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Minimally invasive ^{13}C-breath test to examine phenylalanine metabolism in children with phenylketonuria☆,☆☆

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ABSTRACT

Background: Phenylketonuria (PKU) is an autosomal recessive disorder caused by deficiency of hepatic phenylalanine hydroxylase (PAH) leading to increased levels of phenylalanine in the plasma. Phenylalanine levels and phenylalanine hydroxylase (PAH) activity monitoring are currently limited to conventional blood dot testing. 1-^{13}C-phenylalanine, a stable isotope can be used to examine phenylalanine metabolism, as the conversion of phenylalanine to tyrosine occurs in vivo via PAH and subsequently releases the carboxyl labeled ^{13}C as $^{13}CO_2$ in breath.
Objective: Our objective was to examine phenylalanine metabolism in children with PKU using a minimally-invasive 1-^{13}C-phenylalanine breath test (^{13}C-PBT).
Design: Nine children (7 M: 2 F, mean age 12.5 ± 2.87 y) with PKU participated in the study twice: once before and once after sapropterin supplementation. Children were provided 6 mg/kg oral dose of 1-^{13}C-phenylalanine and breath samples were collected at 20 min intervals for a period of 2 h. Rate of CO_2 production was measured at 60 min post-oral dose using indirect calorimetry. The percentage of 1-^{13}C-phenylalanine exhaled as $^{13}CO_2$ was measured over a 2 h period. Prior to studying children with PKU, we tested the study protocol in healthy children (n = 6; 4 M: 2 F, mean age 10.2 ± 2.48 y) as proof of principle.
Results: Production of a peak enrichment (C_{max}) of $^{13}CO_2$ (% of dose) in all healthy children occurred at 20 min ranging from 17–29% of dose, with a subsequent return to ~5% by the end of 2 h. Production of $^{13}CO_2$ from 1-^{13}C-phenylalanine in all children with PKU prior to sapropterin treatment remained low. Following sapropterin supplementation for a week, production of $^{13}CO_2$ significantly increased in five children with a subsequent decline in blood phenylalanine levels, suggesting improved PAH activity. Sapropterin treatment was not effective in three children whose $^{13}CO_2$ production remained unchanged, and did not show a reduction in blood phenylalanine levels and improvement in dietary phenylalanine tolerance.
Conclusions: Our study shows that the ^{13}C-PBT can be a minimally invasive, safe and reliable measure to examine phenylalanine metabolism in children with phenylketonuria. The breath data are corroborated by blood phenylalanine levels in children who had increased responses in $^{13}CO_2$ production, as reviewed post-hoc from clinical charts.

1. Introduction

Phenylketonuria (PKU; OMIM 261600) is an autosomal recessive deficiency of phenylalanine hydroxylase (PAH) resulting in an accumulation of phenylalanine (Phe) in blood and in the brain [1]. PKU is one of the most common rare disorders and is classified as one of the treatable inborn errors of metabolism presenting with intellectual disability [2,3]. Cognitive/behavioral deficiency is prevented by early institution of a Phe restricted diet supplemented with Phe free amino acid formula [4,5]. The degree of dietary Phe restriction depends on the severity of PKU [6]; Phe restriction ranges from severe (<20 mg/kg/d) in patients

Abbreviations: ^{13}C-PBT, phenylalanine breath test; APE, atom percent excess; BH_4, tetrahydrobiopterin; $F^{13}CO_2$, rate of 1-^{13}C-phenylalanine tracer oxidation; HPA, hyperphenylalaninemia; Phe, phenylalanine; PKU, phenylketonuria; PAH, phenylalanine hydroxylase.

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with severe PKU to up to 50 mg/kg/d in individuals with milder forms of PKU [7].

In clinical practice, Phe restriction is determined depending on the amount of Phe intake which allows for stable blood Phe levels within the therapeutic range of 2–6 mg/dl (120–360 μmol/L). Determination of the individual patient's Phe tolerance may be challenging because it requires daily patient adherence including detailed food records from the patients/caregivers and the general health state of the patient [8].

Sapropterin (Sapropterin dihydrochloride, Kuvan®, Biomarin Pharmaceutical, Novato, CA) is a Phenylalanine hydroxylase cofactor and chaperone [9,10], with the ability to enhance residual enzyme activity and improve dietary Phe tolerance [11–15] and thus improve metabolic control and quality of life for many individuals. Many clinical trials have assessed the effectiveness of sapropterin treatment and concluded that ≥30% decrease in blood Phe levels on sapropterin can be considered clinically significant [16–18]. Not all patients respond to this treatment and due to limited predictive value of mutation analysis [19], and in current clinical practice, most patients are tested with a sapropterin challenge and concomitant monitoring of blood Phe levels for responsiveness [17]. Determination of responsiveness may be challenging particularly in young patients and adolescents whose dietary Phe tolerance is subject to rapid change due to growth spurt as well as in patients with poor adherence to dietary prescriptions and inability to produce exact diet protocols.

