Improved Measurement of Brain Phenylalanine and Tyrosine Related to Neuropsychological Functioning in Phenylketonuria

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Abstract *Introduction:* Researchers hypothesized that in phenylketonuria (PKU) high brain phenylalanine (Phe) levels and low brain tyrosine (Tyr) levels affect neuropsychological functioning. However, traditional magnetic resonance spectroscopy (MRS) yielded uncertain results of brain Phe and could not adequately measure brain Tyr. This pilot study examined the potential of correlated spectroscopy (COSY) to quantify these biomarkers and explain variability in neuropsychological functioning.

Methods: Nine adults with early treated classic PKU received magnetic resonance imaging (MRI) with COSY and a battery of neuropsychological tests. Brain Phe and Tyr in parietal white matter (PWM) were compared to results in gray matter of the posterior cingulate gyrus (PCG).

Results: Brain Phe ranged from 101 to 182 (mean = 136.76 ± 23.77) $\mu\text{mol/L}$ in PCG and 76 to 185 (mean = 130.11 ± 37.88) $\mu\text{mol/L}$ in PWM. Brain Tyr ranged from 4.0 to 7.4 (mean = 5.44 ± 1.01) $\mu\text{mol/L}$ in PCG and 4.1 to 8.4 (mean = 5.90 ± 1.48) $\mu\text{mol/L}$ in PWM. Correlation coefficients were largest for brain Phe PWM and measures of

auditory memory ($\rho = -0.79$), anxiety ($\rho = 0.79$), and executive functioning ($\rho = 0.69$). Associations were in the expected direction, with higher brain Phe and lower brain Tyr related to poorer functioning. The two participants with severe structural MRI abnormalities had low brain Tyr levels in PCG and 3/5 of the participants with moderate to severe MRI abnormalities had higher than average brain Phe levels.

Conclusion: COSY has the potential to quantify brain Phe and Tyr at low concentrations and in specific brain regions. In this pilot study, these biomarkers were associated with indices of neuropsychological functioning. Additional studies are needed to validate the COSY results.

Introduction

Phenylketonuria (PKU, OMIM 261600), an autosomal recessive disorder, affects approximately 1:11,000 individuals in the USA. In PKU, mutations in the gene responsible for the liver enzyme phenylalanine hydroxylase (PAH) result in reduced or absent conversion of phenylalanine (Phe) to tyrosine (Tyr) and subsequently to elevated plasma concentrations of Phe and reduced concentrations of Tyr (Scriver and Kaufman 2001). Untreated PKU results in progressive, neurological decline by 6–12 months of age (Koch et al. 1971). Even with early detection and treatment with a Phe-restricted diet, patients experience neurocognitive deficits (Waisbren et al. 1994) and psychiatric disturbances as they get older (Bildner et al. 2013; Weglage et al. 2013) as well as white matter abnormalities detected through magnetic resonance imaging (MRI) (Mastrangelo et al. 2015).

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Fig. 1 Three-dimensional rendering of the correlated spectroscopy (COSY) spectrum. Phenylalanine (Phe), tyrosine (Tyr), and *N*-acetyl-aspartate (NAA) can be readily visualized and quantified using this method

Hypotheses attribute neuropsychological deficits in PKU to: (1) reduced myelin as observed in MRI and (2) reduced dopamine (a metabolite of Tyr) in the brain (Surtees and Blau 2000). Evidence for the dopamine hypothesis derives indirectly from neuropsychological tests showing impaired functioning on tasks associated with the prefrontal cortex, such as executive functions (Diamond et al. 1997) as well as abnormal MRI findings in brain regions dependent on dopamine (Bodner et al. 2012). However, direct measurement of Phe concentrations in the brain via conventional magnetic resonance spectroscopy (MRS) is challenging due to overlapping spectra of Phe and Tyr, as well as relatively low concentrations of brain Phe (Kreis et al. 2009). Research to improve quantification of brain Phe and Tyr and to understand the impact of these biomarkers is important not only in understanding the pathology in PKU, but also in understanding the biological mechanisms leading to phenotypic variability (Ramus et al. 1999). In addition, quantification of brain Phe and Tyr can lead to individualizing treatment and biomarkers for clinical trials.

