Impact of Dietary Intake on Bone Turnover in Patients with Phenylalanine Hydroxylase Deficiency

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Received: 05 November 2016 / Revised: 12 December 2016 / Accepted: 14 December 2016
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Abstract Phenylalanine hydroxylase (PAH) deficiency is a genetic disorder characterized by deficiency of the PAH enzyme. Patients follow a phenylalanine-restricted diet low in intact protein, and must consume synthetic medical food (MF) to supply phenylalanine-free protein. We assessed relationships between dietary intake and nutrient source (food or MF) on bone mineral density (BMD) and bone turnover markers (BTM) in PAH deficiency. Blood from 44 fasted females 11–52 years of age was analyzed for plasma phenylalanine, serum BTM [CTx (resorption), P1NP (formation)], vitamin D, and parathyroid hormone (PTH). BTM ratios were calculated to assess resorption

relative to formation (CTx/P1NP). Dual energy X-ray absorptiometry measured total BMD and age-matched Z-scores. Three-day food records were analyzed for total nutrient intake, nutrients by source (food, MF), and compliance with MF prescription. Spearman's partial coefficients (adjusted for age, BMI, energy intake, blood phenylalanine) assessed correlations. All had normal BMD for age (Z-score >−2). Sixty-four percent had high resorption and normal formation indicating uncoupled bone turnover. CTx/P1NP was positively associated with food phenylalanine ($r^2 = 0.39$; p -value = 0.017), energy ($r^2 = 0.41$; p -value = 0.011) and zinc ($r^2 = 0.41$; p -value = 0.014). CTx/P1NP was negatively associated with MF fat ($r^2 = -0.44$; p -value = 0.008), MF compliance ($r^2 = -0.34$; p -value = 0.056), and positively with food sodium ($r^2 = 0.43$; p -value = 0.014). CTx/P1NP decreased significantly with age (p -value = 0.002) and higher PTH (p -value = 0.0002). Phenylalanine was not correlated with any bone indicator. Females with PAH deficiency had normal BMD but elevated BTM, particularly resorption. More favorable ratios were associated with nutrients from MF and compliance. Younger females had less favorable BTM ratios. Promoting micronutrient intake through compliance with MF may impact bone metabolism in patients with PAH deficiency.

Synopsis: Bone mineral density was normal in 44 females with PAH deficiency; however, bone turnover markers suggested uncoupling of bone resorption and formation, particularly in younger patients. Adequate nutrient intake from medical food and overall medical food compliance may positively impact bone turnover.

Communicated by: Ertan Mayatepek, MD

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Introduction

Phenylketonuria, also known as phenylalanine hydroxylase (PAH) deficiency (OMIM #261600), is an autosomal recessive inborn error of metabolism (IEM) diagnosed in infants through newborn screening (NBS) (Williams et al. 2008). Patients with PAH deficiency lack sufficient activity of the PAH enzyme (OMIM 612349) due to mutations in the PAH gene. Under normal physiologic circumstances, PAH metabolizes the amino acid phenylalanine to tyrosine. In patients with PAH deficiency, blood phenylalanine can increase to high concentrations and cross the blood-brain barrier, affecting cognitive development in addition to other sequelae (Blau et al. 2010). In addition, deficiency of tyrosine and products of tyrosine metabolism can occur in patients with PAH deficiency.

To prevent the severe effects of high blood phenylalanine and low tyrosine in patients with PAH deficiency, dietary treatment should be implemented immediately. Patients follow a phenylalanine-restricted diet to limit natural (intact) protein. Since protein is required for normal growth and development, patients must consume a synthetic medical formula (medical food) which contains all amino acids in elemental form except phenylalanine. Medical food can supply up to 85–90% of total protein intake in patients with classical PAH deficiency (Camp et al. 2012). Medical food is prescribed to meet 120–140% of patients' total protein needs defined by the Recommended Dietary Allowance (RDA) for age and sex (IOM 2005). Complete medical food also contains other nutrients (carbohydrates, fat, calcium, vitamin D, B12, etc.) that are difficult to consume in adequate amounts with dietary protein restriction (Lammardo et al. 2013). Previous studies have shown that the PAH deficiency diet generally meets and even exceeds requirements for most nutrients (Evans et al. 2014), but there are no reports of nutrient intake by source: food versus medical food. Phenylalanine restriction and medical food are recommended for life in all patients with PAH deficiency (Singh et al. 2014).

Early and continuous dietary treatment of patients with PAH deficiency results in near normal cognitive outcomes (Burgard 2000; Enns et al. 2010). A recent systematic review, however, showed suboptimal outcomes in 140 of 150 studies including at least one primary indicator of health in early treated patients, including low bone mineral density (BMD) (Enns et al. 2010). Since the first report in 1962 of abnormal bone structure in neonates and infants with phenylketonuria, low BMD has been described in male and female patients of all age groups (Feinberg and Fisch 1962). Though inconsistent criteria have been used to define low BMD, osteopenia, and osteoporosis, low BMD may be present in nearly 50% of individuals with PAH deficiency (Modan-Moses et al. 2007). The most recent meta-analysis, however, suggests a much lower prevalence of low BMD of 10% (Demirdas et al.

2015). Although the cause and extent of low BMD is uncertain, low BMD is the precursor to osteoporosis and an increased risk of fractures (Kanis 2002).

