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Meriah S. Schoen Date

Choline Nutriture in Phenylketonuria: Metabolomic Patterns, Dietary Determinants, and Implications for Cognitive Performance

By

Meriah S. Schoen
Doctor of Philosophy

Laney School of Graduate Studies
Nutrition and Health Sciences

_____
Rani H. Singh, PhD, RD
Advisor

_____
Jessica A. Alvarez, PhD, RD
Committee Member

_____
Xiangqin Cui, PhD
Committee Member

_____
Usha Ramakrishnan, PhD
Committee Member

_____
Thomas R. Ziegler, MD
Committee Member

Accepted:

_____
Kimberly Jacob Arriola, PhD
Dean of James T. Laney School of Graduate Studies

_____
Date

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Meriah S. Schoen

B.S., Emory University, 2014

M.S., Georgia State University, 2017

Advisor: Rani H. Singh, PhD, RD

An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Nutrition and Health Sciences

2022

ABSTRACT

Choline Nutriture in Phenylketonuria: Metabolomic Patterns, Dietary Determinants, and Implications for Cognitive Performance

By Meriah S. Schoen

While the severe neurocognitive consequences of Phenylketonuria (PKU) can now be prevented, cognitive deficits are still common in early-treated individuals and cannot be solely attributed to elevated phenylalanine (Phe) concentrations. The purpose of this dissertation was to identify and evaluate an alternate source of cognitive variability in PKU.

First, we performed untargeted metabolomics in females with PKU to examine metabolic perturbations, beyond Phe, that could be contributing to phenotypic variability. PKU plasma metabolite profiles were examined relative to matched controls, and after a 5-day camp intervention. This analysis identified perturbations in choline-containing compounds, which have not been observed before, in addition to notable alterations in fatty acid and amino acid metabolites. These pathways, which could reflect bioenergetic impairment and oxidative stress, improved or fully normalized with positive changes in dietary adherence following a camp intervention.

Based on these metabolomics results, and prior evidence for a positive relationship between choline and cognition, we then characterized usual choline intake distributions in adults and children with PKU. This analysis found that choline adequacy was suboptimal among individuals with PKU [only 10.8% achieved the adequate intake (AI)], but improved with the use of pharmacotherapies that allow for partially-restricted or fully-unrestricted protein intake. Choline fortified medical foods were required to achieve the AI among individuals maintaining a Phe-restricted diet.

To determine the functional effects of suboptimal choline intake, we evaluated the relationship between total choline intake and working memory performance in early-treated adults with PKU and demographically matched controls. Across the full sample, working memory performance did not differ based on choline intake, and this pattern was not modified by diagnosis. Nevertheless, working memory performance was positively associated with concurrent total choline intake in a subsample of PKU participants with optimal metabolic control in middle childhood and adulthood.

Overall, this dissertation substantiates choline as a nutrient of concern in PKU and suggests that suboptimal choline nutriture may be contributing to the cognitive variability in early-treated individuals with PKU. To confirm and build upon these findings, larger observational studies and controlled trials are needed, in addition to more frequent clinical monitoring of choline intake.

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ACKNOWLEDGEMENTS

First, I would like to thank my mentor and dissertation advisor, Dr. Rani Singh, who has allowed me to think and grow independently, while also guiding me to pursue clinical research in a thoughtful and creative manner. Her ceaseless efforts to improve the lives of individuals with genetic metabolic disorders are inspiring, and continue to imbue meaning in the many hours of data collection and analysis that we have completed together. Her consistent support for me, both personally and professionally, have been invaluable over the last five years.

I would also like to thank my committee members, Drs. Usha Ramakrishnan, Jessica Alvarez, Thomas Ziegler, and Xiangqin Cui for their patience, guidance and encouragement during the completion of this dissertation. Their unique knowledge and insights have helped me to become a better scientist.

The sixth chapter of this dissertation could not have been completed without the expertise and guidance of Dr. Shawn Christ and Dr. Kelly Boland. I appreciate the opportunity to have collaborated with such talented scientists.

The completion of this dissertation would also not be possible without the unwavering love and support of my husband, Mike. He has been a consistent source of encouragement, comfort, joy, and comedic relief through the many stages of this process. I am forever grateful to have found such an incredible life partner.

My parents, Marlene and Marc Schoen, and my brother, Nathan, also deserve more gratitude than I can put into words. They have always encouraged me to pursue a career that I am passionate about, and have been fully supportive of my path to achieve this. Their love has no limits, and has shaped me to be the person I am today.

I would also like to acknowledge Dr. Michelle Lampl, who gave me the skills and confidence to pursue a PhD. Her early mentorship fundamentally changed my career, and I am beyond grateful for the opportunity to have worked with her.

Additionally, I would like to acknowledge the research participants who have donated their invaluable time, and the Singh research team, which has supported all components of this project. I feel so fortunate to have collaborated with a team of such dedicated, compassionate, and hard-working individuals.

Finally, I would like to thank other family, friends, and NHS colleagues who have been tremendous sources of inspiration, joy, and support.

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CHAPTER 1: INTRODUCTION

Phenylketonuria (PKU; OMIM 261600) is an inherited metabolic disorder characterized by impaired or deficient activity of the phenylalanine hydroxylase (PAH) enzyme, which metabolizes phenylalanine (Phe) to tyrosine (1). Early initiation and lifelong adherence to a Phe-restricted diet can prevent Phe accumulation and the development of severe neurological sequelae (2). Nevertheless, treated individuals with PKU still exhibit lower intelligence quotients (3, 4), impaired executive functions (e.g. processing speed, attention, working memory) (5, 6), and a higher prevalence of psychiatric disorders compared to their healthy counterparts (7, 8). These residual effects have been predominantly attributed to poor treatment adherence (9); however, recent studies on early-treated individuals with PKU have reported substantial interindividual variability in cognitive performance that has not been consistently related to Phe control (10-12). This suggests that there are other relevant factors contributing to PKU pathophysiology, which at present, are largely undefined (13). The overarching objective of this dissertation was to identify and evaluate an alternate source of cognitive variability in PKU. To meet this objective, this dissertation had two goals and the following specific aims.

The first goal of this dissertation was to identify metabolic perturbations, beyond Phe, that could be contributing to the cognitive variability identified in early-treated individuals with PKU. To address this goal, this dissertation utilized untargeted high-resolution metabolomics with the following specific aim:

Specific Aim 1 (Chapter 4): Compare the PKU plasma metabolome to healthy, matched controls and examine how the PKU metabolome shifts with short-term changes in treatment adherence.

We hypothesized that plasma metabolite abundance would differ between PKU and controls, and changes with short-term alterations in treatment adherence.

This metabolomics investigation identified notable perturbations in metabolic pathways related to the essential nutrient choline, which has received limited consideration in prior PKU research. Given choline plays a critical role in neurological development and cognitive function through its vital functions in cell membrane integrity (14), one-carbon metabolism (15), and neuromodulation (16), this finding warranted further evaluation. The second goal of this dissertation was focused on characterizing the usual intake of choline and its association with cognitive performance in early treated individuals with PKU. The following specific aims were conducted to meet this objective:

Specific Aim 2 (Chapter 5): Characterize choline consumption in adults and children with PKU by comparing usual choline intake distributions to a national reference sample, and identifying treatment- and diet-related factors that modulate choline needs.

We hypothesized that individuals with PKU would have lower total usual intakes for choline and methyl-donor/co-factor nutrients that impact choline metabolism relative than a natrional reference. Secondarily, we hypothesized that individuals with PKU who were managed with pharmacotherapies would have higher usual choline intake than individuals with PKU who were managed solely with dietary therapy.

Specific Aim 3 (Chapter 6): Evaluate the association between concurrent choline intake and working memory performance in adults with PKU and demographically matched controls, and assess whether this relationship differs by diagnosis and is modified metabolic control.

We hypothesized that there would be a positive relationship between concurrent choline intake and working memory performance, and that higher concurrent choline intake would be associated with a larger improvement in working memory performance among participants with PKU compared to controls. Secondarily, we hypothesized that the positive association between concurrent choline intake and working memory performance would not depend on metabolic control among individuals with PKU.

To address these aims, this dissertation includes three original research studies. Each of these studies utilize a unique PKU cohort that will be described in detail within each chapter. Relevalent literature on the pathophysiology of PKU and the functions of choline are reviewed in Chapter 2. Chapter 3 provides additional detail on sample selection, data collection, and analytic approaches utilized in the original research studies. Chapters 4-6 include the three original research studies, which address the aforementioned specific aims. Chapter 7 describes key findings from this reseaerch, strengths and limitations, clinical and publich health applications, and future directions for research.

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CHAPTER 2: BACKGROUND

This chapter reviews previous research that is relevant for this dissertation. First, background is provided on the epidemiology, diagnosis, and pathophysiology of phenylketonuria (PKU). Second, this chapter discusses the phenotypic variability in cognitive outcomes that has previously been identified in PKU. Next, this chapter reviews metabolomics as a novel approach for investigating this variability, and summarizes prior metabolomics research in PKU. The remainder of this section provides the current evidence to support nutrition, and specifically choline nutriture, as an important contributor to neurocognitive differences in PKU.

2.1 Epidemiology and Diagnosis of PKU

PKU is the most common inborn error of metabolism. The global prevalence of this disorder is approximately 1:23,930 newborns; however, this varies widely across geographic regions and ethnic groups (1). Recent estimates suggest that the prevalence is highest in Italy (1:4,000) and lowest in Thailand (1:227,273) (1). The prevalence in the United States (US) falls between these countries, and is roughly 1:25,000 (1). Within the US, PKU is more common among newborns of American Indian, Alaska Native, and Northern European descent. No differences in incidence have been reported based on sex (2).

Diagnosis of PKU typically occurs in the neonatal period due to the implementation of newborn screening (NBS) in most countries. NBS was initiated in 1966, and involves the collection of a single drop of blood from an infant's heel 24-72 hours after birth (3, 4). This blood is then transferred to a filter paper card, which is presently analyzed using tandem mass spectrometry (MS/MS) to quantify concentrations of phenylalanine (Phe) and tyrosine (Tyr) (5). A positive screening result is defined by elevated Phe concentrations and a high Phe to Tyr ratio. While screening cut-off levels differ across laboratories, the average limits for an abnormal result

are Phe > 130 µmol/L and Phe:Tyr >3 (6). PKU diagnoses are made after confirmatory testing with plasma amino acid analysis (7).

2.2 Pathophysiology of PKU

The elevated Phe concentrations identified in PKU can be attributed to pathogenic variants in the phenylalanine hydroxylase (*PAH*) gene, which is located on chromosome 12 (8). These mutations are inherited in an autosomal recessive manner, and result in a reduction or complete deficiency of the hepatic PAH enzyme, which catalyzes the hydroxylation of Phe to Tyr (9) (**Figure 2-1**). The primary biochemical changes associated with this metabolic defect are excess plasma Phe, elevated phenyl ketones (phenylpyruvate, phenylacetate, phenyllactate), and mild Tyr deficiency (3). If left untreated, these metabolic perturbations lead to decreased brain volume, hypomyelination, frequent seizures, and severe intellectual disability (10, 11).

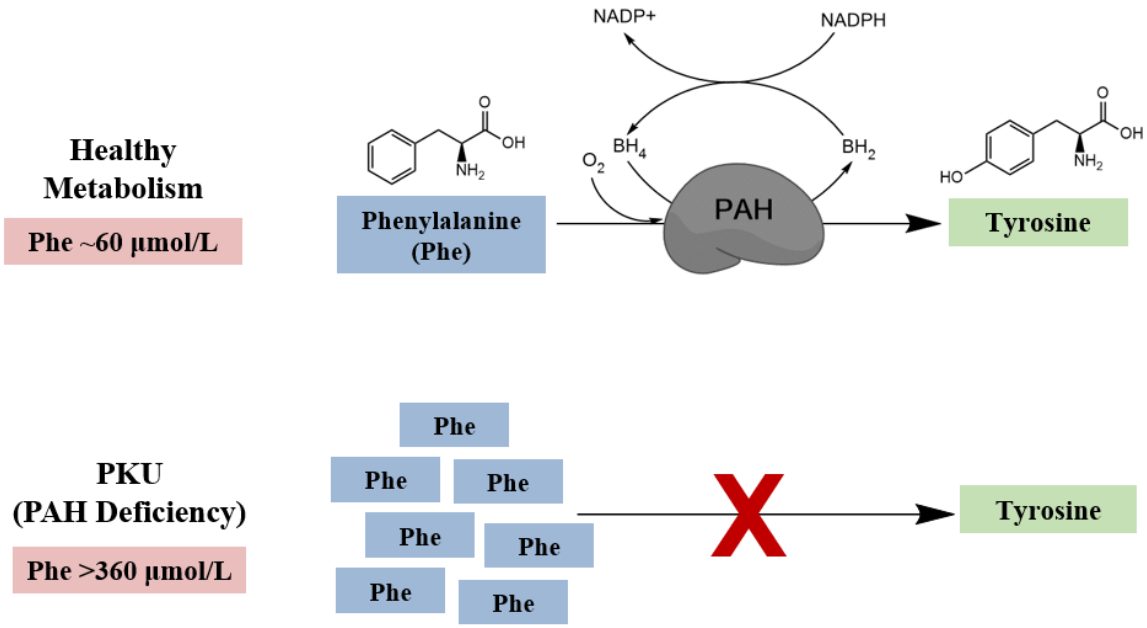

Figure 2-1. Pathophysiology of PKU.

Abbreviations: PAH, Phenylalanine hydroxylase; PKU, Phenylketonuria; Phe, phenylalanine

While Phe has been identified as the primary neurotoxin contributing to these manifestations (12), the underlying mechanisms have not been completely elucidated. Nevertheless, Phe accumulation has been reported to directly and indirectly modulate cerebral protein synthesis, neurotransmitter activity, energy metabolism, lipid metabolism, oxidative stress, calcium homeostasis, gene methylation, and the structural integrity of both white and gray matter regions of the brain (3, 13, 14).

Specifically, Phe may decrease cerebral protein and neurotransmitter synthesis by competitively inhibiting the transport of other large neutral amino acids (LNAAs), such as tryptophan (Trp) and Tyr, across the blood-brain barrier via the LAT1 transporter (15-17). Phe may also impact these pathways by inhibiting Tyr and Trp hydroxylase, which are the enzymes that catalyze the synthesis of dopamine and serotonin (18). Phe has additionally been reported to inhibit the enzymatic activity of pyruvate kinase (19), hexokinase (20), creatine kinase (21), and the mitochondrial respiratory complexes I-III (22), which affect glucose metabolism and ATP generation. These bioenergetic defects may secondarily enhance the production of reactive oxygen species (23), which disturb redox balance and contribute to the enhanced lipid peroxidation observed with excess Phe (24). Beyond energy metabolism, Phe has additionally been reported to down regulate the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol biogenesis (25). As cholesterol is essential for myelin synthesis, this mechanism may directly contribute to white matter disturbances (26). Additional structural perturbations in both white and gray matter may be the result of Phe-driven alterations in neuronal dendritic outgrowth (27) and synaptic connectivity (28).

2.3 Neurocognitive Profile of Early-Treated PKU

Despite early diagnosis and the immediate initiation of dietary treatment, neurologic alterations are still commonly found among early-treated individuals with PKU (29). Based on conventional structural imaging studies, the most consistent neurologic findings are white matter lesions in the posterior periventricular regions of the brain (30). These lesions have been found to extend into more anterior and subcortical brain regions with increased age and decreased metabolic control (31, 32). Additional insight has been provided by diffusion tensor imaging (DTI), an advanced magnetic resonance imaging (MRI) approach, which has identified differences in the white matter microstructural integrity between individuals with PKU and healthy controls in regions that demonstrate normal signal intensity on traditional MRI T2-weighted images (33-35). Recent studies have additionally reported changes within gray matter structures of the brain (36), including the basal ganglia (37) and cerebellum (38).

In addition to these structural impairments, previous research suggests that functional neural connections are also affected in early-treated individuals with PKU. When utilizing functional magnetic resonance imaging (fMRI) in tandem with a working memory, early-treated individuals with PKU have shown decreased functional connectivity within the pre-frontal cortex and between brain regions relative to healthy controls (39). Impaired neural connections have additionally been identified in "task-negative" brain regions (e.g., medial prefrontal cortex, posterior cingulate cortex, and lateral parietal cortex), which are known to be more active during rest than task performance (40). Prior research suggests that these impairments are moderated by metabolic control (40), and may occur even in the absence of structural damage (39).

Abnormalities in the aforementioned structural and functional components of the brain are postulated to contribute to the subtle, but consistent cognitive deficits reported in early

treated individuals with PKU (13). These include a decrease in overall intellectual functioning (41) and impairments in executive functions, motor control, and emotional regulation (42-44). Although the findings from cognitive assessments have not always been consistent, due to heterogeneity among measurement tools and small sample sizes (45), prior research suggests that working memory may be one of the most affected executive function domains among early-treated individuals with PKU (46). This deficit, however, seems to be larger for the executive component of working memory, which manipulates and updates information maintained by the storage component of working memory (i.e., short-term memory) (44). Given the importance of an intact prefrontal cortex and dopaminergic activity for optimal working memory, decreased performance in this domain is thought to reflect reduced non-Phe LNAA concentrations (47).

2.4 Variability in the Cognitive Profiles of Early-Treated Individuals with PKU

Despite the consistent neurocognitive patterns that have been identified among early-treated individuals with PKU as a group, there is tremendous interindividual variability in cognitive performance (48). This may reflect the wide spectrum of PAH activity, which derives from more than 1000 *PAH* variants (49), and/or age-dependent differences in the vulnerability of the brain to Phe concentrations (50, 51). Phe variation, in addition to average Phe concentration, can also lead to relevant differences in cognitive outcomes (52). Longitudinal studies, however, have demonstrated that the neurocognitive variability in PKU cannot solely be explained by Phe control (48, 53, 54). When comparing IQ across the first two decades of life in early-treated patients with PKU, Manti et al. (48) found that 26% of the participants who had maintained good life-long metabolic control demonstrated a significant decrease in IQ, while 72% of participants with poor life-long metabolic control had maintained or improved IQ. Mastrangelo et al. (55) also reported an inconsistent relationship between metabolic control and the progression of white

matter abnormalities in several of their study participants. After accounting for age and long-term quality of metabolic control before or between MRI examinations, about 30% of white matter abnormalities remained unexplained. This evidence suggests that there are other important factors that may modulate the neurotoxic effects of Phe, or independently contribute to the observed structural and functional variability among early-treated patients with PKU.

2.5 Metabolomics Approaches to Investigate Phenotypic Variability in PKU

High resolution metabolomics offers a novel approach to identify and comprehensively examine potential metabolic sources of neurocognitive variability. This assessment method captures low molecular weight molecules (<2000 Da) in a biological fluid, cell, or tissue, and can be utilized in a targeted or untargeted manner (56). The measurement of a specific group of metabolites for clinical monitoring or NBS represents a targeted metabolomics approach (57); whereas, untargeted metabolomics aims to measure all detectable metabolites in a given sample. This produces a large (typically >1000 metabolites), chemically diverse dataset with both known and unknown compounds (58). Unlike targeted metabolomics, measured analytes cannot be absolutely quantified and compared to laboratory references when utilizing an untargeted approach (59). Hence, this methodology is most useful for gaining a broader understanding of disease pathophysiology, examining metabolic interactions, and comparing the biochemical effects of different treatments (60).

Several prior studies have utilized untargeted metabolomics for these purposes in PKU. In studies by Mütze et al. (61) and Stroup et al.(62), metabolomics has been employed to examine metabolite profile differences between early-treated individuals with PKU and healthy controls. Among children with PKU, Mütze et al. (61) found notable metabolite alterations reflective of the reduced fat intake and altered beta-oxidation. This included lower abundances of

activated fatty acids and free carnitine, in addition to a higher abundance of γ-linolenic acid, a precursor of arachidonic acid. Among adults and adolescents with early-treated PKU, Stroup et al.(62) identified lower abundances of docosahexanoic acid (DHA) and eicosapentanoic acid (EPA), and a higher ratio of total n-6 to n-3 fatty acids compared to controls (62).

Untargeted metabolomics has additionally been used to detect metabolic differences associated with the use of distinct dietary treatments. In two studies comparing amino acid medical foods (AA-MF) and glycomacropeptide medical foods (GMP-MF), the consumption of AA-MF was associated with increased abundances of microbiome-derived compounds from Tyr (63), Trp metabolites from the kynurenine pathway (63), and trimethylamine N-oxide (TMAO) (62). Collectively, these findings suggested that both Tyr and carnitine are less bioavailable in AA-MF compared with GMP-MF due to increased degradation by intestinal microbes.

Metabolomics has also been applied to identify biomarkers of disease progression in PKU. In a study conducted by Wild et al. (64), metabolite profiles of plasma and urine samples were analyzed. In the plasma, several metabolites were found to have a significant inverse correlation with Phe. These included arginine, tyrosine, 2-aminobutyric acid, propionylcarnitine, creatine, and aminoadipic acid. This analysis further revealed urinary biomarkers that were reflective of poor dietary adherence, such as Phe catabolites (phenylpyruvic acid, phenylacetylglutaine, hydroxyphenylacetic acid), derivatives of Tyr (e.g. cresol sulfate, phenylsulfate), micronutrients (e.g. folic acid, vitamin B12), and imidazole lactic acid.

2.6 Suboptimal Nutriture as a Source of Phenotypic Variability in PKU

Dietary factors may affect cognitive function through their impact on energy metabolism, redox balance, neurotransmitter synthesis, synaptic transmission, membrane fluidity, and gene expression (65). Nutrient intakes and status, however, differ markedly in PKU due to the highly

restrictive nature of the prescribed diet. To prevent Phe accumulation, individuals with PKU limit their consumption of Phe-containing foods and obtain other essential amino acids through Phe-free elemental AA-MF or GMP-MF. Among individuals with classical PKU, protein tolerance is usually less than 10g of intact protein per day, which makes the contribution of medical food essential for meeting protein needs (66). To ensure the intake of other nutrients is adequate, many medical foods are also fortified with synthetic vitamins, minerals, and essential fatty acids foods (67, 68). Despite this supplementation, nutrient intakes and biochemical status may still be suboptimal due to poor medical food adherence, reduced bioavailability of chemically derived nutrients, and varied nutrient composition across different medical foods (69).

In prior PKU studies, suboptimal biochemical status has been reported for several neurologically important nutrients including docosahexaenoic acid, eicosapentaenoic acid, cholesterol, iron, selenium, vitamin B12, and zinc (69-72). Few of these studies, however, have also included information on dietary intake, medical food compliance, and medical food composition, which are important for clarifying differences in biochemical status. In a recent study that evaluated dietary intake, the consumption of 11 micronutrients (biotin, choline pantothenate, vitamin D, vitamin E, potassium, calcium, zinc, iodine, magnesium, selenium) would be below the dietary reference intakes without the consumption of AA-MF or GMP-MF. This study additionally found that potassium and choline intakes would be inadequate in 93% and 40% of patients, respectively, even with the consumption of fortified medical foods (70). These findings suggest that fortified medical foods are vital for ensuring optimal nutrient status and neurological health, and that choline may be a nutrient of concern in PKU.