In vivo stable isotope techniques may provide a sensitive and robust tool for determination of changes in PAH activity in response to such treatments. Matalon et al. [20] were able to determine in vivo PAH activity in humans measuring ^{2}H-phenylalanine conversion to ^{2}H-tyrosine in plasma. However, this is an invasive procedure requiring repeated blood samples and administration of large dosages of phenylalanine (10–200 mg/kg). A less invasive breath test based on conversion of 1-^{13}C-phenylalanine to tyrosine and the subsequent release of the carboxyl labeled ^{13}C to $^{13}CO_2$ in breath, has been used to identify phenylalanine tolerance in PKU subjects [12,21–23]. The earlier breath tests had varying doses of 1-^{13}C-phenylalanine, and sapropterin loading to measure responsiveness to treatment. Furthermore, no measurements of actual CO_2 production were made and some of the observed differences in $^{13}CO_2$ in breath could be explained by changes in CO_2 production during the study days [24].

The current study was designed as a first step to systematically establish the use of 1-^{13}C-phenylalanine and its oxidation to $^{13}CO_2$ as a minimally invasive technique that could be used routinely as part of each clinic visit to examine in vivo phenylalanine metabolism and to identify PAH activity in PKU children in response to treatment with sapropterin.

2. Methods and materials

2.1. Subjects

We studied six apparently healthy children (4 M: 2 F) ranging in age from 7 to 17 years (Table 1) and nine clinically stable children with PKU (7 M: 2 F) in the age range of 8 to 17 years (Table 2) who are followed by the Biochemical Diseases Clinic at the British Columbia Children's Hospital, Vancouver. For classification of the severity of PKU, we used the criteria based on pretreatment blood phenylalanine concentrations — Classic PKU: >20 mg/dl (>1200 μmol/l); moderate PKU: 15–20 mg/dl (900–1200 μmol/l); mild PKU: 10–15 mg/dl (600–900 μmol/l); mild hyperphenylalaninemia (HPA): <10 mg/dl (<600 μmol/l) [25]. PAH genotype was not available in the patients included in this study, as mutation analysis has not historically been part of the diagnostic confirmation in our clinic. All children were ensured to be free of any concurrent illness (cold, flu like symptoms) at the time of the study. The healthy children participated in the study once, while the PKU children were studied once prior to start of sapropterin therapy, and once after a minimum of a week of sapropterin therapy (~20 mg/kg/day). All procedures were reviewed and approved by the Research Ethics Board at the University of British Columbia and the British Columbia Children's Hospital. All participants provided written informed consent before participating in this study.

Table 1
Characteristics of healthy children[a].

Subject	Age (y)	Gender	Weight (kg)	Height (cm)	BMI (kg/m^2)	Fat free mass (kg)	Fat mass (%)
HC01	9	M	27.4	130.5	16.2	21.5	21.7
HC02	7	M	22	119.5	15.3	16.7	24.1
HC04	9	M	41.2	137.6	21.6	25.9	37.1
HC05	12	M	44	157.8	17.63	29.4	33.3
HC07	17	F	65	162	24.77	37.7	42
HC08	13	F	49.4	155.8	20.30	33.4	32.4

[a] Healthy children were selected to represent the wide range in age of PKU children recruited for the study (see Table 2).

2.2. Study principle

The study design was based on the oxidation of L-1-^{13}C-phenylalanine, a stable isotope tracer, to ^{13}CO2 to examine phenylalanine metabolism. The principle of the ^{13}C-phenylalanine breath test (^{13}C-PBT) is based on quantifying the enzyme dependent (phenylalanine hydroxylase, PAH) conversion of L-1-^{13}C-phenylalanine to tyrosine and the subsequent catabolism of tyrosine to release the carboxyl labeled ^{13}C as $^{13}CO_2$ in breath (Fig. 1).

2.3. Experimental setting

Participants were instructed to arrive for the study day after an overnight fast (~12 h) to standardize measurements at the Clinical Research Evaluation Unit at BC Children's Hospital. Basic anthropometric measurements (body weight and height) were recorded and a brief study day questionnaire was administered to collect information on medical, diet and physical activity history. Baseline breath samples were collected to determine natural background ^{13}C abundance (Fig. 2). Participants received an oral dose of 6 mg/kg of L-1-^{13}C-phenylalanine (99 atom% ^{13}C enrichment, Cambridge Isotope Laboratories Inc., Andover, MA) dissolved in sterile water. Participants remained fasted and rested in the unit for the entire period of the study to eliminate variability in CO_2 production. Breath samples in triplicates were collected at 20,40,60,80,100 and 120 min after oral administration of the labeled isotope. During each study visit, the rate of carbon dioxide production (VCO_2) was measured for 20 min, one hour after the oral isotope dose using an indirect calorimeter (Vmax Encore, Viasys Healthcare Inc. Yorba Linda, CA). Assessment of body composition was performed using the Bioelectrical Impedance Analyzer (BIA-Quantum IV, RJL Systems, MI). Fat free mass and fat mass was calculated using the manufacturer's software system (RJL Systems, Body Composition Analysis V.2.1).

2.4. Sample collection and analysis

Breath samples were collected in disposable glass Exetainer® tubes (Labco Limited, Buckinghamshire, UK) using a collection mechanism that permits removal of dead air space (QuinTron Instrument Company, Inc. Milwaukee, WI) [26]. All breath samples were stored at room temperature until analyzed. Expired $^{13}CO_2$ enrichment was measured using a continuous flow isotope ratio mass spectrometer (IRMS, Isoprime Ltd, Cheadle, UK) and expressed as atom percent excess (APE) when compared against a reference standard of compressed CO_2.

Table 2
Characteristics of children with phenylketonuria (PKU).

