

Plasma metabolomic profile changes in females with phenylketonuria following a camp intervention

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ABSTRACT

Background: There remains a limited understanding of the metabolic perturbations, beyond phenylalanine (Phe) metabolism, that contribute to phenotypic variability in phenylketonuria (PKU).

Objectives: This study aimed to characterize changes in the PKU plasma metabolome following a 5-d metabolic camp intervention and to compare PKU profiles with those of matched healthy controls.

Methods: In 28 females (aged 12–57 y), fasting plasma samples were collected on the first (day 1) and final (day 5) days of camp to measure metabolic control and to complete untargeted metabolomic profiling. Three-day dietary records were collected to assess changes in dietary adherence and composition. Univariate (Wilcoxon signed-rank and Mann–Whitney *U* test) and multivariate (random forest, hierarchical clustering) analyses were performed to identify clinical and metabolic features that were associated with the intervention and disease state.

Results: Relative to healthy controls, Phe catabolites, ketones, and carnitine- and glycine-conjugated fatty acids were elevated in females with PKU at baseline, whereas fatty acylcholine metabolites were substantially lower. After the camp intervention, plasma Phe concentrations decreased [median change: $-173 \mu\text{mol/L}$ (IQR: -325 , $-28 \mu\text{mol/L}$)] and 70% of PKU participants demonstrated improved dietary adherence by decreasing Phe intake and/or increasing medical food consumption. This was accompanied by a shift in abundance for 223 metabolites ($q < 0.05$). Compounds associated with the metabolism of Phe, fatty acids, and choline contributed most to profile differences between camp days 1 and 5.

Conclusions: In females with PKU, untargeted metabolomics identified prominent perturbations in amino acid and lipid metabolites associated with bioenergetic impairment and oxidative stress. Choline-conjugated lipids could have fundamental roles in these pathways and they have not been previously evaluated in PKU. A short-term camp intervention was effective for improving or fully normalizing the abundance of the identified discriminatory metabolites. *Am J Clin Nutr* 2022;115:811–821.

Keywords: phenylketonuria, PKU, metabolomics, metabolic control, choline, oxidative stress

Introduction

Phenylketonuria (PKU; Online Mendelian Inheritance in Man #261,600) is an inherited metabolic disorder of amino acid metabolism that results in absent or defective phenylalanine hydroxylase (EC 1.14.16.1) enzyme activity (1). To prevent Phe accretion and the associated neurocognitive deficits, PKU management has traditionally required a Phe-restricted diet with Phe-free or Phe-reduced amino acid (AA-MF) or glycomacropeptide (GMP-MF) medical foods to meet protein requirements (2, 3). More recently, however, 2 adjunct pharmacotherapies, sapropterin dihydrochloride (Kuvan; BioMarin Pharmaceutical Inc) and pegvaliase (Palynziq; BioMarin Pharmaceutical Inc), have become available to maintain Phe concentrations closer to or within the therapeutic range ($120\text{--}360 \mu\text{mol/L}$) while

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Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

Supplemental Figures 1 and 2 and Supplemental Tables 1–4 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/ajcn/>.

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Abbreviations used: AA-MF, Phe-free amino acid medical food; ESI, electrospray ionization; FC, fold change; FDR, false discovery rate; GMP-MF, Phe-reduced glycomacropeptide medical food; HCA, hierarchical clustering analysis; MURDOCK, Measurement to Understand Reclassification of Disease of Cabarrus and Kannapolis Study; PCA, principal component analysis; PKU, phenylketonuria; RF, random forest; RP, reverse phase; UPLC, ultra-performance liquid chromatography.

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allowing for a more liberal or unrestricted intake of dietary protein. Despite the early initiation and continuous use of these management strategies, many individuals with PKU still exhibit abnormalities in brain morphology (4–6), experience a higher incidence of psychiatric illness, and demonstrate notable cognitive impairments, particularly in executive function (7, 8).

Although the importance of Phe is undisputed, it is well recognized that several vitamins, minerals, trace elements, and fatty acids can also contribute to the pathophysiological changes in PKU (9). Changes in treatment adherence and/or treatment modality (e.g., diet therapy compared with adjunct pharmacotherapy) can alter both Phe control and the intake of other neurologically relevant nutrients. Yet, prior studies have been unable to evaluate how these factors interact to ultimately impact treatment outcomes. High-resolution metabolomics is a useful approach for evaluating metabolic changes associated with the confluence of multiple, complex exposures. Previous studies have leveraged this method to cross-sectionally evaluate the pathophysiology of PKU (10, 11) and determine the biochemical impact of different dietary treatments (12–15). These studies, however, have not assessed how the PKU metabolome shifts in response to short-term changes in treatment adherence. This inquiry is significant because it could identify notable changes in the metabolic milieu, beyond that of Phe metabolism, which could contribute to the phenotypic variability in PKU.

The present study aims to fill this gap by evaluating metabolite profile changes in females with PKU who participated in a 5-d dietary intervention in a camp setting. This camp provides a unique intervention model that has previously been found to improve dietary adherence, social support, and nutritional status (16). To further explore the impact of this camp intervention, the present study used untargeted metabolomics to analyze changes in the plasma profiles of females with PKU between the first and final days of camp, and to compare PKU profiles with those of healthy controls at baseline and the end of camp. We hypothesized that plasma metabolite abundance would differ after the camp intervention and discriminate between PKU participants and controls.

Methods

Sample and study design

The Emory Metabolic Camp (Atlanta, GA) was established in 1995 as a research-based camp, and provides a 5-d experience in a supervised domicile environment for adolescent and adult females with PKU or other inborn errors of metabolism (e.g., maple syrup urine disease). A detailed description of the camp and its objectives has been published (16). Briefly, metabolic camp provides a multicomponent approach for improving disease management through group counseling, enrichment activities, educational seminars, and the provision of medical food and low-protein modified foods. Additionally, most meals are prepared on site by a certified chef and registered dietitian, who provides a wide range of food items to meet each participant's specific protein goals. Camp counselors, who are trained research registered dietitians, teach campers how to assemble meals that comply with their dietary prescriptions. Prior evaluation of the camp has demonstrated that these unique components

collectively enhance knowledge, reduce barriers to treatment adherence, and promote social support.