A potential method to overcome limitations of conventional spectroscopy is two-dimensional shift Correlated Spectroscopy (COSY). This method has been used for the unambiguous identification of cerebral metabolites that could not be detected using conventional MRS methods due to spectral overlap (Thomas et al. 2001; Lin et al. 2012). By obtaining multiple acquisitions at different echo times, a second chemical shift domain allows for metabolites to be identified by two chemical shifts instead of just one based on scalar coupling of different proton groups. The concentration of the metabolite is therefore shown in the third dimension. By visualizing COSY data in three dimensions, smaller resonances that would have been obscured by larger resonances can be measured (Fig. 1). Different brain regions separating white and gray matter tissue can be

assessed with this method as a smaller voxel can be used. In addition, the COSY method can measure Tyr (Ramadan et al. 2011), and other amino acids (Lin et al. 2015).

The aims of this pilot study were to: (1) demonstrate the potential of the COSY method in quantitative measurement of Phe and Tyr in distinct regions of the brain and (2) determine if there is a relationship between these biomarkers and measures of neuropsychological functioning.

Methods

Overview

Nine adults with early treated classic PKU detected by newborn screening (four men, five women; mean age 29 ± 4 years) comprised the sample. Eligibility required pretreatment/off-diet blood Phe concentration above $1,200 \mu\text{mol/L}$, Phe tolerance of less than 300 mg/day , or genotype associated with classic PKU. Study participants were identified and recruited by health care providers (psychologists, dieticians, or metabolic physicians) who followed the patients at Boston Children's Hospital. One sibling pair with identical genotypes for the PAH gene was included in the sample.

Blood draws, neurological examinations, and neuropsychological testing were conducted at the Boston Children's Hospital Clinical Translational Studies Unit (CTSU). The Committee on Clinical Investigations at Boston Children's Hospital approved the study and all participants provided written informed consent.

Magnetic Resonance Imaging and Spectroscopy

MRI and MRS were performed on a 3T Siemens scanner with a 32-channel coil at Boston Children's Hospital. Participants were given ample time to become familiar with the procedures and offered earplugs and/or headphones to block out the sound. The structural MRI sequences included a volumetric multiecho T1-weighted three-dimensional (3D) magnetization-prepared rapid gradient-echo (MP-RAGE) sequence (slice thickness 1 mm , $20\text{--}25 \text{ cm}$ FOV), axial T2-weighted sequence (slice thickness 2.5 mm skip 0 , $20\text{--}22 \text{ cm}$ FOV), axial T2-weighted FLAIR (slice thickness 4 mm skip 0 , 20 cm FOV), and diffusion-weighted images (35 directions, B values: 0 and $1,000 \text{ s/mm}^2$).

Structural MRI sequences including the diffusion-weighted images were reviewed by a fellowship trained board certified pediatric neuroradiologist (SPP), who was blinded to the MR spectroscopy data, clinical examination, and blood levels of Phe and Tyr. The MRI scans were assessed for the degree of T2 prolongation and diffusion restriction and each finding was classified into mild, moderate, and severe

categories. The MRI appearance was classified into these categories by combining the degree of T2 prolongation, diffusion restriction, and degree of parenchymal volume loss indicated by sulcal and extraaxial space prominence. The “mild” category included scans of subjects with hazy T2 prolongation in the periventricular and deep white matter without diffusion restriction and no significant prominence of the extraaxial spaces. The “moderate” category included scans with more pronounced T2 prolongation and scattered areas of mildly restricted diffusion in some of these areas of T2 prolongation and mild sulcal and extraaxial space prominence. The “severe” category included scans with confluent areas of T2 prolongation and decreased diffusion and generalized parenchymal volume loss in the supratentorial and infratentorial brain indicated by moderate sulcal and extraaxial space prominence.

COSY was used to quantify brain levels of Phe and Tyr. Spectroscopy was performed using both 1D MRS (PRESS; TR 2 s, TE 30 ms, 64 avgs) and COSY (TR 1.5 s, initial TE 30 ms, 64 increments of 0.8 ms, 8 avgs). MRS in the gray matter region, posterior cingulate gyrus (PCG) ($3 \times 3 \times 3 \text{ cm}^3$), and parietal white matter (PWM) ($4 \times 3 \times 2 \text{ cm}^3$) was acquired with unsuppressed water reference (Thomas et al. 2001; Ramadan et al. 2011; Lin et al. 2012).