Subject and severity[a]	Age (y)	Gender	Weight (kg)	Height (cm)	BMI (kg/m[b])	Fat free mass[b] (kg)	Fat mass (%)
PKU01 (c)	13	M	66.7	166.3	24.1	44.8	32.9
PKU03 (mHPA)	10	M	39.7	152	17.2	27.7	30.3
PKU04 (mHPA)	8	M	23	126	14.5	18.2	21
PKU05 (mHPA)	15	F	51	158	20.4	31.3	38.6
PKU06 (mHPA)	17	M	63.7	159	25.2	40.3	36.8
PKU09 (c)	9	M	26.3	132.4	15.0	22.2	15.5
PKU10 (c)	12	M	43.3	149.7	19.3	31	28.4
PKU11 (mHPA)	14	F	54.3	157.6	21.9	34.2	36.9
PKU12 (mHPA)	15	M	52	168.2	18.4	40.2	22.7

[a] Criteria based on pretreatment blood phenylalanine concentrations – (c) = classic/severe PKU; (mHPA) = mild hyperphenylalaninemia.
[b] Fat free mass measured using bioelectric impedance analysis (BIA).

2.5. Calculations

Phenylalanine oxidation was measured as the rate of 1-^{13}C-phenylalanine tracer oxidation, $F^{13}CO_2$ (μmol/kg/h):

$$F^{13}CO_2 = (FCO_2)(ECO_2)(44.6)(60)/(W)(0.82)(100),$$

where FCO_2 is the CO_2 production rate (ml/min) on each study day as measured by indirect calorimetry, ECO_2 is the $^{13}CO_2$ isotopic enrichment above baseline (APE) obtained from breath samples at each time point, W is the body weight (kg) of each subject, 44.6 (μmol/l) and 60 (min/h) are constants used to convert FCO_2 to μmol/h, 0.82 is the correction factor for carbon dioxide retained by the body due to bicarbonate fixation, and 100 is used to convert APE to a fraction [27]. In order to quantify the tracer oxidation, the % of dose oxidized was calculated as $(F^{13}CO_2$/isotope dose) * 100.

2.6. Statistical analysis

1-^{13}C-Phenylalanine oxidation as $^{13}CO_2$ (% of dose) was the primary outcome measure, as this best describes the whole body oxidation capacity for phenylalanine. Area under the curve (AUC) for each subject's $^{13}CO_2$ oxidation from t_0 to t_{120}, the time to reach maximum $^{13}CO_2$ oxidation (t_{max}) and the maximum peak enrichment in $^{13}CO_2$ oxidation (C_{max}) was calculated. A Paired t-test was used to compare the $^{13}CO_2$ oxidation before and after sapropterin treatment. All values are presented for individual subjects, and significance was set at $P < 0.05$. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc, CA).

3. Results

3.1. Subject characteristics

The demographic and anthropometric characteristics of the controls and the PKU patients are shown in Table 1 and 2. Subject characteristics were comparable between the healthy controls and the PKU children. BMIs were within normal values for age in all subjects; body composition measures were also comparable among all children except for PKU06 who had low FFM and high fat mass when compared with reference values [28].

3.2. % Dose oxidized of 1-^{13}C-phenylalanine

Production of a peak enrichment (C_{max}) of $^{13}CO_2$ (% of dose) in all healthy children occurred at 20 min ranging from 17–29% of dose,

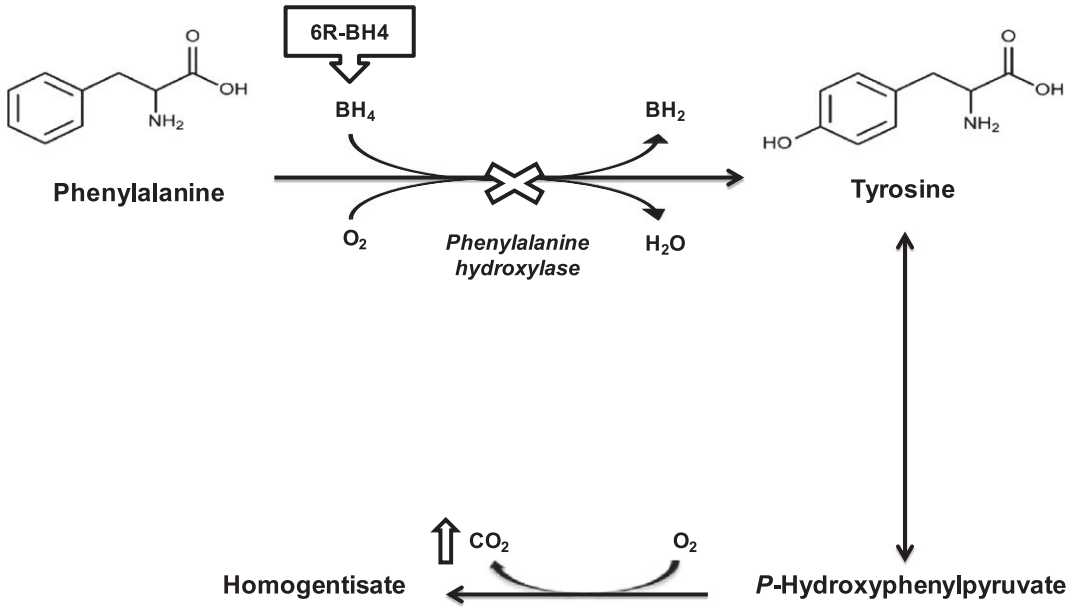

Fig. 1. Concept of the ^{13}C phenylalanine breath test (^{13}C-PBT). ^{13}C-PBT uses the oxidation of a stable isotope labeled amino acid 1-^{13}C-Phenylalanine to indicate whole body phenylalanine metabolism. The test is based on quantifying the enzyme (phenylalanine hydroxylase, PAH) dependent conversion of 1-^{13}C-phenylalanine to tyrosine and the subsequent catabolism of tyrosine to release the carboxyl labeled ^{13}C as $^{13}CO_2$ in breath.