For the present study, differences in metabolite abundance based on the intervention and disease state were the primary outcome measures. Changes in Phe concentration and dietary composition were evaluated as secondary outcomes within the PKU cohort. Inclusion criteria included a diagnosis of PKU and an adequate volume of stored plasma, which was available for 28 of the 46 females who consented to research during the 2016 and 2017 Emory Metabolic Camps (**Supplemental Figure 1**). Using 2 plasma samples from each included female (total $n = 56$), changes in plasma metabolites were evaluated between day 1 and day 5 of camp. At both time points, PKU metabolite profiles were compared with healthy controls—matched on age, sex, and self-reported race—whose samples were provided by the Duke University Measurement to Understand Reclassification of Disease of Cabarrus and Kannapolis (MURDOCK) biorepository (Kannapolis, NC). All procedures were in accordance with the ethical standards of the Emory University Institutional Review Board. Written informed consent was obtained from all adult participants and the legal guardians of pediatric participants. Assent was additionally obtained from all pediatric participants.

Data collection

Participant demographics, medications, and medical history were obtained via paper packets that were mailed to each camper's home several weeks prior to camp. After completion, packets were reviewed by the camp research coordinator with each participant to ensure the reported information was accurate. Each participant's registered dietitian or health care provider was also contacted to obtain a current diet prescription. At baseline (camp day 1) and end line (camp day 5), anthropometry (height, weight, hip and waist circumferences), 3-d dietary records, and fasting plasma samples were collected at the Georgia Clinical & Translational Science Alliance Clinical Research Center at Emory University Hospital. Plasma samples were frozen at -80°C and shipped to Metabolon (Metabolon Inc, Research Triangle Park, NC) and LabCorp (Laboratory Corporation of American, Burlington, NC) for metabolomics and plasma amino acid analysis, respectively. Three-day dietary records were reviewed and analyzed by a registered dietitian using MetabolicPro 1.0 diet analysis software.

For the MURDOCK control subjects, 28 frozen EDTA plasma aliquots were sent to Metabolon to be analyzed with the PKU samples. Qualitative questionnaires were utilized to obtain general information on diet; however, this information was not sufficient to quantify the nutrient composition of control diets.

Metabolomics analysis

Untargeted metabolomics analysis was completed by Metabolon on deidentified plasma samples. Prior to analysis, methanol and centrifugation were used to facilitate protein precipitation and recover chemically diverse metabolites. All samples were spiked with noninterfering standards for quality control. Sample extracts were then divided into fractions and analyzed using 3 different methods to optimize the capture of

both hydrophilic and hydrophobic metabolites: 1) reverse phase ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (RP/UPLC-MS/MS) with positive ion mode electrospray ionization (ESI); 2) RP/UPLC-MS/MS with negative ion mode ESI; and 3) hydrophilic interaction chromatography UPLC-MS/MS with negative ion mode ESI. For all methods, a Waters ACQUITY UPLC was used with a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer interfaced with a heated ESI source and Orbitrap mass analyzer (35,000 mass resolution). The scan range covered was 70–1000 *m/z*. Raw data were extracted, peak-identified, and quality control processed using Metabolon's hardware and software. Peaks were quantified using AUC and normalized for interday variability using block correction. After quantification, a total of 892 compounds were identified via comparison with Metabolon's proprietary library of authenticated standards or recurrent unknown entities. All annotations met the criteria for a level 1 or level 2 identification confidence score as defined by the Metabolomics Standards Initiative (17).

Data processing and statistical analysis

Prior to analyzing the metabolomics data, metabolic features were normalized to the volume extracted, missing values were imputed using *k*-nearest neighbors (18), and feature intensities were converted to \log_{10} -normalized values. Statistical analyses were performed using MetaboAnalyst 4.0 and 5.0 (19), SAS (version 9.4; SAS Institute), and R (version 4.0.3; R Foundation for Statistical Computing). Statistical significance was based on 2-sided hypothesis tests and an α value <0.05 . For univariate tests conducted on the metabolomics data, the false discovery rate (FDR) procedure of Benjamini and Hochberg (20) was applied to collectively adjust *P* values for multiple comparisons. For these analyses, FDR-adjusted *q* values <0.05 were considered statistically significant unless specified otherwise.

Data visualization and differential abundance analysis

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were used to visualize relations among samples in the PKU and control groups, as well as within the PKU group. To identify metabolites that differentiated between disease states (PKU compared with matched controls) and the camp intervention (PKU day 1 compared with PKU day 5), The Mann–Whitney *U* test and the Wilcoxon signed-rank test were used, respectively. Within the PKU sample, subgroup analyses based on age (adults compared with pediatric participants) and treatment (diet compared with adjunct pharmacotherapy) were planned a priori and conducted using the Mann–Whitney *U* test. For these analyses, PKU participants taking sapropterin or pegvaliase were combined into a single adjunct pharmacotherapy group, given that only 4 of the 28 participants were taking pegvaliase.

In the aforementioned independent, 2-group comparisons, metabolites were considered to significantly differ between the groups if FDR adjusted *P* values were <0.05 and geometric mean fold changes (FCs) were $> \pm 1.5$ ($\log_{10}\text{FC} > \pm 0.176$). For paired comparisons (e.g., camp day 5 compared with camp day

1), differentiating metabolites were also required to meet the FC criteria in $>75\%$ of the PKU sample.

Pathway analysis and feature selection

After filtering metabolites using univariate tests, metabolic pathway testing was conducted using the hypergeometric test (21). This method tested for overrepresentation of pathways in a selected group of metabolites that met the *q*-value and FC criteria specified above. This test calculated *P* values based on the hypergeometric distribution. Given the interdependency between the pathways and the limited number of pathways under study, we used a stringent *P*-value cutoff ($P < <0.01i>$) to partially account for multiple testing, rather than adjusting the *P* values to FDR.

