cary, NC). The fit of combined effects of both independent factors was calculated by multiple regression of the natural logarithm (ln) of amino acid concentrations in dependence of p.p. sampling delay and daily protein intake. Analyse-it 3 for Excel software was used for these calculations (Analyse-it Software Ltd., Leeds, UK). In addition, the fit of each dependant variable by linear regression was controlled separately. R software (Koenker and Bassett 1978) was used for computing the fit at the 10th percentile.

Values of $p < 0.05$ were considered significant. Half-life was calculated by: $T_{1/2} = -\ln(2)$/estimate of the regression coefficient of the sampling delay.

Results

Post-absorptive Kinetics of Amino Acid Concentrations in Plasma of Healthy Infants: Effect of p.p. Sampling Delay and Amount of Daily Protein Intake

The kinetic parameters of samples taken between 2.25 and 4.5 h p.p. differed among amino acids. The results of multivariate regressions are shown in Tables 1 and 2 for

Table 1 Kinetic parameters of plasma amino acids sampled at 1 month of age between 2.25 and 4.5 h p.p. fed formula of low protein content

			Intercept at $t = 0$ (constant)			Regression coefficient of p.p. sampling delay (h)						Regression coefficient of daily protein intake				
ln amino acids	n	R^2	Estimate	Lower 95%CI	Upper 95%CI	Estimate	Lower 95%CI	Upper 95%CI	Beta	p	$T_{1/2}$ (h)	Estimate	Lower 95%CI	Upper 95%CI	Beta	p
2-Aminobutyrate	102	0.006	2.656	2.307	3.004	−0.0102	−0.099	0.079	−0.023	0.8204	68	−0.00749	−0.0275	0.0125	−0.075	0.4589
Alanine	102	0.225	6.377	6.092	6.662	−0.1904	−0.263	−0.118	−0.461	< ***0.0001***	**3.6**	0.00760	−0.0088	0.0239	0.082	0.3588
Arginine	102	0.051	4.889	4.599	5.178	−0.0823	−0.156	−0.008	−0.217	***0.0294***	**8.4**	0.00425	−0.0124	0.0209	0.050	0.6131
Asparagine	102	0.144	4.128	3.912	4.343	−0.1068	−0.162	−0.052	−0.359	***0.0002***	**6.5**	0.00650	−0.0059	0.0189	0.097	0.2992
Aspartate	102	0.007	2.730	2.205	3.254	−0.0422	−0.176	0.092	−0.063	0.5328	16	0.00769	−0.0224	0.0378	0.051	0.6134
Citrulline	102	0.032	2.929	2.595	3.262	0.0762	−0.009	0.161	0.176	0.0786		0.00442	−0.0147	0.0236	0.045	0.6476
Glutamate	102	0.001	4.554	4.022	5.087	−0.0037	−0.140	0.132	−0.005	0.9571	188	0.00432	−0.0262	0.0349	0.028	0.7798
Glutamine	102	0.001	6.362	6.180	6.543	−0.0042	−0.050	0.042	−0.018	0.8577	165	−0.00084	−0.0112	0.0096	−0.016	0.8733
Glycine	102	0.011	5.586	5.392	5.781	−0.0269	−0.077	0.023	−0.107	0.2866	26	−0.00061	−0.0118	0.0106	−0.011	0.9140
Histidine	102	0.043	4.538	4.372	4.705	−0.0387	−0.081	0.004	−0.178	0.0733	18	0.00464	−0.0049	0.0142	0.095	0.3371
Isoleucine	102	0.208	4.594	4.300	4.889	−0.1865	−0.262	−0.111	−0.441	< ***0.0001***	**3.7**	0.00824	−0.0087	0.0251	0.087	0.3363
Leucine	102	0.158	5.137	4.875	5.400	−0.1377	−0.205	−0.071	−0.377	< ***0.0001***	**5.0**	0.00850	−0.0066	0.0236	0.103	0.2662
Lysine	102	0.178	5.594	5.378	5.810	−0.1086	−0.164	−0.053	−0.357	***0.0002***	**6.4**	0.01364	0.0012	0.0260	0.199	***0.0314***
Methionine	102	0.116	3.707	3.460	3.953	−0.1013	−0.164	−0.038	−0.302	***0.0019***	**6.8**	0.01035	−0.0038	0.0245	0.137	0.1501
Ornithine	102	0.135	4.885	4.575	5.196	−0.1458	−0.225	−0.066	−0.342	***0.0004***	**4.8**	0.01059	−0.0072	0.0284	0.111	0.2412
Phenylalanine	102	0.074	4.080	3.840	4.320	−0.0777	−0.139	−0.016	−0.244	***0.0135***	**8.9**	0.00757	−0.0062	0.0214	0.106	0.2781
Proline	102	0.117	5.477	5.241	5.713	−0.1097	−0.170	−0.049	−0.342	***0.0005***	**6.3**	0.00037	−0.0132	0.0139	0.005	0.9563
Serine	102	0.090	5.148	4.949	5.346	−0.0800	−0.131	−0.029	−0.301	***0.0023***	**8.7**	−0.00069	−0.0121	0.0107	−0.011	0.9050
Threonine	102	0.184	5.455	5.182	5.728	−0.1430	−0.213	−0.073	−0.371	< ***0.0001***	**4.8**	0.01646	0.0008	0.0321	0.190	***0.0395***
Tryptophan	96	0.009	4.288	4.022	4.553	−0.0328	−0.102	0.036	−0.097	0.3486	21	−0.00048	−0.0155	0.0145	−0.007	0.9492
Tyrosine	102	0.027	4.564	4.270	4.857	−0.0625	−0.137	0.012	−0.164	0.1013	11	−0.00157	−0.0184	0.0153	−0.018	0.8541
Valine	102	0.102	5.303	5.058	5.549	−0.0997	−0.162	−0.225	−0.301	***0.0021***	**7.0**	0.00649	−0.0076	0.0206	0.087	0.3629
Aspartate + Asparagine	102	0.115	4.373	4.163	4.583	−0.0918	−0.146	−0.038	−0.322	***0.0010***		0.00566	−0.0064	0.0177	0.088	0.3547
Glutamate + Glutamine	102	0.0004	6.525	6.387	6.663	−0.0007	−0.036	0.035	−0.004	0.9698		−0.00080	−0.0087	0.0071	−0.020	0.8409

R^2 coefficient of determination, *Beta* standardized Beta, ***Italics*** significant result, *p.p.* postprandial (after end of last feed), $T_{1/2}$ half-life

Table 2 Kinetic parameters of plasma amino acids sampled at 4 months of age between 2.25 and 4.5 h p.p. fed formula of low protein content

			Intercept at $t = 0$ (constant)			Regression coefficient of p.p. sampling delay						Regression coefficient of daily protein intake				
lnAA	n	R^2	Estimate	Lower 95%CI	Upper 95%CI	Estimate	Lower 95%CI	Upper 95%CI	Beta	p	$T_{1/2}$ (h)	Estimate	Lower 95%CI	Upper 95%CI	Beta	p
2-Aminobutyrate	79	0.046	2.922	2.636	3.208	−0.0374	−0.1162	0.0414	−0.106	0.3476	19	−0.0115	−0.0258	0.0027	−0.181	0.1113
Alanine	79	0.114	6.268	5.961	6.575	−0.1305	−0.2151	−0.0459	−0.333	***0.0029***	**5.3**	−0.0026	−0.0179	0.0127	−0.037	0.7341
Arginine	79	0.114	4.989	4.693	5.286	−0.1268	−0.2084	−0.0451	−0.334	***0.0028***	**5.5**	−0.0019	−0.0167	0.0128	−0.028	0.7947
Asparagine	79	0.169	4.101	3.851	4.351	−0.1341	−0.2030	−0.0651	−0.406	***0.0002***	**5.2**	0.0060	−0.0065	0.0185	0.100	0.3408
Aspartate	79	0.055	2.896	2.477	3.315	−0.0892	−0.2046	0.0263	−0.172	0.1281	7.8	−0.0140	−0.0348	0.0069	−0.149	0.1866
Citrulline	79	0.028	2.768	2.408	3.128	0.0716	−0.0275	0.1708	0.163	0.1544		0.0022	−0.0157	0.0202	0.028	0.8046
Glutamate	79	0.105	4.987	4.524	5.450	−0.0546	−0.1824	0.0731	−0.093	0.3972	13	−0.0325	−0.0556	−0.0094	−0.305	***0.0064***
Glutamine	79	0.028	6.250	6.051	6.449	−0.0258	−0.0807	0.0292	−0.106	0.3537	27	0.0060	−0.0039	0.0160	0.137	0.2303
Glycine	79	0.016	5.210	5.019	5.402	0.0004	−0.0525	0.0532	0.002	0.9895		0.0053	−0.0043	0.0148	0.126	0.2737
Histidine	79	0.089	4.574	4.384	4.764	−0.0590	−0.1114	−0.0065	−0.246	***0.0281***	**12**	−0.0067	−0.0161	0.0028	−0.154	0.1656
Isoleucine	79	0.202	4.564	4.298	4.830	−0.1599	−0.2333	−0.0865	−0.445	$<$***0.0001***	**4.3**	−0.0022	−0.0155	0.0111	−0.034	0.7428
Leucine	79	0.214	5.153	4.902	5.404	−0.1571	−0.2263	−0.0878	−0.460	$<$***0.0001***	**4.4**	−0.0013	−0.0139	0.0112	−0.022	0.8316
Lysine	79	0.197	5.542	5.297	5.787	−0.1453	−0.2127	−0.0778	−0.442	$<$***0.0001***	**4.8**	0.0049	−0.0073	0.0171	0.083	0.4248
Methionine	79	0.214	3.817	3.539	4.095	−0.1664	−0.2431	−0.0898	−0.441	$<$***0.0001***	**4.2**	−0.0078	−0.0217	0.0060	−0.115	0.2648
Ornithine	79	0.041	4.578	4.257	4.899	−0.0745	−0.1631	0.0140	−0.189	0.0976	9.3	−0.0042	−0.0202	0.0118	−0.059	0.6004
Phenylalanine	79	0.116	4.246	3.990	4.501	−0.0990	−0.1694	−0.0287	−0.303	***0.0064***	**7.0**	−0.0081	−0.0208	0.0047	−0.137	0.2103
Proline	79	0.112	5.476	5.206	5.746	−0.1011	−0.1755	−0.0267	−0.293	***0.0084***	**6.9**	−0.0088	−0.0223	0.0046	−0.142	0.1940
Serine	79	0.156	5.203	5.026	5.380	−0.0785	−0.1273	−0.0296	−0.338	***0.0020***	**8.8**	−0.0076	−0.0165	0.0012	−0.182	0.0884
Threonine	79	0.091	5.295	4.988	5.601	−0.1126	−0.1971	−0.0282	−0.291	***0.0096***	**6.2**	−0.0044	−0.0197	0.0108	−0.063	0.5651
Tryptophan	70	0.076	4.480	4.222	4.738	−0.0830	−0.1542	−0.0118	−0.274	***0.0231***	**8.4**	0.0026	−0.0100	0.0151	0.049	0.6817
Tyrosine	79	0.054	4.645	4.303	4.986	−0.0956	−0.1898	−0.0014	−0.226	***0.0467***	**7.3**	−0.0032	−0.0202	0.0138	−0.042	0.7089
Valine	79	0.152	5.377	5.165	5.588	−0.1041	−0.1623	−0.0459	−0.377	***0.0006***	**6.7**	−0.0039	−0.0144	0.0066	−0.078	0.4616
Aspartate + Asparagine	79	0.197	4.397	4.180	4.614	−0.1301	−0.1901	−0.0702	−0.445	$<$***0.0001***		0.0012	−0.0096	0.0120	0.023	0.8254
Glutamate + Glutamine	79	0.056	6.544	6.402	6.687	−0.0411	−0.0804	−0.0019	−0.233	***0.0404***		−0.0008	−0.0079	0.0063	−0.026	0.8159

R^2 coefficient of determination, *Beta* standardized Beta, ***Italics*** significant result, *p.p.* postprandial (after end of last feed), $T_{1/2}$ half-life

blood samples taken at 1 and 4 months of age, respectively. Biological half-life is also shown. The equations with the factors of multiple regressions are presented in Table 3. The sample distribution of amino acids was logarithmic; thus the natural logarithms (ln) of the amino acid concentrations were used for calculations. For infants of 1 month of age all the regression parameters of sampling delay of the ln of essential amino acids had a significant negative slope except for total tryptophan (Table 1). This was also found for the following non-essential amino acids: alanine, arginine, asparagine, ornithine, proline, serine and the sum of aspartate and asparagine. The regression coefficients of daily protein intake were significant and positive only for lysine and threonine at 1 month of age.

At 4 month of age the significance of regression coefficients of sampling delay differed from those at 1 month insofar as the regression coefficient of ornithine was not significant anymore ($p = 0.098$); the coefficients of histidine, tryptophan and tyrosine were significant as well as the sum of glutamine and glutamate (Table 2). At 4 months of age the sole significant coefficient of protein intake/d was that of glutamate ($p = 0.006$). Citrulline concentration increased during the post-absorptive phase; the lowest level was reached during the first hour after the end of last feed (Haschke-Becher et al. 2016).

There were not enough data for calculating the kinetics during the initial absorption of the gut (0–2.0 h) and after 5.5 postprandial hours when concentrations of many amino acids tended to increase presumably by incipient proteolysis.

Kinetic parameters of both independent variables calculated separately by univariate linear regression are presented in the electronic supplemental material (Tables 5–8).

Effect of the Independent Variables on the Mean of Amino Acid Concentrations

The standardized squared Beta values were higher for the p.p. sampling delay than for the protein intake/day. At 1 month of age the percentage of the total variance which exceeded 10% and can be attributed to the sampling delay was found for: alanine (21%), isoleucine (20%), leucine (14%), threonine, asparagine, lysine (13% each), ornithine and proline (each 12%). At 4 months the effect of sampling delay was 21% for leucine, 20% for isoleucine and lysine, 19% for methionine, 17% for asparagine, 14% for valine and 11% for arginine and alanine. The effect of protein intake per day on total variance was lower than 5% for all the amino acids, both at 1 and 4 months of age.

Univariate regression was done for each independent variable on the same set of data as used for the multivariate regression. As expected, most estimates differed between the two methods. The differences were, however, minor.

Procedure to Avoid the Pitfall of Overestimated Amino Acid Concentrations

The goal was to avoid the pitfall of overestimating amino acid concentrations sampled during the post-absorptive phase (2.25–4.5 h). We estimated by extrapolation to trough level (5 h) the ln of those amino acids which showed a significant p.p. drop in healthy infants fed formula with low protein content. We defined lower limits at the 10th percentile for such amino acids and back-transformed the logarithmic results into μmol/L in dependence of sampling delay during the exponential drop and at trough level (Tables 4 and 5). These data of healthy infants aged 1 or 4 months may be used for evaluating amino acid results (μmol/L) of controls made on patients aged 1 or 4 months fed low protein formula to make sure that there is no risk of protein malnutrition, e.g. if albumin is below the reference limits.

Discussion

The quantitative effects of p.p. sampling delay indicate that during the post-absorptive phase of 2.25–4.5 h the effect of sampling delay on the ln of essential amino acids (except total tryptophan) and of some non-essential amino acids leads to a significant negative slope, while several non-essential amino acids in plasma are not significantly affected by the p.p. sampling delay. Ornithine concentration is affected by the sampling delay at 1 month of age ($p < 0.001$), but not at 4 months of age ($p = 0.098$). This supports quantitatively the hypothesis that the flux direction at 1 month of ornithine to proline synthesis prevails (Haschke-Becher et al. 2016) due to the high demand of proline for collagen I synthesis. Since the proline content in the formula is lower than in breast-milk additional proline should be provided. Collagen I synthesis further requires glycine which is not affected by sampling delay in contrast to its precursor serine. Hydroxyproline, as a posttranslational product of proline is needed as well; the ln of proline and of hydroxyproline correlate positively (Spearman $\rho = 0.422$ and 0.454 at 1 and 4 months, respectively; $p < 0.001$ for both).

The effect of the actual daily intake of low protein formula is significant for lysine and threonine in infants of 1 month of age and at 4 months solely for glutamate (precursor of Δ1-pyrroline 5-carboxylate and proline or ornithine). It is not clear if the significant effects of intake of lysine and threonine at 1 month of age are due to a

Table 3 Equations of multiple regression of low protein formula-fed healthy infants

Infants aged 1 month	Infants aged 4 months
lnABA = 2.656 − (0.0102 × Sampling delay) − (0.007493 × Prot.Intake/d)	lnABA = 2.922 − (0.0374 × Sampling delay) − (0.01152 × Prot.Intake/d)
lnALA = 6.377 − (0.190 × Sampling delay) + (0.00760 × Prot.Intake/d)	**lnALA = 6.268 − (0.131 × Sampling delay) − (0.002617 × Prot.Intake/d)**
lnARG = 4.889 − (0.0823 × Sampling delay) + (0.00425 × Prot.Intake/d)	**lnARG = 4.989 − (0.1270 × Sampling delay) − (0.001935 × Prot.Intake/d)**
lnASN = 4.128 − (0.107 × Sampling Delay) + (0.00650 × Prot.Intake/d)	**lnASN = 4.101 − (0.134 × Sampling delay) + (0.005996 × Prot.Intake/d)**
lnASP = 2.73 − (0.0422 × Sampling delay) + (0.00769 × Prot.Intake/d)	lnASP = 2.896 − (0.08920 × Sampling delay) − (0.01396 × Prot.Intake/d)
lnCIT = 2.929 + (0.0762 × Sampling delay) + (0.00442 × Prot.Intake/d)	lnCIT = 2.768 + (0.0716 × Sampling delay) + (0.002234 × Prot.Intake/d)
lnGLU = 4.554 − (0.00370 × Sampling delay) + (0.00432 × Prot.Intake/d)	lnGLU = 4.987 − (0.0546 × Sampling delay) − (0.03251 × Prot.Intake/d)
lnGLN = 6.362 − (0.00419 × Sampling delay) − (0.000838 × Prot.Intake/d)	lnGLN = 6.25 − (0.0258 × Sampling delay) + (0.006032 × Prot.Intake/d)
lnGLY = 5.586 − (0.0269 × Sampling delay) − (0.000610 × Prot.Intake/d)	lnGLY = 5.21 + (0.00035 × Sampling delay) + (0.005289 × Prot.Intake/d)
lnHIS = 4.538 − (0.0387 × Sampling delay) + (0.00464 × Prot.Intake/d)	**lnHIS = 4.574 − (0.0590 × Sampling delay) − (0.006662 × Prot.Intake/d)**
lnILE = 4.594 − (0.187 × Sampling delay) + (0.00824 × Prot.Intake/d)	**lnILE = 4.564 − (0.160 × Sampling delay) − (0.002195 × Prot.Intake/d)**
lnLEU = 5.137 − (0.138 × Sampling delay) + (0.00850 × Prot.Intake/d)	**lnLEU = 5.153 − (0.157 × Sampling delay) − (0.001341 × Prot.Intake/d)**
lnLYS = 5.594 − (0.109 × Sampling delay) + (0.0136 × Prot.Intake/d)	**lnLYS = 5.542 − (0.145 × Sampling delay) + (0.004911 × Prot.Intake/d)**
lnMET = 3.707 − (0.1019 × Sampling delay) + (0.0104 × Prot.Intake/d)	**lnMET = 3.817 − (0.166 × Sampling delay) − (0.007815 × Prot.Intake/d)**
lnORN = 4.885 − (0.146 × Sampling delay) + (0.01060 × Prot.Intake/d)	lnORN = 4.578 − (0.0745 × Sampling delay) − (0.004225 × Prot.Intake/d)
lnPHE = 4.08 − (0.0777 × Sampling delay) + (0.00757 × Prot.Intake/d)	**lnPHE = 4.246 − (0.0991 × Sampling delay) − (0.008068 × Prot.Intake/d)**
lnPROL = 5.477 − (0.110 × Sampling delay) + (0.000374 × Prot.Intake/d)	**lnPROL = 5.476 − (0.101 × Sampling delay) − (0.008848 × Prot.Intake/d)**
lnSER = 5.148 − (0.0800 × Sampling delay) − (0.000687 × Prot.Intake/d)	**lnSER = 5.203 − (0.0785 × Sampling delay) − (0.007643 × Prot.Intake/d)**
lnTHR = 5.455 − (0.143 × Sampling delay) + (0.0165 × Prot.Intake/d)	**lnTHR = 5.295 − (0.113 × Sampling delay) − (0.004426 × Prot.Intake/d)**
lnTRP = 4.288 − (0.0328 × Sampling delay) − (0.000483 × Prot.Intake/d)	**lnTRP = 4.48 − (0.0830 × Sampling delay) + (0.00259 × Prot.Intake/d)**
lnTYR = 4.564 − (0.0625 × Sampling delay) − (0.00156 × Prot.Intake/d)	**lnTYR = 4.645 − (0.0956 × Sampling delay) − (0.003201 × Prot.Intake/d)**
lnVAL = 5.303 − (0.0997 × Sampling delay) + (0.00649 × Prot.Intake/d)	**lnVAL = 5.377 − (0.104 × Sampling delay) − (0.00391 × Prot.Intake/d)**
lnASP + ASN = 4.373 − (0.0918 × Sampling delay) + (0.00566 × Prot.Intake/d)	**lnASP + ASN = 4.397 − (0.130 × Sampling delay) + (0.001204 × Prot.Intake/d)**
lnGLU + GLN = 6.525 − (0.000674 × Sampling delay) − (0.0008038 × Prot.Intake/d)	**lnGLU + GLN = 6.544 − (0.0411 × Sampling delay) − (0.0008319 × Prot.Intake/d)**

Bold characters p significant (<0.05) for sampling delay, *Prot. Intake* intake of g protein/d, *sampling delay* postprandial delay (h) after end of last feed

Table 4 Tenth percentile of significant amino acid concentrations (μmol/L) depending on p.p. sampling delays with 95 % confidence intervals (CI) in infants aged 1 month

p.p. sampling delay (h)	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50	5.00[a]
Alanine	320[b] (312;328)	308 (302;328)	297 (291;302)	286 (281;290)	275 (270;280)	265 (259;270)	255 (249;262)	246 (238;253)	236 (228;245)	228 (218;238)	211 (200;223)
Arginine	85 (75;97)	84 (76;93)	83 (76;90)	82 (76;89)	81 (74;88)	80 (72;89)	79 (70;89)	78 (67;89)	77 (64;92)	76 (62;94)	74 (56:97)
Asparagine	43 (37;49)	41 (37;46)	40 (36;44)	38 (35;42)	37 (34;41)	36 (32;40)	34 (30;39)	33 (28;39)	32 (26;39)	31 (24;39)	28 (21;38)
Isoleucine	50 (48;53)	49 (49;51)	48 (46;49)	46 (45;48)	45 (43;47)	44 (42;46)	42 (40;45)	41 (38;44)	40 (37;43)	39 (35;43)	37 (32;43)
Leucine	102 (92;113)	99 (91;108)	97 (90;104)	94 (88;100)	92 (86;98)	89 (82;97)	87 (79;96)	85 (75;96)	83 (71;96)	81 (68;96)	77 (61;96)
Lysine	191 (169;215)	187 (169;206)	183 (169;198)	179 (166;192)	175 (161;189)	171 (155;188)	167 (149;188)	163 (142;188)	160 (135;189)	156 (128;190)	149 (116;193)
Methionine	27 (23;31)	26 (23;29)	26 (23;28)	25 (23;27)	24 (22;27)	24 (21;26)	23 (20;26)	22 (19;27)	22 (18;27)	21 (17;27)	20 (15;27)
Ornithine	77 (64;93)	75 (65;87)	73 (64;83)	71 (63;80)	69 (61;78)	67 (58;78)	65 (55;78)	64 (51;79)	62 (48;80)	60 (44;82)	57 (38;85)
Phenylalanine	40 (36;44)	40 (37;43)	40 (38;43)	40 (38;42)	40 (38;43)	40 (37;43)	40 (36;44)	40 (36;45)	40 (35;46)	40 (34;47)	40 (33;49)
Proline	138 (124;154)	137 (125;149)	135 (126;145)	134 (125;143)	132 (123;142)	131 (120;143)	130 (117;144)	128 (113;146)	127 (109;148)	126 (105;150)	123 (98;155)
Serine	113 (98;130)	111 (99;124)	109 (99;120)	107 (98;117)	105 (96;115)	103 (92;115)	101 (88;116)	99 (84;118)	98 (80;119)	96 (76;121)	92 (68;121)
Threonine	149 (126;177)	143 (124;164)	137 (122;154)	132 (119;147)	127 (113;142)	122 (106;139)	117 (99;138)	112 (92;138)	108 (85;137)	104 (78;137)	96 (66;138)
Valine	123 (107;141)	123 (110;138)	123 (112;135)	124 (113;135)	124 (113;136)	124 (111;139)	124 (108;143)	125 (105;147)	125 (102;152)	125 (99;158)	126 (93;169)

[a] Trough level
[b] Tenth percentile (lower; upper 95 % confidence limit)

Table 5 Tenth percentile of significant amino acid concentrations (μmol/L) depending on p.p. sampling delay in infants aged 4 months

p.p, sampling delay (h)	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50	5.00
Alanine	270[a] (231;316)	266 (234:302)	261 (235;291)	257 (233;283)	253 (228;281)	249 (220;281)	245 (211;284)	241 (201;289)	237 (191;294)	233 (181;300)	226[b] (163;312)
Arginine	85 (77;94)	81 (74;88)	77 (71;82)	73 (68;77)	69 (64;74)	65 (60;71)	62 (56;68)	59 (52;66)	56 (48;64)	53 (45;82)	48 (39;59)
Asparagine	38 (34;42)	37 (34;40)	36 (33;38)	34 (32;37)	33 (31;36)	32 (30;35)	31 (28;35)	30 (27;34)	29 (25;34)	28 (24;34)	27 (21;33)
Histidine	66 (59;73)	65 (59;71)	64 (59;69)	63 (59;67)	62 (58;67)	61 (56;67)	61 (55;67)	60 (53;68)	59 (51;69)	58 (49;69)	57 (45;71)
Isoleucine	52 (48;57)	50 (47;54)	48 (45;51)	46 (44;48)	44 (42;47)	42 (40;45)	40 (37;44)	39 (35;43)	37 (33;42)	36 (31;41)	33 (27;39)
Leucine	99 (95;103)	95 (92;98)	90 (88;93)	86 (84;88)	82 (80;84)	79 (76;81)	75 (72;78)	72 (69;75)	68 (65;72)	65 (61;69)	60 (55;64)
Lysine	161 (140;186)	154 (137;172)	146 (133;161)	140 (128;153)	133 (121;146)	127 (114;142)	121 (106;139)	115 (98;136)	110 (90;134)	105 (83;132)	95 (71;128)
Methionine	23 (19;28)	22 (19;26)	21 (18;24)	20 (18;22)	19 (17;21)	18 (16;21)	17 (14;20)	16 (13;20)	15 (12;20)	15 (11;20)	13 (9;20)
Phenylalanine	39 (36;42)	39 (36;41)	38 (36;40)	38 (36;40)	37 (36;39)	37 (35;39)	37 (34;40)	36 (33;40)	36 (32;40)	36 (31;40)	35 (30;41)
Proline	137 (125;149)	133 (124;143)	130 (123;138)	127 (120;134)	124 (117;131)	121 (113;129)	118 (109;128)	115 (104;127)	113 (100;127)	110 (96;126)	105 (88;125)
Serine	122 (114;130)	119 (113;126)	116 (111;122)	114 (109;118)	111 (106;116)	108 (103;114)	106 (100;113)	103 (96;111)	101 (93;110)	99 (89;109)	94 (82;108)
Threonine	119 (114;124)	115 (111;119)	111 (108;114)	108 (105;110)	104 (101;107)	101 (97;104)	97 (93;101)	94 (90;99)	91 (86;96)	88 (82;94)	82 (75;90)
Tyrosine	54 (44;68)	54 (45;65)	54 (46;62)	53 (46;61)	53 (46;61)	53 (44;63)	52 (42;65)	52 (40;67)	52 (38;70)	51 (36;73)	51 (32;80)
Valine	131 (102;143)	128 (120;138)	126 (119;134)	124 (117;130)	121 (115;128)	119 (111;127)	116 (107;126)	114 (103;126)	112 (100;126)	110 (96;126)	106 (88;126)

[a] Tenth percentile (lower; upper 95 % confidence limit)
[b] Trough level

relatively high concentration of these amino acids in formulas as compared to breast-milk and/or to reduced utilization for protein synthesis.

