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Translating evidence to practice: a systematic and clinical analysis of bone health in phenylketonuria

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Translating evidence to practice: a systematic and clinical analysis of bone health in phenylketonuria

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MS RD, Case Western Reserve University, 2010

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An abstract of
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Abstract

Since the 1960s, newborn screening has allowed early detection of phenylketonuria (PKU), an inborn error of metabolism characterized by the inability to break down phenylalanine. Patients are treated immediately and for life with a low-protein diet and elemental medical food. Secondary effects of PKU are surfacing including low bone mineral density (BMD). A systematic review of 13 studies examining BMD in early-treated patients found lower mean BMD Z-score than normal, but within normal range defined by the International Society for Clinical Densitometry (ISCD) as Z-scores>-2. Most studies used incorrectly applied definitions for osteopenia and osteoporosis. To assess characteristics associated with BMD Z-score in patients with PKU, a study of 88 patients was conducted. BMD Z-score was positively associated with dietary vitamin D, calcium and medical food intake and compliance with medical food prescription. BMD Z-score was negatively correlated with dietary carbohydrate, sugar, caffeine, glycemic load and prescribed medical food. Models were developed to estimate BMD Z-score using predictors significantly correlated with BMD Z-score (p-value<0.10) and estimates were compared to DXA Z-scores. One model predicted Z-scores with 66.7% sensitivity and an AUC of 0.83 and included medical food compliance, medical food intake, caffeine intake, and blood bone-specific alkaline-phosphatase. The model must be validated in a separate set of patients before being used clinically to screen for patients that need DXA scans. Primary data were collected from 44 patients with PKU to examine unreported exposures, bone turnover markers (BTM), and BMD. All patients had normal BMD; however, 67% had elevated bone resorption but normal formation suggesting uncoupling of bone turnover. Nutrient intake met requirements for nutrients except vitamin D. The majority of protein, vitamin D, calcium and zinc intake came from medical food. Two dietary patterns were developed and the pattern considered compliant with medical food was associated with lower bone turnover. Physical activity was not associated with BTM or BMD. Together, results suggest BMD is not as compromised as hypothesized in patient with PKU, and well within the normal range for age and sex. Dietary intake and body composition drive variation in BMD instead of PKU related factors like phenylalanine concentrations.

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Chapter 1: Introduction

1.1 Background and Significance

Since its implementation as one of the first public health screening programs in the United States in 1963 [1], newborn screening (NBS) has succeeded in identifying infants with inborn errors of metabolism (IEM) immediately at birth [2]. NBS involves a simple heel prick in newborns to detect abnormally elevated concentrations of metabolites, including amino acids, in a single drop of blood [3]. The first IEM included in the NBS panel, phenylketonuria (PKU), also referred to as phenylalanine hydroxylase deficiency (PAH deficiency, OMIM 261600), is one of the most widely known and studied NBS disorders [2]. Patients with phenylketonuria have mutations in the gene which codes for phenylalanine hydroxylase (PAH), an enzyme produced by the liver that is responsible for metabolizing phenylalanine to tyrosine (Figure 1-1) [4].

PKU follows an autosomal recessive pattern of inheritance with an incidence of approximately 1 in every 10,000 to 15,000 births in the United States [5, 6]. Patients are classified with classical PKU (most severe), moderate PKU, mild PKU, or mild hyperphenylalaninemia (HPA) depending on blood phenylalanine concentrations prior to treatment [6]. The severity of the over 500 mutations identified in the PAH gene may also be used to classify patients. Heterozygotes, individuals who do not have PKU but carry a single mutation in the PAH gene, do not have elevated phenylalanine concentrations [7]. The PAH enzyme normally requires a cofactor known as BH_4 or tetrahydrobiopterin to metabolize phenylalanine [6] and BH_4 deficiencies can also result in high phenylalanine concentrations. BH_4 deficiency is ruled out before a diagnosis of PKU is confirmed [8].

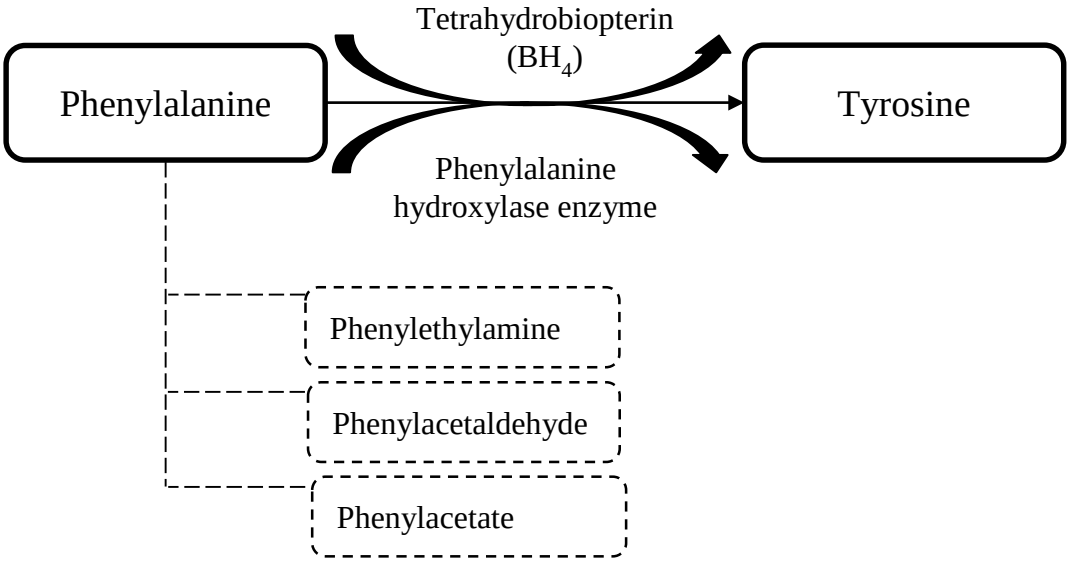

Figure 1-1. Normal metabolism of phenylalanine to tyrosine by the PAH enzyme

Untreated PKU causes severe neurocognitive, social, physical and behavioral abnormalities resulting from high blood phenylalanine and phenylalanine metabolites which can cross the blood-brain barrier [9]. Noted effects of PKU in untreated patients are growth failure, microcephaly, seizures and intellectual impairment [6]. Patients may also be at-risk for deficiencies in downstream products of tyrosine metabolism including the neurotransmitters dopamine, norepinephrine and epinephrine [10]. Early detection of PKU through NBS has, however, provided an opportunity for intervention within the first few days of life to prevent severe complications [11]. Treatment of infants with PKU begins immediately and consists of medical and dietary interventions [12]. The centerpiece of treatment is a low-protein diet to restrict the intake of phenylalanine found in foods including meats, cheese, beans and peas, and other high-protein foods [13]. Dietary intake of phenylalanine increases blood phenylalanine concentrations and brain phenylalanine concentrations [14].

Just as important as intact protein restriction, individuals with PKU must consume a supplementary medical food multiple times each day to provide a phenylalanine-free source of protein, necessary for normal growth and development [15]. Medical food, discussed in detail in Chapter 2, is an elemental source of all amino acids except phenylalanine and comes in a variety of forms including powder, ready-to-drink and gel [13]. Medical food intake results in lower blood phenylalanine concentrations and better protein status in patients with PKU [16, 17]. Thus, the goal of dietary treatment is twofold: to prevent increases in blood phenylalanine which can cross the blood-brain barrier and damage white and gray matter of the brain by restricting intact protein intake, and to maintain nutritional status by providing protein and energy to meet requirements through medical food [12]. Achieving both goals is a difficult balance and even the best clinicians struggle to ensure patients understand dietary recommendations and the short-term and long-term consequences of non-compliance [18]. Dietary treatment and its challenges by life-stage are discussed in Chapter 2.

Patients who have the financial, medical and social support to maintain long-term dietary compliance do not develop severe consequences of PKU; however, secondary effects are emerging in children and adults [19, 20]. One secondary effect is abnormal bone health [21, 22]. Since an initial publication by Feinberg and Fisch in 1962 citing striations of long bone in neonates with PKU [23], reports of poor bone health have surfaced in all groups of patients. Generally, bone mineral density (BMD), a measure of the amount of calcium and other minerals per square centimeter of bone is used to assess bone health [24]. BMD is the most common indicator of bone health because low BMD increases the risk of fractures [25]. In healthy children, one standard deviation decrease in

BMD doubles the risk of new fractures at any site [26]. In adults, risk is calculated at defined sites, for example a one standard deviation decrease in spine BMD is associated with a 2.3-fold increase in the risk of spinal fracture [27]. In male and female patients of all age groups with PKU, BMD Z-scores are lower compared to age and sex matched reference groups [22, 28, 29]. In younger patients, prevalence of low BMD ranges from 23-33% depending on definitions used [29, 30]. Adolescent and adult patients with PKU have an estimated prevalence of osteopenia and osteoporosis of 45-50% [31-33].

Low BMD in younger patients, including those who have followed a low-protein diet since birth, is of particular concern [28]. Early life is a critical period to maximize peak bone mass which occurs around 18 years of age in females and 20 years of age in males [34]. Approximately 60% of an individual's osteoporosis risk is attributable to peak bone mass [35]; the higher the peak bone mass, the lower the risk of osteoporosis later in life. Young adults with PKU may never reach their potential peak bone mass, predisposing the development of later conditions such as osteoporosis and fractures [36]. Reduced peak bone mass would cause a lower BMD even before normal age-related declines due to external factors such as genetics, nutrition and medications which compose the additional 40% of osteoporosis risk [35].

Many factors related to growth and physical development affect BMD and peak bone mass, examined in detail in the Fels Longitudinal Study in healthy boys and girls [37]. Height exerts a large impact on BMD and bone mineral content (BMC) [38]. Height in patients with PKU is, however, statistically comparable to peers based on Z-scores [39, 40]. Pubertal status and age at menarche also influence BMD in healthy individuals [41], but age at menarche is unreported in females with PKU. Finally, skeletal age is a

comprehensive measure of bone development compared to normal chronological development and data suggest skeletal age is identical to chronological age in patients with PKU [42]. Combined, these findings suggest impaired or delayed growth and development do not likely underlie differences in BMD in patients with PKU compared to healthy peers.

Nutritionally, dietary intake of vitamin D, calcium, phosphorus and protein impact bone health [43]. Evidence linking dietary vitamin D intake directly to BMD is scarce. Most studies focus on the impact of blood vitamin D status on bone parameters. Dietary vitamin D intake is, however, strongly correlated to blood concentrations of 25-hydroxyvitamin D and insufficient blood vitamin D is associated with lower BMD Z-scores in healthy school-aged children [44]. Dietary calcium intake and bone health has been widely studied. Calcium intake is significantly associated with increases in BMC in girls 5-11 years of age and BMD in post-pubertal teenage girls [45]. Dietary phosphorus intake is also associated with BMD, but in a complex manner with calcium. Higher calcium intake with lower phosphorus intake is beneficial to BMC and BMD while higher phosphorus intake with higher calcium intake is detrimental to BMC in young women [46]. In addition, type of nutrient and food source may also have an impact on bone. For example, low vegetable protein intake in healthy young women is associated with lower BMD at the hip and whole body [47]. Protein intake from low-fat milk is associated with higher BMD compared to protein intake from red meat and processed foods [48]. Protein is of particular benefit to bone mass independently and to BMD in the presence of adequate calcium intake, shown in healthy young adults [49]. Six amino acids in particular (alanine, arginine, glutamic acid, leucine, lysine and proline) are

reported to be associated with higher BMD at the spine and forearm [50]. The impact of nutrition on BMD has not been widely studied in patients with PKU.

Dietary patterns, derived through statistical approaches to identify patterns of consumption of food groups within a sample of individuals, is an alternative method to assess the impact of diet on health outcomes [51]. Dietary patterns have been described in other populations in association with bone health. In early post-menopausal women, diets characterized by higher intakes of processed and snack foods were associated with lower BMD while a healthy pattern was associated with decreased bone resorption [52]. In girls and young women, fruit and vegetable intake was associated with significantly higher BMD [53] and bone area of the whole body and radius [54]. In populations with complex nutrient intake, such as in PKU, dietary patterns could help understand how components of the diet interact to affect bone health. To our knowledge, dietary patterns in patients with PKU have not been reported.

It has been difficult for individual studies to assess the extent and clinical significance of low BMD, including long-term risk for osteoporosis and fracture in patients with PKU. Studies have used a variety of methods to measure bone status—from hand radiographs in the earliest reports, to dual energy x-ray absorptiometry (DXA) and blood concentrations of bone turnover markers (BTM) and vitamin D (25-hydroxyvitamin D) in newer investigations. DXA is the most common technique to measure BMD in current clinical practice and research, and the recommended measurement of pediatric bone status [55]. The general conclusion of individual studies utilizing DXA in patients with PKU is reduced BMD with unknown clinical significance, and a 33-62% prevalence of osteopenia using the definition of Z-scores below -1 [31, 36].

Applying the latest recommendations published by the International Society of Clinical Densitometry (ISCD) for low BMD, prevalence of BMD Z-scores ≤ -2 is 19-23% in patients of all ages with PKU [29] (Table 1-1). As reported in Chapter 4, there is no estimated prevalence of low BMD (Z-score≤-2) in a healthy population for comparison.

Table 1-1. Estimates of the prevalence of low bone mineral density (BMD) using a variety of definitions in patients with phenylketonuria

Author (Year)	***Study Type (n)***	***Definitions***	***Results***	***Prevalence***
deGroot (2012) **[29]**	Cross-section (n=53)	Low bone density for age (Z-score ≤ -2)	N=10 (19%)	<-2 = 19%
Mendes (2012) **[30]**	Cross-section (n=13)	Low bone mass (BMO ≤ -2) Osteopenia (BMO ≤ -2.5)	n=2 (15%) n=1 (8%)	<-2 = 23%
Coakley (2013) **[56]**	Cross-section (n=57)	At-risk BMD (Z-score -1 to -2.5) Low BMD (Z-score < -2.5)	n=16 (28%) n=3 (5%)	<-1 = 33%
Lage (2010) **[57]**	Cross-section (n=47)	Osteopenia (Z-score -1 to -2.5) Osteoporosis (Z-score < -2.5)	n=13 (28%) n=6 (13%)	<-1 = 41%
Modan-Moses (2007) **[36]**	Cross-section (n=31)	Osteopenia (Z-score -1 to -2.5) Osteoporosis (Z-score < -2.5)	n=11 (38.7%) n=2 (6.5%)	<-1 = 45%
Barat (2002) **[58]**	Cross-section (n=13)	Osteopenia (Z-score < -1)	n=8 (62%)	<-1 = 62%
Zeman (1999) **[31]**	Cross-section (n=44)	Lumbar BMD (Z-score -1 to -2.5) Lumbar BMD (Z-score < -2.5)	n=14 (32%) n=6 (14%)	<-1 = 46%

Low BMD is not an adverse outcome itself, but is a significant risk for fractures [59]. A single study has examined self-reported fracture risk in 85 patients, estimating those with PKU eight years and older have 2.6 times [95% confidence interval (CI) 1.1-6.1] the risk of fractures compared to sibling controls [60]. A recent systematic review found approximately 20% of patients with PKU reported a history of fracture, though circumstances were not reported by three of the six studies and all were cross-sectional [61]. In studies reporting circumstances, all fractures resulted from significant trauma (24 of 139 subjects, 17%), thus it is difficult to determine the impact of PKU on fracture risk.

Recent studies have reported bone turnover makers (BTM), measurable derivatives of the physiological process of bone turnover in patients with PKU [29, 33, 40, 62-67] (Table 2). Many BTM assays are available to measure bone formation by osteoblasts and bone resorption by osteoclasts [68, 69] [70]. Based on an extensive review of the literature, the International Osteoporosis Foundation (IOF) and the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) Bone Marker Standards Working Group developed recommendations for two gold-standard bone turnover markers: C-terminal cross-linking telopeptide of type I collagen (CTx) to reflect bone resorption and procollagen type I N propeptide (P1NP) to reflect bone formation [71]. The IOF-IFCC reported these bone turnover markers accurately reflect resorption and formation and should be included in future studies of bone turnover [71]. There are no studies reporting P1NP and CTx concurrently in patients with PKU. All six studies shown in Table 1-2 measured alkaline phosphatase and five of the six measured osteocalcin.