To further select features that distinguished PKU participants from controls, random forest (RF) was used (22). For this analysis, metabolite importance was estimated based on a permutation procedure (23). Top metabolites were selected by thresholding the importance scores at the elbow point of the curve of importance scores (Supplemental Figure 2).

Diet and metabolite correlations

Within the PKU sample, changes in nutrient intake and treatment adherence from baseline to day 5 were evaluated using the Wilcoxon signed-rank test. To determine if these changes were associated with shifts in metabolite abundance, Spearman rank correlation was used among nutrients and metabolites that significantly altered with the intervention.

Results

Baseline demographic and treatment characteristics are detailed in Table 1. The PKU cohort comprised 15 adult and 13 pediatric females. Camp participants were predominantly Caucasian with a median (IQR) age of 18 (15, 22) y. Although 15 (53.6%) of the 28 participants were receiving adjunct pharmacotherapies (sapropterin or pegvaliase), 10 (66.7%) were still consuming AA-MF or GMP-MF, and 7 (46.7%) had Phe concentrations that exceeded the recommended therapeutic range (120–360 $\mu\text{mol/L}$). In the full sample, 18 (64.3%) participants had Phe concentrations that exceeded the desired range, and the median (IQR) plasma Phe concentration was 591.5 $\mu\text{mol/L}$ (223.5, 1075 $\mu\text{mol/L}$). Of the 28 participants, 20 (12 on diet therapy, 6 on sapropterin therapy, and 2 on pegvaliase therapy) had complete dietary intake and prescription data that were used to evaluate baseline dietary treatment adherence. Within this subsample, 80% demonstrated nonadherence to their dietary prescription at baseline due to excess consumption of Phe ($n = 10$), suboptimal medical food intake ($n = 2$), or both ($n = 4$). In the matched controls, the median (IQR) BMI was 22.5 kg/m^2 (20.7, 25.3) for adults and the BMI percentile was 74% (51%, 81%) for pediatric participants. Median BMI was higher in PKU adults (28; IQR = 21.3, 34.3) and pediatric participants (84%; IQR = 75, 87%); however, there were no notable differences between the matched pairs ($P = 0.09$). Based on self-reported medical history collected by MURDOCK, no controls had significant medical conditions. One control, however, was later found to have cystic fibrosis through analysis of participant medications. Because this was not identified prior to metabolomics analysis, the matched

TABLE 1 Baseline demographic and clinical characteristics for PKU participants and healthy controls whose samples were used for metabolomics analysis¹

Variable	Adult PKU (<i>n</i> = 15)	Pediatric PKU (<i>n</i> = 13)	Matched controls (<i>n</i> = 28)
Age, y	22 (19, 24)	15 (14, 15)	18 (18, 22)
Caucasian ethnicity, <i>n</i>	15 (100.0)	12 (92.3)	27 (96.4)
Camp year, <i>n</i>			
2016	5 (33.3)	4 (30.8)	–
2017	10 (66.7)	9 (69.2)	–
PKU therapy, ² <i>n</i>			
Diet only	7 (46.7)	6 (46.2)	–
Sapropterin dihydrochloride	4 (26.7)	7 (53.9)	–
Pegvaliase	4 (26.7)	0 (0)	–
BMI, ³ kg/m ²	28 (21.3, 34.3)	–	22.5 (20.7, 25.3)
BMI percentile ³	–	84 (75, 87)	74 (51, 81)
Plasma Phe, μmol/L	603 (105, 1335)	580 (321, 834)	–
Plasma Tyr, μmol/L	42 (33, 47)	42 (29, 52)	–
Plasma Phe:Tyr ratio	17.2 (4.0, 27.5)	16.7 (5.9, 28.4)	–
Nonadherence to diet Rx, ⁴ <i>n</i>	11 (55.0)	9 (45.0)	–
Phe intake above Rx, <i>n</i>	3 (27.8)	7 (77.8)	–
Medical food intake below Rx, <i>n</i>	2 (18.2)	0 (0)	–
Both, <i>n</i>	3 (27.3)	1 (11.1)	–

¹Values for continuous variables are medians (IQR); for categorical variables, numbers within parentheses represent percentages. AA-MF, Phe-free amino acid medical food; GMP-MF, Phe-reduced glycomacropeptide medical food; PKU, phenylketonuria; Rx, prescription.

²Twenty-three of the 28 participants were consuming medical foods (*n* = 13 on diet therapy only, *n* = 9 on sapropterin, and *n* = 1 on pegvaliase). Four participants were consuming GMP-MF (*n* = 2 on diet therapy only, *n* = 2 on sapropterin) and 2 were consuming a combination of GMP-MF and AA-MF (both were on diet therapy only).

³BMI is reported for participants ≥ 18 y of age (*n* = 15); BMI percentile is reported for participants < 18 y of age (*n* = 13) and determined using the CDC calculator.

⁴Reported only for a subsample (total *n* = 20; *n* = 11 adult, *n* = 9 pediatric) with complete diet and treatment prescription information.

pair involving this control was not excluded from the analytic sample.

Baseline plasma metabolome based on disease state, treatment status, and age group

PCA demonstrated distinct clustering of metabolic features by disease state; however, there was minimal separation between adults and pediatrics in both the PKU and control groups (Figure 1A). Univariate analysis identified 265 metabolites that differed (*q* < 0.05) by disease state (Supplemental Table 1). HCA among the top 25 metabolites showed increased expression of compounds derived from Phe, glutathione, glycine, purine, and microbiome-associated tryptophan metabolism. The branched-chain amino acid leucine and several choline derivatives, including fatty acylcholines, glycerophosphocholines, and lysophospholipids, were prominently downregulated in PKU participants compared with controls (Figure 2A). To further select features that distinguished PKU participants from controls, overrepresentation analysis and RF were performed on 166 of the metabolites identified by the Mann–Whitney *U* test whose abundance was > 1.5-fold or < 0.667-fold in PKU participants relative to controls. Within this subset, significant enrichment was identified for 13 metabolic pathways (Figure 2B). Fatty acid metabolism, and particularly pathways involving carnitine conjugated analytes, were the most enriched in the PKU group, whereas fatty acylcholine and plasmalogen metabolism were more enriched in controls. From the RF algorithm, 25 metabolites were identified as effective classifiers of disease state (Figure 2C).