As shown in the equations of multiple regression (Table 3) the coefficients of sampling delay are more than 10 times higher than the coefficients of actual daily protein intake. Despite our demand to the supervisors of the original studies to obtain blood samples later than 3.5 h after the last feed, this recommendation was ignored. In fact 82% of the samples were taken before 3.75 h p.p. at 1 month and 80% at 4 months of age. This is due to feeding the infants on-demand. We wonder if after 3.5 h satiety was not reached any more with the low protein formula.

Conclusion

For the interpretation of plasma amino acid concentrations data on the postprandial delay of sampling must be obtained and taken into account; otherwise low amino acid concentrations could be missed in patients.

Take-Home Message

For the interpretation of plasma amino acid concentrations data of the postprandial delay of sampling must be obtained and taken into account.

Compliance with Ethics Guidelines

Conflict of Interest

Alexander Kainz was funded by the research fund of the central clinical chemistry laboratory (CHUV, Lausanne) for his statistical work.

All authors declare no conflict of interest.

The authors confirm independence from Nestec Inc. The content of the article has not been influenced by any company or institution.

Informed Consent

All procedures were in accordance with the ethical standards of the responsible committees on human experimentation (national and international) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of all infants included in the original studies from which the data of this study were extracted.

Contribution of the Authors

C. Bachmann contributed by the conception, the planning, multiple regression statistics and analysis of the laboratory results, their interpretation as well as drafting and revising of the manuscript. He is the guarantor of the final manuscript.

A. Kainz contributed by the statistics including quantile regression, drafting and revising the manuscript.

E. Haschke-Becher contributed by the conception, the drafting and revision of the manuscript.

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RESEARCH REPORT

A Multiplatform Metabolomics Approach to Characterize Plasma Levels of Phenylalanine and Tyrosine in Phenylketonuria

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Abstract *Background*: Different pathophysiological mechanisms have been described in phenylketonuria (PKU) but the indirect metabolic consequences of metabolic disorders caused by elevated Phe or low Tyr concentrations remain partially unknown. We used a multiplatform metabolomics approach to evaluate the metabolic signature associated with Phe and Tyr.

Material and methods: We prospectively included 10 PKU adult patients and matched controls. We analysed the metabolome profile using GC-MS (urine), amino-acid analyzer (urine and plasma) and nuclear magnetic resonance spectroscopy (urine). We performed a multivariate analysis from the metabolome (after exclusion of Phe, Tyr and directly derived metabolites) to explain plasma Phe and Tyr concentrations, and the clinical status. Finally, we performed a univariate analysis of the most discriminant metabolites and we identified the associated metabolic pathways.

Results: We obtained a metabolic pattern from 118 metabolites and we built excellent multivariate models to explain Phe, Tyr concentrations and PKU diagnosis. Common metabolites of these models were identified: Gln, Arg, succinate and alpha aminobutyric acid. Univariate analysis showed an inverse correlation between Arg, alpha aminobutyric acid and Phe and a positive correlation between Arg, succinate, Gln and Tyr ($p < 0.0003$). Thus, we highlighted the following pathways: Arg and Pro, Ala, Asp and Glu metabolism.

Discussion: We obtain a specific metabolic signature related to Tyr and Phe concentrations. We confirmed the involvement of different pathophysiological mechanisms previously described in PKU such as protein synthesis, energetic metabolism and oxidative stress. The metabolomics approach is relevant to explore PKU pathogenesis.

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Abbreviations

^{1}H NMR	Nuclear magnetic resonance
CV ANOVA	ANalysis Of VAriance testing of cross validated predictive residuals, used to evaluate the robustness of multivariate model
GC-MS	Gas chromatography coupled with mass spectrometry
HCA	Hierarchical cluster analysis

KEGG	Pathway database
LC	Liquid chromatography
METPA	A web metabolomics tool to analyse metabolic pathways
OPLS-DA	Orthogonal partial least-squares discriminant analysis
PLS	Partial least square
Q^2	Parameter to estimate of the predictive ability of the model, used to evaluate the robustness of multivariate model
R^2	Parameter defined as a fraction of the variance explained by a component, used to evaluate the robustness of multivariate model
ROC	Receiver-operating characteristics
UV Scaling	UV scaling is defined by a variable that is centred and scaled to “Unit Variance”, i.e. the base weight is computed as 1/SD, where SD is the standard deviation of variable computed around the mean.
VIP	Variable importance parameters

Introduction

Phenylketonuria (PKU) is a metabolic disorder due to a deficiency in phenylalanine hydroxylase (PAH, RC 1.14.16.1), leading to high concentrations of Phenylalanine (Phe) and low concentrations of Tyrosine (Tyr) in blood and brain. A specific phenylalanine-restricted diet is prescribed following newborn screening but frequently discontinued by adult patients in some countries (Giovannini et al. 2012; Rocha et al. 2013; Jahja et al. 2014). Numerous reports highlighted the neurotoxic role of Phe, thus causing neurological and neuropsychiatric disturbances in PKU patients (van Spronsen et al. 2011; Christ et al. 2012; Bilder et al. 2013). Central mechanisms have been associated with neurotoxicity, such as amino-acid imbalance, reduced glutamatergic transmission, bioenergetics deficit, oxidative stress, impairment of lipid and protein metabolism, disruption of calcium homeostasis (Schumacher et al. 2008; Martynyuk et al. 2010; Schuck et al. 2015), but the interactions between these mechanisms await elucidation. The same questions are raised by the low concentrations of tyrosine responsible for the depletion of catecholamines related to brain dysfunction (McKean 1972; Christ et al. 2010; de Groot et al. 2010; Harding et al. 2014). Thus, a better knowledge of the metabolic pathways indirectly disturbed by both high Phe and low Tyr concentrations may be beneficial to better understand the pathogenesis of the disease, and finally to adjust the nutritional care (van Spronsen et al. 2009; Hennermann and Querfeld 2013; Longo et al. 2015; Schuck et al. 2015; Turki et al. 2015). Metabolomics is a powerful strategy to ascertain metabolic signatures from a combination of small molecules in biological fluids. One of the main objectives of the metabolomics approach is to improve understanding of pathophysiology, especially based on the non-a priori metabolic signature. Gas chromatography coupled with mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) is often used to perform metabolomics studies, but rarely in combination. The aim of our study was then to characterize the metabolic disturbances associated with plasma Phe and Tyr concentrations using a multiplatform targeted metabolomics approach. We used a combination of three analytical methods, namely routine determination of amino-acid profiles using amino-acid analyzer, GC-MS and NMR in order to obtain the most comprehensive metabolic pattern.

Material and Methods

Patients

Ten adult PKU patients (1–10) and ten age- and sex-matched healthy (11–20) controls were prospectively recruited from September 2014 to February 2015. This study has been approved by the ethics committee of Tours Hospital (Comité de Protection des Personnes). All subjects were informed and signed a consent. No subject has Phe-restricted diet at the time of inclusion. Clinical data available for their routine clinical follow-up were collected.

Samples Collection

Blood and urinary samples were collected at the inclusion visit after a night fasted. Blood samples were quickly centrifuged (2,500 g, 10 min). Urine samples were centrifuged in the same conditions. All samples were split into three aliquots and stored at −20°C until analysis. Blood samples were used to analyse amino-acids by aminotac, and urine samples were used to analyse (1) amino-acids by aminotac, (2) organic acids by GC-MS and (3) metabolomics profile by NMR. A brief description of these techniques is detailed below and additional material (document named “Additional Material” in supplemental data) provides complementary information about these analytical methods.

Amino-Acids Chromatography

We determined concentrations of amino-acid by Jeol Aminotac 500 analyzer, based on the ion-exchange chromatography with a post-column derivatization with ninhydrin and colorimetric detection. We used 500 μL of blood

and urine samples to perform the analysis. The determination of concentrations was based on the peak areas of amino-acids compared to that of internal standard.

Urinary Organic Acids Chromatography

Organic acids were extracted from urine by liquid/liquid extraction using ethyl acetate. The organic phase was evaporated (SpeedVac, ThermoFisher). We used two internal standards (4 phenylbutyric acid and heptadecanoic acid in acetonitrile). The derivatization was based on a silylation, by adding *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and incubation for 40 min at 80°C. The samples were then injected in GC-MS (QP2010 PLUS (Shimadzu)). We normalized the peaks of metabolites to the internal standard and the urinary creatinine concentration.

NMR Analysis

Urine samples were thawed at room temperature and centrifuged at 4,000 g for 10 min. 500 µL of supernatant was added with 100 µL of deuterated phosphate buffer 0.2 M (pH 7.4) and 8 µL of external reference (TSP). All the ^{1}H-NMR spectra were obtained on a Bruker Avance III HD NMR spectrometer (Bruker, SADIS, Wissembourg, France) operating at 700 MHz for ^{1}H resonance frequency using an inverse detection 5 mm ^{1}H-^{13}C-^{15}N cryoprobe.

Statistical Analysis

Multivariate Analysis

The plasma and urine signatures explored by the 1 or 3 analytical methods previously described were implemented in statistical software to analyse the merged data. The models were built to explain Phe and Tyr concentrations, and we completed the analysis by a discrimination of PKU and control group. For all the models, we excluded all metabolites directly modified by PKU (Phe, Tyr and derived organic acids).

We performed a multivariate analysis using Simca-P + -13, as done previously (Blasco et al. 2014). Principal component analysis (PCA) (Kemsley et al. 2007) was first performed as unsupervised clustering to identify similarities or differences between sample profiles.

Orthogonal partial least-squares discriminant analysis (OPLS-DA) is a supervised analysis that aims at evaluating the variations in metabolites between groups: the variation in the measured data was partitioned into two blocks by the program; one containing variations that correlated with the class identifier (PKU/controls) and the other containing variations that were orthogonal to the first block and thus did not contribute to discrimination between groups. OPLS-DA finds the algorithm of metabolites that will enable the best discrimination of the groups (PKU patients and controls) (Madsen et al. 2010).

After these multivariate analyses (PCA, OPLS-DA), we created a score plot to visualize the OPLS-DA model: it represents points corresponding to the different subjects defined by an algorithm of selected metabolites. We show only the models using the more robust algorithm of metabolites to separate the groups. We also characterized the contribution of each metabolite to the separation of the groups using a loading plot. The metabolites with large values of w*c (loading plot) (positive or negative) are highly correlated with Phe or Tyr values. The position of the metabolites in the loading plot enables to explain the group of subjects represented in score plot. The most discriminant metabolites called variable importance parameters (VIP) ranked the compounds according to their contribution to the model.

The quality of the models was described by different parameters: the CV ANOVA, R^2 and Q^2 values. R^2 is defined as a fraction of the variance explained by a component. Cross validation of R^2 gives Q^2. Q^2 is an estimation of the predictive ability of the model. The OPLS-DA was cross validated by withholding one-seventh of the samples in seven successive simulations such that each sample was omitted once in order to guard against over fitting. The set of multiple models resulting from the cross validation was used to calculate jack-knife uncertainty measures (Westerhuis et al. 2010).

Supplemental material (see "Additional Material" in supplemental data) provides complementary information.

Based on the same strategies, we also used partial least square (PLS) models to explain the outcome in question (i.e. concentrations of plasma phenylalanine and tyrosine analysed by aminotac, considered as the reference method). We also performed a hierarchical cluster analysis (HCA).

We compared the metabolic pathways identified from PLS analysis (to explain Phe and Tyr concentrations) and OPLS-DA (to explain PKU diagnosis) and we highlighted the common metabolites between all these approaches.

Univariate Analysis

We performed the univariate analyses on the VIPs as a complementary means to improve understanding of mechanistic pathways involved in the disturbance of phenylalanine metabolism. We compared metabolites concentrations between PKU and controls with Wilcoxon tests for categorical variables and we used Spearman tests to evaluate the correlation between quantitative variables (Phe or Tyr versus other metabolites). We applied a correction (Bonferroni) to the p value to correct for multiple statistical tests. We performed statistical analyses with JMP

statistical software version 7.0.2 (SAS Institute, Cary, North Carolina). We used KEGG pathway database (http://www.genome.jp/kegg/pathway.html) and METPA web application (http://metpa.metabolomics.ca/MetPA/faces/Home.jsp) to discuss the highlighted metabolic pathways. To focus on the most relevant data, we highlighted only the pathways that had significant Holm p and a pathway impact >0.10. The pathway impact value is the cumulative percentage from the matched metabolite nodes. Even if all the pathways having a significant Holm p are relevant, the threshold of 0.1 was fixed to focus our results and discussion only on the main metabolic pathways.

Results

Patients

The characteristics of PKU patients are provided in Table 1. The concentrations of Phe and Tyr are reported in Fig. S1 and will be used for further metabolomics models. Overall, the mean (± standard deviation) concentration of plasma Phe was 679.9 ± 157.5 μmol/L and the mean concentration of Tyr was 56.5 ± 3.0 μmol/L (Fig. S1). As expected we found higher concentrations of Phe (1,325 ± 465 vs 63 ± 15 μmol/L) and lower concentrations of Tyr (43 ± 9 vs 65 ± 11 μmol/L) in PKU patients, as compared to controls.

Metabolites Analysis

At the end of samples analysis, we detected 131 metabolites: 26 amino-acids measured in plasma and 105 molecules measured in urine: 27 organic acids measured by GC-MS, 26 amino-acids measured by the amino-acid analyzer and 52 metabolites measured by NMR. After identification of the main metabolites, we found that 92 out of the 105 urinary metabolites correspond to distinct molecules. Indeed, some metabolites may be detected by different analytical methods. Thus, our dataset used to perform statistical analysis was based on 118 different variables: 26 amino-acids measured in plasma by the amino-acid analyzer and 92 metabolites measured in urine (26 amino-acids measured by amino-acids analyzer, 23 metabolites measured by NMR and 27 by GC-MS and 16 unidentified metabolites measured by NMR). We provided on the supplementary data only the list of the identified metabolites, including metabolites measured both, in urine and plasma (Table S1).

Multivariate Models to Explain Phenylalanine Concentrations

We used UV scaling and logarithmic transformation to minimize the impact of noise or high variance of the variables.

The best model describing the concentrations of Phe was obtained from 13 metabolites.

Six were from plasma: threonine, glutamine, proline, alpha aminobutyric acid, isoleucine and arginine, and seven from urine: glycerate, 3-OH-3-Me-glutarate, methylmalonate, succinate and three unknown molecules: unknown 3, 8, 13 (U3, U8, U13).

The performances of the models are as follows: R^2X(cum): 0.802, R^2Y(cum): 0.973 and Q^2(cum): 0.934. CV ANOVA was 1.4×10^{-6}. The score plot is shown in Fig. 1 and the metabolites contributing to the model are shown in Fig. S2. Patients having high concentrations of Phe (with high $t(1)$ and $t(2)$, on the top right in the Fig. 1) are particularly discriminated from the others based on the high levels of U13, U8 and 3-OH 3-Me-3Glu.

Table 1 Clinical and biological characteristics of PKU patients

Patient	Age	Gender	Type of PKU	Phe concentration (μM)	Tyr concentration (μM)	Age of stopping Phe restricted diet (years)
1	34	F	Classical	1,641	37	6
2	22	M	Mild	1,051	61	16
3	30	F	Classical	1,281	51	5
4	34	F	Classical	1,854	46	5
5	27	F	Mild	1,069	30	5
6	36	M	Classical	1,449	56	5
7	30	F	Classical	1,472	39	5
8	39	M	Classical	1,790	52	5
9	30	F	Mild hyperphenylalaninemia	363	52	8
10	18	M	Mild	1,007	51	13

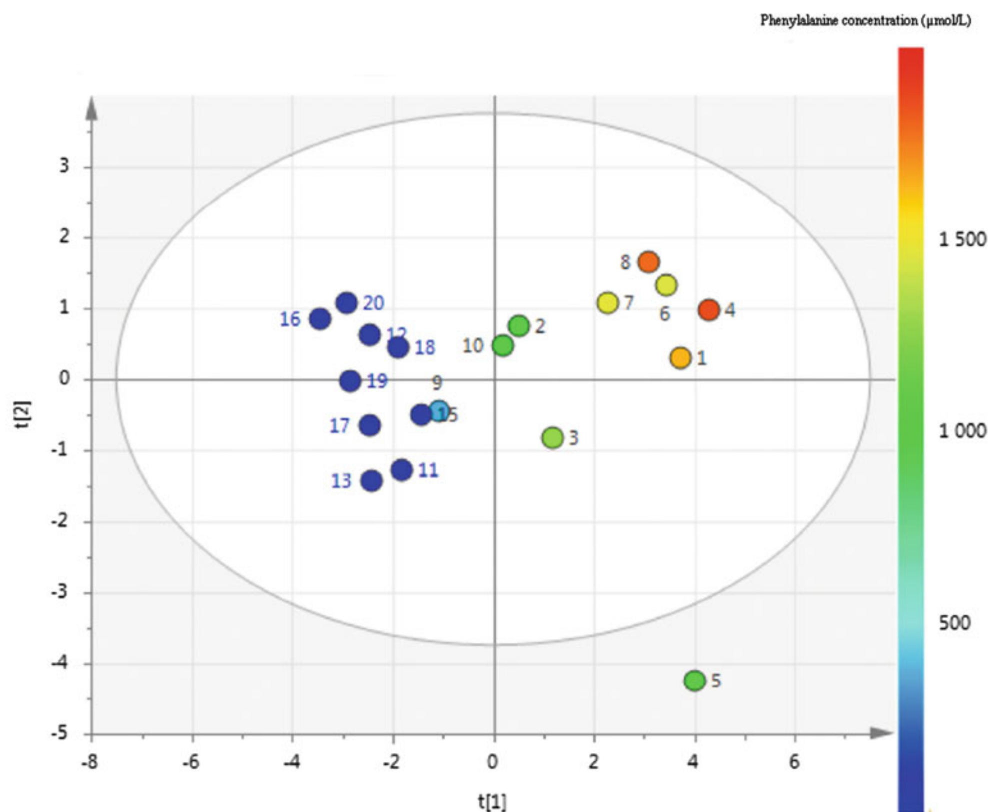

Fig. 1 Score plot based on the partial least square (PLS) models (13 metabolites) to explain Phe concentrations. The intensity of colour is proportional to the concentrations of Phe ($n = 20$, PKU patients from 1 to 10, healthy controls from 11 to 20). To note, the patient 9 is classified within the control group. R^2X(cum): 0.802, R^2Y(cum): 0.973 and Q^2(cum): 0.934, CV ANOVA: 1.4×10^{-6}

The univariate analysis performed on the VIPs and using the p value corrected by Bonferroni (13 metabolites in the model, so cut-off: $p = 0.0004$) showed a significant inverse correlation between glycerate (adjusted R^2 value: 0.61, $p < 0.0001$), alpha aminobutyric acid (adjusted R^2 value: 0.50, $p = 0.0003$), arginine (adjusted R^2 value: 0.56, $p < 0.0001$), glycerate (adjusted R^2 value: 0.61, $p < 0.0001$) and Phe and positive correlation between 3-OH-3-Me-glutarate (adjusted R^2 value: 0.61, $p < 0.0001$) and Phe (Fig. S2).

The dendrogram based on these data showed five different subgroups (Fig. 2a), visualized in the corresponding score plot (Fig. 2b). We noted that one patient was classified within the control group, especially because the concentration of Phe is lower than other patients (373 µmol/L).

Multivariate Models to Explain Tyrosine Concentrations

The best model describing the concentrations of Tyr was obtained from 13 metabolites.

Six from plasma: glutamine, alanine, alpha amino-butyric acid, methionine, isoleucine and arginine, seven from urine: 2-Me succinate, fumarate, isovalerylglycine, pyruvate, 3-OH isovalerate, succinate and one unknown molecule (U11).

The performances of the models are as follows: R^2X (cum): 0.685, R^2Y(cum): 0.922 and Q^2(cum): 0.898. CV ANOVA was 7.8×10^{-7}. The score plot is shown in Fig. 3 and the metabolites contributing to the model are shown in Fig. S3.

The univariate analysis performed on the VIPs and using the p value corrected by Bonferroni (cut-off: $p = 0.0004$) showed a significant positive correlation between glutamine (adjusted R^2 value: 0.51, $p = 0.0003$), methionine (adjusted R^2 value: 0.48, $p = 0.0004$), isoleucine (adjusted R^2 value: 0.54, $p = 0.0001$), arginine (adjusted R^2 value: 0.53, $p = 0.0002$), pyruvate (adjusted R^2 value: 0.57, $p = 0.0001$), succinate (adjusted R^2 value: 0.56, $p = 0.0001$), U11 (adjusted R^2 value: 0.53, $p = 0.0003$) and Tyr.

The dendrogram showed five different subgroups (Fig. S4A), visualized in the corresponding score plot (Fig. S4B). We noted that one control was classified within the PKU group, due the low concentration of Tyr (45 µmol/L).