Table 1-2. Summary of studies reporting bone turnover markers of formation (F)* or resorption (R)* in patients with PKU (n=6)

Author (Year)	***Study Type (n)***	***Bone Turnover Markers***	***Specimen (R/F)****
Koura (2014) **[40]**	Cross-section (n=33)	Bone alkaline phosphatase (BALP) Osteocalcin Carboxy-terminal propeptide of type I collagen (CICP) Osteoprotegerin Receptor activator of nuclear factor kB ligand (RANLK) Deoxypyridinoline (DPD)	Serum-F Serum-F Serum-F Serum-R Serum-R Urine-R
deGroot (2012) **[29]**	Cross-section (n=53)	Alkaline phosphatase (BALP)	Serum-F
Nagasaka (2011) [63]	Cross-section (n=34)	Bone alkaline phosphatase (BALP) Osteocalcin Pyridinoline cross-linked telopeptide of type I collagen (ICTP)	Serum-F Serum-F Serum-R

		N-telopeptide of type I collagen (NTx) Deoxypyridinoline (DPD)	Urine-R Urine-R
Millet (2005) [65]	Cross-section (n=46)	Bone alkaline phosphatase (BALP) Osteocalcin Deoxypyridinoline (DPD)	Serum-F Serum-F Urine-R
Perez-Duenas (2002) [33]	Cross-section (n=28)	Bone alkaline phosphatase (BALP) Osteocalcin	Serum-F Serum-F
Hillman (1996) [67]	Cross-section (n=11)	Bone alkaline phosphatase (BALP) Osteocalcin Procollagen type I carboxy-terminal peptide Tartrate resistant acid phosphatase	Serum-F Serum-F Serum-F Serum-R

Individual cross-sectional and cohort studies have attempted to identify the cause of low BMD in patients with PKU. A number of factors related to PKU etiology have been examined including blood phenylalanine concentrations and phenylalanine variation over time [29], medical food consumption [31], and dietary intake of select nutrients [28], but associations are inconsistent and studies are poorly powered. Published investigations have focused only on the impact of PKU-related factors, leaving out many other important determinants such as nutritional status, physical activity, inflammation, body mass index (BMI) and other measures of adiposity [72, 73]. Figure 1-2 shows hypothesized factors that could interplay to affect BMD. In summary, it has not been determined if PKU-related factors including phenylalanine concentration and dietary treatment, other unexamined variables, or a combination of factors underlie low BMD in patients with PKU.

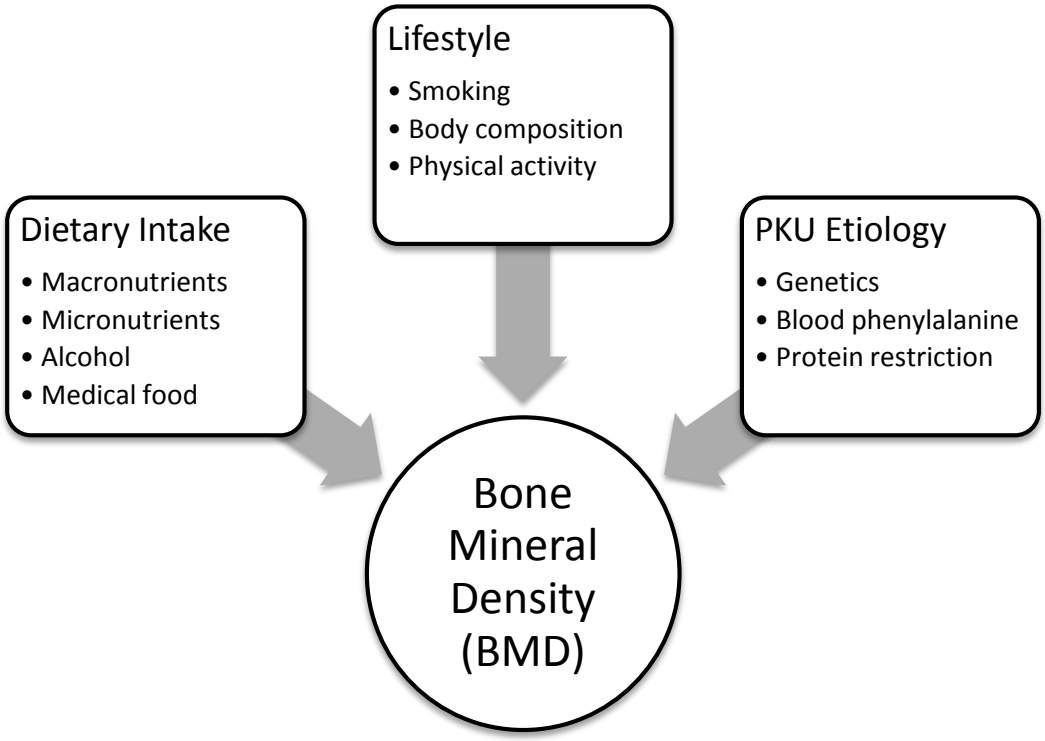

Figure 1-2. Factors that may affect bone mineral density (BMD) in individuals with PKU

1.2 Focus of the Investigation

To address gaps in the literature, a systematic and clinical study of bone health in patients with PKU was conducted. First, a literature review of studies related to bone health in patients with PKU and a meta-analysis of BMD Z-scores was performed to assess the extent and clinical significance of low BMD. Next, a tool to predict BMD Z-scores from routinely measured clinical indicators was developed. The tool was developed as an alternative to DXA for clinicians to use during regular metabolic clinic visits to screen for patients with low BMD who need follow-up DXA scans. Finally, to fill gaps in the literature and to comprehensively assess factors hypothesized to impact BMD in patients with PKU, primary data were collected including demographics, anthropometrics, bone health history and fracture prevalence, dietary intake including medical food and supplement intake, physical activity, blood phenylalanine

concentration, blood vitamin D and parathyroid hormone (PTH), gold-standard bone turnover markers CTx and P1NP, and bone mineral density and BMD Z-scores.

Chapter 2. Nutrition Management of Patients with PKU and its Impact on Bone

2.1 Overall Goals of Treatment

In individuals with PKU, it is hypothesized that the restriction of dietary protein and the reliance on synthetic medical food over the lifespan could lead to low BMD and altered bone turnover [11]. Dietary management begins immediately after birth and is ideally continued over the entire life course [12]. Successful treatment incorporates a balance between limiting intact protein from natural food sources and providing enough medical food to meet protein needs [12]. Clinicians must therefore consider many factors when calculating 1) dietary phenylalanine tolerance of a patient and 2) medical food needed to meet recommendations for protein for age and sex [74]. Additionally, response to medical treatments such as Kuvan (a BH_4 analog) or PEG-PAL may change both dietary phenylalanine tolerance and medical food prescription in some patients. Treatment imbalances including inadequate amount of total protein or medical food that does not meet nutritional needs can lead to suboptimal health outcomes.

Some patients with PKU benefit from vitamin and mineral supplements; however, most medical foods are supplemented with micronutrients [13]. Patients who are not compliant with medical food recommendations may be missing key nutrients, predisposing to deficiencies in macro- and micronutrients. Other factors such as insurance coverage, treatment availability and patients' social support must also be considered when developing dietary recommendations [75].

2.2 Phenylalanine Tolerance and Medical Food Prescription

A variety of mutations can cause PKU resulting in phenotypes ranging from mild to moderate to severe (classical) PKU [76]. Mutation severity affects dietary

phenylalanine tolerance and response to medical treatment options such as tetrahydrobiopterin, as discussed later [76]. Patients with milder mutations, thus more residual PAH enzyme activity, can tolerate more dietary phenylalanine without increases in blood phenylalanine. Patients with more severe mutations, however, have little to no PAH enzyme activity and can tolerate very little dietary phenylalanine without increases in blood phenylalanine concentration above the therapeutic treatment range. The effect of genotype on BMD has been reported in a limited number of patients with no apparent difference in BMD by genotype severity [77]. Differences in BTM by genotype severity have not been reported.

A key component of dietary management of PKU is the dietary phenylalanine prescription, or amount of phenylalanine in milligrams per day that is appropriate for a patient to consume while maintaining acceptable phenylalanine concentrations [12]. Based on phenylalanine concentrations prior to treatment, usually measured during confirmatory testing in early life, dietary phenylalanine tolerance is estimated by a metabolic dietitian. Over time, patients' dietary phenylalanine intake must be assessed concurrently with blood phenylalanine concentrations to evaluate the adequacy of the prescription. Increases in blood phenylalanine above the treatment range (120-360 μmol/L or 2-6 mg/L) indicates the patient's phenylalanine intake is too high and adjustments are made [12]. While phenylalanine concentrations may not correlate with BMD Z-score [29], data suggest fluctuations in blood phenylalanine may play a role in the development of osteopenia and low BMD [58]. Consistent phenylalanine concentrations over time is an important goal in the treatment of all patients with PKU.

Recommendations for medical food intake in patients with PKU are calculated based on dietary phenylalanine prescription. To ensure protein needs are met, the Dietary Reference Intake (DRI) Recommended Dietary Allowance (RDA) for protein are used [74]. The RDA provides exact protein needs for sex and age that have been found to meet the needs of 97% of the healthy American population [78]. Evidence suggests, however, PKU patients' protein needs exceed the RDA by 120-140% to account for the inefficient utilization of elemental amino acids found in medical food by the body [12]. Based on the patient's phenylalanine tolerance (50 milligrams phenylalanine = 1 gram of protein), the amount of protein (grams) allowable from food is calculated [12]. Intact protein recommendations vary greatly from patient to patient and more severe patients may tolerate only 5-7 grams of intact protein per day, just 11-15% of the estimated RDA for women (46 grams/day) [3]. Medical food requirements are then calculated by subtracting the patient's intact protein tolerance from the adjusted RDA [12]. The resulting grams of protein must be supplied by medical food.

Type and form of medical food can be recommended by the RD, but patient and family preference should also be considered. It is important to note differences in type of medical food, whether the formula is complete with other nutrients or contains protein only, discussed in the "Considerations" section. Impact of the type of medical food on bone has been studied in mice models only and results suggest the type of medical food can affect skeletal fragility [79]. Elemental amino acid formulas may result in more brittle and weak femoral bones compared to glycomacropeptide (GMP) based formulas, a low-phenylalanine whey protein that is acceptable for consumption by individuals with PKU.

2.3 Nutrition Management over the Lifespan

Infancy

After a positive newborn screen and confirmatory test, infants are officially diagnosed with phenylketonuria (ICD-9 code 270.1). If the mother prefers, breastfeeding is possible depending on the infant's phenylalanine tolerance, since breastmilk has a lower concentration of phenylalanine than regular cow's milk [80]. Infants who are breastfed must be strictly monitored to ensure phenylalanine concentrations falls within the recommended treatment range (2-6mg/dL, 120-360µmol/L) [81]. Formula designed for infants with PKU such as Periflex Infant may be used to supplement the breastmilk supply through alternate feedings, or for infants who cannot tolerate much breastmilk [82, 83]. Evidence suggests infants who are breastfed have phenylalanine concentrations that are comparable to infants who are formula fed, and are even more likely to have concentrations in the normal treatment range [80].

Early Childhood

Weaning an infant with PKU presents with unique challenges. As the amount of breastmilk or the mother's supply decreases, the addition of medical food to provide adequate protein is required [84]. Generally, weaning infants with PKU follows a pattern similar to World Health Organization (WHO) recommendations for weaning any infant, starting around six months of age or between 17 and 26 weeks [84]. A critical part of weaning infants with PKU is introducing foods low in phenylalanine early to establish healthy food preferences. Examples include strained or pureed fruits and vegetables and low-protein cereal, introduced in increments of 50 mg of phenylalanine (1 exchange) over time. Medical food intake must be monitored frequently and adjusted as needed to

meet protein needs by the patient's dietitian [84]. It is important to establish independent feeding practices and knowledge of appropriate PKU food choices from an early age to enhance compliance with diet throughout life. It is also important to encourage consumption of medical food in equal amounts over the course of the day to maintain phenylalanine concentrations within treatment range [84].

Adolescence

Dietary phenylalanine tolerance and prescription are developed in infancy, but may be revised as the child with PKU reaches adolescence. Recently published recommendations for medical management [85] and nutrition management of PKU [12] are excellent starting points to develop and implement dietary and medical food prescriptions for patients. The RD may use a standard recommendation for phenylalanine per kilogram body weight (phe/kg) [12] and tailor to the individual patient's needs. Over time, phenylalanine concentrations are measured to assess the impact of phenylalanine consumption on maintaining metabolic control [13]. The RD will adjust the dietary phenylalanine prescription to promote blood phenylalanine concentrations within the treatment range (2-6mg/L, 120-360µmol/L) [13].

While most patients fluctuate in compliance with dietary recommendations with age, it is important to make sure a treatment team is always in place to reinforce the importance of PKU management. Transition of care from pediatric to adult is often a major challenge to patients with PKU [86].

Adulthood

While attaining metabolic control is certainly possible in adult patients with PKU, the goals of treatment expands beyond maintaining acceptable phenylalanine

concentrations. New responsibilities including taking control of their own health, starting a family, changes in insurance, and the lack of clinics focusing on adult care may affect even the most dedicated patients. Studies generally report increases in phenylalanine concentrations of approximately 14.5 μmol/L (0.24 mg/dL) per year and a higher degree of fluctuation in phenylalanine concentrations as patients age [14, 87]. Only 55% of adults with PKU over age 16 in Europe report monitoring blood phenylalanine in concordance with country-specific recommendations [88]. In some scenarios, adult patients are unwilling to comply with recommendations or have lost motivation to continue seeking treatment due to financial, social or medical reasons [89]. These barriers create a difficult scenario to effectively monitor and treat adult patients. A particular concern is the adult PKU patient who cannot access medical food, but continues to follow a strict low-protein diet. This pattern predisposes the patient to protein and other nutrient deficiencies that can be detrimental to wellbeing, including bone mineral density [90, 91].

Despite barriers, treatment recommendations remain stringent for adult patients. Blood phenylalanine and tyrosine should be monitored monthly, generally by filter paper, with a full clinic visit one to two times per year including a plasma amino acid assessment [12]. In 2000, the National Institutes of Health (NIH) published the target blood phenylalanine treatment range of 120-900 μmol/L for adult patients over the age of 20 [92]. Evidence and consensus-based recommendations have since been revised and are now 120-360 μmol/L (2-6 mg/dL) over the lifespan to optimize cognitive outcomes [12]. Dietary phenylalanine prescription will likely need adjustments as patients age and the treatment range for blood phenylalanine remains the same.

2.4 Medical Treatment Options

Medical treatment options for patients with PKU have emerged in recent years. BH_4 (sapropterin dihydrochloride, Kuvan®), the co-factor of the affected PAH enzyme, is an option for patients who respond [93]. Responders are generally patients with less severe mutations in the PAH enzyme that still produce residual amounts of PAH. In these patients, BH_4 may reduce blood phenylalanine concentrations by 30% or more. Typically, 32-50% of patients with PKU respond to the drug depending on criteria used to determine responsiveness [94, 95]. A Cochrane review of sapropterin dihydrochloride treatment of patients with PKU published in 2015 included only two studies and reported sapropterin significantly lowers blood phenylalanine concentrations and increases protein tolerance [96].

Unfortunately, no studies have reported the impact of sapropterin therapy on long-term outcomes such as cognition, nutritional status or quality of life [94]. A single analysis suggests long-term growth is not improved by BH_4 compared to a standard phenylalanine-restricted diet [97]. Impact of BH_4 on bone health, however, is more promising. In a study of 43 patients, none of the patients treated with tetrahydrobiopterin (n=14) for an average of seven years developed mineral bone disease (MBD), defined as a T-score below -1 [77]. The study cited higher natural protein intake in those treated with tetrahydrobiopterin, and thus a positive impact on BMD. Another study, however, reported no difference in bone turnover markers (BTM) between patients treated with tetrahydrobiopterin compared to non-treated patients [62]. Understanding the impact of BH_4 on long-term outcomes, including bone health, is critical to evaluating the overall impact of BH_4 as a treatment option.

Glycomacropeptide (GMP) and large neutral amino acids (LNAA) are other alternative therapies for patients with PKU. GMP is a natural protein found in cheese that is naturally low in phenylalanine and has been used in a variety of medical foods [98, 99]. It has acceptable satiety and allows for a higher percentage of protein in a patient's diet to come from intact sources (up to 70%) compared to traditional medical foods (only 20-30% of protein from intact sources) [98]. Studies examining metabolic control of patients who consume GMP-based medical food compared to traditional amino acid based medical foods are needed [98]. LNAA therapy (tyrosine, tryptophan, methionine, valine, isoleucine, leucine, and histidine) is hypothesized to block the transport of phenylalanine across the blood-brain barrier, as LNAA utilize the same transporter [100]. In several trials, LNAA tablets were supplemented (0.25-0.5g/kg) and patients continued to consume a normal phenylalanine-restricted diet [101, 102]. Estimated reduction in blood phenylalanine was 25-39% from baseline, suggesting LNAA have a potential role in treatment. There are no studies in humans on the impact of GMP or LNAA therapies on BMD or other indicators of bone health. Other emerging therapies are in developmental phases including cell directed therapy; stem-cell transplant of hepatocytes, cells in the liver responsible for producing PAH enzyme; and gene therapy of liver cells [13]. These treatment options have only been tested in animal models and are not currently available for patients.