Within the PKU sample, few metabolites significantly differentiated between age groups, with the exception of the amino acid

sarcosine (mean log₁₀FC: −0.25; 95% CI: −0.34, −0.17) and the dicarboxylic amine, iminodiacetate (mean log₁₀FC: −0.30; 95% CI: −0.40, −0.20), which were both less abundant in adults. When considering treatment differences, there were 43 metabolites that differed between PKU participants on diet and adjunct pharmacotherapies (*P* < 0.05); however, none were significant after FDR adjustment (Supplemental Table 2). This finding was further supported by PCA, which did not identify distinct metabolite profiles across PKU treatment groups or medical foods (GMP-MF compared with AA-MF) (Figure 1B).

Changes in biochemical control and dietary composition associated with the metabolic camp intervention

After the 5-d camp intervention, participants experienced a notable shift in Phe control (*P* < 0.0001). The median reduction in plasma Phe was 173 μmol/L (IQR: −325, −28 μmol/L). This shift did not significantly differ based on age group (*P* = 0.72) but was more pronounced in females who were solely on diet therapy [median plasma Phe change diet therapy: −313 μmol/L (IQR: −345, −176 μmol/L); median plasma Phe change adjunct pharmacotherapies: −76 μmol/L (−246, 15 μmol/L)]. Although plasma Phe concentrations remained above the therapeutic range (120–360 μmol/L) for 16 participants (57.1% of total), 9 females maintained Phe concentrations within the recommended range throughout the camp intervention, and 2 were able to shift their Phe concentrations into the desired range. In 1 participant who exhibited good Phe control at baseline, Phe concentrations were > 360 μmol/L after the intervention.

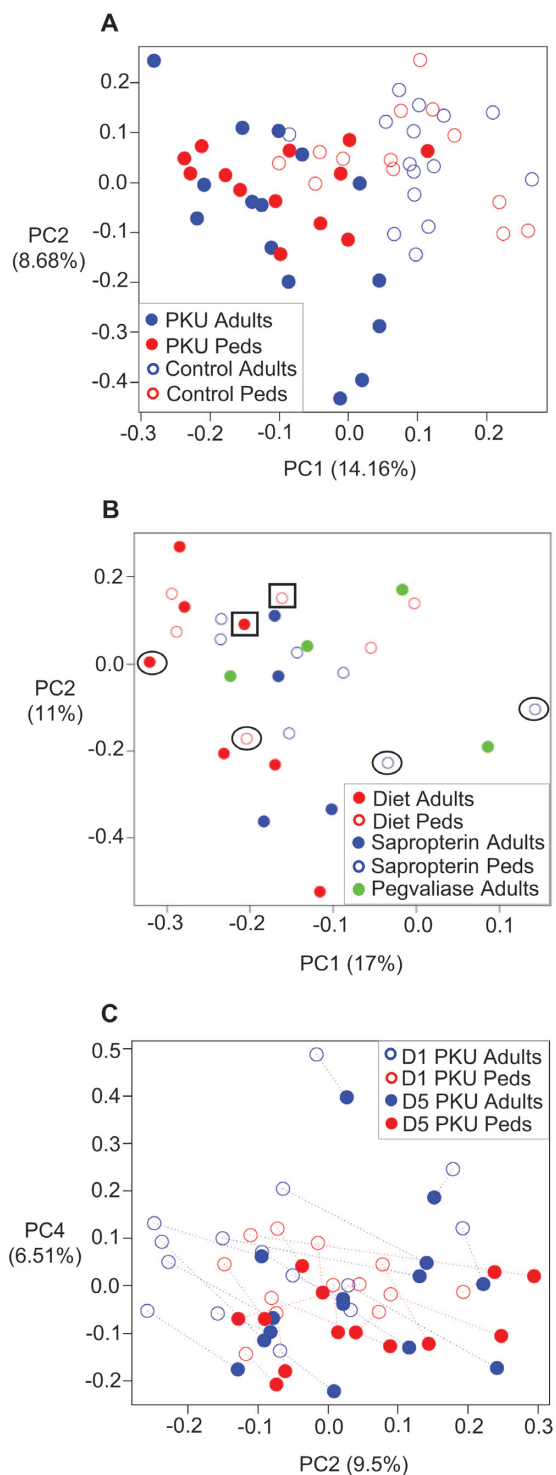


FIGURE 1 Principal component analysis (PCA) of untargeted plasma metabolomics data collected from healthy controls ($n = 28$) and PKU participants ($n = 28$). (A) PCA score plot of control and PKU samples from day 1 of camp, according to age group. (B) PCA score plot of PKU samples from day 1 of camp according to treatment and age groups. Samples with black circles ($n = 4$) represent participants consuming glycomacropeptide medical foods and samples with black squares represent participants consuming a combination of glycomacropeptide and amino-acid medical foods ($n = 2$). (C) PCA score plot of PKU samples on days 1 and 5 of camp, according to age group. Paired samples are connected with a dotted line. D1–D5, days 1–5; PC, principal component; Peds, pediatrics; PKU, phenylketonuria.

Changes in plasma Phe concentrations were accompanied by important alterations in diet composition. Within the subsample of participants with complete diet and prescription information ($n = 20$ total), 70% improved dietary adherence by increasing medical food intake ($n = 5$) or decreasing Phe consumption ($n = 9$) in alignment with prescribed amounts. These improvements were observed in participants on both diet therapy and adjunct pharmacotherapies, and a reduction in Phe consumption was the most prominent change in both groups (**Supplemental Table 3**). By the final day of camp, 65% of the participants were meeting their prescription goals for both dietary Phe and medical food intake. This overall shift in adherence did not significantly change macronutrient or micronutrient distribution, and nutrient intakes remained below the age-specific recommendations. Nevertheless, there was a notable decrease in the consumption of protein from intact food sources ($\Delta = -4.7$ g; 95% CI: $-7.8, -1.5$ g), which was paralleled by a decrease in dietary Phe. Dietary fiber intake ($\Delta = 3.15$ g; 95% CI: 0.29, 6.01 g) also increased over the intervention period, and might reflect greater consumption of fruits and vegetables (**Table 2**).

