NORMAL PLASMA FATTY ACID CONCENTRATIONS AMONG YOUNG CHILDREN WITH PHENYLKETONURIA

by

Stacey M. LaVoie

A THESIS

Presented to the Department of Dietetics & Nutrition

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Master of Science

May 2007

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

Stacey M. LaVoie

has been approved

[redacted]
Mentor/Advisor

[redacted]
Member

[redacted]
Member

TABLE OF CONTENTS

Acknowledgements pg. ii

Abstract pg. iv

Chapter 1: Hypothesis & Specific Aims pg. 1

Chapter 2: Background & Significance pg. 2

Chapter 3: Subjects & Methods pg. 12

Chapter 4: Results pg. 19

Chapter 5: Discussion pg. 29

References pg. 41

Appendices

A. Consent Form – PKU pg. 44

B. Consent Form – Control pg. 49

C. HIPAA Research Authorization pg. 54

D. Lab Protocol: Plasma & Erythrocyte Separation pg. 59

E. Lab Protocol: Plasma Fatty Acid Analysis - PFB Esters pg. 61

F. Convenient Times Schedule Form pg. 64

G. 24-Hour Diet Recall Interview Script pg. 68

H. Food Product Fatty Acid Composition Form pg. 77

ACKNOWLEDGEMENTS

This study was supported by the Pediatric Nutrition Practice Group of the American Dietetic Association, the BioAnalytical Shared Resource/Pharmacokinetics Core in the Department of Physiology and Pharmacology at Oregon Health & Science University (OHSU), and the Clinical & Translational Research Center PHS Grant 1 UL1 RR024120-01.

Thank you to Martha Duffy, Kathleen Huntington and the staff at the Metabolic Clinic of the Child Development and Rehabilitation Center for aiding in the recruitment of eligible subjects with PKU and for your flexibility and patience during the recruitment, consent and data collection process. Thank you to the laboratory staff at Doernbecher Children's Hospital (DCH) for assisting with the blood collection. Thank you to the Day Surgery Clinic at DCH for aiding in the recruitment of eligible control subjects, for your flexibility and patience during the recruitment, consent and data collection process, and especially to the anesthesiologists for your willingness to assist with the blood collection. And, thank you to Dr. Dennis Trune and your laboratory staff for providing us access and use of your hematocrit centrifuge.

Thank you to Dr. Bill Connor, Dr. Cary Harding and Dr. Diane Stadler for agreeing to serve on my committee. Your constructive feedback, your encouragement and your dedication to research have made an invaluable contribution to the success of this study.

Thank you to my friends and family, especially my mom and dad, for believing in me, encouraging me, and supporting me throughout this journey.

Finally, thank you to Dr. Melanie Gillingham for your friendship, your sacrifice and for mentoring, encouraging and supporting me throughout this project. Your commitment to excellence and your passion for learning have made an invaluable contribution to this study and to my personal growth as a student and a researcher.

ABSTRACT

The long-chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA; C22:6n-3) and arachidonic acid (ARA; C20:4n-6) have important physiological implications and are required for optimal somatic growth and brain development, particularly in the early years of life (1-4). Agostoni et al. (5) observed that children with phenylketonuria (PKU) have very low intakes of LCPUFA and thus rely on endogenous synthesis of ARA and DHA through elongating and desaturating reactions. While several studies have observed reduced levels of essential fatty acids (EFA) in subjects with PKU, researchers disagree on whether the observations are significantly lower and thus suggestive of EFA deficiency compared to unaffected control subjects (1, 5-7).

The objective of this study was to determine if children with PKU have lower plasma EFA concentrations than healthy, sex- and age-matched control subjects. The study also investigated whether the variation in plasma fatty acid concentrations could be explained by variation in type and amount of dietary fat consumed. Dietary records were used to determine if adequate intakes of linoleic acid (LA) and α-linolenic acid (ALA), EFA precursors for ARA and DHA, were consumed by subjects with PKU compared to subjects consuming a normal U.S. childhood diet.

In this case-control study, dietary intake of energy and macronutrients, and fatty acid concentrations in total plasma lipid of twenty-one children with PKU and twenty sex- and age-matched, healthy control children were compared. Subjects with PKU had a significantly lower intake of dietary protein (% total energy, p=0.048; g protein/kg body weight, p=0.029; g protein/1000 kcals, p=0.048), and significantly higher intake of

polyunsaturated fat (% total energy, p=0.003; g polyunsaturated fat/1000 kcals) compared to controls.

Subjects with PKU had significantly higher concentrations in total plasma lipid of C14:0 (p=0.024), C18:0 (p=0.010), C18:1 (p=0.007), LA (p=0.006), ALA (p=0.006), and the sum of the ω-6 (p=0.023), monounsaturated (p=0.021) and polyunsaturated fatty acids (p=0.024) compared to controls. Multivariate linear regression analysis found dietary protein and polyunsaturated fat intake to be a significant predictor of LA and ALA concentrations in total plasma lipid. ARA concentrations in total plasma lipid were associated with gender, and negatively associated with age. Concentrations in total plasma lipid of DHA, and the sum of ω-3 and ω-6 fatty acids were negatively associated with age and dietary protein and positively associated with polyunsaturated fat intake.

This study did not observe EFA deficiency, as evidenced by reduced EFA concentrations in total plasma lipid, among young children with PKU. Analysis of diet and EFA concentrations in total plasma lipid showed that a higher intake of LA and ALA among young children with PKU compared to controls was associated with normal ARA and DHA concentrations in total plasma lipid and is most likely related to the contribution from the phenylalanine-free medical formula.

CHAPTER 1

HYPOTHESIS & SPECIFIC AIMS

Hypothesis

We hypothesized that children with phenylketonuria (PKU) consume inadequate amounts of the essential fatty acids (EFA) linoleic acid (LA; C18:2), α-linolenic acid (LNA; C18:3), arachidonic acid (ARA; C20:4), and docosahexaenoic acid (DHA; C22:5) because of a comprehensive dietary restriction of phenylalanine-containing foods. Low dietary intake of EFAs would result in significantly reduced concentrations of these fatty acids in plasma.

Specific Aims

The primary aim of this study was to determine if infants and young children with PKU have low EFA concentrations in plasma suggesting EFA deficiency compared to sex- and age-matched controls. A secondary aim was to investigate whether the diet of subjects with PKU provided inadequate amounts of LA, LNA, ARA, and DHA as a result of the comprehensive dietary restriction of phenylalanine-containing foods used to treat PKU.

CHAPTER 2

BACKGROUND & SIGNIFICANCE

Phenylketonuria

Phenylketonuria (PKU) is an autosomal recessive disorder affecting approximately 1:15,000 live births in North America (8) and is characterized by elevated blood phenylalanine concentration due to phenylalanine hydroxylase deficiency. Approximately three hundred new cases nationwide and five new cases in Oregon are diagnosed each year. Phenylalanine is one of nine essential amino acids and as such must be supplied exogenously by the diet. Under normal physiological conditions, phenylalanine hydroxylase (PAH) converts phenylalanine to tyrosine, which in turn is used to synthesize two important neurotransmitters—dopamine and norepinephrine. In addition to its essential role in the nervous system, phenylalanine is also important in endogenous protein synthesis, memory and learning. Deficiency of phenylalanine may result in failure to thrive and mental retardation (5). In classic PKU, PAH is completely or nearly completely deficient, resulting in an inability of the body to metabolize the essential amino acid phenylalanine and thus, tyrosine becomes a conditionally essential amino acid. If untreated, PKU results in accumulation of phenylalanine and phenylalanine metabolites in the blood and body tissues (8-9).

Just as phenylalanine deficiency can lead to failure to thrive and mental retardation, chronically elevated blood phenylalanine concentration can also lead to behavioral impairments, and physical and mental retardation (9). Elevated levels of blood phenylalanine block transport of tyrosine and tryptophan, amino acid precursors to the

neurotransmitters dopamine and serotonin, into the brain (10). Dopamine and serotonin play an important physiological role in the brain, having a significant affect on mood and learning (10). Thus, if untreated, individuals with PKU are at an increased risk of developing microcephaly, mental retardation, behavioral impairments, and psychomotor disturbances.

Treatment of PKU is centered on dietary intervention aimed at comprehensive dietary restriction of phenylalanine-containing foods (i.e. high protein foods) and supplementation with a phenylalanine-free medical formula. The goal of nutrition therapy is to provide adequate calories, protein, vitamins and minerals to support normal growth and development, and to maintain serum phenylalanine concentration between two and six mg/dl (120-360 μmol/L) to prevent phenylalanine deficiency and/or phenylalanine "toxicity." Meeting protein and amino acid needs in children with PKU is a delicate balance between natural protein and the phenylalanine-free medical formula. Although comprehensive dietary restriction of high protein foods such as meat, eggs, poultry, fish, cheese and milk is essential to maintaining blood phenylalanine levels within the acceptable range, some natural protein is necessary to prevent phenylalanine deficiency, which may result in failure to thrive and mental retardation. Daily intake of a phenylalanine-free medical formula, based on L-amino acids, supplemented with tyrosine, and which may contain added carbohydrate, fat, vitamins, and minerals is fundamental to the successful dietary management of PKU (11). Phenylalanine-free medical formulas supply approximately 80-90% of required dietary protein and other nutrients (11-12), and most are a significant source of polyunsaturated fat in the form of linoleic acid (LA, C18:2) and α-linolenic acid (ALA, C18:3).

Essential Fatty Acids

Essential fatty acids have many important functions in the body. They are structural components of all tissues and are indispensable for cell membrane synthesis. Long-chain polyunsaturated fatty acids (LCPUFA) are found in high concentrations in the brain, retina, and other neural tissues and serve as specific precursors for eicosanoids that regulate numerous cell and organ functions (2). In particular, the LCPUFAs docosahexaenoic acid (DHA; C22:6) and arachidonic acid (ARA; C20:4) have important physiological implications and are required for optimal somatic growth and brain development, particularly in the early years of life (1-4).

The presence of large quantities of LCPUFAs in the brain and retina has prompted researchers to investigate differences in plasma and erythrocyte levels of ARA and DHA in breastfed infants as compared to infants fed standard infant formula; the rationale being that human milk provides optimal nutrition to the developing infant and historically, standard infant formulas have not contained ARA and DHA. Preformed polyunsaturated fatty acids (PUFA), including ARA and DHA, are especially important in neurological development. Several studies have demonstrated that the endogenous synthesis of ARA and DHA in newborns is insufficient to support optimal mental development unless either human milk or ARA and DHA supplemented infant formula is provided (13-15). Furthermore, clinical trials have shown that plasma and erythrocyte levels of ARA and DHA in infants fed standard infant formula containing only LA and ALA, the eighteen-carbon precursors to ARA and DHA, are significantly lower than in infants fed standard infant formula supplemented with ARA and DHA and breastfed infants (13-15).

Recent randomized clinical trials of infant formula composition have provided evidence that DHA may confer an advantage in cognitive development (13). DHA in the diets of preterm or term infants has been associated with higher mental developmental scores, higher psychomotor developmental scores, and better problem-solving skills (13). Following a randomized trial of early dietary supply of LCPUFAs in term infants, Birch et al. (13) reported that supplementation of standard infant formula with DHA and ARA was associated with a mean increase of seven points on the Mental Developmental Index (MDI) of the Bayley Scales of Infant Development, 2nd edition. Furthermore, both the cognitive and motor subscales of the MDI showed a significant developmental age advantage in the supplemented group over the control group (13). As a result of these findings, standard infant formulas supplemented with ARA and DHA became commercially available in the United States in February 2002 (15).

Essential Fatty Acids and PKU

Despite the rationale for adding LCPUFAs to standard infant formula, metabolic formulas used to treat infants and children with inborn errors of metabolism such as PKU have not traditionally been supplemented with ARA and DHA. The first ARA and DHA supplemented medical formula for infants with PKU will become available in the United States in 2007. Furthermore, dietary restriction of phenylalanine is characterized by a low intake of animal based foods and a high intake of plant based foods. LCPUFAs such as ARA and DHA are found preformed only in animal foods and are abundant in eggs, meat and fish, rich sources of protein and phenylalanine. Consequently, as a result of their dietary restriction of phenylalanine, children with PKU have low intakes of LCPUFAs

and rely on endogenous synthesis from the eighteen-carbon precursors, ARA from LA and DHA from ALA, through elongating and desaturating reactions (16).

Several studies have reported reduced levels of EFAs in children with PKU following a low phenylalanine diet (4-5, 7, 17), however, researchers disagree on whether the difference is statistically significant and thus suggestive of EFA deficiency (1, 5-7). Acosta et al. (6) reported no clinically significant, consistent difference in fatty acid levels in plasma or erythrocytes between subjects with PKU and their unaffected siblings. Conversely, Poge et al. (7) and Agostoni et al. (1, 5) concluded that subjects with PKU undergoing comprehensive dietary therapy demonstrated a significant reduction in ω-3 fatty acids, most likely resulting from lower intake of preformed ω-3 fatty acids compared to unaffected control subjects.