2.7 Functions and Metabolism of Choline

Choline is a quaternary amine that serves as building block for a diverse set of metabolites that are integral for neurological development and function (40). Free choline undergoes four main metabolic reactions: oxidation, phosphorylation, acetylation, and base exchange (73). Choline can be oxidized to betaine by the mitochondrial enzyme, choline dehydrogenase. Betaine can then serve as a methyl-donor for L-homocysteine, which aids in the regeneration of L-methionine and the production of dimethylglycine (74). Alternately, choline can be phosphorylated by choline kinase in the CDP-pathway to synthesize phosphatidylcholine (75), an important intermediate in the biosynthesis of membrane phospholipids. Choline can additionally be acetylated by choline acetyltransferase to form the neurotransmitter, acetylcholine (76). Finally, choline can be substituted for serine, ethanolamine, or inositol present within endogenous phospholipids (77); however, this reaction is infrequent and may not be metabolically relevant (73).

2.8 Maintenance of Choline Nutriture

To carry out choline's essential functions, choline nutriture is maintained through pathways of acquisition, depletion, and recycling (**Figure 2-2**). Choline is predominantly acquired through dietary sources in the form of free choline or choline phosphatides, such as phosphatidylcholine, sphingomyelin, phosphocholine, and glycerophosphocholine (78). The richest food sources of choline are animal products, including eggs, liver, beef, chicken, and fish animal (79-81). Smaller amounts of choline can also be synthesized through the sequential methylation of phosphatidylethanolamine by the enzyme phosphatidylethanolamine-N-methyltransferase (PEMT) or recycled through the catabolism of acetylcholine and phosphocholine (73). PEMT is most active in the liver, but is also expressed in the kidney, testes,

heart, lungs, adrenal glands, erythrocytes, spleen, and brain (82, 83). The primary paths by which choline is depleted include oxidation to betaine and excretion in the bile (73, 84).

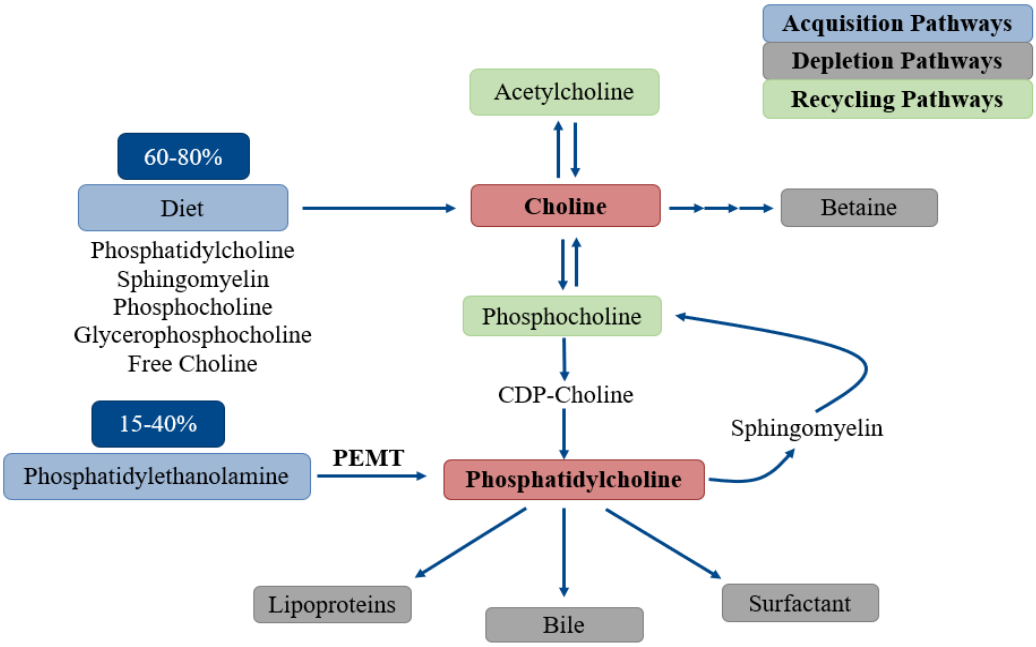

Figure 2-2. Pathways that maintain choline nutriture.

Abbreviations: PEMT, phosphatidylethanolamine-N-methyltransferase

2.9 Choline Needs May Be Higher in PKU

There are several factors that may contribute to a higher dietary requirement for choline in PKU (**Figure 2-3**). First, choline is most concentrated within animal products that also have high Phe content. As Phe must be restricted in PKU, these food items are often not consumed. Instead, individuals with PKU obtain choline predominantly through the consumption of fortified medical foods, which include choline bitartrate or choline chloride. These products, however, may not be sufficient to meet choline needs based on one prior study by Stroup et al. (85), which found that most participants were unable to achieve the choline AI, despite regular consumption of medical foods. This study additionally identified lower concentrations of betaine among PKU participants, which may be indicative of suboptimal choline status (85).

Another important factor contributing to choline needs is the PEMT enzyme, which catalyzes the endogenous synthesis of choline (73). Previous research has demonstrated that PEMT can be induced by estrogen (86, 87) and down regulated by several common single nucleotide polymorphisms (88). PEMT activity may be further modulated by poor metabolic control, and specifically the Phe-derivative, Phenylacetate, which is elevated in PKU (89). Evidence for this hypothesis comes from one prior study which demonstrated that Phenylacetate can inhibit the estrogen-signaling pathway (90).

The dietary requirement for choline may additionally be affected by the oxidation of choline into betaine, which serves as an important methyl donor for the regeneration of S-adenosylmethionine (74). This function can also be modified by other nutrients that serve as methyl-donors or cofactors in one-carbon metabolism, including vitamins B12, B6, folate and methionine (91). As prior research has reported suboptimal intakes and/or status for these nutrients in PKU (66, 92, 93), more choline may be required for betaine formation in this population.

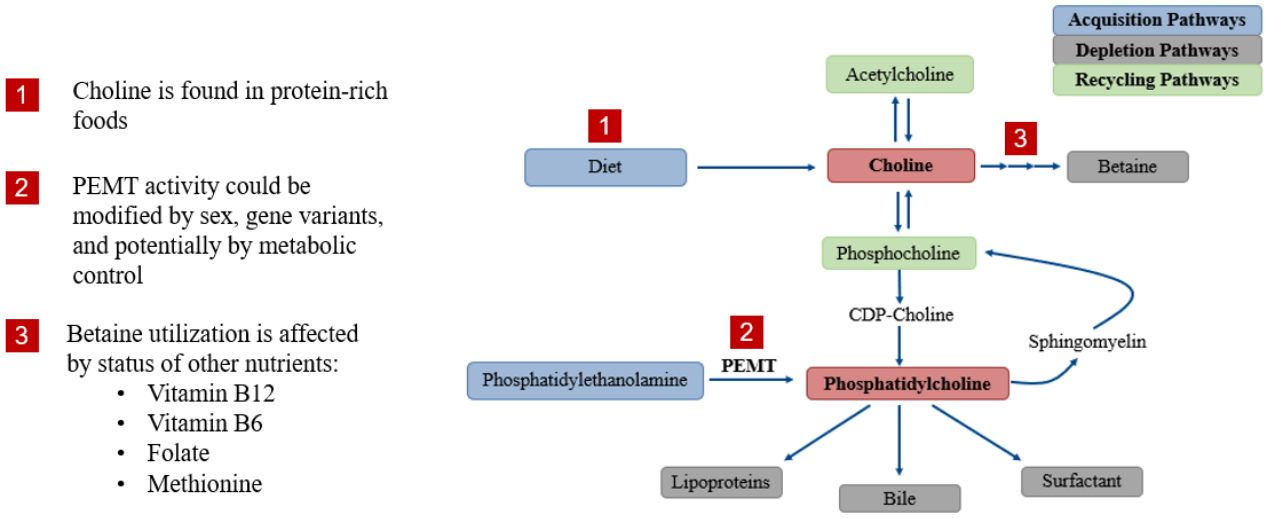

Figure 2-3. Factors that Modulate the Dietary Requirement for Choline.

Abbreviations: PEMT, phosphatidylethanolamine-N-methyltransferase

2.10 Choline Nutriture May Contribute to the Pathophysiology of PKU

Insufficient concentrations of choline-containing compounds can result in structural, neurometabolic, and regulatory consequences that contribute to the pathophysiological mechanisms underlying neurocognitive impairment in PKU. More specifically, choline serves as a precursor for phospholipids (e.g., phosphatidylcholine, sphingomyelin, ceramide) that are indispensable for the synthesis and structural integrity of cellular membranes and myelin sheaths throughout the body and brain (94). Decreased concentrations of these phospholipids due to suboptimal choline intake may contribute to demyelination and white matter abnormalities that have been reported in PKU (95). Alterations in phospholipid content and composition may also lead to mitochondrial dysfunction and increased production of reactive oxygen species, which may contribute to the enhanced oxidative damage that has also been identified in PKU (96). As choline can be oxidized to the methyl-donor betaine, choline availability may additionally contribute to the differential methylation patterns and subsequent changes in gene expression that have been observed in and rodent models (97) and individuals with PKU (98).

2.11 Choline Nutriture May Affect Cognitive Performance PKU

There is a growing body of evidence to support the sustained neuroprotective effects of choline during the perinatal period. McCann et al. (99) previously reviewed numerous rodent studies, which have demonstrated that choline supplementation during development enhances hippocampal electrophysiological responsiveness, increases neuron size, protects against neurotoxic agents, and improves performance on more complex cognitive tasks. The evidence from human studies remains less robust due to differences in study design, sample sizes, and the choline indicator used; however, several have provided compelling results. A controlled feeding study conducted by Caudill et al. (100) found that higher maternal choline intake (930 vs 480

mg/d) during the third trimester of pregnancy improves infant processing speed and visuospatial memory at 4, 7, 19, and 13 months of age. This study additionally reported faster reaction times among infants who were born to mothers that experienced longer durations of supplementation at the lower level of choline intake (480 mg/d) (100). The children from this cohort were further evaluated at 7 years of age in a study by Bahnfleth et al. (101), which reported lasting benefits of increased maternal choline intake on memory, problem solving, and attention. The findings of this randomized controlled trial align with the observations of Boeke et al. (102), who found improved memory performance among children at 7 years of age whose mothers achieved the AI for choline during pregnancy.

Fewer studies have evaluated the cognitive effects of choline nutriture in childhood or adulthood, which are the focus of this dissertation, and the findings have been mixed. A cross-sectional analysis of children 5 years of age from the Seychelles Child Development Nutrition Study did not identify any significant relationships between biochemical measures of choline status and neurodevelopmental outcomes (103). Positive results, however, have been found among healthy young adults who participated in a short challenge study. After supplementation with choline bitartrate, Naber et al. (104) found decreased pupil size (a marker of cholinergic function) and improved visuomotor performance. These findings have been complemented by Knott et al. (105), who found that processing speed, working memory, verbal learning, verbal memory, and executive function improved among healthy males that demonstrated poor baseline cognitive performance and were given a single dose of choline (as CDP-choline). Research has been limited in healthy older adults, but Poly et al. reported interesting findings in a sample from the Framingham Offspring Cohort (106). In this study concurrent choline intakes were positively

associated with improved verbal and visual memory, while historical choline intakes were inversely associated with white matter hyperintensity (106).

2.12 Summary and Overall Significance

The residual neurocognitive deficits identified in early-treated individuals with PKU are currently hypothesized to derive from the neurotoxic effects of Phe, which subsequently result in structural, metabolic, and functional alterations in the brain. Concentrations and variability in blood Phe, however, have not been found to consistently predict cognitive outcomes in PKU. The overall objective of this dissertation was to identify and evaluate an alternate source of cognitive variability in PKU. As untargeted metabolomics may provide a novel approach for this inquiry, this methodology was leveraged in the first original research study of this dissertation (Chapter 4) to identify metabolic perturbations, beyond Phe, that could be contributing to the cognitive variability in PKU. Based on the findings of this investigation, the second and third original research studies of this dissertation (Chapters 5 and 6) characterized choline intake in and the cognitive correlates of this essential nutrient in PKU.

2.11 References

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CHAPTER 3: EXTENDED METHODS

This chapter provides an overview of the PKU cohorts that were evaluated in our three original research studies (Chapters 4-6). This chapter also includes additional information on data collection procedures and assessment techniques that were beyond scope of a traditional journal publication.

3.1 Overview of Study Cohorts

Given PKU is a rare disease, and this dissertation aimed to obtain samples with age and treatment diversity, this dissertation leveraged data from several current and prior studies that have assessed children, adolescents, and adults. Data on individuals with PKU was obtained from the following studies: The Emory Metabolic Camp Study, The Emory Kuvan Study, The MNP4PKU Study, the Emory MNT4P Study, The Pegvaliase Studies at Boston Children's Hospital, and the remPKU Study. Healthy control participants/reference populations were also acquired from three different cohorts: The Duke Murdock Study, the National Health and Nutrition Examination Survey (NHANES), and the remPKU Study.

3.1.1 PKU Cohorts

The Emory Metabolic Camp Study is a 5-day intervention that provides dietary management, lifestyle coaching, and socialization to adolescent and adult females with PKU (12-60 years of age). This camp has occurred annually at Emory University since 1994, and has collected data on various measures of nutritional status, diet quality, treatment adherence, bone denisty, and impressions of neuropsychological health (1-4). While the camp research agenda alters every year to accommodate new research questions, dietary data and plasma amino acids are consistently collected. All camp participants complete a dietary record during the 3 days leading up to camp and for 3 days during the camp. Fasting plasma amino acids are collected on

the morning of the first (day 1) and final (day 5) days of camp. For the metabolomics analysis in our first original research study (Chapter 4), stored plasma samples were used from females who participated in the 2016 or 2017 metabolic camps. Dietary and demographic data from females who participated in the camp between 2013-2015 were also utilized for our second original research study (Chapter 5). We specifically included participants who were solely on dietary therapy or sapropterin dihydrochloride treatment (with or without dietary therapy).

The Emory Kuvan Study was an investigator-initiated clinical trial that evaluated the impact of a tetrahydrobiopterin analog, sapropterin dihydrochloride (Kuvan®, BioMarin Pharmaceutical Inc., Novato, CA, USA) on plasma Phe and several other health outcomes (e.g., quality of life, neurotransmitter concentrations, nutritional status, bone mineral density) (5-9). This study enrolled 58 children and adults with PKU who were 4-50 years of age. The inclusion criteria were a medical diagnosis of phenylalanine hydroxylase (PAH) deficiency, a minimum age of 4 years, no consumption of sapropterin in the 8 weeks prior to study initiation, and no self-reported co-morbidities (e.g. diabetes, hypertension, etc.). After enrollment, participants were evaluated over a year, with study visits at baseline, 6 months, and 12 months. This trial was completed in 2010. Dietary and demographic data from this study were utilized for our second original research study (Chapter 5). For participants who were deemed sapropterin responders, we utilized data from the 3rd study visit (12 months) or long-term study follow-up (>12 months). For participants who were not sapropterin responders, we utilized baseline data (sapropterin naïve).

The MNP4PKU Study is a clinical observational study that began in October 2019 at Emory University and is still currently in progress. This study aims to evaluate the long-term impact of the FDA-approved enzyme substitution therapy, pegvaliase (Palynziq®, BioMarin

Pharmaceutical Inc., Novato, CA, USA), on diet quality, nutritional status, neurological health, and the global metabolome of adults with PKU. This study involves the collection of biweekly filter papers and diet records, in addition to study visits at baseline (Palynziq naïve), pegvaliase response (denoted by significant reductions in plasma Phe concentration and diet liberalization), and long-term response (one year after second study visit). The inclusion criteria for this study are a minimum age of 16 years and enrollment in the Palynziq Risk Evaluation and Mitigation Strategy (REMS) program. Pregnancy and diagnoses for other genetic metabolic disorders exclude participants from this study. Demographic and dietary data from this study were utilized for our second original research study (Chapter 5). Specifically, we utilized data from participants who had been on pegvaliase treatment for $\geq$ 1 year, achieved pegvaliase response, and were no longer consuming any medical food.

The MNT4P study enrolls participants who are involved in the Medical Nutrition Therapy for Prevention (MNT4P) program at Emory University. MNT4P was developed to serve as a bridge program, providing supplies (e.g., medical foods, low-protein modified foods, and monitoring supplies), nutrition counseling, and insurance navigation for patients with inherited metabolic disorders. The aims of the associated research study are (1) to evaluate the impact of MNT4P services on dietary treatment compliance, growth, hospitalizations, and travel time/costs, and (2) to compare participant satisfaction between MNT4P services that are offered in-person and via telemedicine. Dietary, sociodemographic, and treatment-related data are gathered at enrollment, after MNT4P visits, and through chart review of Emory medical records. For our second research study (Chapter 5), we utilized demographic and dietary data from children and adults who are currently or previously enrolled in MNT4P, and did not already have records being utilized from the aforementioned Emory research cohorts.

Boston's Children's Hospital (BCH) has also conducted two studies focused on pegvaliase treatment in adults with PKU. The first is a cross-sectional study that took place from March 2019 through December 2020. The aim of this study was to evaluate the nutritional status of individuals with PKU who had been treated with pegvaliase for one or more years, or who had followed an unrestricted diet for 6 or more months while on pegvaliase treatment (10). Inclusion criteria for this study were a diagnosis of PKU, age between 18 and 65 years, treatment with pegvaliase, and unrestricted protein intake that met or exceeded the recommended dietary allowance (RDA) for protein (0.8 g/kg/d) at the time of evaluation (11). The second BCH study is a prospective, longitudinal study that was initiated in March 2019 and is currently active. The primary aim of this study is to evaluate changes in protein intake within each participant after pegvaliase treatment. Adults (18-65 years) are eligible to participate in this study if they have been diagnosed with PKU, are following a protein restricted diet (<0.8 g/kg/d), and have recently initiated or plan to initiate ($\pm$ 90 days) pegvaliase treatment. This study evaluates participants across three visits: baseline (within 90 days of initiating treatment), 9 months after treatment initiation, and 15 months after treatment initiation. To obtain additional participants for our second research study (Chapter 5), we utilized demographic and dietary data from individuals who were enrolled in either the cross-sectional or longitudinal BCH cohorts and had been managed with pegvaliase for $\geq$ 1 year and were no longer consuming medical food.

The remPKU study was a collaboration between Emory University and the University of Missouri. This remote, cross-sectional study measured dietary intake, metabolic control, and working memory performance in adults (18-40 years) with PKU utilizing web-based neuropsychological assessment. Individuals with PKU were eligible to participate if they were diagnosed as newborns and immediately managed with dietary treatment, but were excluded if

they reported history of neurologic compromise, major medical conditions unrelated to PKU (e.g., closed head injury, multiple sclerosis), or current/previous pegvaliase treatment. Data collection was initiated in January 2021 and completed in September 2022. The data from this project was utilized for our third research study (Chapter 6).

3.1.2 Control Cohorts

For our first study (Chapter 4), healthy controls were matched to each PKU participant based on age and sex. Data and biospecimens for these controls were acquired from the MURDOCK biorepository, which is managed by Duke University and funded by the David H. Murdock Foundation (12). This community-based longitudinal cohort was recruited from a 20-zip code region in the southeastern United States centered on the City of Kannapolis and encompassing Cabarrus County in North Carolina, and aims to capture changes in health and wellness over time. At enrollment, MURDOCK participants complete a demographic and health questionnaire, physical exam, and biospecimen collection (blood and urine). For each of the matched controls included in the present dissertation, we obtained demographic information and a plasma sample for metabolomics analysis. This biorepository was deemed a good match for our research given both the PKU participants and controls predominantly resided within the same geographic region of the country, which would result in similar environmental and health exposures.

For our second study (Chapter 5), data from the National Health and Nutrition Examination Survey (NHANES) was used as a reference population. This cross-sectional survey is administered by the National Center for Health Statistics (NCHS) and uses a multistage probability cluster design to obtain a representative sample of the noninstitutionalized, civilian US population. NHANES began collecting data on choline

intakes in 2005-2006, and this dissertation utilized data from the 2015-2018 cycles. To mirror the demographic characteristics of our PKU sample, this dissertation included NHANES participants who were 4–70 years of age and had completed two nonconsecutive 24 h recalls. Individuals who were pregnant or nursing were excluded. NHANES was selected as a reference population for this project given the prior national estimates of usual choline intake were also based on data collected through NHANES. This dissertation analyzed a more recent cycle of NHANES data to build upon prior analyses and provide a more recent estimate of choline adequacy in the US.

For our third study (Chapter 6), healthy control participants were recruited and evaluated as a component of the remPKU study protocol. These participants were recruited through advertisements placed in the University of Missouri's campus-wide weekly email announcements, a database of research volunteers maintained by the Clinical Neuropsychology Laboratory at the University of Missouri, and unaffected contacts of PKU participants with similar ages and sociodemographic backgrounds. Controls completed all of the same procedures as the PKU participants, with the exception of filter paper collection for phenylalanine (Phe) analysis.

3.2 Dietary Assessment Methods

3.2.1 Collection of Dietary Intake Data

The main independent variable of interest in our second and third studies (Chapters 5 and 6) was total choline intake. In our second study, choline intake data was pooled across several, independent PKU cohorts given a consistent approach was utilized for dietary data collection. All studies used 3-day dietary records which provide more precise estimates of nutrient intake than 24-hour recalls (13). Prior to the completion of diet records, each PKU cohort was

instructed on how to estimate portion sizes and provide adequate detail on the consumption of food items, beverages, medical foods, and dietary supplements. Important details requested included the time of consumption, method of preparation, and the restaurant or brand, if applicable. After collection, diet records were reviewed with each participant by a trained research registered dietitian to obtain any missing details that would affect the accuracy of the nutrient analysis. If participants did not complete a three-day dietary record on the day of a study visit (as determined by each study protocol), then the registered dietitian would collect a 24 h recall utilizing the multi-pass methodology (14).

3.2.2 Nutrient Analysis

Two different nutrient analysis programs were utilized in this dissertation. MetabolicPro™ was utilized in our first study, which analyzed the dietary intakes of females with PKU before and after a camp intervention. This web-based, HIPAA compliant software was created by Genetic Metabolic Dietitians International (GMDI) for metabolic dietitians. MetabolicPro™ contains food composition data from the U.S. Department of Agriculture's National Nutrient Databank for Standard Reference, in addition to manufacturer-provided information on medical foods and low-protein modified foods (15).

For our second and third research studies, nutrient analysis was completed using the Nutrition Data System for Research (NDSR 2020, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, USA). NDSR is linked to a nutrient database that has been developed and maintained by the Nutrition Coordinating Center at the University of Minnesota (16). This database is often regarded as one of the most comprehensive programs for dietary analysis. It includes over 18,000 foods and provides values for 178 nutrients, nutrient ratios and other food components. The USDA Nutrient Data Laboratory is the primary source of nutrient

values and nutrient composition. Despite the generally wider application of NDSR, this software does not contain the specialized nutritional products consumed by individuals with PKU or other inborn errors of metabolism. To combat this issue, we added these products to the NDSR database utilizing the manufacturer-provided nutritional fact sheets for each medical food.

3.2.3 Estimating Mean Nutrient Intake

In our first and third studies (Chapters 4 and 6), which had small sample sizes (<100), a within-person mean was calculated for choline and other nutrients of interest across three or two days of dietary intake, depending on data availability. This methodology, often referred to as the "mean method," is sufficient for describing the mean usual nutrient intake of a sample. However, this method may provide biased estimates for the distribution of usual intake, inadequacy, and excess if only a small number of replicate diet records have been collected (17). This bias can primarily be attributed to the considerable within-person variability associated with short-term measures of dietary intake, and is most troublesome for nutrients that are consumed episodically with large day-to-day variations in intake (18).