Changes in the PKU plasma metabolome associated with the metabolic camp intervention

PCA demonstrated minimal separation of PKU plasma samples based on the intervention (day 1 compared with day 5); however, interindividual sample segregation was evident (**Figure 1C**). These shifts were further substantiated by the Wilcoxon signed-rank test, which identified 232 metabolites (26% of total identified) that significantly changed with the camp intervention (**Supplemental Table 4**). Among these metabolites, there were no remarkable differences in the directionality or magnitude of change between adult and pediatric participants (all $q > 0.05$). The change in 1 metabolite, phenylacetate, significantly differed ($q = 0.01$) between PKU treatment groups. After the intervention, the abundance of this Phe catabolite decreased in both groups, but the decline was greater in participants receiving diet therapy (mean $\log_{10}FC$: -0.42 ; 95% CI: $-0.53, -0.30$) compared with those receiving adjunct pharmacotherapies (mean $\log_{10}FC$: -0.09 ; 95% CI: $-0.39, 0.02$).

To further identify metabolites that most prominently changed with the intervention, FC criteria were applied to the discriminatory compounds obtained from the Wilcoxon signed rank test. Seven of the 232 metabolites were found to have an $FC > \pm 1.5$ in $>75\%$ of the sample (**Figure 3**). The most substantial fold decrease and fold increase were exhibited by phenylacetylcarnitine (mean $\log_{10}FC$: -0.45 ; 95% CI: $-0.56, -0.35$) and palmitoylcholine (mean $\log_{10}FC$: 0.32; 95% CI: 0.19, 0.45), respectively. Relative to controls, the abundances of 3-hydroxybutyrate, tridecenediote, palmitoylcholine, and the Phe catabolites became more similar to controls after the 5-d intervention. This trend was echoed among almost all of the amino acids and lipids that differentiated PKU samples from controls at baseline (**Supplemental Table 1**).

Correlations between nutrient intake and metabolite changes

To determine if the identified metabolic shifts were related to changes in dietary intake, correlations were examined