Bone mineral density is a good indicator of bone status, but bone turnover markers (BTM) are valuable indicators of dynamic bone remodeling (Lee and Vasikaran 2012). The by-products of bone turnover may provide insight into acute bone metabolism (Wheater et al. 2013) including resorption by osteoclasts and formation by osteoblasts (Naylor and Eastell 2012; Burch et al. 2014). Gold-standard markers of bone resorption (CTx) and formation (P1NP) (Lee and Vasikaran 2012) have not been measured in patients with PAH deficiency, or with factors such as dietary intake, compliance with dietary treatment (phenylalanine restriction and medical food), and blood phenylalanine concentrations. The primary aim of this study was to assess the impact of dietary intake and nutrient source (food versus medical food) on BMD and gold-standard BTM (Lee and Vasikaran 2012) in patients with PAH deficiency. Secondary aims were to assess differences across age categories and correlations among bone biomarkers.

Subjects and Methods

Subjects

Females 11 years of age and older attending a research-based camp for girls and women with PAH deficiency from 2013 to 2015 were eligible for this study. Subjects also had the option to attend camp but not participate in the study. Exclusion criteria included those not participating in camp research, those with a diagnosis of maple syrup urine disease (MSUD), those who were pregnant, and those who the PI did not feel comfortable enrolling (i.e., could not understand the study, abnormalities in pre-camp physical). The study was approved by the Emory University Institutional Review Board (IRB) and data were collected in compliance with Health Insurance Portability and Accountability (HIPAA) guidelines. Phone consents (adults) and assents (<18 years) were performed with a study coordinator to explain all components of the study, including blood collection to dual energy X-ray absorptiometry (DXA) scans. Subjects also had time to ask questions about the study.

Study Design

All subjects attended study visits on the first day of camp at either the Clinical Research Network (>18 years of age) at Emory University Hospital or Children's Healthcare of Atlanta – Egleston Pediatric Research Center (<18 years). Seasonality was consistent for all participants since all visits occurred during the summer in Atlanta, Georgia. All subjects

were fasting for at least two and a half hours before study visits. A history and physical was performed and research nursing staff assessed anthropometrics, vitals and collected urine and blood samples. Urine pregnancy tests were completed and documented as negative prior to performing DXA scans. A trained phlebotomist drew venous blood for all blood samples and a research laboratory technician performed preliminary processing and aliquoting of the samples.

Dietary Intake

Three-day diet records were submitted by all subjects and reviewed individually with a metabolic Registered Dietitian (RD) to ensure completeness and accuracy. If subjects did not bring a completed 3-day food record to camp, the RD performed a 24-h recall. All food records and recalls were analyzed by a single RD using Nutrition Data System for Research (NDSR) (NCC 2011). All foods and medical foods that were missing from the database were requested through the NDSR administrators at the University of Minnesota, Nutrition Coordinating Center. The 3 days of dietary intake were averaged to calculate median daily intake of each nutrient. Median nutrient intake from medical food (not including low-protein foods) over the 3-day record was also calculated. Median nutrient intake from medical food was subtracted from median total nutrient intake to calculate median nutrient intake from food sources.

The following nutrients were investigated based on known or proposed relationship with bone health: energy, fat, carbohydrates, protein, vitamin A, vitamin D, vitamin B12, calcium, phosphorus, magnesium, iron, zinc, selenium, sodium, and cholesterol. Nutrient intakes were compared to age- and gender-specific Dietary Reference Intakes (DRI) (Institute of Medicine (U.S.) Subcommittee on Interpretation and Uses of Dietary Reference Intakes et al. 2000) to assess adequacy of reported dietary intake. For nutrients with an established RDA, total nutrient intake was compared to RDA as percent RDA. For nutrients without an established RDA, nutrient intake was compared to the Adequate Intake (AI) as percent AI. For each nutrient, the percent of RDA met from food sources and the percent of RDA met from medical food was also calculated.

DXA Scans

All study subjects had a dual energy X-ray absorptiometry (DXA) scan at the Winship Cancer Institute by the same technician on a GE Lunar Prodigy machine. DXA measured bone mineral content (BMC, g), bone mineral density (BMD, g/cm²), and body composition [fat mass (g), % fat mass, lean mass (g), % lean mass]. BMD Z-scores were

calculated by DXA software to compare BMD to the average for a healthy person of the same age and sex. International Society for Clinical Densitometry (ISCD) standards recommend using Z-scores to assess BMD in pediatric subjects, pre-menopausal women, and men under 50 years of age (ISCD 2013a, b). Using ISCD recommendations, subjects were categorized as having normal BMD (Z-score > -2) or low BMD (Z-score < -2) (ISCD 2013a, b).

Laboratory Samples

Plasma amino acids were analyzed at Emory Genetics Laboratory via ion exchange chromatography. Aliquots of serum were sent on dry ice to Maine Medical Center Research Institute to measure bone turnover markers [bone-specific alkaline phosphatase (BALP), C-terminal telopeptide of type 1 collagen (CTx), pro-peptide of type 1 collagen (P1NP), vitamin D (25-hydroxyvitamin D) and intact parathyroid hormone (PTH)] using Immunodiagnostic System's (IDS) multi-discipline automated system (IDS-iSYS). The system, one of only two validated methods to accurately measure CTx and P1NP, uses a single sample of serum to measure all five markers using chemiluminescence techniques (Cavalier et al. 2012a, b; Morovat et al. 2013). CTx and P1NP were selected as the two best markers of resorption and formation, respectively, based on a literature review by the International Osteoporosis Foundation and the International Federation for Clinical Chemistry in 2013 (Johansson et al. 2014). CTx and P1NP have diurnal variation with concentrations peaking in the morning, thus all blood samples were collected between 8:30 a.m. and 12:00 p.m. to minimize variation (Qvist et al. 2002).