In 2001, Acosta et al. (6) reported on dietary intake and blood levels of fatty acids in treated subjects with PKU. The study evaluated whether the diet of subjects with PKU provided adequate intakes of LA and ALA and whether or not subjects with PKU undergoing dietary therapy could form adequate amounts of ARA and DHA from the respective precursors. Between April 1995 and June 1999, a cross-sectional study of patients between one and thirteen years of age with classic PKU (n=28) and their non-PKU sibling (n=26) closest in age was conducted. Subjects with PKU were prescribed one of four phenylalanine-free medical formulas: Phenex® Amino Acid-Modified Medical Food (Ross Products Division, Abbott Laboratories, Columbus, Ohio), Phenyl-Free® (Mead Johnson Nutritionals, Evansville, Indiana), XP Maxamaid® or XP Maxamum® (Scientific Hospital Supplies, Ltd., Liverpool, England). Subjects with PKU ingested the prescribed medical formula for a minimum of three consecutive months prior

to a blood draw. The twenty-six siblings ingested a normal mixed diet. Diet diaries were maintained for all study participants for three days prior to the blood draw. Selection criteria for subjects with PKU included: (1) diagnosed as having classic PKU, (2) receiving Phenex®, Phenyl-Free®, XP Maxamaid® or XP Maxamum® as their primary protein source, (3) maintenance of diet prescription, (4) free of congenital malformations, (5) parental agreement to maintain a three-day diet diary on each participant before the blood draw, (6) parental consent, and (7) participant was not ill and was not receiving any medications. Diet assessment of EFA intake was based on the three-day diet diary, which included brand names and labels for all processed foods consumed. Energy intake and percentages of energy as protein and fat were calculated and approximate intakes of LA and ALA were estimated. A two to five hour post-prandial blood sample was obtained. Fatty acids were analyzed by capillary column gas liquid chromatography and flame ionization detection (GC/FID) and amino acids were analyzed by ion exchange chromatography. Mean energy intake did not differ between groups. Protein (% energy) ranged from 10.7±0.6% by siblings to 12.7±1.2% by Phenex-fed patients. Siblings ingested a greater percentage of energy as fat (34.8±1.3%) than the Phenyl-Free-fed (19.5±1.2%) and the XP Maxamaid- and XP Maxamum-fed groups (19.8±1.2%). Phenex-fed patients had significantly ($p<0.01$) greater mean values for plasma ALA (wt%) when compared with their siblings. A trend toward significance ($p<0.05$) was noted in the Phenex-fed patients, where mean plasma LA (wt%) was greater than in their siblings. In the Phenyl-Free-fed patients, mean values for plasma ALA (wt%) were lower than in their siblings ($p<0.05$). Acosta et al. (6) concluded that no patient in the study exhibited EFA deficiency, and noted that siblings' ingestion of animal protein containing

ARA and DHA accounted for their greater weight percent of these plasma and erythrocyte fatty acids. Additionally, given that subjects with PKU did not ingest fatty acids greater than eighteen carbons, in vivo synthesis from LA and ALA appeared to occur, as evidenced by ARA and DHA found in plasma and erythrocytes of subjects with PKU.

In 2003 Agostoni et al. (16) reported on the relationship between LCPUFA status and neurodevelopment through the first twelve months of life in subjects with PKU. The study investigated the association between LCPUFA status at diagnosis of PKU and neurodevelopment through the first twelve months of life, and assessed whether any differences in LCPUFA status existed between breastfed and bottlefed infants during the first few days on life. The study was an observational, prospective study of twenty infants (9 females and 11 males) with PKU consecutively admitted to the Department of Pediatrics, San Paolo Hospital, Milan, Italy. Subjects with PKU met the following criteria: weight at birth $\geq$ 2500g, gestational age 36-42 weeks, and singleton birth. Furthermore, infants tested positive for PKU at the neonatal screening and were diagnosed as having PKU resulting from PAH deficiency. Infants were classified as either breastfed (n=12) or bottlefed (n=8) based on whether the infant was breastfed or bottlefed from birth up to diagnosis. Mean age at diagnosis was twenty days in the breastfed group and nineteen days in the bottlefed group. Breastfeeding was discontinued in the first month of life in five of the twelve breastfed infants and during the second month of life in the remaining seven breastfed infants. At diagnosis, subjects were placed on an individualized dietary regimen designed to maintain plasma phenylalanine levels below 360 μmol/L. The dietary regimen was deprived of LCPUFA-containing foods,

provided high intake of carbohydrate, low intake of saturated fat, low intake of cholesterol, and was based on phenylalanine-free protein products and vegetables with a low phenylalanine content. Total plasma fatty acids were analyzed via high-resolution gas chromatography flame ionization detection (GC/FID). Individual polyunsaturated fatty acids were identified via pure reference compounds and mass spectrometry and expressed as a weight percent of fatty acid methyl esters. LA, ALA, ARA, eicosapentaenoic acid (EPA) and DHA were assessed. Developmental assessments were conducted through the first twelve months of life. Outcome measures included the Bayley Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) at five and twelve months, and visual function at twelve months. There was a significant difference between the breastfed and bottlefed groups for plasma ARA, DHA, and ALA (mean difference, percentage of total lipids; 3.4%, 0.7%, -0.2%, respectively) at diagnosis of PKU. Higher PDI scores were associated with mean values of plasma ARA (wt%) at diagnosis, but not with any other fatty acid. Agostoni et al. (16) concluded that early LCPUFA intake and status were associated with neural and visual performance through the first twelve months of life. Furthermore, there was a weak association between plasma LCPUFA status at diagnosis and visual function at twelve months of life. Thus, it was suggested that feeding breast milk in the first month or two of life might positively contribute to the infant's later developmental outcome, possibly resulting from the dietary provision of LCPUFAs in breast milk. Therefore, given the importance of LCPUFAs, dietary deficiency of LCPUFAs may be a contributing factor and further compromise neurological development in children with PKU.

In addition to mental and psychomotor developmental impairment (16, 18), studies have also observed visual impairment in children with PKU (19-20). DHA, in particular, has significant effects on photoreceptor membranes involved in the signal transduction process, rhodopsin activation, and rod cone development (21). One study observed that pattern-reversal visual evoked potentials generated by different spatial frequencies and visual contrasts elicited a significantly longer P100 latency in subjects with PKU compared to control subjects (20). Another study observed that visual evoked potentials indicated a positive effect of dietary LCPUFA, particularly DHA, on the visual function of preterm and term infants (22). At baseline, children with PKU had poorer DHA status and prolonged P100 wave latencies than healthy children of comparable age. Following twelve months of supplementation with LCPUFAs, a significant increase in DHA (wt%) in erythrocyte lipids and a decrease in P100 wave latencies were observed (22).

Measurement of Essential Fatty Acids

The literature pertaining to EFA status in children has traditionally expressed EFA status qualitatively versus quantitatively, thus leaving a gap in the knowledge concerning EFA status in subjects with PKU. In the literature reviewed, EFA status was reported as a relative weight percent (wt%) ratio of individual plasma fatty acids in lipid to total lipid fatty acids (i.e. qualitative analysis) versus the absolute concentration (μmol/L) of the individual plasma fatty acids (i.e. quantitative analysis). The ratio of fatty acids provides valuable information on the proportion of fatty acid eighteen-carbon precursors elongated and desaturated, but the effects of fatty acid deficiency are most

likely related to the absolute concentration of fatty acids. Gillingham et al. (23) reported on children with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and concluded that a very-low fat diet used to treat this disorder resulted in low concentrations of all fatty acids while the relative percentage of fatty acids was normal. Quantitative analysis allows the investigator to determine the absolute concentration of plasma fatty acids, thus providing a more accurate criterion by which to diagnose fatty acid deficiency (24). The purpose of this study was to examine the incidence of EFA deficiency in young children with PKU, as evidenced by plasma EFA concentrations (μmol/L), thereby attempting to bridge the gap present in the existing literature.

CHAPTER 3

SUBJECTS AND METHODS

Study Design

This was a case-control study conducted at a single institution (Oregon Health & Science University, OHSU, Portland, Oregon). Dietary intake of energy and macronutrients, and plasma concentrations of essential fatty acids of forty-one infants and young children were compared. Twenty-one subjects with PKU were selected from those undergoing routine follow-up of PKU at the Metabolic Clinic in the Department of Pediatrics at OHSU. Twenty sex- and age-matched (±1 year) control subjects were recruited from children undergoing routine surgery at the Day Surgery Clinic at Doernbecher Children's Hospital, Portland, OR.

Control subjects were judged to be healthy and free from disease, recent illness or hospitalization other than day surgery admission at the time of consent by parent/guardian report. Exclusion criteria included: receiving nutrition via a nasogastric or gastrostomy tube, medical history significant for any chronic medical condition, recent weight loss greater than five pounds, and/or consumption of fish oil nutritional supplements.

The study protocol was reviewed and approved by the Human Subjects Institutional Review Board of OHSU. The consent form for subjects with PKU, the consent form for control subjects, and the Authorization for the Creation, Use, and Disclosure of Protected Health Information for Institutional Review Board Approved Research (HIPAA Research Authorization) are included as appendices (Appendix A, B,

and C, respectively). All specimens and data collected were coded with a unique study identifier and stored in locked laboratory facilities. Computer files of collected data were password protected and subjects' names or other personal health information were de-identified.

Blood Analysis

Blood samples (3 ml) were obtained by venipuncture using sterile technique during a routine blood draw from children with PKU, and at the time of IV placement for anesthesia from control subjects. Blood samples were collected in test tubes containing ethylenediaminetetraacetic acid (EDTA) and plasma and erythrocytes were separated and stored separately until the time of analysis (Appendix D). Plasma and erythrocytes were separated by centrifugation and plasma was transferred to 2mL cryogenic vials and stored at -80°C until the time of analysis. Erythrocytes were washed with phosphate buffered saline and centrifuged again. The saline layer was removed and discarded and erythrocytes were washed a second time. Following the second wash the saline layer was removed and discarded and erythrocytes were transferred to 2mL cryogenic vials and stored at -80°C until the time of analysis.

Analysis of fatty acid concentrations in total plasma lipid was conducted in the Bioanalytical Shared Resource/Pharmacokinetics Core Facility (BSR/PK Core) in the Department of Physiology and Pharmacology at OHSU using the methods of Lagerstedt et al. (24) (Appendix E). Internal standards of deuterated free fatty acids, including d3C10:0, d3C14:0, d3C16:0, d3C18:0, d3C20:0 and d4C22:0, were added to each plasma sample prior to extraction. Fatty acids in plasma lipid were hydrolyzed with hydrochloric

acid (HCl) followed by sodium hydroxide (NaOH). After neutralization with HCl the free fatty acids generated were extracted with hexane. Fatty acids were then derivatized to their pentafluorobenzyl-esters (PFB-esters) and quantified by gas chromatography-mass spectroscopy (GC/MS) using a DSQ II Single Quadrupole GC/MS (ThermoFisher Scientific, Inc., Waltham, Massachusetts) operating in the negative ion chemical ionization mode with methane as the reagent gas. The fatty acid PFB-esters were separated on a DB-5ms capillary column (30 m x 0.25 mm x 0.25 μm film thickness; ThermoFisher Scientific, Inc., Waltham, Massachusetts) with helium as the carrier gas. Fatty acids analyzed included C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:4, C20:5 and C22:6 and were detected using selected ion monitoring for the corresponding free fatty acid resulting from the loss of PFB. Each fatty acid was matched to the deuterated internal standard closest in length and retention time. Peak area ratios of known amounts of standard fatty acids and the internal standards were used to generate calibration curves to quantify unknowns using Xcalibur software (Thermo Fisher Scientific, Inc., Waltham, Massachusetts). Individual fatty acid peaks were compared to internal standards of known concentration and identified by molecular mass and retention time. The sums of ω-3, ω-6, saturated, monounsaturated, and polyunsaturated fatty acids were calculated for each subject by adding together the absolute concentrations of the respective individual fatty acids within each summed group.

Weight

Weight was measured at the time of consent. Young children were weighed on a stand-on scale in light clothing and without shoes (Scale-Tronix 5002, White Plains, New

York). Infants were weighed on a pediatric scale (Scale-Tronix 4800, White Plains, New York).

Diet Analysis

At the time of study enrollment, the parent or guardian of each subject was asked to complete a Food Interview Convenient Times Schedule form (Appendix F). The purpose of this form was to identify when the parent/guardian of the study participant would be available to participate in a 24-hour diet recall telephone interview. The interviews were unannounced and a standard, multiple-pass approach script was used to conduct the interviews (Appendix G). Three separate 24-hour dietary recall telephone interviews, including two non-consecutive weekday recalls and one weekend day recall were conducted for each study participant. Dietary intake of energy, macronutrients, saturated fat, monounsaturated fat, and polyunsaturated fat was determined for each day and the three-day average was calculated. Nutrient analysis was performed using The Food Processor SQL 9.7.3 (ESHA, Salem, Oregon). Nutrient information for phenylalanine-free medical formulas and low-protein foods was obtained from the company and entered into the database, as were any new food products reported in the diet records of control subjects. Many food items in the ESHA database included total and saturated fat content but not monounsaturated and polyunsaturated fat content. For foods supplying more than one gram of total fat per serving, the amount of saturated, monounsaturated and polyunsaturated fat contributing to the total fat content was calculated using data from the manufacturer's website or the USDA National Nutrient Database for Standard Reference. If the information on fatty acid composition was not

available, the estimated fatty acid content was calculated based on the ingredient list of that product. Ingredients were recorded on the Food Product Fatty Acid Composition Form (Appendix H) and the composition of that ingredient was obtained from the USDA National Nutrient Database for Standard Reference. The contribution of each ingredient to the total fat composition was estimated based on the known amount of total and saturated fat reported. For the majority of diet recalls analyzed, the sum of the grams of saturated, monounsaturated and polyunsaturated fat intake equaled at least 90% of the total fat intake.

Statistical Analysis

Power calculations using mean published normal values (expressed as relative weight percent) of DHA (2.15±1.66) and ARA (9.62±6.95) suggested a sample size between eleven and twenty-six subjects was required to detect at least a 0.5 weight percent difference at $p<0.05$ with 80% power.

Descriptive Analysis: Descriptive analysis of demographic and anthropometric data of subjects with PKU and sex- and age-matched control subjects was conducted and reported as the mean and standard error of the mean. Weight-for-age percentiles were based on the National Center for Health Statistics (NCHS) 2000 Centers for Disease Control and Prevention (CDC) Growth Charts (United States) and weight-for-age percentiles and z-score values were calculated using EpiInfo™ (Version 3.3.2, NCHS CDC). Estimated energy requirement (EER) was calculated using Doernbecher Children's Hospital Nutritional Standards for Assessment. The study population was subdivided into three age groups: 9-35 months of age, 3-4 years of age, and 5-7 years of

age. Within each age group, the means and standard error of the means of subjects with PKU and the sex- and age-matched control subjects were calculated for age, weight (kg), weight-for-age percentiles and z-score values. The study population was then further subdivided by gender and the means and standard error of the means were calculated.

