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The Effect of Intact Protein from Foods and Phenylalanine Free Medical Foods on Large Neutral Amino Acids in Patients with Phenylketonuria.

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This thesis, THE EFFECT OF INTACT PROTEIN FROM FOODS AND PHENYLALANINE FREE MEDICAL FOODS ON LARGE NEUTRAL AMINO ACIDS IN PATIENTS WITH PHENYLKETONURIA, by Ann Berry was prepared under the direction of the Master's Thesis Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree Master of Science in the Byrdine F. Lewis School of Nursing and Health Professions, Georgia State University. The Master's Thesis Advisory Committee, as representatives of the faculty, certify that this thesis has met all standards of excellence and scholarship as determined by the faculty.

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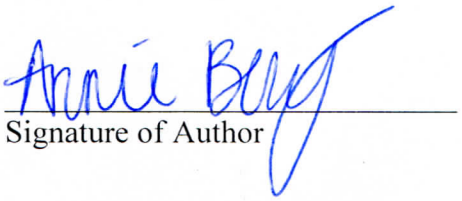

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ABSTRACT

THE EFFECT OF INTACT PROTEIN FROM FOODS AND PHENYLALANINE FREE MEDICAL FOODS ON LARGE NEUTRAL AMINO ACIDS IN PATIENTS WITH PHENYLKETONURIA

by

Ann Berry

Objective: The primary aim of this retrospective cohort study was to determine the association between the source of dietary protein intake and the sum of plasma concentration of large neutral amino acids (LNAA) in patients with Phenylketonuria (PKU). A secondary aim of the study was to examine the effect of dietary compliance on plasma concentration of LNAA. **Methods**: The analysis included combined participant data from two previous studies conducted at the Emory University School of Medicine. Subjects are males (n=34) and females (n=43) with PKU ages 4-50 years. A Student t-test was used to compare total combined plasma LNAA (excluding tryptophan and phenylalanine) by dietary compliance status (alpha=0.05). Correlation statistics were used to determine the association between the ratio of reported intact food protein to medical food protein on plasma levels of LNAA. Multiple regression analysis was used to examine the contribution of intact protein to medical food protein ratio and other variables to plasma LNAA. **Results:** The median ratio of intact protein to medical food protein reported was 0.354 (IQR: 0.188, 0.914). Median percent of PHE intake over the PHE intake recommendation was 31.64 (Interquartile range [IQR]; 7.44, 104.98). Plasma concentration of LNAA did not differ significantly between those with plasma PHE levels within the therapeutic range $\leq$360 μmol/L (compliant; 611.7 μmol/L [n=19]) vs levels above the therapeutic range (non-compliant; 595.3 μmol/L [n=47]); p=0.613). There was an inverse marginal correlation between the ratio of intact protein to medical food protein and plasma concentration of LNAA for those who were compliant (r = -0.436, r = 0.1) although the association was not statistically significant (p=0.08).

No correlation was found for patients who were non-compliant. Regression analysis revealed that plasma concentration of LNAA was not significantly affected by the ratio of intact protein to medical food protein ratio, age, or gender. **Conclusions**: Although not statistically significant, a negative trend was observed between plasma LNAA concentration and the intact protein to medical food protein ratio in patients compliant with the PHE prescription. This suggests that the ratio of intact dietary protein to protein coming from medical food, as reported by patient diet records, may promote increased plasma LNAA levels in the effective treatment of PKU. The majority of the sample (74%) were non-compliant with diet based on plasma PHE levels. Future studies are needed to determine the consequences of non-compliance by decreased intake of medical food protein or increased intake of intact protein on plasma LNAA concentration and downstream health effects.

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ABBREVIATIONS

AV	Assigned Value
BMI	Body Mass Index
cm	centimeter
DRI	Dietary Reference Intake
EAA	Essential Amino Acid
GMP	Glycomacropeptide
kg	kilogram
L	liter
LNAA	Large Neutral Amino Acid
m	meter
µmol	micromole
PAH	Phenylalanine Hydroxylase
PHE	Phenylalanine
PKU	Phenylketonuria
TYR	Tyrosine

CHAPTER I

THE EFFECT OF INTACT PROTEIN FROM FOODS AND PHENYLALANINE FREE MEDICAL FOODS ON LARGE NEUTRAL AMINO ACIDS IN PATIENTS WITH PHENYLKETONURIA

INTRODUCTION

Phenylketonuria (PKU) is a rare metabolic disorder in which the body does not utilize the essential amino acid (EAA) phenylalanine (PHE). The condition affects up to 1 in 20,000 people in the United States.[1] Phenylketonuria is an autosomal recessive genetic disorder in which the enzyme, phenylalanine hydroxylase (PAH), produced in the liver, is deficient.[1] The lack in activity of PAH causes PHE to build up in the blood and body tissues which is toxic to the central nervous system, most notably the brain.[2] If untreated at birth, infants with PKU can suffer from neurological complications including intelligence quotient loss, memory loss, problems with concentration, mood alterations, and in serious cases severe mental retardation.[1] However, when PKU is detected at birth through Newborn Screening and a proper therapeutic diet is followed, the child can live a normal healthy life.[1]

Phenylketonuria is treated with a strict low PHE diet and a synthetic, PHE free, amino acid-based nutritional formula that serves as the major dietary protein source.[1,2] The goal of the diet is to keep patient blood PHE levels in between 120-360 μmol/L level which is needed for normal development and throughout the lifespan.[1] National dietary guidelines for patients with PKU are available[3], but whether patients with PKU are meeting these guidelines is not known. PHE intake is necessary for the body to make tyrosine which then is responsible for making proteins and brain chemicals such as L-

dopa, epinephrine, norepinephrine, and thyroid hormones.[4] Therefore, some PHE must be consumed from the diet, but high protein foods such as poultry, eggs, fish, meat, cheese, milk, and legumes should be avoided. As a result, dietary intake of other EAAs is limited.[1] These other EAAs include histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, and valine.[5] Essential amino acids are critical nutrients in the diet because they cannot be synthesized by the body. The supplemental formulas provided to patients with PKU are meant to provide these essential amino acids without PHE. However, it is unknown whether these formulas, along with a low protein diet provide the dietary requirements for essential amino acids.