Other Variables

Subjects also completed a bone health questionnaire to assess previous fractures, medications, concurrent conditions that could affect bone, vitamin D and multivitamin supplement use, and pubertal status [pre-menstrual, menstrual (regular or irregular), or post-menopausal]. All participants also completed surveys on demographics, medical information, current dietary and medical food prescriptions, and health insurance.

Statistical Analysis

All statistics were performed using Statistical Analysis Software (SAS) version 9.4 (SAS 2013). Since the study was a cross-sectional pilot analysis, a power calculation was not performed. The primary dependent variables included: CTx, P1NP, BMD and BMD Z-score and the

primary independent variables included: median nutrient intake by source (total, food, and medical food) and plasma phenylalanine. A BTM ratio (CTx/PINP) was calculated to assess the relative degree of bone resorption to formation (Fardellone et al. 2014) with a higher ratio indicating higher resorption compared to formation. Normality of each variable was assessed using the Shapiro-Wilk test. If outliers in any primary dependent variables were identified during exploratory analyses, statistical tests were performed including and excluding the outlier and compared. If results were similar, outliers were retained in final analyses.

Descriptive statistics including prevalence and 95% confidence intervals (CI) were calculated for the following categories: age based on DRI-defined groups [11–13, 14–18, >19 years (Ryan-Harshman and Aldoori 2006)], BMI [underweight <18.5, normal weight 18.5–24.9, overweight 25–29.9, and obese >30 (kg/m²)], menstrual status (pre-menstrual, regular menstrual cycle, and irregular menstrual cycle), pharmaceutical use [no medications, treatment with Kuvan, treatment with pegylated ammonia-lyase (PEG-PAL)], reported fracture or break (yes or no), and BMD Z-score category [normal (>−2) or low (<−2)]. Continuous variables including dietary intake by source and biomarkers (plasma phenylalanine, vitamin D, PTH, BALP, CTx, and PINP) were described by mean and standard deviation (or median and interquartile range for non-normally distributed variables). Biomarkers were also compared to clinical reference ranges for females by age group and determined to be low, normal, or high. For biomarkers found to be higher or lower than the reference range, mean difference and mean percent difference from the reference range were calculated using the upper or lower limit as appropriate.

Paired t-tests (or Wilcoxon signed rank tests for non-parametric data) were performed to determine if nutrient intake from food differed from nutrient intake from medical food for each nutrient. Nutrients with greater than 50% of daily intake from medical food were noted. Pearson's (or Spearman's for non-parametric data) partial correlation coefficients were calculated to examine associations between nutrient intake by source and BTM and BMD, adjusting for age, BMI, total energy intake, and plasma phenylalanine. Pearson's or Spearman's partial correlation coefficients were also calculated to examine associations between medical food prescription and medical food compliance (% of prescription consumed) and dietary phenylalanine prescription and dietary phenylalanine compliance (% of prescription consumed), and BTM and BMD. A correlation matrix was also constructed to examine relationships between all bone-related parameters included in this study (CTx, PINP, CTx/PINP ratio, BALP, PTH, vitamin D, BMD, and BMD Z-score), adjusting for age and BMI.

To compare independent variables (CTx, PINP, CTx/PINP, BMD, BMD Z-score) by age category, each non-normal variable was first log-transformed. Linear regression was then used to control for BMI and blood phenylalanine. Tukey's adjustment for multiple comparisons was applied (criterion *p*-value <0.01) to calculate significant differences by age category.

Results

Study Subjects

Over 3 years of Metabolic Camp, 97 females were eligible for the study. The following participants were excluded: 9 (9%) had MSUD; 20 (21%) did not choose to participate in research, and 24 (25%) had already enrolled in the study in 2013 or 2014. Forty-four females with PAH deficiency were included in the final study, ranging from 11 to 54 years of age (Table 1). Fifteen (34%) were overweight or obese. Ninety-three percent had reached menarche, though 46% of these women reported an irregular cycle. The majority of subjects (*n* = 34, 77%) had phenylalanine concentrations above the therapeutic range, defined as 120–360 μmol/L (2–6 mg%) (Singh et al. 2014), even though 16 (36%) were taking phenylalanine-lowering pharmaceuticals. Nearly half (*n* = 20) reported at least one previous fracture.

All subjects had normal BMD for age (Z-score >−2). Nine (20%) had a BMD Z-score between −1 and −2. CTx, marker of bone resorption, was above normal (Cavalier et al. 2012a, b) in 81% (*n* = 34) of the sample while PINP, marker of bone formation, was normal in 83% (*n* = 35) (Cavalier et al. 2012a, b). Normal reference ranges have not been established for CTx or PINP by assay manufacturers, thus comparisons were made to two studies calculating mean CTx and PINP by age groups and 95% confidence intervals to designate “high” (above the upper limit), “normal” (within 95% CI) and “low” (below the lower limit). On average, CTx was 0.79 ng/mL (342%) above the upper limit of the 95% confidence interval. The majority of the sample (*n* = 27; 64%) had high CTx and normal PINP indicating a possible uncoupling of normally coupled bone turnover.