Group Comparisons: Statistical analysis was conducted using SPSS 15.0 (Chicago, Illinois). Between group comparisons of dietary intake and plasma fatty acid concentrations were conducted using independent sample t-tests. Differences were considered statistically significant when the *p*-value was <0.05.

Multiple Linear Regression: Correlation analysis and backward selection multiple linear regression analysis were conducted to assess the relationship between dietary intake of total fat, saturated fat, monounsaturated fat and polyunsaturated fat, and plasma EFA concentrations. Correlations were considered significant when the *p*-value was <0.05. Each of ten dependent variables was analyzed, including plasma concentrations of C18:2 (linoleic acid, LA), C18:3 (α-linolenic acid, ALA), C20:4 (arachidonic acid, ARA), C20:5 (eicosapentaenoic acid, EPA), C22:6 (docosahexaenoic acid, DHA), total ω-3 fatty acids (ALA, EPA, DHA), total ω-6 fatty acids (LA, ARA), total saturated fatty acids (C14:0, C16:0, C18:0), total monounsaturated fatty acids (C14:1, C16:1, C18:1), and total polyunsaturated fatty acids (LA, ALA, ARA, EPA, DHA). Independent variables used in the model included: group status (PKU/Control), age, gender, weight (kg), and dietary intake of energy, protein, carbohydrate and either total fat, saturated fat, monounsaturated fat, or polyunsaturated fat, expressed per kilogram of body weight (kcal/kg, g pro/kg, g CHO/kg and either g total fat/kg, g sat fat/kg, g mono fat/kg, or g poly fat/kg, respectively). Independent variables were removed from the model if the *p*-

value was >0.01. Insignificant variables were removed one at a time in order of insignificance and the model was re-analyzed with the remaining variables. This process was repeated until only significant associations remained in the equation. Residual plots and probability-probability plots were used to verify assumptions for a multivariate linear regression model, including normal distribution, equal variance, and linearity.

CHAPTER 4

RESULTS

Descriptive Analysis

Forty-six subjects, twenty-one with PKU and twenty-five controls, were consented to participate in this study. Blood samples were collected from forty-four subjects, twenty-one subjects with PKU and twenty-three control subjects. Blood samples were not obtained from two subjects because IV access was not established. These subjects were not included in the study. Analysis of fatty acid concentrations in total plasma lipid was completed for all forty-four blood samples. Complete dietary intake information was collected for twenty-one subjects with PKU and twenty control subjects. Subject distribution is presented in Figure 1. Subject characteristics are presented in Table 1.

Three control subjects, two males three years of age and one female one year of age, were excluded from the dietary analysis due to completing one or fewer of the 24-hour diet recalls; one due to inaccurate contact information provided on the Convenient Times Schedule Form (Appendix F) and two due to multiple unsuccessful attempts to contact the parent or guardian. Blood samples collected from these subjects were analyzed and included in the between-group comparisons of fatty acid concentrations in total plasma lipid, but subject data was excluded from all other statistical analyses, including descriptive statistics, correlation analysis and multivariate linear regression. For another two subjects, one subject with PKU and one control subject, two of the three diet recalls were completed, one weekday and one weekend day recall. Both subjects were

twelve months of age or less and total dietary intake was consistent for each of the two days analyzed; thus it was assumed that the third diet recall would not have significantly altered the average nutrient intake and the average intake for the two days was included in the analyses.

There was no significant difference in mean age, weight, weight for age percentile, and weight for age z-score between subjects with PKU and control subjects within each age group.

Comparisons of Fatty Acid Concentrations in Total Plasma Lipid

Individual fatty acid concentrations in total plasma lipid and the sums of the ω-3, ω-6, saturated, monounsaturated and polyunsaturated fatty acids were compared between groups and are presented in Table 2 and Table 3, respectively. Subjects with PKU had significantly higher concentrations in total plasma lipid of C14:0 (p=0.024), C18:0 (p=0.010), C18:1 (p=0.007), LA (p=0.006), ALA (p=0.006), ω-6 fatty acids (p=0.023), monounsaturated fatty acids (p=0.021) and polyunsaturated fatty acids (p=0.024) compared to sex- and age-matched controls.

Comparisons of Dietary Macronutrient Intake

Dietary intake of energy and macronutrients was compared between groups and is presented in Table 4. Subjects with PKU had a significantly lower protein intake (% total energy, p=0.048; g protein/kg body weight, p=0.029; g/1000 kcals, p=0.048), and a significantly higher polyunsaturated fat intake (% total energy, p=0.003; g/1000 kcals,

p=0.003) compared to sex- and age-matched controls. There were no other significant differences between the two groups.

Correlation Analysis

There was a significant positive correlation between dietary polyunsaturated fat intake and LA concentration (R^2=0.440, p=0.004), and dietary polyunsaturated fat intake and ALA concentration in total plasma lipid (R^2=0.443, p=0.004). Age was negatively correlated with ARA and DHA concentrations (R^2=-0.382, p=0.014 and R^2=-0.447, p=0.003, respectively). Negative correlations of age and ARA concentration, and age and DHA concentration were observed in subjects with PKU but not in sex- and age-matched controls (Figures 2 and 3). A potentially outlying value of ARA concentration in total plasma lipid was observed in one subject with PKU, however, the negative correlation of age and ARA concentration in subjects with PKU was significant whether or not this value was excluded. Thus, the value was included in the correlation analysis.

Multivariate Linear Regression Analysis

The linear regression models for each of the fatty acids in total plasma lipid identified are presented in Table 5. Multivariate linear regression analysis found dietary protein and polyunsaturated fat intake to be a significant predictor of LA and ALA concentrations. LA and ALA concentrations were negatively associated with age and dietary protein, and positively associated with dietary polyunsaturated fat intake. ARA concentrations were associated with gender, and negatively associated with age. DHA concentrations and the sums of the concentrations of the ω-3 fatty acids and ω-6 fatty

acids were negatively associated with age and dietary protein and positively associated with polyunsaturated fat intake.

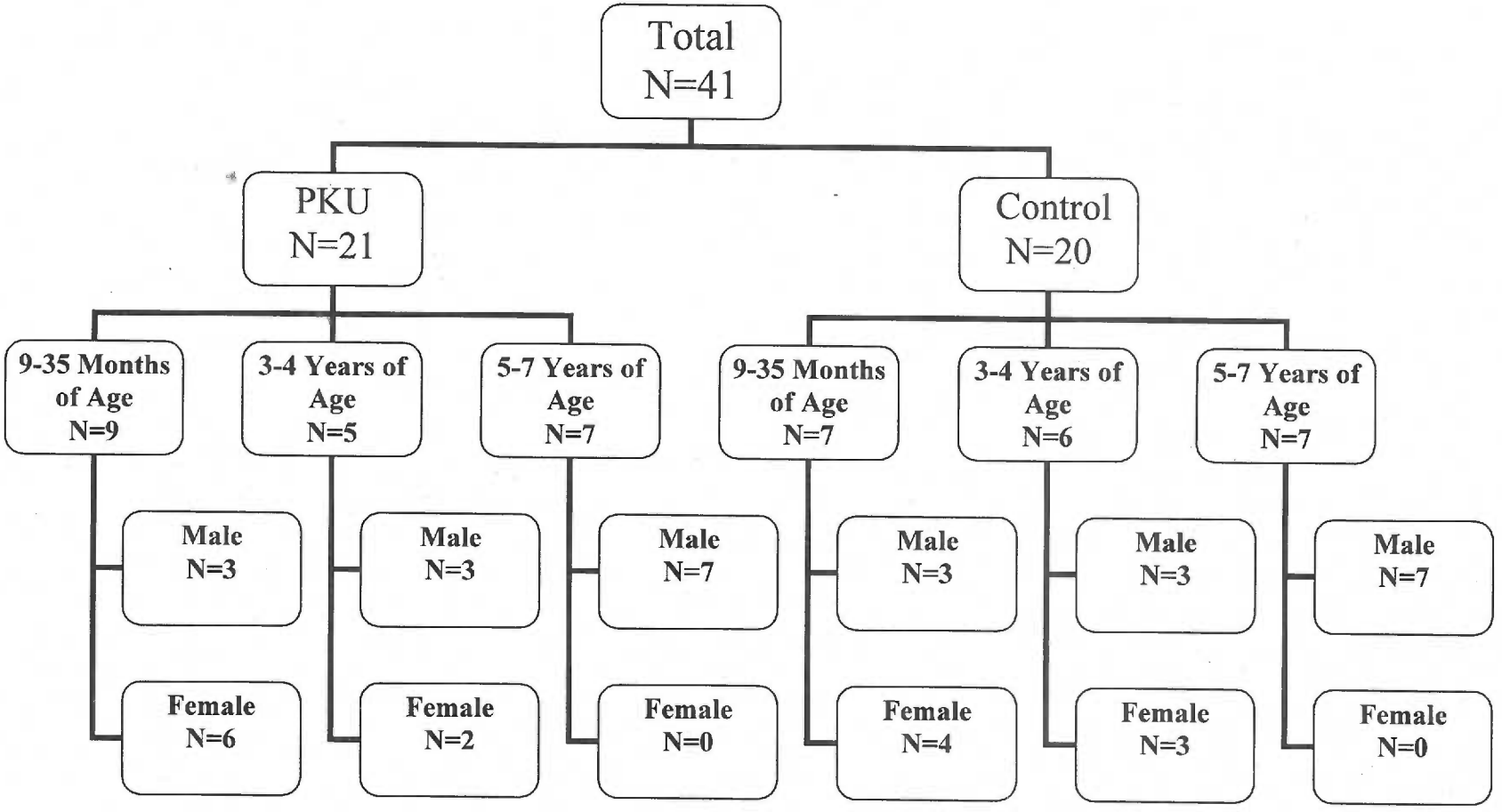

Figure 1. Distribution of subjects. A blood sample and complete dietary intake information were collected from forty-one subjects, twenty-one subjects with PKU and twenty control subjects. Subject groups were subdivided into three age groups: 9-35 months of age, 3-4 years of age, and 5-7 years of age and then further subdivided by gender. An additional three control subjects (not shown above), two males three years of age and one female one year of age, were included in between-group comparisons of fatty acid concentrations in total plasma lipid, but were excluded from all other statistical analysis.

Table 1. Subject characteristics.

	PKU				Control			
	Birth-35 months	**3-4 Years**	**5-6 Years**	**Total**	**Birth-35 months**	**3-4 Years**	**5-6 Years**	**Total**
	Mean ± SD				Mean ± SD			
N	9	5	7	21	7	6	7	20
Male	3	3	7	13	2	3	7	12
Female	6	2	0	8	5	3	0	8
Mean Age (years)	1.8±0.8	3.8±0.5	6.2±0.6	3.7±2.0	1.8±0.7	4.2±0.5	6.1±1.0	4.0±2.0
Mean Wt. (kg)	12.0±2.6	17.4±1.0	23.2±2.4	17.0±5.4	11.2±3.7	17.3±1.9	22.2±5.3	16.9±6.0
Mean Wt./Age %ile	57%±29.1%	70%±18.9%	68%±26.4%	64%±25.6%	19%±25.6%	60%±28.9%	57%±36.1%	46%±34.7%
Wt./Age z-score	0.22±0.88	0.63±0.70	0.82±1.21	0.51±0.96	-0.50±2.34	0.24±0.96	0.25±1.23	-0.01±1.61

SD, standard deviation of the mean.
Weight/Age %ile, weight-for-age percentiles, based on the National Center for Health Statistics (NCHS) 2000 Centers for Disease Control and Prevention (CDC) Growth Charts (United States).
Weight/Age z-score, weight-for-age z-score value.

Table 2. Means of individual fatty acid concentrations in total plasma lipid of 21 children with PKU and 23 sex- and age-matched controls.

	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2 (LA)	C18:3 (ALA)	C20:4 (ARA)	C20:5 (EPA)	C22:6 (DHA)
	Mean ± Std. Error of Mean (μmol/L)										
PKU (n = 21)	800 ± 155	6 ± 1	2444 ± 212	139 ± 18	1101 ± 90	1154 ± 131	1370 ± 128	38 ± 4	404 ± 47	48 ± 6	84 ± 11
Control (n = 23)	440 ± 39	8 ± 1	2167 ± 88	158 ± 14	861 ± 24	773 ± 45	969 ± 65	27 ± 2	367 ± 31	36 ± 4	72 ± 7
Sig. (2-tailed)	0.024*	0.204	0.218	0.406	0.010*	0.007*	0.006*	0.006*	0.506	0.094	0.382

C14:0, Myristic Acid; C14:1, Myristoleic Acid; C16:0, Palmitic Acid; C16:1, Palmitoleic Acid; C18:0, Stearic Acid; C18:1, Oleic Acid; LA, Linoleic Acid; ALA, α-Linolenic Acid; EPA, Eicosapentaenoic Acid; ARA, Arachidonic Acid; DHA, Docosahexaenoic Acid
*Significant at $p<0.05$

Table 3. Means of sums of fatty acid concentrations in total plasma lipid of 21 children with PKU and 23 sex- and age-matched controls.

	ω-3 FA	ω-6 FA	Saturated FA	Monounsaturated FA	Polyunsaturated FA
	Mean ± Std. Error of Mean (μmol/L)				
PKU (n = 21)	169 ± 19	1774 ± 170	4345 ± 442	1299 ± 144	1943 ± 185
Control (n = 23)	135 ± 11	1335 ± 87	3467 ± 132	938 ± 57	1470 ± 93
Sig. (2-tailed)	0.110	0.023*	0.054	0.021*	0.024*

FA, fatty acids
n-3 FA = sum of α-Linolenic Acid (C18:3), Eicosapentaenoic Acid (C20:5), Docosahexaenoic Acid (C22:6)
n-6 FA = sum of Linoleic Acid (C18:2), Arachidonic Acid (C20:4)
Saturated FA = sum of Myristic Acid (C14:0), Palmitic Acid (C16:0), Stearic Acid (C18:0)
Monounsaturated FA = sum of Myristoleic Acid (C14:1), Palmitoleic Acid (C16:1), Oleic Acid (C18:1)
Polyunsaturated FA = sum of Linoleic Acid (C18:2), α-Linolenic Acid (C18:3), Arachidonic Acid (C20:4), Eicosapentaenoic Acid (C20:5), Docosahexaenoic Acid (C22:6)
*Significant at $p<0.05$

Table 4. Mean ± SEM estimated daily energy intake, %EER, protein, carbohydrate, fat, saturated fat, monounsaturated fat, and polyunsaturated fat expressed as a percentage of total energy and as grams per 1000 kcals. of 21 children with PKU and 20 sex- and age-matched controls.