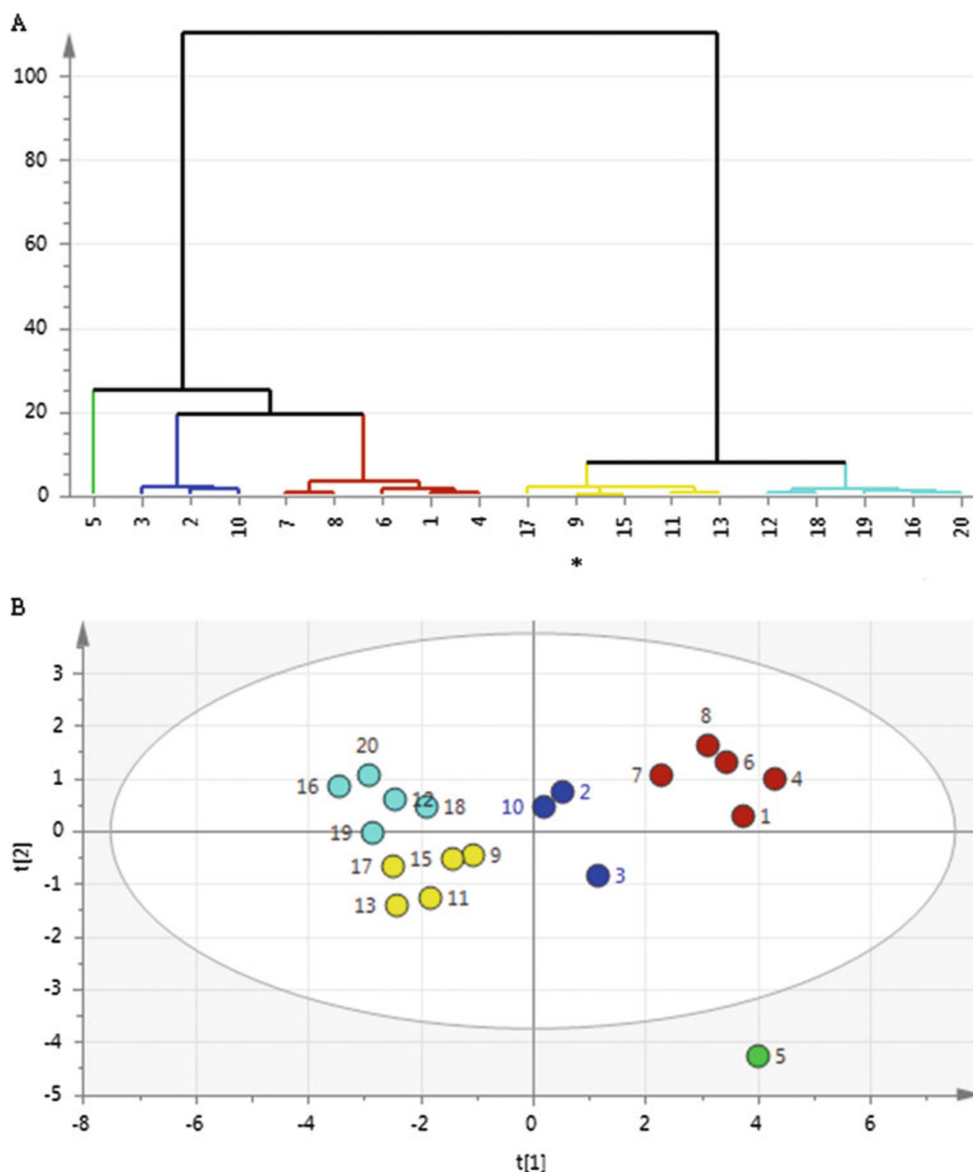

Fig. 2 (**a**) Dendrogram obtained from hierarchical cluster analysis (HCA) based on the 13 relevant metabolites used in the partial least square (PLS) model to explain Phe concentrations, and showing 5 subgroups of subjects; the *X* axis represents the patients and the *Y* axis the distance between the clusters; (**b**) Score plot characterized by the same colours as identified in the dendrogram. To note, the patient 9 (*asterisk*) is classified within the control group

Multivariate Models to Explain PKU

The best OPLS-DA model was based on 12 metabolites: six from plasma: threonine, glutamine, proline, alpha amino-butyric acid, methionine and arginine, and six from urine: glycerate, 2-OH isobutyrate, 3-OH isovalerate, 3-Me-2-oxovalerate, succinate and one unknown molecule (U10) (Fig. S5). R^2X(cum): 0.719, R^2Y(cum): 0.837 and Q^2(cum): 0.761. CV ANOVA was 0.0003.

Common Metabolites Explaining Phe and Tyr Concentrations

We found four common molecules with the two PLS models to explain Phe and Tyr concentrations and the OPLS model to explain PKU (Fig. 4). The most relevant pathways involved in the different models are shown in Fig. 4 (defined by a significant p-value and a pathway impact >0.1). The ways found in the three models

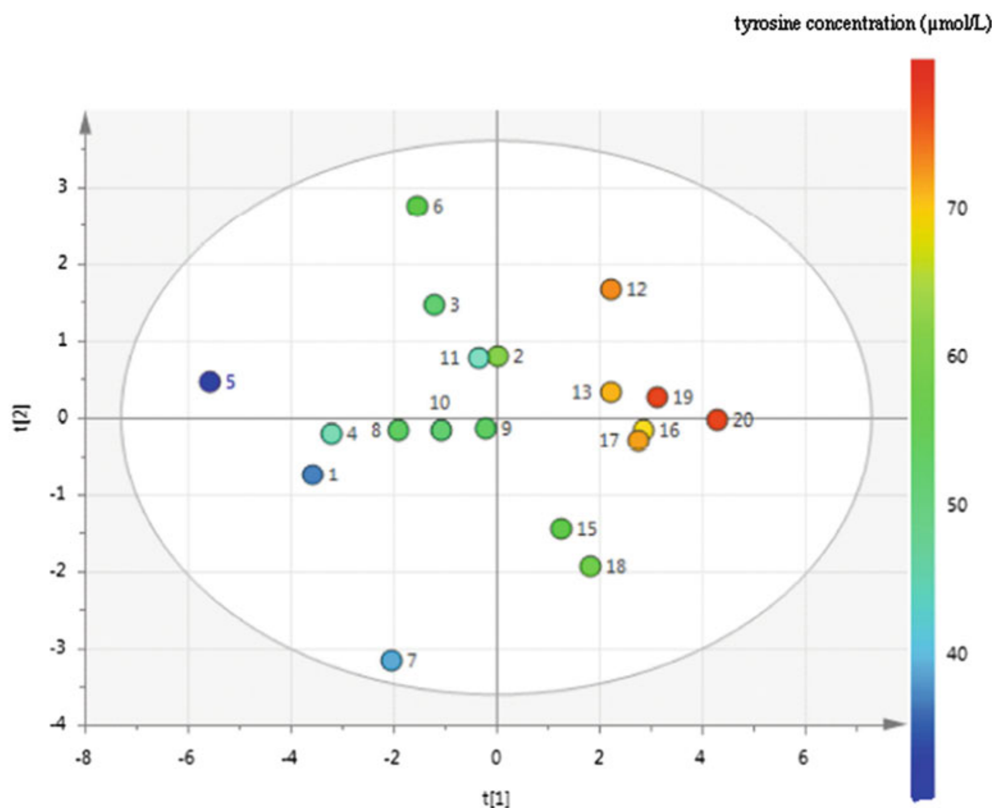

Fig. 3 Score plot based on the partial least square (PLS) models (13 metabolites) to explain Tyr concentrations. The intensity of colour is proportional to the concentrations of Tyr ($n = 20$, PKU patients from 1 to 10, healthy controls from 11 to 20). R^2X(cum): 0.685, R^2Y(cum): 0.922, Q^2(cum): 0.898, CV ANOVA: 7.8×10^{-7}

correspond to the metabolism of Arg and Pro, and also of Ala, Asp and Glu metabolism.

Discussion

According to the partially unknown mechanisms associated with neurotoxicity in PKU, we hypothesized that a metabolomics profile of different subjects characterized by a large distribution of Phe and Tyr concentrations may be relevant to identify metabolic pathways disturbances.

Few studies reported metabolomics approach in PKU and they especially focused on new diagnosis biomarkers of this disease (Denes et al. 2012; Janeckova et al. 2012; Xiong et al. 2015) to develop a diagnosis from less invasive samples (for example, from urine collection or dried spot) or to limit false positive cases, for example. A recent metabolomics approach based on GC-MS aimed at developing a new standard to diagnose PKU from urine samples. They reported 19 differentially expressed compounds in PKU patients (for example, phenylacetic acid or phenylpyruvic acid) with a relevant receiver-operating characteristic (ROC) analysis (Xiong et al. 2015), thus validating the metabolomics approach from urine to diagnose PKU. They observed a high specificity (93.6%) and sensitivity (100%) from the ROC curve based on urinary phenylacetic acid concentrations. They noted a low false positive rate (6.4%) that could be improved from the use of additional specific markers. Interestingly, one other study evaluated the metabolic influence of the long term PKU diet (Mutze et al. 2012). The present study is the first metabolomics study in PKU based on multiplatform analysis and only focused on the pathophysiology of the disease.

The combination of several analytical methods adds complexity but might be the currently most expeditious approach in a disease with perturbations focused on metabolism. The present approach is based on the combination of two methods usually performed in routine practice to help in the diagnosis of metabolic disease or to adapt patients' diet or therapeutics. We added the exploration of urine samples by NMR, using a 700 MHz NMR spectrometer which is very sensitive and reproducible and actually rarely used for metabolomics approaches. We applied a targeted analysis to obtain a limited number of metabolites, mostly identified and with sufficient signal to be representative of physiological and pathological variations. A combination of different "omics" approaches has never been performed for a PKU condition, but has been tried in

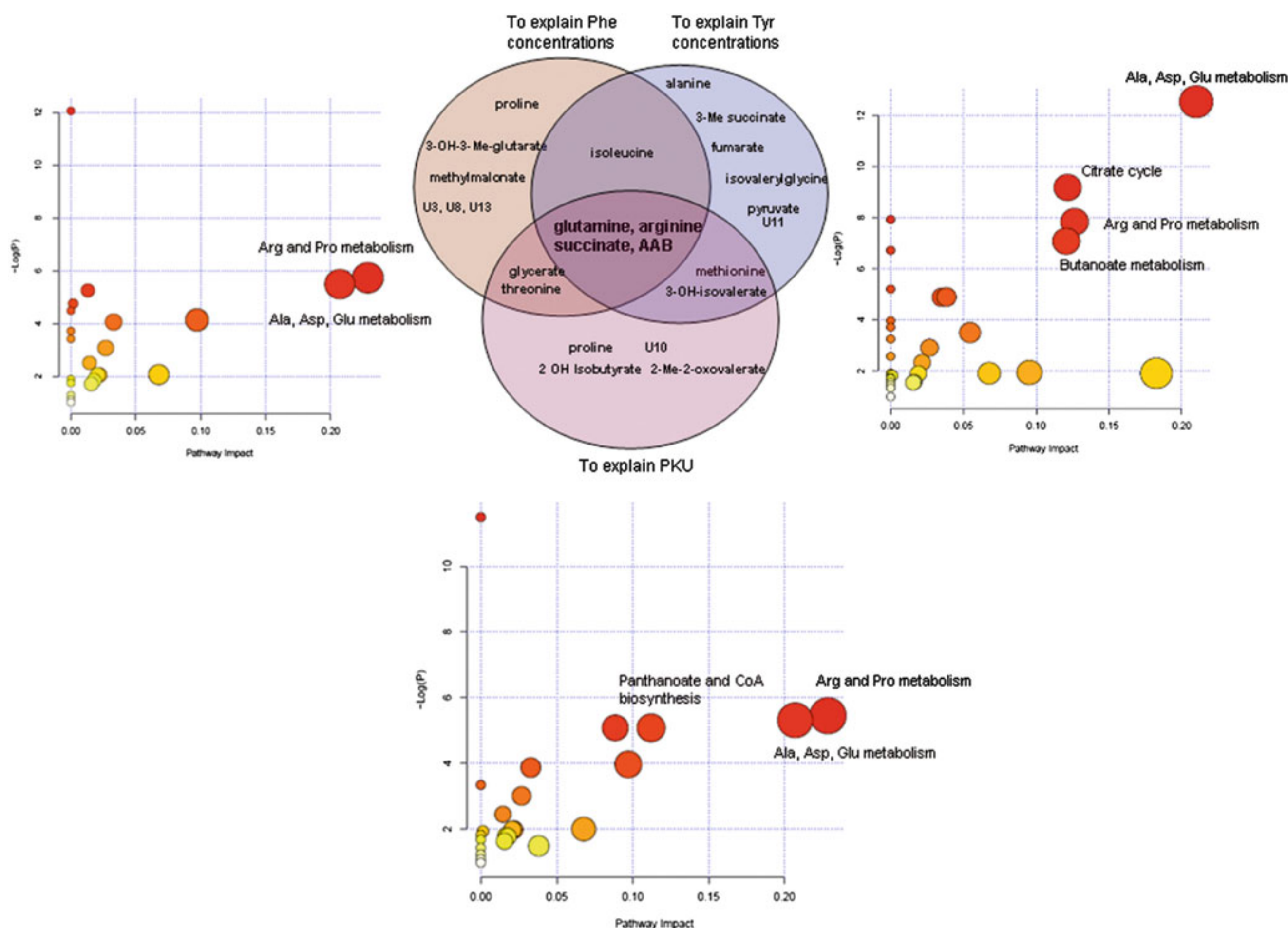

Fig. 4 Venn diagram showing metabolites identified in the partial least square (PLS) models to explain Phe concentration (the *left circle*), Tyr concentration (the *right circle*) and Orthogonal partial least-squares discriminant analysis (OPLS-DA) to explain clinical status (the *bottom circle*). This diagram highlights the common and specific metabolites according to the objectives of the models. The metabolic pathways identified by METPA web application from the metabolites noted in the Venn diagram are shown for each model: PLS to explain Phe concentration (graph on the *left*), Tyr concentration (graph on the *right*) and OPLS-DA to explain clinical status (graph at the *bottom*). We noted on the figures only the most relevant pathways defined as significant Holm p and pathway impact >0.1

other fields (Weckwerth 2008). One of the limitations of this strategy is the inadequate number of metabolites compared to a limited samples size. In our study, we used merged data, and we optimized the different multivariate models to obtain the better performances with a limited number of metabolites (always <13), thus avoiding misinterpretation. Moreover, we validated our PLS modeling based on concentrations values of Phe and Tyr by the OPLS-DA model.

The different multivariate models enable a correct classification of subjects, except the subjects 9 and 11. The multivariate models performed to explain Phe concentrations revealed a clear discrimination of patients (except the subject 9), even after exclusion of metabolites directly altered by PKU. Some of discriminant metabolites included in the models were also significantly associated with Phe concentrations after univariate analysis. HCA enabled us to identify some subgroups of subjects, especially the patient 5, who seems different and the subject 9 who is classified with controls. Importantly, the statistical analysis of outsiders performed using SIMCA analysis did not reveal any outsider in the included subjects (data not shown). After a deeper analysis of the clinical characteristics of the patients, we noted that the patient 5 has also type 1 diabetes, thus explaining the different metabolic profile despite the high concentrations of Phe. To note, the OPLS-DA discriminating PKU patients from controls also found the patient 5 as an outlier. The patient 9 has a low concentration of Phe (363 μM) compared to other PKU patients, so associated metabolism modification is similar to that of controls.

Inhibition of tyrosine transport across the presynaptic membrane by phenylalanine (McKean 1972; Knudsen et al. 1995), and decreased urinary excretion of dopamine and serotonin or their metabolites (Curtius et al. 1972) have

been largely reported in the literature since many years but the metabolic perturbation associated with Tyr in PKU context remains partially unknown. To explain Tyr concentrations, the best fit was obtained from 13 metabolites, with excellent performances. However, we observed a bigger dispersion of subjects in the score plot. We noted that the patient 5, but also the 7 are quite different (Fig. 3). The control 11 was misclassified, that could be explained by its particularly low concentration of Tyr compared to other controls. The score plots of both PLS models showed that the dispersion of subjects is higher within the group of PKU patients, thus highlighting the heterogeneity of these patients for the discriminant metabolites. According to the misclassified subjects, the potential impact of diet has to be taken into account. In this study, subjects were fasted and the analysis of the amino-acids profile suggests that subjects respected the conditions of samples collection. However, the major role of diet on metabolic explorations has to be considered to avoid misinterpretation. Moreover, fasting, as other physiological challenges increased inter-individual variations, even in similar subjects (Krug et al. 2012). These findings may explain some of our unexpected results.

The analysis of the common metabolites found in both PLS models and the OPLS-DA model revealed an important role of glutamate, succinate, arginine and alpha aminobutyric acid. METPA analysis highlighted the involvement of Arg, Pro, and Ala, Asp, Glu metabolism.

One of the most described pathophysiological ways in PKU is the defect in central nervous system protein synthesis (de Groot et al. 2015). Numerous authors highlighted a significant negative correlation between plasma Phe levels and the cerebral protein synthesis rate, based on positron emission tomography in PKU patients, and experiments in animal or in vitro models (Schuck et al. 2015). We observed a relation between different amino-acids largely involved in protein synthesis and Phe or Tyr (such as alanine, isoleucine). Alpha aminobutyric acid is an amino-acid not taking part in protein synthesis but is considered as a non-specific biomarker of liver, malnutrition or protein catabolism (Chiarla et al. 2011). An old report showed that alpha aminobutyric acid concentration was significantly higher (Thalhammer et al. 1980) in PKU that is consistent with our findings. This observation needs complementary data to understand the role of this metabolite in PKU pathophysiology.

Moreover energy metabolism disturbance was reported in PKU, including pyruvate kinase, hexokinase, other enzymes dependent or not from metal ions, and also ketonic bodies metabolism (Schuck et al. 2015). The inhibition of glucose-6-phosphate dehydrogenase activity by phenylpyruvic acid has also been reported, thus leading to an impairment of NADPH production (Rosa et al. 2012). Importantly, some author observed a significant decrease of succinate dehydrogenase and mitochondrial respiratory chain in cerebral cortex of rats with hyperphenylalaninemia (Rech et al. 2002). These data are consistent with the involvement of succinate in our different models. To note the alteration of mitochondrial respiratory chain by phenylalanine is still debated (Hargreaves et al. 2002; Rech et al. 2002; Kyprianou et al. 2009). Even if succinate is a non-specific metabolite of energetic disturbance, this finding merits more explorations. As all these patients were not under rigorous Phe-restrict diet therapy, we suggest that these observations are probably not due to a poor nutritional status.

We showed that arginine was a relevant metabolite to explain Phe and Tyr concentration. Arginine has numerous roles in inflammation response (Rath et al. 2014), growth hormone regulation (Merimee et al. 1965), wound repair (Williams et al. 2002), glucose metabolism (Piatti et al. 2001) and so on. Among the different effect of arginine, its link with oxidative stress seems one of the most relevant mechanisms in PKU. Indeed, NO produced from L-arginine by NO synthases is largely involved in oxidative stress described in PKU patients (Ribas et al. 2011; Sanayama et al. 2011).

Interestingly, some metabolic pathways were highlighted only in models to explain Tyr concentrations (Fig. 4, citrate cycle, butanoate metabolism) but they were closely linked with that associated with Phe.

The main perspectives of this pilot study are to pursue on a larger cohort composed of patients from childhood to adult age. We may assess the evolution of metabolome that may be related to the Phe and Tyr equilibrium as well as the effect of age. As metabolism is largely age-associated and as metabolomics provide a global metabolism view without a priori, we have to plan some longitudinal studies to explore the evolution of the altered metabolic pathways during several years. As metabolomics profile may be influenced by gender and age (Thevenot et al. 2015), the robustness of this present study and the followings is based on the matching between PKU patients and controls. Importantly, such kinds of study will not be able to interpret the metabolism disturbance directly influenced by Phe, but it would establish a specific metabolic profile using high throughput techniques of PKU patients, deeply linked to the disequilibrium of Phe and Tyr (Kaufman 1976).

Conclusion

Importantly, we observed metabolism alterations in plasma and urine, although most studies are focused on brain metabolism to explain PKU pathogenesis. This pilot study

based on a new strategy of pathophysiology exploration based on multiplatform metabolomics to analyse biological fluids is promising for further experiments. The approach based on easily available samples enabled us to obtain a large metabolic pattern without a priori and we identified the involvement of relevant pathways, thus opening new perspective of pathogenesis understanding.

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Synopsis

The metabolomics approach based on a multiplatform strategy is promising to improve the knowledge of PKU pathogenesis.

Compliance with Ethics Guidelines

Conflict of Interest Statements

Blasco H, Veyrat-Durebex C, Bertrand M, Patin F, Labarthe F, Henique H, Emond P, Andres CR, Antar C, Landon C, Nadal-Desbarats L and Maillot F declare no conflict of interest

All the authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsor

Informed Consent

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

Contribution of Authors

Hélène Blasco performed the statistical analysis, interpreted the data and wrote a part of the manuscript

Charlotte Veyrat-Durebex: acquired data

Franck Patin: performed the statistical analysis, interpreted the data and wrote a part of manuscript

Labarthe F: critically revised the manuscript for important intellectual content

Bertrand M: acquired NMR data

Hélène Hénique: acquired clinical data

Patrick Emond: acquired GC-MS data

Christian R Andres: critically revised the manuscript for important intellectual content

Catherine Antar: pre-treated and integrated NMR data

Céline Landon: acquired NMR data

Lydie Nadal-Desbararats: pre-treated, integrated NMR data and identified metabolites

François Maillot: recruited patients, interpreted data and critically revised the manuscript for important intellectual content

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CASE REPORT

Japanese Male Siblings with 2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase Deficiency (HSD10 Disease) Without Neurological Regression

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Abstract 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (HSD10 disease) is a rare X-linked disorder caused by a mutation in the *HSD17B10* gene. Fewer than 30 patients with this disorder have been reported worldwide. The classical infantile form of HSD10 disease is characterized by a progressive neurodegenerative course with retinopathy and cardiomyopathy, although HSD10 disease has broad clinical heterogeneity. However, several male patients have not shown neurological regression. Here, we describe two Japanese siblings with HSD10 disease without neurological regression. A 4-year-old boy presented with unconsciousness due to severe hypoglycemia. Laboratory testing on admission showed mild metabolic acidosis and mild hyperammonemia. Urinary organic acid analysis in the acute phase showed elevated excretion of 2-methyl-3-hydroxybutyric acid, tiglylglycine, and ketones. However, 2-methylacetoacetate was not elevated. HSD10 disease was suspected based on urinary organic acid data. The patient had a novel hemizygous c.470C>T (p.A157V) mutation in the *HSD17B10* gene. His mother was a heterozygous carrier of this mutation. The patient's older brother also had the c.470C>T (p.A157V) mutation. Neurological development was normal at the time of evaluation. The pilot newborn screening results using tandem mass spectrometry of the proband were reevaluated retrospectively and showed a high C5:1 carnitine level of 0.070 nmol/mL (upper cutoff limit, 0.05 nmol/mL) and a normal C5-OH carnitine level of 0.290 nmol/mL (upper cutoff limit, 1.0 nmol/mL). His affected brother and another patient with the atypical form of HSD10 disease having p.A154T also showed elevated C5:1 but not C5-OH in serum acylcarnitine analysis. Thus, these data suggested that some patients with this disorder may be identified using newborn screening.

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Introduction

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (HSD10 disease) is a rare X-linked disorder caused by a mutation in the *HSD17B10* gene (Zschocke et al. 2000; Ofman et al. 2003). Fewer than 30 patients have been reported worldwide. Classical infantile form of HSD10 disease is characterized by a progressive neurodegenerative course with retinopathy and cardiomyopathy, leading to death at the age of 2–4 years or later (Zschocke 2012); however, this disorder has wide clinical heterogeneity. Only three patients in two families have been reported to have the atypical presentation of the disease, without neurological regression (Rauschenberger et al. 2010; Fukao et al.

2014). Moreover, one patient without neurological regression during childhood was reported to develop Parkinsonism in adulthood, as an abstract form (Lorea et al. 2015).

Here, we describe the fourth and fifth case of HSD10 disease without neurological regression in two Japanese siblings. The proband developed ketotic hypoglycemia three times at 4 years of age.

Case Presentation

A 4-year-old Japanese boy, who had been well and exhibited normal development until admission, presented with unconsciousness. One day before admission, he had a high fever and appetite loss. His parents were unrelated, and he had a 10-year-old brother and an 8-year-old sister. Both of his siblings exhibited normal growth and development.

On physical examination on admission, the patient had a height of 90.5 cm (−1.1 SD), body weight of 12.7 kg (−1.7 SD), heart rate of 114 bpm, and body temperature of 36.8°C. The Glasgow Coma Scale was E4V1M1. Laboratory testing on admission showed mild metabolic acidosis (blood gas pH, 7.337; pCO_2, 33.8 mmHg; HCO_3-, 17.7 mM), mild hyperammonemia (156 μM), and severe hypoglycemia (blood glucose less than 1.1 mM). Other data were as follows: white blood cell count, 26,900/μL; hemoglobin, 12.5 g/dL; blood urea nitrogen, 0.57 mM; aspartate aminotransferase, 49 IU/L; lactate dehydrogenase, 353 IU/L; lactate, 2.7 mM; total ketone bodies, 5.4 mM; and insulin, 2.2 μIU/mL. Three minutes after bolus infusion of 18 mL of 20 % glucose, blood glucose was increased to 6.61 mM, and the patient became conscious. After continuous glucose infusion, his symptoms improved, and he began to take oral food. On day 2 of hospitalization, the patient exhibited duodenal ulcers, with complaints of abdominal pain and melena. He was successfully medicated and was discharged from the hospital on day 22 of hospitalization. Urinary organic acid analysis in the acute phase showed elevated excretion of 2-methyl-3-hydroxybutyrate to 830 μg/mg Cr (mean ± SD, 5.1 ± 5.3) and tiglylglycine to 252.6 μg/mg Cr (1.1 ± 0.6). However, 2-methylacetoacetate was not elevated (0.1 μg/mg Cr; 1.0 ± 1.6). Urinary organic acid analysis 1 month later under nonsymptomatic conditions also showed increased excretion of 2-methyl-3-hydroxybutyrate (91.8 μg/mg Cr) and tiglylglycine (207.7 μg/mg Cr). HSD10 disease was suspected based on urinary organic acid data and harmonized elevation of blood ketone bodies with hypoglycemia.

Mutation analysis was then carried out at the genomic level. The results showed that the patient had a novel hemizygous c.470C>T (p.A157V) mutation in the *HSD17B10* gene. His mother was a heterozygous carrier of this mutation, and his older brother also had the c.470C>T (p.A157V) mutation. This mutation was not present in a population-based variation database consisting of 1,208 Japanese individuals (Human Genetic Variation Database, www.genome.med.kyoto-u.ac.jp/SnpDB/index.html).

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (2M3HBD) activity was assayed using fibroblasts, as previously described (Zschocke et al. 2000). 2M3HBDH activity was low (0.25 ± 0.05 nmol/min/mg protein; control, 1.33 ± 0.08 nmol/min/mg protein). Immunoblot analysis showed that fibroblasts from patients with p.A157V or p.A154T mutations had similar levels of HSD17B10 protein as control fibroblasts (Fig. 1).