Large neutral amino acid (LNAA) levels may also play an important role in the treatment of PKU. Large neutral amino acids include the EAAs histidine, isoleucine, leucine, methionine, threonine, tryptophan, phenylalanine and valine as well as the non-essential amino acid tyrosine.[6] Consumption of LNAAs will increase levels of these amino acids in the bloodstream and may reduce blood PHE levels and the concentration of PHE in the brain.[6] Adequate concentrations of LNAAs are important as they have a common transport system with PHE at the blood-gut barrier as well as the blood-brain barrier.[6] All LNAAs compete for similar carrier proteins at these locations and it is believed that higher concentrations of LNAAs may inhibit transporters from taking up PHE at these sites.[6] However, it is unknown whether there is an association between dietary intact protein intake or the protein composition of medical formula with plasma levels of LNAAs in those with PKU.

PAH differs between patients with PKU resulting in varied tolerance to foods containing PHE.[7] Patients with mild PHE restrictions can consume more high protein

foods and may be getting more essential amino acids through the diet, including LNAA. The goal of this research study is to describe the dietary intake of individuals with PKU and assess whether they are receiving the recommended intake of energy and total protein intake along with adequate LNAA in the diet. The sample population includes patients who participated in two studies conducted by Rani H. Singh, PhD RD and Teresa Douglas, PhD at the Emory University School of Medicine. The first study included patients (>4 years of age) with PAH deficiency (Sapropterin Study). The second study included female patients ages 12 and older with PAH deficiency who attended the Emory University Metabolic Nutrition Program's Metabolic Camp in the summer of 2016. We set out to determine intake adequacy by analyzing patient diet records of food and formula intake and examining their serum levels of large neutral amino acids (LNAA) including valine, isoleucine, methionine, leucine, tyrosine, histidine, threonine, and tryptophan.[6] Associations were investigated for enteral intake and plasma concentrations to determine if the protein source as well as compliance to the therapeutic diet has a relationship on those with PAH deficiency.

The purpose of this study is to determine whether people with PKU are meeting recommended nutrition intake guidelines and to examine the effect that amino acid supplements and intact food sources of protein have on plasma LNAA levels in patients with PKU. These data will be used to determine whether further intervention is necessary to meet total energy and dietary protein needs as well as assess the effect of intake of LNAAs on plasma concentrations.

Specific Aim 1: Compare energy and total dietary protein from both intact and medical foods to energy and protein intake recommendations in the Nutrition Management Guidelines for Phenylketonuria (PKU, PAH deficiency).

Research Hypothesis 1A: Protein and energy intake will be higher than recommended intake guidelines for individuals with PAH deficiency.

Null Hypothesis 1: Protein and energy intake of individuals with PAH deficiency will meet not meet recommended intake guidelines.

Specific Aim 2: To determine the association between enteral intake and plasma concentrations of LNAAs as affected by dietary compliance and protein source in individuals with PAH deficiency.

Research Hypothesis 2A: Individuals with PAH deficiency who are strictly adherent to therapeutic diet will have a higher plasma concentration of LNAA than those who are less compliant.

Null Hypothesis 2A: Dietary and plasma concentration of LNAA will not differ by dietary compliance in children with PKU.

Research Hypothesis 2B: Dietary ratio of intact protein to medical food protein as sources of LNAA will predict plasma concentrations of LNAA in patients with PAH deficiency.

Null Hypothesis 2B: Large neutral amino acid dietary intake does not affect plasma concentration of LNAA regardless of dietary source.

CHAPTER II

LITERATURE REVIEW

Phenylketonuria and Dietary Treatment

Phenylketonuria is an inborn error of metabolism in which the activity of the enzyme phenylalanine hydroxylase (PAH) is deficient (Figure 1).[8] Phenylalanine hydroxylase converts PHE into tyrosine which then produces brain chemicals such as L-dopa, epinephrine, norepinephrine, and thyroid hormone.[4] The lack of PAH activity in those with PKU leads to PHE accumulation in the blood and can cause symptoms such as mental retardation, physical disability, and concentration difficulties.[9] In order to prevent these symptoms, dietary treatment is put into effect immediately upon diagnosis via Newborn Screening. Treatment includes a lifelong low PHE diet, including foods naturally low in protein such as fruits and vegetables, as well as specially formulated foods specifically made for those with PKU. A PHE-free amino acid formula (or medical food) is also consumed daily. Those with PKU are allotted an individualized amount of PHE each day depending on his/her PAH activity affecting PHE tolerance. The amino acid formula is prescribed according to how much natural protein the patient can consume as well as the age, height and weight of the individual. The amino acid mixture provides the patient with the daily requirement of protein as well as vitamins, minerals, and trace elements that may be under consumed in the restricted diet.

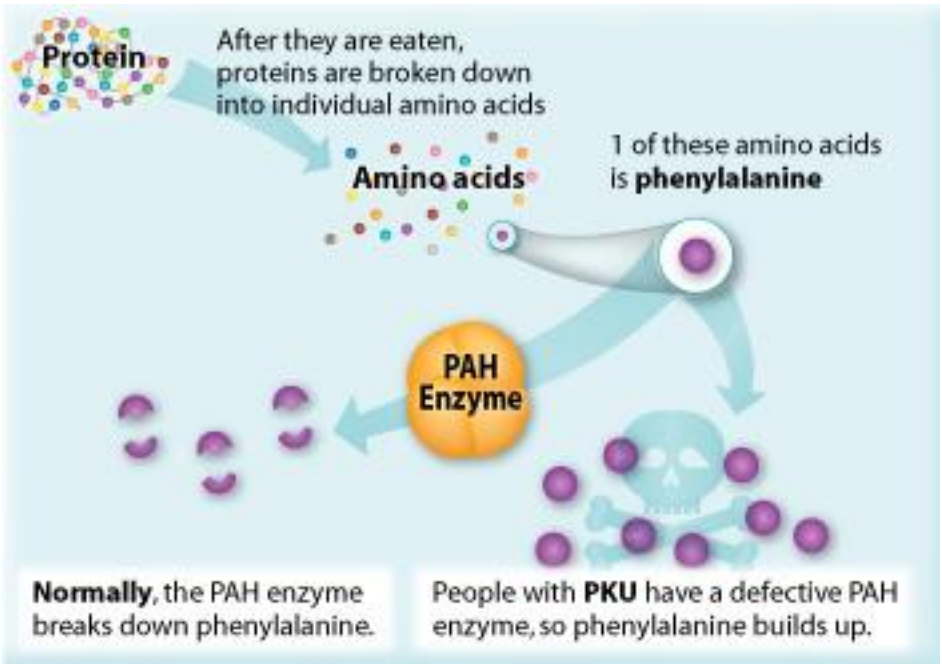

Figure 1. Metabolism of Phenylalanine Hydroxylase (PAH) in Phenylketonuria (PKU).[8]