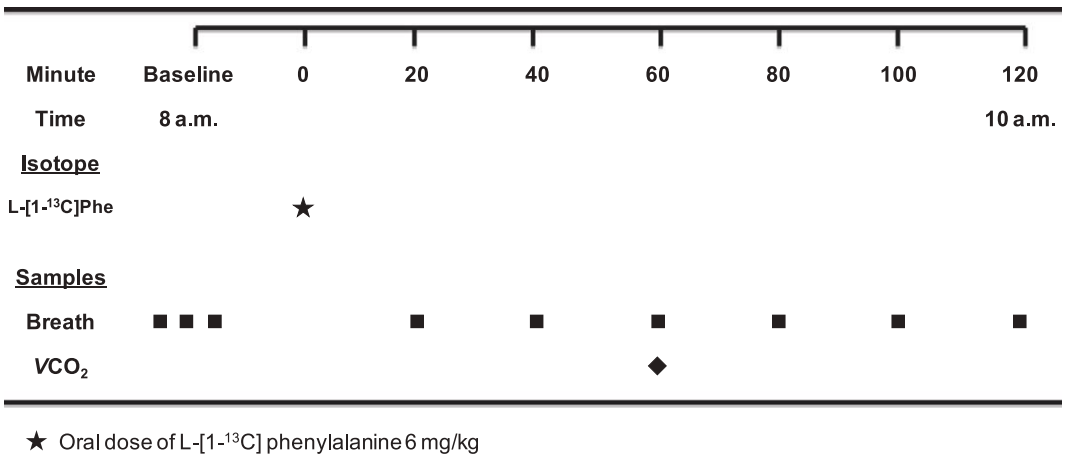

Fig. 2. Study day protocol for the ^{13}C-phenylalanine breath test (^{13}C-PBT).

with a subsequent return to ~5% by the end of 2 h (Fig. 3). These findings indicate that the oral dose of 1-^{13}C-phenylalanine was sufficient and reached the liver at 20 min and was metabolized to tyrosine with a first-pass effect.

Production of $^{13}CO_2$ from 1-^{13}C-phenylalanine in all children with PKU prior to sapropterin treatment remained low, except in one subject (PKU04) who had peak enrichment (C_{max}) at t_{40} of 4.82%, reflecting residual PAH activity in the liver (Fig. 4A). Following sapropterin supplementation for a week, production of $^{13}CO_2$ significantly increased in five children (PKU03, 05, 06, 11 and 12) suggesting improved PAH activity (Fig. 4B). Two children (PKU09, and 10) had decreased $^{13}CO_2$ production (C_{max} of 0.62–0.3%, and 0.23–0.07%, which was non-significant and significant respectively) following sapropterin supplementation; both values representing negligible PAH activity, compared to the 5–15% peak enrichment (C_{max}) in PKU03, 05, 06, 11 and 12. PKU04, who had moderate $^{13}CO_2$ production prior to sapropterin supplementation showed a non-significant increase in peak enrichment (C_{max}: 4.82% to 6.71%), although the time to reach maximum enrichment (t_{max}) improved from t_{40} to t_{20}.

3.3. Blood phenylalanine levels and dietary phenylalanine tolerance before and after sapropterin supplementation

Blood phenylalanine levels were obtained from the medical records of each of the PKU subjects as a post-hoc chart review. Blood phenylalanine levels (where available, for approximately 6 months prior to treatment and 6 months post treatment with sapropterin) were extracted for the nine children who completed both pre and post sapropterin breath tests (Table 3). Six PKU subjects (PKU03, 04, 05, 06, 11 and 12) who had an improved $^{13}CO_2$ production in the ^{13}C phenylalanine breath test during sapropterin treatment, also had a reduction of blood phenylalanine levels in response to sapropterin treatment (range: 48% to 69%, median 54%) compared to baseline value. Three out of the six subjects (PKU 03, 11,12) who showed a reduction in blood phenylalanine concentrations on treatment also had an additional increase (2–3 fold) in the daily dietary phenylalanine tolerance (Table 3). Sapropterin treatment was not effective in 3 patients (PKU 01, 09, 10) whose $^{13}CO_2$ production remained unchanged on treatment and did not show a

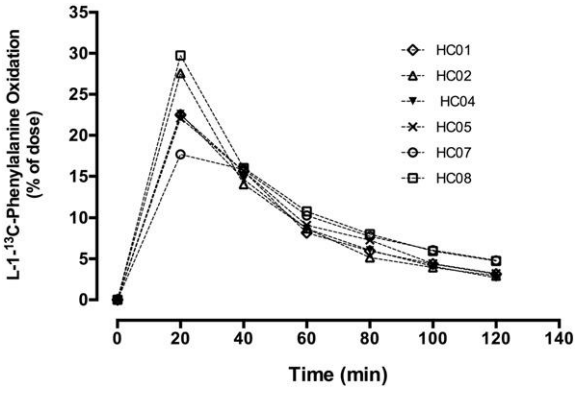

Fig. 3. ^{13}C-phenylalanine breath test (^{13}C-PBT) in healthy children. 1-^{13}C-phenylalanine oxidation (% of dose) during the minimally invasive two-hour study protocol. AUC_{120}: area under the curve for $^{13}CO_2$ oxidation from t_0 to t_{120}; t_{max}: time to reach maximum $^{13}CO_2$ oxidation; C_{max}; maximum peak enrichment in $^{13}CO_2$ oxidation.