3.2.4 Estimating the Distribution of Usual Intake

Many statistical models have now been developed to remove within-person variance and estimate the distribution of usual intake. The most commonly used approaches include the National Research Council Method, the Institute of Medicine (NRC/IOM) method, the Iowa State University Method (ISU), and the National Cancer Institute (NCI) method (19-21). While each of these methods have differences, they all apply the same general approach which involves three steps (18). First, nutrient intakes are adjusted for relevant confounding factors (e.g. recall/record sequence, day of the week, seasonal effects) and transformed to approximate a normal distribution. Second, nutrient intakes are adjusted using a shrinkage factor based on the

within- and between-subject variance components of the transformed data. In the third and final step, nutrient data is back-transformed to its original scale and percentiles of the usual intake distribution are estimated. To carry out these methods, a subset of the sample is required to have 2 or more nonconsecutive 24-hour recalls or consecutive consumption days from a dietary record.

This methodology was applied in Chapter 4 of this dissertation in order to estimate the distribution of usual total choline intakes in both a PKU cohort and healthy reference (NHANES) population. Of the many methods available, we chose to apply the NCI method, given this approach allows for the incorporation of covariates into the modeling procedure (20, 22). This was particularly useful for a relatively small PKU sample (N=120) given sub-groups could be specified as covariates. This removed the need to stratify our sample into small groups based on age and treatment status in order to obtain sub-group specific estimates of usual choline intake. An additional reason that we applied the NCI method was that it has been used to estimate usual choline intake in prior NHANES survey cycles (23, 24). To continue updating the literature on usual choline intake for the general population, without adding additional sources of variability, we chose to maintain a consistent statistical approach.

3.3 Cognitive Assessment Methods

In Chapter 6 of this dissertation, we evaluated overall intellectual functioning and working memory performance. Study visits were conducted over a HIPAA-compliant cloud-based video and phone conferencing system, where web-based versions of the Cambridge Neuropsychological Test Automated Battery (CANTAB) (25) and the Wechsler Adult Intelligence Scale 4th edition (WAIS-IV) (26) were administered. During these assessments,

participants were asked to find a quiet location, and report any distractions or issues that may have affected their performance.

3.3.1 Assessment of Overall Intellectual Functioning

To estimate overall intellectual ability, the Matrix Reasoning subtest from the WAIS-IV (26) was administered.

3.3.2 Assessment of Visuospatial and Verbal Working Memory

Four subtests of CANTAB were used to evaluate visuospatial working memory and related executive functioning skills: Spatial Span (SSP), Paired Associates Learning (PAL), Rapid Visual Information Processing (RVP), and Spatial Working Memory (SWM). Each of the four CANTAB subtests yielded age-, sex-, and education level-normed standard z-scores ($M = 0$, $SD = 1$).

SSP assesses visuospatial working memory by asking participants to observe white boxes that change colors on their screen. Participants reported the color change sequence, initially in order and subsequently in reverse. With each trial, the number of boxes changing color was increased. The outcome from this test was the average of the z-scores for the longest forward span sequence recalled and the longest backward span sequence recalled.

PAL evaluates visual memory and learning by utilizing the same white boxes as the SSP. In this subtest, however, the boxes were “opened” (represented by the switch from a filled box to an outline of a box), with some boxes revealing a pattern that moved to the middle of the screen. The participants were then asked to select the box that originally contained the pattern. The outcome variable for this test was calculated by averaging the z-scores for total errors and number of times the participant chose the correct stimulus on their first attempt for each trial.

RVP measures sustained attention by asking participants to identify specific 3-digit sequences that appear across their computer display. During this test, participants were given a continuous stream of digit stimuli (100 digits/minute) and asked to press the spacebar when the target sequence was detected. As the test progressed, participants had to update and shift functions in order to identify multiple target sequences. The outcome variable for RVP was the average of z-scores for A' (a signal detection measure of a participant's sensitivity to the target sequence, regardless of response tendency) and probability of false alarms.

SWM evaluates the retention and manipulation of visuospatial information. During this test, participants used a process of elimination to identify one yellow token hidden within a set of colored boxes. As the number of boxes increased, this test became more difficult. SWM's outcome variable was the average of z-scores for total errors and strategy use (number of times the participant begins a new trial with a different box).

For all four of these tests, prior research has found that on average, individuals with PKU demonstrate demonstrated lower performance worse than unaffected individuals (27-29).

3.3.3 Assessment of Verbal Working Memory

To evaluate verbal working memory, the Digit Span (DS) subtest from WAIS-IV was administered. During this test, participants were orally presented with a series of digits in pseudo-random order. Participants were then asked to verbally recall the sequence as presented (forward condition), in reverse (backward condition), and in order from smallest to largest (sequencing condition). To increase difficulty, the number of digits presented was systematically increased over the course of the task. The overall score for this test was based on performance across the forward, backward, and sequencing trials. This score was then converted to an age-

normed standard scaled score (M = 10, SD = 3). Scaled scores were then converted to z-scores to facilitate comparison with the other working memory tasks.

3.4 Collection of Historical Phenylalanine Records

An index of dietary control (IDC) was calculated in Chapter 6 to obtain a measure of historical metabolic control from early childhood (0-5 years), middle childhood (6-11 years), adolescence (12-17 years), and adulthood (18+ years). This computation was additionally used to create a lifetime composite of metabolic control. The methodology for this calculation is described in Chapter 6 and previously in literature (30). While this data is critical for advancing our understanding of PKU, several challenges are associated with collection, particularly among individuals who have been managed at many different clinical centers.

To obtain historical Phe data from the adult participants evaluated in Chapter 6, several steps were required. First, medical authorization forms were obtained from each participant's metabolic clinic, and signed by the participant. After receiving authorization from all participants, these forms were faxed or emailed to the respective clinics with cover letters entailing the specific data (e.g. Phe concentrations from plasma amino acid analysis or filter paper blood spots) and time-frame (e.g. May 1992 – August 2022) required. Particular detail was needed for this step, given medical records were often managed by an external company that did not have advanced knowledge about the patient or specific types of medical/laboratory data. After submitting this information, frequent and consistent communication was required with the medical team and the external medical record service. While several participant records were successfully obtained through this process, we were not able to acquire complete records for all participants. This issue was most common among individuals who has received care from multiple metabolic clinics throughout their lifetime given the aforementioned process needed to

be completed separately for each clinic. Additionally, several clinics had destroyed or had limited access to previous paper medical records after switching to electronic systems.

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CHAPTER 4: Plasma Metabolomic Profile Changes in Females with Phenylketonuria (PKU) Following a Camp Intervention

Specific Aim 1

Meriah S. Schoen, MS, RD[1,2] and Rani H. Singh, PhD, RD, LD[2]

[1]Nutrition and Health Sciences, Laney Graduate School, Emory University, Atlanta, GA, USA

[2]Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, USA

*Am J Clin Nutr (*2022), 115(3):811-821. doi: 10.1093/ajcn/nqab400.

4.1 Abstract

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

Objective: This study aimed to characterize changes in the PKU plasma metabolome following a five-day Metabolic Camp intervention and to compare PKU profiles with those of matched healthy controls.

Design: In 28 females (12-57 years of age), fasting plasma samples were collected on the first (day one) and final (day five) 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, while fatty acylcholine metabolites were substantially lower. After the camp intervention, plasma Phe concentrations decreased [median change (IQR): -173 μmol/L (-325, -28)] 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 one and five.

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 may have fundamental roles in these pathways and 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.

4.2 Introduction

Phenylketonuria (PKU; OMIM #261600) is an inherited metabolic disorder of amino acid metabolism that results in absent or defective phenylalanine hydroxylase (PAH; 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, two adjunct pharmacotherapies, sapropterin dihydrochloride (KUVAN®, BioMarin Pharmaceutical Inc., Novato, CA, USA) and pegvaliase (PALYNZIQ®, BioMarin Pharmaceutical Inc., Novato, CA, USA), have become available to maintain Phe concentrations closer to or within the therapeutic range (120-360 μmol/L) while 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).

While the importance of Phe is undisputed, it is well recognized that several vitamins, minerals, trace elements, and fatty acids may also contribute to the pathophysiological changes in PKU (9). Changes in treatment adherence and/or treatment modality (e.g. diet therapy vs. 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 as it could identify notable changes in the metabolic milieu, beyond that of Phe metabolism, which may 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 five-day 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 to 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.

4.3 Methods

4.3.1 Sample and Study Design

The Emory Metabolic Camp (Atlanta, GA) was established in 1995 as a research-based camp, and a provides a five-day 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 have been previously described (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 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 out of the 46 females who consented to research during the 2016 and 2017 Emory Metabolic Camps **(Supplementary Figure 4-1)**. Using two plasma samples from each included female (total N=56), changes in plasma metabolites were evaluated between day one and day five of camp. At both time points, PKU metabolite profiles were compared to 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.

4.3.2 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 one) and end line (camp day five), anthropometry (height, weight, hip, and waist circumferences), three-day dietary records, and fasting plasma samples were collected at the Georgia CTSA Clinical Research Center at Emory University Hospital. Plasma samples were frozen at -80°C and shipped to Metabolon (Metabolon Inc., Research Triangle Park, NC, USA) and LabCorp (Laboratory Corporation of American, Burlington, NC, USA) 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 ethylenediamine tetraacetic acid (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.

4.3.3 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 non-interfering standards for quality control. Sample extracts were then divided into fractions and analyzed using three different methods to optimize the capture of both hydrophilic and hydrophobic metabolites: (1) reverse phase ultra-performance liquid chromatography 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 (HILIC) 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 electrospray ionization source and Orbitrap mass analyzer (35,000 mass resolution). The scan range covered was 70-1000 mass/charge (m/z). Raw data was extracted, peak-identified, and quality control processed using Metabolon's hardware and software. Peaks were quantified using area-under-the-curve and normalized for inter-day variability using block correction. After quantification, a total of 892 compounds were identified via comparison to Metabolon's proprietary library of authenticated standards or recurrent unknown entities. All annotations met the criteria for a level one or level two identification confidence score as defined by the Metabolomics Standards Initiative (17).

4.3.4 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 (23), SAS (version 9.4; SAS Institute, Cary, NC), and R (version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was based on two-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 (19) 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.

4.3.5 Data Visualization and Differential Abundance Analysis

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were used to visualize relationships among samples in the PKU and control groups, as well as within the PKU group. To identify metabolites that differentiated between disease states (PKU versus

matched controls) and the camp intervention (PKU day one versus PKU day five), The Mann Whitney U Test and the Wilcoxon Signed-Rank Test were used, respectively. Within the PKU sample, subgroup analyses based on age (adults versus pediatric participants) and treatment (diet versus 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 only four of the 28 participants were taking pegvaliase.

In the aforementioned independent, two-group comparisons, metabolites were considered to significantly differ between the groups if FDR adjusted p-values were <0.05 and geometric mean fold changes were $> \pm 1.5$ (log10 fold change $> \pm 0.176$). For paired comparisons (e.g. camp day five versus camp day one), differentiating metabolites were also required to meet the fold change criteria in more than 75% of the PKU sample.

4.3.6 Pathway Analysis and Feature Selection

After filtering metabolites using univariate tests, metabolic pathway testing was conducted using the hypergeometric test (20). This method tested for over-representation of pathways in a selected group of metabolites that met the q-value and fold change 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 ($p<0.01$) 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 (21). For this analysis, metabolite importance was estimated based on a

permutation procedure (22). Top metabolites were selected by thresholding the importance scores at the elbow point of the curve of importance scores **(Supplementary Figure 4-2)**.

4.3.7 Diet and Metabolite Correlations

Within the PKU sample, changes in nutrient intake and treatment adherence from baseline to day five 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.

4.4 Results

Baseline demographic and treatment characteristics are detailed in **Table 4-1**. The PKU cohort was comprised of 15 adult and 13 pediatric females. Camp participants were predominantly Caucasian with a median (IQR) age of 18 (15,22) years. 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 µmol/L). Among 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 µmol/L (223.5,1075). Of the 28 participants, 20 (12 on diet therapy, six on sapropterin therapy, and two on pegvaliase therapy) had complete dietary intake and prescription data that were used to evaluate baseline dietary treatment adherence. Within this subsample, 80% demonstrated non-adherence to their dietary prescription at baseline due to excess consumption of Phe (n=10), suboptimal medical food intake (n=2), or both (n=4). Among the matched controls, the median (IQR) body mass index (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 (IQR) BMI was higher among PKU adults [28 kg/m^2 (21.3,34.3)] and pediatric participants [84% (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. As this was not identified prior to metabolomics analysis, the matched pair involving this control was not excluded from the analytic sample.

4.4.1 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 4-1A)**. Univariate analysis identified 265 metabolites that differed ($q<0.05$) by disease state. 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 to controls **(Figure 4-2A)**. To further select features that distinguished PKU participants from controls, over-representation 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 4-2B)**. Fatty acid metabolism, and particularly pathways involving carnitine conjugated analytes, were the most enriched among the PKU group, while fatty acylcholine and plasmalogen metabolism were more enriched among controls. From the RF algorithm, 25 metabolites were identified as effective classifiers of disease state **(Figure 4-2C).**

Within the PKU sample, few metabolites significantly differentiated between age groups, with the exception of the amino acid sarcosine [mean log10FC (95% CI): -0.25 (-0.34, -0.17)]

and the dicarboxylic amine, iminodiacetate [mean log10 FC (95% CI): -0.30 (-0.40, -0.20)], which were both less abundant among 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. This finding was further supported by PCA, which did not identify distinct metabolite profiles across PKU treatment groups or medical foods (GMP-MF vs. AA-MF) **(Figure 4-1B)**.

4.4.2 Changes in Biochemical Control and Dietary Composition Associated with Metabolic Camp Intervention

After the five-day 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). This shift did not significantly differ based on age group ($p=0.72$) but was more pronounced among females who were solely on diet therapy [median plasma Phe change (IQR) diet therapy: -313 µmol/L (-345, -176); median plasma Phe change (IQR) adjunct pharmacotherapies: -76 (-246, 15)]. Although plasma Phe concentrations remained above the therapeutic range (120-360 µmol/L) for 16 participants (57.1% of total), nine females maintained Phe concentrations within the recommended range throughout the camp intervention, and two were able to shift their Phe concentrations into the desired range. In one participant who exhibited good Phe control at baseline, Phe concentrations exceeded 360 µmol/L after the intervention.

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 among participants on both diet therapy and adjunct

pharmacotherapies, and a reduction in Phe consumption was the most prominent change among both groups **(Supplementary Table 4-1)**. 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 (Δ= -4.7g, 95% CI: -7.8, -1.5), which was paralleled by a decrease in dietary Phe. Dietary fiber intake (Δ=3.15g, 95% CI: 0.29, 6.01) also increased over the intervention period, and may reflect greater consumption of fruits and vegetables **(Table 4-2).**

4.4.3 Changes in the PKU Plasma Metabolome Associated with Metabolic Camp Intervention

PCA demonstrated minimal separation of PKU plasma samples based on the intervention (day one versus day five), however, inter-individual 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**.** 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 one 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 among participants on diet therapy [mean log10 FC (95% CI): -0.42 (-0.53, -0.30)] compared to those on adjunct pharmacotherapies [mean log10 FC (95% CI): -0.09 (-0.39, 0.02)].

To further identify metabolites that most prominently changed with the intervention, fold change criteria were applied to the discriminatory compounds obtained from the Wilcoxon

Signed Rank Test. Seven of the 232 metabolites were found to have a fold change greater than ± 1.5 in more than 75% of the sample **(Figure 4-3)**. The most substantial fold decrease and fold increase were exhibited by phenylacetylcarnitine [mean log10 fold change (95% CI): -0.45 (-0.56, -0.35)] and palmitoylcholine [mean log10 FC (95% CI): 0.32 (0.19, 0.45)], respectively. Relative to controls, the abundance of 3-hydroxybutyrate, tridecenediote, palmitoylcholine, and the Phe catabolites became more similar to controls after the five-day intervention. This trend was echoed among almost all of the amino acids and lipids that differentiated PKU samples from controls at baseline.

4.4.4 Correlations between Nutrient Intake and Metabolite Changes

To determine if the identified metabolic shifts were related to changes in dietary intake, correlations were examined between the three nutrients (dietary fiber, intact protein, dietary Phe,) and seven 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 only a subgroup of the PKU sample had complete dietary data and this was an exploratory analysis, unadjusted results are reported in **Table 4-3.** 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).

4.5 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 five-day 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. As these metabolites represent alternate oxidation or conversion products of non-oxidized acyl-CoA esters, elevated abundances among PKU participants may indicate mitochondrial β-oxidation and/or the 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 may reflect the inhibition of fuel utilization pathways and the use of alternate energetic substrates (25).

Prior literature suggests that these metabolic alterations may 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 may indirectly trigger the aforementioned bioenergetic changes by enhancing the production of reactive oxygen species (26) and modulating the activity of the plasma membrane Ca2+ -ATPase (27). The resulting accumulation of ROS and calcium not only enhance cellular

vulnerability to oxidative damage (28), but may 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 to 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 is 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). As this activation can release calcium from the cells, these metabolites may 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 H_2O_2-induced cytotoxicity (33). As perturbed calcium homeostasis is a potential source of oxidative stress and neurological dysfunction in PKU (34), low acylcholine abundance may represent a previously unrecognized form of antioxidant suppression. This may derive from suboptimal choline intakes, which has been identified in one prior PKU study (13) and is evident in the present sample (90% of PKU participants had intakes less than the AI), 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 five-day Metabolic Camp intervention, participants demonstrated notable biochemical and dietary changes. Both Phe concentrations and Phe intake decreased, while the

consumption of dietary fiber increased. In parallel with these changes, there was 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 post-camp profiles. As expected, the decrease in plasma Phe led to a robust reduction in all phenylketones, which may have influenced the observed decrease in dicarboxylates, carnitine-conjugated fatty acids, and 3-hydroxybutyrate. By day five 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 may have derived from an increase in fatty acylcholines and choline-containing phospholipids, with a concomitant reduction in the tryptophan (Trp)-derived Kynurenine metabolites, picolinate and xanthurenate. An increased abundance of choline derivatives may 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-hydroxyanthranillic acid, quinolinic acid) that enhance oxidative stress and apoptosis (38). Trp may also have been transformed by the gut microbiota into several bioactive indole molecules, including N-acetyltryptophan and indolpriopionate, 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 may be associated with fluctuations in the bacterial ecology of the gut microbiome. While additional research is needed to clarify these microbial shifts, the enhanced fiber intake during camp may have contributed (40).

Among 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. While this study included several PKU participants on 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 are 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 may 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 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 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 nutriture on the metabolome and neurocognitive outcomes in PKU.

4.6 References

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4.7 Tables and Figures

Table 4-1. Baseline Demographic and Clinical Characteristics for PKU participants and Healthy Controls whose samples were used for metabolomics analysis

Variable[1]	Adult PKU (n=15)	Pediatric PKU (n=13)	Matched Controls (n=28)
Age, *y*	22 (19,24)	15 (14,15)	18 (18,22)
Caucasian Ethnicity, *N*	15 (100.0)	12 (92.3)	27 (96.4)
Camp Year, *N*			
2016	5 (33.3)	4 (30.8)	--
2017	10 (66.7)	9 (69.2)	--
PKU Therapy[2], *N*			
Diet Only	7 (46.7)	6 (46.2)	--
Sapropterin Dihydrochloride	4 (26.7)	7 (53.9)	--
Pegvaliase	4 (26.7)	0 (0)	--
BMI[*b*], *kg/m*^{3}	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], N	11 (55.0)	9 (45.0)	--
Phe Intake Above Rx, *N*	3 (27.8)	7 (77.8)	
Medical Food Intake Below Rx, *N*	2 (18.2)	0 (0)	--
Both, *N*	3 (27.3)	1 (11.1)	--

[1]Values for continuous variables are medians (IQR); For categorical variables, numbers within parenthesis represent percent.
[2]Twenty-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 two were consuming a combination of GMP-MF and AA-MF (both were on diet therapy only)
[3]BMI (kg/m^2) is reported for participants $\geq$ 18 years of age (n=15); BMI percentile is reported for participants <18 years of age (n=13) and determined using the CDC calculator.
[4]Reported only for a subsample (N total=20; n=11 adult, n=9 pediatric) with complete diet and treatment prescription information.

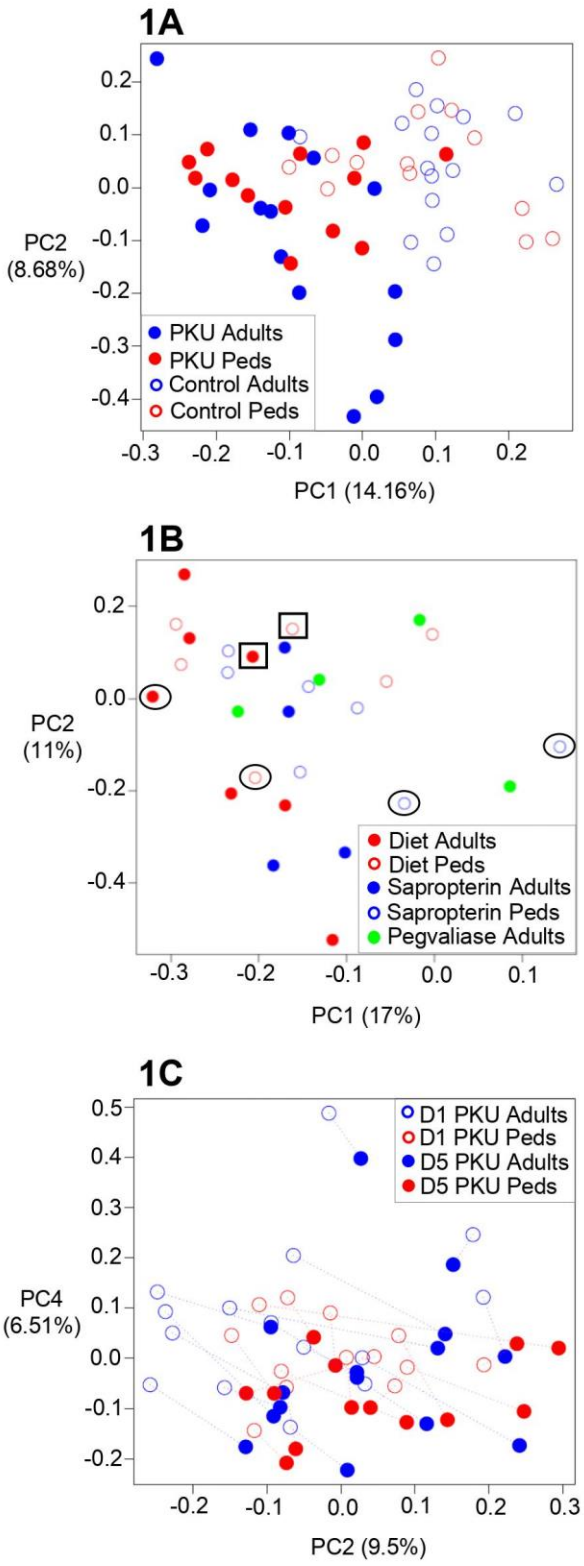

Figure 4-1. Principle component analysis (PCA) of untargeted plasma metabolomics data collected from healthy controls (n=28) and PKU participants (n=28). **(A)** PCA score plot of control and PKU samples from day one of camp, according to age group. **(B)** PCA score plot of

PKU samples from day one 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 one and five of camp, according to age group. Paired samples are connected with a dotted line.