Pilot newborn screening using tandem mass spectrometry at 5 days of age showed C5:1 levels of 0.070 nmol/mL and C5-OH of 0.290 nmol/mL. Screening criteria for beta-ketothiolase deficiency at newborn screening were 0.05 nmol/mL for C5:1 and 1.0 nmol/mL for C5-OH. Reexamination showed C5:1 of 0.090 nmol/mL and C5-OH of 0.590 nmol/mL. Therefore, screening criteria for beta-ketothiolase deficiency was not carried out and was judged as normal at that time.

The patient developed two additional ketotic hypoglycemic episodes at 4 years of age. His neurological development was normal, with an IQ of 90 (Wechsler Intelligence Scale for Children). No abnormal findings were identified in echocardiographic or ophthalmological examinations.

Urinary and blood analyses were also performed for his older brother at 11 years of age. Urinary organic acid analysis showed elevated 2-methyl-3-hydroxybutyrate to 25.0 μg/mg Cr and tiglylglycine to 44.1 μg/mg Cr. Serum acylcarnitine profile analysis showed an elevated C5:1 of 0.108 nmol/mL (mean ± SD, 0.012 ± 0.005) and a normal C5-OH of 0.06 nmol/mL (0.06 ± 0.03). His IQ was 74, classifying him as borderline intelligence; however, he was enrolled in normal classes in school and had never experienced any apparent neurological problems.

Discussion

Typical HSD10 disease is suspected when patients with neurological degeneration or psychomotor retardation show similar urinary organic acid or blood acylcarnitine profiles with beta-ketothiolase deficiency. Our patient developed severe ketotic hypoglycemia, and HSD10 disease was suspected based on metabolic evaluation of this episode. His neurological development was normal, and he had still not exhibited any neurological symptoms at the latest follow-up (5 years of age). Moreover, his older brother was shown to have the same mutation and an IQ indicating borderline intelligence at 11 years of age, without the occurrence of neurological problems. The older brother had never experienced hypoglycemic episodes.

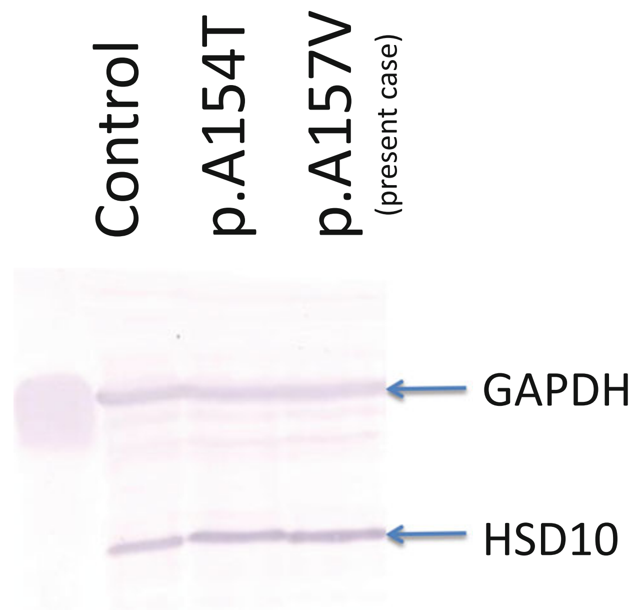

Fig. 1 Immunoblot analysis: Fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Immunoblot analysis for 2M3HBD was carried out using anti-rat 2M3HBD antibodies and antihuman glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (sc-25778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a reference. *Each lane* represents 30 μg of fibroblast extracts

Based on urinary organic acid profiles and normal mitochondrial acetoacetyl-CoA thiolase activity in the patient's fibroblasts, we assumed that the function of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was blocked in this patient. In accordance to this, we identified a novel hemizygous mutation c.470C>T (p.A157V) in the *HSD17B10* gene, which encodes the HSD10 protein, a multifunctional mitochondrial 17beta-hydroxysteroid dehydrogenase and one of the three components of mitochondrial RNaseP. The latter is essential for mitochondrial tRNA processing (Holzmann et al. 2008). Several studies have shown that defects in 2-methyl-3-hydroxybutyryl-CoA dehydrogenase activity are not associated with clinical severity of HSD10 disease, whereas defects in RNaseP function are associated with clinical severity (Rauschenberger et al. 2010; Yang et al. 2009).

Patients with HSD10 disease exhibit broad clinical heterogeneity. Zschocke subdivided HSD10 patients into four groups: the neonatal form, infantile form, juvenile form, and atypical presentation, as shown in Table 1 (Zschocke 2012). The classical presentation, which is called the infantile form, is characterized by a progressive neurological regression and cardiomyopathy, leading to death. However, several patients have been described who did not show neurological regression, a presentation classified as the atypical presentation (Table 2). A patient with the c.495A>C (p. Q165H) mutation was shown to have normal development up to his current age of 5 years, although he previously exhibited growth retardation and feeding difficulties during infancy. Moreover, his male cousin, who had the same mutation, achieved normal neurodevelopment until his current age of 8 years (Rauschenberger et al. 2010). We recently reported another patient with the c.460G>A (p.A154T) mutation who showed no neurological regression until his current age of 6.5 years (Fukao et al. 2014). Thus, in this report, we described an additional two patients with c.470C>T (p.A157V) mutations, leading to the atypical presentation of HSD10 disease. However, another patient with the p.A158V mutation was reported to have developed Parkinsonism and was diagnosed with HSD10 disease at the age of 27 years (Lorea et al. 2015). Because the main characteristic of these patients was the lack of neurological regression during childhood, we propose that these patients should be classified as having a nonregressive form of HSD10 disease. Some patients may have developmental delays or neurological problems without neurological regression. Therefore, careful follow-up is needed for young patients without neurological regression because we cannot exclude the possibility of neurological problems and other symptoms later in life.

The mutations identified in the four patients with atypical HSD10 (Table 2) are located in close proximity in the tertiary structure of the HSD17B10 protein subunit (PDB ID: 2O23). Moreover, the catalytic triad of this subunit is formed by Ser155, Tyr168, and Lys172. All of these four mutations are very close to one of the catalytic residues. Thus, catalytic activity is expected to be affected by these mutations. Polyphen2 software suggests that

Table 1 Clinical presentation of HSD10 disease and mutations in the *HSD17B10* gene

Classification	Number of previous cases	Typical mutations	Clinical presentation	Reference
Neonatal form	3	p.N247S, p.R226Q, p.D86D	Immediate postnatal onset of severe metabolic lactic acidosis, little psychomotor development, seizures, progressive cardiomyopathy, death in the first month of life	Perez-Cerda et al. (2005), García-Villoria et al. (2009), and Rauschenberger et al. (2010)
Infantile form	More than 13	p.R130C, p.P210S, p.L122V	Normal development in the first 6–18 months of life, followed by a progressive neurodegenerative course with retinopathy and cardiomyopathy, leading to death at the age of 2–4 years or older	Zschocke et al. (2000), Ensenauer et al. (2002), Sutton et al. (2003), Sass et al. (2004), Perez-Cerda et al. (2005), Cazorla et al. (2007), Lenski et al. (2006), García-Villoria et al. (2009), Rauschenberger et al. (2010), and Zschocke (2012)
Juvenile form	1	p.E249Q	Normal development in the first 5 years and slow deterioration of speech and motor skills linked to measles infection	Olpin et al. (2002)
Nonregressive form	Six cases from four families, including the siblings in the current report	p.R165H, p.A154T, p.A157V, pA158V	Normal development in childhood, with typical urinary organic acid profiles and potential transient metabolic decompensation	Rauschenberger et al. (2010), Fukao et al. (2014), and Lorea et al. 2015; Present case

Table 2 Mutations and clinical presentation of nonregressive form of HSD10 disease

Mutation	First presentation	Neurological problem	Family members with the same mutation	Reference
p.Q165H	Pre- and postnatal failure to thrive	None at 8 years of age	Brother and male cousin	Rauschenberger et al. (2010)
p.A154T	Five-day period of appetite loss and vomiting with severe ketoacidosis due to gastroenteritis at 6 years of age	None at 7 years of age		Fukao et al. (2014)
p.A158V	Parkinsonism at 27 years of age	None before diagnosis		Lorea et al. (2015)
p.A157V	Unconsciousness due to severe hypoglycemia at 4 years of age	None at 5 years of age	Older brother	Present case

p.A157V may be classified as probably damaging. Additionally, immunoblot analysis showed that fibroblasts from patients with p. A157V or p.A154T mutations had similar levels of HSD17B10 protein as control fibroblasts (Fig. 1) (Fukao et al. 2014). These mutations may affect catalytic activity but not stability.

Our proband and the patient with the p.A154T mutation developed ketotic hypoglycemic episodes several times, leading to further urinary organic acid analyses and thereby facilitating the final diagnosis (Fukao et al. 2014). However, it is not clear whether patients with HSD10 disease may tend to develop ketotic hypoglycemia owing to the lack of sufficient cases; further studies are needed to determine the associations between ketotic hypoglycemia and HSD10 disease.

Notably, C5:1 was particularly elevated, whereas C5-OH was within the upper cutoff limit in newborn screening in our probands. Such high C5:1 levels were not observed in more than 10,000 newborns in Gifu, Japan (unpublished observation). However, the patient's older brother and our other patient with the p.A154T mutation also showed elevated C5:1 but not C5-OH in serum acylcarnitine analysis. Thus, these data suggest that this disorder may be identifiable during newborn screening.

Conclusion

Here, we present two male siblings with HSD10 disease without neurological regression (the nonregressive form) having a novel hemizygous c.470C>T (p.A157V) mutation in the *HSD17B10* gene. The younger sibling had several ketotic hypoglycemic events at 4 years of age, whereas the older brother showed no symptoms. The nonregressive

form of HSD10 disease may not be as rare as was previously thought, and careful follow-up is needed for young patients with atypical HSD10 disease.

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Take-Home Message

Patients of HSD10 disease without neurological regression during childhood may not be as rare as was previously thought.

Conflict of Interest

Shohei Akagawa, Toshiyuki Fukao, Yuko Akagawa, Hideo Sasai, Urara Kohdera, Minoru Kino, Yosuke Shigematsu, Yuka Aoyama, and Kazunari Kaneko declare that they have no conflicts of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the family for inclusion in this report. The molecular study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University, Gifu, Japan, and carried out with written approval.

Compliance with Ethics guidelines

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

Author Contributions

SA is the attending doctor of the siblings. YA, UK, and MK followed and treated the patients with SA. YS chemically diagnosed these patients by GC-MS. TF, HS, and YA performed molecular analysis and contributed to molecular diagnosis. TF, YS, and KK helped SA to diagnose and treat the patient as advising doctors. SA and TF contributed to the conception and design of this report. YS and KK contributed to drafting of the article. SA, TF, YS, YA, and KK contributed to critical editing of the article. All authors approved the final version of the article.

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RESEARCH REPORT

Newborn Screening for Vitamin B_6 Non-responsive Classical Homocystinuria: Systematical Evaluation of a Two-Tier Strategy

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Abstract *Background*: In classical homocystinuria (HCU, MIM# 236200) due to the deficiency of cystathionine β-synthase (EC 4.2.1.22) there is a clear evidence for the success of early treatment. The aim of this study was to develop and evaluate a two-tier strategy for HCU newborn screening.

Methods: We reevaluated data from our newborn screening programme for Qatar in a total number of 125,047 neonates including 30 confirmed HCU patients. Our hitherto existing screening strategy includes homocysteine (Hcy) measurements in every child, resulting in a unique dataset for evaluation of two-tier strategies. Reevaluation included methionine (Met) levels, Met to phenylalanine (Phe) ratio, and Hcy. Four HCU cases identified after database closure were also included in the evaluation. In addition, dried blood spot samples selected by Met values >P97 in the newborn screening programs in Austria, Australia, the Netherlands, and Taiwan were analyzed for Hcy.

Results: Met to Phe ratio was found to be more effective for first sieve than Met, sorting out nearly 90% of normal samples. Only 10% of the samples would have to be processed by second-tier measurement of Hcy in dried blood spots. As no patient with HCU was found neither in the samples investigated for HCU, nor by clinical diagnosis in the other countries, the generalization of our two-tier strategy could only be tested indirectly.

Conclusion: The finally derived two-tier algorithm using Met to Phe ratio as first- and Hcy as second-tier requires 10% first-tier positives to be transferred to Hcy measurement, resulting in 100% sensitivity and specificity in HCU newborn screening.

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Abbreviations

CBS	Cystathionine β-synthase
DBS	Dried blood spots
ESI-MS/MS	Electrospray ionization-tandem mass spectrometry
HCU	Classical homocystinuria
Hcy	Homocysteine

HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Met	Methionine
Phe	Phenylalanine

Introduction

Homocystinuria (HCU, MIM# 236200) is an autosomal recessive disorder usually caused by deficiency of cystathionine β-synthase (CBS, EC 4.2.1.22, Mudd et al. 1964), leading to highly increased plasma and urine concentrations of homocysteine (Hcy) as well as increased levels of plasma methionine (Met). Clinical symptoms in untreated or late treated patients include Marfan-like appearance, progressive myopia and lens dislocation, thromboembolism, epilepsy, and mental retardation (Mudd et al. 1964, 2001). Early treatment with high doses of pyridoxine and/or methionine restriction combined with betaine administration has proven to be effective in preventing many complications of this disease (Mudd et al. 1964, 2001).

There is considerable evidence that early detection and treatment can prevent the clinical consequences of the enzyme deficiency; therefore, newborn screening for the disorder has been advocated (American College of Medical Genetics Newborn Screening Expert Group 2006; Schulze et al. 2009). HCU leads to hypermethioninemia as an additional metabolic marker (Huemer et al. 2015; Wong et al. 2016, Region 4 Stork [R4S] collaborated website, http://www.clir-r4s.org). Newborn screening for HCU by measurement of Met in dried blood spots (DBS) is being performed in some countries but has poor sensitivity. In Ireland, 4 of 25 cases were missed, including one pyridoxine-responsive patient. The false negative-screening in the other three cases might be attributed to breast-feeding, due to low Met concentrations in breast milk and some infant formulas (Naughten et al. 1998). It is likely that pyridoxine-responsive patients, which account for about half of all CBS-deficiency cases, are not detected by measurement of methionine in neonates; there is no substantiated report of such detection (Huemer et al. 2015).

Based on data from newborn screening programs, the worldwide prevalence of pyridoxine non-responsive HCU has been estimated to be approximately 1 in 335,000 (Mudd et al. 2001) but varies from 1:900,000 (Japan, Naughten et al. 1998), 1:400,000 (Germany, Aymé and Hivert 2011) to 1:65,000 (Ireland, Yap and Naughten 1998) and 1:58,000 (Australia, Naughten et al. 1998) the latter at a time when the Australian population had a high proportion with Irish ancestry. We previously reported a high prevalence of about 1:3,000 in Qatar. In this highly consanguineous population HCU is caused primarily by homozygosity for the mutation p.R336C (c.1006C>T) in the CBS gene (Bener and Hussain 2006; El-Said et al. 2006; Zschocke et al. 2009; Gan-Schreier et al. 2010).

Based on the newborn screening samples of 125,047 children born in Qatar, obtained over a 7-year period from July 2006 to June 2013 using the parameters Met, Phe and Hcy measured by high-throughput liquid chromatography–tandem mass spectrometry (LC-MS/MS) in all samples, we developed a strategy using Met to Phe ratio as first-tier, and Hcy values as second-tier screening parameters for HCU.

In addition, samples from newborn screening programs in Austria, Australia, the Netherlands, and Taiwan were used to test the generalizability of the suggested two-tier strategy.

Materials and Methods

Study Design

The primary goal of the present study was to develop a two-tier strategy for screening for HCU. Screening of 125,047 samples from Qatar between 2006 and 2013 for HCU on the basis of Hcy alone resulted in 30 confirmed cases. In a reevaluation of the data initially obtained from the regular newborn screening (Met, Phe) as well as from the Hcy determination by LC-MS/MS performed in all newborns in Qatar we used in a first approach Met as first-tier and Hcy as second-tier parameters. As this strategy resulted in a rather insufficient model with a cut-off for Met at P62 to achieve 100% sensitivity, in a second approach the Met to Phe ratio was used as first-tier variable. As phenylalanine metabolism is not affected by HCU, Phe normalizes Met, i.e., individuals with overall higher or lower amino acid profiles would have normal ratios, and only individuals with Met values deviating from the average profile would become noticeable. Again Hcy was used as a second-tier parameter.

The secondary goal was to investigate samples from the newborn screening programs of Australia, the Netherlands, Austria, and Taiwan to test the generalizability of the two-tier algorithms. All samples exceeding the top 3% (>P97) of methionine concentrations in DBS were punched into sealed microtiter membrane plates (96 wells; Millipore Multi Screen Filter Plates 0.22 µm, Darmstadt, Germany), stored at 4°C, and sent to the screening laboratory in Heidelberg, Germany, for measurement of Hcy. No cases of

HCU were identified in these samples. The confirmed HCU cases from Qatar were then processed together with this second data set using the Met and Hcy two-tier algorithm previously applied on the Qatar data set. The study was approved by the respective local ethical committees.

Determination of Methionine and Phenylalanine in Dried Blood Spots

Blood spots taken between the 36th and 72nd hour of life were sampled on screening cards (Neonatal Screening Card; Whatman 903, GE Healthcare Europe GmbH, Freiburg, Germany) and dried at room temperature. Met and Phe were analyzed as their butyl esters on a triple quadrupole tandem mass spectrometer with [$^2H_3$]methionine and [$^2H_3$]phenylalanine as internal deuterated standards as previously described (Lindner et al. 2007 and the references therein). All other participating laboratories performed amino acid and acylcarnitine profiling using the above-mentioned derivatization step and subsequent ESI-MS/MS measurements (Australia and Austria: Wilcken et al. 2003; Taiwan: Huang et al. 2006; and the Netherlands: Rizzo et al. 2003).

Determination of Homocysteine in Dried Blood Spots

Following a formerly reported technique (Gan-Schreier et al. 2010), Hcy concentrations were quantified from the same blood samples in 3.2 mm punch-outs by LC-MS/MS using a modified stable isotope dilution technique (Gempel et al. 2000) and [$^2H_4$]homocysteine (Fluka, Sigma Aldrich, Taufkirchen, Germany) as an internal standard.

A Quattro micro tandem mass spectrometer (Waters, Germany) fitted with a Z-Spray ion source was used for all analyses. The instrument was operated in electrospray positive ion mode coupled to a Rheos 2000 HPLC system using an Atlantis™ HILIC Silica 2.1 × 50 mm, 3 μm (Waters, Eschborn, Germany) for separation. All aspects of system operation and data acquisition were controlled using Masslynx NT v3.5 software.

Data Analysis

For comparison of distributions of Met, Hcy, and Met to Phe values between samples with the Brown–Forsythe Levene-type test, R function “levene.test” in software package lawstat (Gastwirth et al. 2013) of statistical program R (R Core Team 2015) was used with the setting ‘correction.method = “zero.correction”’. All other statistical analyses were performed using SPSS 22 (SPSS Inc., Chicago, IL).

Results

HCU Screening Based on Hcy

In Qatar, from July 2006 until June 2008 a total of 29,791 neonates (12,152 = 40.8% Qatari; 17,639 = 59.2% non-Qatari) were screened for HCU. Starting from July 2008, information concerning ethnic origin was suppressed. For 95,256 neonates screened from July 2008 portions for the Qatari population (38,856 corresponding 40.8%) as well as for the non-Qatari population (56,400 corresponding 59.2%) were estimated on the basis of the data from 2006 until 2008. For all further calculations the total sample of 125,047 screened neonates was divided into a total sample of 51,008 Qatari children and 74,039 of non-Qatari children.

During the whole period of the study 132 cases of vitamin B_{12} deficiency and 30 confirmed patients with HCU were identified. Vitamin B_{12} deficiency was confirmed in 59 subjects in the Qatari subsample and 73 subjects in the non-Qatari subsample. The 30 patients with classical HCU were found by Hcy screening using the LC-MS/MS method. HCU patients were exclusively of Qatari origin (Table 1).

In the one-tier Hcy screening approach the cut-off for Hcy was 10 μmol/l (99.0 percentile) until March 2009 and 12 μmol/l (99.2 percentile) thereafter. In confirmed HCU patients Hcy levels in the first blood spot ranged from 14.1 to 97.0 μmol/l for Hcy. Compared to the healthy Qatari population, the lowest patient value for homocysteine of 14.1 μmol/l was well above the 100 percentile of the healthy population (12 μmol/l) resulting in a sensitivity and specificity of 100% for the cut-off of 12 μmol/l, and a sensitivity of 100% and a specificity of 99.04% (leading to 107 false positives) for the 10 μmol/l cut-off. All cases with Hcy values above the cut-offs were further investigated by molecular genetics identifying 30 confirmed cases (homozygosity for R336C: $n = 29$, D234N: $n = 0$, G347S: $n = 1$). No additional case with HCU has been diagnosed based on clinical symptoms so far (between July 2006 and June 2013).

Development of a Two-Tier Strategy for the Screening of Classical Homocystinuria: Met and Hcy Two-Tier Strategy

We first used Met as first-tier parameter, as it is measured routinely during screening of amino acids by ESI-MS/MS. Met values in the first screening card for patients with confirmed HCU ranged from 21.0 to 262.0 μmol/l, with the lowest value for Met corresponding to the 62nd percentile. In a retrospective approach, the cut-off for Met was set at 20 μmol/l, i.e., below the minimum value of the HCU patients, thus ensuring 100% sensitivity. This first-tier criterion led to a percentage of 38.44 (19,097 cases)

Table 1 Basic description of the data set

Country (period)	Samples (*n*)	Excluded from analysis[a] (*n*)	Missing data[b] (*n*)	Samples included in analysis (*n*)	Cases HCU	*N* for prevalence[c]	Prevalence
Qatari (July 2006–June 2008)	12,152	952	0	11,200	7	12,152	1:1,736
Qatari (July 2008–June 2013)[d]	38,856	377	0	38,479	23	38,856	1:1,689
Qatari (July 2006–June 2013)	51,008	1,329	0	49,679	30	51,008	1:1,700
Non-Qatari (July 2006–June 2008)	17,639	2,177	0	15,462	0	17,639	<1:17,639
Non-Qatari (July 2008–June 2013)[d]	56,400	862	0	55,538	0	56,400	<1:56,400
Non-Qatari (July 2006–June 2013)	74,039	3,039	0	71,000	0	74,039	<1:74,039
Australia (2009–2010)	4,344	452	96	3,796	0	144,800[c]	<1:144,800
Austria (2009–2010)	1,795	246	440	1,109	0	59,833[c]	<1:59,833
Taiwan (2010)	206	2	0	204	0	6,867[c]	<1:6,867
The Netherlands (2009–2010)	1,812	10	1,485	317	0	60,400[c]	<1:60,400

[a] Preterm sample, other diagnosis, medication, material insufficient, and post mortem sample
[b] Met and/or Phe missing
[c] Calculated as (*n* of samples/3) $\times$ 100
[d] Estimated on the distribution of screening period July 2006–June 2008

Table 2 Results of first- and second-tier strategies with cut-offs trimmed for 100% sensitivity

	First-tier			Second-tier: Hcy = 12 μmol/l (P100)		
	Sensitivity	Specificity	No. (%) of positives	Sensitivity	Specificity	No. of false positives
Met > 20 (=P61)	1	0.616	19,097 (38.44)	1	1	0
Met/Phe > 0.77 (=P98.8)	1	0.988	611 (1.23)	1	1	0
Met/Phe > 0.61 (=P94)	1	0.942	2,885 (8.81)	1	1	0
Met/Phe > 0.56 (=P89)[a]	1	0.898	5,105 (10.28)	1	1	0

Number of samples that go into first-tier: 49,679 including 30 homocystinuria patients
[a] Including four additional HCU cases

suspected cases of HCU (specificity = 0.616, see Table 2) if the cut-off was trimmed for a 100% sensitivity. Only samples with first-tier values above the respective cut-offs entered the second-tier measuring Hcy. As this approach was judged to be inefficient, a second strategy, using the Met to Phe ratio as first- and Hcy as second-tier was developed and tested.

Development of a Two-Tier Strategy for the Screening of Classical Homocystinuria: Met to Phe and Hcy Two-Tier Strategy

Phe values of confirmed cases ranged from 27.0 to 73.0 μmol/l and Met to Phe ratios from 0.78 to 4.68. The lowest Met to Phe ratio corresponded to the 98.8th percentile of controls. Using a cut-off of 0.77 for the Met to Phe ratio as first-tier led to a percentage of 1.23 (611 cases) of suspected HCU (specificity = 0.988, Table 2). Again, only samples with first-tier values above the respective cut-offs entered the second-tier using Hcy values as diagnostic criterion. The 132 cases of vitamin B_{12} deficiency identified by first-tier Hcy were sorted out by the Met to Phe ratio cut-off during the first-tier.