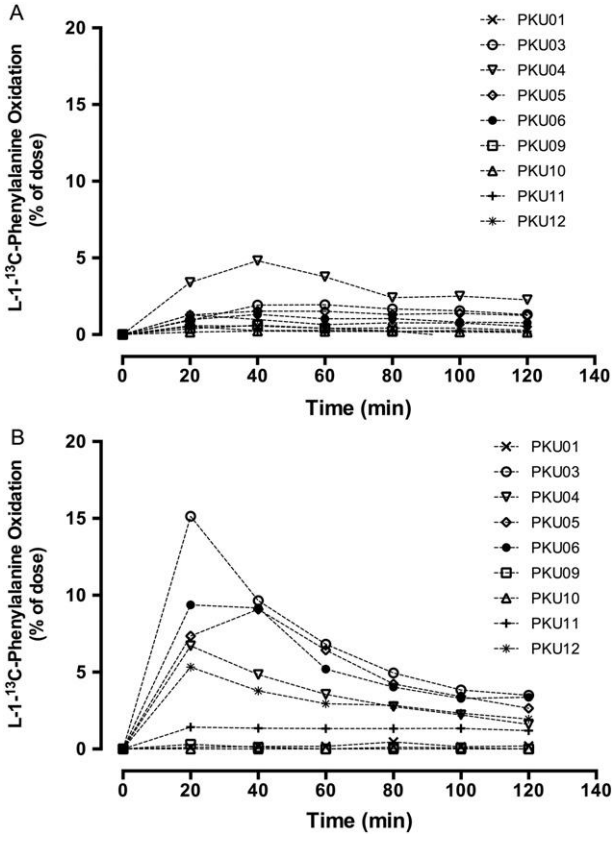

Fig. 4. ^{13}C-phenylalanine breath test (^{13}C-PBT) in children with phenylketonuria (PKU). A. 1-^{13}C-phenylalanine oxidation (% of dose) in PKU children prior to treatment with sapropterin (Kuvan®). B. 1-^{13}C-phenylalanine oxidation (% of dose) in PKU children after treatment with sapropterin (Kuvan®). AUC_{120}: area under the curve for $^{13}CO_2$ oxidation from t_0 to t_{120}; t_{max}: time to reach maximum $^{13}CO_2$ oxidation; C_{max}; maximum peak enrichment in $^{13}CO_2$ oxidation.

Table 3
Blood phenylalanine concentrations in children with PKU before and after treatment with sapropterin.

Blood phenylalanine concentrations (mg/dl)[a]				Phenylalanine tolerance (mg/d)			
Subject	Sapropterin dose at time of breath test (mg/kg/d)	Baseline (4–6 months pre-treatment)	Treatment (4–6 months post-treatment)	% reduction in blood Phe concentrations from baseline	Baseline (4–6 months pre-treatment)	Treatment (4–6 months post-treatment)	Response to ^{13}C-phenylalanine breath test*
PKU01	20	6.36 ± 2.14	4.81 ± 3.81	24%	495–570	495–570	−
PKU03	20	3.82 ± 0.44	1.99 ± 0.35	48%	1350	2700	++
PKU04	20	5.69 ± 1.01	1.74 ± 0.35	69%	**	**	++
PKU05	19	5.74 ± 1.20	1.77 ± 0.18	69%	**	**	++
PKU06	20	6.60 ± 1.08	3.22 ± 1.23	51%	**	**	++
PKU09	17	4.56 ± 1.05	3.76 ± 2.34	18%	330	330	−
PKU10	20	1.60 ± 0.62	4.89 ± 2.23	©	350–450	350–450	−
PKU11	19	9.23 ± 1.71	4.35 ± 1.21	53%	550	1100	+
PKU12	18	3.18 ± 1.41	1.47 ± 0.49	54%	1500	5000	++

*Response to ^{13}C-phenylalanine breath test (based on Fig. 4B) and defined as: ++ = maximum peak enrichment in $^{13}CO_2$ oxidation of >5% post sapropterin therapy; + = maximum peak enrichment in $^{13}CO_2$ oxidation of >1% post sapropterin therapy; − = maximum peak enrichment in $^{13}CO_2$ oxidation of <1% post sapropterin therapy.
**mHPA — 3 of the patients were on liberal diet prior to treatment with sapropterin with poor metabolic control. Upon treatment, blood Phe concentrations are within therapeutic range and patients continue to be on liberal diet.
©Unexplained increase in blood Phe concentrations on sapropterin treatment possibly due to liberalization of diet.
[a] Values are mean ± SD; n = 4–22 blood dots per child collected over 2 weeks–6 months.

reduction in blood phenylalanine levels and improvement in dietary phenylalanine tolerance (Fig. 5).