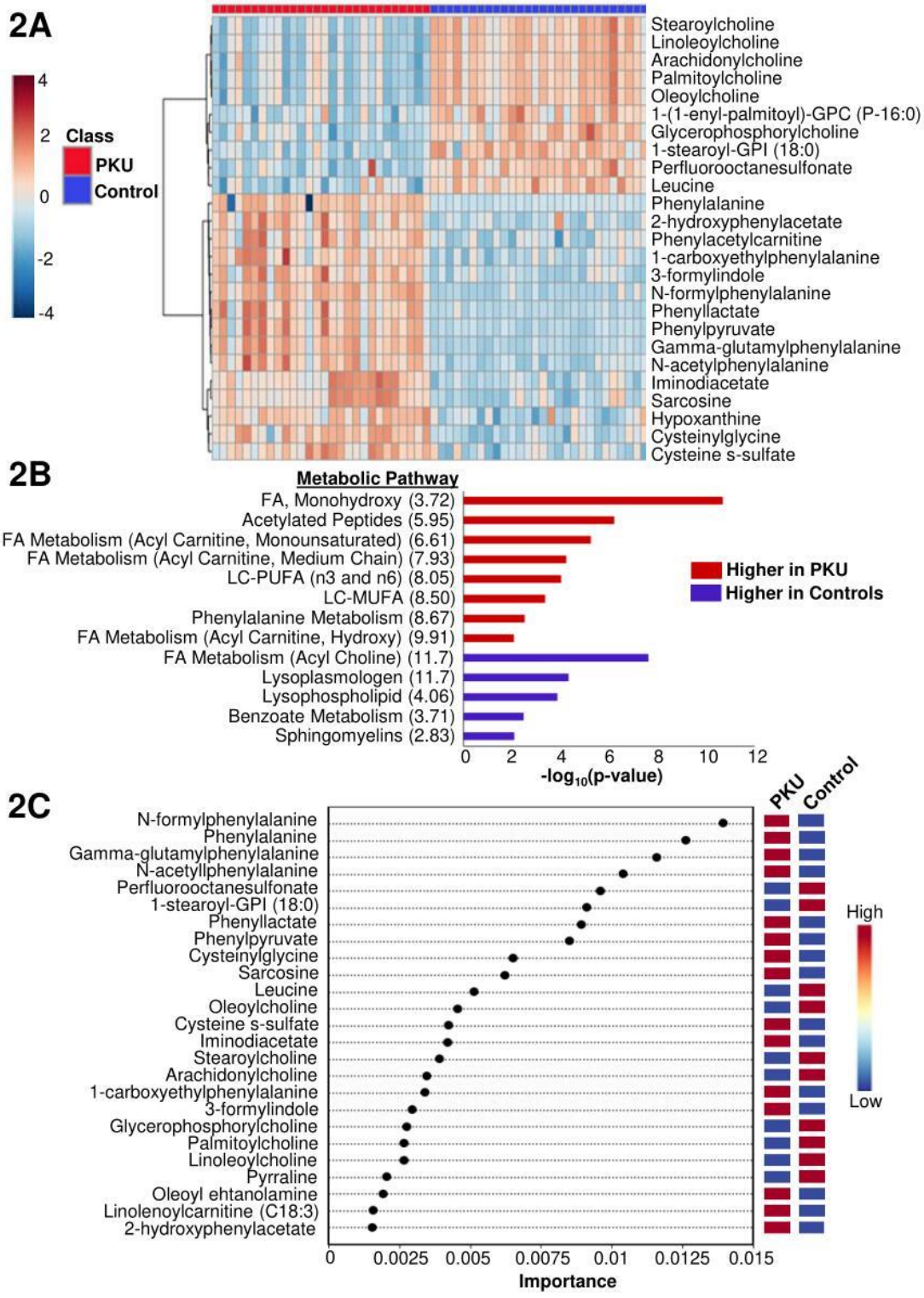

Figure 4-2. Differences in plasma metabolites between PKU participants (n=28) and healthy, matched controls (n=28) **(A)** HCA of the top 25 metabolites that differed between PKU participants on day one of camp and matched controls based on q-values from the Mann Whitney

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 over-representation 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 fold change 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 one of camp.

Abbreviations: FA, fatty acid; FC, fold change; HCA, hierarchical clustering analysis; MUFA, monounsaturated fatty acid; PKU, Phenylketonuria; PUFA, polyunsaturated fatty acid; RF, random forest

Table 4-2. Nutrient Intakes for PKU Participants on and Day 1 and Day 5 of Metabolic Camp[1]

Nutrients[2]	Day 1		Day 5		
	Intake	**Meeting DRI (%)[3]**	**Intake**	**Meeting DRI (%)[3]**	***P*[4]**
Total Energy (Kcal)	1474.1 (1361.7, 1644.2)	15	1659.3 (1100.9, 2185.6)	40	0.50
Kcal from Protein (%)	13.9 (12.0, 17.4)	85	13.5 (11.3, 16.1)	80	0.78
Kcal from Carbohydrate (%)	53.5 (50.0, 61.5)	85	59.0 (53.5, 65.0)	80	0.06
Kcal 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 B12 (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 (7.0, 23.5)	75	0.79

[1]Reported for a subsample (n=20) with complete diet and treatment prescription information
[2]Values are medians (IQR)
[3]Reference for percentage of total calories from protein, carbohydrates and fat is the acceptable macronutrient distribution range (AMDR) (23); reference for dietary fiber and all micronutrients is the dietary reference intakes (DRIs) (23); 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.
Abbreviations: DFE, Dietary Folate Equivalents; PKU, Phenylketonuria

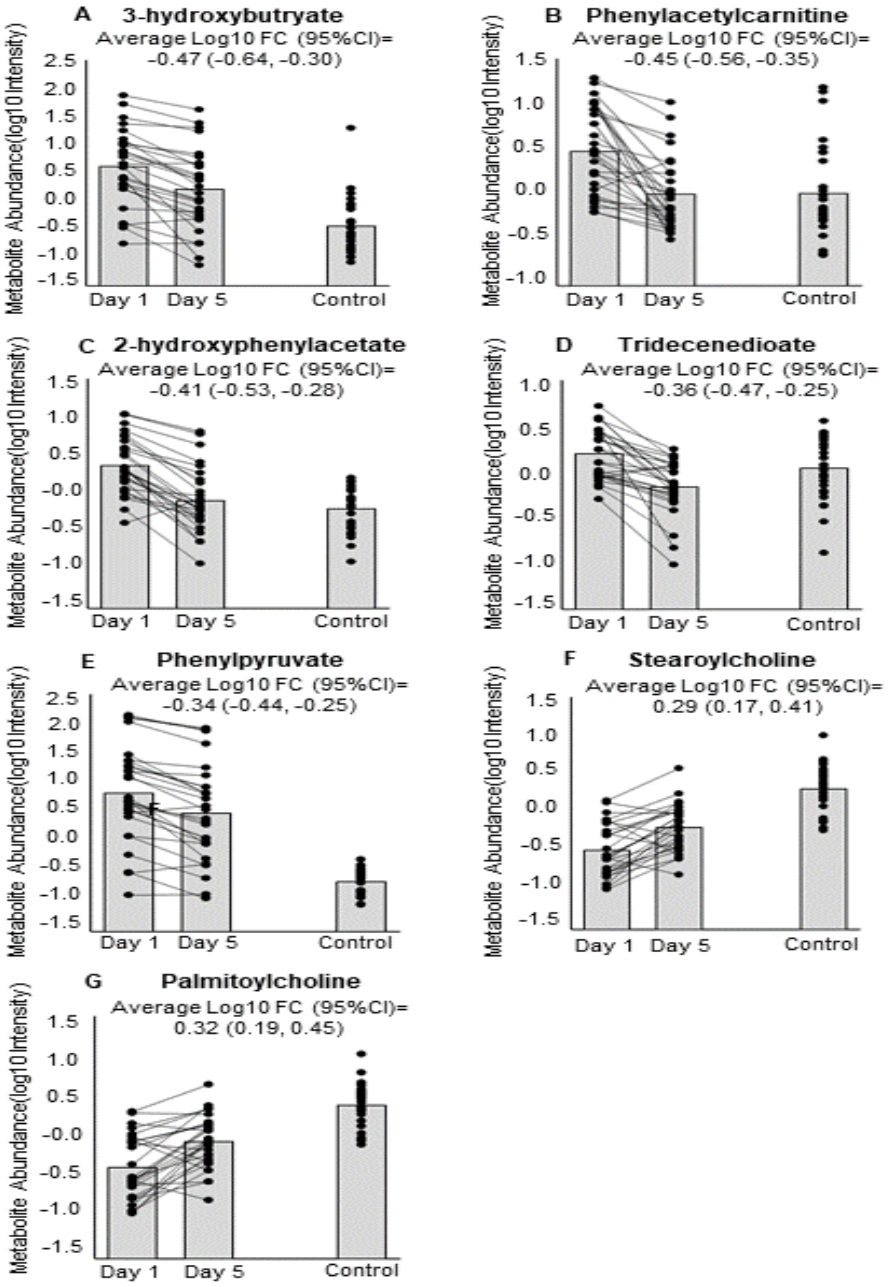

Figure 4-3. Scatter plots for seven metabolites that significantly (q<0.05) and consistently (log10 FC > 0.176 or < -0.176 in more than 75% of the sample) altered among PKU participants (n=28) with the camp intervention based on the Wilcoxon Signed-Rank test: **(A)** 3-hydroxybutyrate, **(B)** Phenylacetylcarnitine, **(C)** 2-hydroxyphenylacetate, **(D)** Tridecenediote, **(E)** Phenylpyruvate, **(F)** Stearoylcholine, and **(G)** Palmitoylcholine. Average metabolite abundances among PKU participants (n=28) and controls (n=28) are depicted in grey bars. **Abbreviations:** FC, fold change; PKU; Phenylketonuria

Table 4-3. Correlations between Nutrient and Metabolite Changes[1]

	Phe (mg)		Intact Protein (g)		Dietary Fiber (g)	
Metabolite	r_s	*p*	r_s	*p*	r_s	*p*
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	0.7
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

[1]Results are derived from pairwise Spearman Rank Correlation analyses on 20 females with PKU.
[2]P-values are unadjusted. All FDR adjusted p-values were not statistically significant (q>0.2).
Abbreviations: Phe, Phenylalanine

4.8 Supplementary Tables and Figures

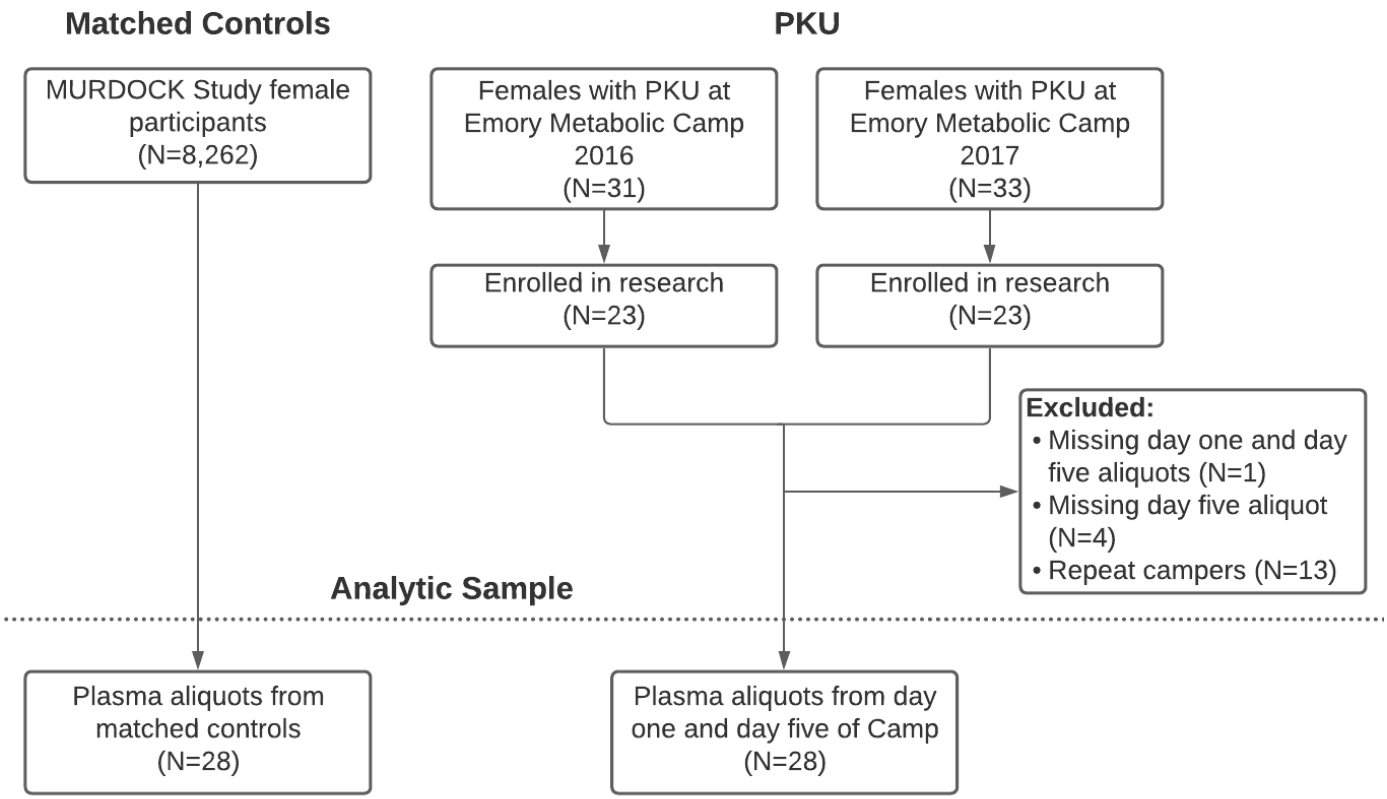

Figure S4-1 (supplementary). Flow Chart of inclusion/exclusion criteria for selecting the final analytical sample 28 females with PKU from the Emory Metabolic Camp and 28 matched controls from the Duke MURDOCK Cohort Study.

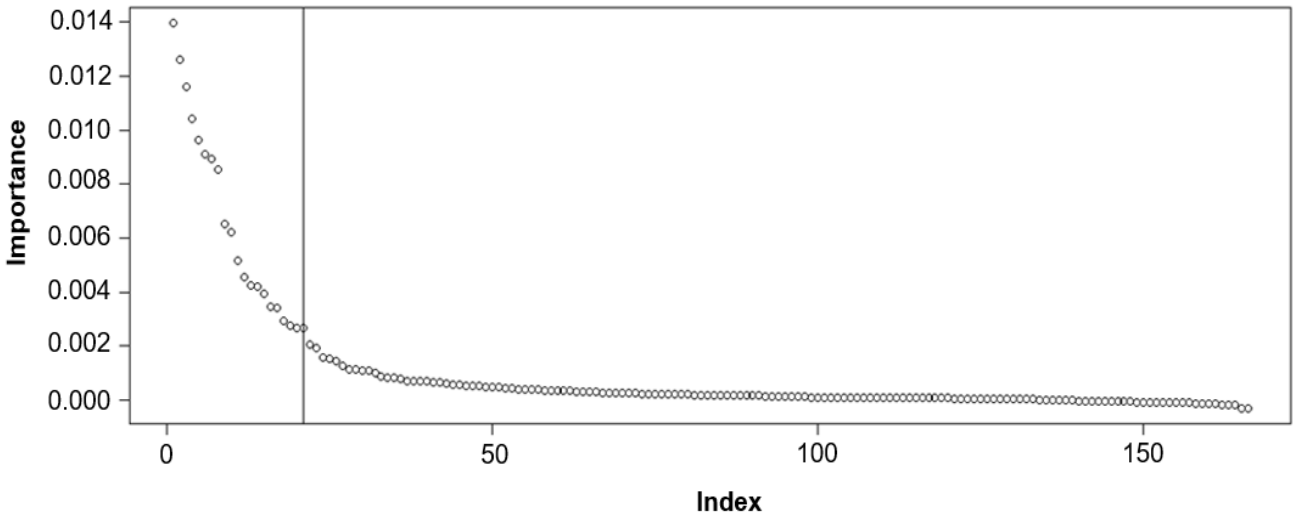

Figure S4-2 (supplementary). The tree-based machine learning method Random Forest was used to select metabolic features that differentiated PKU plasma profiles on day one of camp from matched controls. Each dot represents one of the 166 metabolites that were initially selected based on a q-value <0.05 from the Mann Whitney U Test and a geometric mean fold change > $\pm$ 1.5. Metabolites are indexed based on their importance scores, which were determined by a permutation procedure. Twenty-five metabolites were selected as important differentiators between PKU and controls by thresholding the importance scores at the elbow point of the curve. This threshold is represented by the solid vertical line.

Table S4-1 (supplementary). Nutrient Intakes for PKU Participants on Day 1 and Day 5 of Metabolic Camp Stratified by Treatment Group[1]

Nutrient[2]	Diet Therapy (n=12)	Adjunct Therapy (n=8)	*P*-value[3]
Total Energy (kcal)			
Day 1	1430.9 (1279.1, 1794.6)	1510.1(1404.0, 1644.2)	0.62
Day 5	1805.4 (1476.3, 2257.5)	1331.1 (994.1, 1908.1)	0.19
Change[4]	131.4 (-193.2, 594.9)	-134.5 (-543.1, 342.7)	0.28
Kcal from Protein (%)			
Day 1	13.8 (12.0, 17.4)	14.7 (12.0, 18.3)	0.62
Day 5	13.5 (11.8, 15.8)	13.03 (8.8, 17.3)	0.79
Change[4]	0.5 (-2.9, 2.0)	-1.0 (-3.4, 1.2)	0.52
Kcal from Carbohydrate (%)			
Day 1	58.5 (50.0, 64.0)	52.5 (48.5, 55.5)	0.23
Day 5	60.5 (55.5, 66.0)	57.0 (51.5, 61.5)	0.39
Change[4]	7.5 (4.0, 13.0)	4.0 (0.0, 7.0)	0.97
Kcal from Fat (%)			
Day 1	28.0 (23.5, 32.5)	27.0 (25.5, 31.0)	1.00
Day 5	25.5 (23.5, 30.5)	28.5 (25.5, 33.0)	0.22
Change[4]	-3.0 (-5.0, -1.0)	1.0 (-2.5, 6.0)	0.35
Dietary Fiber (g)			
Day 1	9.45 (8.5, 15.8)	7.75 (6.6, 9.4)	0.06
Day 5	12.1 (9.9, 18.3)	9.4 (7.0, 15.5)	0.35
Change[4]	1.3 (-2.1, 3.2)	2.4 (0.9, 6.1)	0.50
Total Protein (g)			
Day 1	57.7 (41.7, 68.6)	56.6 (44.0, 64.6)	0.97
Day 5	57.7 (41.7, 68.6)	47.4 (29.0, 57.5	0.10
Change[4]	0.5 (-2.3, 15.5)	-3.9 (-13.9, -0.5)	0.10
Intact Protein (g)			
Day 1	14.0 (9.9, 19.1)	24.1 (11.5, 44.1)	0.22
Day 5	10.2 (6.4, 12.9	12.1 (7.0, 35.6)	0.60
Change[4]	-2.8 (-6.1, 0.5)	-5.3 (-8.7, -2.5)	0.28
Medical Food Protein (g)			
Day 1	42.7 (17.5, 57.0)	20.3 (0.0, 50.9)	0.35
Day 5	47.0 (41.0, 57.0)	19.0 (1.7, 41.6)	0.07
Change[4]	0.0 (0.0, 14.7)	0.0 (0.0, 1.7)	0.39
Phenylalanine (mg)			
Day 1	628.5 (398.5, 843.0)	809.5 (513.0, 1887.0)	0.31
Day 5	396.5 (240.0, 578.5)	530.5 (309.0, 1609.5)	0.39
Change[4]	-248.5 (-382.0, -25.5)	-190.0 (-329.0, -137.0)	1.00
Vitamin D (IU)			
Day 1	572.1 (257.0, 708.3)	228.4 (48.7, 437.6)	0.06
Day 5	537.6 (278.7, 740.2)	131.4 (53.6, 494.3)	0.06

Change[4]	50.6 (-27.8, 145.6)	0.4 (-39.3, 17.3)	0.28
Vitamin B12 (µg)			
Day 1	4.8 (2.6, 9.8)	4.1 (1.7, 7.8)	0.57
Day 5	6.3 (3.4, 9.5)	2.4 (1.3, 8.6)	0.22
Change[4]	0.7 (-0.2, 2.2)	-0.6 (-1.1, 0.4)	0.09
Choline (mg)			
Day 1	221.0 (147.0, 314.0)	141.0 (76.0, 181.5)	0.05
Day 5	253.5 (171.5, 370.5)	165.0 (82.5, 197.5)	0.05
Change[4]	20.0 (-44.0, 36.5)	-1.0 (-10.0, 43.5)	0.97
DFE (µg)			
Day 1	869.7 (421.2, 1536.5)	678.9 (475.1, 1147.4)	0.57
Day 5	1097.8 (541.0, 1497.9)	511.6 (277.5, 1135.0)	0.17
Change[4]	13.7 (-71.8, 194.6)	-117.8 (-291.0, 86.8)	0.13
Calcium (mg)			
Day 1	1369.5 (720.0, 1749.5)	831.5 (411.0, 1502.0)	0.25
Day 5	1474.0 (1018.0, 1871.0)	555.0 (382.0, 1678.0)	0.19
Change[4]	92.0 (-39.0, 251.0)	16.0 (-130.5, 123.5)	0.31
Iron (mg)			
Day 1	20.3 (13.0, 27.0)	15.1 (10.1, 22.3)	0.35
Day 5	26.4 (16.7, 27.0)	9.6 (7.4, 22.7)	0.05
Change[4]	0.6 (-3.1, 6.2)	-2.7 (-4.6, -0.25)	0.10
Magnesium (mg)			
Day 1	396.5 (239.5, 501.0)	261.5 (180.5, 397)	0.13
Day 5	446.5 (295.0, 479.5)	169.5 (126.5, 433.5)	0.07
Change[4]	1.0 (-46.0, 55.5)	-21.0 (-54.0, 18.0)	0.45
Selenium (µg)			
Day 1	71.4 (40.5, 90.0)	56.9 (42.3, 68.1)	0.52
Day 5	66.1 (48.8, 94.8)	53.5 (34.1, 69.7)	0.47
Change[4]	0.1 (-6.6, 13.8)	-6.6 (-14.0, 8.1)	0.43
Potassium (mg)			
Day 1	2762.5 (1872.5, 3811.0)	2349.5 (1655.0, 2832.5)	0.25
Day 5	2894.5 (2248.5, 3302.5)	1643.0 (1244.0, 2949.0)	0.13
Change[4]	-132.0 (-311.0, 312.5)	-45.0 (-671.0, 87.5)	0.82
Zinc (mg)			
Day 1	15.0 (9.0, 25.0)	11.5 (6, 19.5)	0.45
Day 5	18.5 (10.5, 25.0)	7.0 (4.5, 21)	0.10
Change[4]	0.0 (-1.5, 4.0)	-2.0 (-3.5, -0.5)	0.07

[1]Reported for a subsample (n=20) with complete diet and treatment prescription information
[2]Values are medians (IQR)
[3]p-values compare nutrient intakes between PKU treatment groups for camp day 1, camp day 5, and the change in intake with the intervention (Day 5 – Day 1) based on the Mann Whitney U Test.
[4]Change represents the absolute difference between nutrient intake on day 5 and day 1 of camp.
Abbreviations: DFE, Dietary Folate Equivalents; PKU, Phenylketonuria

CHAPTER 5: Characterization of Choline Nutriture Among Adults and Children with Phenylketonuria

Specific Aim 2

Meriah S. Schoen[1,2], Usha Ramakrishnan[3], Jessica A. Alvarez[4], Thomas R. Ziegler[4], Xiangqin Cui[5], Rani H. Singh[2]

[1]Nutrition and Health Sciences, Laney Graduate School, Emory University, Atlanta, GA, USA

[2]Department of Human Genetics, Emory University, Atlanta, GA, USA

[3]Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA, USA

[4]Department of Medicine, Emory University, Atlanta, GA, USA

[5]Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, GA, USA

*Nutrients (*2022), 14(19):4056. doi: 10.3390/nu14194056.