Generalizability of the Two-Tier Approach

A strict test of the generalizability of the two-tier approach using the samples from Australia, Austria, the Netherlands, and Taiwan would require confirmed HCU cases identified in these screening programmes. However, no case of HCU

was found in the samples analyzed for this study, nor was any clinically identified case reported during the observation period. To test the generalizability of our algorithm using the data of the samples defined by the upper 3% of the Met distributions of neonates screened in Australia, Austria, the Netherlands, Taiwan, and Qatar (Table 1 and Supplemental Table 1) we developed the following approach. First, we compared the upper 3% of values in the Qatari and non-Qatari subsamples from July 2006 to June 2008. There were only small differences between the medians as well as the distributions of Met and Hcy (Met: median test: $\chi^2(\mathrm{df} = 1,\ N = 753) = 1.78,\ p = 0.182$, Cramer's $V = 0.049$, 95%-CI[−0.023; 0.120]; Brown–Forsythe Levene-type test for distribution: $W(\mathrm{df}_1 = 1,\ \mathrm{df}_2 = 751) = 4.33,\ p = 0.038$, Cramer's $V = 0.076$, 95%-CI[0.004; 0.146] – Hcy: median test: $\chi^2(\mathrm{df} = 1,\ N = 753) = 0.12,\ p = 0.729$, Cramer's $V = 0.013$, 95%-CI[−0.059; 0.084]; Brown–Forsythe Levene-type test for distribution: $W(\mathrm{df}_1 = 1,\ \mathrm{df}_2 = 751) = 1.87,\ p = 0.172$, Cramer's $V = 0.050$, 95%-CI[−0.022; 0.121]). Cramer's V gives a standardized difference between sample characteristics, small values indicating small differences. As in studies with large samples even small differences easily become significant, effect sizes and their confidence intervals are recommended to evaluate the practical significance of a result (Lin et al. 2013; Nuzzo 2014). Following Cohen's (Cohen 1988) effect sizes were classified as small (values >= 0.1), medium (>=0.3), or large (>=0.5). All Vs comparing Qatari and non-Qatari samples were very small. Therefore, for further analyses subsamples from Qatar were merged.

In a second step comparing Met values >P97 across Qatar, Australia, Austria, the Netherlands, and Taiwan (Supplemental Fig. 1), we found a medium size difference between medians (median test: $\chi^2(\mathrm{df} = 4,\ N = 8{,}638) = 4648.30,\ p < 0.001$, Cramer's $V = 0.367$, 95%-CI [0.348; 0.385]) but not for distributions (Brown–Forsythe Levene-type test for distribution: $W(\mathrm{df}_1 = 4,\ \mathrm{df}_2 = 8{,}633) = 287.81,\ p < 0.001$, Cramer's $V = 0.091$, 95%-CI[0.070; 0.112]). Significance for medians was only due to Australian data showing higher Met values than in all other countries, which were rather similar (median test: $\chi^2(\mathrm{df} = 3,\ N = 4{,}842) = 163.03,\ p < 0.001$, Cramer's $V = 0.106$, 95%-CI[0.078; 0.134]; Brown–Forsythe Levene-type test for distribution: $W(\mathrm{df}_1 = 3,\ \mathrm{df}_2 = 4{,}838) = 308.80,\ p < 0.001$, Cramer's $V = 0.146$, 95%-CI[0.118; 0.173]).

Also differences between Met to Phe ratios (Supplemental Fig. 2) as well as between Hcy values (Supplemental Fig. 3) in blood samples selected if Met > P97 from Qatar, Australia, Austria, the Netherlands, and Taiwan were small (Met/Phe: median test: $\chi^2(\mathrm{df} = 4,\ N = 8{,}637) = 317.92,\ p < 0.001$, Cramer's $V = 0.096$, 95%-CI[0.075; 0.117]; Brown–Forsythe Levene-type test for distribution: $W(\mathrm{df}_1 = 4,\ \mathrm{df}_2 = 8{,}632) = 333.43,\ p < 0.001$, Cramer's $V = 0.098$, 95%-CI[0.077; 0.119]; Hcy: median test: $\chi^2(\mathrm{df} = 4,\ N = 8{,}638) = 555.19,\ p < 0.001$, Cramer's $V = 0.127$, 95%-CI[0.106; 0.147]; Brown–Forsythe Levene-type test for distribution: $W(\mathrm{df}_1 = 4,\ \mathrm{df}_2 = 8{,}633) = 70.80,\ p < 0.001$, Cramer's $V = 0.045$, 95%-CI[0.024; 0.066]).

In summary, distributions of Met values and Met to Phe ratios for Met > P97 were similar across all countries allowing us to conclude that Qatar data are comparable to those of other countries and a generalization of our results found for Qatar could be justified. The definite test of the generalizability of the two-tier strategy, however, is not possible, as this would require access to the complete data set of Met to Phe ratios.

Determination of a Cut-Off for the Met to Phe Ratio

Using a Met to Phe ratio of 0.77 as a cut-off given the lowest Met to Phe ratio of confirmed HCU patients being 0.78 would not be conservative enough, as repeated measurement of the same sample might be at risk to be lower than 0.77 and therefore would miss the case. The standard error of prediction (σ_{iI}) allows calculation of the interval for a repeated measurement (Xi) of a blood sample having the result XI (Dudek 1979). It is calculated as $\sigma_{iI} = \sigma_i(1 - r_{iI}{}^2)^{1/2}$, σ_i being the standard deviation of all measurements and r_{iI} the reliability of the measurement device. Quality control samples of four different concentrations for Met and Phe used as internal standards for MS/MS experiments were used to calculate the reliability (Hopkins 2000) resulting in a value of 0.994. These quality controls are the regular quality control samples which were measured each day before starting the amino acid and acylcarnitine profiling.

The mean of Met to Phe ratios of all 30 confirmed cases obtained from the initially measured data obtained from the regular screening program was 1.55 with a standard deviation of 0.77 and was used to estimate σ_i, leading to a standard error of prediction of 0.084. The expected true value of the lowest Met to Phe ratio is calculated as $1.55 + ((0.78 - 1.55) \times 0.994) = 0.784$. Now the 95% CI for 0.784 can be calculated as $0.784 \pm 1.96 \times 0.084$ resulting in an interval of]0.62–0.95[, with the lower bound corresponding to P 94.7. Using 0.61 (P94) as a new cut-off for Met to Phe ratio as a first-tier would still result in a sensitivity of 1. Specificity would slightly decrease to 0.942 remitting 2,885 samples to the second-tier Hcy measurement of the screening process.

Discussion

Newborn screening for classical homocystinuria performed by measurement of Met concentrations in dried blood spots has an insufficient sensitivity. The exact proportion of cases missed by newborn screening for methionine is unknown. In the present study taking the 99.5th Met centile (42 μmol/l) as a cut-off, 4 out of 34 (11.8%) affected babies would have been missed. Met cut-off values as low as 40 μmol/l have been proposed to yield higher sensitivity (Huemer et al. 2015 and the references therein: McHugh et al. 2011; Turgeon et al. 2010) but methionine seems to be to insecure as a first-tier parameter. The false negative rate is likely to be higher with early (<5 days) than with later blood sampling (5–7th day of life). Therefore, early screening, due to early discharge from the maternity unit, can be expected to influence the proportion of cases missed by methionine screening. Breast-fed babies may also have significantly lower methionine levels because of low concentrations of methionine in breast milk compared to some infant formulas (Naughten et al. 1998). Taken together, Met alone is not an adequate parameter for screening for classical homocystinuria.

Hcy can be measured in dried blood spots by LC-MS/MS for HCU screening with 100% sensitivity and specificity (Gan-Schreier et al. 2010; Alodaib et al. 2012). However, this approach requires measuring all samples with this particular and elaborated method. Our screening strategy for Qatar was so far based on Hcy as first-tier in all samples. As this is the only newborn screening programme in the world with Hcy measured in all subjects we could use a unique data set for evaluation of different second-tier strategies in newborn screening for HCU. We could demonstrate that using Met as first- and Hcy as second-tier is also a strategy with 100% sensitivity and specificity, if the cut-off for Met is set at a sufficiently low level. Compared to the Hcy alone strategy the number of samples requiring Hcy measurement is reduced by 61.6% in this second-tier strategy. However, Met and Phe are already measured by ESI-MS/MS in NBS for aminoacidopathies without additional cost and effort. Sensitivity is also increased by using the Met to Phe ratio which adjusts to the above-mentioned different protein intake (Bowron et al. 2005). Using the Met to Phe ratio as first-tier reduces the total number of samples requiring Hcy measurement by 89.7% in our retrospective data evaluation. Therefore, Met to Phe ratio as first-tier and Hcy as second-tier appears to be the optimal strategy. In the generalizability study we could demonstrate that the distributions of Met, Met to Phe ratio, and Hcy for samples selected if Met $>$ P97 were rather similar between Qatari and non-Qatari samples from Qatar, as well as between Australia, Austria, Taiwan, and the Netherlands. However, the direct test comparing complete distributions of Met to Phe ratios remains to be done.

In retrospect although all HCU cases could be detected by first-tier Met and second-tier Hcy in the data sets selected by the upper 3% of Met distributions (corresponding to a first-tier screening using P97 for Met) from Australia and Austria, one case would not have been included in the data sets from Taiwan and the Netherlands, where one more had a Met value equal to the cut-off.

Our data allow the calculation of the prevalence for HCU in Qatar among the Qatari population, giving an estimate of 1:1,700, stable across different observation periods (Table 1, see also Gan-Schreier et al. 2010). As not a single case was found in any of the other samples from other countries, only upper limits for prevalence estimates can be derived from the data sets. Merging all non-Qatari subgroups included in our analysis results in a prevalence estimate for HCU lower than 1:345,939, which is in agreement with some previously published estimates (see introduction and Skovby et al. 2010, which estimated the frequency of classical CBS to 1:344,000).

One limitation is that CBS deficiency in the Qatari population is predominately caused by homozygosity for a frequent mutation (Bener and Hussain 2006; El-Said et al. 2006; Zschocke et al. 2009; Gan-Schreier et al. 2010). Therefore our results are not necessarily transferable to other populations with different genetic backgrounds. The generalizability of the approach should be prospectively evaluated in future studies in other populations with high incidences of CBS deficiency, e.g., the Irish population.

As expected according to the genetic background of the Qatari population, all patients detected in this study were pyridoxine non-responsive. Therefore, we cannot draw conclusions about detection of pyridoxine-responsive CBS patients by the proposed strategy in other populations. Based on the higher frequency of pyridoxine-responsive CBS patients in historical cohorts compared to cohorts detected by newborn screening (Huemer et al. 2015) current newborn screening strategies have been reported to miss the majority of pyridoxine-responsive CBS patients.

After we had finished all calculations leading to the cut-off for the Met to Phe ratio of 0.61 four more confirmed cases with HCU have been identified by one-tier Hcy screening (Hcy: 57, 82, 100, 79 μmol/l). Met to Phe ratios of these cases were 0.94, 0.96, 0.73, 1.54, i.e., all these cases would have also been detected by the suggested two-tier algorithm. However, the case with a Met to Phe ratio of 0.73 constitutes a new minimum (Fig. 1). This is comparable with the recommended cut-off value (0.75–0.97) presented previously as a summary of the laboratory markers for newborn screening of homocystinurias and methylation defects in the R4S collaborative project (Huemer et al. 2015). The lower bound of the interval for a repeated measurement of the sample with the Met to Phe ratio $= 0.73$ based on now 34 confirmed cases with HCU is 0.57. Therefore, for a sensitivity of 1, the new cut-off had

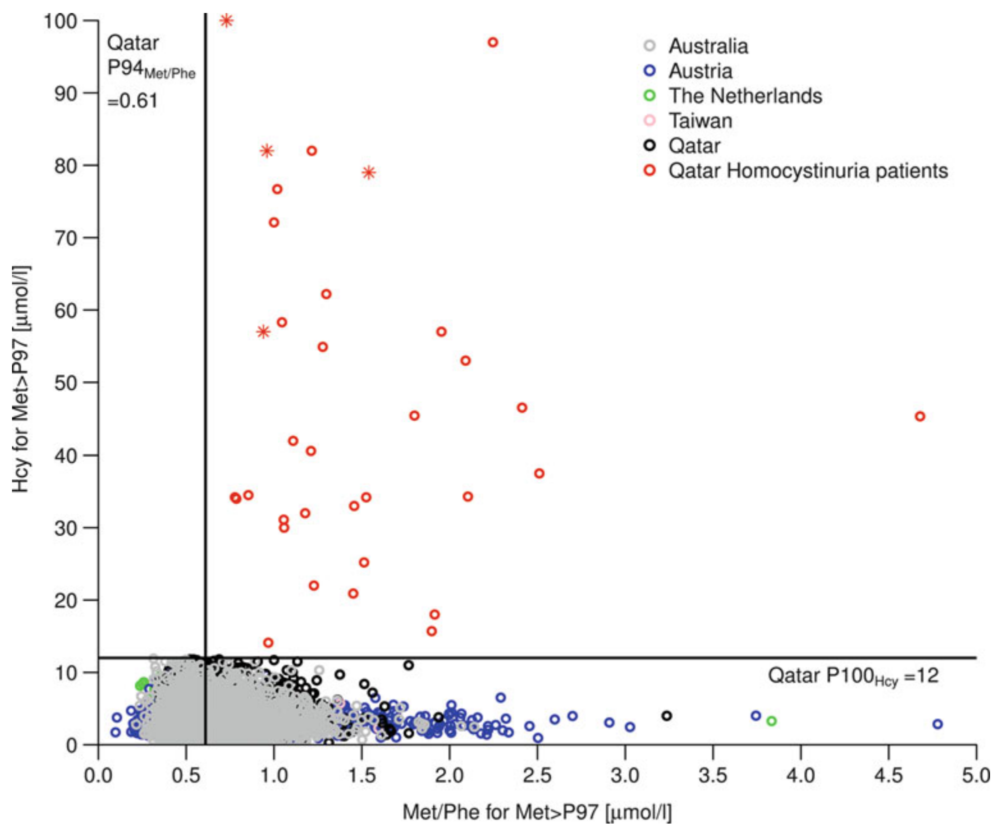

Fig. 1 Met/Phe and Hcy for upper three percent of Met distribution of all samples and for Qatar homocystinuria patients; *red stars* indicate cases with confirmed HCU found after data analysis

to be changed to 0.56 (corresponding to P89), resulting in a specificity of 0.898 or 10.28% first-tier positives to be transferred to Hcy determination in the same dried blood sample.

Conclusion

In conclusion, three options have been evaluated retrospectively for neonatal screening for HCU. In a one-tier strategy samples of all newborn have to be investigated for Hcy, which has a sensitivity and specificity of 100% but is an elaborated method. Using Met as first-tier would result in nearly 40% of samples to be measured for Hcy in a second-tier strategy. Finally, using the Met to Phe ratio as first-tier would result in ~10% of samples which have to be analyzed for Hcy. We have validated a reliable strategy to screen for HCU using MS/MS for the first-tier Met to Phe ratio and LC-MS/MS for Hcy as second-tier.

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Synopsis

Systematical evaluation and subsequent implementation of a second-tier test for homocysteine in dried blood spots improve the specificity and positive predictive value for classical homocystinuria screening.

Author Contributions

Jürgen G. Okun, Hongying Gan-Schreier, and Kathrin V. Schmidt participated in the design of the study and were involved in the experimental setup of the study. Junmin Fang-Hoffmann, Gwendolyn Gramer, Ghassan Abdoh, Tawfeg Ben-Omran, Noora Shahbeck, Hilal Al Rifai, and Abdul Latif Al Khal were involved in the newborn screening process and clinical evaluation of patients. Chuan-Chi Chiang, David C. Kasper, and Bridget Wilcken provided the upper 3% methionine samples of their

newborn screening programs. Gisela Haege and Peter Burgard performed the statistical analyses and made substantial contributions to conception and interpretation of the study. Georg F. Hoffmann and Jürgen G. Okun initiated the study, participated in its design and coordination, and drafted the manuscript.

Conflict of Interest

Jürgen G. Okun, Hongying Gan-Schreier, Kathrin V. Schmidt, Junmin Fang-Hoffmann, Ghassan Abdoh, Tawfeg Ben-Omran, Noora Shahbeck, Hilal Al Rifai, Abdul Latif Al Khal, Gisela Haege, Chuan-Chi Chiang, David C. Kasper, Bridget Wilcken, Peter Burgard, and Georg F. Hoffmann declare to have no conflict of interest.

Compliance with Ethics Guidelines

This article does not contain any studies with human or animal subjects. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

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RESEARCH REPORT

Management of an LCHADD Patient During Pregnancy and High Intensity Exercise

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Abstract In this report we describe a female Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency (LCHADD) patient who suffered from severe exercise intolerance. At age 34, the patient became pregnant for the first time. After an uneventful first 32 weeks of pregnancy she developed sinus tachycardia (resting heart rate 120–134 bpm) and lactate and creatinine kinase levels increased (3.3 mmol/L and 264 U/L, respectively). Increasing MCT supplementation (dose and frequency of administration) lowered heart rate and improved biochemical parameters. At 34 weeks the heart rate rose again and it was decided to deliver the child by caesarean section. Postpartum both mother and child did well.

Prior to pregnancy, she performed exercise tests with different doses of medium chain triglycerides (MCTs) to establish a safe and effective exercise program (baseline test, second test with 10 g MCTs and third test with 20 g of MCTs). In the MCT supplemented tests the maximal power output was 23% (second test) and 26% (third test) higher, while cardiac output at maximal power output was the same in all three tests (~15.8 L/min).

In conclusion, this is the first report of pregnancy in an LCHADD patient, with favourable outcome for both mother and child. Moreover, in the same patient, MCT supplementation improved cardiac performance and metabolic parameters during high intensity exercise. Using impedance cardiography, we got a clear indication that this benefit was due to improved muscle energy generation at high intensity exercise, since at the same cardiac output a higher power output could be generated.

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Introduction

Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency (LCHADD, OMIM 600890) is a rare inborn error of long chain fatty acid metabolism, resulting in mitochondrial dysfunction with subsequent energy shortage as well as toxic effects of fatty acid metabolites (den Boer et al. 2002). Clinically, this results in hypoketotic hypoglycaemia, metabolic acidosis, cardiomyopathy and liver disease presenting in infancy (den Boer et al. 2002). In those patients who survive early childhood, the main characteristics are fatigue and exercise intolerance, retinopathy and less common peripheral neuropathy (Spiekerkoetter 2010). Prognosis in early treated patients is generally favourable (Sim et al. 2002), but still not all patients survive into adulthood and long term complications are common.

Acute metabolic decompensation, leading to elevated CK concentrations or rhabdomyolysis, acidosis and eventually hypoglycaemia, can be triggered by fasting, infection

or other causes of increased energy demand. Treatment consists of immediate supply of extra energy in the form of carbohydrates and medium chain triglycerides (MCTs) and strict limitation in intake of long chain fatty acids (Spiekerkoetter et al. 2009). Carnitine supplementation can be considered, though it may theoretically increase levels of hydroxyacylcarnitines, one of the toxic fatty acid metabolism intermediates that accumulate in LCHADD (Spiekerkoetter et al. 2010). Given the restriction in long chain fatty acid intake additional essential fatty acids (especially docosahexaenoic acid) should be given (Spiekerkoetter et al. 2010).

Exercise increases energy demand and therefore supplementation of additional energy before exercise initiation can be necessary in LCHADD patients to prevent complications. MCTs unique kinetics, with faster intestinal as well as mitochondrial uptake compared to long chain fatty acids, make them a very effective energy source. In the small intestines MCTs can diffuse passively into the portal system (as opposed to LCT that require active uptake into the lymphatic system), without modification (Bach and Babayan 1982). MCT uptake into the mitochondrion does not require the formation of acylcarnitines and MCTs can thus be used as a source of energy in LCHADD patients. A study in nine LCHADD patients showed a positive effect of MCT supplementation (0.5 g/kg lean body mass) on exercise tolerance (Gillingham et al. 2006). A second study in 11 patients showed a lower steady state heart rate at the same workload after using pre-exercise MCT supplementation (0.5 g/kg lean body mass) compared to isocaloric carbohydrate supplementation in long chain fatty acid oxidation disorders (Behrend et al. 2012). In addition, a positive effect of an odd chain triglyceride, triheptanoate, on exercise performance in a single LCHADD patient has been reported (Karall et al. 2014). Since no cardiac output measurements were performed, these studies cannot distinguish between a positive effect of MCTs at the level of the heart versus the skeletal muscle.

To our knowledge, there are no published reports of pregnancy in an LCHADD patient, though pregnancies in other fatty acid oxidations, e.g. CPT2 deficiency, MCADD and VLCADD have been described in several case reports (Ramsey and Biggio 2005; Santos et al. 2007; Yamamoto et al. 2015).

Case Description

We here describe a 34-year-old female LCHADD patient who was diagnosed in early childhood when she presented with hypotonia, hypoglycaemia and failure to thrive. This was first attributed to a systemic carnitine deficiency. At age 10 the correct diagnosis of LCHAD deficiency was established based on the presence of a typical acylcarnitine pattern in plasma, reduced enzyme activity and genetic testing (homozygous for the c.1528G $>$ C mutation). With dietary management, she did quite well during childhood, experiencing only mildly reduced exercise tolerance compared to peers. From age 21 onwards her exercise capacity has declined considerably, her last measured maximal uninterrupted walking distance was 200 m. She did try to exercise frequently, but often suffered from post-exercise myalgia. Further symptoms consisted of poor eyesight due to retinitis pigmentosa (OD 2/60: able to count fingers at 2 m distance, OS: can see hand movements only) and peripheral neuropathy. On echocardiography her cardiac function was normal, but resting heart rate was elevated at outpatient clinic visits ($\pm$100 bpm). Because of intermittent episodes of palpitations she was treated with low dose beta-blockade (50 mg slow acting metoprolol once daily).

The patient adhered to a long chain triglyceride restricted, high carbohydrate diet, supplemented with MCTs. Her weight was 65.5 kg, total energy intake 2,151 kcal per 24 h of which 9% came from long chain fatty acids, 21% from medium chain fatty acids, 58% from carbohydrates and 15% from protein. Protein intake was ample at 1.5–2 g/kg bodyweight. She used the following supplements: MCTs three times 10 g daily (MCT Procal, Vitaflo, UK), essential fatty acids once daily (KeyOmega, one dose containing 200 mg of arachidonic acid and 100 mg of docosahexaenoic acid, Vitaflo, Liverpool, UK), maltodextrin six times daily 5 g (Fantomalt, Nutricia, the Netherlands), 30 g of uncooked cornstarch at nighttime and levocarnitine 1 g three times daily.

Pregnancy and Birth

At age 34 the patient became pregnant for the first time. Early on in pregnancy the beta-blockade treatment was discontinued because of its potential negative effects on placental function. At 10 weeks pregnancy duration maternal echocardiography showed a structurally normal heart with normal left and right ventricular function. 24 hour ECG Holter monitoring at 17 weeks showed sinus rhythm with a mean heart rate of 94 bpm without arrhythmia.

During pregnancy the caloric intake was gradually increased by increasing both carbohydrate (simple and complex) and MCT intake. During the first 31 weeks of pregnancy there were no specific complaints or symptoms and biochemical parameters remained stable. Fetal development, biometry and structural ultrasonography were unremarkable.

At 32 weeks pregnancy duration the patient developed complaints of intermittent, mostly nightly, palpitations and

a reduction in exercise tolerance. Resting heart rate was 120 bpm (sinus rhythm). There were no signs of cardiac decompensation. Reinstitution of low dose beta-blockade for 1 week did not have any effect on the complaints or the heart rate and at 33 weeks she was admitted to the cardiac care unit with a resting heart rate of ~120/min. Both plasma lactate and CK levels (both previously in the normal range) were mildly elevated (3.3 mmol/L and 264 U/L, respectively). We increased the dose and the number of administrations of MCT supplementation from 40 to 60 g/day (in six doses). The next day her heart rate was lower (~100 bpm), palpitations diminished and CK levels normalised. This improvement lasted 12 days, after which heart rate rose again and the patient felt worse. After a multidisciplinary discussion and consulting the patient and her partner it was decided to deliver the child by caesarian section at 34 + 1 weeks. Her weight at that time was 74.9 kg, total energy intake 3,230 kcal per 24 h of which 8% came from long chain fatty acids, 23% from medium chain fatty acids, 55% from carbohydrates and 14% from protein. During pregnancy, levels of long chain hydroxyacylcarnitines dropped initially, but at 34 weeks the levels were similar to the preconceptional levels. For example, the C16OH-carnitine level was 0.20 μmol/L preconceptionally, 0.09 μmol/L at 17 weeks of pregnancy and 0.17 μmol/L at 34 weeks of pregnancy. Free carnitine levels remained normal throughout pregnancy.

To prevent metabolic decompensation the patient continued taking her oral MCT supplements right up to surgery and 10% glucose infusion (2 L per 24 h) was started on the ward. Premedication consisted of oral metoclopramide and oral ranitidine (aspiration-prophylaxis). Standard non-invasive heart rhythm, blood pressure and oxygen saturation monitors were applied.

Because of the need for frequent blood samples and continuous blood pressure monitoring, an arterial line was placed in the right radial artery. Normal saline preload was administered intravenously. To prevent interference with the patient's serum lactate levels, ringers lactate infusion was avoided. A combined spinal epidural was placed in sitting position. After spinal injection, a phenylephrine infusion was started to prevent hypotension. The patient was placed supine in the left lateral tilt position. An adequate sensory block was reached 10 min after spinal injection.

Surgery commenced and a baby girl was born 5 min after incision. Routine prophylactic antibiotics were injected intravenously after clamping of the cord. An oxytocin bolus was avoided to prevent sudden extreme contraction of the uterus musculature, which might cause metabolic derangement. An oxytocin infusion of 10 IU in 500 mL of glucose 5% solution was infused over 2 h. Estimated intra-operative blood loss was 300 mL.

The patient remained haemodynamically stable throughout the whole procedure. Postoperative analgesia was delivered by means of intravenous paracetamol and a patient controlled epidural infusion. The patient was admitted to the intensive care unit for postoperative monitoring. The following day, she was returned to the obstetric high care unit in good condition.

Postpartum the patient did clinically very well. Biochemically there was slight further increase in plasma lactate (maximal level 4.9 mmol/L) and a drop in bicarbonate (lowest level 16.1 mmol/L) which corrected spontaneously over the next few days. Oral energy intake (carbohydrates and MCTs) was high (3,230 kcal per 24 h) the first days after delivery and IV glucose administration could be discontinued after 1 day. The patient left the hospital 4 days postpartum in good clinical condition. 14 weeks postpartum she was in good clinical condition, had no palpitations or other complaints and her resting heart rate was ~82 bpm using 25 mg of metoprolol.