Dietary Intake

Table 2 shows dietary intake of individual nutrients including source (food or medical food). Total intakes of protein, vitamin D, calcium, and zinc were predominantly from medical food (>50% of total intake). Patients reported a median medical food prescription of 45.0 g of protein

Table 1 Characteristics of females with PAH deficiency ($n = 44$)

	Mean (SD)	<i>N</i>	Percent	95% CI
Demographics				
Age (years)	20.1 (10.0)			
11–13		7	15.9	4.7, 27.2
14–18		25	56.8	41.6, 72.1
>19		12	27.3	13.6, 41.0
BMI (kg/m ²)	25.5 (8.9)			
Underweight (<18.5)		2	4.5	0, 11.0
Normal (18.5–24.9)		27	61.4	46.4, 76.3
Overweight (25–29.9)		8	18.2	6.3, 30.0
Obese (>30)		7	15.9	4.7, 27.2
BMD Z-score Category	0.045 (0.141)			
Normal (>−1)		35	79.5	67.1, 92.0
Between −1 and −2		9	20.5	8.0, 32.9
Low for chronological age (<−2)		0	0	–
Laboratory measures				
Plasma phenylalanine (μmol/L)	777.3 (424.7)			
Below treatment range (<120)		2	4.5	0, 11.0
Normal (120–360)		8	18.2	6.3, 30.0
Above treatment range (>360)		34	77.3	64.4, 90.2
Serum 25-hydroxyvitamin D (ng/mL)	37.6 (14.3)			
Normal (>30)		28	66.7	51.8, 81.5
Insufficient (20–30)		12	28.6	14.3, 42.8
Deficient (<20)		2	4.8	0.0, 11.5
Carboxyterminal telopeptide of type I collagen (CTx) (ng/mL) ^a	0.947 (0.68)			
Low for age		0	0	–
Normal for age		8	19.0	6.7, 31.4
High for age		34	81.0	68.6, 93.3
Aminoterminal propeptide of type I collagen (P1NP) (ng/mL) ^b	171.5 (200.5)			
Low for age		1	2.4	0, 7.2
Normal for age		35	83.3	71.6, 95.1
High for age		6	14.3	3.2, 25.3

^a CTx indicates active bone resorption; 95% confidence intervals of mean CTx concentrations (ng/mL) in females (Cavalier et al. 2012a, b): 10–<12 years (0.388–1.094), 12–<14 years (0.082–0.650), 14–<16 years (0.538–0.300), 16–<18 years (0.0251–0.148), 18–<20 years (0.0241–0.118), premenopausal women (0.03–0.66)

^b P1NP indicate active bone formation; 95% confidence intervals of median P1NP concentrations (ng/mL) in females (Morovat et al. 2013): 9–12 years (386–1,070), 13–16 years (59.3–672), 17–20 years (25.2–160), premenopausal women (13.7–71.1)

(IQR 28.4–56.6) per day and a median phenylalanine allotment of 450 mg per day (IQR 75–825). Median intake of most nutrients exceeded the RDA/AI by an average of 147% (excluding cholesterol intake). Only median vitamin D and cholesterol intake fell below the RDA/AI (75% of RDA and 5% of AI, respectively). Thirty-six percent ($n = 16$) of subjects reported taking a multivitamin and/or vitamin D supplement, in addition to their prescribed medical food.

Differences in source of nutrients were compared between those under age 18 and adults. A higher percent of vitamin D came from medical food in the younger age group compared to the older age group (76% versus 52%; p -value = 0.04); all other nutrients were comparable. Though median vitamin D intake was 75% of the RDA, 80% of subjects' vitamin D intake fell below the RDA of 600 IU/day, two-thirds of whom were in the youngest age group (Ross 2011).

Table 2 Intake of individual bone-related nutrients and source from 3-day food records

	Total intake		Food intake		Medical food intake		
	Median (IQR)	% RDA/AI	Median (IQR)	% RDA/AI	Median (IQR)	% RDA/AI	<i>p</i> -value ^a
Macronutrients							
Total energy (kcal)	1,532 (531)	–	1,139 (554)	–	277 (423)	–	<0.01
Fat (g)	50.7 (35.9)	–	35.6 (24.3)	–	4.9 (19.6)	–	<0.01
Carbohydrates (g)	218.7 (70.9)	168 (55)	187.5 (60.7)	141 (41)	21.0 (44.2)	27 (34)	<0.01
Protein (g) ^b	56.8 (34.0)	126 (61)	17.6 (20.0)	54 (50)	30.4 (34.8)	92 (101)	0.13
Micronutrients							
Vitamin A (IU)	4,810 (4,350)	219 (183)	2,685 (2,597)	120 (158)	1,452 (2,513)	91 (115)	<0.01
Vitamin D (mcg) ^{b,c}	11.2 (10.4)	75 (69)	0.6 (3.4)	5 (27)	6.4 (9.8)	61 (72)	<0.01
Vitamin B12 (mcg) ^b	5.1 (5.7)	213 (238)	0.8 (2.2)	27 (49)	2.9 (6.1)	153 (254)	<0.01
Calcium (mg) ^b	1,235 (984)	102 (82)	255 (424)	24 (36)	655 (1,103)	79 (88)	<0.01
Phosphorus (mg)	1,240 (801)	110 (80)	443 (283)	43 (50)	581 (930)	77 (82)	0.16
Magnesium (mg)	391 (252)	110 (82)	134 (83)	39 (29)	164 (300)	77 (81)	0.11
Iron (mg)	20.9 (15.6)	142 (111)	6.7 (5.9)	53 (52)	8.6 (16.8)	87 (111)	0.21
Zinc (mg) ^b	16.3 (16.3)	191 (195)	3.4 (3.4)	41 (45)	8.4 (17.3)	136 (197)	<0.01
Selenium (mcg)	70.1 (51.0)	128 (114)	27.8 (35.0)	61 (89)	27.4 (43.5)	78 (84)	0.74
Sodium (mg)	2,800 (1,244)	187 (83)	1,868 (1,081)	121 (52)	425 (1,125)	43 (73)	<0.01
Cholesterol (mg) ^d	14.4 (37.6)	5 (13)	14.4 (37.7)	5 (13)	0 (0)	0 (0)	<0.01