		PKU (n=21)	Control (n=20)	Sig. (2-tailed)
		Mean ± Std. Error of Mean		
Energy				
	total kcal/day	1461 ± 91	1614 ± 132	0.342
	kcal/kg/day	91 ± 7	100 ± 7	0.391
	%EER	93% ± 7%	106% ± 7%	0.209
Protein				
	% total energy	11% ± 1%	13% ± 1%	0.048*
	g/kg/day	2.4 ± 0.2	3.3 ± 0.3	0.029*
	g/1000 kcals	27 ± 2	33 ± 2	0.048*
Carbohydrate				
	% total energy	57% ± 2%	57% ± 2%	0.907
	g/1000 kcals	144 ± 4	143 ± 4	0.907
Total Fat				
	% total energy	32% ± 2%	30% ± 1%	0.375
	g/1000 kcals	35 ± 2	33 ± 2	0.375
Saturated Fat				
	% total energy	10% ± 0%	11% ± 0%	0.391
	g/1000 kcals	11 ± 1	12 ± 1	0.391

%EER = actual (kcal/day)/EER (kcal/day); estimated daily energy intake as a percentage of estimated energy requirement (EER), based on age and weight
*Significant at $p<0.05$

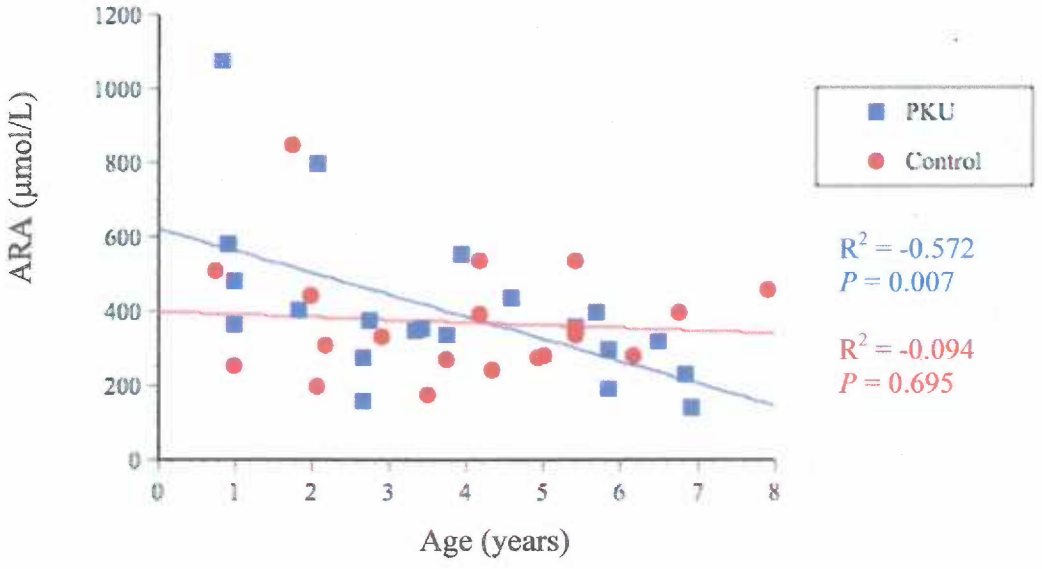

Figure 2. Concentrations in total plasma lipid of ARA negatively correlated with age in 21 children with PKU, but were not significantly correlated with age in 20 control children. R^2, correlation coefficient; p, significance (p-value).

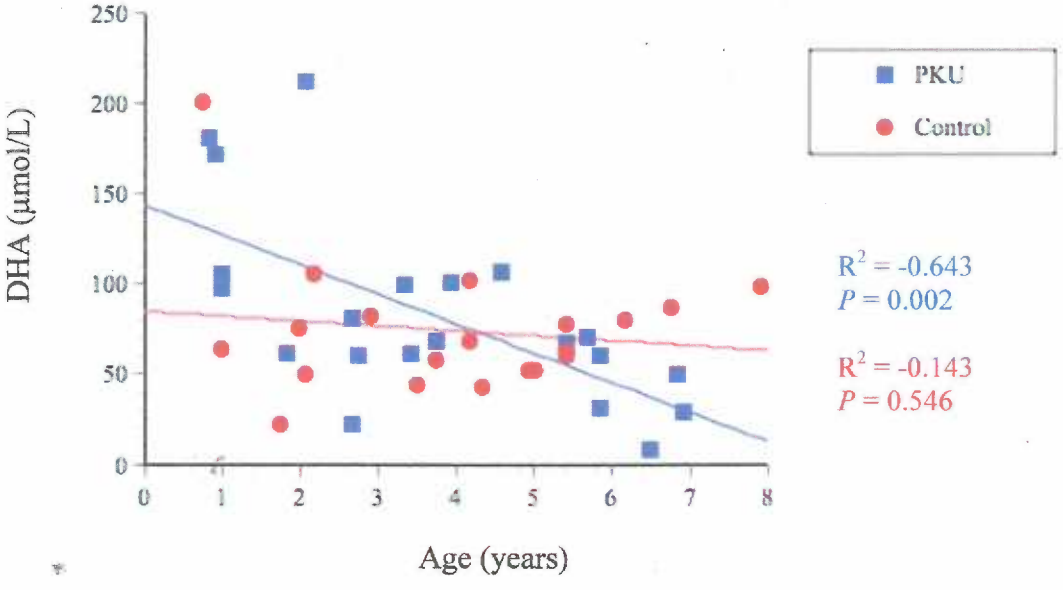

Figure 3. Concentrations in total plasma lipid of DHA negatively correlated with age in 21 children with PKU, but were not significantly correlated with age in 20 control children. R^2, correlation coefficient; p, significance (p-value).

Table 5. Final linear regression models.

Dependent Variable	Independent Variable	Intercept	Slope Est.	Sig.	R^2 Model
C18:2 (LA)					
		1283.282			0.201
	g Protein/kg		-179.229	0.010	
	g Poly Fat/kg		585.019	0.016	
C18:3 (ALA)					
		36.093			0.209
	g Protein/kg		-5.031	0.007	
	g Poly Fat/kg		15.739	0.017	
C20:4 (ARA)					
		629.335			0.211
	Gender		-113.748	0.085	
	Age (years)		-50.814	0.003	
C22:6 (DHA)					
		131.741			0.353
	g Protein/kg		-14.498	0.011	
	g Poly Fat/kg		42.513	0.035	
	Age (years)		-10.393	0.002	
ω-3 Fatty Acids					
		236.755			0.313
	g Protein/kg		-26.285	0.006	
	g Poly Fat/kg		69.012	0.039	
	Age (years)		-14.160	0.011	
ω-6 Fatty Acids					
		2107.508			0.225
	g Protein/kg		-219.414	0.015	
	g Poly Fat/kg		623.169	0.051	
	Age (years)		-87.669	0.091	
PUFA					
		2344.263			0.244
	g Protein/kg		-245.700	0.011	
	g Poly Fat/kg		692.181	0.044	
	Age (years)		-101.829	0.068	

LA, Linoleic Acid; ALA, α-Linolenic Acid; ARA, Arachidonic Acid; DHA, Docosahexaenoic Acid

ω-3 Fatty Acids = sum of α-Linolenic Acid, Eicosapentaenoic Acid, Docosahexaenoic Acid

ω-6 Fatty Acids = sum of Linoleic Acid, Arachidonic Acid

PUFA, polyunsaturated fatty acids = sum of Linoleic Acid, α-Linolenic Acid, Arachidonic Acid, Eicosapentaenoic Acid, Docosahexaenoic Acid

Slope Est. = the estimated amount that the mean difference in the dependent variable (plasma concentration of LA, ALA, ARA, DHA, ω-3 Fatty Acids, ω-6 Fatty Acids, or PUFAs) increases per unit increase or decrease (indicated by the sign ± of the slope est.) in the independent variable (grams of dietary protein, carbohydrate, or polyunsaturated fat per kilogram of body weight, and/or age).

CHAPTER 5

DISCUSSION

Summary & Conclusions

This case-control study compared dietary intake of energy and macronutrients, and concentrations of EFAs in total plasma lipid of twenty-one children who were healthy but had PKU and twenty sex- and age-matched children who were healthy and unaffected by PKU. The primary objective of this study was to determine if infants and young children with PKU have low concentrations of EFAs in total plasma lipid suggesting EFA deficiency compared to control children. A secondary aim was to investigate whether the diets of children with PKU provided adequate amounts of LA, ALA, ARA, and DHA, despite the comprehensive dietary restriction of phenylalanine-containing foods used to treat PKU.

The primary study hypothesis was rejected as the mean differences in EFA concentrations in total plasma lipid were either insignificant or significantly higher in subjects with PKU compared to controls. The secondary study hypothesis was also rejected. Dietary analysis and EFA concentrations in total plasma lipid showed that a higher dietary intake of polyunsaturated fat among children with PKU compared to unaffected controls was associated with normal concentrations of ARA and DHA in total plasma lipid. The higher dietary intake of PUFAs among children with PKU is most likely related to the significant contribution of these nutrients by the phenylalanine-free medical formula. Daily intake of a phenylalanine-free medical formula containing L-amino acids, and which oftentimes contains added carbohydrate, fat, vitamins, and

minerals is fundamental to the dietary management of PKU (11). While the present study did not report on the percentage of total energy supplied by the phenylalanine-free medical formula in subjects with PKU, diet recall interviews indicated that all study participants with PKU were ingesting the prescribed amount of their medical formula daily during their study enrollment. Previous studies have reported that phenylalanine-free protein substitutes supply approximately 80-90% of required dietary protein and other nutrients (11-12), and most are a significant source of polyunsaturated fat in the form of LA and ALA. Thus, the contribution of the phenylalanine-free medical formula to the total fat intake, and in particular LA and ALA intake is highly influential.

Dietary restriction of phenylalanine in the treatment of individuals with PKU is characterized by a low intake of animal based foods and a high intake of plant based foods, and in the absence of a fat-containing phenylalanine-free medical formula is also typically low in fat. Rose et al. (25) compared the effects of a fat-supplemented phenylalanine-free formula versus a fat-free phenylalanine-free formula on the fatty acid status of children with PKU and observed that total fat and ALA intakes were lower in the fat-free phenylalanine-free formula group compared to the fat-supplemented phenylalanine-free formula group.

All but one subject in the present study were prescribed a fat-supplemented phenylalanine-free medical formula that supplied between two and four grams of total fat per one hundred calories. The prescribed phenylalanine-free medical formulas included: Phenyl-Ade® Drink Mix and Phenyl-Ade® MTE Amino Acid Blend (Applied Nutrition Corp., Cedar Knolls, New Jersey), Phenyl-Free® 2 (Mead Johnson Nutritionals, Evansville, Indiana), Phenex®-1 and Phenex®-2 (Ross Products Division, Abbott

Laboratories, Columbus, Ohio), and Periflex® (Nutricia North America, Gaithersburg, Maryland). Only Phenyl-Ade® MTE Amino Acid Blend does not contain added fat. Schultz and Bremer (26) evaluated the nutritional adequacy of the nutrient intake of subjects between twelve and twenty-five years of age with PKU and observed that the diet was relatively low in fat (26). Furthermore, the study noted that the primary fat sources of subjects with PKU were vegetable oils, butter, margarine and small amounts of cream. Vegetable fats provided most of the PUFAs, mainly LA, thus raising additional concern about the quality of fat in the diets of children with PKU.

The present study showed a statistically significant negative correlation between age and ARA concentration in total plasma lipid, and age and DHA concentration in total plasma lipid in subjects with PKU, but a non-significant correlation in control subjects, thus suggesting a reduction in ARA and DHA concentrations with increasing age in children with PKU that is not observed in unaffected children. One possible explanation for this relationship is that while ARA and DHA concentrations in total plasma lipid are within the normal range during the first six years of life, as children with PKU age, a high intake of LA and ALA cannot compensate for the limited supply, if any, of preformed ARA and DHA in the diet. A second possibility is that as children with PKU age, the contribution of the phenylalanine-free medical formula as a percent of total nutrient intake declines and thus, the intake of PUFAs declines. The phenylalanine-free medical formula is the predominant source of protein in the diets of children with PKU, replacing the protein they would otherwise obtain from traditional protein-rich foods, including foods of animal origin. The dietary prescription of phenylalanine-free medical formula is based on the protein requirements of the child. As the child ages, protein needs per

kilogram of body weight decline. Thus, lower amounts of phenylalanine-free medical formula, as a percent of total nutrient intake, are needed to meet the child's estimated protein requirements. This study suggests that the phenylalanine-free medical beverage is a significant source of total fat and, in particular, polyunsaturated fat in the form of LA and ALA in addition to protein. The decline in formula intake as a percent of energy results in a decline in the percent of protein and PUFA intake, as well as LA and ALA, thereby reducing endogenous synthesis of ARA and DHA from these precursors. The negative association of EFA concentrations and dietary protein intake observed in the linear regression models reflect this decrease in percent of energy intake from the phenylalanine-free medical formula as the child ages. Protein intake and PUFA intake vary inversely among subjects with PKU, and both serve as a marker of consumption of the phenylalanine-free medical formula. If this pattern continues into adolescence it might predict that older children and adolescents with PKU are at the highest risk for EFA deficiency due to the lower intake of phenylalanine-free medical formula as a percent of their total dietary intake and thus lower PUFA intake.