2.5 Other Considerations

As a result of strict, long-term dietary treatment, there are concerns for issues such as nutrient deficiencies [103], impaired growth [104] and obesity [105] in patients with PKU. A patient's overall nutritional status is generally linked to the degree of

compliance with medical food [106] and total protein intake [107]. Micronutrient inadequacies have been reported for zinc, selenium, iron, vitamin B12 and folate [103]. Rohde et al. identified three groups of patients with PKU particularly at-risk for micronutrient deficiencies: 1) patients who are not consuming medical food, 2) patients with a protein supply from medical food less than 0.5 grams per kilogram body weight, and 3) patients with a total protein intake of less than 120% of recommendations [107].

The Collaborative Study of Children Treated for Phenylketonuria (PKUCS), conducted in the 1970s, did not find impaired growth of treated children with PKU [108], but investigations in more recent years report children are significantly shorter than their healthy counterparts who do not have PKU [42, 109]. Rocha et al. reported continuously treated patients with PKU can, however, achieve normal growth [39] with high-quality and consistent treatment. Overweight and obesity are other nutrition-related issues reported in pediatric and adult patients [110]. Estimated prevalence of overweight (BMI $\geq 85^{th}$ percentile) in pediatric patients is 40% [105, 111], slightly higher than the general population estimate of 31.8% [112]. A review of eight PKU treatment centers found females with PKU are particularly vulnerable to excess adiposity, both children and adults [113]. The estimated prevalence of overweight in females is 55% and obesity prevalence is 33%, 1.8 and 2.1 times higher than the rate in the general U.S. female population [110].

In 2015, the Genetic Metabolic Dietitian International (GMDI) and the Southeast Newborn Screening and Genetics Regional Collaborative (SERC) published nutrition management guidelines for patients with PKU including recommendations for monitoring bone health [12]. Recommendations for laboratory and anthropometric

parameters to monitor, specimens needed, and frequency are detailed in Table 2 of the manuscript to prevent potential complications of dietary treatment outlined above. It is recommended to monitor BMD by DXA every three to five years in patients 8-18 years of age and every five years in adults.

In conclusion, dietary treatment of patients with PKU is complex and requires multiple clinic visits and diligence on the part of the patient and the clinician. Maintaining blood phenylalanine concentrations within the therapeutic range, and achieving optimal nutritional status are the main goals of treatment. The impact of long-term nutrient intake on nutritional status, growth and other outcomes such as bone health should be examined in future investigations in patients with PKU.

Chapter 3. Methods

This dissertation was a mixed methods study on bone health in patients with PKU and included 1) a literature review and meta-analysis, 2) a mathematic modeling and correlation analysis, and 3) a cross-sectional study to collect primary data. The Methods chapter will explain the flow of the dissertation, justify methods used for each chapter, and supplement material which follows in Chapters 4-6.

3.1 Literature Review

After an unofficial search for literature on bone health in patients with PKU, the need for a comprehensive and systematic review was recognized. In 2012, a systematic review of bone health in patients with PKU was initiated at Emory University. During this effort, a simultaneous effort was being conducted by a research team at the Academic Medical Center in Amsterdam, the Netherlands. The two research teams, complementary in vision and project aims, decided to collaborate to complete the systematic review and include a meta-analysis. The Emory research team aimed to conduct a systematic review of BMD, bone turnover markers, and the prevalence of osteopenia and osteoporosis in patients with PKU, while the Academic Medical Center team aimed to conduct a meta-analysis of BMD Z-scores in early-treated patients with PKU.

Independent literature searches were performed by each research team with different inclusion and exclusion criteria, detailed in Chapter 4. The inclusion of only early-treated patients with PKU in the Academic Medical Center criteria differed from Emory's criteria to include early and late-treated patients. In the final manuscript, both research teams' criteria were applied and only articles including early-treated patients were included. To develop the outline and content of the systematic review, the Preferred

Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement was used [114]. BMD Z-score was selected as the primary outcome for meta-analysis since it is most commonly reported and is standardized for age and sex. Studies reporting BMD Z-score at any site (femoral hip, lumbar spine or total body BMD) were included. Bone mineral content (BMC) and bone turnover markers (BTM) were also included in the qualitative systematic review, but have not been reported in sufficient numbers to include in a quantitative meta-analysis. Prevalence of low BMD (generally reported as osteopenia and osteoporosis) and definitions were also extracted from included articles.

Each research team used a separate method to assess the quality of included articles. The Emory research team utilized an “Evidence Analysis” approach developed by the Academy of Nutrition and Dietetics (AND) and adapted by Dr. Rani Singh as part of the SERC-GMDI effort to develop evidence and consensus-based nutrition management guidelines for IEM [12, 115]. To assess risk of bias, quality criteria checklists (QCC) were completed for each included article. Separate QCCs are available for primary research articles and review articles and both QCCs include four relevance questions and 10 validity questions with sub-questions. Questions address study design, quality of methods and data collection, statistical analyses, potential for bias, and other key study elements recommended by the Agency for Healthcare Research and Quality (AHRQ) [116]. A scoring algorithm is then applied based on responses to the 10 validity questions and articles are scored positive, negative or neutral. Key results related to bone indicators and quality ratings were abstracted onto a standardized overview table by the same individual.

The Amsterdam research team used the Scottish Intercollegiate Guidelines Network (SIGN) checklists to assess quality and risk of bias of included articles. Checklists are available for different types of studies including cohort, case-control, randomized controlled trials, and reviews/meta-analyses [117]. Unlike the QCC utilized in the GMDI-SERC process, there are separate SIGN checklists for each study design. Based on the checklist and the reviewer's opinion, a quality score is assigned to studies: high quality, acceptable quality, or low quality. Using a standard overview table, evidence was abstracted by both authors on the Amsterdam research team. Overview table contents from both research groups were compared and combined to develop a final overview table used for qualitative results.

Though two methods were used to assess quality and abstract content from included articles, results were very similar between the two groups (see Results section, Chapter 4). It appears that the GMDI-SERC method of Evidence Analysis using QCCs to assess study quality is comparable to the established SIGN checklists, an internationally recognized quality assessment method, at least to differentiate low quality from high or acceptable quality studies. A comparison of the two methods can be found in Table 3-1.

Table 3-1. Comparison of the GMDI-SERC Evidence Analysis Process and the Scottish Intercollegiate Guidelines Network (SIGN) Checklist process of quality assessment

GMDI-SERC Evidence Analysis Process	***Scottish Intercollegiate Guidelines Network (SIGN) Checklists***
Adapted from the Academy of Nutrition and Dietetics (AND)	Original resource, not adapted
Questions based on AHRQ domains for research studies[a]	Questions based on principles of the GRADE process[b]
3 checklists (1 for non-humans) • Primary research (humans or animals) and review article	6 checklists • RCT, cohort, case-control, review/meta-analysis, diagnostic, economic
63 questions per checklist • 4 relevance, 10 validity questions with sub-questions	Number of questions vary by checklist • Range from 11 (economic) to 17 (cohort)

Answers to checklist questions: Yes No Unclear N/A	Answers to checklist questions: Yes No Don't know Does not apply
Quality ratings assigned according to answers to specific questions	Quality ratings assigned subjectively based on checklist and reviewer judgement
Quality ratings: - (negative quality) o (neutral quality) + (positive quality)	Quality ratings: o (low quality – most criteria not met) + (acceptable – most criteria met) ++ (high quality – majority of criteria met)

[a]AHRQ: Agency for Healthcare and Research Quality [116]
[b]GRADE: Grading of Recommendations Assessment, Development and Evaluation [118]

The GMDI-SERC process involves fewer checklists with more questions compared to SIGN checklists which include checklists specific to each study type with fewer, more specific questions. The GMDI-SERC method includes an objective measure of quality, requiring specific answers to specific questions to assign quality ratings. SIGN, on the other hand, requires an overall subjective assessment of the completed checklist and general study by the reviewer to designate study quality. Both methods have pros and cons, but both are appropriate in nutrition research. A full guide to the AND Evidence Analysis Process is available online (https://www.andeal.org/files/Docs/2012_Jan_EA_Manual.pdf), as is the SIGN Methodology Handbook (http://www.sign.ac.uk/pdf/qrg50.pdf).

3.2 Reporting of bone health in the literature

During the process of developing the systematic review, a critical error in the literature database of bone health in patients with PKU was discovered. Most studies used BMD Z-scores to report prevalence of osteopenia and/or osteoporosis in cohorts of patients consisting of pediatric and adolescent subjects. Two sets of recommendations have been developed for reporting BMD by Z-score or T-score category, the World Health Organization's (WHO) traditional definitions [119] and the International Society of Clinical Densitometry's (ISCD) definitions (Table 3-2). According to the WHO, T-

scores should be used to indicate the presence of osteopenia (T-score between -2.5 and -1) or osteoporosis (T score ≤ -2.5). ISCD recommends WHO T-score definitions should be used in adults only [120] and states that the diagnosis of osteopenia is not clinically acceptable for pediatric patients, pre-menopausal women or men under 50, the population included in all studies in PKU [121]. Moreover, to correctly identify pediatric and pre-menopausal patients with osteoporosis, a clinical fracture must be present with low BMD [121]. ISCD recommends the use of Z-scores to identify pediatric patients, pre-menopausal women and men under 50 with low BMD using a cut-off of -2 to indicate patients with low BMD (also termed low BMD for chronological age).

Table 3-2. ISCD and WHO Definitions for Low Bone Mineral Density (BMD)

	International Society for Clinical Densitometry (ISCD) 2013 [120, 121]		***World Health Organization (WHO) 2010 [119, 122]***	
	Pre-menopausal women, men<50	Post-menopausal women, men>50	Children	Adults
Low BMD[a]	Z-score ≤-2	—	—	—
Osteopenia	Term not recommended	T-score -1 to -2.5	—	T-score -1 to -2.5
Osteoporosis	Z-score ≤-2 and fracture	T-score ≤-2.5	—	T-score ≤-2.5

[a]Low BMD for chronological age also correct terminology — not defined

None of the studies included in the meta-analysis reported T-scores and only one study reported low BMD using ISCD guidelines (Z-scores ≤-2), the correct classification for the population studied [120]. Most studies included the classification of subjects as osteopenic, also against ISCD guidelines. Of the studies noting osteoporosis in patients with PKU, none included the presence of a fracture [121]. Future studies must use the correctly applied and population-relevant definitions of low BMD before conclusions can be drawn on bone status in patients with PKU.

During the literature search and review, the authors also noted that while bone turnover markers (BTM) were reported by a few studies (Table 1-2), most measured a

variety of markers in serum or urine instead of gold-standard BTM recommended by the IOF and the IFCC [123]. IOF/IFCC recommend one marker of bone formation [serum procollagen type I N propeptide (P1NP)] and one marker of bone resorption [serum C-terminal telopeptide of type I collagen (CTx)] as gold-standard indicators of bone turnover in clinical studies. The National Bone Health Alliance (NBHA) initiated a project to standardize BTM collection and harmonize analysis procedures in the United States and to establish evidence-based reference ranges for P1NP and CTx [124]. BTM collected in this dissertation, described in Chapter 6, will be contributed to this effort.

Bone turnover is a constant and dynamic process that directs the building, maintenance or breakdown of both types of bone in the body, cortical and trabecular [125]. When bone is broken down by osteoclasts, osteoblasts must act to fill the resulting resorption pits with collagen fibrils to build new bone. This process, known as bone remodeling, is tightly coupled through menopause in women. Regulation of the four phases of remodeling occurs through the receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG) system. Remodeling results in newly formed bone which takes approximately four to six months to complete. CTx is a proteolytic fragment of bone collagen that is released by osteoclast activity and considered a reliable marker of bone resorption [126]. P1NP, on the other hand, is a protein cleaved by proteases outside of osteoblasts during the synthesis of new collagen and is considered a reliable marker of bone formation [127].

Studies have investigated the utility of BTM in predicting fracture risk in post-menopausal women and conclude high levels of some biomarkers are independently associated with an increased risk of osteoporotic fractures [128]. In children, cross-

sectional evidence suggests BTM may not be associated with BMD [129], or bone gain over a period of 18 months [130]. A single study suggested BTM are higher in healthy children with repeated fractures than children without fractures, but the fractures themselves may have caused increases in BTM [131]. Other studies examining the association between BTM and fractures in children have not been published. Repeated measures of BTM and a longer period of assessment may be necessary to fully understand the relationship between BTM and BMD, especially in children. In patients with PKU, the clinical utility of BTM is unexamined. Until BTM are further assessed, alternative methods to assess risk of fractures are needed.

3.3 Developing a predictive model to estimate BMD

Results of the systematic review and meta-analysis of bone health in patients with PKU highlight several important research gaps. First and foremost, BMD is statistically lower in patients with PKU compared to normal for age and sex (BMD Z-score=0), but mean Z-scores are well within normal for age and sex. Z-scores do, however, indicate some patients with PKU, particularly those who are not compliant with dietary recommendations and/or do not consume adequate medical food, have lower BMD than expected for age. In order to identify these patients, GMDI-SERC guidelines provide monitoring recommendations for regular DXA scans [12]. Data examining actual monitoring rates of BMD in patients with PKU are only available from European clinics. A survey of 12 European countries showed only 1 of 14 clinics monitor bone density in children under 10 years, and 5 of 14 monitor bone density in adolescents 10-16 years of age as part of regular practice [132]. Even in adults, nearly 60% of clinics are not monitoring bone density regularly.

3.4 Survey of Metabolic Dietitians to Assess Current Monitoring of Bone Health

To assess if and how metabolic clinics in the U.S. are monitoring bone health in patients with PKU, a survey was developed and sent to dietitians who subscribe to a Metabolic Dietitian's listserv. Survey instructions encouraged all dietitians who received the invitation to complete the survey, even if their clinic does not currently monitor bone health. Response rate could not be calculated since the listserv active membership is difficult to estimate. After one month of availability on Survey Monkey, results were tallied and responses were calculated as prevalence (number of positive responses/total number of responses).

A total of 32 individuals participated, all of whom were registered dietitians, representing 29 clinics. The survey found that the majority of clinics reported measuring any indicator of bone health (including BMD) as needed (34%) or less than once per year (25%) in patients with PKU. Half of respondents indicated BMD is part of their clinical assessment of bone health, while only one respondent indicated BTM are included in assessment. Vitamin D, however, is being measured in most clinics (88%). Monitoring any parameter of bone health increased by age group of patients, for example, 50% of clinics reported assessing bone in children 2-10 years of age while 72% reported assessing bone in adults 18 years and older.

For most questions, monitoring bone was defined as the measurement of any indicator and only 31% of clinics reported following GMDI recommendations for regular DXA scans. Encouragingly, all respondents indicated they believe it is important to assess bone health in patients with PKU. Clearly, a gap exists between evidence-based

recommendations and clinical practice, and until a solution is implemented, a simple alternative method of bone assessment would be useful.

In summary, we know a subset of patients with PKU have low BMD and may be at-risk for fractures, but clinics are not monitoring bone health as recommended. Additional data on long-term outcomes of patients with low BMD are likely needed before clinics begin implementing DXA scans for all patients. In the meantime, routinely assessed factors correlated to BMD Z-score have been identified in patients with PKU, outlined in Chapter 5. These indicators may be useful to identify patients at-risk for low BMD. Chapter 5 describes the development of predictive models to estimate BMD Z-scores from subsets of routinely collected patient parameters correlated with Z-scores. With further validation, the models could be useful for clinicians who do not have easy access to DXA machines to easily screen for patients who should receive DXA.

3.5 Primary data collection

Following the developing of predictive models, two important concepts were evident: 1) BTM may be useful to assess bone status and/or predict BMD and need to be assessed, and 2) gaps still exist in assessing key factors related to bone health in patients with PKU. To address these concerns, a protocol to collect primary data from patients attending a Metabolic Camp for girls and women with PKU was developed, approved by Emory IRB and implemented. Starting in 2012, data were collected over three consecutive years of camp. Goals of primary data collection were to 1) assess BMD and BTM utilizing gold standard methods of dual energy x-ray absorptiometry (DXA) and Immunodiagnostic Systems (IDS) iSYS Multi-Discipline Automated System, respectively [133]; 2) examine the impact of novel factors including comprehensive

dietary intake, compliance with dietary prescription of medical food and phenylalanine and physical activity on BTM and BMD; and 3) draw conclusions on the state of bone health in patients with PKU and propose a mechanism underlying low BMD.

Before data were collected, the study team contacted the National Bone Health Alliance (NBHA) to explain the project and its relevance to their current Bone Marker Standardization Project [124]. The NBHA supported the project since its two gold standard markers, P1NP and CTx, were included. Immunodiagnostic Systems (IDS), manufacturers of one of two validated assays to measure P1NP and CTx, agreed to collaborate with the study team and provided support for biomarker measurement using the iSYS Multi-Discipline Automated System. The iSYS also measured bone-specific alkaline phosphatase (BALP), 25-hydroxyvitamin D, and parathyroid hormone (PTH) in the same sample of 0.5 mL of serum. For CTx (assay range 0.033-6.0 ng/mL), interassay variability was 6.2% and intra-assay variability was 3.2%. For P1NP (assay range 2-230 ng/mL), interassay variability was 4.6% and intra-assay variability was 3.2%. For 25-hydroxyvitamin D (assay range 6-126 ng/mL), interassay variability was 8.0% and intra-assay variability was 6.8%. The study team agreed to provide data to the NBHA upon publication of this dissertation to contribute to the Bone Marker Standardization Project. This is the first report of CTx and P1NP in patients with PKU and with DXA and fracture prevalence. In the future, BTM could be incorporated into routine clinical monitoring to detect patients at-risk for low BMD or patients who should receive follow-up DXA scans.