Nutrient Requirements for People with Phenylketonuria

The latest nutrition management guidelines for PKU were released in 2016.[3] The guidelines give specific nutrient requirements for clinicians treating patients with PKU. The first recommendation is to determine the amount of intact protein needed to provide a patient's recommended intake of PHE (Table 1). Adequate protein is necessary to promote anabolism and to maintain proper blood concentrations of PHE. The second recommendation is to determine total protein intake, including intact protein and protein from the amino acid mixture prescribed. The recommended total protein intake for people with PKU includes 50% more protein than the Dietary Reference Intakes (DRI) for infants birth to age 4 years (1.5 g/kg/day for infants, 1.1 g/kg/day for 1-3 years) and 20-40% more protein than the DRI for those older than 4 years of age (0.95 g/kg/day for 4 – 13 years, 0.85 g/kg/day for 14 – 18 years) .[3] The L- amino acids that are in medical

formula are oxidized more quickly and therefore lead to an increased protein recommendation.[3] Thirdly, the amino acid tyrosine should be supplemented if blood tyrosine levels are consistently low.[3] Finally, those with PKU also should meet the DRI values for other macronutrients, micronutrients, and energy.[3] The goal of these recommendations is for individuals with PKU to maintain a blood PHE level of 120-360 μmol/L while meeting all of their nutritional needs.[3]

Table 1. Guidelines for Energy, Phenylalanine, Tyrosine and Protein for Individuals with PKU*

Age	Energy (kcals/kg/day)	PHE (mg/day)	TYR (mg/day)	Protein (g/kg/day)
Infant to <4 years				
0 to <3 months	102	130-430	1100-1300	2.5-3.0
3 to <6 months	82	135-400	1400-2100	2.0-3.0
6 to 9 months	80	145-370	2500-3000	2.0-2.5
9 to <12 months	80	135-330	2800-3500	2.0-2.5
1 to <4 years	**	200-320	2800-3500	1.5-2.1
After early childhood				
4 to 5 years	65-70	200-1100	4000-6000	120-140% DRI for age
6 to 7 years	61-64			
8 years	59			
9 to 11 years	42-49			
12 to 13 years	40-44			
14 to 16 years	33-39			
17 to 18 years	31-37			

PHE – phenylalanine, TYR – tyrosine, PKU – phenylketonuria
Kcal – kilocalories, kg – kilograms, mg – milligrams, g - grams
*Adapted from Singh et al. (2016) and Texas Children's Hospital Pediatric Nutrition Reference Guide[3,10]
**Energy requirements: 12 to <36 months (82 kcal/kg/day); 3 years (82-85 kcal/kg/day)

Multiple amino acid mixtures are available on the market (Table 2). The clinician and the patient can determine which product would be most applicable to the patient's needs. The amino acid mixture should be selected based on the recommended nutrient intake for the patient and considering adherence by the patient. If an incomplete amino acid mixture is chosen, a vitamin, mineral, energy and/or fat supplement should be incorporated in appropriate if adequate amounts are not available in the diet.[3] Medical formula should be consumed throughout the day in order to maintain consistent blood PHE levels.[3]

Table 2. Enteral medical products for Infants, Children, Adolescents and Adults with PKU

Composition →	**Amino acids, fat, carbohydrate, vitamins, and minerals**	**Amino acids, carbohydrate, vitamins, and minerals**	**Amino acids**	**Glyco-macropeptide**	**Large neutral amino acids**
Nutrient profile	Most complete	Most vitamins and minerals, no fat	Few or no vitamins and minerals and no fat	Variable depending on product, contains PHE	Variable depending on product
Energy/ protein ratio (kcal/ protein)	High to medium	Medium to low	Low	Variable	Low
Forms	Powder	Powder, ready-to-drink	Powder, capsules, tablets	Powder, ready –to-drink, bars, pudding	Powder, tablets
Products designed for infants	Periflex Infant, Phenex-1, Phenyl-Free 1	None	None	None	None
Products designed for children	Periflex Junior, Phenex-2, PhenylAde Essential, Phenyl-Free 2	Lophlex, PhenylAde 40, PKU Coolers, PKU Gel, Maxamaid XP	None	Bettermilk <12, Complete Bars <12, Restore	Lanaflex
Products designed for adolescents and adults	Periflex Advance, Phenex-2, PhenylAde Essential, Phenyl-Free 2, Phenyl-Free 2HP	Lophlex LQ, PhenylAde RTD, PhenylAde 40, PhenylAde 60, PKU Coolers, Maxamum XP	PhenylAde Amino Acid Blend and Amino Acid Blend MTE, Phlexy-10	Bettermilk 12+, Complete bars, Restore, Restore Lite, Swirl	PheBloc

*Adapted from Singh et al. (2014)[11]

The Therapeutic Diet and Essential Amino Acid Levels

Several studies have been conducted to determine whether a restricted diet results in nutrient deficiencies in patients with PKU. There is also research on the effects of glycomacropeptide (GMP) based medical formulas, a low PHE whey protein, compared

to traditional synthetic amino acid formulas. However, there is limited research on the effect of the level of PHE restriction, the type of amino acid mixture used, and how these variables affect plasma LNAA concentrations.

Research has been conducted on the effect of adding LNAAs in the PHE free amino acid mixture on plasma amino acid levels. In a study by Schindeler et al. (2007), the researchers found that blood isoleucine, leucine, threonine, tyrosine, valine, lysine, and histidine levels were all highest when the subjects were receiving the LNAAs with their amino acid mixture. [12] They also reported that methionine was higher in the phase in which patients were taking the LNAAs but not when they consumed their standard amino acid formula without LNAAs. They observed this in patients who were consuming a formula that was low in methionine vs. a 267% increase in methionine and a 127% increase in histidine with LNAA supplementation. These results show that formula composition and intake may have an effect on LNAA amino acid levels.