^a Wilcoxon signed rank test, unadjusted differences between mean intake from food and medical food

^b >50% of intake from medical food

^c 5 mcg of dietary vitamin D is equivalent to 200 International Units (IU)

^d Cholesterol based on recommended intake of <300 mg/day (no defined AI/RDA) (IOM 2005)

Correlation Between Nutrient Intake and Bone Markers

Table 3 shows correlations between nutrient intake by source and bone indicators adjusted for age, BMI, plasma phenylalanine, and total energy intake. P1NP (bone formation) was correlated positively with food energy ($r^2 = 0.44$), and negatively with total magnesium intake ($r^2 = -0.43$) and medical food compliance ($r^2 = -0.53$). CTx/P1NP was positively correlated ($p < 0.05$) with energy ($r^2 = 0.41$), zinc ($r^2 = 0.41$), and phenylalanine ($r^2 = 0.39$) from food sources. CTx/P1NP was negatively correlated ($p < 0.01$) with medical food fat intake ($r^2 = -0.44$) and positively with sodium intake from food ($r^2 = 0.43$). BMD was negatively correlated with cholesterol from food ($r^2 = -0.42$), despite the low reported median. BMD Z-score was marginally positively correlated with sodium intake from food ($r^2 = 0.39$, p -value = 0.014), but no other nutrient. Dietary phenylalanine intake was not associated ($p > 0.01$) with BTM, BMD, or Z-score.

Differences Across Age Categories

As a secondary aim, we examined variables by age group given the wide range of age in the sample (Table 4). BMI significantly increased with age (p -value <0.01), by nearly

10 kg/m² from the youngest group to the oldest group. As expected, BMD was higher in the oldest group compared to the youngest group (mean = 1.150 g/cm² vs 1.008 g/cm², p -value = 0.016).

All BTM (BALP, CTx, and P1NP) decreased with age (p -value <0.0001), as expected based on the physiological decrease in bone turnover with aging (Adami et al. 2008). CTx/P1NP ratio also decreased significantly with age (p -value = 0.0014) suggesting younger patients have a higher degree of uncoupling in the direction of higher resorption, that is opposite to normal bone metabolism in adolescent females (Levine 2012). CTx decreased by 77% from the youngest to the oldest age group while P1NP decreased by 90%, indicating P1NP decreases more over time than CTx in patients with PAH deficiency. Blood phenylalanine was also significantly different over age categories with those in the middle age group (14–18 years), having higher concentrations than both their younger and older peers.

Associations Between Bone Biomarkers

As a final aim, we examined associations between biomarkers, adjusting for age, BMI, and plasma phenylalanine. CTx and P1NP had a strong negative association with age ($r^2 = -0.72$ and -0.84 , p -value <0.001) and a marginal

Table 3 Source of nutrient intake and spearman's partial^a correlation coefficients with bone turnover markers, BMD and BMD Z-score