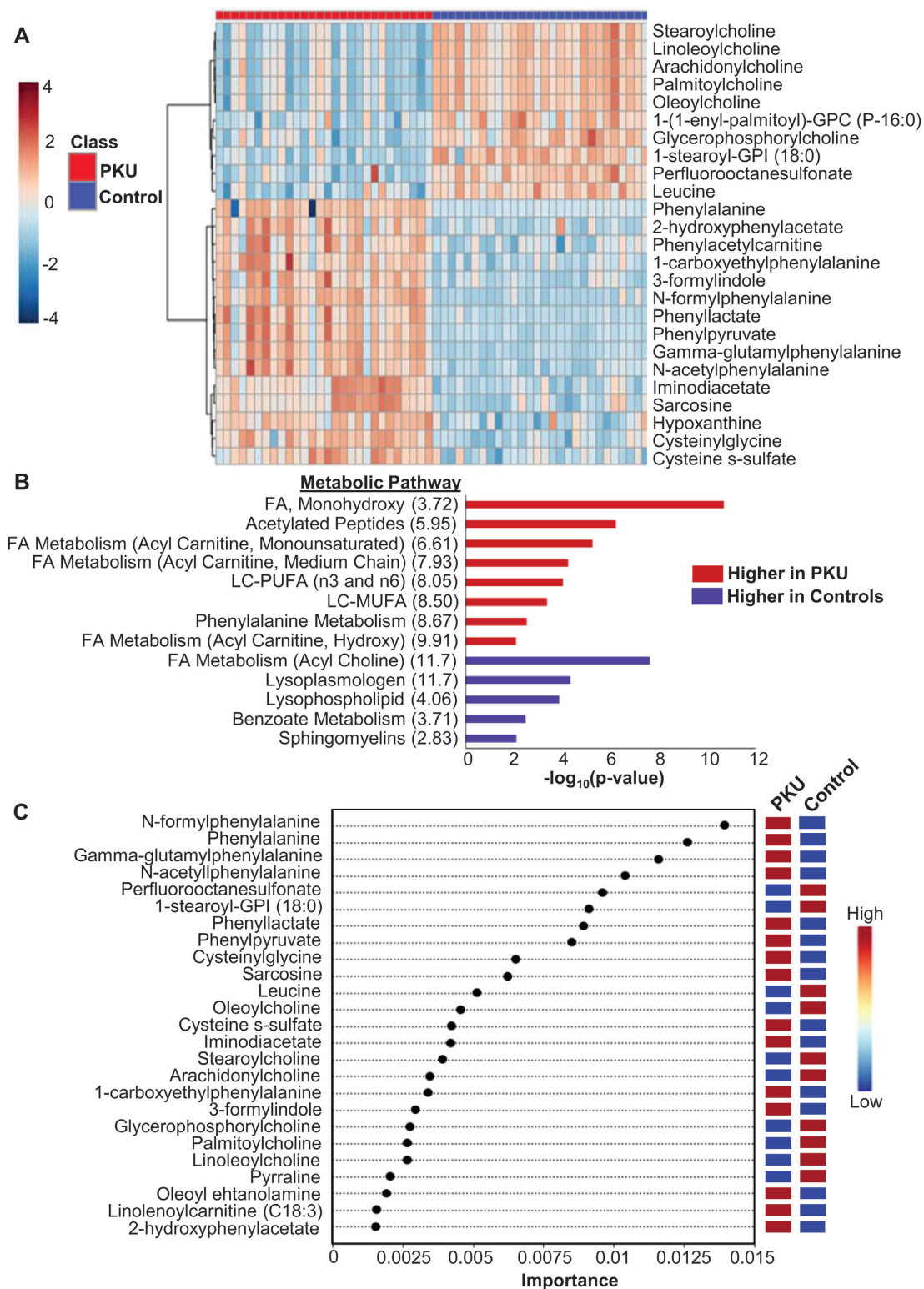


FIGURE 2 Differences in plasma metabolites between PKU participants ($n = 28$) and healthy, matched controls ($n = 28$). (A) HCA of the top 25 metabolites that differed between PKU participants on day 1 of camp and matched controls based on q -values from the Mann–Whitney U test. For class, red represents PKU and blue represents controls. For the expression level of each metabolite, red represents high and blue represents low. (B) Metabolic pathways selected by overrepresentation analysis performed on 166 metabolites that differentiated PKU participants from controls based on q -value (< 0.05) and FC criteria (FC $> \pm 1.5$ -fold). P values were calculated based on the hypergeometric distribution and only pathways with a $P < 0.01$ are represented. Fold enrichment is in parentheses next to each pathway name. (D) The top 25 metabolites selected by RF based on a permutation procedure performed on the 166 metabolites that met the q -value and FC criteria (detailed above). Metabolites are ordered based on feature importance with an expression heatmap, which indicates high (red) or low (blue) expression in controls and PKU participants on day 1 of camp. FA, fatty acid; FC, fold change; GPC, glycerophosphocholine; GPI, glycosylphosphatidylinositol; HCA, hierarchical clustering analysis; LC, long chain; MUFA, monounsaturated fatty acid; PKU, phenylketonuria; PUFA, polyunsaturated fatty acid; RF, random forest.

TABLE 2 Nutrient intakes for PKU participants on day 1 and day 5 of metabolic camp¹

Nutrients ²	Day 1		Day 5		<i>P</i> ⁴
	Intake	Meeting DRI, ³ %	Intake	Meeting DRI, ³ %	
Total energy, kcal	1474.1 (1361.7, 1644.2)	15	1659.3 (1100.9, 2185.6)	40	0.50
Kilocalories from protein, %	13.9 (12.0, 17.4)	85	13.5 (11.3, 16.1)	80	0.78
Kilocalories from carbohydrate, %	53.5 (50.0, 61.5)	85	59.0 (53.5, 65.0)	80	0.06
Kilocalories from fat, %	27.5 (25.0, 31.5)	75	26.5 (24.0, 31.5)	85	0.64
Dietary fiber, g	8.9 (8.1, 12)	0	11.2 (8.8, 17.1)	5	0.02
Total protein, g	56.6 (43.9, 68.6)	25	55.3 (47.3, 61.4)	30	0.78
Intact protein, g	14.7 (9.9, 27.9)	–	11.1 (6.4, 17.1)	–	0.006
Medical food protein, g	39.5 (13.5, 54.6)	–	43.5 (18.9, 53.5)	–	0.30
Phenylalanine, mg	709.0 (401.5, 1044)	65	464.5 (240.0, 761)	70	0.007
Micronutrients					
Vitamin D, IU	378 (123.6, 594.6)	25	448.2 (131.4, 595.3)	25	0.44
Vitamin B-12, µg	4.7 (2.1, 9.0)	75	5.3 (2.2, 9.2)	75	0.25
Choline, mg	184.0 (107.0, 225.5)	10	202.0 (135.5, 267.5)	10	0.49
DFE, µg	753.1 (447.9, 135.6)	80	839.9 (352.8, 1378.8)	75	0.81
Calcium, mg	1204.5 (537.5, 1671)	50	1285.0 (555.0, 1809.5)	60	0.20
Iron, mg	19.6 (10.7, 25.6)	65	19.8 (9.6, 26.5)	65	0.76
Magnesium, mg	342.0 (196.0, 469.0)	55	377.5 (169.5, 471.5)	65	0.82
Selenium, µg	65.2 (41.9, 83.2)	55	63.0 (41.0, 83.9)	65	0.81
Potassium, mg	2565.0 (1800.5, 3116.5)	50	2611.5 (1643.0, 3229.0)	50	0.37
Zinc, mg	13.5 (7.0, 25)	75	13.0 (7.0, 23.5)	75	0.79

¹Reported for a subsample ($n = 20$) with complete diet and treatment prescription information. DFE, dietary folate equivalents; PKU, phenylketonuria.

²Values are medians (IQR).

³Reference for percentage of total calories from protein, carbohydrates, and fat is the acceptable macronutrient distribution range (19); references for dietary fiber and all micronutrients are the DRIs (19); reference for total protein and phenylalanine intakes is the Genetic Metabolic Dietitians International PKU Nutrition Management Guidelines (2).

⁴*P* values compare day 1 and day 5 nutrient intakes within each participant based on Wilcoxon signed-rank tests.

between the 3 nutrients (dietary fiber, intact protein, dietary Phe) and 7 metabolites (3-hydroxybutyrate, phenylacetylcarnitine, 2-hydroxyphenylacetate, tridecenedioate, phenylpyruvate, stearoylcholine, palmitoylcholine) that prominently altered with the camp intervention. When applying an FDR threshold of 0.2, no significant associations were found. Given that only a subgroup of the PKU sample had complete dietary data and this was an exploratory analysis, unadjusted results are reported in **Table 3**. Changes in 2-hydroxyphenylacetate, which decreased in abundance after the intervention, were moderately associated with decreased intakes of Phe ($r_s = 0.52$; $P = 0.02$) and intact protein ($r_s = 0.50$; $P = 0.03$). This analysis also identified a tendency for tridecenedioate, which decreased with the intervention, to decline less as dietary fiber intakes increased ($r_s = 0.44$; $P = 0.05$).

Discussion

Given the wide range of phenotypic variability in PKU, it is critical to develop intervention programs and monitoring strategies that target more than blood Phe. The present study leveraged both common clinical indicators (diet records, plasma amino acids) and high-resolution metabolomics to investigate short-term changes in the plasma metabolome of females with PKU following a 5-d camp intervention. This comprehensive analytic approach lends new insight to the findings of a prior study on the same camp model (16) by elucidating key metabolic

pathways, beyond Phe metabolism, that altered with the camp intervention.