4. Discussion

The objective of the current study was to establish in a pediatric population, a minimally invasive and sensitive test to measure phenylalanine metabolism in vivo. The results from the healthy children confirmed that the ^{13}C-PBT study protocol was sufficient and robust to detect phenylalanine disposal within a 2 h study period. Furthermore, in PKU children, who are responsive to pharmacologic treatment (sapropterin therapy), the ^{13}C-PBT was able to identify reliably the whole body in vivo oxidative capacity of phenylalanine metabolism.

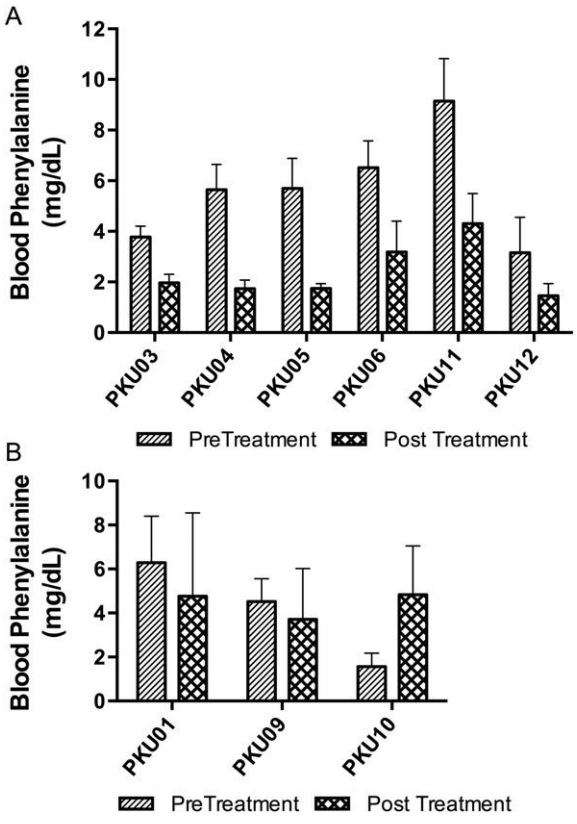

Fig. 5. Blood phenylalanine concentrations in children with phenylketonuria (PKU). A. Blood phenylalanine concentrations in PKU children who had increases in $^{13}CO_2$ after sapropterin treatment. B. Blood phenylalanine concentrations in PKU children who had no change in $^{13}CO_2$ after sapropterin treatment.

Previous studies have used similar breath tests in PKU children to identify responders to sapropterin treatment. Muntau et al. [22] studied, 38 children with PKU aged 1 day–17 years for period of one year. The experiment was based on phenylalanine (100 mg/kg) and tetrahydrobiopterin (20 mg/kg) loading followed by measurement of 1-^{13}C-phenylalanine oxidation to $^{13}CO_2$ within a 24 h period. The authors' concluded that sapropterin improves PAH activity in patients with mild HPA, and the breath test reliably identified patients who were responsive [22]. Okano et al. [12] studied 20 PKU patients with a wider age range (1–23 y) and used a 10 mg/kg of 1-^{13}C-phenylalanine dose, but had a restrictive maximum amount (200 mg). Their study also involved sapropterin loading (10 mg/kg) for two days, as well on the study day [12], and the results indicated a wide range in phenylalanine oxidative capacity which was reflective of the type of mutation in the PAH gene. Both the studies did not measure actual CO_2 production rates, and basal metabolic rate. It has been shown previously that the endogenous CO_2 production rates by each subject could explain some of the observed differences in ^{13}C-Breath tests [24]. In the current study all values were corrected by study day measured CO_2 production rates. Furthermore, the varying amounts of doses provided make it difficult to compare different responses among PKU patients. Our study was designed to test a constant dose of 1-^{13}C-Phenylalanine, measure actual CO_2 production rates, conduct the study under a standardized condition (fasting) in all subjects and the first step in making the ^{13}C-PBT routine, as part of regular PKU visits by patients.

The ^{13}C-PBT is reflective of PAH activity in the liver, but is more representative of whole body phenylalanine oxidative capacity, and as such represents the clinical phenotype. The current test protocol of 2 h, with a peak enrichment (C_{max}) occurring consistently at 20 min in healthy children suggests that the oral dose of 1-^{13}C-phenylalanine is absorbed in the intestine, and primarily metabolized in the liver as a first pass effect. The 1-^{13}C-label is transferred to 1-^{13}C-tyrosine by PAH, and subsequently converted to p-hydroxyphenylpyruvate, and the ^{13}C is released as $^{13}CO_2$ during the formation of homogentisate (Fig. 1). Comparison of the peak enrichment and the time to achieve peak enrichment between healthy children (Fig. 3) and PKU children (Fig. 4A) suggests that the clearance rate of phenylalanine is clearly different prior to sapropterin treatment. After sapropterin therapy the C_{max} was at 20 min in most children who were responsive to the treatment (Fig. 4B). While measurement of blood phenylalanine levels offer a more global picture of phenylalanine metabolism, the stable isotope based breath test offers more dynamic and patient specific details of in vivo disposal of

phenylalanine. PKU is a complex and heterogeneous disorder, with almost 500 different mutations identified in the PAH gene. While genotypes which are more responsive to sapropterin therapy have been identified and classified [14], the genotype–phenotype correlations are not perfect. Thus the ^{13}C-PBT adds value to the clinical management of PKU children.