	CTx (ng/mL)		P1NP (ng/mL)		CTx/P1NP		BMD		BMD Z-score	
	<i>r</i> ²	<i>p</i> -value	<i>r</i> ²	<i>p</i> -value	<i>r</i> ²	<i>p</i> -value	<i>r</i> ²	<i>p</i> -value	<i>r</i> ²	<i>p</i> -value
Total energy (kcal)	0.14	0.39	0.35	0.03	0.16	0.34	−0.22	0.17	−0.09	0.57
Medical food	−0.19	0.36	−0.13	0.45	−0.28	0.08	0.11	0.49	−0.05	0.75
Food	0.33	0.047	0.44	0.006	0.41	0.011	−0.39	0.015	0.01	0.94
Total fat (g)	−0.15	0.37	−0.13	0.45	−0.24	0.16	−0.05	0.76	0.24	0.15
Medical food	−0.26	0.13	−0.28	0.10	−0.44	0.008	0.24	0.14	0.04	0.81
Food	0.02	0.89	−0.04	0.80	0.09	0.60	−0.31	0.056	0.22	0.18
Total carbohydrates (g)	0.24	0.16	0.24	0.15	0.14	0.42	−0.14	0.41	0.08	0.64
Medical food	−0.26	0.12	−0.32	0.057	−0.37	0.03	0.26	0.12	−0.02	0.93
Food	0.38	0.02	0.35	0.03	0.36	0.03	−0.25	0.14	0.12	0.47
Total protein (g)	−0.14	0.42	−0.39	0.019	−0.19	0.28	0.10	0.55	−0.23	0.17
Medical food	−0.25	0.14	−0.39	0.017	−0.33	0.047	0.20	0.23	−0.06	0.70
Food	0.19	0.26	0.03	0.87	0.25	0.14	−0.15	0.36	−0.06	0.71
Vitamin A (IU)	0.03	0.84	−0.09	0.58	−0.02	0.92	0.28	0.09	−0.20	0.24
Medical food	−0.21	0.21	−0.30	0.08	−0.37	0.03	0.29	0.09	0.04	0.81
Food	0.13	0.46	0.02	0.91	0.13	0.46	0.11	0.50	−0.17	0.31
Total vitamin D (mcg)	0.07	0.69	−0.12	0.49	−0.10	0.57	0.17	0.31	0.02	0.90
Medical food	−0.10	0.56	−0.25	0.14	−0.24	0.15	0.30	0.07	0.01	0.94
Food	0.18	0.28	0.05	0.77	0.29	0.09	−0.04	0.82	0.00	0.98
Total vitamin B12 (mcg)	−0.07	0.69	−0.20	0.25	−0.27	0.12	0.25	0.14	0.11	0.52
Medical food	−0.22	0.20	−0.27	0.12	−0.37	0.02	0.28	0.09	0.06	0.73
Food	0.07	0.68	−0.06	0.71	0.11	0.52	−0.24	0.14	0.19	0.25
Calcium (mg)	−0.15	0.39	−0.34	0.04	−0.35	0.04	0.15	0.36	−0.10	0.54
Medical food	−0.22	0.21	−0.34	0.04	−0.36	0.03	0.28	0.08	0.01	0.96
Food	0.15	0.38	−0.06	0.71	0.04	0.83	−0.24	0.14	−0.10	0.53
Iron (mg)	−0.04	0.81	−0.28	0.10	−0.21	0.22	0.26	0.12	0.08	0.64
Medical food	−0.21	0.22	−0.32	0.06	−0.34	0.045	0.28	0.08	0.02	0.90
Food	0.29	0.09	0.10	0.55	0.26	0.12	−0.14	0.39	0.17	0.32
Magnesium (mg)	−0.14	0.42	−0.43	0.008	−0.27	0.10	0.21	0.20	−0.12	0.48
Medical food	−0.21	0.22	−0.36	0.03	−0.34	0.04	0.24	0.15	0.01	0.97
Food	0.21	0.22	−0.08	0.63	0.26	0.13	−0.03	0.89	−0.11	0.52
Phosphorus (mg)	−0.15	0.38	−0.35	0.03	−0.28	0.10	0.24	0.15	−0.12	0.47
Medical food	−0.22	0.20	−0.35	0.04	−0.35	0.03	0.30	0.07	0.00	0.99
Food	0.23	0.17	0.09	0.61	0.27	0.12	−0.24	0.15	0.01	0.94
Selenium (mcg)	0.05	0.78	−0.09	0.60	−0.06	0.73	0.11	0.51	0.08	0.63
Medical food	−0.19	0.26	−0.34	0.04	−0.33	0.049	0.26	0.11	0.04	0.83
Food	0.32	0.06	0.16	0.34	0.35	0.03	−0.16	0.34	0.09	0.59
Zinc (mg)	−0.05	0.78	−0.21	0.23	−0.23	0.17	0.27	0.11	0.01	0.97
Medical food	−0.23	0.18	−0.33	0.05	−0.34	0.04	0.28	0.08	0.02	0.91
Food	0.40	0.016	0.24	0.16	0.41	0.014	−0.18	0.28	0.05	0.75
Sodium (mg)	0.13	0.46	0.15	0.38	0.07	0.71	−0.03	0.86	0.32	0.048
Medical food	−0.23	0.18	−0.30	0.07	−0.38	0.02	0.26	0.11	0.04	0.80
Food	0.34	0.046	0.28	0.10	0.43	0.008	−0.33	0.04	0.39	0.014
Cholesterol (mg)	0.21	0.21	0.31	0.059	0.21	0.23	−0.41	0.011	−0.03	0.86
Medical Food	—	—	—	—	—	—	—	—	−0.00	0.98
Food	0.21	0.21	0.32	0.046	0.20	0.24	−0.42	0.008	−0.02	0.91

(continued)

Table 3 (continued)

	CTx (ng/mL)		P1NP (ng/mL)		CTx/P1NP		BMD		BMD Z-score	
	r^2	p -value	r^2	p -value	r^2	p -value	r^2	p -value	r^2	p -value
Phenylalanine (mg)	0.31	0.07	0.29	0.08	<i>0.39</i>	<i>0.02</i>	−0.20	0.22	0.02	0.91
Medical food	—	—	—	—	—	—	—	—	—	—
Food	0.31	0.06	0.30	0.08	<i>0.39</i>	<i>0.017</i>	−0.21	0.21	0.02	0.90
Medical food prescription (g)	0.02	0.91	0.10	0.56	0.22	0.21	0.13	0.48	−0.06	0.75
Medical food compliance (%)	−0.25	0.16	−0.53	0.001	−0.34	0.056	0.16	0.38	−0.10	0.60
Phe prescription (mg)	0.16	0.37	−0.05	0.77	0.06	0.72	−0.12	0.51	0.18	0.32
Phe compliance (%)	0.06	0.75	0.22	0.23	0.16	0.38	−0.18	0.32	−0.00	0.99

Correlations significant at $p < 0.05$ in *italics*; Correlations significant at $p < 0.001$ **bolded**