Exercise Testing

Approximately 6 months prior to her pregnancy, the patient was referred to the rehabilitation outpatient clinic because she wanted to improve her endurance capacity. She completed three incremental cycle ergometry exercise tests over the course of 1 month to examine the effects of MCT supplementation on exercise tolerance and cardiac function, in order to establish a safe training program with adequate dietary advice. The patient performed a symptom limited exercise stress test. This standard ramp test started with a 2 min rest period followed by 4 min of unloaded cycling as warming-up. Next, the workload increased with a slope of 1 W/5 s. The subject was instructed to cycle until exhaustion with a pedal frequency of 60–80 revolutions per minute (rpm). The loaded phase was terminated when pedalling frequency dropped below 60 rpm after which a 5 min unloaded recovery phase ended the exercise test. During warming-up, workload and recovery phase of the exercise protocol, VO_2 and VCO_2 were measured (Oxycon Pro, Carefusion, Houten, the Netherlands). Using these respiratory indices the respiratory exchange ratio (RER) was calculated (VCO_2/VO_2). The RER was then used to determine the ventilatory threshold (RER = 1), at which point lactate starts to accumulate in the bloodstream. Expiratory VCO_2 rises exponentially after this point, as a byproduct of lactate buffering in the bloodstream, to prevent the occurrence of severe acidosis.

Stroke volume and cardiac output were assessed during the test using impedance cardiography (SM-ICG, PhysioFlow PF05 Lab1, PhysioFlow, France). Blood was drawn at rest, just before reaching the ventilatory threshold and at

maximal power output. The first test was performed without MCT supplementation, the second test with 16 g of MCT Procal supplement (containing 10 g of MCTs, total 112 kcal) and the third test with 32 g (containing 20 g of MCTs, total of 224 kcal). The MCT supplement was taken 25 min prior to the test. The diet was otherwise unchanged and body weight remained stable between the tests.

The observed peak oxygen consumption in the baseline test was only 40% of predicted (using the Fairbairn equation which takes into account age, gender and weight) (Fairbarn 1994). Compared to the baseline test, in the second test (using 10 g of MCTs) maximal power output was increased by 25% (70 vs 88 W) and maximal oxygen consumption by 26% (956 mL/min vs 1,209 mL/min). At maximal workload, in the second test the stroke volume was lower and maximal heart rate higher, resulting in the same cardiac output at maximal power output. Thus, the 25% higher power output in the second test was reached at the same cardiac output (Table 1).

When comparing the second to the first exercise test, power output at the ventilatory threshold was more than doubled (64 vs 28 W) and the oxygen consumption per Watt produced was much lower (16 vs 28 W). No further improvement of the increase in MCT dose from 10 to 20 g on power output or cardiac parameters was observed.

In the MCT supplemented second and third test, both at rest and during exercise, plasma lactate concentrations were lower compared to the baseline test. The same was true for plasma creatinine kinase levels (Table 2). There were no clear differences between the tests in plasma glucose, ammonia and free fatty acid (FFA) concentrations (Table 2). Plasma beta-hydroxybutyrate was higher in the second test, but this increase was not observed in the third test (Table 2).

Table 1 Physiological and cardiac measurements during exercise tests

Test	1	2	3
Grams of MCTs	0	10	20
Maximal power output (W)	70	88	86
Time to exhaustion (mm:ss)	12:21	13:37	13:30
Power output at RER = 1 (W)	28	64	40
Heart rate rest (bpm)[a]	96 ± 3	93 ± 4	112 ± 3
Heart rate max (bpm)	150	160	174
VO_2 max (mL/min)	956	1,209	1,245
VO_2/W at RER = 1 (mL/min)	27	16	19
RER rest	0.99	0.93	1.02
RER max	1.18	1.26	1.24
Stroke volume rest (mL)[a]	97 ± 4	88 ± 6	80 ± 2
Stroke volume at max power (mL)[b]	111 ± 4	100 ± 1	92 ± 1
Cardiac output rest (L/min)[a]	9.4 ± 0.5	8.2 ± 3.1	9.0 ± 0.4
Cardiac output at max power (L/min)[b]	15.6 ± 0.5	15.9 ± 0.3	15.9 ± 0.2

Heart rate max maximal heart rate measured during test, *VO_2 max* the highest VO_2 measured during the test, *RER max* RER measured at the highest power output during the test

[a] Average of 21 measurements

[b] Average of 3 measurements

Discussion

MCTs are an ideal source of energy to be used prior or during exercise since, in contrast to long chain fatty acids, they do not delay gastric emptying and are rapidly taken up and transported to peripheral tissues (Beckers et al. 1992; Jeukendrup and Saris 1996). Combining MCTs with carbohydrates accelerates MCT oxidation without negatively affecting endogenous carbohydrate oxidation. The positive effect of MCT supplementation on exercise performance in long chain fatty acids disorders has been described before, but it remained unclear whether this effect was due to improved metabolic efficiency at the level of the heart, the skeletal muscle or both (Gillingham et al. 2006; Behrend et al. 2012).

During exercise, carbohydrates and fatty acids are the main source of energy for skeletal muscle and the heart. In terms of substrate preference, the heart favours fatty acids over glucose, both at rest and during exercise. Fatty acids

Table 2 Biochemical measurements during exercise tests

Test	1	2	3
Glucose rest (mmol/L)	5.5	4.5	4.3
Glucose max (mmol/L)	5.0	5.2	5.1
CK rest (IU/L)	183	135	110
CK max (IU/L)	197	139	115
Lactate rest (mmol/L)	1.7	0.9	0.8
Lactate max (mmol/L)	3.5	2.4	1.9
NH_3 rest (μmol/L)	41	<10	16
NH_3 max (μmol/L)	30	22	39
FFA rest (mmol/L)	0.21	0.27	0.32
FFA max (mmol/L)	0.31	0.26	0.24
BHB rest (mmol/L)	0.10	0.29	0.14
BHB max (mmol/L)	0.10	0.22	0.12

CK creatine kinase, *NH_3* ammonia, *FFA* free fatty acids, *BHB* betahydroxybutyric acid, *Rest* before starting exercise, *max* at maximal exercise capacity

become a more important energy source during high intensity and prolonged exercise as muscle and liver glycogen stores are gradually depleted (Massicotte et al. 1992; van Loon et al. 2001). Given the preference of the heart for fatty acids as fuel and the fact that acute dilated cardiomyopathy can develop in LCHADD patients at times of acute metabolic stress (Dyke et al. 2009) we expected the improvement in exercise performance after MCT supplementation in our patient to be due to improved cardiac performance. However, after MCT supplementation maximal cardiac output was the same, but maximal power output increased considerably. This points in the direction of more efficient ATP generation at the level of the skeletal muscle. The more than doubled power output at ventilatory threshold after MCT supplementation indicates improved aerobic oxidation. Since we used a high intensity exercise protocol aimed at reaching exhaustion, we cannot exclude a positive effect of MCT supplementation on cardiac function at lower intensity exercise. In addition to the positive effect of the MCTs, the small amount of protein present in the MCT supplement (2 g in the second test and 4 g in the third test) may have had an anaplerotic effect in energy.

In this report, we describe for the first time a pregnancy in an LCHADD patient. Pregnancies in the mothers carrying a fetal with LCHADD or mitochondrial trifunctional protein deficiency (MTP deficiency) can be complicated by acute fatty liver of pregnancy or HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome (Ibdah et al. 1999; Yang et al. 2002). This is thought to be due to the spill of toxic fatty acid oxidation intermediates from the fetal and/ or placenta into the maternal circulation (Strauss et al. 1999). In the pregnancy here described, LCHADD or MTP deficiency in the foetus was highly unlikely since molecular diagnostics showed that the patient's partner did not carry a mutation in the relevant genes. What the risk of these pregnancy complications is in a mother who is homozygous for an LCHADD mutation, carrying a heterozygous foetus, is unknown. We closely monitored our patient throughout pregnancy and no signs of fatty liver of pregnancy or HELPP were found.

The pregnancy was uneventful up to 32 weeks when the increase in heart rate and the slight changes in biochemical parameters indicated the onset of metabolic decompensation. Most likely, the increase in heart rate was a result of a higher oxygen demand resulting from peripheral energy shortage and not a sign of cardiomyopathy, since repeated echocardiography showed no signs of either dilatation of the heart or of decreased cardiac muscle function. Increasing the dose and the number of administrations of MCTs initially lowered heart rate and improved the patient's well-being. However, at 34 weeks the heart rate increased again and the patient was feeling less well. It was then decided to not risk full metabolic decompensation, but to deliver the child. We debated whether a caesarean section or assisted vaginal deliverance would give the best outcome for both mother and child, since in, for example, patients with cardiomyopathy assisted vaginal birth gives better maternal outcome compared to caesarean section (Ruys et al. 2013; Ruys et al. 2014) because of substantial haemodynamic instability during surgery. Since we wanted to prevent further metabolic derangement resulting from muscle activity during a vaginal delivery we chose to perform a caesarean section.

In conclusion, this is the first report of pregnancy in an LCHADD patient, with favourable outcome for both mother and child. Moreover, in the same patient, MCT supplementation improved cardiac performance and metabolic parameters during high intensity exercise. Our results indicate that at high intensity exercise this benefit is a result of improved aerobic muscle energy generation. This finding needs to be confirmed in a larger patient cohort.

Take Home Message

This report describes the first documented pregnancy in an LCHADD patient, with favourable outcome for both mother and child.

Compliance with Ethics Guidelines

Conflict of Interest

D.C.D. van Eerd, M. Langeveld, I.A. Brussé, V.F.R. Adriaens, R.T. Mankowski, S.F.E. Praet and M. Michels 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 (Medical Ethics Committee Erasmus MC) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the patient described in this case report.

Contributions of Individual Authors

Data collection: DCDvE, RTM, SFEP and MM; analysis and interpretation of the data: DCDvE, IAB, RTM, SFEP, MM and ML; drafting the article: DCDvE, IAB, VFRA, RTM and ML; critically revising the article: SFEP

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CASE REPORT

Rare Case of Hepatic Gaucheroma in a Child on Enzyme Replacement Therapy

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Abstract *Background*: We present a 6 year old boy with type I Gaucher treated from 16 months with ERT, developing focal Gaucheroma in the liver at 3.5 years.

Case: The subject presented at 13 months of age with anaemia, thrombocytopenia and hepatosplenomegaly. Gaucher disease was confirmed by leucocyte enzyme assay. A homozygous change: c.1193G>A (p.Arg398Gln) in the GBA gene was identified. He had normal neurology with normal saccades. Imiglucerase was administered at 60 IU/kg/fortnight from 15 months as per Australian regulations with good clinical response. At 3.5 years hepatic ultrasound demonstrated a nodular cystic lesion measuring 7 × 5.3 × 5.1 cm in the right lobe of liver, confirmed on MRI. Biopsy demonstrated acellular hyaline necrosis, portal–portal bridging fibrosis and nodules of Gaucher cells. Cystic fluid comprised necrotic debris and Gaucher cells. Further evaluation over 18 months including repeat MRI, biopsy, alpha-fetoprotein monitoring and whole-body FDG-Pet scan demonstrate no malignancy.

Conclusion: GD is the most common lysosomal storage disorder. The aetiology, natural history and optimal management strategy of rare Gaucheroma in paediatric cases has not been defined particularly in regards to malignancy risk.

Introduction

Gaucher disease (GD) is the commonest lysosomal storage disorder and is caused by deficiency of lysosomal glucocerebrosidase resulting in accumulation of undegraded glucosylceramide and other glycolipids in the reticuloendothelial system (Brady et al. 1965). Phenotypically GD presents as a clinical spectrum ranging from an asymptomatic to a perinatal lethal disorder. It has been traditionally classified into three types – GD 1 (non-neuropathic form), GD 2 (acute neuropathic form) and GD 3 (chronic neuropathic form). GD1 is the commonest type comprising of 95% of all cases (Charrow et al. 2000) and although considered non-neuropathic, peripheral neuropathy (Biegstraaten et al. 2010) and Parkinson's disease (Tayebi et al. 2001) have been documented in this subset. Most of the patients with GD1 are symptomatic in childhood (Charrow et al. 2004) and often present with hepatosplenomegaly, haematological manifestations (anaemia and thrombocytopenia) or bone disease. Early onset correlates well with severe disease progression (Kaplan et al. 2006). GD2 typically presents within 2 years of age with a rapidly progressive course resulting in death by 2–4 years of age. In GD 3 patients usually survive till third or fourth decade. The current recommendation for use of enzyme replacement therapy (ERT) in GD1 has shown to help significantly with anaemia, thrombocytopenia, bone pain and bone crises (Weinreb et al. 2002; Anderson et al. 2014). The possibility of localised deposition of Gaucher cells in various organs has been described in adults. Such a well-delineated lesion has been termed Gaucheroma historically, although there does not seem to be a very clear definition outlined so far in literature. Radiological follow-up of splenic lesions has been documented in the paediatric cases earlier with most showing complete radiological recovery with ERT (Chippington et al. 2008). However increased risk of malignancies especially haematological is well documented in adult patients with GD (Mistry et al. 2013). The development of solid tumours in adults with type 1 GD

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has also been reported in the lung, bone, spleen, breast, prostate, colon, brain and liver (Xu et al. 2005): all of them being organs that harbour Gaucher cells. Specifically looking at liver – 3 cases of hepatocellular carcinoma in Gaucher disease have been reported so far (Xu et al. 2005), all in adults. Multiple lesions on radiological examination and HbsAg positivity were present in the case reported in this paper. The other patient included in the review (Xu et al. 2005, patient described in reference 5) had a singe echodense hepatic lesion with metastatic peritoneal deposits having had a previous normal AFP and being HbsAg negative. Thus there are do not appear to be clear diagnostic indicators for predicting HCC in patients with GD. To our knowledge there are no cases of a hepatic Gaucheroma described in a paediatric population. What significance this holds in terms of malignancy risk remains unexplored as well. We thus report a case of Gaucheroma noted in a 3.5-year-old boy after being on ERT for nearly 2 years and his follow-up till 6 years of age.

History

A 13-month-old male infant 4th child to consanguineous parents of Lebanese background presented with abdominal distension for 2 months. His development was normal and he was in good health otherwise. There was no significant family history of note. On examination he had liver palpable 7 cm below the right costal margin and spleen was palpable 15 cm along its long axis below the left costal margin. He also had anaemia (Hb 74 g/L, ref 105–138) and thrombocytopenia (Plat 83 × 10 9/l, ref 150–600) noted with mild elevation of liver enzymes (AST 72 μ/L, ref 10–50; GGT 51 μ/L ref 15–45). Systemic examination including saccadic eye movements was normal. Malignancy was suspected and hence bone marrow biopsy was performed, demonstrating several foamy histiocytes on the aspirate. Leucocyte enzyme assay confirmed decreased beta glucocerebrosidase activity (<10 pmol/min/mg protein (ref 600–3,200)) and serum chitotriosidase level was markedly elevated at 11,700 nmol/h/mL (ref 3.6–78) in keeping with GD. At 15 months of age he was commenced on ERT with Imiglucerase at 60 IU/kg/fortnightly. Genetic testing subsequently showed a homozygous change: c.1193G>A (p. Arg398Gln) in the GBA gene. At 3.5 years a routine hepatic ultrasound demonstrated a nodular cystic lesion in the right lobe of liver. This measured 7 × 5.3 × 5.1 cm in size and was confirmed on MRI (Fig. 1). Biopsy demonstrated hyaline necrosis, portal–portal bridging fibrosis and nodules of Gaucher cells (Fig. 2). Alpha fetoprotein was 2 kIU/l (ref range 0–6). Cystic fluid comprised necrotic debris and Gaucher cells. Further evaluation over 18 months including repeat MRI (Fig. 3), biopsy, serial alpha-fetoprotein monitoring and whole-body FDG-Pet scan demonstrate no evidence of malignancy. The patient remained stable throughout and his serum chitotriosidase levels have also shown a steady decline with treatment with the last level being 2,400 nmol/h/mL at 4 year 4 months of age, indicative of positive response to ERT. Subsequently the reference laboratory has been testing plasma glucosylsphingosine levels and our patients have ranged from 230 to 330 (ref <10 nmol/L).

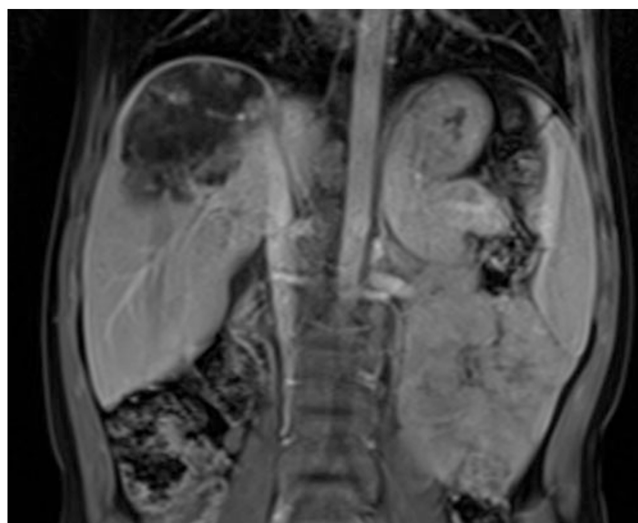

Fig. 1 Large subcapsular heterogeneous lesion 7 × 5.3 × 5.1 cm in the right lobe of liver with multiple satellite lesions

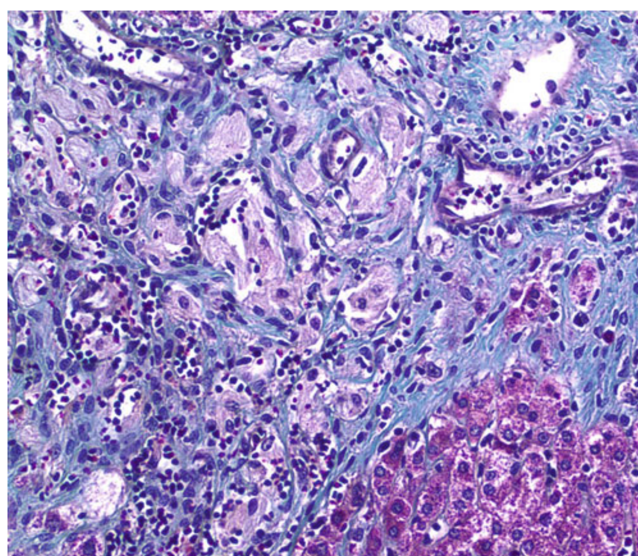

Fig. 2 Histopathology of liver biopsy with Masson Trichrome staining shows a nodule of Gaucher histiocytes (*light pink*) surrounded by fibrosis (*green*) and lymphocytes. Normal hepatocytes (*magenta*) are at the bottom right

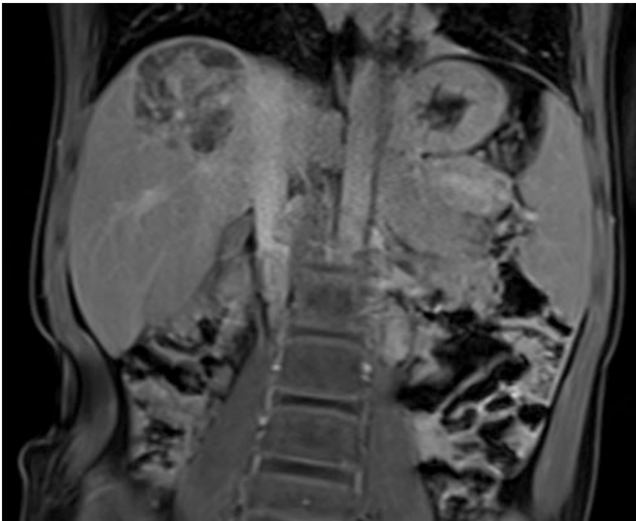

Fig. 3 Interval reduction in right lobar hepatic mass, 4.6 × 3.8 × 5.1 cm with diffuse dystrophic calcification within the mass

Discussion

Management of GD in terms of ERT, dose and therapeutic goals is well established based on previous studies (Weinreb et al. 2002; Pastores et al. 2004; Anderson et al. 2014). Hepatic Gaucheroma has only been reported in a 23-year-old adult as a differential for focal nodular hyperplasia (Poll 2009). It is unclear if this adult was receiving ERT and if any follow-up was documented. We know that haematological and solid organ malignancies occur in adults with Gaucher disease. Although the exact aetiology of increased incidence of malignancies in type 1 is unknown, it is believed that accumulated glucocerebroside stimulates the immune system predisposing to haematological carcinogenesis and damage to the immunosurveillance system of T lymphocytes may contributes to solid organ tumours (Shiran et al. 1993; Bertram et al. 2003).

The exact approach to management of a solid organ lesion remains unclear, more so in paediatric population. Serum chitotriosidase level was trending downwards when this lesion was detected. In our patient, liver biopsy was done twice and was in keeping with a benign process. Options include observation, adjunctive or single substrate reduction therapy, resection of primary lesion, partial hepatectomy or liver transplantation. All of these therapeutic options were considered, but none has proven utility in this situation. Funding authorities in Australia do not reimburse doses above 60 IU/kg fortnightly and hence this prescribed dose/kg has been maintained and conservative management of the lesion has continued. Serial alpha-fetoprotein measurements remained normal. Anti-imiglucerase antibody levels were not performed but continuing on the same dose/kg of enzyme resulted in decrease in the size of the lesion with dystrophic calcification within it (Figs. 1 and 3). There were two stellate lesions noted inferior to the main mass which we will follow up on serial imaging. The spectre of looming potential malignancy is a heavy burden for families to bear from early childhood and this has led to huge psychological and emotional impact on the family. Unfortunately with rare disorders having even rarer manifestations it is difficult to explain unquantifiable risks of these findings to the family and for the family to fathom what lies ahead of them.

Conclusion

Hepatic Gaucheroma is a rare entity in children. We believe this is the first case described in paediatric population. The lesion in our patient was picked up incidentally on routine screening. Our patient has shown features of decrease in size of the lesion with continuation of ERT with no features of malignancy established both pathologically and based on serum markers. More such cases with longitudinal follow-up need to be reported to establish the natural course of these rare lesions.

Compliance with Ethics Guidelines

Conflict of Interest Statement

All authors, Sophy Korula, Penny Owens, Amanda Charlton and Kaustuv Bhattacharya, declare that they have no conflict of interest.

Informed consent was obtained from the patients parents and permission from institutional ethics committee has also been obtained as per the hospital guidelines.

Contributors

Manuscript writing – SK.

Manuscript editing – KB, AC.

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RESEARCH REPORT

Newborn Screening Programmes in Europe, Arguments and Efforts Regarding Harmonisation: Focus on Organic Acidurias

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Abstract *Background*: The state of newborn screening (NBS) programmes for organic acidurias in Europe was assessed by a web-based questionnaire in the EU programme of Community Action in Public Health 2010/2011 among the – at that time – 27 EU member states, candidate countries, potential candidates and three EFTA countries.

Results: Thirty-seven data sets from 39 target countries were analysed. Newborn screening for glutaric aciduria type I (GA-I) was performed in ten, for isovaleric aciduria (IVA) in nine and for methylmalonic aciduria including cblA, cblB, cblC and cblD (MMACBL) as well as for propionic aciduria (PA) in seven countries. Samples were obtained at a median age of 2.5 days and laboratory analysis began at median age of 4.5 days. Positive screening results were mostly confirmed in specialised centres by analysis of organic acids in urine. Confirmation of a positive screening result usually did not start before the second week of life (median ages: 9.5 days [IVA], 9 days [GA-I], 8.5 days [PA, MMACBL]) and was completed early in the third week of life (median ages: 15 days [IVA, PA, MMA], 14.5 days [GA-I]). Treatment was initiated in GA-I and IVA at a median age of 14 days and in MMACBL and PA at a median age of 15 days.

Conclusion: NBS for organic acidurias in Europe is variable and less often established than for amino acid disorders. While for GA-I its benefit has already been demonstrated, there is room for debate of NBS for IVA and especially PA and MMACBL.

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Abbreviations

EFTA	European Free Trade Association
ESPE	European Society for Paediatric Endocrinology
FYROM	Former Yugoslav Republic of Macedonia
GA-I	Glutaric aciduria type I, OMIM 231670 deficiency of glutaryl-CoA dehydrogenase
GP	General practitioner
IVA	Isovaleric aciduria, OMIM 243500 deficiency of isovaleryl-CoA dehydrogenase
ISNS	International Society for Neonatal Screening
MMACBL	Methylmalonic aciduria including cblA, cblB, cblC and cblD defects OMIM 251000 methylmalonic aciduria, methylmalonyl-CoA mutase deficiency OMIM 251100 methylmalonic aciduria, cblA type OMIM 251110 methylmalonic aciduria, cblB type OMIM 277400 methylmalonic aciduria and homocystinuria, cblC type OMIM 277410 methylmalonic aciduria and homocystinuria, cblD type
NBS	Newborn screening

PA	Propionic aciduria OMIM 232050 deficiency of propionyl-CoA carboxylase subunit β OMIM 232000 deficiency of propionyl-CoA carboxylase subunit α
SSIEM	Society for the Study of Inborn Errors of Metabolism

Introduction

In 2010/2011 regulation and practice of population newborn screening (NBS) for rare disorders were surveyed among – at that time – 27 EU member states (NBS in Belgium is different in the Flemish and in the French part, therefore contributed two data sets), five candidate countries (Croatia, Former Yugoslav Republic of Macedonia, Iceland, Montenegro, Turkey), four potential candidates (Albania, Bosnia Herzegovina, Kosovo, Serbia) and three EFTA countries (Norway, Switzerland and Liechtenstein) through a tender of the European Commission within the EU programme of Community Action in Public Health (Burgard et al. 2012; Loeber et al. 2012).