The use of stable isotope based breath tests in clinical conditions are well known, with the ^{13}C-urea breath test for the detection of H Pylori infection in the stomach being the most well established as a routine test [29]. 1-^{13}C-phenylalanine oxidation to $^{13}CO_2$ is also used as a test for chronic liver disease [30] and gastric emptying [31]. While it is likely that measurement of blood phenylalanine levels will remain the key measure of clinical management, there is a need for a routine, simple test to help better manage PKU children. Future work is necessary before a standardized protocol can be recommended for routine use in PKU children. For example, validation studies of different isotope doses within the same subject, prediction of CO_2 production values versus actual measured values, length of study protocol etc. need to be tested. New treatment modalities, such as polyethyleneglycol-phenylalanine ammonia lyase (PEG-PAL), are constantly being explored for the management of PKU children [32]. Thus newer stable isotope based dynamic tests would be required to help understand and manage patients with PKU.

In summary, the current study is a first step in establishing the ^{13}C-PBT as a minimally invasive and dynamic test that could potentially be used as a routine test as part of each clinic visit in PKU children to establish treatment response. The results suggest that the 2 h-stable isotope based breath test provides reliable data on whole body in vivo phenylalanine metabolism. In six of the nine PKU children tested, an increase in $^{13}CO_2$ production from 1-^{13}C-phenylalanine was measured due to sapropterin treatment. The breath data are corroborated by decline in blood phenylalanine levels in the same children who had increased responses in $^{13}CO_2$ production, as reviewed post-hoc from clinical charts. Future work should focus on further validation of the ^{13}C-PBT and whether it could be useful in the clinical management of children with PKU.

Acknowledgments

RE and SS contributed to the study design and development; AT and GM collected and analyzed the data; KU, BC and AG contributed to patient recruitment, extraction of data from clinical charts; AT, GM, KU, BC, AG, SS and RE contributed to writing the manuscript; RE had primary responsibility for final content. All authors read and approved the final manuscript.