^a Correlation coefficients adjusted for age, BMI, total calorie intake, and blood phenylalanine

Table 4 Differences in key variables by age group in females with PAH deficiency

	Age 11–13 ($n = 7$) Mean (SD)	Age 14–18 ($n = 25$) Mean (SD)	Age >19 ($n = 12$) Mean (SD)	p -value ^d
Demographics				
Body mass index (kg/m ²)	22.8 (5.1) ^a	23.1 (5.7) ^a	32.2 (12.4) ^b	0.0054
Bone mineral density (g/cm ²)	1.008 (0.111) ^a	1.090 (0.094)	1.150 (0.096) ^b	0.0920
BMD Z-score	−0.428 (1.163)	−0.276 (1.009)	0.400 (1.065)	0.5705
Percent with fracture or break	57.1%	36.0%	58.3%	0.28
Biomarkers				
Phenylalanine (μmol/L)	470 (252) ^a	871 (415) ^c	762 (464) ^b	0.1875
25-hydroxyvitamin D (ng/mL)	28.2 (9.2)	40.3 (14.6)	36.4 (14.8)	0.1934
Parathyroid hormone (pg/mL)	29.6 (15.5)	25.7 (8.6)	32.1 (14.9)	0.7746
Bone-specific alkaline phosphatase (μg/L)	84.1 (59.4) ^b	29.4 (18.9) ^a	20.6 (7.6) ^a	<0.0001
C-terminal cross-linking telopeptide of type I collagen (ng/mL)	1.88 (0.86) ^c	0.95 (0.52) ^b	0.43 (0.23) ^a	<0.0001
Procollagen type I N propeptide (ng/mL)	546.4 (288.9) ^c	133.8 (89.2) ^b	52.7 (24.3) ^a	<0.0001
CTx/P1NP ratio	0.075 (0.036) ^b	0.042 (0.035)	0.017 (0.012) ^a	0.0014

^a significantly less than b

^a significantly less than c

^b significantly less than c

^d Mantel-Haenszel Chi-Square test with Tukey's post-hoc p -value adjustment, controlling for BMI and plasma phenylalanine

association with BMI ($r^2 = -0.31$ and -0.32 , p -value <0.05). Blood 25-hydroxyvitamin D negatively correlated with PTH concentration ($r^2 = -0.40$, p -value = 0.01) as expected, but no other biomarkers. Bone turnover markers CTx, P1NP and BALP had strong positive correlations; the strongest correlation was between CTx and P1NP ($r^2 = 0.74$, p -value <0.0001). The ratio of CTx/P1NP had a strong negative association with age ($r^2 = -0.55$, p -value = 0.002) and PTH ($r^2 = -0.59$, p -value = <0.0001), adjusting for BMI and blood phenylalanine. Blood phenylalanine and BMD Z-score had no significant correlation with any marker of bone metabolism.

Discussion

In this sample of females with PAH deficiency, dietary intake of most nutrients exceeded RDA/AIs, but nutrients related to bone health (protein, vitamin D, calcium, zinc and vitamin B12) were consumed predominantly through medical food. Nearly two in three had normal or low P1NP (formation) but high CTx (resorption) indicating an uncoupling of bone turnover. Younger patients had a less favorable turnover ratio, with higher resorption compared to formation, than their older counterparts. BTM was impacted by dietary intake, but not blood phenylalanine, and nutrients from medical food correlated

to more favorable BTM ratio than nutrients from food sources. Higher intake of food phenylalanine correlated to a BTM ratio favoring resorption (p -value = 0.017) while better compliance with medical food correlated to a BTM ratio favoring formation (p -value = 0.056) suggesting following diet is critical.

DXA showed normal BMD for age in all subjects and a mean total BMD Z-score of 0.045 (range -1.9–2.3) contradicting low BMD found in previous studies (Carson et al. 1990; Zeman et al. 1999; Perez-Duenas et al. 2002; Koura et al. 2011) but in line with more recent reports (Demirdas et al. 2015; Geiger et al. 2016). We defined low BMD by ISCD criteria (Z-score < -2) instead of alternative definitions used in previous studies (Demirdas et al. 2015). Prevalence of previous fractures, though cause was not assessed, was higher compared to population estimates for adolescent females (44% vs an estimated 19.4–33.9%) (Chevalley et al. 2012; Faje et al. 2014) and adult females (47.4% vs 25.3%) (Young et al. 2011), and much higher than the 20% fracture rate reported in a systematic review of males and females with PAH deficiency (Hansen and Ney 2014). Though BMD was normal in all subjects, uncoupled bone turnover, particularly in younger patients, and high prevalence of vitamin D insufficiency, still suggests potential abnormalities in bone health and vitamin D status in females with PAH deficiency.

We found higher bone turnover was associated with nutrients from food, and lower bone turnover was associated with nutrients from medical food and compliance. Since nutrients are likely co-correlated, we emphasize significant findings indicate an overall correlation between multiple nutrients in medical food and bone turnover. We hypothesize medical food compliance is associated with a more favorable BTM ratio because its high content of nutrients improves PINP suppression. PINP is a fragment of collagen released by osteoblastic cells as collagen is incorporated into the bone matrix, and an indicator of bone formation (Wheater et al. 2013). Increasing medical food compliance may increase PINP more than CTx, decreasing CTx/PINP ratio, promoting bone formation and the attainment of peak bone mass in adolescents or maintaining BMD in adults.