Essential amino acid supplementation has been shown to be vital in the treatment of PKU. However, the quantity and quality prescribed can differ among metabolic clinics. A study conducted in Europe assessed how different clinics prescribe the amount of protein to be received for each PKU patient.[13] The researchers determined that only 46% of the participating centers calculated an "inefficiency factor" and allowed for a mean 20% of additional protein to make sure that the patient was receiving adequate protein and remained in positive nitrogen balance. They found that 64% of centers recommended giving the amino acid mixture three times a day and 17% recommended four times a day. [13] Most centers recommended L-amino acid formula with added

vitamins and minerals with only three centers recommending GMP products and 17% recommending LNAA supplementation. [13]

Recently, GMP has been introduced as an alternate PKU low PHE amino acid source. GMP is a 64-amino acid glycosylated peptide that is a byproduct of cheese production that remains in the whey portion.[14] Glycomacropeptide does not contain any aromatic amino acids such as PHE. However, GMP can be contaminated with other whey proteins and provide some PHE when used in commercial products. Glycomacropeptide is two to three times higher in isoleucine, threonine, and valine and does not contain all amino acids to be a complete source of protein for patients with PKU.[14] Studies have shown that a low protein diet with GMP provides 70% of intact protein intake from the GMP products as well as from fruits and vegetables vs. a low protein diet with synthetic amino acid mixture that provides only 20% of intact protein from fruits and vegetables.[14] Glycomacropeptide has been shown to increase the total amino acids in plasma when compared to synthetic amino acid supplements due to a slower transit time in the digestive system with intact proteins vs. synthetic proteins.[14,15] It has also been proven to increase the LNAA isoleucine and threonine and this has shown a reduced level of plasma PHE concentration in mice studies.[15] Further research is needed in order to determine what supplementation products are best for providing total repletion of the vital nutrients required by a patient with PKU when following a strict low PHE diet.

Large neutral amino acids compete for the same carrier sites at the blood brain barrier as PHE, therefore, inhibiting PHE passage into the brain when levels of LNAAs are high.[3] Limited research on LNAA supplementation has shown improvements in blood

tyrosine levels and decreases in brain PHE using Magnetic Resonance Spectroscopy.[3,16] Best practices for LNAA supplementation have not yet been determined. However, the majority of recommendations support the use of 20-30% of the DRI protein requirement (0.8 g/kg/day) from LNAAs, and the remaining 70-80% from intact dietary protein.[3] LNAA supplementation has shown to have a role in PKU treatment. However, it is still unknown whether there is a correlation between intact dietary protein and type of medical formula with plasma LNAA levels.

A study conducted by Rohde et al. (2014) reported that patients with PKU may be at greater risk for micronutrient and essential amino acid deficiencies. This study evaluated 3-day diet records that included intact dietary intake and amino acid medical food.[10] Due to the higher intake of natural protein, this study aimed to identify whether or not dietary intake without medical food placed the patients at greater risk for nutrient deficiencies. The study included two groups, patients who received medical food vs. those who did not. The researchers reported that total protein intake was not significantly different between the groups.[10] However, PHE intake was significantly lower (P=0.006) in the group that received medical food. [10] No essential amino acid deficit was found in either group.[10]

Another study by Schulpis et al. (2011) showed differences in serum amino acid levels after subdividing patients with PKU into two groups depending on their mean annual PHE blood concentrations.[8] Both groups received the same amino acid supplemental formula (PKU2Prima) which was enriched with vitamins and trace elements but contained no PHE.[8] Group A was defined as the "relaxed diet group" who had not had good control over their serum PHE levels.[8] Group B was defined as those

patients who “strictly adhered to the diet”. The researchers reported no statistically significant difference in the total protein intake in the two groups.[8] However, the analysis did not include essential amino acid intake.

Pimental et al. (2014) evaluated the protein value in 16 low PHE recipes formulated for patients with PKU.[17] The researchers found that all of these products had low protein content and therefore did not meet essential amino acid requirements. The results of this study pointed out that amino acids are not equally distributed among protein sources. High positive correlations were found between total protein and PHE as well as other amino acids such as leucine, methionine, isoleucine, proline, histidine, and glycine (R=0.9842, 0.9811, 0.9464, 0.9275, and 0.9132, respectively). [17] These results reveal that by restricting PHE in the diet, some essential amino acids are not provided in sufficient quantity without proper intake of a PHE free amino acid formula.

The literature review reveals that adequate energy and protein intake, as well as LNAA concentrations, are important in the monitoring and treatment of PKU. The aims of this study are to examine the intake of energy and protein and LNAA plasma concentrations in the participants at Emory University School of Medicine to evaluate further interventions needed in the treatment of PKU.

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CHAPTER III

MANUSCRIPT IN STYLE OF MOLECULAR GENETICS AND METABOLISM

Introduction

Phenylketonuria (PKU) is a rare metabolic disorder in which the body does not utilize the essential amino acid (EAA) phenylalanine (PHE).[1] Phenylketonuria is an autosomal recessive genetic disorder in which the enzyme, phenylalanine hydroxylase (PAH), produced in the liver, is deficient.[1] The lack in activity of PAH causes PHE to build up in the blood and body tissues which is toxic to the central nervous system, most notably the brain.[2] Phenylketonuria is traditionally treated with a strict low PHE diet and a synthetic, PHE free, amino acid-based nutritional formula that serves as the major dietary protein source.[1,2] The goal of the diet is to keep plasma blood PHE between 120-360 μmol/L, the level which is needed for normal development and cognitive function.[1]

Plasma large neutral amino acid (LNAA) concentration may also play an important role in the treatment of PKU. Large neutral amino acids include the EAAs histidine, isoleucine, leucine, methionine, threonine, tryptophan, phenylalanine and valine as well as the non-essential amino acid tyrosine.[3] Consumption of LNAAs will increase levels of these amino acids in the bloodstream and may reduce blood PHE levels and the concentration of PHE in the brain.[3] Adequate concentrations of LNAAs are important as they have a common transport system with PHE at the blood-gut barrier as well as the blood-brain barrier.[3] All LNAAs compete for similar carrier proteins at these locations and it is believed that higher concentrations of LNAAs may inhibit transporters from

taking up PHE at these sites.[3] However, it is unknown whether there is an association between dietary intact protein intake or the protein composition of medical formula with plasma levels of LNAAs in those with PKU.