All data were collected during Metabolic Camp according to an IRB-approved research protocol. Each year, girls attending Camp must provide three days of detailed dietary intake and blood samples to measure plasma amino acids. Anthropometrics, blood

pressure and temperature are also assessed. Some campers choose to participate in additional research implemented through Camp each year. For this study, several data collection procedures were added to the protocol and consent form including additional blood for biomarker measurement, a urine sample, a DXA scan, and additional research questionnaires. In this study, two questionnaires were required: a bone health history questionnaire and a physical activity questionnaire. All primary data were collected to answer specific gaps identified by the systematic review and meta-analysis described in Chapter 4 and to meet the aims of this dissertation.

Data collected during Metabolic Camp were used for two investigations. Primary data (from 2013 and 2014 Metabolic Camp only) were included in the dataset used to build predictive models outlined in Chapter 5. The remainder of the data for Chapter 5 came from baseline study visits of a separate study conducted by Dr. Rani Singh at Emory Genetics prior to 2010 to test tetrahydrobiopterin (Kuvan) responsiveness in patients with PKU. All methods of data collection were the same between the Kuvan study and the primary data collected for this dissertation. Data from 2013-2015 Metabolic Camps were included in Chapter 6.

3.6 Dissertation Hypotheses

This dissertation sought to fill important gaps in the literature on bone health in patients with PKU with the following hypotheses:

1. Bone mineral density (BMD) Z-scores are significantly lower in patients with PKU compared to reference populations.
    a. The proportion of patients with low BMD is significantly higher than expected in a normal population.

2. Utilizing patient characteristics including age, sex, dietary intake and compliance with medical food prescription as predictors in mathematical models will result in an acceptable estimate of BMD Z-score compared to DXA Z-scores.
    a. Models will be useful enough for clinical settings if DXA is not available or to screen for patients who should receive DXA scans.
3. Bone turnover markers in patients with PKU will be abnormal. In particular, bone resorption will exceed formation.
    a. Dietary deficiencies in calcium and vitamin D will be associated with higher resorption and resorption to formation ratio (CTx/P1NP).
    b. Noncompliance with medical food prescription will also be associated with higher resorption (CTx) and resorption to formation ratio (CTx/P1NP).
    c. Lower body mass index (BMI) will be associated with lower BMD and/or lower bone turnover in patients with PKU.
4. Dietary patterns will be evident in patients with PKU including a group that is compliant with medical food (and underweight) and a group that is not compliant with medical food (and overweight).
    a. Both groups of patients will consume low amounts of fruits and vegetables and high amounts of carbohydrates, fat in the form of snacks and sweets, and potatoes.
    b. Patients who are not compliant with medical food will have higher BMI and phenylalanine concentrations and lower BMD.

c. BMD will be lower and bone turnover will be higher in non-compliant patients.

The following dissertation aims to test these hypotheses.

Chapter 4. Bone health in phenylketonuria: a systematic review and meta-analysis

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4.1 Abstract

Patients with Phenylketonuria (PKU) reportedly have decreased bone mineral density (BMD). The primary aim of this study was to perform a systematic review and meta-analysis to determine the extent and significance of low BMD in early treated patients with PKU. Secondary aims were to assess other bone status indicators including bone turnover markers (BTM) and to define areas for future research. Two research teams (Amsterdam, Netherlands and Atlanta, USA) performed literature searches for articles reporting data on BMD, osteopenia and osteoporosis, BTM or other bone indicators in patients with PKU. Included articles were compared between research teams and assessed for quality and risk of bias. A total of 13 unique articles were included; 11/13 articles reported BMD including a total of 360 patients. Ten out of 11 articles found BMD was significantly lower in patients with PKU. Meta-analyses for total BMD (TBMD; 3 studies; n = 133), lumbar spine BMD (LBMD; 7 studies; n = 247), and femoral neck BMD (FBMD; 2 studies; n = 78) Z-scores were performed. Overall effect sizes were: TBMD −0.45 (95% CI −0.61, −0.28); LBMD −0.70 (95% CI −0.82, −0.57); FBMD −0.96 (95% CI −1.42, −0.49). Definitions of osteopenia and osteoporosis were highly heterogeneous between studies and did not align with World Health Organization standards and the International Society for Clinical Densitometry positions on BMD measurement. Despite individual study findings of low BMD indicating higher risk of osteoporosis, pooled available data suggest reduction in BMD is not clinically important when using standard definitions of low BMD. Results from studies evaluating BTM are inconclusive. Phenylalanine concentration, vitamin D, PTH, and nutrient intake do not correlate with BMD or BTM. We recommend forthcoming studies use standard definitions of low BMD to determine clinical implications of BMD Z-scores below 0, explore cause of low BMD in the subset of patients with low BMD for chronological age (Z-score < −2) and assess fracture risk in patients with PKU.

4.2 Introduction

Phenylketonuria (PKU, ORPHA79254, MIM 261600) is a genetic disorder caused by mutations in the gene coding for phenylalanine hydroxylase (PAH; EC 1.14.16.1). As a consequence, the essential amino acid phenylalanine (Phe) cannot be converted to tyrosine and accumulates in the blood. Phe is transported across the blood–brain barrier and high concentrations can lead to mental retardation and behavioural and physical abnormalities. Implementation of newborn screening to detect PKU across the world since the 1960s has enabled early diagnosis and treatment. Early dietary treatment results in near normalization of outcomes for patients with the disorder [1].

The success of dietary treatment has, however, led to the discovery of secondary issues in the life-long treatment of PKU [1]-[8]. First reported in 1962, one of the complications seen in early and continuously treated patients is abnormal bone status [9]. Initially examined by radiological assessment, Feinberg et al. [9] described calcified spicules of cartilage projecting into the distal metaphyses of growing long bones in a sample of 33 patients with PKU ranging from infants to young adults. These findings were later supported by Murdoch et al. [10] and led to further studies assessing bone status in PKU by quantitative ultrasound (QUS) [11], peripheral quantitative computed tomography (pQCT) [12] and dual-energy X-ray absorptiometry (DXA) [13]-[17]. Low bone mineral density (BMD), an important risk factor for skeletal fractures, has since been reported by many studies [16],[17].

A recent systematic review reported spine bone mineral density (BMD) was 0.100 g/cm^2 lower (95% CI, −0.110, −0.090 g/cm^2) in 67 subjects with PKU, compared to 161 controls collected from 3 studies [18]. This review, however, has methodological

limitations: ascertainment bias by inclusion of late diagnosed patients who may suffer from cognitive delays and less physical activity potentially affecting the bone outcomes; lack of literature quality appraisal and assessment of bias; and no correction for age, gender and ethnicity on BMD data (based on g/cm^2).

Most studies on bone in patients with PKU agree that bone is affected; however, there are significant gaps in knowledge and no consensus on the degree and implications of bone abnormalities, biological causes and risk-factors for low BMD [4],[5],[14],[19],[20], and the identification of subgroups of patients at-risk for fractures and compromised bone status [13],[20],[21]. To investigate these knowledge gaps, we combined the efforts of two international centers to perform a systematic review on bone status in PKU. Our primary aim was to systematically review the literature concerning bone status in early treated patients with PKU to perform a meta-analysis on BMD, corrected for bias, age and gender. Secondary aims were to assess other indicators of bone status including bone turnover markers (BTM) and to define areas for future research on bone status in PKU.

4.3 Materials and methods

Research question

Two centers for metabolic diseases in Atlanta, Georgia (USA) and Amsterdam (Netherlands) performed separate searches for literature concerning bone health in patients with PKU according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [22]. Both centers included similar research questions, review strategies and proposed outcomes, thus efforts were combined. Whereas the Atlanta research group focused on assessing the effects of nutrient intake, blood Phe concentration, and adjunctive therapy on bone status indicators (search 1); the group in Amsterdam focused on a comprehensive meta-analysis of BMD and an assessment of BTM in early diagnosed patients with PKU (search 2). The protocol for the Atlanta systematic review is registered with the 'International prospective register of systematic reviews' (PROSPERO) as systematic review number CRD42014009176 [23].

Inclusion criteria for search 1 were primary research or review articles, human research including subjects with PKU or hyperphenylalaninemia, and written in English only. Exclusion criteria were articles unrelated to PKU, animal studies, studies including in vitro results only, and studies that did not include BMD, bone mineral content (BMC), bone turnover, or measures of bone metabolism.

Inclusion criteria for search 2 were original studies (randomized controlled trial, cohort or case–control studies) of early diagnosed and treated patients with PKU studying either BMD or BTM with a quality rating of acceptable or better according to quality appraisal. Exclusion criteria were reviews (however reference lists were viewed for

relevant articles), studies that include pregnant patients, articles published in a language other than English or Dutch and articles not meeting the inclusion criteria.

Methodology

Databases

Literature eligible for inclusion in search 1 was retrieved from PubMed and EMBASE databases through a computerized search with assistance from a trained Emory University librarian. The initial search was performed in 2013, with an updated search completed in May 2014 to ensure the inclusion of recently published articles. As an example, we provide here the MEDLINE® search: (pku[All Fields] OR ("phenylketonurias" [MeSH Terms] OR "phenylketonurias" [All Fields] OR "phenylketonuria" [All Fields])) AND ("bone and bones" [MeSH Terms] OR ("bone" [All Fields] AND "bones" [All Fields]) OR "bone and bones"[All Fields] OR "bone" [All Fields]). All articles and abstracts retrieved through PubMed and EMBASE searches were downloaded in PDF format through Emory University open access or requested through Illiad, a document-delivery service, if full text was not available.

A computerized search with the help of a trained University of Amsterdam librarian in MEDLINE®, EMBASE and The Cochrane Library [24] was performed for search 2. The databases were searched initially in October 2013 and last in June 2014. No limits were used in the searches. As an example, we provide here the search used in MEDLINE®: ("Phenylketonurias" [mh] OR phenylketon* [tiab] OR "PKU" [tiab] OR hyperphenylalaninaemia[tiab] OR hyperphenylalaninemia [tiab]) AND ((minerals[mh] OR mineral*[tiab] OR "Bone Diseases, Metabolic" [Mesh] OR "Osteoporosis" [Mesh] OR osteoporosis [tiab] OR "Bone Density" [Mesh] OR "Bone Demineralization,

Pathologic" [Mesh] OR "Bone Resorption" [Mesh] OR "Bone Development" [Mesh]) OR "Bone Remodelling" [Mesh] OR osteolysis [tiab] OR decalcification [tiab] OR bone [tiab] OR bones [tiab]).

MEDLINE® contains references of articles published since 1966, the majority of which are published in the USA. EMBASE also contains articles published in Europe, with references dating back to 1976. The Cochrane library contains over 250,000 records of Cochrane Controlled Trials [24].

Screening literature

Retrieved titles and abstracts were screened for inclusion eligibility and applicability by one researcher for search 1 and two separate researchers for search 2. Articles not related to the research question or not meeting inclusion criteria were discarded and the reason for exclusion was noted. Remaining articles were screened as full text and included in the final analysis if they met inclusion criteria. Abstracts concerning conference meetings were included in the search to prevent publication bias; however abstracts not containing adequate information related to research questions were discarded. Bibliographies of all included articles and of review articles that were excluded from the meta-analysis were reviewed for missed relevant articles.

Data extraction

Two investigators extracted data from all included articles for search 1 (author KEC) and for search 2 (author SD) using validated abstraction forms [Genetic Metabolic Dietitian International (GMDI)/Southeast Regional Collaborative (SERC) Evidence Abstract Worksheet (search 1) [25], Cochrane Renal Group protocol guidelines appendix 4 (search 2) [26]]. Data extracted included characteristics of study populations and

control groups, study design, outcome measures, results, and limitations. Outcome measures of bone status were BMD [total body (TBMD), lumbar spine (LBMD) and/or femoral bone (FBMD)]; BTM; BMC; incidence or prevalence of osteopenia, osteoporosis, low BMD, or fractures; vitamin D and/or parathyroid hormone (PTH) status; and other indicators. DXA is the preferred and most commonly reported method to measure BMD in both children and adults [27]-[29]. Studies of bone in patients with PKU primarily report DXA estimates of BMD; however, other techniques such as pQCT, QUS and several X-ray methods used to measure BMD are available and are compared elsewhere [30],[31]. We included studies measuring BMD using any recognized method.

Quality appraisal search 1

The Academy of Nutrition and Dietetics Evidence Analysis Process (AND EA Process [32]) was adapted by the GMDI/SERC effort to create nutrition management guidelines [25] for inborn disorders of metabolism and applied as the foundation for search 1. The AND Evidence Analysis Process provides a method to abstract data and assign a quality grade to primary and review articles retrieved through systematic searches. All included articles were reviewed, graded, and abstracted by author KEC, trained in the Evidence Analysis Process through participation in the development of PKU guidelines [33].

Quality criteria checklists (QCCs) were completed for all studies included in search 1. Each QCC included four relevance questions addressing the purpose and applicability of the study and 10 validity questions with a varying number of sub-questions (Tables 4-1 and 4-2). Answers to validity questions were used to assign a quality score of positive, negative or neutral to each article. For a positive quality rating,

specific validity questions including an unbiased selection of patients, comparable study groups (i.e. matched controls for age, height and weight), sufficient description of study intervention and procedures, and clearly defined outcomes were required. Articles that did not meet these validity criteria, but did include other strengths were assigned a neutral quality rating. Articles that did not contain most of the validity components (6 out of 10 or more) received a negative quality rating.

Table 4-1. **Quality criteria checklist used in search 1— Primary research**

	Relevance Questions
1.	Would implementing the studied intervention procedures (if found successful) result in improved outcomes for the patients/clients/ population group? (N/A for some Epidemiological studies)
2.	Did the authors study an outcome (dependent variable) or topic that the patients/clients/population group would care about?
3.	Is the focus of the intervention or procedure (independent variable) or topic of study a common issue of concern to dietetics practice?
4.	Is the intervention or procedure feasible? (N/A for some Epidemiological studies)
	Validity questions
1.	**Was the research question clearly stated?**
1.1	Was the specific intervention(s) or procedure (independent variable(s)) identified?
1.2	Was the outcome(s) (dependent variable(s)) clearly indicated?
1.3	Were the target population and setting specified?
2.	**Was the selection of study subjects/patients free from bias?**
2.1	Were inclusion/exclusion criteria specified (e.g. risk, point in disease progression, diagnostic or prognosis criteria), and with sufficient detail and without omitting criteria critical to the study?
2.2	Were criteria applied equally to all study groups and/or all subjects?
2.3	Were health, demographics, and other characteristics of subjects described?
2.4	Were the subjects/patients a representative sample of the relevant population?
3	**Were study groups comparable?**
3.1	Was the method of assigning subjects/patients to groups described and unbiased? (Method of randomization identified if Randomized Controlled Trial (RCT))
3.2	Was the distribution of disease status, prognostic factors, and other factors (e.g. demographics) at baseline similar across study groups (original or created post hoc)?
3.3	Were concurrent controls used? (Concurrent preferred over historical controls.)

3.4	If cohort study or cross-sectional study, were groups comparable on important confounding factors and/or were preexisting differences accounted for by using appropriate adjustments in statistical analysis? (Criterion may not be applicable in some cross-sectional studies.)
3.5	If case control study, were potential confounding factors comparable for cases and controls? (If case series or trial with subjects serving as own control, this criterion is not applicable.)
3.6	If diagnostic test, was there an independent blind comparison with an appropriate reference standard (e.g., "gold standard")?
4.	**Was method of handling withdrawals described?**
4.1	Were follow-up methods described and the same for all groups and/or all subjects?
4.2	Was the number, characteristics of withdrawals (i.e., dropouts, lost to follow up, attrition rate), and/or response rate (cross-sectional studies) described for each group? (Follow up goal for a strong study is 80%)
4.3	Were all enrolled subjects/patients (in the original sample) accounted for?
4.4	Were reasons for withdrawals similar across groups?
4.5	If diagnostic test, was decision to perform reference test not dependent on results of test under study?
5.	**Was blinding used to prevent introduction of bias?**
5.1	In intervention study, were subjects, clinicians/practitioners, and investigators blinded to treatment group, as appropriate?
5.2	Were data collectors blinded for outcomes assessment? (If outcome is measured using an objective test, such as a lab value, this criterion is assumed to be met.)
5.3	In cohort study or cross-sectional study, were measurements of outcomes and risk factors blinded?
5.4	In case control study, was case definition explicit and case ascertainment not influenced by exposure status?
5.5	In diagnostic study, were test results blinded to patient history and other test results?
6.	**Were intervention/therapeutic regimens/exposure factor or procedure and any comparison(s) described in detail? Were intervening factors described?**
6.1	In RCT or other intervention trial, were protocols described for all regiments studied?
6.2	In observational study, were interventions, study settings, and clinicians/provider described?
6.3	Was the intensity and duration of the intervention or exposure factor sufficient to produce a meaningful effect?
6.4	Was the amount of exposure and, if relevant, subject/patient compliance measured?
6.5	Were co-interventions (e.g., ancillary treatments, other therapies) described?
6.6	Were extra or unplanned treatments described?