Post-processing of the COSY data quantified the levels of Phe as the primary measure using Felix NMR. Secondary measures included Tyr. Values for Phe and Tyr reported here were expressed in unadjusted terms in addition to ratios to illustrate the capability of measuring low elevations. For the 2D COSY spectra, metabolite concentrations were calculated from the volume of the peak and calibrated to the LCModel estimation. Felix NMR was used to measure the volume of Phe and Creatine (Cr) resonances from the 2D COSY spectra. The Felix NMR was used at the D1 settings: data size to 512, spectrometer frequency of 123.23, and a sweep of 2,000.0. Next, the D2 settings were set to 64, spectrometer frequency 123.23, and a sweep width of 1,250.0. The acquisition mode was set to magnitude, as well as the acquisition in the D2. Next, the window function was set to “skewed sinebell²,” along with CNV based solvent suppression at a value of 30. The phase shift and the skew parameter were set to 0.0 and 0.3, respectively. Consequentially, the D2 window function was set to sinebell² with a phase shift of 0.0. Using the chemical shift of creatine 3.02 (Govindaraju et al. 2000), the resulting COSY data were referenced. Using the chemical shifts of Phe 7.32 and Tyr 7.18, a dba was created to measure the peaks of Phe and Tyr.

Neuropsychological Evaluation

All participants received a brief neuropsychological evaluation in order to further describe the sample and identify functional outcomes sensitive to variations in brain Phe and Tyr. The Wechsler Abbreviated Scale of Intelligence

(WASI) (Wechsler 1999) was administered for IQ determination. The Beery Visual Motor Integration Test, Sixth Edition (VMI) (Beery et al. 2010) provided a measure of visual motor skills. The California Verbal Learning Test – Adult Version (CVLT-A) (Delis et al. 2000) measured verbal learning and auditory memory. The Delis–Kaufman Executive Functioning Verbal Fluency subtest measured aspects of processing speed (Delis et al. 2001). The Behavior Rating Inventory of Executive Function – Adult Version (BRIEF-A) (Gioia et al. 2000) provided a self-reported index of executive functioning and the Beck Anxiety Inventory (Beck and Steer 1993) and Beck Depression Inventory, Second Edition (Beck et al. 1996), provided self-reported indices of mood. Participants also completed a self-report measure of day-to-day functioning, the Adaptive Behavior Assessment System, Second Edition (ABAS-II) (Harrison and Oakland 2003).

Serum Measurements

For determination of the plasma amino acids, Phe and Tyr, venipuncture was performed by nurses at the Clinical Translational Studies Unit (CTSU) and sent to a CLIA approved laboratory.

Neurological Examinations

All participants were evaluated with a standardized neurological examination performed by the two adult neurologists on the research team.

Statistical Analysis

Results from COSY were derived from direct measurement of the crosspeak volume and are presented as means with standard deviations. Due to the small sample size, exploratory analyses were conducted using nonparametric statistical methods. The Mann–Whitney test was used to compare COSY results derived from the ratios of Phe/creatine and Tyr/creatine in individuals with PKU and a group of nine healthy adults who comprised an historical control group. Spearman’s correlation coefficients were used to identify associations between COSY and laboratory results and Phe and Tyr concentrations in gray and white matter regions. Preliminary analyses, also using Spearman’s correlation coefficients, examined associations of blood and brain Phe and Tyr concentrations with neuropsychological test results.

Results

To validate the COSY method, several studies were conducted prior to scanning research participants. Solutions of the Phe and Tyr, or phantoms, were made up in 250 ml

Fig. 2 COSY measures of Phe and Tyr in phantoms (*top*) and human subjects (*bottom*)

MR compatible spheres at physiological concentrations with creatine at 10 mM to serve as a chemical shift reference. Along with these amino acids, creatine was measured for normalization purposes. The resulting COSY volumes of Phe and Tyr were normalized with their respective creatine volumes. Characterization of downfield resonances including Phe and Tyr was validated using phantom solutions of different concentrations to ensure the specificity of the COSY measurements (Fig. 2). Scans were conducted with phantoms with concentrations consisting of 0.01, 0.1, 1, and 1 mM at the chemical shifts of $F2 = 7.35 \pm 0.016$ and $F1 = 7.50 \pm 0.27$ ppm. For Tyr, the chemical shifts of $F2 = 7.17 \pm 0.008$ and $F1 = 6.87 \pm 0.0019$ ppm were measured. The same MRS protocol was used for phantom and human studies. Results of the phantom studies showed good correlations between cross-peak volumes and the concentrations of Phe ($r = 0.98$) and Tyr ($r = 0.84$).

To further validate COSY, average measurements of Phe and Tyr from de-identified human brain MRI scans with COSY were used. Nine healthy adults (ages 28 ± 3 years and matched for sex and age within 3 years of the enrolled study participants with PKU) had previously participated in

a similar study that obtained measurements of brain Phe and Tyr in the PCG. For the previous study, brain Phe and Tyr results were expressed as ratios, with creatine as the denominator to account for variability in brain volumes. One of the subjects was scanned repeatedly five times with the same protocol to ensure reproducibility of the methods. Reproducibility of these measures in the human subject scanned repeatedly showed a variation of 9% for Phe and 22% for Tyr. The increased variability of Tyr is likely due to its lower concentration.