5.1 Abstract

Choline is an essential nutrient for brain development and function that is attained through high-protein foods, which are limited in the phenylalanine-restricted diet of people with phenylketonuria (PKU). This study compared choline consumption among individuals with PKU to a reference sample from the National Health and Nutrition Examination Survey (NHANES), and identified treatment and diet-related factors that may modulate choline needs. Participants were individuals with PKU (N=120, 4-61 years) managed with dietary therapy alone (n=49), sapropterin dihydrochloride for ≥1 year (n=38), or pegvaliase for ≥1 year with no medical food (n=33). NHANES participants were not pregnant or nursing and came from the 2015-2018 cycles (n=10,681, 4-70 years). Dietary intake data were used to estimate total usual intake distributions for choline, and mean probability of adequacy (MPA) was calculated as a summary index of nutrient adequacy for four methyl-donor/co-factor nutrients that impact choline utilization (folate, vitamin B12, vitamin B6, and methionine). Only 10.8% (SE: 2.98) of the total PKU sample (14.7% [SE:4.03] of children; 6.8% [SE: 2.89] of adults) achieved the adequate intake (AI) for choline, while 12.2% (SE:0.79) of the NHANES sample met the recommended level. Adults receiving pegvaliase were the most likely to exceed the AI for choline (14.82% [SE: 4.48]), while adults who were on diet therapy alone were the least likely (5.59% [SE: 2.95]). Without fortified medical foods, individuals on diet therapy and sapropterin did not achieve the AI, and MPA for other methyl donor/co-factor nutrients was reduced. More frequent monitoring of choline intake and increased choline fortification of medical foods could benefit this population.

5.2 Introduction

Phenylketonuria (PKU; OMIM #261600) is autosomal recessive disorder characterized by a deficiency of the phenylalanine hydroxylase (PAH; EC 1.14.16.1), an enzyme required to metabolize the amino acid phenylalanine (Phe) [1]. Untreated PKU leads to Phe accretion and subsequent significant changes in brain development and function [2]. PKU has traditionally been managed with a Phe-restricted diet combined with Phe-free or Phe-reduced amino acid (AA-MF) or glycomacropeptide medical foods (GMP-MF) to achieve protein adequacy while maintaining Phe within the recommended therapeutic range (120-360 µmol/L) [3,4]. Beyond providing essential amino acids, several medical foods are also fortified with synthetic vitamins, minerals, and essential fatty acids to provide a complete nutritional profile [5,6]. Nevertheless, nutrient inadequacies (e.g. docosahexaenoic acid, eicosapentanoic acid, cholesterol, potassium) still occur [7,8] and may be a particular concern among patients who have transitioned to liberalized or fully normalized diets with the use of new pharmacotherapies [9,10]. As suboptimal nutriture may contribute to cellular dysfunction, including the residual neurocognitive deficits observed in PKU [11,12], it is critical to further evaluate micronutrient intakes and status in this population.

Given the neurocognitive deficits observed in PKU, it is important to ensure adequate intakes of nutrients that play a critical role in neurotransmission and related processes. One such nutrient is choline, which serves as a precursor for phospholipids (e.g., phosphatidylcholine, sphingomyelin, ceramide) that are indispensable for the synthesis and structural integrity of myelin sheaths and cellular membranes throughout the brain and body [13] **(Figure 5-1)**. In the brain, decreased concentrations of these phospholipids due to suboptimal choline intake may result in demyelination, white matter abnormalities, and mitochondrial dysfunction [14,15].

These structural and functional alterations may impair executive cognitive processes and enhance oxidative damage, both of which have been reported among individuals with PKU [16-18]. As choline can also be oxidized to the methyl-donor betaine, choline availability may additionally contribute to the differential epigenomic methylation patterns and subsequent changes in gene expression that have been observed in human [19] and rodent models [20] of PKU. Despite the important role of choline in neurocognitive development, there is a paucity of data on the effect of decreased choline consumption in patients with PKU.

Choline has limited endogenous synthesis [21] and is concentrated in high-protein foods [22], such as beef, chicken, fish and eggs, which are generally restricted in a PKU diet due to high Phe content. Thus, fortified medical foods are the predominant source of choline for individuals with PKU. The only study to have previously assessed choline intake among individuals with PKU [8] identified suboptimal choline intakes in 40% and 63% of participants, respectively, who regularly consumed GMP-MF and AA-MF. When considering the choline contribution solely from natural foods, no individuals in this cohort were capable of meeting the adequate intake (AI) level [8]. This is concerning given medical food compliance is typically low [23] and adjunct pharmacotherapies are now available that allow medical food consumption to be significantly reduced or discontinued. Beyond treatment regimen, choline nutriture may be further perturbed by suboptimal intake of other methyl-donor or co-factor nutrients that impact choline utilization, including the micronutrients folate, vitamin B12, vitamin B6, and the essential amino acid methionine. Poor intake of any of these nutrients may increase the dietary requirement for choline by increasing its utilization as a methyl donor and decreasing endogenous choline synthesis [24-26].

The objective of this study was to evaluate usual choline intake among individuals with PKU and the role of two factors that may modulate the dietary requirement for this nutrient: (1) treatment regimen, and (2) the concurrent intake of other nutrients that affect choline metabolism.

5.3 Materials and Methods

5.3.1 Study Participants

Dietary, biochemical, and demographic data for adults and children with PKU (4-61 years of age) were extracted from the research records of five clinical observational studies that were conducted at Emory University (Atlanta, GA) and Boston Children's Hospital (BCH; Boston, MA). To assess the impact of treatment regimen on usual choline intake, participants were categorized into three groups based on the use of diet therapy only, the synthetic tetrahydrobiopterin therapy, sapropterin dihydrochloride (Kuvan®, BioMarin Pharmaceutical Inc, Novato, CA, USA), and the enzyme replacement therapy, pegvaliase (Palynziq®, BioMarin Pharmaceutical Inc, Novato, CA, USA). For inclusion in the sapropterin or pegvaliase groups, participants were required to have diet records that had been collected >1 year after treatment initiation. Medical food discontinuation was additionally required for inclusion in the pegvaliase group.

Data from the 2015-2018 National Health and Nutrition Examination Survey (NHANES) was used as a reference population. This cross-sectional survey is administered by the National Center for Health Statistics (NCHS) and uses a multistage probability cluster design to obtain a representative sample of the noninstitutionalized, civilian U.S. population [27]. To mirror the demographic characteristics of the PKU sample, the present study included NHANES participants 4-70 years of age who completed two nonconsecutive 24-hour recalls. Individuals

who were pregnant or nursing were excluded. Study protocols for both the PKU and NHANES cohorts were approved by the respective Research and Ethics Review Boards (Emory, BCH, NCHS), and written informed consent was obtained from all participants. The final analytic sample included 120 participants with PKU (n=49 on diet therapy alone, n=38 on sapropterin, n=33 on pegvaliase) and 10,681 NHANES controls.

5.3.2 Quantification of Nutrient Intake from Food and Supplemental Sources

Participants in the PKU cohort completed diet records over two (n=6) or three (n=114) consecutive days, with detailed descriptions of all foods, beverages, supplements, and medical foods consumed. At the time of collection, records were reviewed for accuracy and completeness by a trained research registered dietitian. If participants had several complete diet records with at least two consecutive 24-hour recalls, the record with the most recent collection date was selected for the present study. Records were then analyzed using the Nutrition Data System for Research (NDSR 2020, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN) to determine dietary and supplementary intake of choline, vitamin B12, vitamin B6, folate, and methionine. While the NDSR Database contains over 18,000 foods [28], it does not include nutritional products that are critical for PKU treatment. This includes Phe-free or reduced Phe medical foods, which provide 85-90% of the protein needs for an individual with PKU, and low protein modified foods, which are common products made with low-protein flours [29]. Given the importance of these products, 19 medical foods were added to the database using the manufacturer-supplied nutrient information. For 33 low protein modified foods, recipes were created using ingredients within the NDSR database that contain composition data for choline and the other nutrients of interest. Any additional food items that were not found within the NDSR database were substituted for nutritionally comparable foods using the following set of

nutrient tolerances per 100 g of food: 85 kcal, 2.5 g of fat, 100 mg of sodium, 10 g of carbohydrates, 5 g of protein, and 50 mg of phenylalanine [30].

For the NHANES cohort, diet and supplement data were obtained from two 24-hour recalls collected using the automated multiple-pass method [31]. The first recall was collected in person at the mobile examination center, while the second recall was collected by telephone three to 10 days later. For each recall day, choline intake from food sources was determined based on nutrient values from the USDA's Food and Nutrient database for Dietary Studies [32]. Supplemental choline consumption was derived from the dietary supplement questionnaire, which evaluates the use of vitamins, minerals, herbs, and other supplements over the 30 days preceding the dietary recall interview. Mean daily intake of supplemental choline was calculated based on the amount of choline taken across all supplements and the number of days each supplement was used.

5.3.3 Estimation of Usual Intake

Daily nutrient intake data was extrapolated from the 24-hr recalls and 3-day food records completed by NHANES and PKU participants, respectively. The use of two different diet data collection strategies was deemed appropriate based on a recent study [33], which found that the two strategies resulted in equivalent intake distributions for regularly consumed nutrients. Total usual nutrient intakes for choline, vitamin B12, vitamin B6, folate, and methionine, were estimated using the National Cancer Institute (NCI) method [34].This approach uses Box-Cox transformed consumption-day data in a mixed effects model to adjust for relevant covariates and estimate within- and between-person variability. Using Monte-Carlo simulations, the estimates from this model were then used to derive usual intake distributions for populations and subgroups [35]. The present study estimated usual nutrient intake from both diet and

supplemental sources using the "shrink-then-add" approach [36], and incorporated three covariates in the mixed model: sequence of the recall/record, day of the week that consumption data were collected, and subgroup (age and PKU treatment groups). For the PKU sample, nutrients from medical food were differentiated from dietary supplements (DS). The relative contributions of medical food and DS to total usual nutrient intakes were calculated at the population level by dividing usual supplement intake and medical food intake by the total usual nutrient intake.

5.3.4 Estimation of Nutrient Probability of Adequacy

For choline, nutrient adequacy and excess intake was assessed at the individual level using the cut-point method by comparing usual intakes to age- and gender-specific AI levels and tolerable upper intake levels (UL) [37]. The probability approach was used to estimate the probability of usual intake adequacy (PA) for folate, vitamin B12, vitamin B6, and methionine. Using the estimated average requirement (EAR) and standard deviation (SD) from the Institute of Medicine requirement distributions [38], PA was calculated as the percent of the requirement distribution that falls below an individual's usual intake. Mean probability of adequacy (MPA) was calculated for each individual in both the PKU and NHANES groups as an average of the PA values for folate, vitamin B12, and vitamin B6. Mean probability of adequacy with methionine (MPAm) was also calculated for the PKU group, but not NHANES given dietary data for methionine was not available. Both MPA and MPAm were used as summary indices of nutrient adequacy for the four nutrients that impact choline utilization.

5.3.5 Statistical Analysis

All statistical analyses were completed in SAS (version 9.4, SAS Institute, Cary, NC). The steps required for usual intake estimation were carried out using the SIMPLE Macro [39],

which links three macros developed by the NCI. Standard errors for intake estimates were approximated by a simple bootstrap using 250 replicate weights for the PKU sample. For the NHANES samples, standard errors were estimated using Fay's balanced repeated replication, and 32 replicate weights were generated with a factor of 0.3. Replicate weights for both samples were computed from the initial sampling weights. For NHANES, 4-year weights were constructed from the dietary two-day sample weights. For PKU, sample weights were calculated based on the demographic and treatment profile of the PKU population at the Emory Genetics Clinic, which this study assumed was representative of the United States PKU population.

To attain nutrient intake estimates that were representative of the United States population and could be compared to prior research [40], this study chose not to match PKU participants to a small sub-sample of participants from NHANES. Instead, the PKU and NHANES groups were evaluated separately using descriptive statistics to compare demographic characteristics and usual intakes by age (<18 years and >18 years) and treatment status (for the PKU sample). No inferential statistics were employed for group comparisons given different sampling strategies were used to generate the PKU and NHANES populations.

5.4 Results

Demographic and treatment characteristics of the PKU and NHANES samples are described in **Table 5-1**. Adults with PKU in all three treatment groups were predominantly female and younger on average compared to their unmatched NHANES counterparts. Children with PKU in the diet therapy group were also predominantly female, but were older than the NHANES sample. Overweight and obesity were more common among individuals with PKU in both age categories, with adults receiving pegvaliase (81.8%) and children receiving sapropterin (47.0%) demonstrating the highest prevalence. Within the PKU sample, there were notable

differences in adherence across treatment groups, as denoted by plasma Phe concentrations. On average, children managed with both sapropterin and diet therapy had better adherence (lower Phe concentrations) and reported increased medical food consumption compared to adults on the corresponding treatments. Seventy-four individuals (n=49 diet therapy, n=25 sapropterin) within these treatment groups had medical food prescriptions and reported using 18 different medical foods (Table S1). Thirteen of these products contained choline and other nutrients that impact choline metabolism, however, the amount of choline was highly variable (range: 67 mg-530 mg/100 g medical food). Five medical foods were not fortified with any micronutrients or contained only select minerals and trace elements. Four adults (n=2 diet therapy, n=2 sapropterin) and two children (both sapropterin) who were prescribed "nutritionally complete" medical foods did not report medical food consumption during the recall period. Six adults on diet therapy and two adults receiving sapropterin were adherent with their formula prescription, but were solely consuming medical foods that lacked choline and other relevant nutrients.

5.4.1 Estimated Usual Choline Intake

Estimated mean usual total choline intakes for individuals 4-70 years of age in the PKU and NHANES populations were 230.81 (SE: 14.82) and 324.0 (SE: 2.84) mg/day, respectively. Usual intakes for individuals with PKU stratified by treatment groups are reported in Table 2 for adults and Table 3 for children. On average, adults in both the PKU and NHANES samples consumed more choline than their pediatric counterparts. Adults, however, were less likely to achieve the AI, compared to children. In the unaffected population, only 22% (SE:1.08) of children (4-17 years) and 9.5% (SE: 0.92) of adults (18-70 years) exceeded the AI for choline. When accounting for all sources of choline (food, medical food, dietary supplements), adults with PKU on sapropterin or pegvaliase had a higher prevalence of choline adequacy than adults

from NHANES. The opposite was observed among adults on diet therapy and all children with PKU. Across the full PKU sample, adults receiving pegvaliase were the most likely to exceed the AI for choline [14.82% (SE: 4.48)] while adults who were solely on diet therapy were the least likely [5.59% (SE: 2.95)]. Usual choline intake did not exceed the UL in any of the subgroups evaluated.

Among patients with PKU on diet and sapropterin therapies, medical food contributed 38% to 72% of total choline intake **(Figure 5-2)**. Choline was not obtained from any other dietary supplements. Without the consumption of choline-fortified medical foods, individuals in these treatment groups were not be able to achieve the AI **(Table 5-2 and Table 5-3)**.

5.4.2 Mean Probability of Adequacy (MPA) for Nutrients that Affect Choline Metabolism

When considering total intake from food sources, medical food, and dietary supplements, median MPA of the methyl-donor nutrients that impact choline utilization was 100% among adults and children in the PKU and NHANES cohorts, suggesting that there was a very low probability of inadequate intake **(Table 5-4)**. While dietary supplements contributed 40%, on average, to intakes of these micronutrients and amino acids among the PKU pegvaliase group (Figure 1), median MPA and MPAm did not exhibit significant change when solely considering intake from food sources. The NHANES cohort exhibited the same trend. Among the diet therapy and sapropterin groups, however, the exclusion of medical foods and dietary supplements resulted in a notable decrease in median MPA and MPAm. This was particularly evident among children in the diet therapy group, who obtained 57% to 85% of their methyl-donor nutrient intake from medical foods **(Figure 5-2)**.

5.5 Discussion

The importance of dietary choline for neurological development and sustained cognitive function has been supported by animal models and human studies [41-43]. Yet data on the adequacy of dietary intakes of choline remain limited, especially among populations with an increased risk of choline deficiency and neurocognitive deficits, such as PKU. This descriptive study is the first to estimate usual choline intake among adults and children with PKU across three therapies that could modulate choline needs. Our findings suggest that the overall prevalence of choline adequacy was lower in the PKU sample than the US population from NHANES; however, the proportion of individuals able to exceed the AI for choline was relatively low in both populations. This aligns with previous assessments of choline intake in NHANES [22,40], and suggests that educational efforts and supplement consumption remain limited and/or are not substantially impacting choline intake. Our observations also complement one prior study in PKU [8], which also found that individuals on sapropterin and/or dietary treatment were not able to achieve the AI for choline without the consumption of fortified medical foods. This study advances the previous research by using the NCI method to estimate usual choline intake distributions and separately characterize consumption patterns by treatment group. These treatment-specific estimates lend insight into the impact of diet liberalization and normalization on nutrient adequacy.

Across PKU treatment groups, adults on diet therapy were the least likely to achieve choline intakes above the AI. There are several factors that may have contributed to this finding. The first is poor treatment adherence, which was evident in the group's elevated median Phe concentration, and could have derived from the inconsistent intake of fortified medical foods and/or the overconsumption of food items lacking choline. The low prevalence of choline

adequacy also may have been affected by the intake patterns of six patients (35% of the group) who exhibited good treatment adherence but were not consuming choline-fortified medical foods or multivitamin/mineral supplements. Alternately, some patients in this group were consuming fortified medical foods as prescribed, but the amount of choline in these products may not have been sufficient to compensate for the lack of choline in their protein-restricted diets. Given treatment nonadherence has been found to increase with age in PKU [23], and may impact choline status, it would be beneficial to start monitoring choline intake more regularly in this group. Moreover, medical food formulations may benefit from higher amounts of added choline given most mainstream multivitamin/multimineral supplements still do not contain choline [44], and thus cannot serve as an alternate nutrient source for this population.

Adults receiving pegvaliase, who were consuming an unrestricted diet, were found to have the highest prevalence of choline intake above the AI. The proportion of patients meeting this recommendation was slightly higher than NHANES, suggesting that patients managed with pegvaliase require less supplemental choline than their counterparts on sapropterin or diet therapy to match the intake levels of their unaffected peers. Both groups also had similar usual intakes for the methyl-donor and co-factor nutrients that impact choline metabolism. Median MPA and MPAm were 100% among adults on pegvaliase and NHANES controls, and both indices did not notably change when excluding the nutrient input from dietary supplements. Micronutrient and protein adequacy for 18 of the 33 individuals in the pegvaliase group had been previously evaluated in another study [45], which found that nutrient intakes for most patients met or exceeded the dietary reference intake (DRI), and that overall diet quality (assessed by the Healthy Eating Index) was nearly equivalent to the 2015-2016 NHANES sample. The present

study expands these findings by identifying similar patterns for a group of essential nutrients that were previously not assessed.

Although the pegvaliase group was able to consume adequate amounts of the methyl donor and co-factor nutrients solely through diet, the other PKU therapy groups could only attain equivalent MPA and MPAm scores when medical food and dietary supplements were consumed. Without these supplements, patients on diet therapy and sapropterin may not have the required methyl donor concentrations to compensate for suboptimal choline intakes. This is a concern given the present study identified a low prevalence of choline adequacy even with the consumption of fortified medical foods. Poor metabolic control could further increase the risk of choline deficiency given high Phe levels may limit the endogenous synthesis of choline. This could occur through the increased production of phenylacetate, a phenylketone that has been reported to inhibit the estrogenic induction of the rate-liming enzyme, phosphatidylethanolamine-N-methyltransferase [46,47]. While future research is required to directly assess how choline synthesis is modulated by Phe control, the present findings emphasize the importance of patient education for maintaining treatment adherence in PKU, and subsequently increasing choline consumption and the intake of nutrients that support choline metabolism

As choline status may be an important contributor to differences in neurocognitive outcomes among individuals with PKU [12], this analysis provides foundational insight into the dietary and pharmacological factors that impact choline adequacy in this population. A limitation of this study is that dietary intakes were not determined in tandem with biochemical measures. The utility of this, however, remains unclear as there are important limitations associated with choline biomarkers [48]. For example, plasma choline has not been found to reflect the diet at

very low intake levels [47,49] and plasma betaine concentrations, which may be a better marker of dietary intake, does not accurately represent tissue concentrations [50]. Through the use of metabolomics and isotope dilution, emerging studies can better estimate the relationship between choline intake and choline status to provide new clinically useful biomarkers [51]. Another shortcoming of the present study was the potential measurement error with self-reported dietary data. This may have resulted in an underestimation or overestimation of both micronutrient and amino acid intakes, which could have been further impacted by our collection strategy for dietary supplements. For the PKU sample, dietary supplements were collected on the day of each diet record, which may not be as representative of long-term intake as the 30-day questionnaire administered by NHANES. The impact of our assessment technique may be minimal given choline is not included in most dietary supplements, and there remains limited knowledge regarding best practices for supplement reporting [36].

Despite these limitations, the present analysis was strengthened by the application of statistical models that adjust dietary intakes for the effects of random measurement error, which allowed for the estimation of usual intake distributions. An additional strength was the comparison of PKU intake distributions to a nationally representative sample of unaffected individuals. This provided prospective on the normalization of nutrient intake based on PKU therapy and age group. Although this study's sample was relatively large given PKU is a rare disease, future analyses would benefit from additional participants so that differences in intake trends by sex could also be assessed.

5.6 Conclusions

In summary, this study demonstrated that adults and children with PKU who are managed on phenylalanine-restricted and liberalized diets were unable to achieve the AI for choline

without the consumption of medical food. Future research is needed to determine if suboptimal choline intake affects cognitive function, particularly among individuals on diet therapy and sapropterin with poor treatment adherence.

5.5 References

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5.6 Tables and Figures

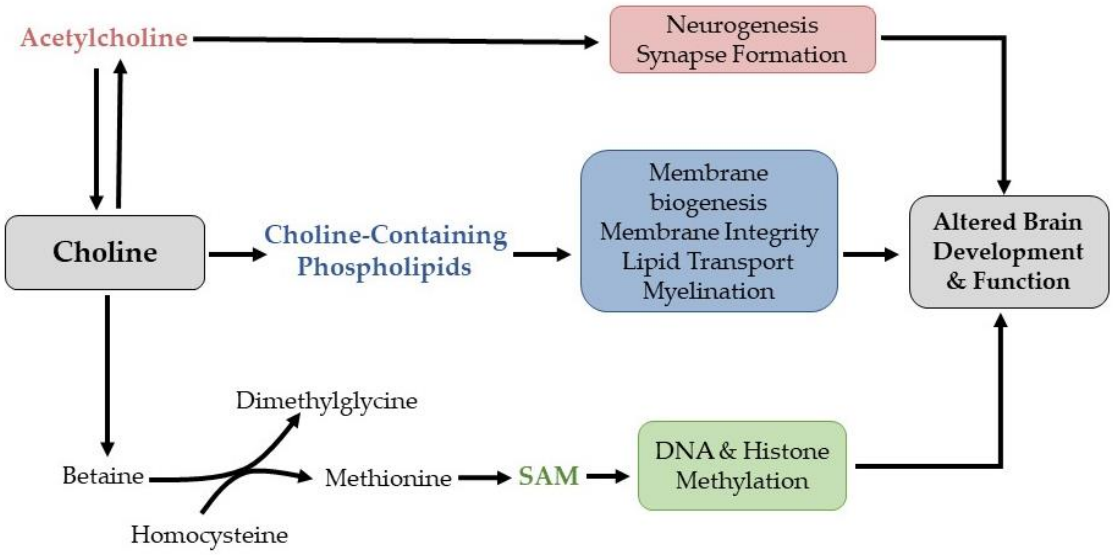

Figure 5-1. Choline serves as precursor for the following metabolites which impact brain development and function: (1) acetylcholine, a neurotransmitter that is involved in neurogenesis and synapse formation; (2) choline-containing phospholipids (e.g. phosphatidylcholine, sphingomyelin) which contribute to membrane biogenesis, lipid transport, and myelination; (3) betaine, which participates in one-carbon metabolism and aids in the regeneration of S-Adenosylmethionine (SAM). SAM is a methyl donor for both DNA and histone methylation.