6.7	Was the information for 6.4, 6.5, and 6.6 assessed the same way for all groups?
6.8	In diagnostic study, were details of test administration and replication sufficient?
7.	**Were outcomes clearly defined and the measurements valid and reliable?**
7.1	Were primary and secondary endpoints described and relevant to the question?
7.2	Were nutrition measures appropriate to question and outcomes of concern?
7.3	Was the period of follow-up long enough for important outcome(s) to occur?
7.4	Were the observations and measurements based on standard, valid, and reliable data collection instruments/tests/procedures?
7.5	Was the measurement of effect at an appropriate level of precision?
7.6	Were other factors accounted for (measured) that could affect outcomes?
7.7	Were the measurements conducted consistently across groups?
8.0	**Was the statistical analysis appropriate for the study design and type of outcome indicators?**
8.1	Were statistical analyses adequately described the results reported appropriately?
8.2	Were correct statistical tests used and assumptions of test not violated?
8.3	Were statistics reported with levels of significance and/or confidence intervals?
8.4	Was "intent to treat" analysis of outcomes done (and as appropriate, was there an analysis of outcomes for those maximally exposed or a dose-response analysis)?
8.5	Were adequate adjustments made for effects of confounding factors that might have affected the outcomes (e.g., multivariate analyses)?
8.6	Was clinical significance as well as statistical significance reported?
8.7	If negative findings, was a power calculation reported to address type 2 error?
9.	**Are conclusions supported by results with biases and limitations taken into consideration?**
9.1	Is there a discussion of findings?
9.2	Are biases and study limitations identified and discussed?
10.	**Is bias due to study's funding or sponsorship unlikely?**
10.1	Were sources of funding and investigators' affiliations described?
10.2	Was there no apparent conflict of interest?

Table 4-2. **Quality criteria checklist used in search 1 — Reviews**

	Relevance Questions
1.	Will the findings of the review, if true, have a direct bearing on the health of patients?
2.	Is the outcome or topic something that patients/clients/population groups would care about?

3.	Is the problem addressed in the review one that is relevant to dietetics practice?
4.	Will the information, if true, require a change in practice?
	Validity questions
1.	Was the research question clearly focused and appropriate?
2.	Was the search strategy used to locate relevant studies comprehensive? Were the databases searched and the search terms use described?
3.	Were explicit methods used to select studies to include in the review? Were inclusion/exclusion criteria specified and appropriate? Were selection methods unbiased?
4.	Was there an appraisal of the quality and validity of studies included in the review?
5.	Were specific treatments/interventions/exposures described? Were treatments similar enough to be combined?
6.	Was the outcome of interest clearly indicated? Were other potential harms and benefits considered?
7.	Were processes for data abstraction, synthesis, and analysis described? Were they applied consistently across studies and groups? Was there appropriate use of qualitative and/or quantitative synthesis? Was variation in findings among studies analyzed? Were heterogeneity issues considered? If data from studies were aggregated for meta-analysis, was the procedure described?
8.	Are the results clearly presented in narrative and/or quantitative terms? If summary statistics are used, are levels of significance and/or confidence intervals included?
9.	Are conclusions supported by results with biases and limitations taken into consideration? Are limitations of the review identified and discussed?
10.	Was bias due to the review's funding or sponsorship unlikely?

Assessment of bias search 1

QCC-derived quality ratings reflected the likelihood of bias in each study. Those rated positive were unlikely to contain significant bias, while those with neutral quality ratings included some elements likely to produce bias. Negative quality ratings indicated that bias in the study was very likely and these articles were excluded from the review.

Quality appraisal search 2

Quality appraisal and assessment of bias were performed for search 2 on all assessed full text articles by two separate researchers (SD and AMB) and outcomes were discussed. The 'Scottish Intercollegiate Guidelines Network' (SIGN) checklists were

used [34] to assess quality based on the study design (RCT, cohort or case–control study). SIGN checklists are based on the Grading of Recommendations Assessment, Development and Evaluation (GRADE) [35] approach. Articles were appraised as of low, acceptable or high quality and those assessed as low quality were excluded from the review.

Assessment of bias search 2

Quality ratings reflected the likelihood of bias in each study. Those rated high quality were unlikely to contain significant bias, while those with acceptable quality ratings included or did not include some elements likely to produce bias. Low quality ratings indicated bias in the study was likely and these studies were not included in the review.

Statistical analysis

Z-scores and T-scores for BMD are calculated to clinically assess an individual's bone status. T-scores describe the number of standard deviations (SD) by which a patient's BMD differs from the expected mean value in a healthy young adult. The World Health Organization (WHO) defines osteopenia in adults as a T-score between -1 and -2.5, and osteoporosis as a T-score below -2.5 [29]. Z-scores describe the number of SDs by which the BMD in an individual differs from the mean value expected for age and sex. For children, Z-scores are mostly used. The International Society for Clinical Densitometry (ISCD) states that the diagnosis of osteoporosis in children, premenopausal women and males under 50 years of age should not be based on densitometric criteria alone. Instead, a BMD Z-score below -2 is defined as "BMD below the expected range for age" or "low BMD for chronological age" and cannot be defined as osteoporosis

unless coupled with a significant fracture history [28]. The ISCD does however stress that Z-scores above -2 do not preclude the possibility of skeletal fragility. Most recent studies of bone health in PKU report BMD Z-scores only [14],[36], because the patient population is relatively young and pediatric patients are assessed together with adult patients. BMD can be measured at a variety of locations and we included studies that measured BMD in total body, spine and femur. Spinal BMD reflects BMD in trabecular bone, and femoral BMD is significant for cortical bone [37].

Qualitative and quantitative analyses were performed to assess BMD in patients with PKU. Qualitative analysis was performed to review evidence on bone health and assess the prevalence of low BMD in patients with PKU. Quantitative analysis was performed in the form of a meta-analyses to analyze whether BMD Z-scores are different in patients with PKU than reference values (deviant from 0 SD of reference). If the full text of an article did not contain BMD or BMD Z-scores, the authors were contacted to obtain data. The meta-analysis was performed in Review Manager [38]; a fixed effects model or random effects model was used to pool the patient-based data. The choice for selecting a fixed effects model or a random effects model was based on heterogeneity of the data per meta-analysis. Low heterogeneity between studies led to the use of a fixed effects model, and high heterogeneity to the use of a random effects model to pool data [39],[40]. Heterogeneity was tested by calculating I^2 (heterogeneity is low when I^2 is $\leq 25\%$, moderate if $25 \leq 50\%$ and high if $\geq 75\%$) [39]. The presence of publication bias was visually assessed by means of a funnel plot and calculation of Egger's test with statistical software (IBM SPSS statistics 20) [40]. By using outcomes from a specific

population (early diagnosed and treated patients with PKU), it should be noted that the effect cannot be extrapolated to other patients with PKU.

To assess BMD Z-scores considered low for chronological age (below -2), we used a normal distribution curve to estimate prevalence in a healthy population and developed a PKU-specific normally distributed curve based on estimated effect size for LBMD. Since these estimates are hypothetical, we also calculated prevalence in a normal population using the 2007–2008 National Health and Nutrition Examination Survey (NHANES) [41] data. We limited the analysis to participants 8–45 years of age with lumbar spine and femoral neck BMD measurements since NHANES does not collect data in children under eight years of age, and included studies in this review do not report on patients over the age of 45 years (older patients are often late diagnosed). Z-scores for age and sex were calculated using the Centers for Disease Control and Prevention's (CDC) references [42] for lumbar spine and femoral neck BMD. We used the computer program SAS-Callable SUDAAN 11.0.1 to calculate weighted population prevalence of low BMD for chronological age (Z-score <-2), taking into account primary sampling units and strata. To calculate final prevalence, we limited analyses to a subpopulation of non-Hispanic Caucasian participants only.

All other outcomes including BTM, BMC, and blood vitamin D and PTH status were evaluated qualitatively. Overview tables of results were generated to summarize results and draw conclusions. Factors examined in association with bone-related outcomes were included in overview tables with statistical methods, direction of association, and statistical and clinical significance. If analyses controlled for variables, these were also noted. Factors significantly associated with bone-related outcomes in

multiple studies were noted to identify potential underlying causes of low BMD. Finally, all data collected and summarized through quality appraisal and overview tables were used to identify gaps in the bone health evidence base in PKU.

4.4 Results

Study selection

Twenty-three articles were included in this review as a result of study selection criteria for search 1, resulting from 437 initially identified records from both EMBASE and MEDLINE® (Figure 4-1).

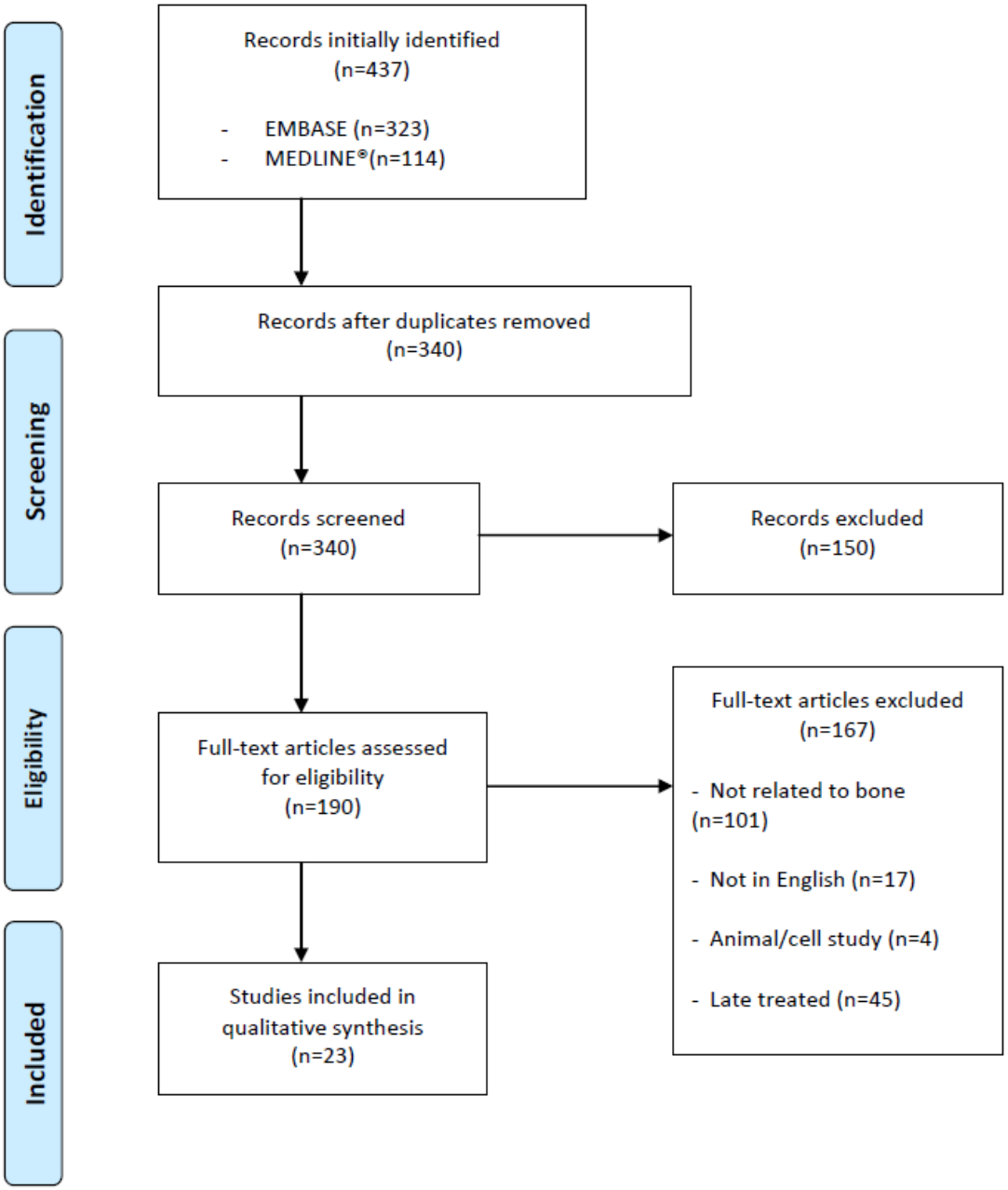

Figure 4-1. Inclusion process flow diagram for search 1 (PRISMA 2009[[22]]).

A total of 13 articles were included for search 2 after selection from 418 initially identified records from EMBASE, The Cochrane Library and MEDLINE® (Figure 4-2).

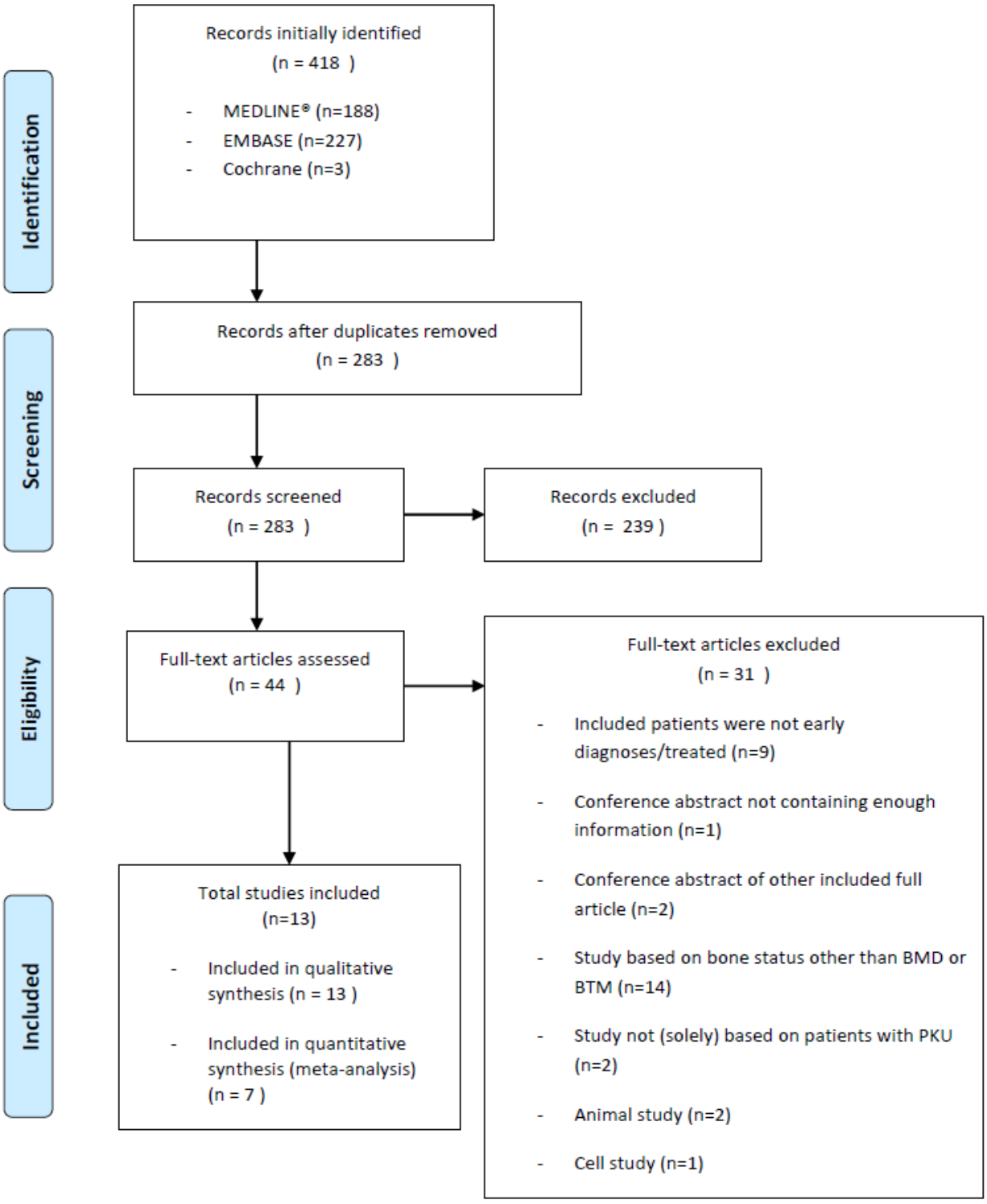

Figure 4-2. Inclusion process flow diagram for search 2 (PRISMA 2009[[22]]).