Comparisons with our results in individuals with PKU were in the expected direction for brain Phe. The mean brain Phe/creatinine ratio in individuals with PKU was 0.039 ± 0.008 , significantly higher than the brain Phe/creatinine ratio in controls which was 0.025 ± 0.004 (Mann–Whitney test, $p = 0.02$). Mean brain Tyr/creatinine ratios did not differ significantly between the PKU group and controls (0.0085 ± 0.0027 and 0.0076 ± 0.0018 , respectively). However, given the low concentration of Tyr and its variability, it is likely that there are not a sufficient number of subjects to show a difference, if it exists.

All participants with PKU were identified by newborn screening and treated with a Phe-restricted diet since infancy. None had comorbid physical health conditions and all were living independently. Four participants discontinued treatment in middle childhood, but eight were currently on a Phe-restricted diet, albeit with varying levels of metabolic control. Four received Tyr supplementation and one young woman (Participant #9) reported that she was taking supplemental large neutral amino acids (LNAAs), including Tyr, but did not restrict her protein intake.

Table 1 summarizes results from the participants with PKU. Blood and brain Phe and Tyr levels varied among these treated individuals. Blood Phe ranged from 277 to 1,512 $\mu\text{mol/L}$. Brain Phe ranged from 101 to 182 $\mu\text{mol/L}$ in PCG (gray matter region) and from 76 to 185 $\mu\text{mol/L}$ in PWM (white matter region). Blood Tyr ranged from 17 to 131 $\mu\text{mol/L}$. Brain Tyr ranged from 4.0 to 7.4 $\mu\text{mol/L}$ in gray matter and from 4.1 to 8.4 $\mu\text{mol/L}$ in white matter. Spearman rank correlations indicated that blood Phe correlated with blood Tyr ($\rho = -0.94$, $p < 0.01$) with higher blood Phe levels associated with lower blood Tyr. Blood levels of Phe and Tyr did not correlate significantly with brain levels of these biomarkers. Blood Phe levels were on average 6.7 times higher than brain Phe PCG levels and 7.0 times higher than brain Phe PWM levels. Blood Tyr levels were on average 12 times higher than brain Tyr levels in the PCG and 11 times higher than brain Tyr levels in the PWM. Brain Phe did not differ in white and gray matter regions, while Tyr levels tended to be higher in white matter than in gray matter.

Table 1 Description of the sample, biomarkers, neurological exam, and neuropsychological testing results

Participant #	1	2	3	4	5	6	7	8	9	Mean or %	SD
Sex	F	F	F	M	M	F	M	M	F	55% females	–
Age in years	32	25	38	32	31	27	22	27	31	29.44	4.72
Diet discontinued at some time in past?	Yes	No	Yes	No	Yes	No	Yes	No	Yes	55% Yes	
On diet?	Some	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	78% Yes	–
Supplements	No	No	No	Tyr	Tyr	No	No	Tyr	LNAA	44% Yes	–
Blood Phe $\mu\text{mol/L}$	1,189	771	862	277	708	1,336	865	743	1,512	918.08	373.27
Brain Phe (PCG) $\mu\text{mol/L}$	138	157	125	101	135	113	143	182	137	136.76	23.77
Brain Phe (PWM) $\mu\text{mol/L}$	185	118	138	76	161	109	149	78	159	130.11	37.88
Blood Tyr $\mu\text{mol/L}$	17	74	28	131	115	55	18	90	54	64.68	41.49
Brain Tyr (PCG) $\mu\text{mol/L}$	5.6	5.6	4.0	6.3	4.5	5.1	4.9	7.4	5.0	5.44	1.01
Brain Tyr (PWM) $\mu\text{mol/L}$	4.6	8.4	5.3	4.1	5.8	7.3	4.4	7.2	6.0	5.90	1.48
Neurological exam	Normal	Normal	Normal	Normal	Brisk reflexes	Normal	Postural tremor	Postural tremor	Normal	67% Normal	–
MRI changes	Moderate	Moderate	Mild	None	Moderate	Severe	Mild	Mild	Severe	89% Yes	–
Periventricular abnormality	Moderate	Moderate	Mild	Mild	Moderate	Severe	Mild	Mild	Severe	100% Yes	–
Restricted diffusion in white matter	Mild	Mild	None	None	Mild	Severe	Mild	None	Severe	67% Yes	–
Neuropsych. test											
WASI FS IQ	64	98	98	101	103	106	112	116	129	103.00	17.71
Verbal Fluency	12	12	–	12	12	12	12	9	15	12.00	1.60
Visual Motor Integration	77	103	92	103	103	–	103	92	107	97.50	9.94
Visual Perception	92	81	–	45	97	–	97	97	–	84.83	20.48
CVLT-1	–2.50	–1.00	–	–1.50	–1.00	–1.5	–1.00	0.00	0.05	–1.06	0.83
CVLT-5	–2.00	1.00	–	0.00	–1.00	0.00	–0.05	0.50	–1.50	–0.38	1.02
BRIEF-A, GEC	60	49	59	46	71	37	38	46	62	52.00	11.60
ABAS-II, GAC	94	116	96	110	118	108	115	113	96	107.33	9.50
Beck Anxiety	9	1	2	0	11	–	–	2	21	6.57	7.63
Beck Depression	12	1	14	0	7	1	0	2	2	4.33	5.36