Data collection covered five domains of an NBS programme, and results have been reported previously (Burgard et al. 2012; Loeber et al. 2012; Cornel et al. 2011).

This article specifies the state of NBS programmes for organic acidurias in Europe in 2010, regarding four organic acidurias: glutaric aciduria type I (GA-I), isovaleric aciduria (IVA), propionic aciduria (PA) and methylmalonic acidurias including inherited deficiencies of methylmalonyl-CoA mutase as well as of cblA, cblB, cblC and cblD deficiency (MMACBL).

NBS for these disorders aims to detect elevations of characteristic acylcarnitines by tandem mass spectrometry and subsequent calculation of acylcarnitine ratios (Lindner et al. 2006, 2008; Ensenauer et al. 2011; Chace et al. 2001).

In IVA, MMACBL and PA patients may already develop life-threatening metabolic decompensation during the first days of life (Deodato et al. 2006; Vockley and Ensenauer 2006; Kölker et al. 2015a, b). Therefore, NBS process times are key performance indicators.

Methods

A web-based questionnaire asked for current practice of NBS and its possible regulation by directives and/or by guidelines (Burgard et al. 2012; Loeber et al. 2012).

In each country respondents nominated by European professional societies (ISNS; SSIEM; ESPE) reported data for all disorders screened for according to the national NBS panel (Loeber et al. 2012). The survey started in August 2010 and was closed on January 14, 2011; reference date for all data is September 1, 2010. Values given for time points of obtaining NBS samples, conforming diagnosis and start of treatment are mostly experts' estimates for single disorders or groups of disorders, but not calculated measures based on individual measurements. Therefore standard deviations cannot be communicated. Process times were not differentiated by different methods to confirm a diagnosis.

Results

Description of the Data Set

From 39 target countries, 37 data sets were available for analysis (response rate 92.5%). Data sets were not available for Albania (NBS was not established), Liechtenstein (NBS is performed in Switzerland and data are included in the Swiss data set) and Kosovo (the questionnaire was not returned), but it was known by personal communication that newborn screening, if functional, does not include organic acidurias. Newborn screening for GA-I was performed in ten countries and for IVA in nine countries; MMACBL and PA were screened for in seven countries (Table 1).

Confirmation of Screening Results

Nine of ten countries answered that positive screening results for GA-I were confirmed by specialised centres but rarely also by local hospitals or by general practitioners/paediatricians in practice (2/10) (Table 1). Similar results were obtained for IVA: in 9/9 countries positive screening results are confirmed by specialised centres (Table 1). MMACBL and PA screening results are confirmed by specialised centres (7/7 countries), but rarely also in local hospitals (1/7 countries) or by general practitioners or paediatricians in private practice (1/7 countries) (Table 1). Screening results are confirmed most frequently by analyses of organic acids in urine. However, in the majority of countries, additionally mutation analysis and/or enzyme analysis is performed for the confirmation of diagnosis (Table 1).

Process Times

In most countries there is (written) information for prospective parents and informed consent is asked at the time of blood sampling (Burgard et al. 2012). Blood spots are taken at a median age of 2.5 days and analysis in the screening laboratory starts at median age of 4.5 days (Table 2).

Table 1 Confirmation of newborn screening and feedback of screening results

Countries	Point of confirmation			Method of confirmation			Feedback of screening results			
	Specialised centre	Local hospital	GP/ Paed.	Metabolites	Enzyme activity	Mutation analysis	To screening lab	To registry	Only diagnosis	Detailed results
Disorder: GA-I										
Austria	50%	50%		X	X	X	X			X
Belgium (Flemish part)	X			X			X		X	
Czech Republic	X			X	X	X	X		X	
Denmark	X			X	X	X	X		X	
Germany	X			X	X	X	X			X
Hungary	50%		50%	X			X	X		X
Netherlands	na			X	X	X	na	na	na	na
Portugal	X			X	X	X	X			X
Spain	X				X	X	X		X	
Iceland	X			na	na	na	X			X
Disorder: IVA										
Austria	50%	50%		X	X	X	X			X
Belgium (Flemish part)	X			X			X		X	
Czech Republic	X			X	X	X	X		X	
Germany	X			X		X	X			X
Hungary	50%		50%	X			X	X		X
Netherlands	50%	50%		X	X	X	na	na	na	na
Portugal	X			X	X	X	X			X
Spain	X				X	X	X		X	
Iceland	X			na	na	na	X			X
Disorder: MMACBLC										
Austria	50%	50%		X	X	X	X			X
Belgium (Flemish part)	X			X			X		X	
Denmark	X			X	X	X	X		X	
Hungary	50%		50%	X			X	X		X
Portugal	X			X	X	X	X			X
Spain	X				X	X	X		X	
Iceland	X			na	na	na	X			X
Disorder: PA										
Austria	50%	50%		X	X	X				
Belgium (Flemish part)	X			X			X		X	
Denmark	X			X	X	X	X		X	
Hungary	50%		50%	X			X	X		X
Portugal	X			X	X	X	X			X
Spain	X				X	X	X		X	
Iceland	X			na	na	na	X			X

na data not available, *GP* general practitioner, *Paed.* paediatrician

Confirmation of a positive screening result usually does not start before the second week of life and is completed early in the third week of life (Table 2). Treatment starts in GA-I and IVA at a median age of 14 days (range 8–23 days) and in MMACBL and PA at a median age of 15 days (range 14–23 days) (Table 2).

Information Flow Between NBS Domains

In all countries with NBS for organic acidurias, results of the diagnostic confirmatory process are reported back to NBS laboratories (Table 1), following local guidelines (Table 3 Part II). Type and extent of transmitted data are varied, ranging from communication of only the confirmed diagnosis to reporting detailed results (Table 1).

Regulations

A guideline on how to confirm the diagnosis is usually available mostly on a national level, but usually written by local heads/directors of the institution executing the confirmation test – in contrast a national directive concerning the same topic is rarely available (Table 3 Part I). Where to confirm a diagnosis is also predominantly regulated by guidelines, mostly on a national level and less frequently by a directive (Table 3 Part I). The same holds true for the time needed to confirm a diagnosis (Table 3 Part II). Reporting positive results is also more often regulated by guidelines than by directives; few countries have guidelines as well as directives (Table 3 Part II). In Germany, for example, there is a national directive concerning NBS for GA-I, but a guideline endorsed by a national professional society is also in place (Kölker et al. 2011).

Discussion

The major aim of this study was to describe the concepts and state of established NBS programmes for organic acidurias in Europe. In contrast to amino acid disorders, organic acidurias are less often included in European NBS disease panels (Burgard et al. 2012). Therefore experience about the impact of different logistics on outcome of these disorders is scarce.

Our study reveals that performance and, confirmation of positive NBS results, and start of treatment for organic acidurias in Europe is variable. This may result in inappropriate interventions, delayed treatment, increased disease burden and finally health inequities and increased costs. Further studies shall provide systematic evaluation of existing NBS programmes for their impact on patients' outcomes and also costs and health equity. Particular attention should be paid to the time schedule of the NBS process for these conditions (Kelm and Tanksley 2015).

Since patients with a classical organic aciduria such as IVA, MMACBL and PA are at risk to already present early in the neonatal period with a putatively life-threatening metabolic decompensation, it is important to accomplish NBS as early as possible in order to start treatment and care ideally in the still asymptomatic patient. In our survey, treatment in these disorders was reported to start after 15 days of life. This is clearly too late for most patients with a neonatal metabolic decompensation. Furthermore the confirmatory diagnostic process needs to be tailored to avoid therapy in patients with mild phenotypes which is especially recognised in IVA (Ensenauer et al. 2004).

The European Network of Experts on Newborn Screening (EUNENBS) delivered a consensus document with recommendations how to develop NBS in the European Union based on the results of the survey (Cornel et al. 2014). Within these recommendations GA-I is considered as a disorder with lower prevalence, not too difficult to test and prove health gain, IVA is a candidate disorder where NBS is more challenging according to the criteria by Wilson and Jungner (1968), and NBS for MMACBL or PA is not recommended at all.

NBS and subsequent treatment of GA-I according to an international guideline have significantly improved the neurological outcome of affected individuals (Heringer et al. 2010; Kölker et al. 2006, 2007, 2011), and NBS is considered a cost-effective diagnostic strategy for countries with healthcare systems comparable to Germany (Pfeil et al. 2013). According to this international guideline, it is crucial to start treatment early before the manifestation of irreversible neurologic damage (Kölker et al. 2011). Treatment has to be started early in the diagnostic process, i.e. immediately after demonstration of elevated concentrations of 3-hydroxyglutaric acid and already in advance of the final confirmation of the diagnosis by demonstration of two disease-causing mutations or impaired enzyme activity (Kölker et al. 2011). In our survey only in the Netherlands and in Germany, pre-emptive treatment of GA-I patients starts before the end of the confirmation process as recommended (Kölker et al. 2011). In Fig. 1a we illustrate the current practice mostly employed and show our proposed model (Fig. 1b). In concordance with an international guideline (Kölker et al. 2011), we strongly advocate starting pre-emptive treatment in the setting of a metabolic centre as soon as the suspicion of an organic aciduria arises from newborn screening and before the diagnosis is finally confirmed. This is important in GA-I, but may even be more crucial in MMACBL, PA and IVA, where neonatal decompensation is more frequent.

For IVA, successful experience with NBS programmes has been published (Ensenauer et al. 2011; Grünert et al.

Table 2 Process times of newborn screening

Countries	DBS sampling Mean age (days)	NBS lab analysis Age at start (days)	Confirmation		Treatment Mean start (days)
			Mean start (days)	Mean end (days)	
Disorder: GA-I					
Austria	2.3	3.8	11	14	14
Belgium (Flemish part)	2.2	4.2	10	12	14
Czech Republic	2.5	4.5	6	8	8
Denmark	2.5	4.5	5	12	na
Germany	2.2	3.2	11	16	14
Hungary	2.5	5.5	na	na	na
Netherlands	3.8	4.8	10	21	10
Portugal	2.2	4.2	8	na	na
Spain	3.8	7.8	8	15	15
Iceland	3	6	9	23	23
Median	2.5	4.5	9	14.5	14
Disorder: IVA					
Austria	2.3	3.8	11	14	14
Belgium (Flemish part)	2.2	4.2	10	12	14
Czech Republic	2.5	4.5	6	8	8
Germany	2.2	3.2	11	16	14
Hungary	2.5	5.5	na	na	na
Netherlands	3.8	4.8	10	21	10
Portugal	2.2	4.2	8	na	na
Spain	3.8	7.8	8	15	15
Iceland	3	6	9	23	23
Median	2.5	4.5	9.5	15	14
Disorder: MMACBL					
Austria	2.3	3.8	11	14	14
Belgium (Flemish part)	2.2	4.2	10	14	14
Denmark	2.5	4.5	5	15	15
Hungary	2.5	5.5	na	na	na
Portugal	2.2	4.2	8	na	na
Spain	3.8	7.8	8	15	15
Iceland	3	6	9	23	23
Median	2.5	4.5	8.5	15	15
Disorder: PA					
Austria	2.3	3.8	11	14	14
Belgium (Flemish part)	2.2	4.2	10	12	14
Denmark	2.5	4.5	5	15	15
Hungary	2.5	5.5	na	na	na
Portugal	2.2	4.2	8	na	na
Spain	3.8	7.8	8	15	15
Iceland	3	6	9	23	23
Median	2.5	4.5	8.5	15	15

na data not available, *DBS* dried blood spot

Table 3 Regulation of the confirmation process

Countries	How to confirm NBS					Where to confirm diagnosis?				
	Guideline	Application	Directive	Application	Authors	Guideline	Application	Directive	Application	Authors
Disorder: GA-I PART I										
Austria	X	National			PS	X	National			PS
Belgium (Flemish part)	na					X	National			PS
Czech Republic	X	Regional			LH	X	National	X	National	HA
Denmark	X	National	X	National	HA, LH	X	National	X	National	HA, LH
Germany	X	National			PS	X	National	X	National	HA, PS
Hungary	X	National			LH	X	Regional			LH
Netherlands	na			na		na		na		
Portugal	X	National				X	Regional			
Spain	na		na			na		na		
Iceland	X	Regional	X	Regional	LH	X		X	National	LH
Disorder: IVA PART I										
Austria	X				PS	X	National			PS
Belgium (Flemish part)	na		na			X	National			PS
Czech Republic	X	Regional			LH	X	National	X	National	HA
Germany	X	National			PS	X	National	X	National	HA, PS
Hungary	X	National			LH	X	Regional			LH
Netherlands	X					X		X		HA,PS
Portugal	X	National				X	Regional			
Spain	na		na			na		na		
Iceland	X	Regional	X	Regional	LH	X		X	National	LH
Disorder: MMACBL PART I										
Austria	X	National			PS	X	National			PS
Belgium (Flemish part)	na		na			X	National			PS
Denmark	X	National	X	National	HA, LH	X	National	X	National	HA, LH
Hungary	X	National			LH	X	Regional			LH
Portugal	X	National				X	Regional			
Spain	na		na			na		na		
Iceland	X	Regional	X	Regional	LH	X		X	National	LH

Disorder: PA PART I										
Austria	X	National			PS	X	National			PS
Belgium (Flemish part)	na		na			X	National			PS
Denmark	X	National	X	National	HA, LH	X	National	X	National	HA, LH
Hungary	X	National			LH	X	Regional			LH
Portugal	X	National				X	Regional			
Spain	na		na							
Iceland	X	Regional	X	Regional	LH	X		X	National	LH

	Recommended age to confirm a suspicious NBS result					Feedback of final diagnosis				
Countries	Guideline	Minimum age (days)	Maximum age (days)	Application	Authors	Guideline	Application	Directive	Application	Authors
Disorder: GA-I PART II										
Austria	na					X	National			PS
Belgium (Flemish part)	X	1		National	LH	X	Regional	X	Regional	HA, PS
Czech Republic	na					X	Regional			LH
Denmark	na					X	National	X	National	HA, LH
Germany	X			National	HA,PS			X	National	HA
Hungary	na					X	National			LH
Netherlands	na					na		na		
Portugal	na					X	National			
Spain	X	3	20	National	PS	X	Regional	X	Regional	LH
Iceland	X			National	LH	X	National			LH
Disorder: IVA PART II										
Austria	na					X	National			PS
Belgium (Flemish part)	X	1		National	LH	X	Regional	X	Regional	HA, PS, LH
Czech Republic	na					X	Regional			LH
Germany	X			National	HA, PS			X	National	HA
Hungary	na					X	National			LH
Netherlands	X		14	National	PS	X				PS
Portugal	na					X	National			
Spain	X	3	20	National	PS	X	Regional	X	Regional	LH
Iceland	X			National	LH	X	National			LH
Disorder: MMACBL PART II										
Austria	na					X	National			PS
Belgium (Flemish Part)	X	1		National	LH	X	Regional	X	Regional	HA, PS, LH

(continued)

Table 3 (continued)

Countries	Recommended age to confirm a suspicious NBS result					Feedback of final diagnosis				
	Guideline	Minimum age (days)	Maximum age (days)	Application	Authors	Guideline	Application	Directive	Application	Authors
Denmark	na					X	National	X	National	HA, LH
Hungary	na					X	National			LH
Portugal	na					X	National			
Spain	X	3	20	National	PS	X	Regional	X	Regional	LH
Iceland	X			National	LH	X	National			LH
Disorder: PA PART II										
Austria	na					X	National			PS
Belgium (Flemish part)	X	1		National	LH	X	Regional	X	regional	HA, PS, LH
Denmark	na					X	National	X	National	HA, LH
Hungary	na					X	National			LH
Portugal	na					X	National			
Spain	X	3	20	National	PS	X	Regional	X	Regional	LH
Iceland	X			National	LH	X	National			LH

na data not available, *LH* local head of institution responsible for confirmation of NBS, *PS* professional society, *HA* health authority

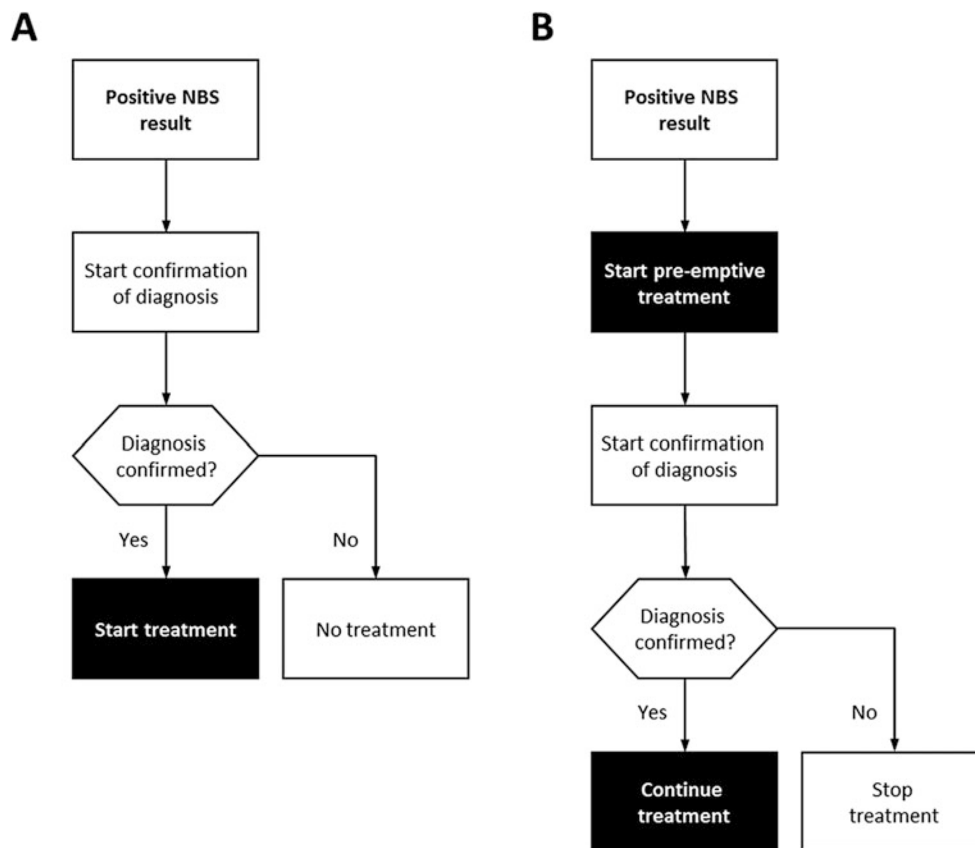

Fig. 1 Current practice after positive NBS result (**a**) versus a proposed model recommending pre-emptive treatment after a positive NBS result is received (**b**). This should be initiated and supervised by a metabolic centre with the major aim to minimise the risk of a neonatal metabolic decompensation in patients with organic acidurias during the confirmatory diagnostic process

2012a, b; Lindner et al. 2011), and the natural history is favourable as long as early neonatal brain damage can be prevented by adequate therapy (Grünert et al. 2012a, b). However, about half of the individuals with a positive screening result for IVA are homozygous for mutation c.932C>T in the *IVA* gene, presumably associated with a benign phenotype (Ensenauer et al. 2004), for whom the benefit of screening is questionable.

The impact of NBS on history and outcome of PA and MMACBL was thought to be low due to the fact that many patients will have already presented clinically before the results of NBS are available (Leonard et al. 2003). In line with this, a study demonstrated that NBS for PA did not affect the outcome of these patients (Grünert et al. 2012a, b). However, there still is controversy whether PA and MMACBL patients with a late disease onset, i.e. presenting with first symptoms after the newborn period, might benefit from NBS (Dionisi-Vici et al. 2006; Couce et al. 2011). Follow-up studies on a larger number of patients are required to address this question.

In 2011, E-IMD (EAHC no. 2010 12 01; www.eimd-registry.org), an observational patient registry including organic acidurias, has been initiated. It currently includes detailed follow-up data of more than 1100 patients (Kölker et al. 2015a, b).

Taking the start of confirmation as a surrogate parameter for the time when an organic aciduria can be suspected from NBS, percentages of patients identified before onset of symptoms can be estimated. For example, in Denmark confirmation of NBS for MMACBL starts on day 5 after birth (Table 2), which is the earliest among all European countries performing NBS screening for MMACBL. At that time at least 25% of all MMACBL patients have already been diagnosed by clinical symptoms [total number of patients: 101; median age at diagnosis (interquartile range): 21 (3–210) days] in the E-IMD sample. Similar results were obtained for PA with at least 25% of all patients being diagnosed until day 5 [total number of patients: 78; median age at diagnosis (interquartile range): 14 (4–90) days].

For IVA, confirmation of NBS is at first initiated at day 6 in the Czech Republic (Table 2); at that time at least 25% of patients have been diagnosed [total number of patients, 29; median age at diagnosis (interquartile range), 8 (5–465) days]. In contrast, since GA-I patients rarely present with symptoms before age 3 months (Kölker et al. 2006), neonatal onset in GA-I has been rarely observed until day

5 in the E-IMD survey. On day 5 confirmation of positive NBS results for GA-I is first started in Denmark [total number of patients: 52; median age at diagnosis (inter-quartile range): 300 (145–428) days].

Although being preliminary, these data support the call for NBS to be fast in intoxication-type organic acidurias such as MMACBL, PA and IVA and to initiate treatment early before completing follow-up investigations. Patients with late-onset forms of these disorders will most likely benefit the most from NBS programmes. This notion is supported by a recently published E-IMD study demonstrating that (1) NBS significantly lowers the age of diagnosis in classic organic acidurias compared to patients with late onset of symptoms identified by selective metabolic testing, (2) improves the neurological outcome in patients with GA-I and cobalamin-nonresponsive MMA and (3) reduces the probability of cardiac manifestation with increasing age in PA patients (Heringer et al. 2015).

However, the decision whether or not to screen for these diseases must be based on many more criteria, including long-term follow-up studies and health economic evaluations.

Conclusion

NBS for organic acidurias in Europe is variable and less often established than for amino acid disorders. While for GA-I its benefit has already been demonstrated, there is room for debate of NBS for IVA and especially PA and MMACBL patients. NBS for intoxication-type organic acidurias has to be fast and treatment has to be started pre-emptively, i.e. even before the diagnosis is finally confirmed.

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Synopsis

NBS for organic acidurias in Europe is variable and less often established than for amino acid disorders. While for GA-I its benefit has already been demonstrated, there is room for debate of NBS for IVA and especially PA and MMACBL defects. For optimal benefit NBS for intoxication-type organic acidurias has to be fast and treatment has to be started pre-emptively, i.e. even before the diagnosis is confirmed.

Authors’ Contributions

Designing, planning and conducting the study: All authors

Collection of data and statistical analysis: Peter Burgard

Manuscript writing: All authors

Guarantor

Peter Burgard

Conflict of Interest

Friederike Hörster, Stefan Kölker, J. Gerard Loeber, Martina C. Cornel, Georg F. Hoffmann and Peter Burgard declare that they have no conflict of interests.

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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. No data on individual patients are included in this study; therefore no informed consent had to be obtained.

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

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RESEARCH REPORT

Whole Exome Sequencing Identifies the Genetic Basis of Late-Onset Leigh Syndrome in a Patient with MRI but Little Biochemical Evidence of a Mitochondrial Disorder

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Abstract Leigh syndrome is a subacute necrotising encephalomyopathy proven by post-mortem analysis of brain tissue showing spongiform lesions with vacuolation of the neuropil followed by demyelination, gliosis and capillary proliferation caused by mutations in one of over 75 different genes, including nuclear- and mitochondrial-encoded genes, most of which are associated with mitochondrial respiratory chain function. In this study, we report a patient with suspected Leigh syndrome presenting with seizures, ptosis, scoliosis, dystonia, symmetrical putaminal abnormalities and a lactate peak on brain MRS, but showing normal MRC enzymology in muscle and liver, thereby complicating the diagnosis. Whole exome sequencing uncovered compound heterozygous mutations in NADH dehydrogenase (ubiquinone) flavoprotein 1 gene (*NDUFV1*), c.1162+4A>C (NM_007103.3), resulting in skipping of exon 8, and c.640G>A, causing the amino acid substitution p.Glu214Lys, both of which have previously been reported in a patient with complex I deficiency. Patient fibroblasts showed a significant reduction in NDUFV1 protein expression, decreased complex CI and complex IV assembly and consequential reductions in the enzymatic activities of both complexes by 38% and 67%, respectively. The pathogenic effect of these variations was further confirmed by immunoblot analysis of subunits for MRC enzyme complexes in patient muscle, liver and fibroblast where we observed 90%, 60% and 95% reduction in complex CI, respectively. Together these studies highlight the importance of a comprehensive, multipronged approach to the laboratory evaluation of patients with suspected Leigh syndrome.