Table 5-1. Characteristics of the NHANES (N=10,681) and PKU (N=120) Study Populations, by Age and Treatment Group.

	PKU			NHANES
	Diet Therapy	**Sapropterin dihydrochloride**	**Pegvaliase**	
Adults (>18 years)	n=17	n=21	n=33	n=7,267
Age, years[2]	21 (18, 26)	27 (22, 34)	36 (32, 44)	43 (30, 56)
Age range, years	18-50	18-45	22-61	18-70
Female, n (%)[1]	12 (70.6)	13 (61.9)	20 (60.6)	3,589 (50.6)
Caucasian, n (%)[1]	16 (94.1)	21 (100.0)	33 (100.0)	2,191 (61.3)
Weight status, n (%)[1]				
Overweight	7 (41.2)	5 (23.8)	11 (33.3)	2040 (30.0)
Obese	4 (23.5)	8 (38.1)	16 (48.5)	2939 (41.5)
Plasma phe (µmol/L)[2]	842.0 (613.2, 951.1)	362.0 (261.0, 533.2)	23.0 (3.0, 218.0)	--------
Taking medical food, n (%)	17 (100.0)	12 (57.1)	0 (0.0)	--------
Children (<18 years)	n=32	n=17	n=0	n=3,414
Age, years[2]	14 (10, 16)	10 (8, 13)	--------	10 (7, 14)
Age range, years	4-17	6-16	--------	4-17
Female, n (%)[1]	23 (71.9)	8 (47.1)	--------	1666 (49.1)
Caucasian, n (%)[1]	31 (96.9)	16 (94.1)	--------	999 (49.6)
Weight status, n (%)[1]			--------	
Overweight	4 (12.5)	4 (23.5)	--------	526 (15.8)
Obese	4 (12.5)	4 (23.5)	--------	719 (21.2)
Plasma phe (µmol/L)[2]	626.0 (323.5, 1082.0)	302.0 (185.0, 397.0)	--------	--------
Taking medical food, n (%)	32 (100.0)	13 (76.5)	--------	--------

[1]Categorical variables are reported as the unweighted frequency and weighted percent.
[2]Continuous variables are reported as the median (IQR).

Table 5-2. Total Usual Intakes and the Estimated Percent (%) of Usual Intakes Above the Adequate Intake (AI) for Choline Among Adults 18-70 Years of Age with PKU (n=71) and Adults from NHANES 2015-2018 (n=7,267).

		Percentile				
Subgroup	**n[1]**	**Mean (SE)**	**25th (SE)**	**50th (SE)**	**75th (SE)**	**% >AI**
NHANES	7,267	341.3 (3.6)	257.9 (3.6)	327.5 (3.5)	409.0 (4.7)	9.5
PKU diet therapy	17					
With MF[2]		203.6 (34.5)	116.4 (18.0)	175.7 (24.0)	263.9 (32.5)	5.6
Without MF[2]		115.3 (13.3)	73.3 (11.0)	101.5 (13.2)	145.6 (17.2)	0.2
PKU sapropterin	21					
With MF[2]		299.4 (37.0)	179.2 (22.2)	268.3 (27.3)	390.6 (37.4)	14.2
Without MF[2]		176.6 (21.4)	114.2 (17.6)	160.4 (20.7)	222.1 (26.7)	0.8
PKU pegvaliase	33	302.3 (28.0)	185.1 (21.8)	273.4 (26.2)	389.5 (35.3)	14.8

[1]Represents the unweighted sample size.
[2]Represents the usual intakes and the estimated percent (%) of usual intakes above the AI for the designated treatment group under two conditions: (1) with reported medical food intake, (2) without medical food (hypothetical).
Abbreviations: AI, adequate intake; MF, medical food; PKU, phenylketonuria.

Table 5-3. Total Usual Intakes and the Estimated Percent (%) of Usual Intakes Above the Adequate Intake (AI) for Choline Among Children 4-17 Years of Age with PKU (n=49) and Children from NHANES 2015-2018 (n=3,414)

Subgroup	n[1]	Mean (SE)	Percentile			% >AI
			25 (SE)	50 (SE)	75 (SE)	
NHANES	3,414	261.2 (2.8)	191.5 (2.9)	249.3 (2.9)	318.2 (3.6)	22.0
PKU diet therapy	32					
With MF[2]		221.0 (16.0)	148.2 (12.7)	211.2 (15.0)	287.0 (24.2)	12.3
Without MF[2]	32	61.6 (5.93)	45.4 (6.0)	58.9 (5.9)	75.6 (7.4)	0
PKU sapropterin	17					
With MF[2]		174.8 (15.1)	105.3 (15.2)	167.4 (15.8)	232.4 (22.4)	6.4
Without MF[2]		96.7 (8.8)	75.6 (9.6)	94.6 (8.9)	115.1 (9.9)	0

[1]Represents the unweighted sample size.
[2]Represents the usual intakes and the estimated percent (%) of usual intakes above the AI for the designated treatment group under two conditions: (1) with reported medical food intake, (2) without medical food (hypothetical).
Abbreviations: AI, adequate intake; MF, medical food; PKU, phenylketonuria.

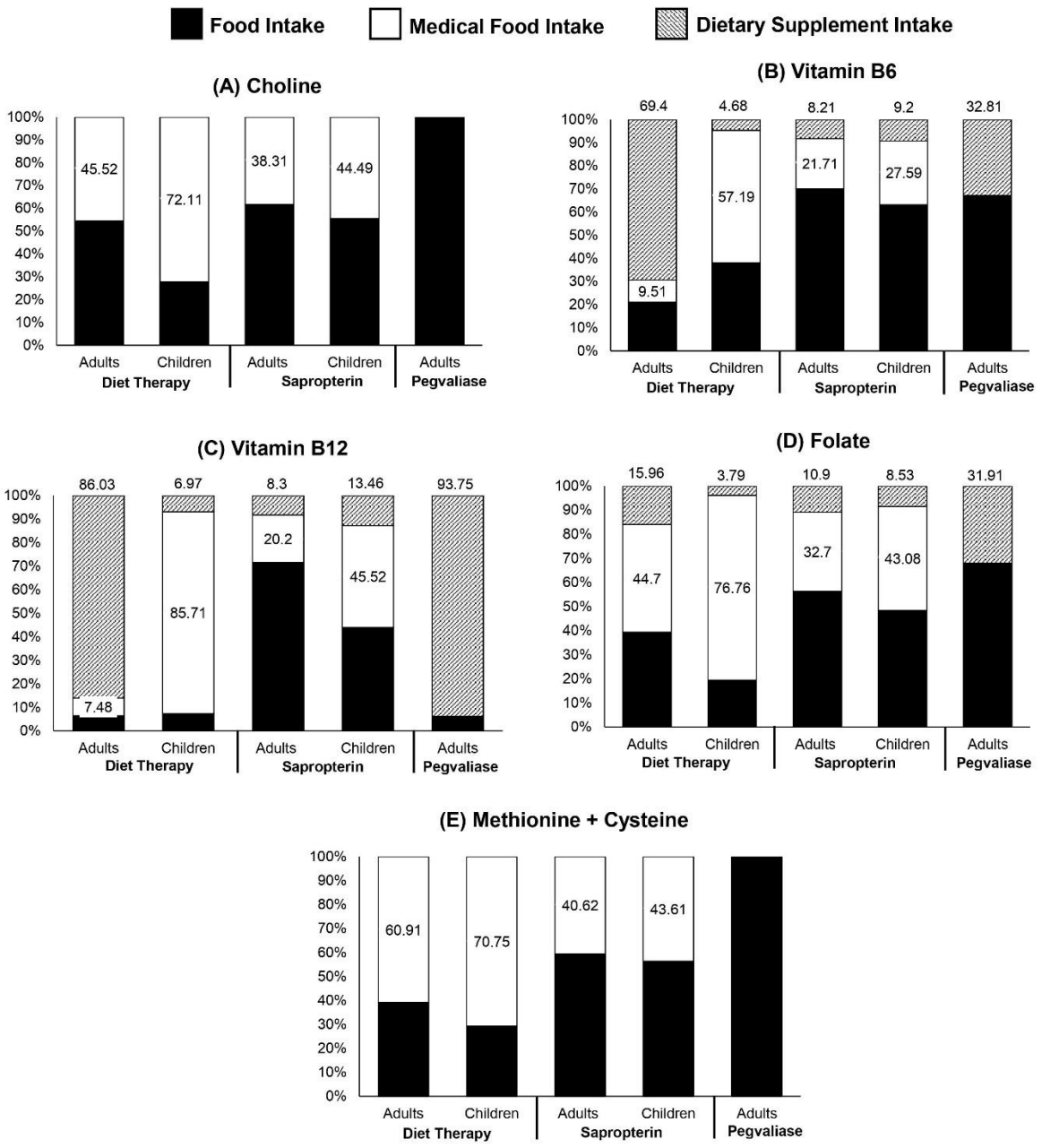

Figure 5-2. The contribution of food sources, medical food, and dietary supplements to total usual intakes for (A) choline, (B) vitamin B6, (C) vitamin B12, (D) folate, and (E) methionine plus cysteine among adults (n=71) and children (n=49) with PKU, stratified by treatment group (diet therapy, sapropterin, and pegvaliase). Percentage values within white bars represent the contribution from medical food and percentage values above each bar represent the contribution of dietary supplements.

Table 5-4. Mean Probability of Adequacy (MPA) of nutrients that affect choline metabolism (vitamin B6, vitamin B12, folate, methionine) among individuals with PKU (n=120) and NHANES participants (n=10,681), by age and treatment group[1].

	Adults			Children		
	n[2]	**MPA[3]**	**MPAm[4]**	**n[2]**	**MPA[3]**	**MPAm[4]**
NHANES	7,267			3,414		
With DS[5]		100 (100,100)	-----		100 (100,100)	-----
Without DS[5]		100 (100,100)	-----		100 (100,100)	-----
PKU diet therapy	17			32		
With MF + DS[5]		100 (100,100)	100 (100, 100)		100 (100,100)	100 (100,100)
Without MF + DS[5]		65.1 (26.7, 96.3)	48.8 (25.2, 72.6)		37.2 (1.6, 64.8)	28.1 (3.2, 51.8)
PKU sapropterin	21			17		
With MF + DS[5]		100 (95.7, 100)	100 (83.9, 100)		100 (94.7, 100)	98.9 (88.1, 100)
Without MF + DS[5]		77.4 (66.7, 100)	74.4 (52.6, 98.5)		66.7 (31.9, 97.3)	73.3 (25.4, 97.3)
PKU pegvaliase	33			0		
With DS[5]		100 (96.1, 100)	100 (97.1, 100)		-----	-----
Without DS[5]		99.8 (80.0, 100)	99.8 (85.0, 100)		-----	-----

[1]Reported as the median (IQR) of the distribution of MPA.
[2]Represents the unweighted sample size.
[3]Probabilities of adequacy for vitamin B6, vitamin B12, and folate were averaged for each participant to estimate MPA.
[4]MPAm was estimated in the same manner as MPA but includes the probability of adequacy for the sum of methionine and cysteine. Dietary data for methionine was not available for NHANES.
Abbreviations: DS, dietary supplements; MF, medical food; MPA, mean probability of adequacy; MPAm, mean probability of adequacy with methionine; PKU, phenylketonuria.
[5]Represents the MPA and MPAm (PKU only) for the designated diagnosis and/or treatment group under two conditions: (1) with reported medical food and dietary supplement intake, (2) without medical food or dietary supplements (hypothetical).

5.7 Supplementary Tables and Figures

Table S5-1 (supplementary). Amount of Choline, Vitamin B12, Vitamin B6, Folic Acid, and Methionine found in 100g or 100mL of the Medical Foods Reported by Participants with PKU

Medical Food	N[1]	Choline (mg)	Vitamin B12 (mcg)	Vitamin B6 (mg)	Folic Acid (mcg)	Methionine (g)
Phenex-2	28	100	5	1.10	425	0.60
Phenylade MTE Amino Acid Blend	10	0	0	0	0	1.89
Phenyl-free 2HP	5	67	3.10	1.29	470	0.88
Phenyl-free 2	4	98	2.40	0.98	350	0.48
Phlexy-10 Drink Mix	4	0	0	0	0	0.85
Phenylade Amino Acid Blend	3	0	0	0	0	1.95
Phenylade 60	3	425	4.20	2.30	700	1.45
Glytactin RTD 15	3	82.4	0.30	0.20	56	0.06
Glytactin Bettermilk	3	375	1.10	1.50	200	0.45
PKU Periflex Advance	2	376	2.70	1.40	430	0.64
Phenylade Essential	2	210	3	1.40	300	0.61
Phenylade GMP Mix-In	2	0	0	0	0	1.17
PKU Cooler	2	102	0.82	0.44	51	0.26
PKU Maxamum	1	430	3.10	1.60	491	0.87
PKU Express	1	530	4.30	2.50	360	1.24
Phenylade 40	1	284	2.80	1.50	468	1.08
PKU Easy Microtabs	1	0	0	0	0	1.42
Camino Pro PKU	1	95.5	0.90	0.30	81.4	0.29

[1]Reflects the number of participants who reported taking a specific medical food. Participants who were consuming two or more medical foods were reported more than once.

CHAPTER 6: Total Choline Intake and Working Memory Performance in Adults with Phenylketonuria

Specific Aim 3

Meriah Schoen[1,2], Kelly Boland[3], Shawn E. Christ[3], Xiangqin Cui[4], Usha Ramakrishnan[5], Thomas R. Ziegler[6], Jessica A. Alvarez[6], Rani H. Singh[2]

[1]Nutrition and Health Sciences, Laney Graduate School, Emory University, Atlanta, GA, USA

[2]Department of Human Genetics, Emory University, Atlanta, GA, USA

[3]Department of Psychological Sciences, University of Missouri, Columbia, MO, USA

[4]Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, GA, USA

[5]Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA, USA

[6]Department of Medicine, Emory University, Atlanta, GA, USA

In preparation for submission to *Molecular Genetics and Metabolism*

6.1 Abstract

Background: Even with early diagnosis and consistent treatment with a phenylalanine (Phe)-restricted diet, many individuals with phenylketonuria (PKU) still exhibit neurological changes and experience deficits in executive function. Suboptimal choline intake may contribute to these impairments, particularly those associated with working memory, but this relationship has not been previously investigated in PKU.

Objectives: To determine if higher choline intake normalizes working memory performance in adults with PKU relative to healthy controls, and to assess whether the association between total choline intake and working memory is dependent on metabolic control in participants with PKU.

Materials and Methods: This was a remote, cross-sectional study of 40 adults with PKU and 40 healthy controls, matched by age, sex, and education level. All study visits were conducted by video and/or phone and included diet collection via 3-day dietary records, assessment of metabolic control via filter paper blood spots, and the evaluation of working memory performance and overall intelligence using the Cambridge Neuropsychological Test Automated Battery (CANTAB) and the Wechsler Adult Intelligence Scale. Total choline intake was quantified from dietary records and a working memory composite was computed by averaging z-scores from each CANTAB task. Historical Phe data was obtained from PKU medical records. ANCOVA and multivariate regression models were used to assess if the relationship between total choline intake and overall working memory differed by diagnosis (PKU vs control), and if the relationship between choline intake and overall working memory was affected by metabolic control (PKU group only).

Results: After adjusting for intellectual ability, overall working memory performance was 0.32 z-scores (95% CI: 0.06, 0.58) lower, on average, in participants with PKU compared to controls.

Scores in both groups were not modified by total choline intake (F_1,75=0.85, p=0.36). In a subsample of PKU participants who demonstrated well-controlled blood Phe levels throughout mid-childhood and adulthood, working memory performance was improved with higher concurrent total choline intake (average change in working memory per 1mg change in choline = 0.002; 95%CI: 0.0002, 0.003; p=0.02). However, there was a relevant, but not statistically significant, trend for the relationship between improved working memory performance and increased choline intake, though weak, as Phe concentrations increased (p=0.10).

Conclusions: Clinical monitoring of choline intake is essential for all individuals with PKU, but may have important implications for working memory function among patients with good metabolic control. Results from this study should be confirmed in a larger, controlled trial with complete data on current and historical Phe measures.

6.2 Introduction

Phenylketonuria (PKU; OMIM #261600) is a rare genetic metabolic disorder characterized by impaired phenylalanine (Phe) metabolism. When disrupted, this pathway produces elevated Phe and reduced tyrosine concentrations, which lead to profound chemical and morphological changes in the brain (1). These changes include reduced neurotransmitter synthesis (2), abnormalities in both white and gray matter (3, 4), and disruptions in the functional connectivity between brain regions (5, 6). While these alterations manifest as severe developmental delays and intellectual disability among individuals with untreated PKU (7), newborn screening has led to a remarkable change in prognosis. With early diagnosis and the prompt initiation of a Phe-restricted diet, severe neurological sequelae can be prevented (1). This, however, has not ameliorated all manifestations of the disorder. Many individuals with PKU still exhibit a decrease in overall intellectual functioning (8), in addition to impaired executive abilities, such as problem solving, inhibitory control, task switching, and working memory (9-11).

These deficits may derive from periods of poor metabolic control due to medical food nonadherence and/or the consumption of excess intact protein. Previous research, however, suggests that current and historical fluctuations in Phe may account for only 43% of the variance in overall cognitive performance of early-treated patients with PKU (12). This suggests that there are other relevant factors that may contribute to phenotypic variability. Beyond blood Phe levels, genotype, age, and inherent individual differences, these factors remain largely unknown (13).

To begin identifying alternate drivers of cognitive variability, the present study investigated one essential nutrient, choline, which affects the brain through several pathways. Choline serves as a precursor for phospholipids, the methyl-donor betaine, and the

neurotransmitter acetylcholine (14). These products play critical roles in cell membrane integrity (15), one-carbon metabolism (16), and neuromodulation (17). The importance of these functions for neurological development and sustained cognitive performance has been demonstrated in both animal and human studies, which have shown that higher prenatal choline intake has long-term benefits for attention and memory (18). In children, this effect has been reported through seven years of age (19, 20), while rodent models have demonstrated improvement across the lifespan (21). Evidence is more limited among healthy human adults, but one prior study has found a positive association between concurrent choline intake and memory, in addition to an inverse association between prior choline intake and white matter hyperintensity volume (22).

Despite choline's potential link to many of the pathophysiological changes in PKU (e.g., demyelination, oxidative damage, differential methylation, impaired neural connectivity) (15, 23-25), few studies have investigated the relationship between choline and neurocognition in the PKU population. The only prior research was conducted in a PKU mouse model, which evaluated choline in the context of a multi-nutrient supplement that also contained uridine monophosphate (UMP), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), phospholipids, folic acid, selenium, and vitamins B12, C, B6, and E (26, 27). Long-term use of this supplement was associated with improved hippocampal synaptic functioning and performance on a novel recognition task among mice maintaining low- or high-Phe diets (26, 27). While there are presently no studies in human subjects with PKU to complement these findings, our prior work using untargeted metabolomics identified substantial shifts in choline-related pathways among individuals with PKU relative to healthy controls (28). Additionally, we have demonstrated that choline intake is suboptimal among both adults and children with PKU (29). Even with the consumption of choline-fortified medical foods, only 10.8% of our previous

PKU sample was able to achieve the sex- and age-specific adequate intake (AI) recommendations (30), which may increase susceptibility to neurocognitive deficits, and particularly working memory impairment.

Given the importance of choline for cognitive function, the objective of this study was to evaluate the relationship between total choline intake and working memory performance in PKU. This study had two specific aims: (1) to determine if higher choline intake normalizes working memory performance in PKU participants relative to controls, and (2) to assess whether the relationship between total choline intake and working memory is dependent on metabolic control in participants with PKU.

6.3 Materials and Methods

6.3.1 Participants

Adults 18-40 years of age with PKU (N=42) were recruited primarily from the Genetics Clinic at Emory University and a database of research volunteers maintained by the Clinical Neuropsychology Lab at the University of Missouri. Recruitment flyers were additionally shared with patient advocacy organizations and registered dietitians at other metabolic clinics throughout the United States. Individuals were eligible if a PKU diagnosis was made and treatment was initiated shortly after birth, as substantiated by patient report or medical records. The PKU cohort was compared to a demographically-matched group of healthy adults (N=41) based on age, sex, and years of education. These controls were recruited from the University of Missouri, the Clinical Neuropsychology Lab database, and the unaffected contacts of PKU participants. Exclusion criteria for both PKU participants and controls included a history of neurologic compromise and major medical conditions unrelated to PKU (e.g., closed head injury, multiple sclerosis). Participants with PKU were excluded if they were currently being treated

with the enzyme substitution therapy pegvaliase (Palynziq®, BioMarin Pharmaceutical Inc, Novato, CA, USA). Out of the 83 enrolled participants, three (2 PKU, 1 control) did not complete study due to scheduling conflicts. The final analytic sample comprised 40 PKU participants and 40 controls.

6.3.2 Design and Procedure

This was a remote, cross-sectional study that was approved by the Research and Ethics Review Boards at Emory University and the University of Missouri, and informed consent was obtained from all participants. Participant visits were conducted over a HIPAA compliant cloud-based video and phone conferencing system. During each study visit, participants were asked to find a quiet, distraction-free location to complete a structured interview, cognitive tests, and diet record review. Participants were asked to report any distractions during the study visit that may have affected their performance.

To assess overall intellectual functioning, the Matrix Reasoning subtest from the Wechsler Adult Intelligence Scale 4th edition (WAIS-IV) (31) was administered. Visuospatial working memory and related executive functioning skills were assessed using four sub-tests from the web-based Cambridge Neuropsychological Test Automated Battery (CANTAB): Spatial Span (SSP), Paired Associates Learning (PAL), Rapid Visual Information Processing (RVP), and Spatial Working Memory (SWM) (32). To evaluate verbal working memory, the Digit Span (DS) subtest from WAIS-IV was administered. Each CANTAB subtest yielded age-, sex-, and education level-normed standard z-scores (M = 0, SD = 1). The overall score for the DS test was based on performance across the forward, backward, and sequencing trials. This score was then converted to an age-normed scaled score (M = 10, SD = 3). Scaled scores were then converted to z-scores to facilitate comparison with the other working memory tasks.

6.3.3 Generation of a Working Memory Composite

The z-scores from the four CANTAB visuospatial working memory tests (SSP, PAL, RVP, SWM) and the DS verbal working memory test were averaged to generate a composite working memory score. In cases where a participant's score on an individual subtest was an outlier (>2.5 SD above sample mean) or a participant reported distractions in the testing environment that may have affected performance, the composite score was based on the remaining four subtests. This resulted in a single subtest being discarded for 6 participants (5 PKU, 1 non-PKU; no participants had more than one problematic subtest).