Abbreviations: SD standard deviation, F female, M male, Tyr tyrosine, LNAA large neutral amino acid, Phe phenylalanine, PCG posterior cingulate gyrus (gray matter region of the brain), PWM periventricular white matter (white matter region of the brain), IQ intelligence quotient, Neuropsych neuropsychological

Neuropsychological tests:

WASI FS IQ: Wechsler Abbreviated Scale of Intelligence, Full-Scale IQ

Verbal Fluency: Delis–Kaplan Executive Function System, Verbal Fluency Subtest (normative mean = 10 ± 3)

Visual Motor Integration: Beery Test of Visual Motor Integration, Sixth Edition (normative mean = 100 ± 15)

Visual Perception: Beery Test of Visual Perception, Sixth Edition (normative mean = 100 ± 15)

CVLT-1: California Verbal Learning Test – Adult Version, Trial 1 (z-scores)

CVLT-2: California Verbal Learning Test – Adult Version Trial 5 (z-scores)

BRIEF-A, GEC: Behavior Rating Inventory of Executive Function – Adults, Global Executive Composite (scores greater than 65 indicate difficulties in executive functions)

ABAS-II, GAC: Adaptive Behavior Assessment System, Second Edition, General Adaptive Composite (normative mean = 100 ± 15)

Beck Anxiety: Beck Anxiety Inventory (Scores 0 – no anxiety; 1–7 minimal anxiety; 8–15 mild anxiety; 16–25 moderate anxiety; and 26–63 severe anxiety)

Beck Depression: Beck Depression Inventory, Second Edition (0 – no depression; 1–13 minimal depression; 14–19 mild depression; 20–28 moderate depression; and 29–63 severe depression)

Table 2 Spearman rank order correlation (ρ) between Phe biomarkers and neuropsychological tests

	Blood Phe	Brain Phe PWM	Brain Phe PCG	Blood Tyr	Brain Tyr PWM	Brain Tyr PCG
WASI Full-Scale IQ	0.23	−0.18	0.18	0.18	0.19	0.02
Beck Depression ^a	0.14	0.55	0.03	−0.31	0.09	−0.37
Beck Anxiety ^a	0.58	0.79	0.13	−0.38	0.13	−0.49
CVLT Trial 1	0.10	−0.05	0.45	0.12	0.32	−0.16
CVLT Trial 5 ^a	−0.41	−0.79	0.30	0.53	0.50	0.51
Visual Motor Integration	0.01	−0.08	−0.17	0.37	0.11	−0.15
ABAS-II (GAC)	−0.60	−0.18	0.28	0.58	0.22	−0.02
BRIEF-A (GAC)	−0.03	0.69	0.03	−0.02	−0.03	−0.34

Abbreviations: Phe phenylalanine, Tyr tyrosine, PCG posterior cingulate gyrus (gray matter region of the brain), PWM parietal white matter (white matter region of the brain), IQ intelligence quotient, WASI Wechsler Abbreviated Scale of Intelligence, Beck Depression Beck Depression Inventory, Second Edition (higher scores indicate greater depression), Beck Anxiety Beck Anxiety Inventory (higher scores indicate greater anxiety), CVLT-1 California Verbal Learning Test – Adult Version, Trial 1, CVLT-2 California Verbal Learning Test – Adult Version Trial 5, ABAS-II, GAC Adaptive Behavior Assessment System, Second Edition, General Adaptive Composite (higher scores indicate better functioning), BRIEF-A, GEC Behavior Rating Inventory of Executive Function – Adults, Global Executive Composite (higher scores indicate greater difficulties in executive functions)

^a Only eight subjects completed these measures

In the one sibling pair (Participants #3 and #5), blood Tyr levels were 27.9 $\mu\text{mol/L}$ (without Tyr supplementation) and 115.2 $\mu\text{mol/L}$ (with Tyr supplementation), but brain Tyr levels were similar (4.0 and 4.5 $\mu\text{mol/L}$ in gray matter; 5.3 and 5.8 $\mu\text{mol/L}$ in white matter, respectively). Both had similar scores on IQ tests and reported emotional disturbance (depression in one, anxiety in the other).