Included studies

Articles included in search 1 and search 2 were combined and 23 unique articles were identified. We found that both study teams included 13 records (57%), listed in Additional file 1: Table S1 with their study characteristics. Studies were published between 1994 and 2013, with the majority published since 2000 (69%). Twelve were cohort studies whereas one was a case–control study. Search 1 included 10 articles that were not included in search 2. Most of these articles were published before 2000 (80%) and identified due to differences in search terms (n = 5) or discrepancy in quality ratings (n = 5). Of these 10 articles, 5 were discarded when appraised as low quality in search 2 and the remaining 5 articles did not address BMD and were therefore excluded in search 2. We limited our analyses to articles included in both searches only (n = 13).

Quality appraisal

Of the 13 included studies, seven (54%) were graded neutral quality and six (46%) were graded positive quality according to the AND Evidence Analysis Process applied during search 1 (Figure 4-3). As defined by SIGN checklists, applied during search 2, the majority of papers were graded acceptable quality (n = 11) and two [12],[21] were high quality (Figures 4-4 and 4-5).

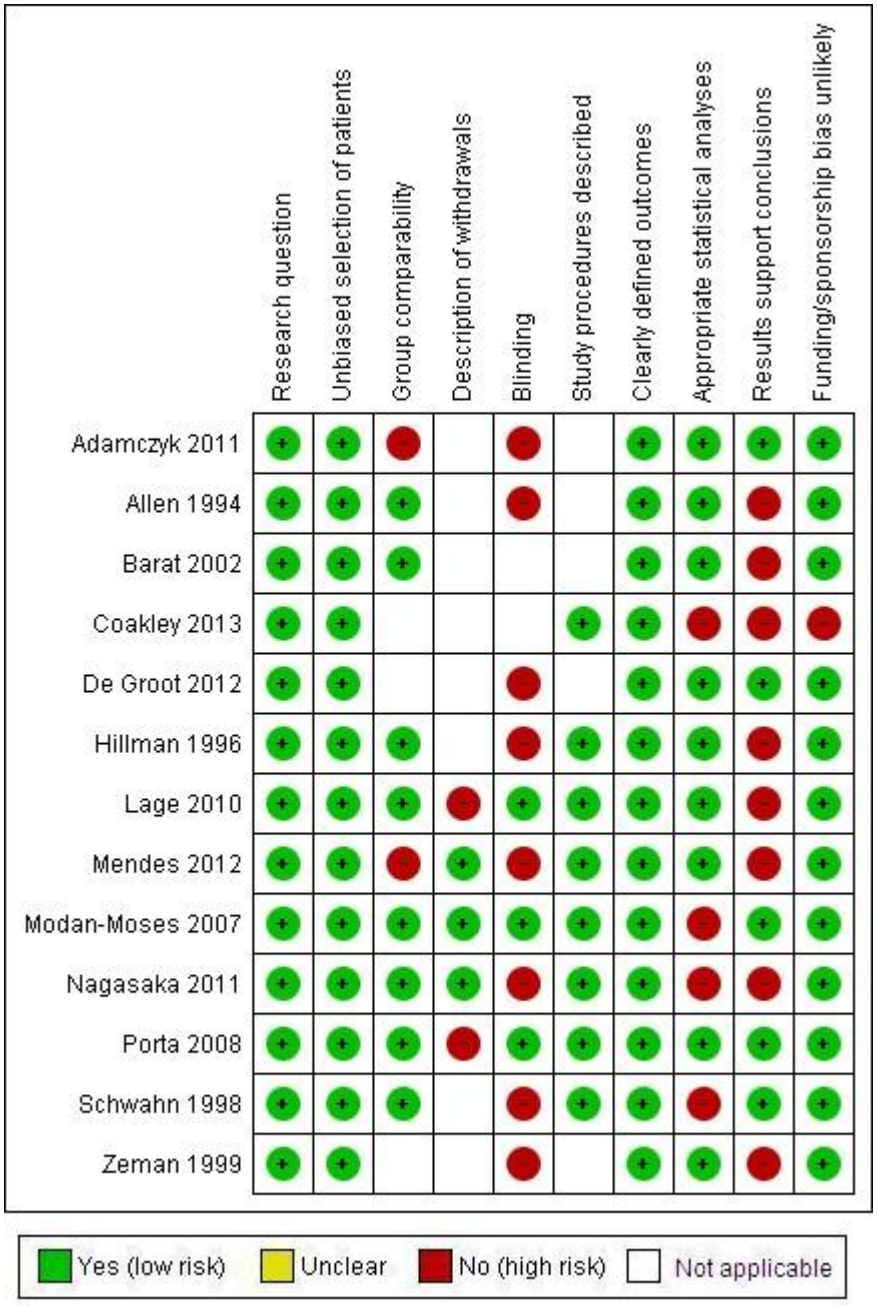

Figure 4-3. Risk of bias summary table search 1.

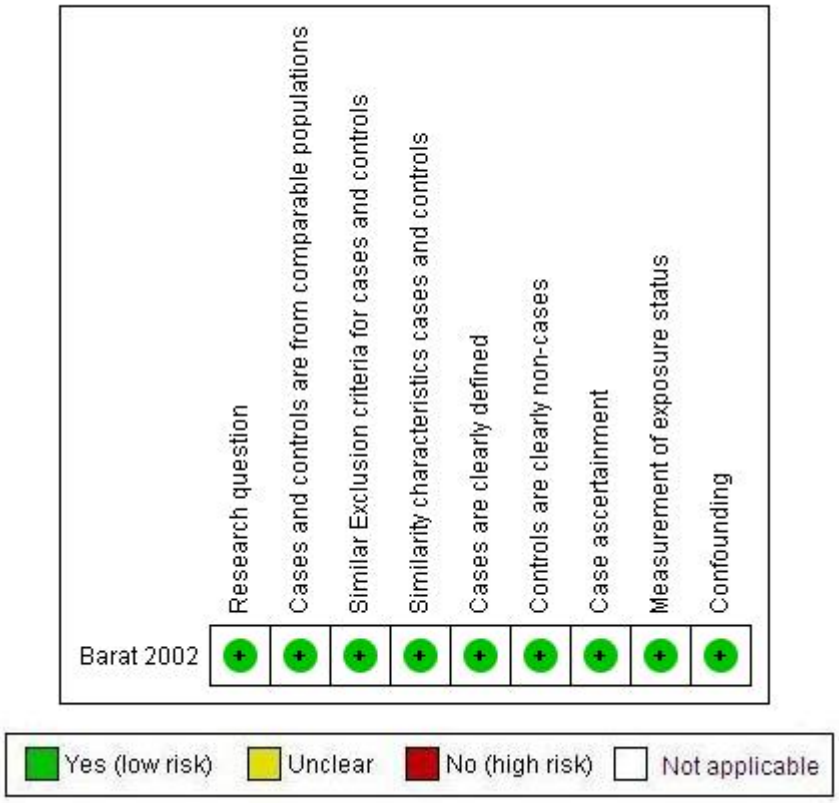

Figure 4-4. Risk of bias summary table case–control study search 2.

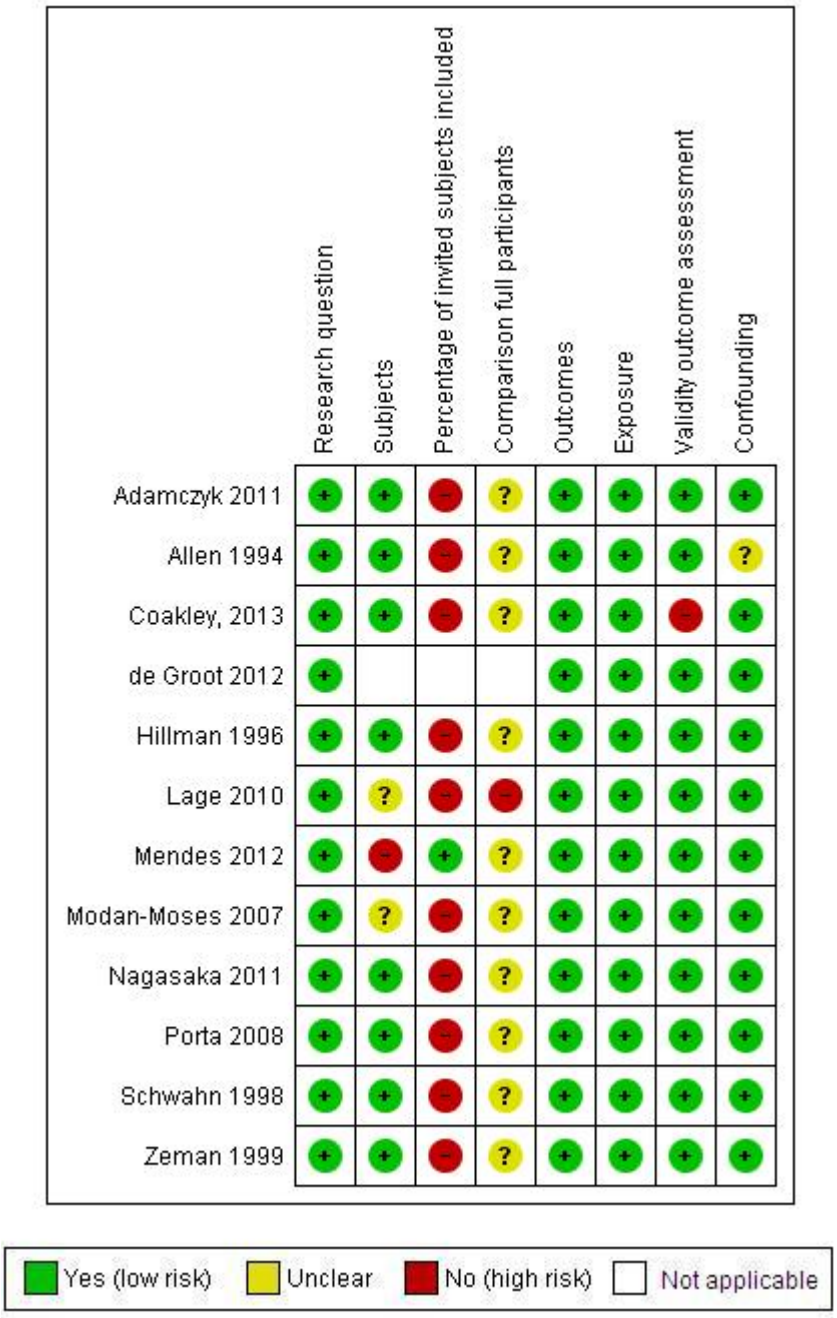

Figure 4-5. Risk of bias summary table cohort studies search 2.

While quality was determined on separate scales by each search team, scores of eight articles (62%) corresponded between AND and SIGN quality scores. Seven of the eight were assessed as neutral quality by AND criteria in search 1 and a corresponding

acceptable quality by SIGN checklists in search 2. One article was graded positive quality by AND criteria and a corresponding high quality by SIGN checklist. The remaining five of the 13 (38%) included studies with quality ratings that did not agree between AND and SIGN scales. All five were scored with the highest quality rating (positive) by AND criteria in search 1, but rated acceptable quality by SIGN checklists in search 2. Most of these papers did not fully describe the number of patients recruited for the study versus the number of actual study, a requirement for a high quality rating on the SIGN checklist. Two articles that were scored positive based on AND criteria also scored very well on the SIGN checklists, but they were retrospective studies, automatically disqualifying them for a rating above acceptable quality [14],[43].

BMD in early treated patients with PKU

A total of 11 articles studied BMD in early treated patients with PKU; 10 cohort studies and one case–control study. Combined, a total of 360 patients (range 11 – 57 per study) were included. Five studies included pediatric patients only, one study selectively included adult patients [44], and 5 studies included pediatric and adult patients. Ten of the 11 studies found that BMD was significantly lower in patients with PKU compared to a reference group or controls. A single study, including children and adolescents, did not find altered BMD in patients with PKU [20].

BMD in pediatric patients with PKU

All 5 studies that included pediatric patients used DXA to measure BMD. Four reported a reduced BMD in patients and one study did not find a significantly altered LBMD when comparing 8 pediatric patients with PKU to a control population [20]. In this study, however, two of the eight (25%) patients had a LBMD Z-score below -2,

meeting the criteria for low BMD for pediatric patients defined by the ISCD [28],[45]. Both are described as adolescent patients not adherent to diet.

Of the 4 articles reporting lower BMD in patients with PKU, Adamczyk et al. [13] described a group of 45 children (mean age 13.8 ± 5.2 years) and concluded that skeletal status is impaired in patients with PKU (mean Z-score LBMD -0.572 ± 1.270 and TBMD -0.117 ± 1.347). They also found that in patients who were sexually mature, those who were non-adherent to diet had a significantly lower BMD than those who adhered to diet. Furthermore, Barat et al. [43] investigated a group of 13 pediatric patients with PKU, reporting a mean LBMD Z-score of -1.36 ± 1.586.

Similarly, a study by Hillman et al. [37] established that BMD at multiple sites was significantly lower in a group of 11 pediatric patients with PKU compared to age-matched controls [LBMD 0.61 ± 1.5 g/cm^2 vs 0.72 ± 0.24 g/cm^2 and FBMD 1.56 ± 0.30 g/cm^2 vs 1.87 ± 0.56 g/cm^2].

Finally, Allen et al. [17] investigated 32 pre-pubertal patients (mean age 7.7 ± 2.3 years) and found significantly lower BMD compared to age-matched, non-PKU controls (TBMD 0.770 ± 0.085 g/cm^2 vs 0.814 ± 0.075 g/cm^2 and LBMD 0.619 ± 0.100 g/cm^2 vs 0.701 ± 0.097 g/cm^2). TBMD of patients with PKU was 97.1% of predicted BMD for children of the same gender and age while LBMD was 92% of predicted BMD. Clinical fracture risk was not directly evaluated by any of the studies

BMD in pediatric and adult patients with PKU

Four of the 5 studies that described a mixed group of pediatric and adult patients used DXA to assess BMD and 1 study [12] used pQCT to assess BMD in the radius. All 5 studies reported altered BMD in patients with PKU.

A conference abstract by Coakley et al. [46] reported TBMD in a population of 57 patients over 4 years of age. The authors found that 16 patients (28%) had a TBMD Z-score between -1 and -2.5 and three patients (5%) had a Z-score below -2.5. TBMD was positively correlated with age (controlling for BMI, sex, metabolic control, and medical food intake) in their population with a mean age of 17.5 years. Similar results were obtained by three other studies. de Groot et al. [14] reported a mean LBMD Z-score of -0.78 ± 1.1 in a group of 53 patients with PKU and low BMD (LBMD Z-score below -2) in 10 patients (19%). A subgroup analysis showed that younger patients had a higher prevalence of low BMD though no significant correlations were established between BMD and age.

Lage et al. [15] investigated BMD in 47 patients with PKU and found a mean Z-score significantly below 0 (mean FBMD Z-score -1.2 ± 1.0; LBMD Z-score -0.4 ± 0.8). A Z-score between -1 and -2.5 was found in 13 patients (28%) and a Z-score below -2.5 in 6 patients (13%) of at least one site. The authors found a negative correlation between age and LBMD in patients 6–10 years of age and a positive correlation between age and FBMD in patients 11–18 years of age.

Zeman et al. [16] studied 44 patients with PKU and described that 14 (32%) had a TBMD Z-score below -1 and 20 patients (45%) had a LBMD Z-score below -1, of whom 6 had a Z-score below -2.5. No correlation between age and LBMD or TBMD was evident.

A final study by Schwahn et al. [12] used pQCT in 14 patients with PKU ages 5–28 years to assess BMD of both spongy and total bone of the non-dominant distal radius. They found that spongy bone BMD was significantly lower in patients with PKU

compared to 14 age, gender, weight and height-matched controls [139.7 ± 23.5 mg/cm^3 vs 169.3 ± 31.5 mg/cm^3]. Mean total bone BMD of the radius in patients with PKU was slightly lower than controls, but not significant. Within the group of PKU patients, TBMD and LBMD were lower in adolescents ages 13–16 years compared to younger children and adults. The authors hypothesized that patients with PKU have altered trabecular bone architecture indicated by low spongy bone BMD and/or altered mineralization, but show minor changes of cortical bone. They emphasize this hypothesis by describing the case of an untreated severely retarded female patient who showed lower BMD, especially of trabecular bone, at 10 years of age, which could not be explained by a history of malnutrition or immobilization.

BMD in adult patients with PKU

The only study included in this review examining exclusively adult patients is by Modan-Moses et al. [44]. In a group of 31 patients, 42% had compromised BMD (Z-score <-1). Mean TBMD Z-scores (-0.474 ± 0.719) and FBMD Z-scores (-0.727 ± 0.66) were significantly lower than expected for individuals of the same sex and age without PKU ($p = 0.002$ and $p < 0.001$, respectively). Mean LBMD was also lower than expected, but not statistically significant.