Strengths

Compared to previously published studies investigating EFA status in children with PKU, the primary strength of the present study was the inclusion of an unaffected, non-relative control population. Acosta et al. (6) reported on dietary intake and blood levels of fatty acids in subjects with PKU compared to their unaffected sibling closest in age. Not only did the study design ignore potential differences arising from gender and age variation, but using siblings as controls is a potentially significant limitation in that

family diet may be appreciably altered from a typical U.S. diet given the comprehensive dietary restrictions of the child with PKU. Therefore, the diet of the unaffected sibling may not accurately represent the typical diet of children in the United States, which consequently will not accurately reflect differences in EFA status between children with PKU and healthy, unaffected children. Agostoni et al. (16) also reported on LCPUFA status in infants, but the study lacked a control group. Agostoni et al. (16) investigated the association between LCPUFA status at diagnosis of PKU and neurodevelopment through the first twelve months of life to assess whether differences in LCPUFA status existed between breastfed and bottlefed infants during the first few days of life, prior to diagnosis of PKU. Of the twelve breastfed infants enrolled in the study, breastfeeding was discontinued in the first month of life for five infants and in the second month of life for the remaining seven infants. Dietary treatment of PKU severely restricts intake of fatty acids greater than eighteen carbons, as natural food sources are typically high in protein. Consequently, many infants with PKU are not exclusively breastfed, but rather are given a prescribed amount of a phenylalanine-free medical formula that supplies the majority of the infant's energy and protein needs for growth and then supplemented with breast milk to provide natural protein to prevent phenylalanine deficiency. Therefore, it can be assumed that the amount of LCPUFAs they receive from breast milk is considerably less than in an exclusively breastfed infant, and the differences observed between a breastfed infant with PKU and a bottlefed infant with PKU might not be as significant as what might be observed between infants with PKU and unaffected, exclusively breastfed control infants.

A second strength of the present study was the evaluation of EFA status as determined by absolute concentrations of fatty acids in total plasma lipid. In the literature reviewed, EFA status was reported as a relative weight percent (wt%) ratio of individual plasma fatty acids in lipid to total lipid fatty acids (i.e. qualitative analysis) versus the absolute concentration (μmol/L) of the individual plasma fatty acids (i.e. quantitative analysis). Qualitative analysis of the ratio of fatty acids provides valuable information on the proportion of fatty acid eighteen-carbon precursors that are elongated and desaturated, but the effects of fatty acid deficiency are most likely related to absolute fatty acid concentrations. Quantitative analysis allows for the determination of the absolute concentration of plasma fatty acids, thus providing a more accurate criterion by which to diagnose fatty acid deficiency (24). If plasma lipids and essential fatty acids are all proportionately decreased, plasma lipids and essential fatty acids expressed as a percent of total lipids will appear normal despite decreased absolute concentrations that may in fact suggest fatty acid deficiency. Gillingham et al. (23) reported on children with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and concluded that dietary restriction of fat used to treat this disorder resulted in reduced concentrations of fatty acids, despite a normal relative percentage of fatty acids. Conversely, if the concentrations of the predominant dietary fatty acids are elevated and the essential fatty acids are normal, expressing the results as a weight percent would suggest a deficiency that does not exist. Our preliminary results, expressed as a weight percent, suggested just that. However, when fatty acids were measured quantitatively, subjects with PKU had normal or elevated plasma fatty acids.

Limitations

Perhaps the most significant limitation of the present study was the collection of blood samples without controlling for whether or not the subject was fasting or non-fasting. Given that the blood samples of control subjects were obtained at the time of IV placement for surgery-related anesthesia, it is known that all control subjects were fasting at the time of collection. However, for the subjects with PKU, whether the child was fasting or non-fasting was not questioned at the time of blood collection and for clinical follow-up of PKU a fasting blood sample is not required. Given that clinic appointments are scheduled throughout the day, and many families travel up to four hours to attend clinic, it can be assumed that that majority of subjects with PKU were not fasting at the time the blood sample was obtained. Furthermore, as is the practice of most children with PKU, a significant portion of the phenylalanine-free medical formula is consumed at breakfast, prior to attending their clinic appointment. This, of course, raises concern that the higher concentrations of EFAs in total plasma lipid observed in subjects with PKU may be influenced by the intake of formula within the four hours prior to the blood draw and may not, therefore, accurately reflect the child's EFA status.

In one unpublished study (MB Gillingham, Oregon Health & Science University, personal communication, 20 March 2007), absolute concentrations of fatty acids in total plasma lipid, and changes in fatty acid concentration from baseline were analyzed after a ten-hour overnight fast and two and four hours after eating a standardized high carbohydrate meal. Data from three healthy adults were reported before and after consumption of a meal composed of 58% carbohydrate, 18% protein, 17% fat and 6% saturated fat. Repeated measures ANOVA did not show a significant post-prandial

change in concentrations in total plasma lipid of C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, LA, ALA, ARA, EPA, or DHA at two hours or four hours, nor was there a significant change observed in fatty acids as a percentage of baseline concentrations between time zero and either two hours or four hours post-prandially. Despite the low sample size, these results suggest that concentrations of fatty acids in total plasma lipid are not significantly impacted by eating a high complex carbohydrate meal. Therefore, it is possible that fasting and non-fasting blood samples are equal and reliable markers of the EFA status of the individual. However, it does not provide information as to whether similar observations would be noted with consumption of a higher fat meal or one more similar to that of a young child with PKU.

In the present study, between-group comparisons showed a trend toward significance for the sum of saturated fatty acid concentrations in total plasma lipid (p=0.085) and multivariate linear regression showed that the single-most significant predictor of saturated fatty acid concentration in total plasma lipid was group status. Based on the dietary fat and saturated fat intake, expressed as grams per kilogram of body weight, among subjects in the present study, we conclude that subjects with PKU do not consume more fat, but have higher circulating concentrations of fatty acids in total plasma lipid as compared to controls. There are two possible explanations for this observation. First, the higher concentrations of circulating fatty acids in total plasma lipid may be impacted by fasting and feeding and thus, represent higher circulating post-prandial lipids. To test this, we plan to analyze fatty acid concentrations in total erythrocyte lipid and compare these to fatty acid concentrations in total plasma lipid. The second possible explanation is that children with PKU have altered lipid metabolism with

increased serum lipid concentrations. Increased lipid synthesis and/or secretion from body stores or decreased clearance of lipids from circulation would lead to higher circulating lipid concentrations. Lower serum cholesterol and higher triglyceride levels have been observed in several studies comparing children with PKU with unaffected controls (26). While analyzing concentrations of fatty acids in total erythrocyte lipid will control for the differences in feeding and fasting between groups, it will not rule out the possibility that subjects with PKU do have altered lipid metabolism. These possible alterations should be investigated.

Traditionally, erythrocyte phospholipid concentrations have been viewed as a more reliable indicator of an individual's EFA status than plasma phospholipid concentrations (27), and a better long-term indicator of EFA status. Specifically, erythrocyte fatty acids are thought to be a better long-term marker of fatty acid intake than plasma fatty acids because the turnover of erythrocyte fatty acids is much longer than that of plasma fatty acids (28). A limitation of this study is that only EFA concentrations in total plasma lipid were analyzed and thus the EFA concentrations reported might not accurately reflect the subjects' long-term EFA status. Vlaardingerbroek et al. (27) compared levels of EFAs and LCPUFAs in erythrocyte and plasma phospholipids and observed that the relative concentrations (weight percent of total lipids) of erythrocyte and plasma EFAs were strongly correlated, thus suggesting that plasma and erythrocyte phospholipids are an equal marker of EFA status and an equal time marker of EFA status. Skeaff et al. (28) measured dietary-induced changes in the fatty acid composition of plasma and erythrocyte lipids as a function of time and observed that the time course of dietary-induced changes in erythrocyte fatty acid

composition is similar to that in plasma lipids, thereby suggesting that the tissue in which fatty acids are assessed should not significantly impact the observed absolute concentration as a function of intake, assuming steady-state conditions.

An additional limitation of this study was the use of a 24-hour recall interview to collect dietary intake information. There are several acceptable methods of dietary intake collection, but each has its own set of limitations. A 24-hour recall interview is limited by the ability of the study participant and/or parent or guardian of the study participant to recall the food, beverages, and quantities consumed in the prior 24-hour period. Furthermore, the inability of the parent or guardian to accurately estimate the amount of food or beverage consumed, as a result of their being away from the child at the time of intake and/or their unfamiliarity with common household measures, cannot be ignored. Additionally, the 24-hour recall reports on a single 24-hour period and may not capture usual intake and/or variety of intake for the subject. To minimize the limitations associated with the 24-hour recall interview, three separate (2 weekdays and 1 weekend day) 24-hour recall interviews were conducted. This approach was designed to aid in capturing both the typical amount of food consumed as well as the variability of the types of food consumed within the study population. Additionally, each 24-hour recall was conducted using a scripted, multiple-pass approach. This approach was employed to facilitate consistent and meaningful data collection, minimize recall bias, and capture foods and beverages that might otherwise be overlooked. Despite the careful design of the nutrient analysis, it is nevertheless recognized that both underreporting and over-reporting of food intake is inherent with this method.

Dietary intake of energy and macronutrients, including saturated, monounsaturated, and polyunsaturated fat was estimated using The Food Processor SQL 9.7.3 (ESHA, Salem, Oregon). A final limitation of the present study was the number and type of food items not included in the ESHA database, as well as the incomplete nutrient breakdown of several food items. Phenylalanine-free medical formulas and low-protein foods for children with PKU were not available in the database. The nutrient information for these products was obtained from the company and entered into the database. Additionally, any new food products reported in the diet records of control subjects were entered into the database. For many food items, the monounsaturated and polyunsaturated fat content needed to be estimated based on available information as several of the foods in the ESHA database included only total and saturated fat content. Manual data entry and estimations of monounsaturated and polyunsaturated fat content introduced some error into the data.

Future Direction

Analysis of fatty acid concentrations in total erythrocyte lipid and further analysis of the impact of fasting versus non-fasting state of the subject on the absolute concentration of fatty acids in total plasma lipid is needed to validate the findings of the present study.

This study showed higher fat intake and higher concentrations of fatty acids in total plasma lipid, particularly PUFAs, in children with PKU compared to unaffected control children. Future studies are needed to investigate the impact of total dietary fat

intake on circulating fatty acid concentrations in children with PKU, as well as the impact of the macronutrient composition of the diet on body composition.

Many of the low-protein foods designed to supplement the diets of children with PKU are high in total fat, with a significant percentage of total fat coming from saturated fat. Detailed analysis of the macronutrient and fatty acid composition of foods designed for patients with metabolic disorders needs to be conducted to ensure the provision of optimal nutrition to a population that is already at a higher risk for nutritional deficiencies.

Given the observations of the present study concerning the negative association between age and ARA concentrations, and age and DHA concentrations among children with PKU, future studies are needed to investigate whether or not this association continues beyond the first six years of life, and if so, whether or not older children with PKU are at risk of developing EFA deficiency. Furthermore, if it is observed that ARA and DHA concentrations in children with PKU continue to decline with age, is this decline associated with a decreased compensatory mechanism of LA and ALA related to a limited supply of preformed ARA and DHA in the diet, or is it related to a decline in the phenylalanine-free medical formula as a percent of total energy? Decreasing intakes of the medical formula would most likely result in a lower intake of LA and ALA and thus, limited precursors for ARA and DHA synthesis. These associations warrant further investigation.

REFERENCES

1. Agostoni C, Marangoni F, Riva E, Giovannini M, Galli C. Plasma arachidonic acid and serum thromboxane B_2 concentrations in phenylketonuric children negatively correlate with dietary compliance. Prostaglandins, Leukotrienes and Essential Fatty Acids, 1997; 56(3):219-222.

2. Connor WE, Neuringer M, Reisbick S. Essential fatty acids: the importance of n-3 fatty acids in the retina and brain. Nutr Rev, 1992; 50(4):21-29.

3. Innis S. The role of dietary n-3 and n-6 fatty acids in the developing brain. Dev Neurosci, 2000; 22:474-480.

4. Moseley K, Koch R, Moser AB. Lipid status and long-chain polyunsaturated fatty acid concentrations in adults and adolescents with phenylketonuria on phenylalanine-restricted diet. J Inherit Metab Dis, 2002; 25:56-64.

5. Agostoni C, Scaglioni S, Bonvissuto M, Bruzzese MG, Giovannini M, Riva E. Prostaglandins, Leukotrienes and Essential Fatty Acids, 2001; 64(2):111-115.

6. Acosta PB, Yannicelli S, Singh R, Eisas LJ, Kennedy MJ, Bernstein L, Rohr F, Trahms C, Koch R, Breck J. Journal of Pediatric Gastroenterology and Nutrition, 2001; 33:253-259.

7. Poge AP, Baumann K, Muller E, Leichsenring H, Schmidt H, Bremer HJ. J Inherit Metab Dis, 1998; 21:373-381.

8. Infante JP, Huszagh VA. Impaired arachidonic (20:4*n*-6) and docosahexaenoic (22:6*n*-3) acid synthesis by phenylalanine metabolites as etiological factors in the neuropathology of phenylketonuria. Molecular Genetics and Metabolism, 2001; 72:185-198.

9. Dyer CA. Pathophysiology of Phenylketonuria. Mental Retardation and Developmental Disabilities Research Reviews, 1999; S:104-112.

10. Moseley K. Fatty Acid Status of Metabolic Patients: To Supplement of Not to Supplement. National PKU News, 2004; Spring/Summer.

11. MacDonald A, Chakrapani A, Hendriksz C, Daly A, Davies P, Asplin D, Hall K, Booth IW. Protein substitute dosage in PKU: how much do young patients need? Arch Dis Child, 2006; 91:588-593.

12. McBurnie MA, Kronmal RA, Schuett VE, Koch R, Azeng CG. Annals of Human Biology, 1991; 18(4):357-368.

13. Birch EE, Garfield S, Hoffman DR, Uauy R, Birch DG. A randomized control trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. Developmental Medicine and Child Neurology, 2000; 42:174-181.

14. Kuratko C, Arterburn L, Hoffman JP, Nelson EB. Importance of arachidonic acid in long-chain polyunsaturated fatty acid-supplemented infant formula. Am J Clin Nutr, 2005; 82:1353-1357.