All study participants exhibited MRI changes in at least one domain, with five individuals rated as having moderate or severe changes overall, five with moderate or severe changes in periventricular regions, and two with severely diminished diffusion in white matter. Neurological examinations were rated as normal, although two individuals exhibited mild postural tremor and one young man had slightly brisk reflexes. Three of the five participants with moderate to severe MRI abnormalities had higher than average brain Phe levels in both white and gray matter. The two participants with severe structural MRI abnormalities (participants #6 and #9) had lower than average brain Tyr levels in gray matter, as well. Participant #9 who reported LNAA supplementation and an unrestricted diet exhibited severely abnormal MRI changes, periventricular abnormalities, and restricted diffusion, despite having a normal neurological exam and the highest IQ in the sample. She suffered clinically significant anxiety and had the highest blood Phe level in the sample, as well as high brain Phe levels in both PCG and PWM brain regions.

IQ ranged from 64 to 129, with all but one participant performing within the average range for the general population (IQ above 85). All but two research participants experienced anxiety or depression, as determined by scores above the cutoff on the self-reported Beck scales or reports

of being treated for these conditions. Verbal processing skills appeared intact, as indicated by scores generally higher than the population norm on the Delis–Kaplan Verbal Fluency subtest (where scores 7–13 represent the average range). Visual motor skills were also within the average range (85–115) for all but the young woman with IQ in the range of intellectual disabilities. Six of the eight individuals receiving the California Verbal Learning Test performed 1 standard deviation or more below the normative mean on the first trial (CVLT-1), indicating poor auditory memory/processing skills. Three of these individuals performed within a standard deviation of the normative mean after hearing the list of words 5 times (CVLT-5), suggesting that the issue may be more related to processing than to memory skills. One research participant received a score above the cutoff indicating executive functioning deficits (scores >65) on the BRIEF-A, but four participants rated themselves higher than the population norm of 50. None of the respondents indicated difficulties in day-to-day functioning as indicated by scores within the average range (85–115) or above on the ABAS-II.

Table 2 presents associations between Phe and Tyr biomarkers and neuropsychological testing results. Spearman rank order correlation coefficients (ρ) were largest for associations between brain Phe PWM and measures of auditory memory (CVLT, $\rho = -0.79$) and the self-reported measures of anxiety ($\rho = 0.79$) and executive functioning ($\rho = 0.69$). In general, associations were in the expected direction, with higher brain Phe and lower brain Tyr related to poorer functioning. The correlations tended to be stronger in white matter than in gray matter. Brain Phe and Tyr were not sensitive indicators of full-scale

IQ and measures of verbal fluency, visual motor integration, and overall adaptive behavior.

Discussion

This pilot study demonstrated the potential of the COSY method to measure even small elevations in brain Phe in white and gray matter. This study also demonstrated that it is possible to differentiate brain Tyr from brain Phe levels.

As measured by COSY, brain Phe and Tyr within different brain regions varied among individuals. If validated in future studies, this finding may provide answers to some of the more vexing questions regarding the specific neuropsychological deficits associated with PKU, despite lifelong treatment. The range of Phe was greater than the range of Tyr in both gray and white matter. This may suggest a capacity for the brain to establish equilibrium in terms of Tyr accumulation. This latter explanation is consistent with our finding that LNAA supplementation in one subject did not appear to result in elevated brain Tyr. Moreover, in our sibling pair, Tyr supplementation in one sibling compared to the other resulted in higher blood Tyr but no difference in brain Tyr. Alternatively, the prefrontal cortex and not the PCG may be the relevant site for investigating variations in Tyr levels in the brain, as has been suggested by previous investigators (Diamond et al. 1997; Bodner et al. 2012). Blood Tyr levels, as well as blood Phe levels, are known to fluctuate depending on recent food intake (Cleary et al. 2013). This potentially could also affect results of COSY measurements of brain Tyr.