At baseline, Phe catabolites and fatty acid derivatives were substantially elevated in PKU participants relative to controls. This was particularly evident for glycine- and carnitine-conjugated fatty acids, which encompassed long-chain, medium-chain, short-chain, hydroxy, and dicarboxylate forms. Because these metabolites represent alternate oxidation or conversion products of nonoxidized acyl-CoA esters, elevated abundances in PKU participants might indicate mitochondrial β -oxidation and/or respiratory chain impairment (24). This observation was further substantiated by increased abundances of the ketone body, 3-hydroxybutyrate, and decreased abundances of the ketogenic amino acids leucine and threonine (Supplemental Table 1). This could reflect the inhibition of fuel utilization pathways and the use of alternate energetic substrates (25).

Prior literature suggests that these metabolic alterations could be a direct or indirect consequence of excess Phe and its toxic catabolites. Rat models of hyperphenylalaninemia have demonstrated that Phe can directly inhibit enzyme activity within all energetic pathways in the brain, including glycolysis, tricarboxylic acid cycle, respiratory transport chain, phosphocreatine-creatine metabolism, and ketone body synthesis and utilization. Alternately, sustained elevations of blood Phe could indirectly trigger the aforementioned bioenergetic changes by enhancing the production of reactive oxygen species (26) and modulating the activity of the plasma membrane Ca^{2+} -ATPase (27). The resulting accumulation of reactive oxygen species and calcium not only enhances cellular vulnerability to oxidative damage (28),

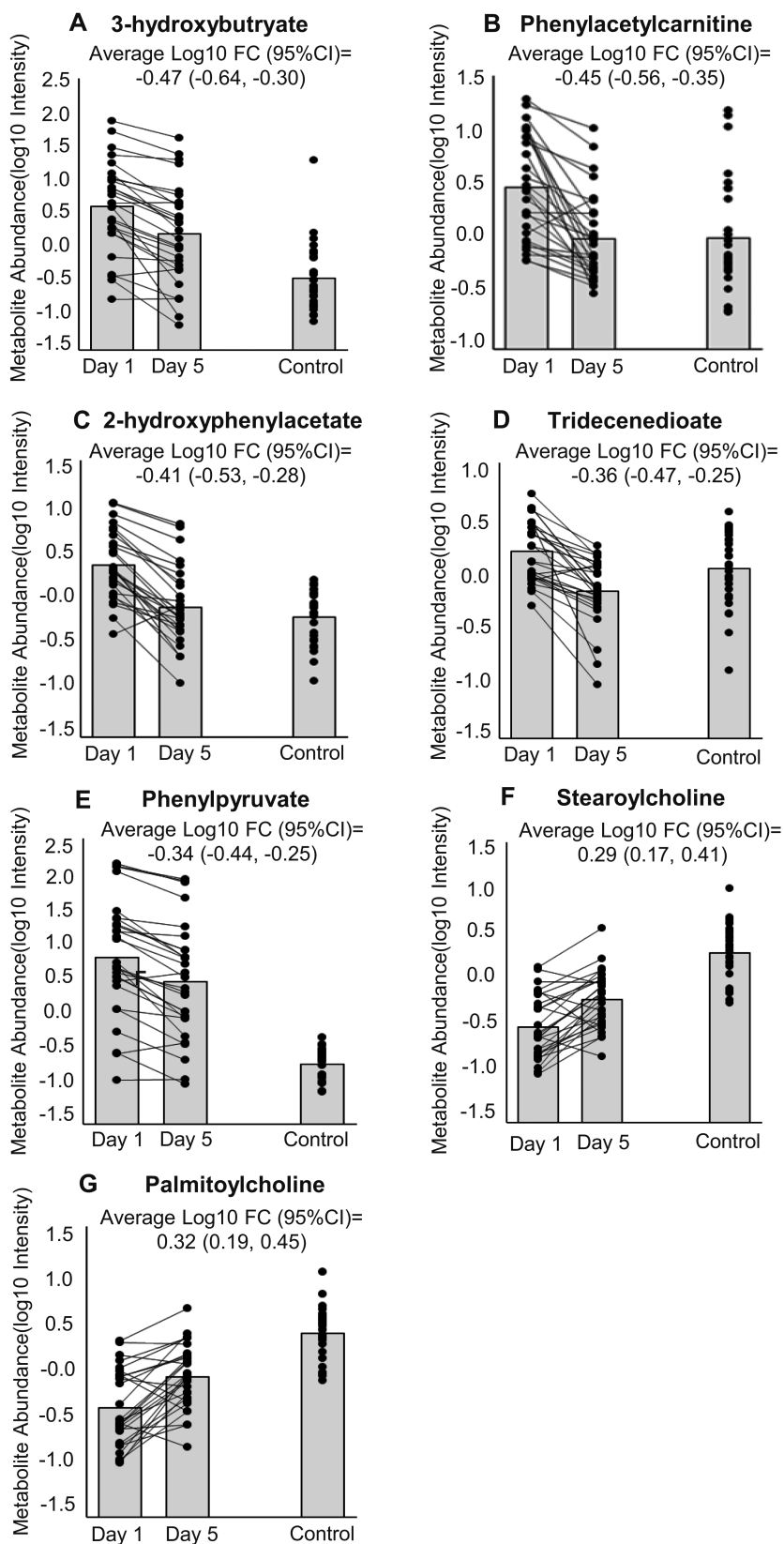


FIGURE 3 Scatter plots for 7 metabolites that significantly ($q < 0.05$) and consistently ($\log_{10}\text{FC} > 0.176$ or < -0.176 in $>75\%$ of the sample) altered in PKU participants ($n = 28$) with the camp intervention based on the Wilcoxon signed-rank test: (A) 3-hydroxybutyrate, (B) phenylacetylcarnitine, (C) 2-hydroxyphenylacetate, (D) tridecenedioate, (E) phenylpyruvate, (F) stearoylcholine, and (G) palmitoylcholine. Average metabolite abundances in PKU participants ($n = 28$) and controls ($n = 28$) are depicted as gray bars. FC, fold change; PKU, phenylketonuria.