15. Koo WWK. Efficacy and safety of docosahexaenoic acid and arachidonic acid addition to infant formulas: can one buy better vision and intelligence? Journal of the American College of Nutrition, 2003; 22(2):101-107.

16. Agostoni C, Verduci E, Massetto N, Radaelli G, Riva E, Giovannini M. Plasma long-chain polyunsaturated fatty acids and neurodevelopment through the first 12 months of life in phenylketonuria. Developmental Medicine and Child Neurology, 2003; 45:257-261.

17. Galli C, Agostoni C, Mosconi C, Riva E, Salari PC, Giovannini M. Reduced plasma C-20 and C-22 polyunsaturated fatty acids in children with phenylketonuria during dietary intervention. J Pediatr, 1991; 119:562-567.

18. Agostoni C, Verduci E, Massetto N, Fiori L, Radaelli G, Riva E, Giovannini M. Long term effects of long chain polyunsaturated fats in hyperphenylalaninemic children. Arch Dis Child, 2003; 88:582-583.

19. Beblo S, Reinhardt H, Muntau AC, Mueller-Felber W, Roscher AA, Koletzko B. Fish oil supplementation improves visual evoked potentials in children with phenylketonuria. Neurology, 2001; 57:1488-1491.

20. Leuzzi V, Rinalduzzi S, Chiarotti F, Garzia P, Trasimeni G, Accornero N. Subclinical visual impairment in phenylketonuria. A neurophysiological study (VEP-P) with clinical, biochemical, and neuroradiological (MRI) correlations. J Inherit Metab Dis, 1998; 21:351-364.

21. Uauy R, Mena P, Rojas C. Essential fatty acids in early life: structural and functional role. Proceedings of the Nutrition Society, 2000; 59(1):3-15.

22. Agostoni C, Massetto N, Biasucci G, Rottoli A, Bonvissuto M, Bruzzese MG, Giovannini M, Riva E. Effects of long-chain polyunsaturated fatty acid supplementation on fatty acid status and visual function in treated children with hyperphenylalanenemia. J Pediatr, 2000; 137:504-509.

23. Gillingham MB, Connor WE, Matern D, Rinaldo P, Burlingame T, Meeuws K, Harding CO. Optimal dietary therapy of long-chain 3-hydroxyacyl-CoA

dehydrogenase deficiency. Molecular Genetics and Metabolism, 2003; 79:114-123.

24. Lagerstedt SA, Hinrichs DR, Batt SM, Magera MJ, Rinaldo P, McConnell JP. Quantitative determination of plasma C8-C26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. Molecular Genetics and Metabolism, 2001; 73:38-45.

25. Rose HJ, White F, MacDonald A, Rutherford PJ, Favre E. Fat intakes of children with PKU on low phenylalanine diets. J Hum Nutr Dietet, 2005; 18:395-400.

26. Schulz B, Bremer HJ. Nutrient intake and food consumption of adolescents and young adults with phenylketonuria. Acta Paediatr, 1995; 84:743-748.

27. Vlaadingerbroek H, Hornstra G. Essential fatty acids in erythrocyte phospholipids during pregnancy and at delivery in mothers and their neonates: comparison with plasma phospholipids. Prostaglandins, Leukotrienes and Essential Fatty Acids, 2004; 71:363-374.

28. Skeaff CM, Hodson L, McKenzie JE. Dietary-induced changes in fatty acid composition of human plasma, platelet, and erythrocyte lipids follow a similar time course. J Nutr, 2006; 136:565-569.

APPENDIX A

CONSENT FORM - PKU

IRB#:_____

MED. REC. NO.:_____

NAME:_____

OHSU **Oregon Health & Science University**
Consent Form

IRB#: 1633

Protocol Approval Date 9/5/2006

MED. REC. NO. _____
NAME _____
BIRTHDATE _____

Complete this section only if clinical services are provided.

OREGON HEALTH & SCIENCE UNIVERSITY
Consent Form

TITLE: Incidence of Essential Fatty Acid Deficiency in Children with Phenylketonuria (PKU)

PRINCIPAL INVESTIGATOR:	Melanie Gillingham, PhD/RD	(503) 494-1682
CO-INVESTIGATORS:	William Connor, MD	(503) 494-2001
	Cary O. Harding, MD	(503) 494-2783
	Diane Stadler, PhD/RD	(503) 494-0168

PURPOSE:

Your child has been invited to be in this research study because your child has phenylketonuria (PKU). The purpose of this study is to determine if children with PKU have an essential fatty acid deficiency.

Essential fatty acids and cholesterol are fats in your food that are important for normal brain development and visual function. Essential fatty acids are also important for normal growth and immune function. This study involves a one-time blood draw and completion of three phone interviews asking you about the foods your child ate the last 24 hours. Your child will be in this study for approximately 3 weeks. Your child's blood sample may be stored for use in other research studies at OHSU. Thirty children with PKU and thirty children without PKU will be enrolled in this study.

PROCEDURES:

Blood samples: One teaspoon of blood will be drawn from a vein in your child's arm to measure essential fatty acid and cholesterol in your child's blood. In the future, samples of your child's blood may be given to researchers at OHSU for other research purposes. The samples will be labeled as described in the **CONFIDENTIALITY** section.

Diet Recalls: You will be asked to complete a Food Interview Convenient Times Schedule form. A person associated with the study will call and ask about the foods your child ate the previous day. The interviewer will ask what kinds of foods your child ate at each meal. She will ask you how the foods were prepared and how much of the foods your child ate. You do not need to prepare or write down foods for this interview. The interviewer will prompt you to think about each meal and recall the foods your child ate.

Each interview will take 30 to 45 minutes. The interviews will be unannounced and will take place during the late morning, afternoon, and early evening hours. Two interviews will occur on a weekday and one interview will occur on a weekend day.

If you have any questions regarding this study now or in the future, contact Dr. Melanie Gillingham (503) 494-1682.

RISKS AND DISCOMFORTS:

Participation in this study may involve some added risks, discomforts or inconveniences. These may include:

Blood Samples: Your child may feel some pain when blood is drawn. There is a small chance the needle will cause bleeding, a bruise, or an infection.

Diet Recalls: Completing the diet records requires some time and effort but poses no added risk or discomfort.

BENEFITS:

You and your child may or may not personally benefit from being in this study. If an essential fatty acid deficiency is detected your child will be referred to your child's metabolic physician for additional follow-up. By serving as a subject in this study, your child may help us learn more about essential fatty acid deficiencies in people with PKU and therefore how to benefit patients in the future.

ALTERNATIVES:

You and your child may choose not to be in this study.

CONFIDENTIALITY:

We will not use your child's name or your child's identity for publication or publicity purposes.

Research records may be reviewed and/or copied by the OHSU Institutional Review Board.

All identifying information about your child will be removed from the blood samples before they are released to any other investigators.

Under Oregon Law, suspected child abuse must be reported to appropriate authorities.

COSTS:

There are no costs to you or your child to participate in this study. You or your child will not be paid for participation.

LIABILITY:

If you believe your child may have been injured or harmed while participating in this research and requires immediate treatment, contact Dr. Melanie Gillingham at 503-494-1682.

The Oregon Health & Science University is subject to the Oregon Tort Claims Act (ORS 30.260 through 30.300). If you suffer any injury and damage from this research project through the fault of the University, its officers or employees, you have the right to bring legal action against the University to recover the damage done to you subject to the limitations and conditions of the Oregon Tort Claims Act. You have not waived your legal rights by signing this form. For clarification on this subject, or if you have further questions, please call the OHSU Research Integrity Office at (503) 494-7887.

PARTICIPATION:

If you have any questions regarding your rights or your child's rights as a research subject, you may contact the OHSU Research Integrity Office at (503) 494-7887.

You do not have to join this or any research study. If you do join, and later change your mind, you may quit at any time. If you refuse to join or withdraw early from the study, there will be no penalty or loss of any benefits to which you are otherwise entitled.

Your child's health care provider may be one of the investigators of this research study, and as an investigator is interested in both your child's clinical welfare and in the conduct of this study. Before entering this study or at any time during the

research, you may ask for a second opinion about your child's care from another doctor who is in no way involved in this project. You do not have to be in any research study offered by your physician.

Your child may be removed from the study if the investigator stops the study.

We will give you a copy of this form.

SIGNATURES:

Your signature below indicates that you have read this entire form and that you agree to be in this study.

OREGON HEALTH & SCIENCE UNIVERSITY
INSTITUTIONAL REVIEW BOARD
PHONE NUMBER (503) 494-7887
CONSENT/AUTHORIZATION FORM APPROVAL DATE

Nov. 7, 2006

Do not sign this form after the
Expiration date of: 9/4/2007

Subject	Date
Parent/Guardian's Signature	Date
Investigator	Date
Signature of Person Obtaining Consent	Date

APPENDIX B

CONSENT FORM - CONTROL

IRB#:_____

MED. REC. NO.:_____

NAME:_____

OHSU **Oregon Health & Science University**
Consent Form

IRB#: 1633

Protocol Approval Date: 9/5/2006

MED. REC. NO. _____
NAME _____
BIRTHDATE _____

Complete this section only if clinical services are provided.

OREGON HEALTH & SCIENCE UNIVERSITY
Consent Form

TITLE: Incidence of Essential Fatty Acid Deficiency in Children with Phenylketonuria (PKU)

PRINCIPAL INVESTIGATOR:	Melanie Gillingham, PhD/RD	(503) 494-1682
CO-INVESTIGATORS:	William Connor, MD	(503) 494-2001
	Cary O. Harding, MD	(503) 494-2783
	Diane Stadler, PhD/RD	(503) 494-0168

PURPOSE:

Your child has been invited to be in this research study because we are collecting information in healthy children. The purpose of this study is to determine if children with a rare disease, called phenylketonuria or PKU have a deficiency of essential fatty acids compared to healthy children who do not have PKU.

Essential fatty acids and cholesterol are fats in your food that are important for normal brain development and visual function. Essential fatty acids are also important for normal growth and immune function. This study involves a one-time blood draw and three phone interviews asking you about the foods your child ate the last 24 hours. Your child will be in this study for 3 weeks. Your child's blood sample may be stored for use in other research studies at OHSU. Thirty children with PKU and thirty healthy children without PKU will be enrolled.

PROCEDURES:

Blood samples: One teaspoon or of blood will be drawn from a vein in your child's arm to measure essential fatty acid and cholesterol in your child's blood. The blood sample will be drawn from an IV after your child has been anesthetized for surgery and is asleep. In the future, samples of your child's

blood may be given to researchers at OHSU for other research purposes. The samples will be labeled as described in the **CONFIDENTIALITY** section.

Diet Recalls: You will be asked to complete a Food Interview Convenient Times Schedule form. A person associated with the study will call you and ask about the foods your child ate the previous day. The interviewer will ask what kinds of foods your child ate at each meal. She will ask you how the foods were prepared and how much of the food your child ate. You do not need to prepare or write down foods for this interview. The interviewer will prompt you to think about each meal and recall the foods your child ate.

Each interview will take 30 to 45 minutes. The interviews will be unannounced and will take place during the late morning, afternoon, and early evening hours. Two interviews will occur on a weekday and one interview will occur on a weekend day.

If you have any questions regarding this study now or in the future, contact Dr. Melanie Gillingham (503) 494-1682.

RISKS AND DISCOMFORTS:

Participation in this study may involve some added risks, discomforts or inconveniences. These include:

Blood Samples: Your child will probably not feel pain when blood is drawn because your child will be "asleep" when the sample is taken and the sample will be taken from the same site that is used for your child's surgery. There is a small chance the needle will cause bleeding, a bruise, or an infection.

Diet Recalls: Completing the diet records requires some time and effort but poses no added risk or discomfort.

BENEFITS:

Your child may or may not personally benefit from being in this study. However, by serving as a subject, your child may help us learn how to better treat people with PKU in the future.

ALTERNATIVES:

You and your child may choose not to be in this study.

CONFIDENTIALITY:

We will not use your child's name or your child's identity for publication or publicity purposes.

Research records may be reviewed and/or copied by the OHSU Institutional Review Board.

All identifying information about your child will be removed from the blood samples before they are released to any other investigators.

Under Oregon Law, suspected child abuse must be reported to appropriate authorities.

COSTS:

There are no costs to you or child to participate in this study. You and your child will not be paid for your participation.

LIABILITY:

If you believe you or your child have been injured or harmed while participating in this research and require immediate treatment, contact Dr. Melanie Gillingham at 503-494-1682.

The Oregon Health & Science University is subject to the Oregon Tort Claims Act (ORS 30.260 through 30.300). If you suffer any injury and damage from this research project through the fault of the University, its officers or employees, you have the right to bring legal action against the University to recover the damage done to you subject to the limitations and conditions of the Oregon Tort Claims Act. You have not waived your legal rights by signing this form. For clarification on this subject, or if you have further questions, please call the OHSU Research Integrity Office at (503) 494-7887.

PARTICIPATION:

If you have any questions regarding your rights or your child's rights as a research subject, you may contact the OHSU Research Integrity Office at (503) 494-7887.

You do not have to join this or any research study. If you do join, and later change your mind, you may quit at any time. If you refuse to join or withdraw early from the study, there will be no penalty or loss of any benefits to which you are otherwise entitled.

Your child's health care provider may be one of the investigators of this research study, and as an investigator is interested in both your child's clinical welfare and

in the conduct of this study. Before entering this study or at any time during the research, you may ask for a second opinion about your child's care from another doctor who is in no way involved in this project. You do not have to be in any research study offered by your physician.

Your child may be removed from the study if the investigator stops the study.

We will give you a copy of this form.

SIGNATURES:

Your signature below indicates that you have read this entire form and that you agree to be in this study.

OREGON HEALTH & SCIENCE UNIVERSITY
INSTITUTIONAL REVIEW BOARD
PHONE NUMBER (503) 494-7887
CONSENT/AUTHORIZATION FORM APPROVAL DATE

Nov. 7, 2006

Do not sign this form after the
Expiration date of: 9/4/2007

_____	_____
Subject	Date
_____	_____
Parent/Guardian's Signature	Date
_____	_____
Investigator	Date
_____	_____
Signature of Person Obtaining Consent	Date

APPENDIX C

HIPAA RESEARCH AUTHORIZATION

OHSU

Oregon Health & Science University
HIPAA Research Authorization

IRB#: __1633__

Protocol Approval Date: 9/5/2006

MED. REC. NO. _____
NAME _____
BIRTHDATE _____

Complete this section only if clinical services are provided.