Phe in white matter as compared to gray matter tended to be associated with neuropsychological testing results (specifically, depression, anxiety, auditory memory, and executive functioning). The opposite tended to be true for brain Tyr, which was more closely associated with executive functioning in gray matter as compared to white matter, consistent with the hypotheses proposed by Diamond et al. (1997).

Our study differed in some respects from previous studies. Pietz et al. (1999) assessed brain Phe in patients with PKU by quantitative 1H MRS during an oral Phe challenge with and without additional supplementation with all other LNAAs. Baseline plasma Phe was $\sim 1,000$ $\mu\text{mol/L}$ and brain Phe was ~ 250 $\mu\text{mol/L}$ in both series, with blood Phe 4 times greater than brain Phe. Significant correlations were found between plasma Phe concentrations and brain Phe only in the group without LNAA supplementation. We did not find close associations between blood and brain Phe levels. Moreover, participants in our study taking supplements, including the one on LNAAs, did not show decreased brain Phe. In the Pietz study, correlations between plasma and brain Phe were significant when specimens were obtained 12–24 h after a Phe loading.

Our values for blood and brain Phe were obtained within a few hours on the same day and without loading doses of Phe.

Möller et al. (2003) used proton MRS and noted that white matter alterations correlated with higher brain Phe and several untreated patients with normal IQ had lower concentrations of brain Phe despite high blood Phe concentrations. All but two of the participants in our study had white matter changes, despite variable brain Phe. The definition of white matter alterations or our inclusion of only early treated individuals may account for these discordant results.

Our results support findings by Weglage et al. (2001, 2002) who studied sibling pairs with disparate intellectual outcomes despite similar blood Phe concentrations. After Phe loading tests (100 mg/kg body weight), the children with the lowest IQ had the highest concentrations of Phe in the brain and higher degrees of white matter abnormalities. Although overall IQ did not correlate with brain Phe in our study, the one participant with intellectual disabilities (IQ = 64) had the highest brain Phe in white matter, a high concurrent blood Phe level, and the lowest blood Tyr level in the sample.

In previous studies using conventional MRS, spectral overlap prevented measurement of Tyr. In addition, these MRS methods encountered poor signal to noise ratios (SNRs), leading to relatively large error coefficients (see Pietz et al. (2002), with reply by Weglage et al. (2002)). Another limitation was the assumption that average brain Phe concentrations in large regions of interest reflected concentrations of brain Phe in smaller distinct regions. This assumption was based on work done by MacKean (1972) and had not been reevaluated. Finally, blood Phe and brain Phe appeared to be linearly related only at lower concentrations. Thus, Phe loading could have distorted the relationship between blood Phe and brain Phe due to potential saturation of Phe in the brain (Möller et al. 2000). Bik-Multanowski and Pietrzyk (2007) exposed many of these limitations in a study of brain MRS in 104 patients (ages 8–29 years) and ten healthy adult controls after an 8–12 h oral Phe load. Using the standard 1.5 T scanner, these researchers found that brain Phe intensity did not exceed the background signal in the majority of patients with blood Phe concentrations below 1,200 $\mu\text{mol/L}$. In the remaining cases, the intensity of signals was proportional to blood Phe concentrations, but could not be quantified due to the high variability of the background spectrum. These authors concluded, “Thus, although magnetic resonance spectroscopy allows semiquantitative assessment of brain phenylalanine signal, routine use of this method seems to be reasonable only in patients with massive hyperphenylalaninemia.”

In 2009, Kreis et al. established a method for measuring brain Phe that addressed some of these concerns. Using the

1.5 T MR scanner (Signa; GE), 28 subjects with PKU (ages 11–55 years) were investigated and in 17 subjects, long-term reproducibility was analyzed. Results indicated determination of brain Phe with a variation in independent sessions of 7 $\mu\text{mol/kg}$ Phe, or a coefficient of variance (CV) of 3%. SNR was optimized through long acquisition times and investigation of a large region of interest (ROI), as described by localization to the ROI performed with PRESS (echo time = 20 ms, repetition time = 2.0 s, acquisitions/spectrum = 256, supraventricular ROI approximately 70 cm^3 , and phase rotation cycle = 16 steps). While an improvement over previous methods, the Kreis method requires a large voxel, which does not allow for differentiation between gray and white matter. Furthermore, this method was optimized only for the detection of Phe and not Tyr.