References

[1] M. van Rijn, M. Hoeksma, P. Sauer, B. Szczerbak, M. Gross, D. Reijngoud, F. van Spronsen, Protein metabolism in adult patients with phenylketonuria, Nutrition 23 (6) (2007) 445–453.
[2] A. Bélanger-Quintana, A. Burlina, C.O. Harding, A.C. Muntau, Up to date knowledge on different treatment strategies for phenylketonuria, Mol. Genet. Metab. 104 (2011) S19–S25.
[3] C.D.M. van Karnebeek, S. Stockler, Treatable inborn errors of metabolism causing intellectual disability: a systematic literature review, Mol. Genet. Metab. 105 (3) (2012) 368–381.
[4] C.R. Scriver, S. Kaufman, Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in: C.R. Scriver, A.L. Baudet, D. Valle, W.S. Sly (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th ed.McGraw-Hill, New York 2001, pp. 1667–1724.
[5] J.B. Shannon, Endocrine and Metabolic Disorders: Inborn Errors of Metabolism, 2nd ed. Omnigraphics, Detroit, MI, 2007.
[6] N. Blau, F.J. van Spronsen, H.L. Levy, Phenylketonuria, Lancet 376 (9750) (2010) 1417–1427.
[7] F. Güttler, P. Guldberg, The influence of mutations on enzyme activity and phenylalanine tolerance in phenylalanine hydroxylase deficiency, Eur. J. Pediatr. 155 (S1) (1996) S6–S10.
[8] A. MacDonald, J.C. Rocha, M. van Rijn, F. Feillet, Nutrition in phenylketonuria, Mol. Genet. Metab. 104 (2011) S10–S18.
[9] N. Blau, Sapropterin dihydrochloride for the treatment of hyperphenylalaninemias, Expert Opin. Drug Metab. Toxicol. 9 (9) (2013) 1207–1218.
[10] H.J. Vernon, C.B. Koerner, M.R. Johnson, A. Bergner, A. Hamosh, Introduction of sapropterin dihydrochloride as standard of care in patients with phenylketonuria, Mol. Genet. Metab. 100 (3) (2010) 229–233.
[11] B.K. Burton, H. Bausell, R. Katz, H. LaDuca, C. Sullivan, Sapropterin therapy increases stability of blood phenylalanine levels in patients with BH4-responsive phenylketonuria (PKU), Mol. Genet. Metab. 101 (2) (2010) 110–114.
[12] Y. Okano, Y. Hase, M. Kawajiri, Y. Nishi, K. Inui, N. Sakai, Y. Tanaka, K. Takatori, M. Kajiwara, T. Yamano, In vivo studies of phenylalanine hydroxylase by phenylalanine breath test: diagnosis of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, Pediatr. Res. 56 (5) (2004) 714–719.
[13] M. Lindner, D. Haas, E. Mayatepek, J. Zschocke, P. Burgard, Tetrahydrobiopterin responsiveness in phenylketonuria differs between patients with the same genotype, Mol. Genet. Metab. 73 (1) (2001) 104–106.
[14] M.R. Zurflüh, J. Zschocke, M. Lindner, F. Feillet, C. Chery, A. Burlina, R.C. Stevens, B. Thöny, N. Blau, Molecular genetics of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, Hum. Mutat. 29 (1) (2008) 167–175.
[15] H.L. Levy, A. Milanowski, A. Chakrapani, M. Cleary, P. Lee, F.K. Trefz, C.B. Whitley, F. Feillet, A.S. Feigenbaum, J.D. Bebchuk, H. Christ-Schmidt, A. Dorenbaum, Sapropterin Research Group, Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomised placebo-controlled study, Lancet 370 (9586) (2007) 504–510.
[16] B.K. Burton, D.K. Grange, A. Milanowski, G. Vockley, F. Feillet, E.A. Crombez, V. Abadie, C.O. Harding, S. Cederbaum, D. Dobbelaere, A. Smith, A. Dorenbaum, The response of patients with phenylketonuria and elevated serum phenylalanine to treatment with oral sapropterin dihydrochloride (6R-tetrahydrobiopterin): a phase II, multicenter, open-label, screening study, J. Inherit. Metab. Dis. 30 (5) (2007) 700–707.
[17] H. Levy, B. Burton, S. Cederbaum, C. Scriver, Recommendations for evaluation of responsiveness to tetrahydrobiopterin (BH4) in phenylketonuria and its use in treatment, Mol. Genet. Metab. 92 (4) (2007) 287–291.
[18] F.K. Trefz, B.K. Burton, N. Longo, M. Martinez-Pardo Casanova, D.J. Gruskin, A. Dorenbaum, E.D. Kakkis, E.A. Crombez, D.K. Grange, P. Harmatz, M.H. Lipson, A. Milanowski, L.M. Randolph, J. Vockley, C.B. Whitley, J.A. Wolff, J. Bebchuk, H. Christ-Schmidt, J.B. Hennermann, for the Sapropterin Study Group. Efficacy of sapropterin dihydrochloride in increasing phenylalanine tolerance in children with phenylketonuria: a phase III, randomized, double-blind, placebo-controlled study, J. Pediatr. 154 (5) (2009) 700–707.
[19] A. Ponzone, F. Porta, A. Mussa, A. Alluto, S. Ferraris, M. Spada, Unresponsiveness to tetrahydrobiopterin of phenylalanine hydroxylase deficiency, Metabolism 59 (5) (2010) 645–652.
[20] R. Matalon, D.E. Matthews, K. Michals, D. Bier, The use of deuterated phenylalanine for the in vivo assay of phenylalanine hydroxylase activity in children, J. Inherit. Metab. Dis. 5 (1982) 17–19.
[21] E.P. Treacy, J.J. Delente, G. Elkas, K. Carter, M. Lambert, P.J. Waters, C.R. Scriver, Analysis of phenylalanine hydroxylase genotypes and hyperphenylalaninemia phenotypes using L-[1-^{13}C] phenylalanine oxidation rates in vivo: a pilot study, Pediatr. Res. 42 (1997) 430–435.
[22] A.C. Muntau, W. Röschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria, N. Engl. J. Med. 347 (26) (2002) 2122–2132.
[23] Y. Okano, K. Takatori, S. Kudo, T. Sakaguchi, M. Asada, M. Kajiwara, T. Yamano, Effects of tetrahydrobiopterin and phenylalanine on *in vivo* human phenylalanine hydroxylase by phenylalanine breath test, Mol. Genet. Metab. 92 (4) (2007) 308–314.
[24] D. Rating, C.D. Langhans, Breath tests: concepts, applications and limitations, Eur. J. Pediatr. 156 (Suppl. 1) (1997 Aug) S18–S23.
[25] N. Blau, J.B. Hennermann, U. Langenbeck, U. Lichter-Konecki, Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies, Mol. Genet. Metab. 104 (Suppl.) (2011) S2–S9, http://dx.doi.org/10.1016/j.ymgme.2011.08.017.
[26] R. Elango, M.A. Humayun, R.O. Ball, P.B. Pencharz, Protein requirement of healthy school-age children determined by the indicator amino acid oxidation method, Am. J. Clin. Nutr. 94 (6) (2011) 1545–1552, http://dx.doi.org/10.3945/ajcn.111.012815.
[27] R.A. Hoerr, Y.M. Yu, D.A. Wagner, J.F. Burke, V.R. Young, Recovery of ^{13}C in breath from NaH$^{13}CO_3$ infused by gut and vein: effect of feeding, Am. J. Physiol. (1989) 426–438.
[28] K.J. Ellis, R.J. Shypailo, S.A. Abrams, W.W. Wong, The reference child and adolescent models of body composition, Ann. N. Y. Acad. Sci. 904 (1) (2000) 374–382.
[29] D.Y. Graham, H.M. Malaty, R.A. Cole, R.F. Martin, P.D. Klein, Simplified ^{13}C-urea breath test for detection of *Helicobacter pylori* infection, Am. J. Gastroenterol. 96 (6) (2001) 1741–1745.
[30] S. Moran, I. Gallardo-Wong, G. Rodriguez-Leal, P. McCollough, J. Mendez, B. Castaneda, P. Milke, J. Jacobo, M. Dehesa, L-[1-^{13}C]phenylalanine breath test in patients with chronic liver disease of different etiologies, Isot. Environ. Health Stud. 45 (3) (2009) 192–197, http://dx.doi.org/10.1080/10256010903083995.
[31] L. Bonfrate, I. Grattagliano, G. Palasciano, P. Portincasa, Dynamic carbon 13 breath tests for the study of liver function and gastric emptying, Gastroenterol. Rep. 1–10 (2014)http://dx.doi.org/10.1093/gastro/gou068.
[32] J. Vockley, H.C. Andersson, K.M. Antshel, N.E. Braverman, B.K. Burton, D.M. Frazier, J. Mitchell, W.E. Smith, B.H. Thompson, S.A. Berry, Phenylalanine hydroxylase deficiency: diagnosis and management guideline, Genet. Med. 16 (2) (2014) 188–200.