HIPAA RESEARCH AUTHORIZATION

AUTHORIZATION FOR THE CREATION, USE, AND DISCLOSURE OF PROTECTED HEALTH INFORMATION FOR INSTITUTIONAL REVIEW BOARD APPROVED RESEARCH

Instructions: This barcode HRA is to be attached to each barcode Consent Form and both submitted to Health Information Services. Investigators please complete information in all headers and the fields below and questions 2-4, 8, 9. If applicable, modify question 6 to match the consent form. Leave subject name and signature areas blank.

Title of Study: Incidence of Essential Fatty Acid Deficiency in Children with Phenylketonuria (PKU) - Banking
Name of Investigator: Melanie Gillingham, PhD
Phone Number: (503) 494-1682
Sponsor: none

This authorization is voluntary, and you may refuse to sign this authorization. If you refuse to sign this authorization, your health care and relationship with OHSU will not be affected. However, you will not be able to enter this research study.

1. This form authorizes Oregon Health & Science University (OHSU) to use and disclose (release) certain protected health information about _____ (name of subject) that we will collect and create in this research study. The description of the information to be used or disclosed and the purposes of the requested use or disclosure are indicated in item number 8 of the authorization form.

2. The persons who are authorized to use and disclose your protected health information are:

   ☒ All investigators listed on page one of the Research Consent Form and others at OHSU who are participating in the conduct of the research protocol
   ☒ The OHSU Institutional Review Board
   ☐ Others: _____

3. The persons who are authorized to receive this information are:
   ☐ The sponsor of this study: _____
   ☒ Federal or other governmental agencies as required for their research oversight and public health reporting in connection with this research study:
   ☒ OHRP ☐ FDA ☐ NIH ☐ Other: _____
   ☐ Others: _____

4. We may continue to use and disclose protected health information that we collect from you in this study until:
   ☐ HIPAA Research Authorization expiration date _____
   -OR-
   ☐ The study is completed _____
   ☒ Indefinitely
   ☐ Other: _____

Document Control No.: IRB-HRA-BAR-01-01
Original Date: 09/14/2004; Revision Date: 8/24/2005
Scan to CO1459

5. While this study is still in progress, you may not be given access to medical information about you that is related to the study until after the research is complete. After the study is completed and the results have been analyzed, you will be permitted access to any medical information collected about you in the study that OHSU maintains in your medical record.

6. You have the right to revoke this authorization and can withdraw your permission for us to use your information and/or tissue or blood sample that identifies you for this research by sending a written request to the Principal Investigator listed on page one of the research consent form. If you do send a letter to the Principal Investigator, the use and disclosure of your protected health information and/or tissue or blood sample that identifies you for this research will stop as of the date he/she receives your request. However, the use and disclosure of information collected before the date of the letter or collected in good faith before your letter arrives is allowed to continue. If you withdraw permission for use of any tissue or blood samples that were collected from you for a genetic research study, they either will be destroyed or stored without any information that identifies you. Revoking this authorization will not affect your health care or your relationship with OHSU..

7. The information about you that is used or disclosed in this study may be re-disclosed and no longer protected under federal law. However, Oregon law restricts re-disclosure of HIV/AIDS information; mental health information; genetic information; and drug/alcohol diagnosis, treatment, or referral information.

8. Description of the information to be used or disclosed and the purposes of the requested use or disclosure:

IRB#:_____

MED. REC. NO.:_____

NAME:_____

HEALTH INFORMATION *(Check as applicable)*	**PURPOSE(S)** *(Enter corresponding letter(s) from Purpose Categories)*
☐ Your complete existing health record **	_____
☐ Limited information from your existing health record** (specify): _____	_____
** If we are requesting existing health records that are located outside of OHSU, you will need to complete an additional authorization to release these records to OHSU.	
THE FOLLOWING CHECKED ITEM(S) WILL BE GENERATED/COLLECTED DURING THE COURSE OF THIS STUDY:	
☐ History and physical examinations	_____
Reports: ☐ Laboratory ☐ Operative ☐ Discharge ☐ Progress	_____
☐ Photographs, videotapes, or digital or other images	_____
☐ Diagnostic images/X-ray/MRI/CT	_____
☐ Bioelectric Output (e.g., EEG, EKG)	_____
☒ Questionnaires, interview results, focus group survey, psychology survey, behavioral performance tests (e.g., memory & attention)	a, b, c
☒ Tissue and/or blood specimens	a, c, e
☐ Other: _____	_____

PURPOSE CATEGORIES

a. To learn more about the condition/disease being studied
b. To facilitate treatment, payment, and operations related to the study
c. To comply with federal or other governmental agency regulations
d. For teaching purposes
e. To place in a repository or information/tissue "bank."
f. Other _____

Document Control No.: IRB-HRA-BAR-01-01
Original Date: 09/14/2004; Revision Date: 8/24/2005
Scan to CO1450

IRB#:_____

MED. REC. NO.:_____

NAME:_____

9. If the information to be used or disclosed contains any of the types of records or information listed just below, additional laws relating to use and disclosure of the information may apply. You understand and agree that this information will be used and disclosed only if you **place your INITIALS** in the applicable space next to the type of information.

_____ Acquired immunodeficiency syndrome (AIDS) or human immunodeficiency virus (HIV) infection information

_____ Drug/alcohol diagnosis, treatment, or referral information

_____ Mental or behavioral health or psychiatric care

_____ Genetic testing information

You will receive a copy of this authorization form after you sign it.

_____

Printed name of Research Subject

_____ _____

Signature of Subject Date

-OR-

_____

Printed name of Subject's Legally Authorized Representative

_____ _____

Signature of Subject's Legally Authorized Representative Date

Description of Relationship to Subject: _____

OREGON HEALTH & SCIENCE UNIVERSITY
INSTITUTIONAL REVIEW BOARD
PHONE NUMBER (503) 494-7887
CONSENT/AUTHORIZATION FORM APPROVAL DATE

Nov. 7, 2006

Do not sign this form after the
Expiration date of: 9/4/2007

Document Control No.: IRB-HRA-BAR-01-01
Original Date: 09/14/2004; Revision Date: 8/24/2005
Scan to CO1450

APPENDIX D

LAB PROTOCOL: PLASMA & ERYTHROCYTE SEPARATION

Separation of Plasma and Erythrocytes & Washing of Erythrocytes

A. Separation of Plasma and Erythrocytes
    a. Place 3ml tube containing blood sample in centrifuge, balance, and centrifuge X 12 min.
    b. Transfer plasma to 2mL cryogenic vials (200-250µL/vial)
    c. Cap and store vials at -80°C
B. Washing of Erythrocytes
    a. Once plasma has been removed, add 2mL 1 X PBS to erythrocytes, mix by inverting 3-4 times
    b. Centrifuge X 5 min.
    c. Remove saline layer into waste container
    d. Add 2mL 1 X PBS to erythrocytes, mix by inverting 3-4 times
    e. Centrifuge X 5 min.
    f. Remove saline layer into waste container
    g. Transfer washed erythrocytes to 2mL cryogenic vials (200-250µL/vial)
    h. Cap and store vials at -80°C

APPENDIX E

LAB PROTOCOL: PLASMA FATTY ACID ANALYSIS - PFB ESTERS

Plasma Fatty Acid Analysis: PFB Esters

A. Tube preparation

1. mix 1 mg/ml BHT in 16X100 glass tube with 2 $ChCl_3$:1 MeOH
2. sonicate internal standard stocks X 15 minutes
3. mix 5 ml of internal standard in volumetric flask with 2 $ChCl_3$:1 MeOH

Fatty Acid:	Stock conc:	µl to 5 ml mix:	µg/ 5 ml:	µg per tube:
d_3C10	1 mg/ml	25	25	1
d_3C14	1 mg/ml	50	50	2
d_3C16	10 mg/ml	50	50	20
d_3C18	10 mg/ml	50	50	20
d_3C20	10 mg/ml	25	25	10
d_4C22	1 mg/ml	50	50	2

4. mix well
5. add 100 µl BHT to tubes
6. add 200 µl internal standard to tubes
7. dry under N

B. Fatty Acid Extraction

a. Standard curve preparation

1. sonicate free FA stocks X 15 min
2. mix 1 ml standard curve in volumetric flask with 2 $ChCl_3$:1 MeOH per attached chart

Fatty Acid:	Stock conc:	µl to 1 ml mix:	µg/ ml mix:
Octanoic C8:0	100 µg/ml	50	5
Decanoic C10:0	100 µg/ml	50	5
Myristic C14:0	1 mg/ml	50	50
Myristoleic C14:1	100 µg/ml	50	5
Palmitic C16:0	50 mg/ml	10	500
Palmitoleic C16:1	1 mg/ml	50	50
Stearic C18:0	5 mg/ml	30	150
Oleic C18:1	10 mg/ml	50	500
Linoleic C18:2	10 mg/ml	50	500
Linolenic C18:3	100 µg/ml	50	5
Arachidonic C20:4	10 mg/ml	15	150
Eicosapentaenoic C20:5	1 mg/ml	50	50
Docosahexaenoic C22:6	1 mg/ml	50	50

3. add 100 µl 2 $ChCl_3$:1 MeOH to blank
4. add standard mix to tubes 2-7 as follows:
    1. tube 2 300 µl
    2. tube 3 200 µl

3. tube 4 100 µl
4. tube 5 50 µl
5. tube 6 25 µl
6. tube 7 10 µl

5. add 25 µl plasma unknowns to remaining tubes

b. mix 90:10 acetonitrile (MeCN):6 N HCl (need 2 ml /tube)
c. add 2 ml 90:10 MeCN:HCl to each tube
d. vortex 30 sec X 2
e. place in oven at 100°C X 45 min
f. mix 90:10 methanol (MeOH):sodium hydroxide (NaOH) (need 2 ml/tube)
g. cool tubes to RT
h. add 2 ml 90:10 MeOH:NaOH to each tube
i. vortex 30 sec X 2
j. place in oven at 100°C X 45 min
k. cool to RT
l. add 350 µl 6N HCl (re-acidify) to each tube
m. add 2 ml hexane to each tube
n. vortex 30 sec X 2
o. spin @ 2100 rpm X 10 min
p. transfer top (hexane) layer to clean conical 13X100 mm tube
q. dry under N
r. mix 90:10 MeCN:pentaflorabenzene (PFB) (need 50 µl per tube)
s. add 10 µl triethylamine + 50 µl 90:10 MeCN:PFB to each tube
t. vortex 30 sec X 2
u. let stand @ RT X at least 30 min
v. add 150 µl 0.1 N HCl + 1 ml hexane to each tube
w. vortex 30 sec. X 2
x. spin @ 600 rpm X 10 min
y. transfer top (hexane) layer to clean 13X100 round bottom tube
z. dry under N
aa. add 1 ml hexane, vortex and collect
bb. fill GC vial insert with 250 µl; save the remaining
cc. load GC vials on the GC/MS and run PFB ester program overnight
dd. calculate absolute concentration of individual fatty acids

1. fatty acid concentrations are reported as nanomoles/25 µL of plasma; to convert to µmol/L, multiply to reported concentration by 1,000 and then divide by 25

APPENDIX F

CONVENIENT TIMES SCHEDULE FORM

INSTRUCTIONS FOR ADMINISTERING THE FOOD INTERVIEW CONVENIENT TIMES SCHEDULE FORM

Overview: This form is used to find out from the parent/caregiver of a subject when he/she will be available to participate in the diet recall interviews with a study investigator.

Component of form to be completed by parent/ primary caregiver: This section of the form has 2 pages:

Page 1 - Food Interview Instruction Sheet,
Page 2 - Food Interview Convenient Times Schedule for the Incidence of Essential Fatty Acid Deficiency in Children with Phenylketonuria (PKU) (to be completed at the Metabolic Clinic/Day Surgery Clinic visit by parent or primary caregiver)

Administration Instructions to be used by study investigator with the parent or primary caregiver:

Administer this form to the parent or primary caregiver at the time of consent.

Ask parent or primary caregiver of the subject to indicate on the Food Interview Convenient Times Schedule the times they would be available to participate in a 30-minute telephone interview. Encourage the parent/caregiver to indicate at least one time slot in each day of the week.

Warn the parent or primary caregiver that if they indicate that they would like to be called at work or on their cellular phone, that they could be interrupted for a 30-minute interview. Investigators should try to encourage people to find times that they can take these calls at home.

If there are any particular dates when a participant is not available (for example, they are available on Mondays in general, but not on Monday the 27th), please add this information to the Notes section.

If the parent or primary caregiver will be out of town during the entire calling period, try to get a number and times when the parent or primary caregiver can be reached.

Review Instructions
Make sure the parent or primary caregiver has chosen at least one time slot per day, and that the form has been marked with "home," "work," or "cell" in the appropriate boxes. Check marks are not acceptable, even if parent or primary caregiver has only listed their home phone number.

FOOD INTERVIEW INSTRUCTION SHEET

You will be receiving 1 phone call each week during weeks two through four following your child's surgery or clinic visit so that we can ask you about the foods your child eats.

During each phone call an interviewer from Oregon Health & Science University will call to ask you what foods and beverages your child ate and drank on the previous day. These calls are done on randomly selected days and are unannounced, so we are not able to tell you what specific day we will be calling, but the interview should only take about 30 minutes to complete.

Each food interview will have 4 parts:

- First, the interviewer will ask for the **time, type of meal, place**, and a **brief list of foods** that your child ate throughout the day.
- Then the interviewer will review the list of foods with you.
- Next, you will be asked for more detail about the **ingredients, preparation**, and **amounts** of each food.
- Then, the **interviewer will repeat** what you have reported to make sure everything is correct. You can add or change the information during the interview.

You don't need to remember all of this. We just wanted to give you an idea of what to expect.

We want to thank you for participating in this part of the project. The information you provide is confidential and is a very important part of the study.