The purpose of this study is to examine the effect that different amino acid supplements and intact food sources of protein have on plasma LNAA levels in patients with PKU. We aim to compare energy and total dietary protein from both intact and medical foods to energy and protein intake recommendations in the Nutrition Management Guidelines for PKU. In addition, we aim to determine the association between enteral intake and plasma concentrations of LNAAs as affected by dietary compliance and protein source in individuals with PAH deficiency. We hypothesize that protein and energy intake will be higher than recommended intake guidelines for individuals with PAH deficiency, that individuals with PAH deficiency who are strictly adherent to therapeutic diet will have a higher plasma concentration of LNAAs than those who are less compliant, and that the dietary ratio of intact protein to medical food protein as sources of LNAAs will predict plasma concentrations of LNAAs in patients with PAH deficiency. These data will be used to determine whether further intervention is necessary to meet total energy and dietary protein needs as well as assess the effect of intake of LNAAs on plasma concentrations.

Methods

Study Population

The study sample included participants from two previous studies conducted by Rani H. Singh PhD, RD and Teresa Douglas, PhD at Emory University School of

Medicine. Part of the sample consisted of females with PKU ages 12 years and older who participated in the Emory Metabolic Camp in the summer of 2016. The other study population consistent of males and females ages 5 and older from the Sapropterin (Kuvan) Study. All patients had been diagnosed with PKU through the Newborn Screening Protocol. Patients with other dietary restrictions such as gluten or lactose intolerance and those with an existing pregnancy were excluded.

Study Design

The project is a secondary retrospective cohort study. Data previously collected as part of the Institutional Review Board (IRB) approved Emory Metabolic Camp protocol and the Sapropterin (Kuvan) Study protocol by Dr. Rani H. Singh and Dr. Teresa Douglas in the Department of Human Genetics, Emory University School of Medicine was used. Variables included demographic characteristics (age in years, gender, and race), anthropometrics (weight in kg, height in cm, head circumference in cm, calculated BMI), nutrient intake from dietary food records, PAH status, and determinants of dietary compliance. Each patient was assigned a random identification number and no personal identifiers were collected. Ethics approval for this secondary analysis study was received from the Georgia State University IRB.

Anthropometric Measures

Weight (kilograms) was measured with a standing scale. Height (centimeters) was determined using a stadiometer. Body mass index (BMI) was calculated using the

following equation weight (kilograms) / height (meters)^2. All measures were done by trained research staff.[4]

Dietary Assessment

All participants at the Emory Metabolic Camp and at the baseline visit for the Sapropterin Study completed a 3-day food record prior to their study visit. A Registered Dietitian from Emory University analyzed each individual's 3- day food record for nutrient intake using Nutrition Data System for Research (NDSR; Nutrition Coordinating Center, University of Minnesota, 2016).[4] In the current study, dietary compliance was determined by blood PHE levels within the normal range ($\leq$360 μmol/L), dietary intake within recommended guidelines as reported on the 3-day food record, and consumption of the amino acid medical food prescribed as reported on the 3-day food record.[4] Phenylalanine was also not included in order to identify the relationship between the beneficial effects of other plasma LNAAs on compliant PHE levels as well as the intact dietary protein to medical formula protein ratio.

Blood Sampling

Green top tubes were used for blood sampling. Plasma amino acids were analyzed using quantitative ion exchange chromatography at Emory Genetics Laboratory and standardized per liter of creatinine.[17] LNAA concentration was calculated using the sum of the individual plasma LNAAs, a method previously used to find benefits of LNAA, including leucine, valine, isoleucine, histidine, methionine, threonine, and tyrosine values.[5] Tryptophan was not included in the calculation as the measure is not

routinely determined and the value was not available for half of the population (n=39). To determine the severity of PAH deficiency based on genotype, venous blood samples were obtained in Sapropterin Study participants and dried blood spots collected on filter paper were obtained in Metabolic Camp participants. Polymerase chain reaction was conducted at the Emory Genetics Laboratory to extract DNA from the blood and filter paper samples. The severity of PAH deficiency was categorized as classical/severe (assigned value = 2) or mild/moderate (assigned value >2) using a previously determined method of AV sum.[4,6]

Statistical Analysis

Frequency statistics was used to describe the demographic, anthropometric, nutrient intake and clinical characteristics of the population. Normality testing was conducted on all continuous variables to determine whether parametric or nonparametric statistics should be used. Descriptive statistics were used to compare dietary intake of the participants with intake recommendations. A student t-test was used to compare LNAA concentration by dietary compliance status. Spearman's rho correlation statistics were used to determine the association between the intact protein to medical food protein ratio and plasma concentration of LNAA. Multiple regression analysis was used to examine the contribution of the intact protein to medical food protein ratio and other variables, such as age and gender, to plasma concentration of LNAA. All statistical analysis were conducted using SPSS (version 23.0, SPSS Inc. Chicago, IL). A P-value of <0.05 was considered statistically significant.

Results

Demographic and Anthropometric Characteristics

The study population included 77 participants (44% male) with a mean age of 18.4 $\pm$ 10.9 years (range, 4 to 50 years). The majority of the population were participants of the Kuvan study (n=62) while the remaining participants attended the Emory Metabolic Camp in the summer of 2016. The median weight of the population was 56.3 kg (Interquartile range [IQR]; 36.6, 79.2), the median height was 158.2 cm (IQR; 142.1, 165.3), and the median BMI was 22.1 kg/m^2 (IQR; 17.8, 28.4).