Our pilot study was limited in its sample size and scope. The validity of COSY in PKU needs further investigation through additional studies in humans (affected individuals and controls) that include COSY under fasting and non-fasting conditions, as well as scans repeated over several days. Attention needs to be given to possible effects related to the LAT1 gene related to transport of amino acids across the blood–brain barrier. Patients with higher values of the Michaelis constant (a lower affinity of the transporter for Phe) and smaller $T_{\text{max}}/V_{\text{met}}$ ratios were found to have lower brain Phe concentrations at a given blood Phe level in the Möller (2003) study. Attention also needs to be given to the effects of long-term as well as concurrent blood Phe exposure. The PCG region was chosen for the current study because of the availability of control scans from another study. As noted above, future studies using COSY should include measurement of Phe and Tyr in the prefrontal cortex. Other LNAAs can and should be included in future studies.

In terms of neuropsychological outcomes, IQ appears to be too “blunt” of a measure for identifying the impact of brain biomarkers and is likely to reflect exposure to Phe during a critical period in early childhood rather than concurrent metabolic status (Waisbren et al. 2007). Rather than focusing on overall functioning, researchers should directly assess processing speed, auditory and visual memory, and executive functioning. Participants should complete questionnaires assessing anxiety and depression as well as health history forms that include prior and current treatments, including psychotherapy and psychotropic medications, since mood disorders appear to be a common symptom in PKU (Clacy et al. 2014) and may be particularly sensitive to brain metabolites.

Despite its limitations, this pilot study suggests hypotheses regarding pathological mechanisms in PKU. As noted, the higher the Phe level within brain tissue, the greater is the perturbation in some aspects of neuropsychological functioning. An important question is whether Phe is elevated in all

cells to the same degree or only in those that are pathological targets of toxicity. In our study, white matter appears to be associated with a variety of neuropsychological functions, suggesting the possibility that the cellular target is an oligodendroglial cell and/or the axon itself. Another important question is whether the toxicity associated with elevated Phe in these cells is due to Phe per se or a consequence of increased influx of Phe at LAT-1 with inhibition of other LNAAs such as Tyr into the cell. With the new COSY methodology, these questions can begin to be answered.

But for now, we conclude that with further validation, COSY will improve measurement of brain Phe and Tyr. Our study suggests that these biomarkers correspond to indices of neuropsychological functioning and that effects of brain Phe and Tyr may vary depending on their concentrations in specific brain regions and the neuropsychological domains evaluated.

Synopsis

Correlated Spectroscopy (COSY) measures brain phenylalanine (Phe) and tyrosine (Tyr) in distinct brain regions and may provide a means for understanding the variability in neuropsychological outcomes found in phenylketonuria (PKU).

Details of the Contributions of Individual Authors

Susan E. Waisbren, PhD, led the research team in planning the study, recruiting participants, conducting neuropsychological evaluations, analyzing and interpreting the data, and drafting the manuscript for publication.

Sanjay P. Prabhu, MD, participated in planning the study, obtaining and interpreting MRI findings, analyzing the data, and drafting the manuscript.

Patricia Greenstein, MD, conducted neurological examinations and participated in planning the study, analyzing the data, and drafting the manuscript.

Carter Petty, MA, conducted statistical analyses and participated in interpreting the results and drafting the manuscript.

Donald Schomer, MD, conducted neurological examinations and participated in planning the study, analyzing the data, and drafting the manuscript.

Vera Anastasoae participated in planning the study, collecting data, and critically reviewing the manuscript.

Kalin Charette participated in planning the study, collecting data, and critically reviewing the manuscript.

Daniel Rodriguez conducted the post-processing of the COSY data in controls, assisted in conducting the phantom studies, and contributed to the manuscript.

Sai Merugumala developed the software to reconstruct and quantify the COSY spectra, participated in the analyzing and interpreting of the data, and contributed to the manuscript.

Alexander P. Lin, PhD, participated in planning the study, obtaining MRI and COSY, conducting post-scanning analyses, analyzing and interpreting the data, and drafting the manuscript.

Dr. Waisbren serves as guarantor for the article.

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Competing Interest Statements

Dr. Waisbren consults to BioMarin Pharmaceuticals, Inc.

Vera Anastasoae serves as research coordinator for studies supported by BioMarin Pharmaceuticals, Inc.

Kalin Charette serves as research coordinator for studies supported by BioMarin Pharmaceuticals, Inc.

Drs. Greenstein, Prabhu, Schomer, and Lin and Mr. Carter, Mr. Rodriguez, and Mr. Merugumala have no competing interests and nothing to declare.

This study was approved by the Committee on Clinical Investigations (Institutional Review Board) at Boston Children's Hospital (IRB-P00003864) and all participants provided written informed consent.

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