6.3.4 Assessment of Total Choline Intakes

In the three days prior to each study visit, PKU and control participants completed diet records with detailed descriptions of all foods, beverages, and supplements consumed. For participants with PKU, medical food consumption and prescriptions were also recorded. During each study visit, diet records were reviewed for accuracy and completeness by a trained research registered dietitian. To estimate the dietary and supplementary intake of choline and the other methyl-donor/co-factor nutrients that impact choline metabolism (vitamin B12, vitamin B6, folate, methionine), dietary analysis was completed using the Nutrition Data System for Research (NDSR 2020, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, USA). Medical foods were added to the NDSR database using manufacturer-supplied nutrient information, and low-protein modified foods were created using ingredients within the NDSR database that contain composition data for choline. Any additional items that were not found within the NDSR database were substituted for nutritionally comparable foods using the following set of nutrient tolerances per 100g of food: 85 kcal, 2.5g of fat, 100mg of sodium, 10g of carbohydrates, 5g of protein, and 50mg of phenylalanine (33). Total nutrient intakes were

calculated by summing intakes from food, dietary supplements, and medical food (for PKU sample). The contributions of food, medical food, and dietary supplements to total choline intake were calculated at the individual level by dividing each participant's intake for each category by total choline intake.

Nutrient adequacy for choline, vitamin B12, vitamin B6, folate, and methionine was determined using the cut-point method, which compares average nutrient intakes to the age- and sex-specific estimated average requirements (EAR) or adequate intake (AI) (34).

6.3.5 Metabolic Control Measures

Multiple measures were used to assess metabolic control. To determine current Phe and Tyr concentrations, participants collected samples of capillary whole blood via filter paper on the day of cognitive assessment. All samples were then sent to PerkinElmer Laboratories for analysis. The most recent Phe measures (collected 1 and 17 days prior to the study visit) were used for two participants who did not successfully submit filter papers. Medical records were also obtained for 35 of the 40 participants to gather historical Phe data. Using all available historical Phe data, an index of dietary control (IDC) was computed as the mean of all half-year median Phe concentrations for early childhood (0-5 years), middle childhood (6-11 years), adolescence (12-17 years), adulthood (18+ years), and lifetime (0-present) (35). In the case of small gaps (e.g., 1-2 years) in records, values were extrapolated using linear regression and Phe levels of adjacent years for purposes of IDC calculation. In cases of larger gaps, the participant was excluded from analysis of the relevant developmental epoch.

6.3.6 Statistical Analysis

Sample characteristics and nutrient intakes were reported as median (IQR) for continuous variables and frequency (percent) for categorical variables. Group profiles were compared using

The Mann-Whitney U Test for continuous variables and the Chi-Square Test of Association for categorical variables. Analysis of Covariance (ANCOVA) was used to assess whether the relationship between total choline intake and working memory performance differed between PKU and control participants. The primary outcome was the working memory composite and the main predictors were diagnosis (PKU versus controls) and total choline intake. For this analysis, total choline intake was converted into a binary variable based on previously published population averages (18). The population average was intended to represent an alternative AI, given the AI established by the Food and Nutrition Board of the National Academy of Medicine was based on a depletion-repletion study in adult men with liver damage as the endpoint for choline deficiency (36). This is in contrast to the AI for other nutrients, which are based on the estimated intake of a healthy group of individuals. Total choline intake greater than the age- and sex-specific population averages was considered high, and total choline intake below these averages was considered low. Demographic or dietary characteristics that were found to have a statistically significant difference between diagnosis groups, and which were associated with the working memory composite, were considered as covariates for the ANCOVA model. To be retained as a covariate, the variable was required to make a notable change in the magnitude (>10%) or direction of the interaction (diagnosis*alternative choline AI) estimate. Only one variable, matrix reasoning (proxy for intellectual ability), met these criteria and was retained in the final model.

To evaluate the association between metabolic control and working memory performance, bivariate correlation analyses were conducted for concurrent Phe, Phe average from the year prior to the study, and all IDC measures (0-5 years, 6-11 years, 12-17 years, 18+ years, lifetime). Multivariate linear regression was then used to evaluate if the relationship

between total choline intake and working memory performance was dependent on metabolic control. For this analysis, both concurrent Phe and total choline intake were retained as continuous variables given data visualization identified linear trends. Prior to analysis, total choline intake was adjusted for total energy using the residual method (37), and the final model was also adjusted for overall intellectual ability based on the aforementioned covariate criteria.

An exploratory multivariate regression analysis was additionally conducted on a subsample of 30 participants who had an IDC measure from middle childhood (6-11 years). This IDC measure was added as a covariate based on statistically significant bivariate correlation results. Variance inflation factors were checked prior to conducting this analysis to ensure that excess collinearity between measures of metabolic control measures would not bias the estimates. All statistical analyses were carried out in SAS 9.4 (version 9.4, SAS Institute, Cary, NC) and R (version 4.1.2). P-values ≤ 0.05 were considered statistically significant and p-values >0.05 and ≤ 0.10 were considered informative trends.

6.4 Results

Characteristics of the PKU and control cohorts are reported in **Table 6-1**. The matched design resulted in similar demographic profiles, however, there were differences in dietary intake between the groups. Among the participants with PKU, median intakes of vitamin B6 and folate were notably higher, while intakes for total fat and the sum of methionine and cysteine were significantly lower than controls. With regard to choline, median (IQR) total intake was lower among participants with PKU [262.4 mg (188.7, 556.1)] compared to controls [(315.8 mg (237.0, 418.4)], but the prevalence of choline adequacy was higher in the PKU cohort (**Figure 6-1**). Although this variability was not statistically significant, the predominant source of choline differed between the groups. On average, participants with PKU obtained 50.8% (SD: 32.4) of

their choline intake from fortified medical foods. This was in contrast to the controls, for which food sources contributed an average of 99.6% (SD: 2.7) to total choline consumption. Dietary supplements were a poor source of choline for both groups. The average contribution was 0.07% (SD: 0.32) for participants with PKU and 0.45% (SD: 2.7) for controls.

Within the PKU cohort, 11 participants (27.5%) were managed with sapropterin dihydrochloride, a synthetic tetrahydrobiopterin treatment that can increase protein tolerance. Only three of the 11, however, did not have medical food prescriptions. Out of the remaining 37 participants with medical food prescriptions, 90% used products that contained choline and other micronutrients, and 72.5% were consuming the amount prescribed. Although 82.8% of the participants with PKU were consuming excess intact protein, median concentrations for concurrent or most recent Phe were 362.4 µmol/L (IQR: 254.5, 585.9), which is only slightly above the therapeutic range (120-360 µmol/L).

6.4.1 Comparison of Working Memory Performance Between Participants with PKU and Controls

Relative to controls, participants with PKU demonstrated a trend toward poorer performance on all working memory tests (**Table 6-2**). Of the five subtests, RVP was the only task that had a notable z-score difference [Δ=0.37 (95% CI: 0.10, 0.65)] between study groups. This trend was reflected in overall working memory performance, which was 0.32 z-scores (95% CI:0.06, 0.58) lower in participants with PKU compared to controls.

6.4.2 Relationship between Working Memory Performance and Metabolic Control

Correlations between measures of metabolic control and overall working memory are reported in **Table 6-3.** Concurrent/most recent Phe was not significantly related to overall working memory performance (r= -0.15, p-value=0.37); however, in a subsample with historical

Phe data, there were moderate inverse correlations with IDC in middle childhood (6-11 years; n=30, r= -0.41, p-value=0.02) and lifetime IDC (n=16, r= -0.59, p-value=0.02).

6.4.3 Relationship between Total Choline Intake, Diagnosis, and Working Memory Performance

ANCOVA was conducted in the full sample (controls + PKU, N=80) to assess whether choline intake above or below the sex- and age-specific population averages modified working memory performance in the PKU and control groups. After adjusting for intellectual ability (matrix reasoning), this model did not identify diagnosis-related differences in the relationship between choline intake and working memory performance (F_1,75=0.85, p=0.36) **(Figure 6-2**). Analysis of main effects also did not demonstrate a significant independent association between choline consumption and working memory performance (F_1,75=0.08, p=0.77).

6.4.4 Relationship between Total Choline Intake, Metabolic Control, and Working Memory Performance

Within the full PKU cohort (N=40), multivariate regression was used to assess if the relationship between choline consumption and overall working memory performance was dependent on metabolic control (reflected by concurrent/most recent Phe concentrations). Given data visualization identified a linear trend between choline intake and working memory across different levels of metabolic control, choline was modeled on a continuous scale. This analysis did not identify a significant relationship between choline intake and working memory performance. A 1 mg/day increase in energy-adjusted total choline intake improved working memory performance by 0.0006 z-scores (95% CI: -0.0007, 0.0018; p=0.35), on average, among individuals with optimal Phe concentrations (120 µmol/L) and average intellectual ability. Phe concentrations did not notably modify this relationship (p=0.50, **Table 6-4**).

Given IDC from middle childhood (6-11 years) was associated with working memory performance in the present sample, a second multivariable regression analysis, which incorporated this measure as a covariate, was performed on a subsample of 30 participants with available IDC data. Using this exploratory model, energy-adjusted choline intake was positively related to working memory performance. Among participants with optimal current and historical Phe, and average intellectual ability, the average estimated improvement in working memory was 0.002 z-scores (95% CI: 0.0002, 0.003; p=0.02) when total choline intake was increased by 1 mg/day. A relevant trend, which was not statistically significant (p=0.10), also became evident in the interaction between choline intake and metabolic control, which suggested that the benefit associated with choline consumption decreased as Phe concentrations increased **(Table 6-4, Figure 6-3).**

To assess whether the identified effects in our second regression analysis could be attributed to the reduced sample rather than the adjustment of historical Phe, we conducted an additional multivariate analysis on the subsample of 30 without including IDC as a covariate. This model explained a greater proportion of the variance in working memory performance (Adjusted R^2 Original Model = 0.36, Adjusted R^2 Alternate Model=0.44) and the main effect for choline reached statistical significance (p=0.05). To evaluate the sources of these changes, characteristics of the reduced sample and the 10 participants with missing IDC data were compared **(Table 6-5)**. There were no characteristics that notably differed between the groups. Further inspection of the regression diagnostics identified one participant who was removed from the alternate model (due to unavailable IDC data) and whose characteristics differed from the pattern identified in the majority of the sample (Cook's D > 0.1). Despite having low Phe concentrations (261 µmol/L) and very high choline intake (777.9 mg/d), this participant had poor

working memory performance (z-score = -0.09). Although the removal of this participant may have impacted the alternate model, adding historical Phe explained an additional 8% of the variance in working memory performance.

6.5 Discussion

Due to the many pathways by which choline impacts the structure and function of the brain (38), and the growing evidence to support choline's long-term effects on memory performance (39-41), this study examined the relationship between total choline intake and working memory in adults with PKU and matched controls. Across the full sample, overall working memory performance did not differ between participants with high versus low total choline intake, and this finding was not modified by diagnosis. This null finding may be attributed to the overall good nutriture among both PKU and control participants. When comparing intakes across the main micronutrients/amino acids that impact choline metabolism (vitamin B12, vitamin B6, folate, and methionine + cysteine), fewer than 15% of both groups were not meeting the EAR for all nutrients. Only 20% of controls and 27.5% of participants with PKU were meeting the AI for choline; however, this is higher than the prevalence reported by previous research in the US population (based on data from the National Health and Nutrition Examination Survey) (42, 43) and our prior study in a larger sample of individuals with PKU (29). In the present sample of participants with PKU, the improved nutrient density that we observed may derive from good adherence to medical food prescriptions (found in 72.5% of sample) and the consumption of medical foods that contained vitamins and minerals (found in 90% of sample). As choline is concentrated in protein-rich foods (44), and most participants were concurrently consuming more intact protein than prescribed, this could also enhance overall nutrient adequacy. The similar pattern observed in controls may be a product of study

recruitment, which occurred predominantly in a university setting and may have resulted in a sample with better health-related behaviors (45).

Underlying metabolic variation may also explain why our findings did not match our hypothesis. The majority of this study's sample were premenopausal women, and this group has a reduced dietary requirement for choline compared to men and postmenopausal women (46, 47). This discrepancy can be attributed to the increased concentrations of estrogen in younger women, which regulate the expression of the *PEMT* gene. This gene encodes the phosphatidylethanolamine n-methyltransferase enzyme that is essential for the endogenous synthesis of choline (48), resulting in a reduced dietary requirement for choline in this demographic. Beyond estrogen, there are several single nucleotide polymorphisms (SNPs) that have been found to modulate choline biosynthesis (49). While this study did not assess genetic variation, it is possible that this sample did not include many individuals with these functional SNPs. Hence, it may not have been possible to identify an association between choline intake and working memory if this sample contained few individuals with metabolic inefficiencies and very low total choline intake (50).

In a subsample of 30 participants with PKU and metabolic control measures from adulthood and middle childhood (6-11 years), we identified a relationship between total choline intake and working memory that was not found in the full sample. In this group, increased total choline intake was associated with higher scores on the working memory composite among participants with optimal (Phe=120 μmol/L) current and historical metabolic control. Although no prior studies have evaluated the cognitive correlates of choline in PKU, this finding complements the results of two previous studies in healthy adults. One was an observational study that found higher performance on verbal and visual memory tasks with increased

concurrent choline intake (40). The second was a randomized, double-blind, crossover trial that found improved processing speed, working memory, verbal learning, verbal memory and executive function among participants who demonstrated poor baseline performance and were supplemented with 5'-diphosphocholine (CDP-choline; a derivative of choline used for phospholipid biosynthesis) (51).

This analysis also identified a statistically non-significant, but clinically interesting trend, which suggested that the positive association between choline intake and working memory was attenuated as Phe concentrations increased (and metabolic control decreased). These findings are in contrast to those of Bruinenberg et al (27), who found that a supplementation with a choline-containing multi-nutrient complex improved memory in PKU BTBR^{Pah2} mice with high Phe concentrations. This lack of congruence may derive from differences in study design (observational versus experimental), the population observed (human versus animal model), or the use of a single versus multi-nutrient approach. Given this study was focused on choline, we solely evaluated the adequacy of other nutrients that are directly involved in choline metabolism (vitamin B12, B6, folate, methionine), and did not identify any deficiencies or associations between these nutrients and working memory performance in the present sample. There are, however, several other nutrients that have been positively associated with working memory in adults (e.g. cholesterol, alcohol, vitamin E, palmitoleic acid, oleic acid, alpha-linoleic acid, linoleic acid, vitamin C, vitamin D) (52), and future research will benefit by expanding the scope of the present study and examining additive nutrient effects.

It is also possible that we did not see any association between choline intake and working memory performance as Phe concentrations increased due to the potential impact of the phenylketone, phenylacetate, on PEMT. Phenylacetate concentrations are increased in patients

with poor metabolic control (53), and one prior study has found that that this metabolite has antiestrogenic properties, which could have implications for PEMT (54). This may substantially increase the dietary requirement for choline among premenopausal women with PKU and high Phe. As few individuals in this sample had very high choline intake in combination with high Phe, this hypothesis could not be further investigated.

While several prior studies have identified deficiencies for neurologically-relevant nutrients in PKU (55), few have evaluated how these nutrients affect cognitive outcomes in this population. This study is the first to evaluate the association between working memory and choline intake in PKU. A strength of this study was the development of a composite measure for working memory which included both visual and verbal components. This allowed for a more comprehensive characterization of working memory performance and reduced the heterogeneity associated with using a single task. This study also used remote assessment for gathering cognitive outcomes, which expanded this study's enrollment to clinics across the US without adding a travel burden for participants. By allowing for a more diverse sample, this methodology also increases the generalizability of our findings.

With these strengths, this study also had several limitations that must be considered. Despite the care taken when collecting and analyzing the dietary data, we cannot eliminate the potential for self-reporting bias, which could have resulted in the over- or underestimation of our main variable of interest. This bias may have been eliminated with the use of a biochemical marker of choline status, but this was not feasible for the present study. Another limitation was the missing IDC data for some participants in the sample. This was important given IDC from 6 to 11 years was associated with current overall working memory in the present study, and represents a key period in which the capacity of working memory significantly increases (56).

For some patients, missing IDC data reflected nonadherence to the PKU monitoring guidelines. For others, the missing data reflected patient movement between clinics (with subsequent loss of records) or the switch to electronic charting, which enhanced the difficulty of accessing paper charts that contained early-life Phe concentrations.

6.6 Conclusions

This study was the first to report that increased total choline intake was related to improved working memory outcomes among adults with PKU who remain in good metabolic control. While the nutrient status of patients with poor metabolic control typically receives more attention, this study suggests that there can be important cognitive benefits of closely monitoring choline intake among patients with Phe levels that are within the therapeutic range. Observational studies with larger samples and controlled trials are needed to confirm the relationship between choline intake, Phe concentrations, and working memory performance.

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6.8 Tables and Figures

Table 6-1. Sociodemographic and Dietary Profiles by Diagnosis Group[1]

	PKU (N=40)	Controls (N=40)	P-value[2]
Female, n (%)	29.0 (72.5)	29.0 (72.5)	1.00
Age (years)	25.0 (21.0, 30.5)	26.0 (21.0, 30.5)	0.70
Education (years)	15.5 (14.0, 16.5)	16.0 (14.5, 17.0)	0.31
Intellectual Ability (Matrix Reasoning Score)	11.0 (8.5, 13.0)	12.5 (10.5, 14.0)	0.03
BMI (kg/m^2)	26.7 (23.0, 29.6)	23.7 (22.0, 27.1)	0.07
Total Energy (kcal/d)	1902.2 (1548.4, 2270.6)	1938.7 (1527.1, 2162.0)	0.78
Total Protein Intake (g/d)	74.1 (60.3, 89.5)	75.0 (61.5, 95.5)	0.39
Medical Food Protein (g/d)	48.9 (29.1, 66.0)	----------	----------
Intact Protein (g/d)	22.4 (15.9, 34.6)	----------	----------
Total Fat (g/d)	60.7 (50.6, 82.3)	78.0 (58.2, 91.2)	0.04
Total Choline (mg/d)	262.4 (188.7, 556.1)	315.8 (237.0, 418.4)	0.53
Total Vitamin B12 (mcg/d)	6.1 (3.3, 11.4)	5.5 (2.8, 8.4)	0.35
Total Vitamin B6 (mg/d)	3.3 (2.0, 4.0)	2.1 (1.5, 3.4)	0.02
Total Folate (mcg DFE/d)	1166.8 (815.9, 1479.1)	547.7 (394.1, 738.0)	<0.0001
Total Methionine + Cysteine (mg/d)	2169.3 (1643.2, 2925.5)	2725.3 (2170.0, 3525.2)	0.01
Most Recent Phe (µmol/L)	362.4 (254.5, 585.9)		
Kuvan Treatment, n (%)	22.0 (27.5)	----------	----------

[1]Continuous variables are reported as median (IQR) and categorical variables are reported as frequency (%).
[2]P-values were derived from the Mann-Whitney U test for continuous variables and the Chi-Square Test of Association for categorical variables.

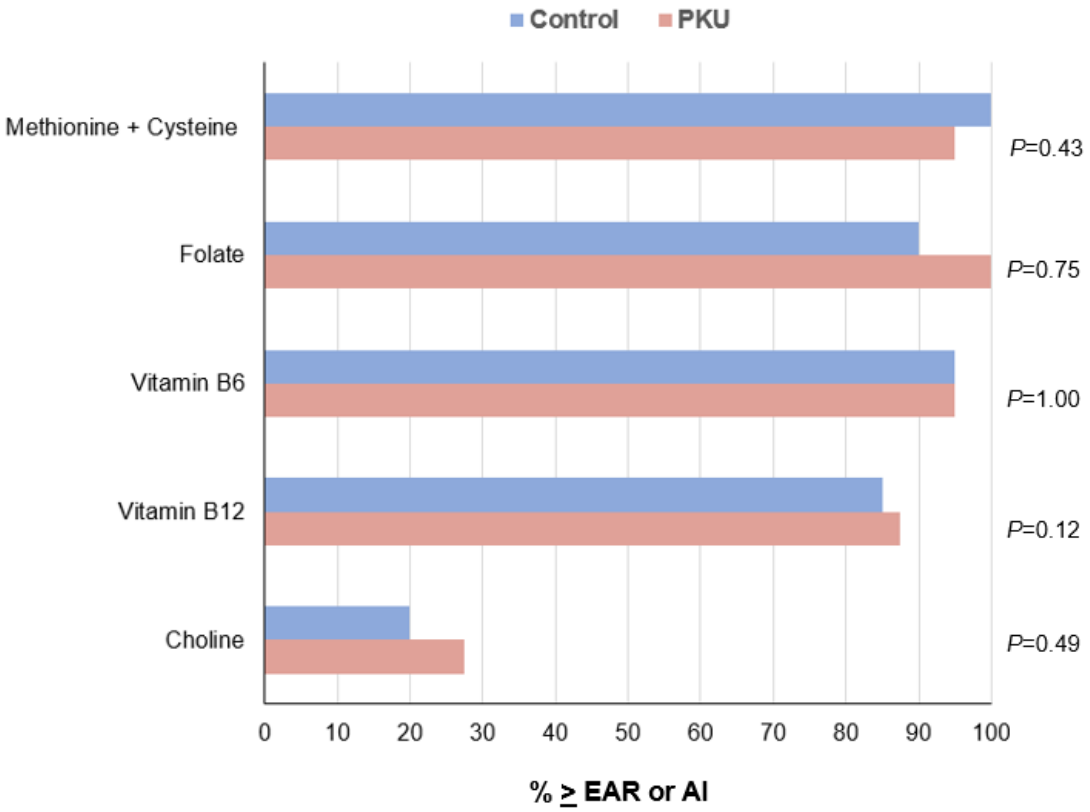

Figure 6-1. Percentages of the PKU and control cohorts with total intakes (food + dietary supplements) at or above the estimated average requirement (EAR) or adequate intake (AI) for choline and the other micronutrients/amino acids that affect choline metabolism (methionine + cysteine, folate, vitamin B12, vitamin B6). P-values reflect the difference in prevalence between the PKU and control groups as determined by the Chi-Square Test of Association or Fisher Exact Test.

Table 6-2. Working Memory Performance in PKU (N=40) and Control (N=40) Participants[1]

Working Memory Outcome	Marginal Mean Z-Score (95% CI)		Average Z-Score Difference (95% CI)	P-value
	PKU	Control		
Composite	0.21 (0.04, 0.39)	0.53 (0.36, 0.71)	0.32 (0.06, 0.58)	0.02
SSP	0.10 (-0.21, 0.41)	0.49 (0.18, 0.80)	0.39 (-0.05, 0.83)	0.08
PAL	0.37 (0.05, 0.69)	0.80 (0.49, 1.12)	0.43 (-0.02, 0.89)	0.06
RVP	-0.01 (-0.20, 0.18)	0.36 (0.17, 0.55)	0.37 (0.10, 0.65)	0.01
SWM	0.35 (-0.01, 0.71)	0.59 (0.23, 0.95)	0.24 (-0.27, 0.75)	0.36
DS	0.16 (-0.12, 0.44)	0.41 (0.13, 0.70)	0.25 (-0.15, 0.66)	0.22

[1]Data Derived from ANCOVA adjusted for overall intellectual ability (Matrix Reasoning).
Abbreviations: DS, Digit Span; RVP, Rapid Visual Information Processing; SSP, spatial span; SWM, Spatial Working Memory; PAL, Paired Associates Learning.