TABLE 3 Correlations between nutrient and metabolite changes¹

Metabolite	Phe, mg		Intact protein, g		Dietary fiber, g	
	r_s	p^2	r_s	p^2	r_s	p^2
2-Hydroxyphenylacetate	0.52	0.02	0.50	0.03	-0.21	0.37
Phenylacetylcarnitine	0.32	0.17	0.25	0.29	0.04	0.88
Tridecenedioate (C13:1-DC)	0.26	0.27	0.08	0.73	0.44	0.05
Phenylpyruvate	0.22	0.35	0.20	0.40	-0.3	0.19
Palmitoylcholine	-0.28	0.23	-0.43	0.06	-0.09	0.70
3-Hydroxybutyrate (BHBA)	-0.23	0.33	-0.30	0.20	0.19	0.43
Stearoylcholine	-0.14	0.54	-0.28	0.23	0.04	0.86

¹Results are derived from pairwise Spearman rank correlation analyses on 20 females with PKU. DC, dicarboxylate; FDR, false discovery rate.

² P values are unadjusted. All FDR adjusted P values were not statistically significant ($q > 0.2$).

but can also change the activity of key enzymes involved in energy metabolism (27).

Contrary to the increasing trend for most fatty acid metabolites, choline-containing phospholipids were significantly lower in PKU participants compared with controls. This was particularly evident among saturated and polyunsaturated long-chain fatty acylcholine compounds, and aligns with the findings of prior metabolomics studies in clinical populations with neurological (29) and immune impairments (30). Although there are limited data on the biological activity of acylcholines, previous studies have demonstrated that they act as agonists for muscarinic and nicotinic acetylcholine receptors (31, 32). Because this activation can release calcium from the cells, these metabolites could have an important role in preventing toxic calcium overload and maintaining cellular redox balance (33). This antioxidant function has been supported by a recent study in human neuroblastoma cells, which demonstrated that long-chain unsaturated acylcholines can bind free radicals and reduce H₂O₂-induced cytotoxicity (33). Because perturbed calcium homeostasis is a potential source of oxidative stress and neurological dysfunction in PKU (34), low acylcholine abundance could represent a previously unrecognized form of antioxidant suppression. This might derive from suboptimal choline intake, which has been identified in 1 prior PKU study (13) and is evident in the present sample (90% of PKU participants had intakes less than the Adequate Intake), or limited endogenous choline production due to the elevated phenylacetate. This phenylketone has been reported to inhibit the estrogenic induction of phosphatidylethanolamine-*N*-methyltransferase (35), a rate-limiting enzyme in choline synthesis (36).

After the 5-d metabolic camp intervention, participants demonstrated notable biochemical and dietary changes. Both Phe concentrations and Phe intake decreased, whereas the consumption of dietary fiber increased. In parallel with these changes, there were also significant alterations in 223 plasma metabolites. Among this diverse group of compounds, metabolite derivatives of Phe, ketone bodies, fatty acids, and choline were the most important for discriminating between pre- and postcamp profiles. As expected, the decrease in plasma Phe led to a robust reduction in all phenylketones, which could have influenced the observed decrease in dicarboxylates, carnitine-conjugated fatty acids, and 3-hydroxybutyrate. By day 5 of camp, the abundance of fatty acid and ketone metabolites more closely aligned with

controls, reflecting enhanced mitochondrial efficiency and the normalization of bioenergetic pathways.

Beyond the influence of Phe, this improvement in energy metabolism could be attributed to shifts in redox balance. This could have derived from an increase in fatty acylcholines and choline-containing phospholipids, with a concomitant reduction in the Trp-derived kynurenine metabolites, picolinate and xanthurenate. An increased abundance of choline derivatives can bolster endogenous antioxidant activity and enhance membrane integrity (33, 37) whereas a reduction of the aforementioned Trp metabolites can decrease the synthesis of neurotoxic compounds (3-hydroxyanthranilic acid, quinolinic acid) that enhance oxidative stress and apoptosis (38). Trp could also have been transformed by the gut microbiota into several bioactive indole molecules, including *N*-acetyltryptophan and indolepropionate, which act as potent antioxidants (39) and were found to increase with the camp intervention (Supplemental Table 4). The increased production of these radical scavengers further supports a change in redox balance, and suggests that the camp intervention can be associated with fluctuations in the bacterial ecology of the gut microbiome. Although additional research is needed to clarify these microbial shifts, the enhanced fiber intake during camp might have contributed (40).

In females with PKU, high-resolution metabolomics identified several pathways, beyond Phe metabolism, that differed from healthy controls and substantially altered with a short-term camp intervention. Although this study included several PKU participants receiving adjunct pharmacotherapies, for which the clinical outcomes remain poorly understood, the detection of treatment-specific metabolic changes was limited by the small sample size and multiple comparisons. These constraints additionally prevented statistical analyses from being adjusted for several relevant factors that could affect the plasma metabolome. Clinical relevance of the reported metabolite shifts is also limited by the methodological challenges associated with untargeted metabolomics, including putative feature identification and relative quantification (41). Despite the benefits of this discovery-oriented approach, future studies are required for validation and confirmation. Future research could also benefit from including structural and functional measures of cognition. It is well recognized that changes in mitochondrial function, redox balance, and choline metabolism have important implications for brain development and function (42, 43), but few studies have

evaluated the utility of their constituent metabolites for predicting neurocognitive performance. This study provides a foundational set of blood biomarkers that can be further explored in a larger and more diverse sample using a targeted metabolomics approach.

In conclusion, the metabolic perturbations identified by this study support several of the pathophysiological mechanisms that have previously been proposed to contribute to phenotypic variability in PKU. Given that the plasma metabolome was the focus of the present study, our findings further demonstrate that the identified metabolic alterations are not relegated to the brain. This work differs from prior studies by identifying shifts in choline metabolism that have not previously been described in PKU. Given that choline is essential for the structural integrity and functionality of the brain (44), and was highly responsive to this short-term intervention, it would be beneficial to further explore the impact of choline nutrition on the metabolome and neurocognitive outcomes in PKU.

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