Prevalence of compromised bone status

Five studies examined the prevalence of low BMD. In cohort studies, prevalence of osteopenia (defined in all papers as a Z-score between -1 and -2.5) ranged from 28–46% [15],[16],[20],[44],[46]. A single study estimated the prevalence of osteopenia retrospectively, finding 62% of children with PKU had a Z-score between -1 and -2.5 at age 12 [43]. The prevalence of osteoporosis (defined in each study as a BMD Z-score

below -2.5) ranged from 5–14% [15],[16],[20],[44],[46]. A single study defined low BMD as a Z-score below -2, consistent with ISCD recommendations, and reported a prevalence of 19% in children and adults [14]. Seven studies included in this review did not report the prevalence of low BMD [12],[13],[17],[19]-[21],[37], and none of the 13 studies reported BMD T-scores.

In known literature databases, we found no reports on low BMD prevalence (Z-score <-2) in a reference population of adolescents or young adults for comparison. A self-performed pilot analysis of weighted NHANES data [41], however, suggests Z-scores between -1 and -2.5 are found in 14.9% (95% CI 12.6–17.4%) at the proximal femur and 14.3% (95% CI 12.1–17.0%) at the lumbar spine in non-Hispanic Caucasians ages 8–45 years. Z-scores below -2.5 were found in an additional 0.13% (95% CI 0.03–0.53%) at the proximal femur and 0.53% (95% CI 0.13–2.10%) at the lumbar spine. The prevalence of low BMD for chronological age, defined by ISCD criteria as a Z-score below -2, was 1.8% (95% CI 1.0–3.3%) at the lumbar spine and 1.6% (0.8–3.0%) at the proximal femur in NHANES data. These findings confirm a normal distribution of BMD in the general population, in which a 2.3% of the population would be expected to have a score below -2 SD.

Height-corrected bone mineral density

Three studies included a height-correction of DXA measured BMD to correct for height bias. Adamczyk et al. [13] reported SD-scores on DXA results with correction for patient height and gender. TBMD and LBMD SD scores were significantly lower in adolescent patients who were not compliant with diet compared to compliant patients.

Total body and lumbar spine BMC SD scores were also significantly lower in non-compliant versus compliant patients.

Allen et al. [17] reported lower LBMD in children with PKU compared to controls, adjusting for height and weight. There was no difference in mean age and SD height and weight scores between the PKU and control children. Based on predictions for LBMD derived from control data, LBMD of the children with PKU was 92% of what was expected.

De Groot et al. [14] report a positive correlation between BMD and height in children with PKU under age 18, but not in adults. They conclude Z-scores of BMD found in their whole study population (n = 53; mean age 16.7 ± 9.1) are not significantly correlated to height and weight.

Blood Phe levels and BMD

Nine studies investigated the correlation between Phe blood levels and BMD [3],[13]-[17],[37],[44],[46], seven of which found no correlation [14]-[17],[37],[44]. de Groot et al. [14] found no significant correlations between BMD and frequency or proportion of Phe blood concentrations below the recommended threshold, or the mean cumulative variation of blood Phe concentrations.

Two studies, however, did find a negative correlation between Phe levels and BMD [13],[43]. First, Barat et al. [43] describe that although mean Phe concentration did not correlate with BMD outcomes, patients with BMD Z-scores below -1 had a significantly higher mean cumulative Phe variation than controls [3.1 ± 0.4 mg/dl versus 2.5 ± 0.4 mg/dl (p-value = 0.006)]. Based on their findings, the researchers suggest variations of Phe concentrations may contribute to lower BMD in children with PKU.

Second, Adamczyk et al. [13] report that among pediatric patients who had reached sexual maturity, those who were compliant to diet had significantly higher BMD Z-scores, and lower plasma Phe levels than non-compliant patients. A regression analysis also showed serum Phe concentration had the most negative influence on BMD values of all variables examined including demographics such as age, sex, and body mass index (BMI).

Dietary intake and BMD

Seven cohort studies examined the impact of total protein and/or medical food protein intake on BMD [13],[15]-[17],[37],[44],[46]. Evidence is consistent for total protein intake with 4 studies reporting no correlation with BMD [17],[37],[44],[46]. Studies assessing medical food protein intake and BMD, however, are inconsistent. Coakley et al. [46] found a positive correlation between medical food prescription (grams of protein per day) and actual medical food intake and TBMD. Zeman et al. [16] reported no correlation between daily intake of Phe-free amino acid mixture per kilogram body weight and TBMD or LBMD Z-scores.

Meta-analysis of BMD in early treated patients with PKU

A meta-analysis was performed on mean BMD Z-scores in the spine (7 studies), whole body (3 studies) and femur (2 studies). All studies used DXA to measure BMD. Pooling of data was performed by using available BMD Z-scores provided by the authors, either in the article [14],[20],[44],[46] or through added information on request per e-mail [13],[15],[43],[46]. A fixed or random effects model of generic inversed variance was used to examine the mean difference between patients with PKU and normal values for healthy age and sex-matched controls (BMD Z-score = 0).

Exploration of heterogeneity

Seven papers measured LBMD for a total of 247 patients [13]-[15],[20],[43],[44],[46]. Mean Z-scores ranged from -1.363 to -0.4 (Figure 4-6). A moderate heterogeneity was observed (I^2 = 59%), justifying the pooling of results and the use of a fixed effects model [39],[47].

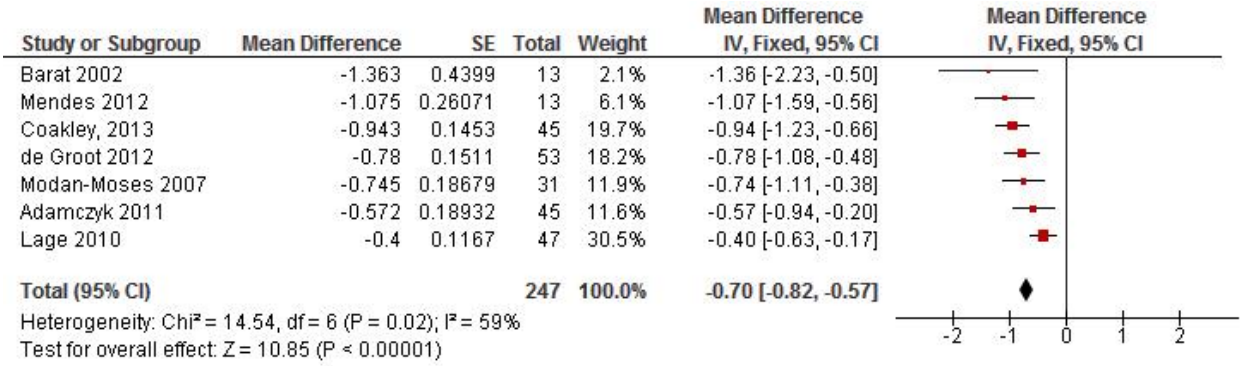

Figure 4-6. Forest plot of LBMD (Z-score) in patients with Phenylketonuria.

(SE = standard error, IV = Inverse Variance, CI = confidence interval).

Three papers measured TBMD for a total of 133 patients [13],[44],[46]. Mean Z-scores ranged from -0.55 to -0.12 (Figure 4-7). A moderate heterogeneity was seen (I^2 = 42%), justifying the pooling of results by the use of a fixed effects model [39],[47].

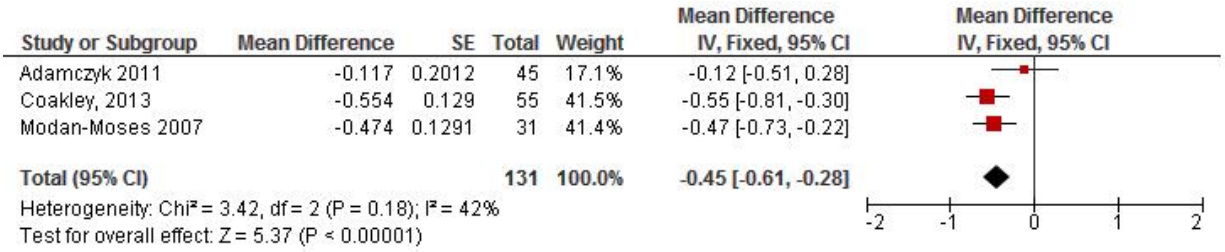

Figure 4-7. Forest plot of TBMD (Z-score) in patients with Phenylketonuria.

(SE = standard error, IV = Inverse Variance, CI = confidence interval).

Two papers were available providing FBMD for a total of 78 patients [15],[44]. Mean Z-scores ranged from-1.2 to-0.727 (Figure 4-8). High heterogeneity between the

two studies was observed ($I^2 = 84\%$), probably due to the low amount of included studies, therefore a random effects model was used to pool patients-based results [39],[47].

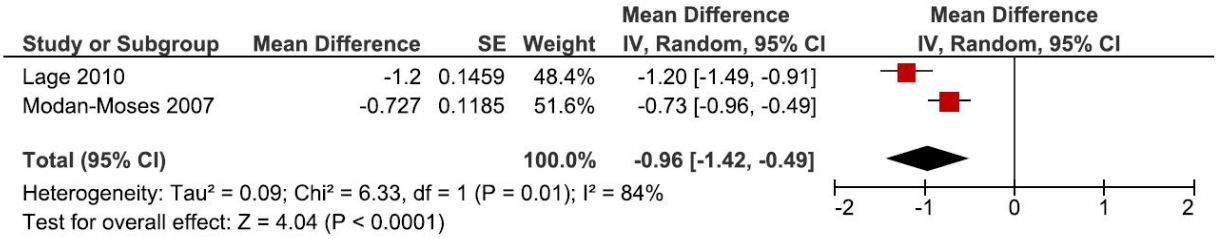

Figure 8. Forest plot of FBMD (Z-score) in patients with Phenylketonuria.

(SE = standard error, IV = Inverse Variance, CI = confidence interval).

Assessment of publication bias

There was no evidence of publication bias for LBMD as visual assessment of the funnel plot (Figure 4-9) shows a symmetrically distributed inversed funnel and Egger's test was not significant ($p = 0.407$). Evaluation of publication bias by funnel plot or Egger's test for TBMD and FBMD was not reliable due to the limited number of studies included.

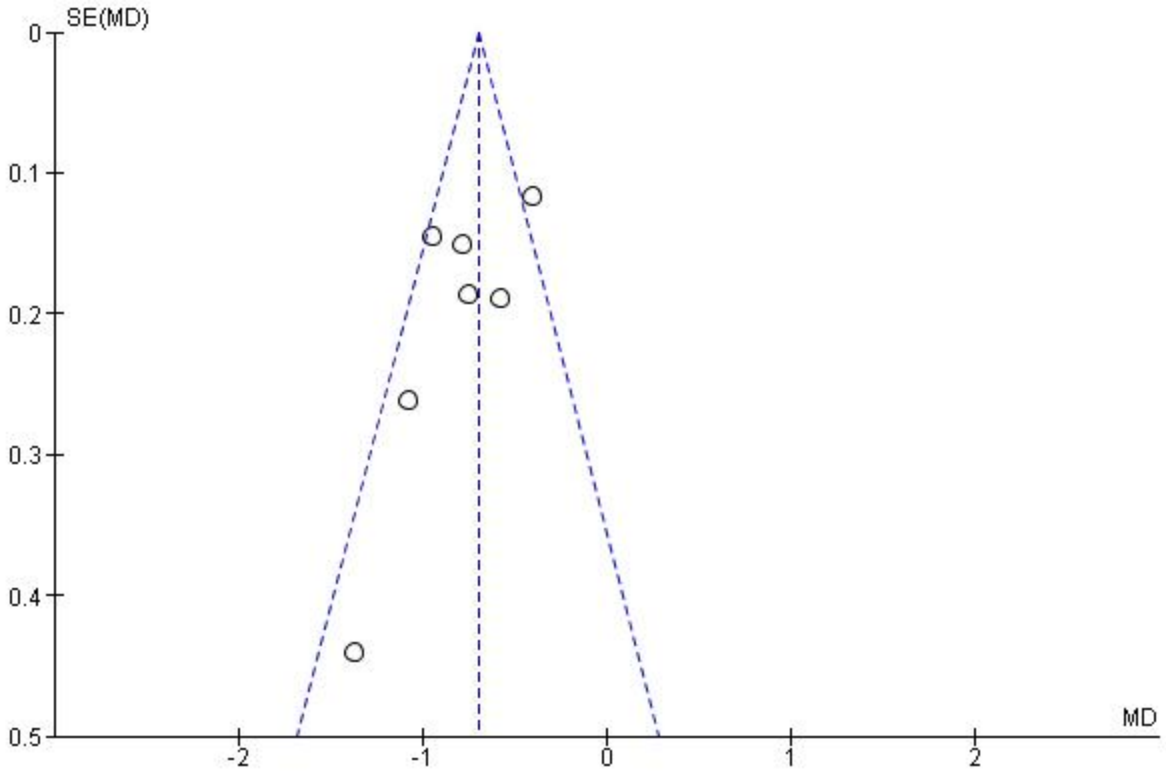

Figure 4-9. Funnel plot LBMD (Z-score) in patients with PKU. (SE = standard error; MD = mean difference) (Egger's test: p = 0.407).

Pooled patient-based BMD

In 247 pooled patients with PKU included in 7 studies, mean LBMD Z-score was -0.70 (95% CI-0.82,-0.57). The overall effect is significantly different (P < 0.00001) from the norm and none of the individual studies crossed the line of no effect (Figure 4-6).

In 133 pooled patients with PKU included in 3 studies, mean TBMD Z-score was -0.45 (95% CI-0.61,-0.28). The overall effect is significantly (P < 0.00001) different from the norm (Figure 4-7). One of the individual studies crossed the line of no effect [13].

In 78 pooled patients with PKU included in 2 articles, mean FBMD Z-score was -0.96 (95% CI-1.42,-0.49). The overall effect is significantly different (P < 0.0001) from the norm and both included studies do not cross the line of no effect (Figure 4-8).

Bone turnover markers in early treated patients with PKU

Four cohort studies examined BTM in patients with PKU. Combined, a total of 110 patients (range 11 – 45 per study) were included. Two studies included only pediatric patients [13],[37], one study included only adult patients [21] and one study included both pediatric and adult patients [48]. All included studies found significant alterations in one or more BTM.

Adamczyk et al. [13] measured 3 bone formation markers including carboxyterminal telopeptide of type I collagen (CTx), bone-specific alkaline phosphatase (bALP) and osteocalcin in serum of 45 pediatric patients with PKU. They compared BTM by subgroups of patients based on sexual maturity and compliance to diet, but did not provide mean values for the group as a whole. Among those compliant with diet, sexually immature patients (Tanner stage below 5) had higher P1NP (10.33 ± 2.97 lg/l vs 6.62 ± 2.10 lg/l) and bALP (75.67 ± 49.60 U/l vs 30.67 ± 37.05 U/l) compared to sexually mature patients.

On the other hand, among sexually mature patients, differences were found between non-compliant and compliant patients including higher bALP (63.0 ± 46.43 U/l vs 30.67 ± 37.05 U/l) and higher osteocalcin (48.87 ± 23.0 ng/ml vs 33.15 ± 11.88 ng/ml). These findings are in line with physiological concentrations of BTM, which are increased during active periods of bone remodeling including growth in childhood and pre-pubertal adolescence [49],[50].

Hillman et al. [37] assessed BTM in 11 children with PKU in comparison to 11 age-matched controls. Bone formation markers bALP (6.1 ± 6.3 U/l vs 13.1 ± 2.0 U/l) and osteocalcin (72 ± 30 U/l vs 126 ± 43 U/l) were significantly lower in patients with PKU

compared to controls, whereas P1NP was lower (290 ± 174 U/l vs 400 ± 159 U/l) but not significant. Bone resorption markers including urinary tartrate resistant acid phosphatase and calcium creatinine ratio did not differ between subjects and controls.

Nagasaka et al. [21] reported BTM in adult patients (n = 34) compared to age-matched controls (n = 36). The bone resorption markers blood pyridinoline cross-linked telopeptide domain of type I collagen, urinary deoxypyridinoline, and urinary N-telopeptide of type I collagen were significantly higher in patients with PKU than in the control group. Blood osteoprotegerin, an inhibitor of bone resorption, was also significantly lower in individuals with PKU. No differences were found in the bone formation markers bALP and osteocalcin between the PKU and control groups.

Porta et al. [48] examined spontaneous osteoclastogenesis, the differentiation of mature osteoclasts from precursors to initiate the process of bone resorption, in pediatric and adult patients with PKU compared to 20 age and sex-matched controls. Their results show that osteoclasts, generated through spontaneous osteoclastogenesis from peripheral blood monocytes, were larger and nearly double in number compared to those of control subjects.