FOOD INTERVIEW CONVENIENT TIMES SCHEDULE

Please complete the following information:

Parent/Caregiver Name:

_____

Home phone: (_____) _____ - _____
Work phone: (_____) _____ - _____

Cellular phone: (_____) _____ - _____

In the table below, please identify each of the times that you will be available for a 20-30 minute interview. For the times when you are available, please write "home," "work," or "cell" in the box to indicate how we can reach you at that time.

Please check at least one time per day. We will try to call during those times.

	Morning			Afternoon				Evening		
DAY	8-9	9-10	10-11	12-1	1-2	2-3	3-4	5-6	6-7	7-8
Monday										
Tuesday										
Wednesday										
Thursday										
Friday										
Saturday										
Sunday										

Notes:

_____
_____
_____
_____
_____

APPENDIX G

24-HOUR DIET RECALL INTERVIEW SCRIPT

OVERVIEW OF THE 24-HOUR DIETARY RECALL INTERVIEW

The purpose of the parent/caregiver telephone interview is to collect a record of the type and amount of foods and beverages consumed by the subject during the previous 24-hour period. To promote consistency in data collection, the phone interview should be conducted with the parent or primary caregiver who assumes most of the responsibility of preparing and serving the study participant's food. The Interviewer (i.e. designated study investigator) is trained to use dietary data collection software to record food intake information. The software includes a food database of over 18,000 foods, including many ethnic foods and over 8,000 brand-name foods. It features built-in, standard interview prompts for obtaining detailed food descriptions, including preparation method and ingredient information.

The interviewer will call the parent/caregiver to conduct a 24–hour dietary recall interview. A standardized multiple-pass approach is used by the interviewer to administer the 24-hour dietary recalls. The interviews are unannounced but take place during late morning, afternoon, and early evening hours on weekday and weekend days as indicated on the "Food Interview Convenient Times Schedule" form completed by the parent or primary caregiver of the participant. Each interview should take no more than 30 minutes to complete.

GENERAL GUIDELINES OF THE 24-HOUR DIETARY RECALL INTERVIEW

Creating Rapport

Because the parent or primary caregiver of the participant is the primary respondent for the 24-hour dietary recall, it is important for the interviewer to be able to motivate him/her to provide complete and accurate information. The interviewer will always remain neutral and not let anything in words or manner express criticism, surprise, approval, or disapproval related to the respondent or his/her responses during the dietary interview. The interviewer will adapt their style and approach to make the respondent comfortable.

Every effort will be made to keep 24-hour dietary recall collection as objective and non-judgmental as possible. The interviewer will avoid congratulating the respondent for providing certain foods or reacting with dismay to reports of other foods. The interviewer can stress that he/she wants to know what the participant really ate and that honesty is appreciated. The interviewer must maintain a demeanor of neutrality to all respondents. The interviewer should look for both verbal and non-verbal responses, be a good listener, and thank the respondent for the information provided.

Confidentiality

The interviewer will gain trust by assuring the respondent before the recall begins that everything the respondent says is all right. Any necessary discussion between the interviewer and study investigators about a specific 24-hour dietary

recall will be conducted in private and not in the presence of others. Furthermore, the participant's personal information (such as contact information) will be kept in a secure location.

Using a script

A standard script is used to introduce the 24-hour dietary recall interview and start the recall process. The script provides continuity between interviews. The remainder of the dietary recall will be guided by interview prompts and is ad hoc in nature due to the variability of respondent comments. The program displays on-line prompts to help the interviewer ask standardized questions.

Probing is the technique used by the interviewer to stimulate discussion and obtain more information. The Interviewer probes when a respondent's response is not meaningful or is incomplete (i.e., when it does not adequately answer the question). Probing can be used to gather information about additional meals and snacks as well as additions to reported foods. The quality of the interview depends a great deal on the interviewer's ability to probe meaningfully and successfully.

CONDUCTING THE INTERVIEW USING THE MULTIPLE-PASS APPROACH

PASS 1: Using the Quick List

The Quick List will be used to collect an outline of the previous day's intake. It is designed to get the respondent to begin to think about what the participant ate and when they ate. Foods and beverages, as reported by the respondent, are entered on the Quick List screen along with time eaten and meal name, if provided by the respondent at this time. Quick List is the first pass in the multiple-pass approach. If the respondent does not volunteer the time of the meal or give a specific meal name during the Quick List, the interviewer need not interrupt to ask for this information. The program will prompt for this information during the third pass.

PASS 2: Reviewing the Quick List

A review of the Quick List permits the interviewer to obtain an overview of the day's intake to note if there are large gaps in time, missed meals or missing beverages, and to insert the meals and or foods recalled during this pass. This review is the second pass of the multiple-pass approach.

PASS 3: Collecting Complete Meal, Food, and Amount Detail

To help the respondent remember what the participant ate, information about the time, name, and location of the meal are provided in the Meal Information Window. Complete descriptive detail for foods and beverages is obtained during the third pass of the multiple-pass approach. The respondent is asked about additions to foods and beverages entered on the Quick List. The interviewer asks probing questions based on the information displayed in the

window and utilizes the food search feature to obtain complete detail for food descriptions, preparation methods, and variable ingredients.

(a) After specifying the food, an open-ended question "How much did your child eat (drink)?" is asked to obtain the amount eaten.

(b) After entering the amount specified by the respondent, the program displays a conversion to a common unit. The interviewer needs to be able to visualize the amount reported and subsequently confirm with the respondent any questionable amounts (e.g., 1 fl. oz. of juice or 4 cups of ice cream). The note field will be used to enter information to confirm atypical amounts as well as other unusual information (e.g., no beverage with a meal or no condiments and/or bread for sandwiches).

PASS 4: Reviewing the recall

(c) The fourth and final pass of the multiple-pass approach occurs after entering all of the food detail. During this review, the interviewer probes for missed meals, beverages, and snacks and any other information that was earlier omitted. Edits are made as needed and notes are provided.

(d) Foods not found in the database are flagged as missing and complete detail is collected from the respondent in a corresponding note field including descriptions of what the food looks like as well as ingredients and the amount eaten.

(e) After the final review, the respondent may ask the participant to provide additional information about the foods and beverages that the respondent was unable to provide during the recall. This is used mainly to confirm amounts identified as very small or very large, large gaps in eating occasions, and missed meals.

THE SCRIPTED 24 HOUR DIETARY RECALL INTERVIEW

The interviewer begins by introducing himself or herself to the identified respondent. The interviewer should be friendly and relaxed. The interviewer should always give neutral responses to whatever the participant tells them.

- The interviewer will say:

"Hi (insert respondent's name). My name is (insert your name). How are you today?"

  - Pause, wait for their response, spend a minute or so to establish rapport, and proceed:

"I'm talking to you today as part of the Incidence of Essential Fatty Acid Deficiency in Children with Phenylketonuria (PKU) study to learn about what your child ate and drank yesterday. I'll enter the information on a computer to get the information that we need. This interview is easy because it's just about what your child ate. There are no right or wrong answers. Whatever he/she ate is okay. Do you have any questions for me? Are you ready? I'm sure you'll do a great job of helping me!"

Entering the Quick List

- The interviewer proceeds by asking the respondent to make a list of all the foods and beverages the child ate or drank yesterday. Say:

"First, we'll make a list in the computer of what your child ate yesterday starting with when he/she got up. Then I will ask you some more questions and we'll figure out how much he/she had to eat. Do you have any questions?"

  - Pause, wait for and respond to questions, and proceed:

"What was the first time (enter child's name) had something to eat or drink?"

  - Enter the response then as needed say:

"What did he/she have at that time?"

  - The interviewer enters the information reported by the respondent on the Quick List screen, not requiring the respondent to give time, meal name, or meal location. The interviewer will use a slash to mark each eating occasion and will prompt later for the time and

meal name. Above all, the interviewer should let the respondent think and say what ever comes to mind about the previous day's intake, avoiding interruptions that may be distracting to the participant.

Reviewing the Quick List

- The interviewer verifies all of the entries on the Quick List and probes for missed items by reading the list back to the respondent and asking:

"I am going to read back what you have told me. Let me know if you want to add or change anything. Can you think of anything else (enter child's name) ate or drank yesterday that we haven't put on the list? Do you know if he/she got up during the night (after midnight) and had anything to eat or drink? Did he/she have any snacks after school or before bed?"

  - Any errors should be corrected, and any additional foods the respondent may report are added at this time.

Collecting Meal Information Detail

- The interviewer begins by saying:

"Next we'll go over our list and I will ask you some questions about each food."

  - The program will bring up the Meal Information window. The interviewer will use this opportunity to ask questions about meal time, meal name, and meal location if this information was not provided earlier during the Quick List.

Asking About Additions

- The interviewer will ask about additions to every food. An on-line prompt will remind you to say:

"The first thing on your list is (inserts the name of each food)."

  - Then, reading from the screen the interviewer will say:

"Did you or your child add anything to the (inserts the name of the food)?"

  - Ask the additions question until you receive a "no" response.

Collecting Complete Food and Amount Detail

- The Food Search window prompts the interviewer for each available level of detail during this third pass. An on-line prompt will remind you to begin by saying:

"What type of (insert name of food) was it?"

- The interviewer continues to define the food, selecting food variables as required on each screen. Unknown should be entered if the respondent cannot describe food in detail (e.g., if it was prepared at a restaurant). An on-line prompt for the amount will remind you to say:

"How much did (enter child's name) eat (drink)?"

- Some foods require additional quantity details, with required fields indicated in yellow. After entering the amount provided by the respondent, the program displays a conversion to a common unit. At this time, the interviewer must be able to visualize the amount reported and confirm as needed any questionable amounts by making reference to other familiar items or recognizable standards. For example, 1/16 of a hamburger should have a note saying, "ate only one bite" or 8 cups of popcorn should have a note saying, "ate entire box at the movies."
- The interviewer should ask if the complete amount described was eaten:

"Was (insert child's name) able to finish that, or the (insert name of food)?"

  - Note: Foods that do not have complete descriptive and/or complete amount information are indicated with a blue question mark to the left of the food. When the interviewer has completely described a food, the program replaces the question mark with a green check mark to the left of each completed item.
  - As the interviewer conducts the 24-hour dietary recall, he/she will provide positive reinforcement by stating:

"You are doing a good job, working hard, a big help," *as appropriate.*

The interviewer should maintain a pleasant tone of voice and avoid responding to the respondent in any negative ways. If it is necessary to ask the respondent to repeat what he/she said, the interviewer should ask him/her to do so in a gentle way and take ownership by saying:

"Sometimes it's hard for me to hear things. Could you please tell me that again?"

Reviewing the Recall

- During the fourth and final pass of the multiple-pass approach, the interviewer will probe for missed meals, beverages, and snacks, making sure no information was inadvertently omitted. The interviewer will try to get a mental picture of the day, looking especially for time gaps of more than four hours between eating. The interviewer should look at the most likely snack times for the participant, for example, after school or work, before bed, etc. Notes should be made to indicate skipped meals or not consuming a beverage or condiments with food. During the review, the interviewer reads back each food and amount, asking for confirmation from the respondent. For example:

"Now we'll go over what I've put in the computer one last time. The first thing that I have is at (insert meal name and time) when (insert child's name) had (insert food name)."

  - When the interviewer notices a large time gap he/she should asks:

"Did he/she have anything to eat or drink after school? Anything before your (insert time e.g., evening meal) and (before bed)?"

  - Additional foods and meals are inserted at any time. If the respondent hesitates and can't remember whether any food was eaten for a long period of time, the interviewer may say:

"Can you think what (enter child's name) was doing (after school, at dinner/supper time, etc.)? Sometimes if we think about where he/she was or whom he/she was with, it helps to remember what was eaten."

  - The process continues until each food has been reviewed.

Completing the Trailer Tab

- When complete, the system presents the Trailer tab and interviewer ends the recall saying:

"Next (insert name of parent/caregiver), in terms of the amount of food (enter child's name) ate, would you say this was close to the amount that he/she usually eats, a lot more than he/she usually eats, or a lot less than he/she usually eats?"

  - This question refers to the overall amount of food for the day, not the type of food. The interviewer records the respondents response to the last question on the Trailer tab. If the respondent reports a lot more, check "considerably more than usual" or if a lot less than usual, check "considerably less than usual." In either case, the

program requires the interviewer to provide a note that briefly states why the intake was not usual. For example, a celebration meal with lots of food or participant not feeling well and not eating much can result in eating a lot more or a lot less than usual. If needed the interviewer can say:

"What makes you say it's (a lot more or a lot less than usual)?"

- The interviewer will determine the reliability of the data. If the dietary recall is unreliable because the respondent was unable to recall one or more meals or for some other reason question the reliability, he/she will click the appropriate button and add the required note. The interviewer does not ask the participant this question, nor share their opinion with them.

Thank the Participant

- The interviewer thanks the participant and ends the recall:

"Thanks so much for your help. Do you have any questions?"

- Pause, wait for and response to questions, and proceed:

"You did a great job and I really enjoyed talking with you."

"Thanks. Bye."

APPENDIX H

FOOD PRODUCT FATTY ACID COMPOSITION FORM

Food Product Fatty Acid Composition Form

Product:	Serving:	Energy:	Total Fat:	Saturated Fat:	Monounsaturated Fat:	Polyunsaturated Fat:
Ingredients:	*Per 100g*	*Energy:*	*Total Fat:*	*Saturated Fat:*	*Monounsaturated Fat:*	*Polyunsaturated Fat:*
Product:	Serving:	Energy:	Total Fat:	Saturated Fat:	Monounsaturated Fat:	Polyunsaturated Fat:
Ingredients:	*Per 100g*	*Energy:*	*Total Fat:*	*Saturated Fat:*	*Monounsaturated Fat:*	*Polyunsaturated Fat:*
Product:	Serving:	Energy:	Total Fat:	Saturated Fat:	Monounsaturated Fat:	Polyunsaturated Fat:
Ingredients:	*Per 100g*	*Energy:*	*Total Fat:*	*Saturated Fat:*	*Monounsaturated Fat:*	*Polyunsaturated Fat:*