Table 6-3. Association between Metabolic Control Measures and Working Memory Performance

Metabolic Control Measure	N	Correlation Coefficient (*r*)[1]	P-value
Concurrent Phe	40	-0.15	0.37
Previous Year Mean Phe	40	-0.17	0.30
IDC Early Childhood (0-5 yrs)	25	-0.19	0.37
IDC Middle Childhood (6-11 yrs)	30	-0.41	0.02
IDC Adolescence (12-17 yrs)	29	-0.13	0.49
IDC adulthood (18+ yrs)	26	-0.14	0.49
IDC Lifetime (0-present)	16	-0.59	0.02

[1]Reflects the Unadjusted Pearson correlation coefficient for metabolic control measures with a parametric distribution and the Spearman correlation coefficient for non-parametric metabolic control measures.
Abbreviations: IDC, Index of Dietary Control; Phe, Phenylalanine; Yrs, years.

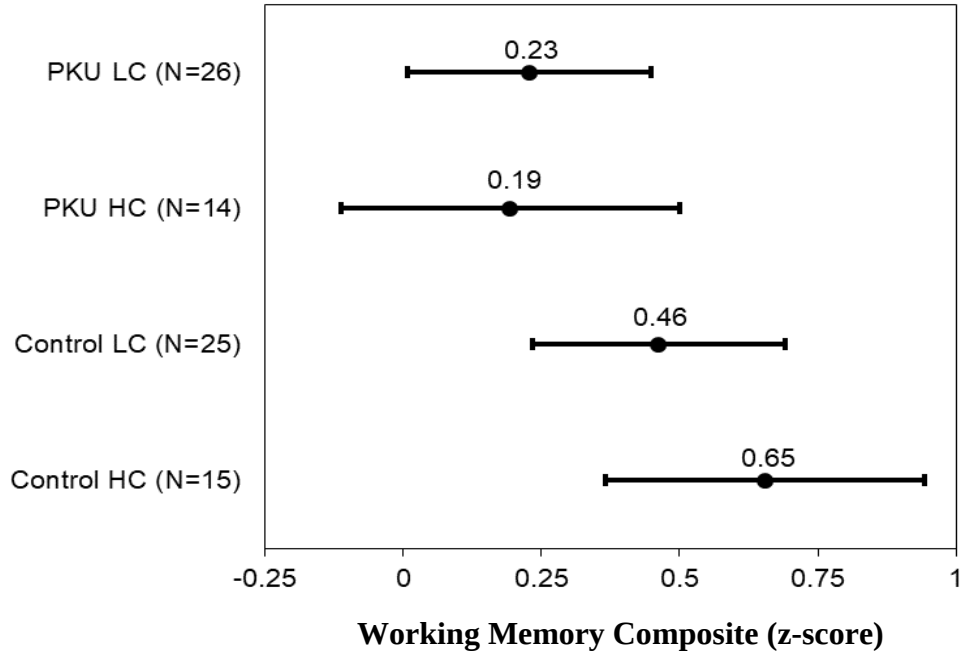

Figure 6-2. Interaction of choline consumption (low and high) and study group (control and PKU) based on ANCOVA for overall working memory performance (reflected by composite measure). Low and high choline consumption represent intake below and above the sex- and age-specific population averages, respectively. Estimated marginal means and 95% confidence intervals are reported for each group. This analysis did not identify choline consumption as a significant effect modifier (p=0.36). Abbreviations: HC, high choline; LC, low choline.

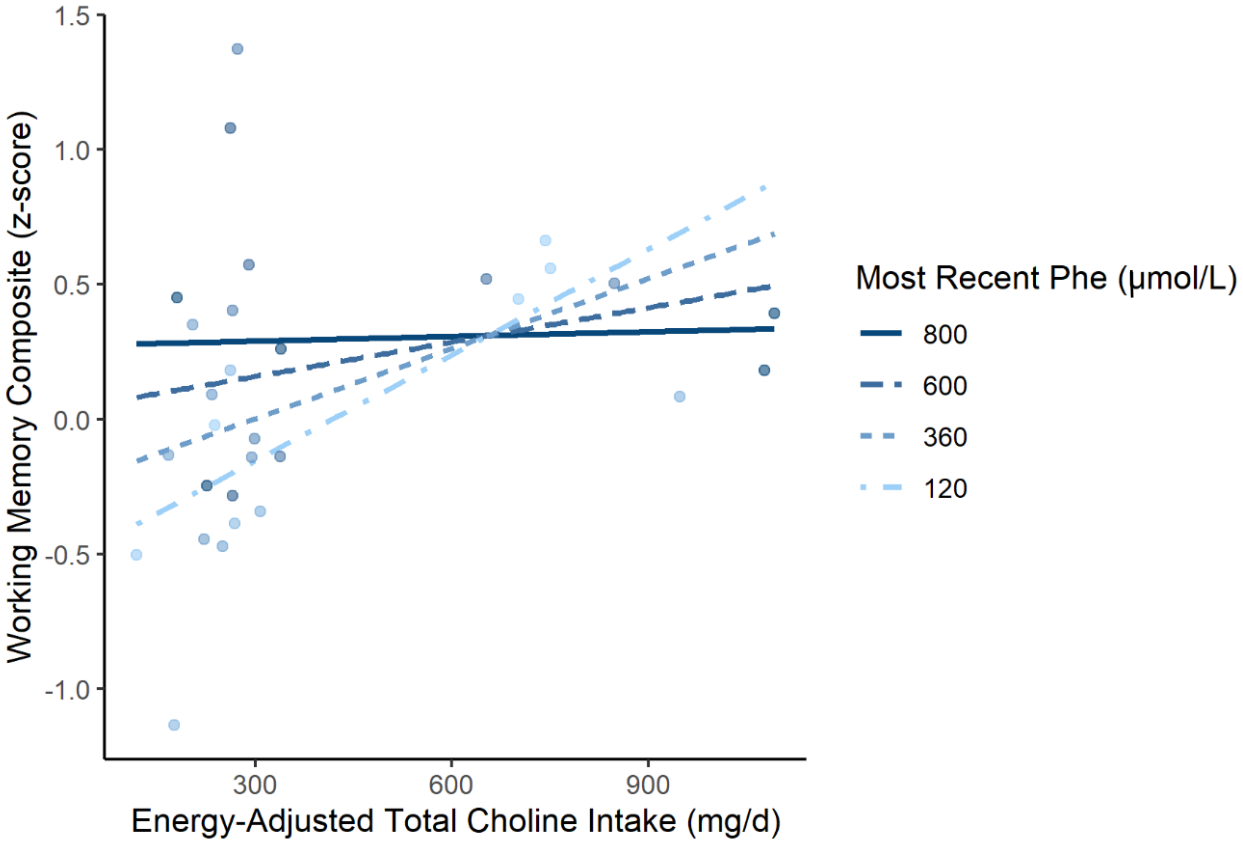

Figure 6-3. Association between working memory performance and energy-adjusted total choline intake across four levels of Phe concentration (120 µmol/L, 360 µmol/L, 600 µmol/L, 800 µmol/L) in a subsample of PKU participants with historical Phe data (N=30). Data points represent observed data adjusted for intellectual ability (matrix reasoning) and IDC from 6 to 11 years.

Table 6-4. Results of the Multivariate Regression Analyses of Working Memory Performance with Energy-Adjusted Total Choline Intake and Metabolic Control[1]

Model	N	Regression Coefficient: Choline Intake (95% CI)[2]	P-value[3]	Regression Coefficient: Choline Intake X Phe (95% CI)[4]	P-value[3]	Adjusted R^2
1	40	0.0006	0.35	-0.0000009	0.50	0.36
2	30	0.001	0.05	-0.000002	0.16	0.44
3	30	0.002	0.02	-0.000002	0.10	0.52

[1]Adjusted for intellectual ability (matrix reasoning) in model 1 (full sample, N=40) and model 2 (reduced sample, N=30); Adjusted for intellectual ability and IDC from 6 to 11 years in model 3 (exploratory analysis with reduced sample, N=30).
[2]Regression coefficient represents the change in working memory performance per 1 mg/day increase in energy-adjusted total choline intake when Phe is optimal (120 µmol/L) and intellectual ability is average.
[3]Significance indicated at $P \leq 0.05$; Trends informative for future research indicated at $P > 0.0.5$ and ≤ 0.1.
[4]Regression coefficient represents the amount by which the association between energy-adjusted total choline intake and working memory performance changes based on a 1 µmol/L increase in most recent Phe concentration.

Table 6-5. Comparison of Characteristics Between Participants with and without IDC Data from 6-11 Years of Age[1]

	With IDC (N=30)	**Without IDC (N=10)**	***P*-value[2]**
Female, n (%)	9 (30)	2 (20)	0.70
Age (years)	25 (21, 30)	25 (21, 33)	0.94
Education (years)	16.0 (14.0, 17.0)	15.0 (15.0, 16.0)	0.69
BMI (kg/m^2)	27.1 (22.7, 29.7)	24.8 (23.2, 28.3)	0.86
Intellectual Ability (Matrix Reasoning Score)	11.0 (9.0, 13.0)	10.0 (7.0, 12.0)	0.43
Most Recent Phe (µmol/L)	384.0 (239.6, 597.7)	335.8 (296.4, 574.1)	0.94
Total Protein Intake (g/d)	74.9 (61.1, 88.4)	67.1 (54.7, 90.6)	0.66
Medical Food Protein (g/d)	22.4 (15.8, 35.8)	23.8 (16.7, 32.0)	0.38
Intact Protein (g/d)	50.0 (38.4, 66.0)	42.0 (18.0, 60.0)	0.75
Total Fat (g/d)	60.2 (50.7, 87.4)	62.6 (46.9, 76.5)	0.94
Total Choline (mg/d)	255.1 (180.6, 510.0)	308.0 (196.8, 671.2)	0.51
Total Vitamin B12 (mcg/d)	5.4 (2.8, 11.4)	7.8 (5.2, 12.7)	0.18
Total Vitamin B6 (mg/d)	3.1 (2.0, 11.3)	3.7 (2.6, 3.9)	0.63
Total Folate (mcg DFE/d)	1083.3 (704.7, 1353.9)	1290.3 (1080.4, 1597.2)	0.16
Total Methionine + Cysteine (mg/d)	2216.7 (1676.0, 2767.7)	2091.7 (1610.3, 3093.3)	0.79
Adherence to Medical Food RX[3]	1.1 (0.8, 1.0)	1.0 (1.0, 1.0)	0.96
Adherence to Intact Protein RX[4]	1.8 (1.1, 2.2)	1.6 (1.2, 3.3)	0.72
Kuvan Treatment, n (%)	16 (53.3)	6 (60.0)	1.00

[1]Continuous variables are reported as median (IQR) and categorical variables are reported as frequency (%).
[2]P-values were derived from the Mann-Whitney U test for continuous variables and the Fisher Exact Test for categorical variables.
[3]Calculated as the ratio of medical food protein equivalents consumed to medical food protein equivalents prescribed. Calculated for N=28 for 'With IDC' group and N=9 for 'Without IDC' group. Two participants with IDC and one participant without IDC did not have medical food prescriptions.
[4]Calculated as the ratio of intact protein (g) consumed to intact protein (g) prescribed.

CHAPTER 7: DISCUSSION AND CONCLUSIONS

Since the introduction of phenylalanine (Phe)-free protein substitutes in the 1960s, treatment for individuals with phenylketonuria (PKU) has greatly evolved (1). Presently, there is a wide array of medical foods which are more palatable, convenient, and nutritionally balanced than previous forms. More recently, PKU management can also include one of two available pharmacotherapies. This includes the synthetic tetrahydrobiopterin cofactor, sapropterin dihydrochloride (KUVAN®, BioMarin Pharmaceutical Inc., Novato, CA, USA), and the enzyme substitution therapy, Pegvaliase (PALYNZIQ®, BioMarin Pharmaceutical Inc., Novato, CA, USA). For many patients, these pharmacotherapies can increase Phe tolerance and improve quality of life (2); however, these treatments still have important limitations. Given sapropterin acts as a cofactor for the PAH enzyme, it is only effective for approximately 25-50% of the PKU population who have residual PAH activity (3). While the efficacy of pegvaliase is not restricted by PAH activity, prescription is only approved for adults and prior research has identified a high incidence of hypersensitivity-related adverse events (4), which lead several patients to discontinue treatment.

Due to these factors, the majority of individuals with PKU are still dependent on lifelong dietary therapy to support their nutrient needs while preventing Phe accretion (5). While continuous treatment can successfully prevent severe neurological impairment, cognitive performance continues to be suboptimal among early-treated individuals with PKU (6). This suggests that dietary treatment should continue to be improved and personalized to optimize cognitive performance and the overall health of this population. This dissertation addressed this need by identifying and evaluating an essential nutrient within the PKU diet, choline, which has

important implications for neurocognitive function but has received minimal prior consideration in the clinical management of PKU.

7.1 Key Findings

Our first study (Chapter 4) used untargeted high-resolution metabolomics to compare the plasma metabolome of females with PKU to matched controls, and investigate changes in the PKU metabolome associated with short-term shifts in dietary adherence. The metabolic features that differentiated PKU participants from controls were Phe catabolites, ketones, carnitine-conjugated fatty acids, glycine-conjugated fatty acids, and fatty acylcholines. With a 5-day camp intervention, PKU participants demonstrated a significant improvement in metabolic control and the abundance of the aforementioned metabolites became more similar to controls. Phe catabolites and fatty acids decreased, while the fatty acylcholines and choline-containing phospholipids increased. The metabolic perturbations observed in this study substantiated prior research, which has reported bioenergetic defects (7) and oxidative stress (8) as pathophysiological mechanisms in PKU, and further added to the literature by identifying alterations in choline metabolism which have not been previously reported or explored.

Given the importance of choline for cognitive function, and the paucity of data in PKU, our second study characterized choline nutriture in adults and children. Total usual choline intake distributions were compared across three PKU treatments (diet therapy only, sapropterin dihydrochloride, and Pegvaliase) and to a national reference population using dietary data from the National Health and Nutrition Examination Survey (NHANES). Only 10.8% of the total PKU sample achieved the adequate intake (AI) for choline, which was slightly lower than the healthy reference population. Additionally, within the PKU sample, individuals on diet therapy and sapropterin would not be able to achieve the choline AI without the consumption of fortified

medical foods. This was particularly evident among adults on diet therapy, who had the lowest total choline intake. Fortified medical foods were also an essential source of other micronutrients and amino acids that are involved in choline metabolism, and thus, play an important role in determining dietary needs for choline.

To understand the implications of suboptimal choline intake, our third study examined the functional correlates of choline consumption. Specifically, this study evaluated the relationship between total choline intake and working memory outcomes in adults with PKU given this cognitive domain has previously been found to have the greatest level of impairment (9). Consistent with prior research, this study identified lower overall working memory performance in participants with PKU compared a group of matched controls (10-14). Among the PKU participants, working memory did not have a strong relationship with concurrent Phe concentrations, but was related to an index of lifetime Phe and metabolic control in mid-childhood (6-11 years of age), a period during which working memory capacity significantly increases (15). After adjusting for metabolic control during this critical period, we identified a significant positive relationship between concurrent total choline intake and working memory in adults who were maintaining good metabolic control. This association, however, diminished as metabolic control worsened, suggesting that choline may not provide cognitive benefits in the presence of high Phe concentrations. Alternately, this could reflect that very high choline intake is required to mitigate the negative consequences of high Phe. This, however, could not be investigated in our sample given only 27.5% of participants were achieving the AI for choline.

7.2 Strengths and Limitations

This dissertation was strengthened by the use of innovative and rigorous methodological approaches for data collection and analysis. With regard to data collection, our third study

evaluated working memory performance using a recently developed, web-based version of the Cambridge Neuropsychological Test Automated Battery (CANTAB). Unlike the traditional in-person battery, this version of the CANTAB could be administered remotely on personal computers or tablets within a participant's home. This provided a cost-effective method to recruit more individuals with PKU and enhance the geographic diversity of our sample. Our success with this assessment approach provides an innovative strategy for future studies focused on individuals with rare diseases.

With regard to data analysis, our first study utilized untargeted metabolomics to evaluate changes within the plasma metabolome associated with acute alterations in dietary adherence. This discovery-oriented approach differs from previous hypothesis-driven studies, which have solely measured Phe concentration and/or Phe variability. By using untargeted metabolomics, we were able to identify relevant biochemical markers, beyond Phe, that reflected meaningful clinical change. Our second study also included important analytic advances from prior research. In this study, we applied statistical models that adjust dietary intakes for the effects of random measurement error (16, 17). This allowed for the estimation of usual choline intake distributions, which could be utilized to more accurately determine the proportion of the PKU sample with choline intakes above the recommended AI.

With these strengths, this dissertation also had important limitations. Cross-sectional designs were utilized to characterize usual choline intake (Chapter 5) and evaluate the relationship between choline intake and working memory performance (Chapter 6). This design prevented us from assessing the stability of choline intake estimates over time, and establishing a causal link between choline intake and working memory. All three studies in this dissertation were also observational. Although it is essential to understand the effectiveness of different PKU

treatments and behaviors outside of clinical trial settings, there are several lifestyle, genetic, and environmental factors that may have confounded the reported estimates and associations. To decrease the impact of these biases, participants with PKU were matched to healthy controls in our first (Chapter 4) and third (Chapter 6) studies, and we adjusted for many potential confounders in the statistical analyses.

Another potential limitation associated with our observational design was selection bias, which could be attributed to specific inclusion/exclusion criteria. This was most relevant for our first (Chapter 4) and second (Chapter 5) studies. Our first study solely included participants from an all-female camp, and our second study had specific criteria for selecting individuals within each PKU treatment group (diet therapy only, sapropterin dihydrochloride, Pegvaliase). Self-selection bias may have also been a concern for the participants enrolled in our third study (Chapter 6). In this study, the adults with PKU were found to have lower concurrent Phe concentrations than our prior studies, and all control participants had working memory composite scores that were above the normative reference average. These sampling issues may reduce the generalizability of our results, and need to be carefully addressed in future research through the recruitment of larger samples and the use of randomization strategies.

Beyond the limitations associated with study design, the use of self-reported dietary intake data was a concern for all three studies. This dietary assessment technique is prone to error due to poor quantification of portion sizes and social desirability bias, which is the tendency of participants to report food choices that appear healthier to the researcher (18). As choline is concentrated in protein-rich foods, and most individuals with PKU are required to restrict protein, it is possible that choline intake may have been underreported. It is also possible that diet analyses underestimated choline intake due to limited choline data within food

composition databases. The most recent update to the US Department of Agriculture database occurred in 2008 and provided choline content for 630 food items (19). Nevertheless, additional work is required in this area to refine estimates of dietary choline.

7.3 Clinical and Public Health Implications

Despite choline's role in several critical functions throughout the brain and body, most health professionals, including registered dietitians, have limited knowledge about the biological importance of this nutrient (20). This dissertation provided the first evidence that this lack of awareness may be associated with negative health consequences for individuals with PKU. We identified perturbed choline metabolism and suboptimal choline intake in this population, suggesting that there is a need for more education among healthcare providers and patients regarding the importance of choline.

This dissertation also found that fortified medical foods are a critical source of choline for patients maintaining a Phe-restricted diet. Not all medical foods, however, provide an equivalent amount of choline, and this variability may have significant implications for cognition. Among adults with PKU who had good historical and concurrent metabolic control, this dissertation identified a significant, positive relationship between total choline intake and overall working memory performance. Although this finding should be replicated in a larger sample, it suggests that choline supplementation practices should be re-evaluated and harmonized. There is presently no guidance on choline fortification for medical foods (21), which makes the clinical monitoring of choline intake an essential practice for building the required evidence base. Additionally, consistent clinical monitoring is important for ensuring that patients are obtaining adequate choline to meet their needs. This practice is particularly relevant for adults who are solely

managed with dietary therapy, given the present research found the lowest usual choline intakes among individuals in this group.

7.4 Future Directions for Research

Given choline has not been explored in prior PKU research, this dissertation was focused on providing foundational evidence on choline adequacy and the relationship between choline and cognition. While we considered choline nutriture in the context of other nutrients that impact choline utilization (e.g. vitamin B12, vitamin B6, folate, methionine), we did not examine other neurologically-relevant nutrients that have historically been suboptimal in PKU (e.g. cholesterol, docosahexaenoic acid, eicosapentaenoic acid, phospholipids, selenium, zinc, vitamin D, vitamin E) (22). Future observational research should consider utilizing an index that reflects the overall adequacy of choline and other neurologically-relevant nutrients. This will allow for a more thorough evaluation of the relationship between nutrient adequacy, metabolic control, and cognitive function in PKU. It would also be beneficial to test the cognitive effects of a multi-nutrient supplement containing choline. This has already been examined in a PKU mouse model with promising results (23), and should be further evaluated in controlled trials among individuals with PKU.

Another related recommendation would be to develop a PKU-specific dietary index that is similar to the healthy eating index (HEI) utilized for the general population (24, 25). This index provides a measure of diet quality by comparing dietary patterns to the Dietary Guidelines for Americans (26). Given the PKU diet generally restricts several adequacy components within the current HEI (25) and includes a large proportion of synthetic nutrients (27), it will be important to create an index that reflects these relevant composition and bioavailability differences. An

index that considers these factors could be useful for clarifying the effects of choline, or all neurologically-relevant nutrients, on cognition within the context of the overall PKU diet.

This dissertation also did not explore the variability in usual intake or cognitive performance associated with different forms of choline. Prior research has identified distinct absorption processes and metabolic functions between the lipid-soluble and aqueous choline molecules, with associated effects on the immune system and cholesterol concentrations (28). These differences may also modulate the impact of dietary choline on the brain, and may be a relevant source of variability in prior research and the present dissertation. Future studies should evaluate both the amount and forms of choline.

Future prospective research should also include biochemical markers of choline status in addition to measures of choline intake. This has been a challenge in prior studies given circulating plasma choline concentrations do not significantly drop among individuals who are severely depleted (29). Betaine, instead, may be a more sensitive marker of choline intake, but has not been found to accurately reflect tissue choline concentrations (30). Continued research in this area will be essential for improving the clinical assessment of choline nutriture.

Throughout this dissertation, we examined how a Phe-restricted diet, adjunct pharmacotherapies, and medical food composition affect total choline intake in PKU. However, these may not be the only pathways by which choline requirements differ in PKU. We have postulated that poor metabolic control (high Phe concentrations) may increase the dietary requirement for choline by modulating the endogenous synthesis of choline. This hypothesis was based on prior research in breast cancer cells, which demonstrated that the Phe catabolite, phenylacetate, has antiestrogenic properties (31). As PEMT, the rate-limited enzyme in choline biosynthesis, is induced by estrogen (32), elevated phenylacetate concentrations may reduce the

activity of this enzyme in premenopausal women. Future research should directly test this hypothesis in individuals with PKU by evaluating changes in PEMT activity as Phe concentrations increase. The information obtained from this analysis could be further expanded by acquiring genotype data for common single nucleotide polymorphisms (SNPs) that also impact PEMT activity (33, 34). When combined with the findings of this dissertation, this additional research will provide a comprehensive understanding of choline needs in PKU.

To ultimately advance the findings of this dissertation, longitudinal studies are needed to understand the combined, long-term impact of choline nutriture and metabolic control during early critical periods of brain development. Prior research suggests that both factors modify critical structural components of the brain during the early postnatal period (35, 36), which subsequently influences intelligence and executive functions (37-39). We expect that these early conditions will influence the effectiveness of dietary interventions later in life.

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