BMD and BMD Z-scores were not associated with any nutrient except for BMD's negative relationship with cholesterol intake from food. Other studies also find no correlations between dietary calcium, protein, and energy intake and BMD, BMD Z-score or BTM in patients of any age (Carson et al. 1990; McMurry et al. 1992; Hillman et al. 1996; Modan-Moses et al. 2007; Lage et al. 2010). Positive correlations have been reported, however, between total calcium and phosphorus intake and BMD Z-score (Perez-Duenas et al. 2002) and calcium and protein from medical food and spine BMD (Geiger et al. 2016). None of these investigations controlled for total energy intake (Willett et al. 1997).

The ratio of bone resorption to formation decreased with age and PTH concentration suggesting uncoupling is more pronounced in younger patients and those with lower PTH. Our findings are opposite of the expected relationship between CTx/PINP and aging. Bone formation should exceed bone resorption prior to attaining peak bone mass in late teenage years. A small sample size could limit the interpretation of this finding, but we believe the higher degree of uncoupling in younger patients warrants further investigation.

The inverse relationship between PTH and CTx/PINP ratio, even after adjustment for age, BMI, and plasma phenylalanine, is difficult to interpret. PTH was positively correlated to PINP in subjects under age 18 but not in adults. Thus, different sets of factors could influence bone turnover in adults compared to children with PAH deficiency including vitamin D concentrations. Eighty-eight percent of adolescents in this study did not meet the RDA for vitamin D and 83% of those with insufficient blood vitamin D were children or adolescents. Given the propensity for younger girls to have higher bone resorption to formation ratios and lower vitamin D concentrations than adults, this may be a particularly high-risk group for developing low BMD and even osteoporosis later in life.

Blood phenylalanine was not significantly correlated with any blood biomarker, but moderately positively correlated with CTx ($r^2 = 0.27$, p -value = 0.09). A single in vitro study found higher phenylalanine concentrations were associated with higher circulating osteoclast precursor cells (Roato et al. 2010) and that T cells, indicators of immune activation, were activating the precursor cells into mature osteoclasts (Teitelbaum 2000). Assessing immune activation with BTM could provide further insight into relationships with blood phenylalanine in future studies.

Our sample of patients with PAH deficiency included a cross-section of females only. Results are not generalizable to males with PAH deficiency, or females under 11 years of age. Subjects were attending a camp for Emory Genetics Clinic patients or were recommended by clinics familiar with Metabolic Camp and may have been more invested in managing their disorder compared to other patients. The nature of this sample may have produced better compliance rates with diet and potentially better bone health. Bias could also exist in 3-day food records as subjects may have changed their usual diet to appear more compliant or recorded false food items and false compliance with medical food (median compliance rate with medical food prescription was 99%). Only 28% of subjects reported a medical food intake of less than 50% of their prescription, but we believe a higher percent were non-compliant. Cause of previous fractures was not assessed and future studies must examine if fractures are caused by typical mechanisms including falls, collisions, and trauma (Hedstrom et al. 2010) or result from low BMD in females with PAH deficiency. Finally, a control group was not

included, thus population-specific reference values were used for comparisons when available.

In future studies, we recommend investigating BTM with other more accessible measures of bone status such as a quantitative heel ultrasound (QUS), in addition to DXA (Faulkner et al. 1994). We also recommend assessing menstrual status and hormone levels in association with bone health. Finally, prospective cohort studies are needed to assess the relationship between bone turnover markers and long-term risk of developing low BMD, fractures and/or osteoporosis. Finally, since key nutrients are coming predominantly from synthetic medical food, not whole foods in most patients with PAH deficiency, bioavailability of nutrients from medical food must be examined.

Adhering to the Genetic Metabolic Dietitians International (GMDI) recommendation to monitor BMD by DXA regularly in PAH deficiency is suggested (Singh et al. 2014). Twenty percent of subjects had a BMD Z-score below -1 , of which long-term clinical implications are unknown. Assessing CTx and P1NP in regular clinical practice could be useful if longitudinal studies show BTM can predict fracture risk. Patients who are not compliant with medical food may be at-risk for nutrient deficiencies and less favorable bone metabolism. Patients should be encouraged to consume medical food as prescribed to promote normal bone turnover and minimize uncoupling and excess resorption.

In conclusion, patients with PAH deficiency may have uncoupled bone turnover. Younger patients and those with low PTH appear to have BTM ratios favoring resorption. Optimal dietary intake, particularly medical food compliance and adequate micronutrient intake, likely impacts bone turnover more than metabolic control in patients with PAH deficiency.

Acknowledgments We would like to thank the National PKU Alliance for support for DXA scans, Immunodiagnostic Systems (IDS) for support for bone turnover marker measurement, and the Atlanta Clinical and Translational Science Institute (ACTSI) for general study support including staff and facilities at Emory University Hospital's Clinical Research Network and Children's Hospital of Atlanta's Pediatric Research Center.

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KEC and RHS designed research; KEC, EIF, and RHS conducted research; KEC, VT, and PWW analyzed data; KEC wrote the paper; KEC and RHS had primary responsibility for final content. All authors read and approved the final manuscript.

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Conflict of Interest Statement

No authors have competing interests related to this body of work.

Funding Details

The National PKU Alliance, Immunodiagnostic Systems, Maine Medical Center Research Institute, National Center for Advancing Translational Sciences of the National Institutes of Health under Award number UL1TR000454, and The Burroughs Wellcome Fund.

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