Nutrient Intake and Dietary Compliance

Median fat, carbohydrate, and protein intakes were 47.9 grams (IQR; 35.9, 64.5), 235.2 grams (IQR; 203.5, 286.3), and 58.2 grams (IQR; 47.8, 70.1), respectively. Median energy intake was 31.588 kcal/kg (IQR; 9.1- 92.1) and median protein intake was 1.108 g/kg (IQR; 0.1- 3). Median dietary protein intake was 14.4 grams (IQR; 9.5, 27.2) and medical formula protein intake was 39.3 grams (IQR; 27, 53.4) grams. A comparison of these intake values to the Nutrition Management Guidelines for PKU are shown in Table 3. Median intake of individual amino acids are shown in Table 4. Medical Formula type was differentiated based on PHE content. The majority of participants (n=64) were consuming a PHE-free formula while 4 participants were consuming a PHE-containing product which would affect total plasma LNAA concentration. Two participants were taking a tyrosine supplement which would also affect total plasma LNAA concentration. The median ratio of intact protein to medical food protein reported was 0.354 (IQR; 0.188, 0.914). The median percent of PHE intake over the PHE intake recommendation

was 31.64 (IQR; 7.44, 104.98) with n=52 of participants who had PHE intake over the recommendation. The majority of the sample (74%, n= 57) were non-compliant with diet based on plasma PHE levels.

Lab and Dietary Large Neutral Amino Acid outcomes

Mean total LNAA plasma concentration was calculated with the sum of the individual plasma LNAA levels without tryptophan and phenylalanine (Table 5).[5] Plasma concentration of LNAA did not differ between those with plasma PHE levels within the therapeutic range ≤360 μmol/L (compliant; 611.7 μmol/L [n=19]) vs. levels above the therapeutic range (non-compliant; 595.3 μmol/L [n=47]); p=0.613). There was a negative correlation between the ratio of intact protein to medical food protein and plasma LNAA concentration for those who were compliant (r = -0.436, *r*=0.1), although the association was not statistically significant (p=0.08) (Figure 2). No correlation was found for patients who were non-compliant. (Figure 3). Regression analysis predicted that only 15% of the variance in plasma LNAA concentration was attributed to the ratio of intact protein to medical food protein ratio (p=0.741), age (p=0.320), or gender (p=0.369).

Discussion

The results of this study are useful for healthcare practitioners who provide energy and protein intake recommendations to people with PKU and also adds to the research literature on LNAA and their role in the treatment of PKU. Participants in our

population exceeded the recommended intakes of total energy and protein intakes, as shown in Table 3, suggesting that people with PKU are meeting their daily dietary requirements. Many participants also exceeded their recommended intake of PHE with a high percent of the sample being categorized as noncompliant based on their plasma PHE levels. Therefore, we reject our null hypothesis that energy and protein intake would not meet recommended intake guidelines for those with PAH deficiency. Plasma LNAA concentrations did not significantly differ by dietary compliance status based on Plasma PHE. However, we observed a negative correlation of borderline significance between the ratio of intact protein to medical food protein and plasma total LNAA concentration for the participants who had plasma PHE levels within the normal range. As a result of these observations, we fail to reject our null hypotheses that LNAA concentrations will not differ by dietary compliance in individuals with PAH deficiency or that the dietary source of LNAAs, as expressed by the ratio of intact protein to medical food protein, would not affect plasma concentrations of LNAAs in patients with PAH deficiency.

The energy and protein intake of our population were consistent with guidelines outlined in the Nutritional Management of Phenylketonuria. The guidelines state that people with PKU between the ages of 15 to 18 years should consume 0.84-0.87 g/kg of protein per day for females and males respectively.[7] Our results show that participants reportedly consumed 1.108 g/kg of protein daily. The energy guidelines state that those between ages 17-18 should consume 31- 37 kcal/kg and our population reportedly consumed 31.588 kcal/kg/day. Rohde et al. (2014) examined EAA deficiency in people with PKU by evaluating the total protein intake in those who consumed medical food vs. those who did not.[8] The researchers found no significant difference in the total protein

intake and no deficit in EAA intake. [8] These findings are consistent with the findings in the current study where a cohort of people with PKU met dietary protein intake recommendations.

Although research on the effect of LNAA intake and brain PHE levels is limited, previous studies have observed a lowering of brain PHE levels measured by Magnetic Resonance Spectroscopy with increased LNAA intake. [3] If confirmed, this may result in a change in medical nutrition therapy recommendations for people with PKU. [7] A study by Schindeler et al. (2007), found that blood isoleucine, threonine, tyrosine, valine, lysine, and histidine levels were all highest when subjects received LNAAs in their amino acid mixtures.[9] This is consistent with the belief that LNAA intake has an impact on plasma LNAA concentrations. A study conducted by Concolino et al. (2017), examined the effectiveness of a medical formula containing LNAAs with lowering plasma PHE levels in those who had levels above 360 μmol/L.[10] The researchers saw a decrease in blood PHE levels from 15.15 to 11.15 after consuming a high LNAA concentration formula for 4 weeks ($p=0.0333$).[10] These study results show that LNAAs can be useful in treating those with non-compliant PHE levels.

The results in the current study show that there is a negative correlation between the ratio of intact protein to medical food protein and large neutral amino concentration in those who had compliant plasma PHE levels. This suggests that those who consume more protein from medical food (resulting in a lower intact protein to medical food protein ratio) may have a higher plasma LNAA concentration. Those who have compliant PHE levels are most likely consuming their medical formula. Most of LNAA consumption comes from the medical formula. Therefore, those who are compliant have a lower intact

protein to medical formula protein ratio and higher plasma LNAA. LNAAs may have benefits that will reduce blood PHE levels and the concentration of PHE in the brain.

We do not yet know if the mean concentration of plasma LNAAs in our population differs from that of a similar age- and gender-matched cohort of people without PKU. A study done by Shriver et al. (1985) studied plasma amino acid levels in normal, healthy adults.[11] The following mean plasma LNAA levels (μmol/L) were found in the study population: threonine 145, isoleucine 64, leucine 133, methionine 24, tyrosine 64, valine 264, and histidine 94.[11] Another study conducted by Lepage et al. (1997) studied proper methods of obtaining plasma amino acid levels in a healthy pediatric population. They found the following plasma LNAA concentrations (μmol/L) in 16 year olds: threonine 104, isoleucine 47, leucine 101, methionine 20, tyrosine 46, valine 178, and histidine 77.[12] Our cohort had LNAA plasma levels of threonine 109.79, isoleucine 48.26, leucine 99.67, methionine 19.35, tyrosine 49.29, valine 201.71, and histidine 72.38 which is very similar to the plasma LNAA levels observed in the pediatric population and just slightly lower than the levels observed in the healthy adult population. These values are included in the plasma LNAA levels in Table 5. The differing values in both of these populations is in the plasma PHE level. In the healthy pediatric population, plasma PHE was 47 μmol/L and in the healthy adults plasma PHE was 58 μmol/L compared to our cohort's plasma PHE of 689.56 μmol/L.[11,12] This proves that higher than normal intakes of LNAA need to be achieved in order to have the beneficial effects of PKU therapy.