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Introduction

Leigh syndrome is largely an early-onset, progressive, neurodegenerative disorder caused by mutations in at least 75 nuclear- and mitochondrial-encoded genes involved in pyruvate metabolism or the mitochondrial respiratory chain complexes (MRC), with the latter mainly involving complex I (CI) and complex IV (CIV) (Lake et al. 2015). Clinical hallmark signs of Leigh syndrome are brainstem dysfunction, symmetrical spongiform lesions in the brain, dystonia, spasticity, movement disorders, visual disturbances, nystagmus, ophthalmoparesis, ptosis, cerebellar ataxia, seizures, scoliosis, bulbar dysfunction and peripheral neuropathy that lead to biochemical signs of mitochondrial dysfunction including lactate elevation in different body fluids, Krebs cycle intermediates in urine and dysfunction of the mitochondrial respiratory chain in different tissues (Lake et al. 2015). Mutations in the nuclear-encoded mitochondrial CI gene *NDUFV1* [MIM 161015] have previously been reported by Schuelke et al. (1999) in a patient with seizures, followed by a report by Benit et al. (2001) in a patient with the same mutations as reported here and a clinical phenotype of Leigh syndrome and CI deficiency (Benit et al. 2001; Schuelke et al. 1999). This gene encodes a 51 kDa subunit of the flavoprotein portion of CI, containing NADH, flavin mononucleotide (FMN) and Fe-S-binding sites (Mimaki et al. 2012). *NDUFV1* is highly evolutionarily conserved and plays a critical role in the catalytic activity of CI (Benit et al. 2001). Dysfunction of CI is the most common oxidative phosphorylation disorder in humans, likely due to the sheer number of subunits (each encoded by a different nuclear or mitochondrial gene) required for its structure, function and assembly, with defects in the CI assembly process commonly at fault (Mimaki et al. 2012).

In a bid to identify the genetic basis of late-onset childhood Leigh syndrome in the proband, we performed whole exome sequencing of DNA from the proband and four unaffected family members and identified compound heterozygous *NDUFV1* sequence variants (NM_007103.3) in the proband. Functional assays of patient fibroblasts revealed reduced levels of NDUFV1 protein, MRC assembly defects and dysfunction of CI and CIV, resulting in a probable reduction in ATP production. These variations have previously been associated with CI deficiency in a patient with moderately elevated plasma lactate levels and clinical features including cerebellar ataxia, persistent seizures, psychomotor regression, strabismus, ptosis and brain atrophy with multiple symmetric areas of hyperintensity in the brainstem (Benit et al. 2001).

Methods and Materials

Proband Summary

The female proband was the second child of unrelated parents of Caucasian Australian background. She had an older and younger brother, both of whom were well. The pregnancy was uncomplicated, and she was born at term by elective lower segment caesarean section due to cephalopelvic disproportion. The birth weight was 3.47 kg (50th percentile) and length was 50 cm (50th–75th percentiles). There were no concerns in the perinatal period, or with early growth and development, and hearing and vision were determined as normal. She was breastfed for the first 3–4 months.

She continued normally until 2 years of life, at which time she had febrile convulsions and subsequently developed a partial left ptosis. This ptosis resolved 2–3 months later. At the age of 7 years, her parents noted that she appeared to use her left arm and leg less than her right limbs. Her parents commented in retrospect that she had always appeared clumsier compared with her older brother. She subsequently developed progressive asymmetric bilateral dystonia of her hands and feet, worse on the right than the left, which was only partially responsive to Baclofen and Botox. Implementation of a ketogenic diet was of no obvious benefit.

Over the following 12 months, her dystonia progressively worsened, chewing became more difficult, she lost the ability to feed herself and her balance and speech deteriorated. She became wheelchair-bound by 10 years of age, developed progressive scoliosis and when last reviewed at the age of 12 years old had a resting tremor, dysarthria and dysphagia, requiring a gastrostomy. Her parents felt that a therapeutic cocktail of thiamine, coenzyme Q10, L-carnitine and biotin had been of some subjective benefit.

A brain MRI performed at the age of 7 years showed symmetrical putaminal lesions, and brain MRS showed a lactate peak (images not shown). A repeat MRI a year later showed progression of the changes in the putamen, as well as involvement of the left body of the caudate and the right quadrigeminal plate (images not shown). Blood and CSF lactate were normal on a number of occasions, as was a urine metabolic screen, serum CPK, copper and ceruloplasmin and liver function tests (results not shown). The clinical evolution together with the brain MRI changes was suggestive of a clinical diagnosis of late-onset Leigh syndrome.

Muscle and liver respiratory chain enzyme activities were found to be normal by spectrophotometric analysis

Table 1 Spectrophotometric MRC enzyme diagnostic data in skeletal muscle, liver and fibroblasts

	Enzyme activity			
	Patient	(Ref range)	% Activity	% CS ratio
Muscle[a]				
CI (nmol/min/mg)	46	19–72	110	53
CII (nmol/min/mg)	121	26–63	269	126
CIII (min/mg)	72.2	12.8–50.9	248	112
CIV (min/mg)	11.88	3.3–9.1	180	86
CS (nmol/min/mg)	273	85–179	212	–
Liver				
CI (nmol/min/mg)	21	8–11	221	113
CII (nmol/min/mg)	166	54–73	272	138
CIII (min/mg)	15.1	5.2–10.3	199	100
CIV (min/mg)	0.78	0.5–0.9	110	56
CS (nmol/min/mg)	54	26–31	193	–
	% Activity			
	Patient	Control		
Skin fibroblasts[b]				
CI in fibroblast	**38***	100		
CIV in fibroblast	**67***	100		

Bold characters indicate clinically significant abnormal values

[a] Patient liver and muscle samples. Enzyme activity data are expressed as nmol/min per mg protein, except for CIII and CIV, which were expressed as /min/mg. Data are also expressed as % CS ratio, which represents % of the normal control mean value when expressed relative to citrate synthase. Complex I (CI), NADH-coenzyme Q1 oxidoreductase; complex II (CII), succinate-coenzyme Q1 oxidoreductase; complex III (CIII), decylbenzylquinol-cytochrome c oxidoreductase; complex IV, cytochrome c oxidase (CIV)

[b] Dipstick MRC enzyme data from cultured fibroblasts. Enzyme activity data are expressed as % residual activity relative to protein (% protein)

*$p < 0.001$

(Table 1). She was negative for the common Leigh syndrome mutations m.8993T>C and m.8993T>G. Subsequent sequencing of the entire mitochondrial genome did not reveal any variants of interest, and fibroblast pyruvate dehydrogenase activity was found to be normal (results not shown).

Whole Exome Sequencing

Genomic DNA was isolated from whole blood collected from the patient, her parents and two unaffected siblings using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. WES and bioinformatics analyses were performed on all samples as previously described (Menezes et al. 2015).

Variants identified by WES analysis were confirmed by Sanger sequencing of DNA from all subjects. Sanger sequencing was performed at the Australian Genome Research Facility (AGRF), Westmead Millennium Institute (Westmead, Australia), using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In Silico Analysis

In silico analyses of variants identified by WES were performed using Sift (http://www.sift.jcvi.org), PolyPhen2 (http://www.genetics.bwh.harvard.edu/pph2/), GVGD (agvgd.iarc.fr/), MutationTaster (www.mutationtaster.org/), PhyloP (http://ccg.vital-it.ch/mga/hg19/phylop/phylop.html), Human Splicing Finder (http://www.umd.be/HSF/) and PhastCons (http://compgen.bscb.cornell.edu/phast/phastCons-HOWTO.html).

Exon Skipping

Total RNA extracted from fibroblasts was converted to cDNA using a Superscript III first-strand cDNA synthesis kit (Invitrogen, Mulgrave, VIC, Australia), following the manufacturer's instructions. Each reaction contained 400 ng of total RNA and 250 ng of random decamers, 200 U of Superscript III and 40 U of RNaseOUT™ (Life Technologies, Carlsbad, CA, USA). Full-length cDNA was generated by incubation at 50°C for 60 min. cDNA was amplified by PCR to determine whether the intronic variant caused exon skipping. PCR products were gel purified (PCR Purification Kit; Qiagen) and sent to AGRF for Sanger sequencing.

Immunoblotting

Fibroblast cells were lysed and protein prepared as previously described (Riley et al. 2013). 20 μg of each protein sample was resolved on a 4–12% Bis–Tris gel (Life Technologies) and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were probed with 1:1,000 anti-NDUFV1 (ab174472, Abcam, Cambridge, United Kingdom) or 1:500 anti-OXPHOS cocktail (consisting of five antibodies, recognising labile subunits from each of the five MRC complexes; ab110411, Abcam). Protein loading was normalised to porin (1:5,000 anti-porin for 2 h at room temperature; ab14734, Abcam). All blots were then probed with either 1:2,500 anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 2 h at room

temperature. Membranes were then incubated with enhanced chemiluminescence (ECL) reagents and exposed to Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Films were scanned using a SRX-101A (Konica Minolta Holdings, Inc., Tokyo, Japan) scanner and analysed with ImageJ version 1.49 (http://rsb.info.nih.gov/ij/).

Protein was extracted from patient and control skeletal muscle and liver biopsy samples as previously described (PMID: 12847163). Relative concentration of protein was estimated by running samples on a 4–12% Bis–Tris gel (Life Technologies) and staining with Coomassie blue staining solution, after which samples were subjected to immunoblotting as above except blots were probed with 1:2,000 anti-mouse IgG horseradish peroxidase for 1.5 h and films were scanned using a CP1000 Processor (Agfa-Gevaert, Mortsel. Belgium).

Spectrophotometric MRC Enzyme Activity

MRC enzyme activities in liver and skeletal muscle biopsies from the proband were determined as previously described (Frazier and Thorburn 2012).

Complex I and IV Dipstick Enzyme Activity Assays in Fibroblasts

CI and CIV enzyme assays were performed on freshly isolated protein lysates from patient fibroblasts using dipstick enzyme assays, following the manufacturer's instruction (Abcam, Cambridge, United Kingdom).

Blue Native Polyacrylamide Gel Electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed on freshly isolated protein lysates from patient fibroblasts as described elsewhere (McKenzie et al. 2007). 20 μg of protein sample was resolved on 4–16% Bis–Tris Protein Gels (Life Technologies, Carlsbad, CA, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, USA). All variables underwent statistical comparisons by Mann–Whitney *U* non-parametric testing. Two-tailed Student's *t*-test of measures was carried out at least in triplicate. Statistical significance was set at $P < 0.05$.

Results

Because of the clinical suspicion of late-onset Leigh syndrome, we initially performed MRC complex spectrophotometric assays in liver and skeletal muscle (Table 1). Since these results were within the reference ranges and the patient had a phenotype strongly indicative of Leigh syndrome, we moved to whole exome sequencing and identified compound heterozygous mutations (c.1162+4A>C and c.640G>A) in the MRC CI flavoprotein subunit encoding gene *NDUFV1*. Both mutations were confirmed by Sanger sequencing, with each parent carrying one of the mutations (Fig. 1a and c). *In silico* review of the c.640G>A variant predicted it to affect protein function with the most damaging score and showed that the p. Glu214 position is completely conserved across many species (Fig. 1b). *In silico* analysis of the c.1162+4A>C variation predicted it to be disease causing, affecting the donor splice site. These variations had very low minor allele frequencies (http://www.1000genomes.org & ExAc http://exac.broadinstitute.org). Amplification of patient cDNA, generated by reverse transcription of patient fibroblast mRNA, showed two bands on agarose gel electrophoresis: an intense higher full-length band and a shorter transcript (faint lower band) (Fig. 1d). Sanger sequencing of the shorter transcript confirmed the skipping of exon 8 (Fig. 1e), and the presence of the missense c.640G>A mutation in the higher full-length band in the patient mRNA.

We examined NDUFV1 protein levels in patient fibroblasts and its effect on MRC complex levels. Immunoblot analysis revealed a 72% reduction in NDUFV1 levels in patient fibroblasts compared to a control (Fig. 2a). Immunoblot analysis of subunits for MRC enzyme complexes, dependent upon MRC complex formation for protein stability, revealed a severe reduction in CI (95%), CIII (45%) and CIV (60%) in patient fibroblasts (Fig. 2b). However, in patient skeletal muscle and liver, only CI was reduced by 90% and 60%, respectively, suggesting that CI was the most vulnerable complex in our patient (Fig. 2c and Supplementary Fig. 1). It is not clear to us what factors may be contributing to the tissue specificity in our patient carrying *NDUFV1* mutations.

Since the *NDUFV1* mutations were only expected to affect CI, we examined MRC assembly in patient fibroblasts by blue native polyacrylamide gel electrophoresis (BN-PAGE). A relative reduction in CI (60%), CIII (35%), CIV (51%) and supercomplex I/III (95%) was observed (Fig. 2d). However, we were unable to examine whether

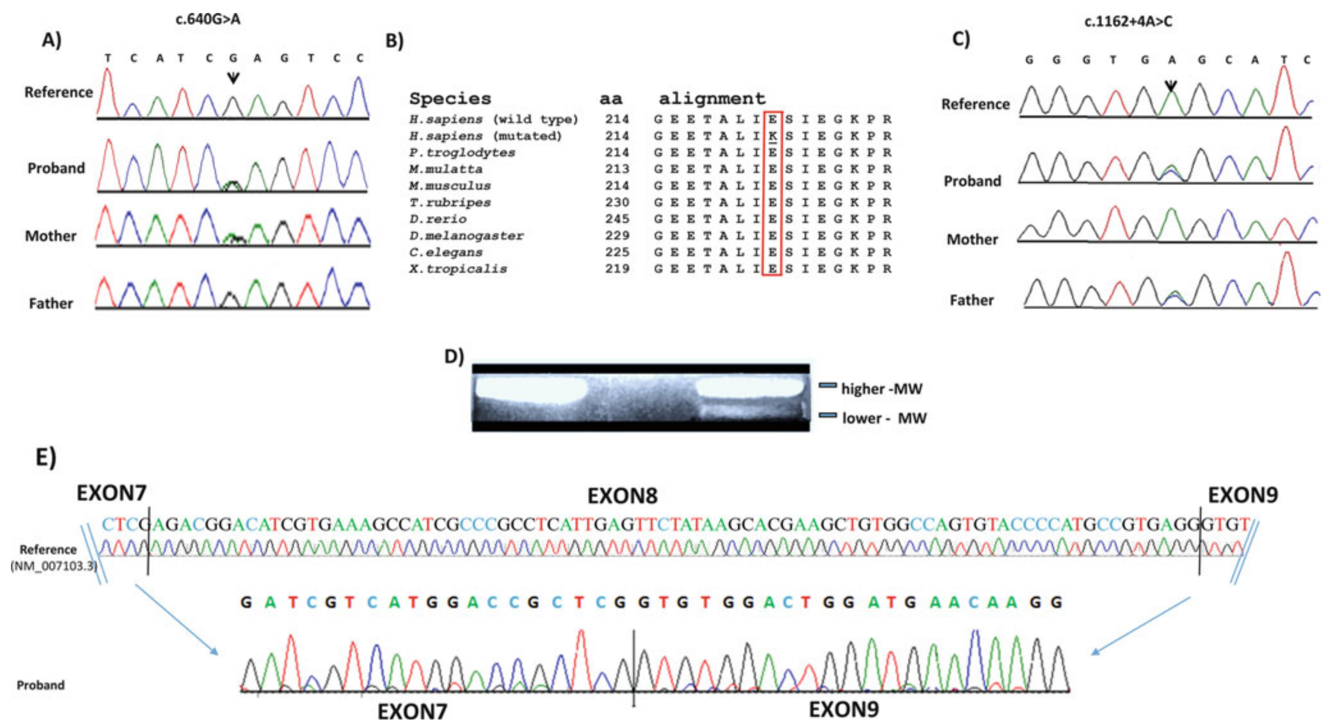

Fig. 1 (**a**) Sanger sequencing profile of *NDUFV1* from the proband and parents showing compound heterozygous mutations in the proband, including c.640G>A (p.Glu214Lys; heterozygous in the mother) and c.1162+4A>C (heterozygous in the father). (**b**) Evolutionary sequence conservation of the altered amino acid residue (p. Glu214) is denoted by a *red rectangle*. (**c**) Amplification of cDNA shows two bands in the proband: an intense higher molecular weight band and a faint lower molecular weight band. (**d**) Sanger sequencing of the faint band from amplified cDNA showing skipping of exon 8 in the patient. (**e**) Representative image of the RT-PCR and sequencing analysis of exon 8 skipping

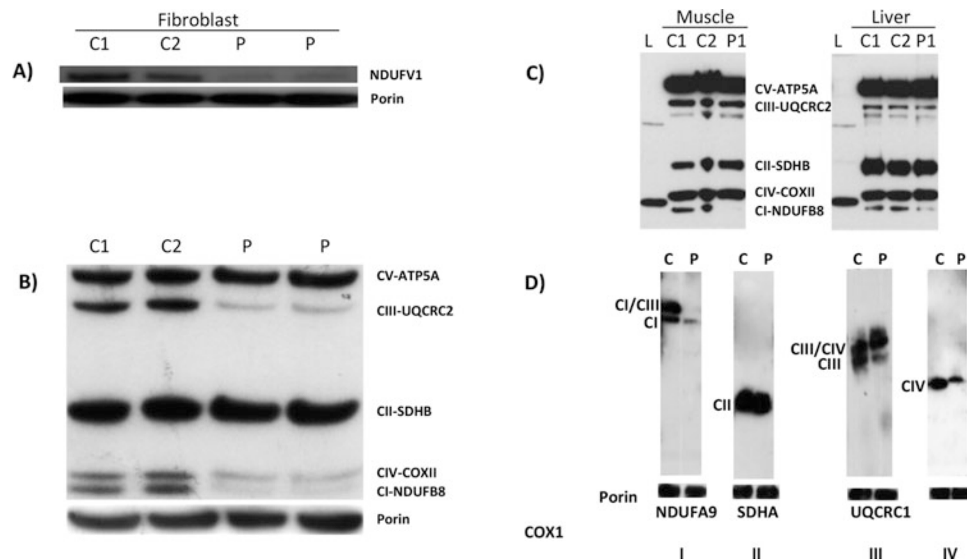

Fig. 2 (**a**) Immunoblot showing reduced NDUFV1 expression in cultured fibroblasts from the proband (P) compared to controls (C). (**b**) Immunoblot showing OXPHOS expression in muscle and liver from the proband (P) compared to controls (C). (**c**) Immunoblot showing OXPHOS expression in cultured fibroblasts from the proband (P) compared to controls (C). (**d**) BN-PAGE immunoblotting of skin fibroblast mitochondria lysed in 1% digitonin, using antibodies against the NDUFA9 subunit of complex I, the SDHA subunit of complex II, the UQCRC1 subunit of complex III and the COX1 subunit of complex IV, shows reduced complexes I, III, IV and supercomplex CI/CIII

these assembly defects were also present in patient skeletal muscle and liver because we did not have enough tissue material to carry out these studies.

In summary, these results confirm the deleterious nature of the *NDUFV1* variants as predicted by *in silico* analyses, with strong evidence that changes at the NDUFV1 subunit level have affected CI assembly in our patient. Mitochondrial respiratory chain enzyme function was then re-evaluated by dipstick enzyme assays to determine the relative specific activity of immunocaptured CI and CIV in patient fibroblast lysates. The results showed reduced activity of both CI and CIV reductions at 67% and 38%, respectively, in patient fibroblasts, relative to controls (Table 1). The reduced CI and CIV activity by dipstick enzyme assay was consistent with reduced CI and CIV levels seen on immunoblotting in patient fibroblast (Fig. 2b–d).

Discussion

This study highlights several challenges faced in the diagnosis of a patient with a clinical picture of Leigh syndrome but normal MRC enzyme activity in clinically unaffected tissues. The findings reinforce the value of using massively parallel sequencing for the identification of the underlying genetic basis of this genetically heterogeneous disorder and demonstrate the importance of confirmatory structural and functional analyses to build an evidence base linking genotype and phenotype.

In most cases, diagnosis of MRC diseases relies on identifying abnormal activity in one or more MRC enzymes (Hui et al. 2006). However, there are limitations in using MRC enzyme activity as a lead diagnostic tool. These relate primarily to dilute aqueous assay systems being an imperfect reflection of the function of membrane-embedded enzymes and the difficulty distinguishing primary from secondary MRC defects, as discussed elsewhere (Thorburn et al. 2004). Hence, only 29 out of 67 suspected Australian cases of Leigh syndrome showed a significant decrease in activity of the MRC complexes, underscoring the notion that enzyme activity investigations should be complimentary and additive to other structural and functional evidence in the diagnosis of MRC disease and should be interpreted with caution (Rahman et al. 1996).

However, implementation of combined MRC enzymology methods such as spectrophotometric assays and polarographic or dipstick enzyme assays is not currently suitable as a frontline diagnostic assay but can be useful for confirmation and evaluation of the effect of novel mutations and to study animal models (Calvo et al. 2010; Wenchich et al. 2003). These complementary methods differ in their methodologies, potentially allowing differential interrogation of the pathways involved (Barrientos et al. 2009; Tang et al. 2013; Wenchich et al. 2003).

Benit et al. (2001) has reported a patient with the same compound heterozygous variations, an onset of seizures at age 1 year, mildly elevated plasma lactate, neurological regression, strabismus, ptosis and a progressive course leading to death by 3 years. The MRI showed the typical bilateral, symmetrical basal ganglia and brainstem lesions. Leigh syndrome was proven histopathologically post-mortem (Benit et al. 2001). They reported CI deficiency in muscle and liver. However, CI was normal in cultured skin fibroblasts (Benit et al. 2001). The patient we describe here had different clinical manifestations compared with the case reported by Benit et al. (2001), with a later onset, slower progression and asymmetrical ptosis and dystonia, but no seizures. The patient is still alive 10 years after diagnosis. It is important to note that although we showed a significant reduction in both complexes I and IV in cultured fibroblasts as well as a significant reduction in complex I in muscle and liver in the current study, the overall evaluation of mitochondrial CI and CIV enzyme activity in the liver, muscle and fibroblasts was not considered helpful for the diagnosis of Leigh disease.

In mammals, all 44 CI subunits must be assembled correctly in the right sequence to form a mature functional complex. Among these components, disease-causing mutations in 14 nuclear-encoded structural subunits of CI (*NDUFS1*, *NDUFS2*, *NDUFS3*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV2*, *NDUFS6*, *NDUFV1*, *NDUFA1*, *NDUFA2*, *NDUFA9*, *NDUFA10*, *NDUFA12*), four assembly factor defects (*NDUFAF2, NDUFAF5, NDUFAF6, FOXRED1*) and 6 mtDNA subunits have previously been linked with Leigh syndrome (Ogilvie et al. 2005; Mimaki et al. 2012; Lemire 2015; Lake et al. 2015). Mutations in four CI subunits (NDUFV1, NDUFS1, NDUFS4 and NDUFS6), all located in the matrix arm (N-module) of CI, lead to CI subassembly complexes of about 830 kDa on BN-PAGE analysis that appear to have lost the matrix arm of CI (Mimaki et al. 2012; Ogilvie et al. 2005; Tuppen et al. 2010; Leong et al. 2012). In our experience, patients and mice with mutations in these genes often show an apparently more severe impact on the amount of fully assembled CI on BN-PAGE than on spectrophotometric CI activity or functional analyses such as ATP synthesis (Leong et al. 2012). This is likely due to the amount of fully assembled CI in the intact inner membrane being higher than implied by BN-PAGE and enzyme studies where the membrane has been disrupted by detergent, freeze-thawing or sonication (Leong et al. 2012), destabilising the assembled CI. All four of these CI subunit genes are expressed ubiquitously, but the impact on CI enzyme activity and function also varies between tissues (Bird et al. 2014). Furthermore, as was seen in our case, mutations in

CI genes can affect the stability of other mitochondrial complexes, which may explain the reduced CIII and CIV levels and low CIV activity in patient fibroblasts (Ugalde et al. 2004; Suthammarak et al. 2010). Some ambiguity in being able to detect isolated CI defects in all patients with mutations in genes like *NDUFV1* is not surprising given the potential variability introduced by the differing severity of individual mutations, genetic background effects and different methods for assessing the activity and assembly of a membrane-embedded complex of 44 proteins that interacts with other membrane complexes.

In summary, we have presented *in silico* studies and abnormalities of the *NDUFV1* mRNA with consequent reductions of CI protein level in skeletal muscle, liver and cultured fibroblasts. The finding of normal CIV and CIII protein levels in the patient skeletal muscle and liver, also observed in a previous study (Benit et al. 2001), emphasises the need to investigate primary tissues, as fibroblasts alone may lead to misleading results. We have also highlighted the assembly defects in OXPHOS complexes CI, CIII, CIV and supercomplex I/III. Considering the limitations in the biochemical diagnosis of a child with clinical manifestations of late-onset Leigh syndrome, implementation of a multifaceted diagnostic pipeline, including whole exome sequencing and complementary structural and functional studies, helped to confirm the pathogenicity of *NDUFV1* mutations in this patient.

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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, as revised in 2000. Informed consent was obtained from all patients included in the study.

Synopsis

A definitive genetic diagnosis was provided by whole exome sequencing and functional studies in a patient with late-onset childhood Leigh syndrome and normal muscle and liver mitochondrial RC enzyme activities.

Conflicts of Interest

Michael Nafisinia, Yiran Guo, Xiao Dang, Jiankang Li, Yulan Chen, Jianguo Zhang, Nicole J Lake, Wendy A Gold, Lisa G Riley, David R Thorburn, Brendan Keating, Xun Xu, Hakon Hakonarson and John Christodoulou declare that they have no conflict of interest.

Details of Contributions of Individual Authors

Michael Nafisinia: design and implementation of structural and functional studies, data analysis and manuscript preparation

Yiran Guo, Xiao Dang, Jiankang Li, Yulan Chen, Jianguo Zhang, Brendan Keating, Xun Xu, Hakon Hakonarson: performance of whole exome sequencing, implementation of bioinformatics, data analysis and manuscript preparation

Wendy Gold, Lisa Riley: contribution to design of structural and functional studies, data analysis and manuscript preparation

Nicole J. Lake: implementation of structural and functional studies

David Thorburn: oversight of spectrophotometric assays, data analysis and manuscript preparation

John Christodoulou: overall oversight of the research project, clinical interface, data analysis and manuscript preparation

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