The current study has some limitations. The majority of participants (74%) were non-compliant as evidenced by plasma PHE levels >360 μmol/L, minimizing the

compliant subgroup sample for our analysis. The homogeneity of our population being non-compliant hinders the ability to fully examine the correlation between source of dietary protein and plasma LNAA concentration. Another limitation was that plasma tryptophan was not able to be calculated into the sum of plasma LNAA concentrations or for diet because the plasma values were not available for the Camp dataset. This may have affected the sum of the plasma LNAA concentration values and the significance of associations in the analysis. However, subgroup analysis where plasma tryptophan was available was still possible. Further, there were 10 subjects who were on other treatments (Kuvan and PegPal) that were not excluded from the sample. This could have altered the compliance as well as dietary protein intake of LNAA. Future research should include separation of the pediatric population from the adult population as well as comparing males to females to accurately report intakes compared to the Nutrition Management Guidelines for PKU as well as anthropometrics such as percentiles of weight, length, and weight/length.

The results of the current study show that people with PKU in the Emory University School of Medicine population are meeting the recommended intake of protein between their combined dietary protein and medical formula intakes. Although not statistically significant, a negative trend was observed between plasma total LNAA concentration and the intact protein to medical food protein ratio in patients compliant with the PHE prescription. This suggests that the ratio of intact dietary protein to protein coming from medical food, as reported by patient diet records, may influence the potential benefits of increased plasma LNAA levels in the treatment of PKU. Most the population (74%) were non-compliant with diet based on plasma PHE levels. Future

studies are needed to determine the consequences of non-compliance by decreased intake of medical food protein or increased intake of intact protein on plasma LNAA concentration.

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APPENDICES

Table 3: Median Protein Intake and Energy Intake compared to Nutrition Management Guidelines for PKU.

Macronutrient Intake	Cohort Result (Mean age 18.4 years $\pm$ 10.9)	Nutrition Management Guidelines for PKU.[3]
Energy (kcal/kg)	31.588	31-37
Protein (g/kg)	1.108	0.84-0.87 (female, male)

Table 4: Reported Intake of Individual Amino Acids

Amino Acid g/day	**Median Intake**	**Interquartile Range (25%, 75%)**
Tryptophan	0.734	0.580, 1.144
Threonine	2.627	2.112, 3.196
Isoleucine	3.193	2.629, 4.286
Leucine	5.546	4.764, 7.013
Lysine	3.813	2.920, 5.111
Methionine	1.170	0.909, 1.521
Cysteine	0.760	0.599, 1.059
Phenylalanine	0.607	0.410, 1.351
Tyrosine	4.624	3.296, 6.063
Valine	3.916	3.256, 5.004
Arginine	3.598	3.018, 4.667
Histidine	1.599	1.239, 1.899
Alanine	3.373	2.696, 4.193
Aspartic acid	3.306	1.943, 6.237
Glutamine	6.847	2.906, 11.751
Glycine	3.075	2.388, 3.869
Proline	4.549	3.946, 5.659
Serine	2.853	2.272, 3.562

g - grams

Table 5: Plasma Levels of Individual Large Neutral Amino Acids

Plasma Amino Acid (μmol/L)	**Mean ± SD**	**Shriver et al (1985)[11]**	**Lepage et al (1997)[12]**
Tryptophan*	44.37 ± 14.15	--	--
Threonine	109.79 ± 42.54	145	104
Isoleucine	48.26 ± 12.75	64	47
Leucine	99.67 ± 23.58	133	101
Methionine	19.35 ± 5.06	24	20
Tyrosine	49.29 ± 18.50	64	46
Valine	201.71 ± 49.28	264	178
Histidine	72.38 ± 14.88	94	77

SD – standard deviation, μmol/L – micromoles per liter

*Plasma values available for only 39 participants in the current study

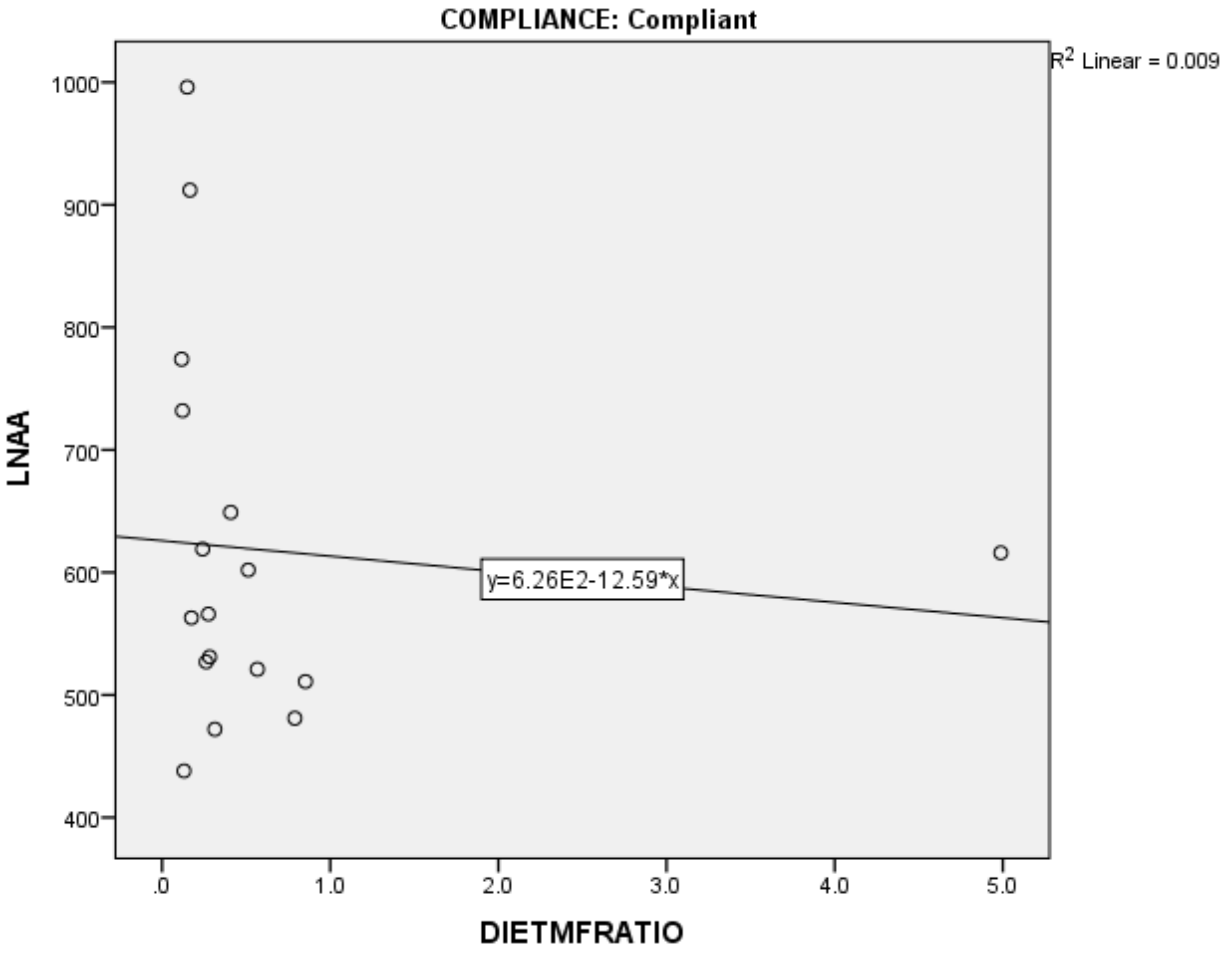

Figure 2. Correlation between the Ratio of Intact Protein to Medical Food Protein (DIETMFRATIO) and Large Neutral Amino Acid (LNAA) Concentration in Participants who were Compliant with their Dietary Phenylalanine Recommendations. P= 0.08

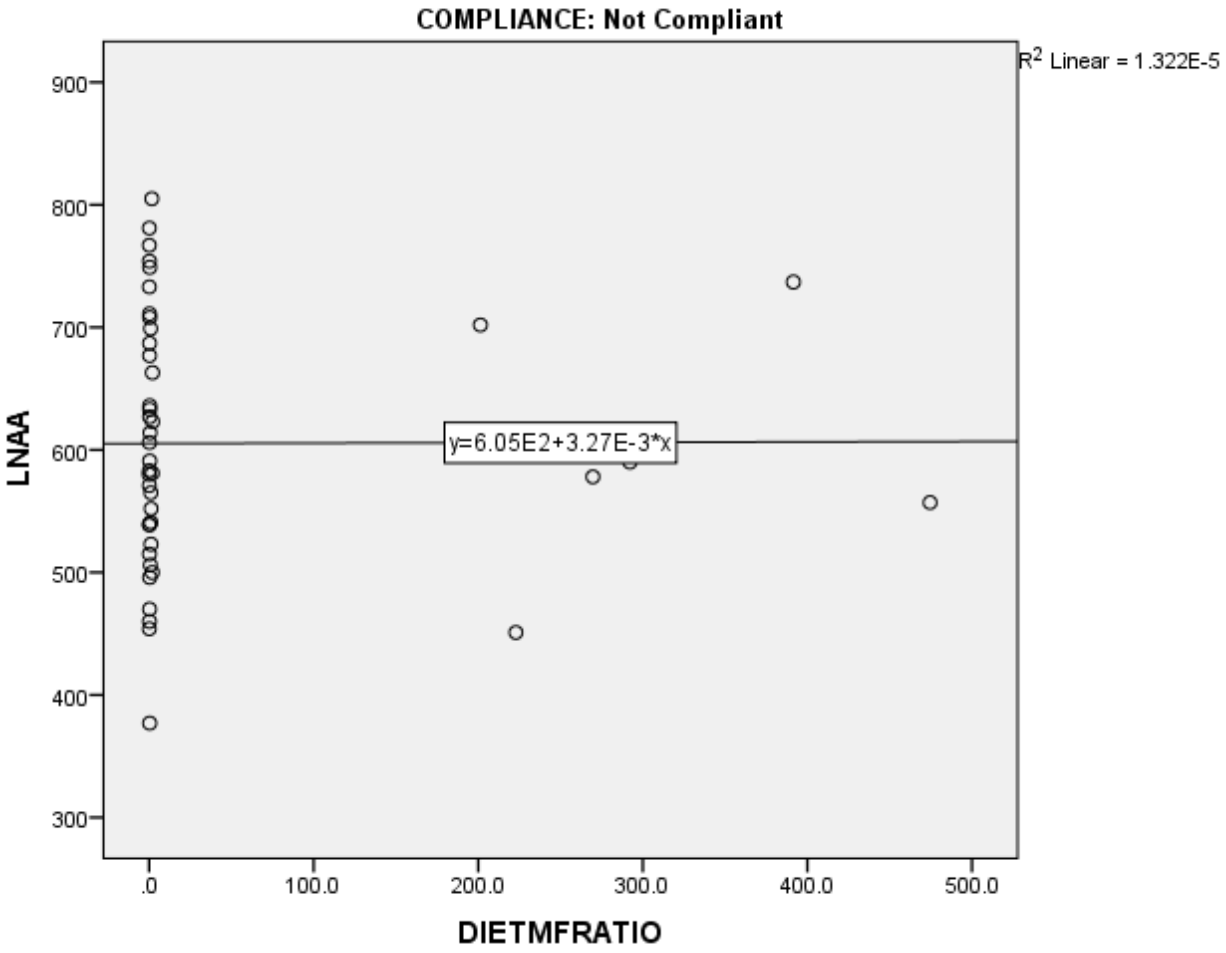

Figure 3. Correlation between the Ratio of Intact Protein to Medical Food Protein (DIETMFRATIO) and Large Neutral Amino Acid (LNAA) Concentration in Participants who were Non-Compliant with their Dietary Phenylalanine Recommendations. P=0.890