Blood Phe levels and BTM

Four studies investigated correlations between blood Phe concentrations and individual BTM [13],[21],[37],[48]. Bone formation markers including bALP and osteocalcin were reported as higher in patients with Phe above recommended levels compared to patients with recommended Phe levels [13]. Moreover, mean serum Phe over a period of one year was significantly correlated with the number of osteoclasts, indicators of active bone resorption, in patients with PKU ($r = 0.576$; p-value = 0.010)

[48]. Other studies report no correlation between serum Phe concentrations and BTM [21],[37].

Other indicators of bone status in early treated patients with PKU

Bone mineral content

BMC was examined in a single study [13] by Adamczyk et al. (2011). The authors reported higher total body BMC and spine BMC in mature patients with concurrent recommended threshold Phe levels at time of measurement compared to mature patients with Phe levels above recommendations. Moreover, in non-compliant patients, the total body BMC to lean body mass (LBM) ratio was reduced, an indicator of increased risk for fragility fractures. In compliant patients, however, the BMC/LBM ratio was not different than expected for age and height.

Vitamin D status in patients with PKU

Six included studies measured blood vitamin D status, all are cohort studies [14],[15],[21],[37],[44],[46]. Among the cohort studies, findings varied by age group. One study of 31 adults with PKU showed that all patients had normal 25-hydroxyvitamin D concentrations, the primary indicator of vitamin D status [44]. Two studies report associations between vitamin D and indicator of bone status in children and adults with PKU [14],[46], but do not mention the prevalence of vitamin D deficiency or insufficiency. A case–control study suggests 25-hydroxyvitamin-D and 1,25-dihydroxyvitamin-D concentrations in children with PKU do not differ from controls matched on sex and age [37]. In male and female adults with PKU on the other hand, 1,25-dihydroxyvitamin-D was reported as significantly higher than in controls and 25-hydroxyvitamin-D was significantly lower than controls [21]. Coakley ea. report a

significant positive association between TBMD and 1,25-dihyroxyvitamin D [46]. All other studies report no correlation between plasma 25-hydroxyvitamin-D and BMD at any site [14],[15],[44].

Parathyroid Hormone (PTH) in patients with PKU

Four of the 13 included studies measured PTH, all are cohort studies [13],[21],[37],[44]. Overall, children with PKU have similar PTH concentrations to healthy controls [37], but differences are reported in subgroups. PTH appears to be significantly higher in non-compliant children and adolescents compared to those with recommended Phe levels [13]. PTH is also reported to be higher in female and male adults with PKU compared to controls, but the difference is not statistically significant in males [21]. PTH above the normal reference range was reported in two of 31 (6%) adults with PKU examined in one study [21].

Other indicators of bone status

Fracture history was examined in a single study. Modan-Moses et al. [44] reported that 4 patients (13%) included in their study had a significant fracture history, though all were the result of physical trauma. Two patients had normal BMD, one had a LBMD Z-score of -1.9, and one had a FBMD Z-score of -2.4. Greeves et al. [51] provided the first investigation of fractures in patients with PKU, reporting the risk of fracture is 2.6 times greater in patients with PKU over 8 years of age compared to controls. Though the study did not meet inclusion criteria for this review, Greeves ea. provides the only estimate of fracture risk in known literature on patients with PKU.

Concentrations of vitamins and minerals related to bone metabolism, including calcium, phosphorus and magnesium, were also measured in several studies.

Calcium was measured in six of the 13 included studies [13]-[15],[21],[37],[44]. Serum calcium was reported as significantly lower in children with PKU compared to controls [37], although no difference in total calcium was found between compliant and non-compliant subgroups of children and adolescents with PKU [13]. In adults with PKU, urinary calcium excretion was significantly higher than in controls [21], though all patients' blood calcium concentrations fell within normal range [44]. A negative correlation between blood calcium and BMD Z-score in individuals with PKU of all ages was reported by de Groot et al. [14], but no correlation between plasma calcium and BMD Z-score was found by Lage et al. [15].

Phosphorus was measured in four of the 13 included studies [14],[21],[37],[44]. Children and adults with PKU were reported to have serum phosphorus concentrations within normal ranges and comparable to healthy reference groups [37],[44]. While no difference in phosphorus concentration was found between adults with PKU and controls [21], children with PKU had lower phosphorus excretion and higher phosphorus reabsorption compared to controls [37]. Children and adults with low BMD Z-scores were described to have higher blood phosphorus concentrations compared to those with normal BMD, but the correlation between blood phosphorus and BMD Z-score was not significant [14].

Two studies examined serum magnesium and found lower concentrations in children with PKU compared to controls [14],[37]. Children and adults with PKU with low BMD also had lower, though not significant, magnesium than those with normal BMD [14]. Magnesium did not correlate significantly with BMD Z-score [14] or any measure of bone status [37] in either study.

4.5 Discussion

BMD in early treated patients with PKU

The results of our qualitative and quantitative review suggest that mean BMD is lower in PKU patients compared to reference groups but within the normal range in most patients, thus the clinical relevance of this finding is questionable.

The meta-analysis of pooled data from 247 patients with PKU shows an overall effect size for LBMD Z-score of -0.70 (95% CI-0.82,-0.57). The overall effect size for TBMD Z-score in 133 patients is also below zero [-0.45 (95% CI-0.61,-0.28)]; however one of the studies [13] shows a large range and crosses the no effect line (Z-score = 0). Because heterogeneity is moderate, it can be assumed the overall effect size is reliable and that TBMD Z-score in patients with PKU is indeed below 0. Our meta-analysis for FBMD shows a similar effect, although heterogeneity of the populations and outcomes in these studies hamper a firm conclusion.

In our qualitative analysis of BMD Z-scores in patients with PKU, we found study-defined prevalence of osteopenia and osteoporosis [15],[16],[20],[44],[46] to be higher than prevalence of comparable Z-score categories in a reference population of adolescents and young adults; however, our meta-analysis of pooled BMD Z-scores reported in patients with PKU challenges this hypothesis. Overall effect sizes of Z-scores for LBMD, TBMD and FBMD calculated in our meta-analyses are categorized as normal by ISCD standards. The 95% confidence intervals of effect size for separate studies do, however, show a number of patients with LBMD Z-scores below -2. Thus, a subset of patients with PKU reported in articles included in this study might have a higher risk for skeletal fragility and fractures. Based on the assumptions that our data are normally

distributed and the overall effect size for LBMD Z-score is -0.70, approximately 10% of early treated patients with PKU may have a LBMD below -2 SD. This means 90% of early treated patients with PKU are not at risk for low BMD, a much better outcome than expected from single studies from the literature. The projected 10% of patients with a Z-score below -2 may be at risk for osteoporosis and may benefit from the same preventative and treatment strategies defined for healthy individuals [52].

A large limitation to these findings is the lack of standardization between individual study's definitions of osteopenia and osteoporosis and clinical diagnostic criteria. In pediatric patients, fracture history must be assessed alongside BMD Z-score before diagnosis can be made. In adult patients, WHO guidelines require T-scores to diagnose osteopenia or osteoporosis. Currently, most studies in patients with PKU report Z-scores, regardless of age groups studied, and only 2 studies report fracture history, but do not mention its relevance to clinical diagnoses. Thus, studies reporting prevalence of osteopenia and osteoporosis in patients with PKU are missing essential information necessary to qualify patients for these diagnoses.

We also examined the impact of Phe status and dietary compliance on BMD. Most studies researching correlations between Phe values and BMD did not find a correlation. Dietary compliance and dietary intake assessed as reported medical food intake [16],[46], total protein [17],[37],[44] or Phe intake [16],[17] were not correlated to BMD or BTM. The impact of overall protein status, including biological value of intact versus medical food protein and percent of total protein derived from medical food, on bone were not considered by any studies.

Age in relation to BMD was examined, but outcomes are very heterogeneous with associations varying across age groups. We are not able to draw conclusions about BMD in different age categories based on the included studies; however children under the age of 10 years and those from 13–16 years of age may have a higher prevalence of low BMD than other age groups [12],[14].

Bone turnover in early treated patients with PKU

Results on bone turnover in PKU were ambiguous, though the 4 studies examining BTM in children and/or adults with PKU found significant alterations in one or more marker. Investigated markers were heterogeneous and populations studied were not similar in age and thus cannot be reliably compared. Examining correlations between Phe concentration and individual BTM provides mixed results. Differences in findings could be due to differences in methods to measure and report Phe and diversity in reported markers. Consensus on the clinical utility of BTM including reliable methods of measurement and reference ranges, and the establishment of markers suitable for (various age groups of) patients with PKU must be established in future investigations.

Other factors related to bone status in PKU

Other indicators of bone status that were investigated in patients with PKU were BMC, vitamin D, PTH, calcium, phosphorus and magnesium concentrations. Most outcomes were reported by a small number of studies with heterogeneous groups of patients and, sometimes, contradictory outcomes. BMC may be reduced in non-compliant individuals with PKU, but the clinical implications of low BMC are unknown. Vitamin D and PTH status do not seem to influence BMD based on found results. Calcium seems to be lower in children with PKU, but the impact on bone is ambiguous. Phosphorus and

magnesium blood levels do not seem to affect bone status. At this time, it is not possible to draw conclusions on these indicators of bone status without additional evidence from high-quality studies in large groups of patients with consistent measurements. Although the results are inconclusive, including additional bone status indicators in future studies could add to the standard evaluation of bone health in patients with PKU of all ages.

Summary of evidence

We examined the strongest current evidence on bone health in patients with PKU. All studies were of adequate to high quality, with low to no risk of bias and included only patients who were early diagnosed and treated. Our results suggest that patients with PKU have lower BMD as shown by the mean effect sizes in our meta-analyses. Clinical significance of these outcomes is debatable as the mean effect size Z-scores are within the range for normal BMD according to ISCD recommendations. Though prevalence of low BMD for chronological age is higher in patients with PKU than in the normal population (estimated 10% vs 2.3%, assuming a normal distribution), definitions used for osteopenia and osteoporosis are highly heterogeneous between studies and ISCD positions and WHO standards are rarely followed. Even though several studies reporting on limited cohorts of patients report osteopenia and hypothesize poor bone health in patients with PKU, our review and meta-analyses of all available data suggests bone is not clinically compromised in most early treated patients with PKU.

With the data at hand, we do not have sufficient evidence to establish conclusions on BTM and other indicators of bone status we examined, nor define relationships between Phe or nutrient intake and bone. Further research with more consistent measurements in larger studies is necessary to provide better insight.

Clinical implications

Mean total body, lumbar spine, and femoral hip BMD Z-scores in patients with PKU are lower than in their healthy peers, but well within the reference range for normal. The clinical relevance of a slightly lower BMD Z-score is unclear. A projected 10% of patients have a BMD Z-score below -2; however 90% of early treated patients with PKU are not at risk for low BMD. Fracture risk must be established before developing final conclusions on bone health in patients with PKU.

In order to evaluate the risk for skeletal fragility or fractures in individual patients, a single assessment of BMD by DXA scan in all adolescent patients with PKU could be considered [33]. Patients with a BMD Z-score above -2 may not require additional follow-up; however patients with low BMD for chronological age (Z-score below -2) and/or a significant fracture history may need follow-up.

For prevention and treatment of low BMD, factors related to bone health in healthy individuals may be applied to prevent low BMD in patients with PKU. We suggest following recommendations for the general population outlined in the 'National Osteoporosis Foundation's Clinician's Guide to Prevention and Treatment of Osteoporosis' to preserve bone strength [53]. In particular, an adequate intake of calcium and vitamin D, lifelong participation in regular weight-bearing and muscle-strengthening exercise, cessation of tobacco and excess alcohol use if applicable, and treatment of risk factors for falling are also appropriate recommendations for patients with PKU.

Future studies

Forthcoming studies will need to establish whether slightly lower BMD from an early age increases the risk for osteoporosis or fractures acutely or long-term.

Furthermore, for patients with low BMD, preventative and treatment strategies to improve BMD in PKU should be defined. To harmonize evidence, where to measure BMD; valid markers of bone turnover; and definitions of osteopenia/osteoporosis, metabolic control, dietary compliance and protein intake must be concretely defined and standardized and related to fracture risk. Finally, studies are needed of factors impacting BMD that may not be related to PKU such as physical activity.

Strengths and limitations

One strength of this review is the inclusion of only early-diagnosed and treated patients. By excluding studies on patients who were late diagnosed and at-risk for nutrient deficiencies and potentially impairments in physical activity, which are known to have a negative impact on bone status, we excluded significant potential ascertainment bias. Another strength is that two metabolic centers from two continents performing independent searches reached the same conclusion before combining efforts. Finally, we were able to include a large group of patients in our meta-analysis by contacting authors personally to request data, resulting in meta-analyses of Z-scores from multiple BMD sites.

Our study also has some limitations. There were differences in methodology between study team 1 and study team 2 during individual literature searches, quality and risk of bias assessment, and data extraction, though standardized tools were used by each study team. After comparing data extraction, overview tables were compared and essentially identical. Therefore data extraction was easily and justifiably combined.

Neither search included “fractures” as a clinical outcome in search terms; however, search 1 was broad and captured all literature related to bone in PKU including articles related to fractures.

A small number of the included articles report a correlation between BMD and height of the patient. This is a known restriction of BMD measured by DXA [28]; patients with lower height for age may be falsely diagnosed with a low BMD. Based on the data published however, we were not able to provide height adjusted BMD Z-scores for our pooled data.

Finally, all included studies reported osteopenia and osteoporosis based on Z-scores, contrary to ISCD positions which do not recommend these diagnoses in pediatric patients. Instead, the ISCD recommends the term “low BMD for chronological age” when Z-scores are less than or equal to -2, and does not recommend the diagnosis of osteoporosis without a clinically significant fracture history [28]. These are important caveats to the current literature in patients with PKU and important evidence that criteria for low BMD, osteopenia, and osteoporosis must be concretely defined.

Conclusions

BMD in early diagnosed and treated patients with PKU is below healthy population average but within normal range. These findings are important to provide preliminary evidence that bone does not appear to be compromised to the extent previously hypothesized. However, while the overall effect size of BMD Z-scores are between -0.4 and -1 in patients with PKU, there is lack of data on a corresponding higher risk of fracture in these patients.

Other indicators of bone status in early treated patients with PKU are inconclusive due to the small number of studies and the heterogeneity in groups examined and measurement methods. Though we now conclude that low BMD does not seem to be an exaggerated concern in patients with PKU, research is needed on the effect of the PKU diet on bone, the reliability of bone turnover markers in bone assessment, and a concrete estimate of fracture risk in patients with PKU.

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4.6 References

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Modeling correlates of low bone mineral density in patients with phenylalanine hydroxylase deficiency

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5.1 Abstract

Phenylalanine hydroxylase (PAH) deficiency is an inherited metabolic disorder requiring life-long restriction of dietary protein and phenylalanine-free medical food. Low bone mineral density (BMD) is reported, but factors associated with BMD Z-score (standard deviations from normal) are unknown. We examined associations between clinical and dietary parameters and total BMD Z-score in PAH deficiency patients, and developed models to predict Z-score. Data collected from patients >4 years of age (n=88; mean age=18.8 y; 61% female) included demographic, clinical, laboratory and dietary intakes. Adjusted Spearman's correlation coefficients were calculated between parameters and TBMD Z-score, measured by dual energy x-ray absorptiometry (DXA). Parameters approaching significance (p-value<0.10) were candidate predictors for four linear regression models predicting TBMD Z-score. To validate, model-predicted Z-scores were compared to DXA Z-scores. Mean TBMD Z-score was -0.326; 18 (20.4%) had Z-score<-1. Z-scores were positively correlated with dietary vitamin D, calcium and medical food intake and compliance with prescription, and negatively with dietary carbohydrate, sugar, caffeine intake, glycemic load, and prescribed medical food (grams protein/day; p-value<0.05). The best model included medical food compliance, medical food intake, caffeine intake, and bone-specific alkaline phosphatase (r-square=0.364). This model predicted Z-score category [normal or low (<-1)] with sensitivity=66.7%, likelihood ratio=14.7, and AUC=0.83 compared to TBMD Z-score. No subjects had low BMD for chronological age (Z-score<-2). Compliance with medical food prescription was the strongest predictor of TBMD Z-score. One model, if validated in a separate sample of patients with more cases of low BMD, showed potential to estimate TBMD Z-score using routine clinical patient parameters.

5.2 Introduction

Phenylalanine hydroxylase (PAH) deficiency, previously known as phenylketonuria (PKU) (OMIM: 26160) is an autosomal recessive disorder of amino acid metabolism [85]. Mutations in the gene coding for the enzyme phenylalanine hydroxylase (EC 1.14.16.1) cause insufficient or absent catabolism of the amino acid phenylalanine to tyrosine [134]. As a result, concentrations of blood phenylalanine and its phenylketone byproducts increase, damaging the brain and other organs. Deficiencies of tyrosine and its downstream products including neurotransmitters such as epinephrine may also result. PAH deficiency is part of the newborn screening panel in the United States with a prevalence of approximately 1 in 10,000 in Caucasians [6].

Management of PAH deficiency begins immediately and requires a life-long low-protein diet to restrict phenylalanine-containing foods such as meat and dairy [135]. To maintain normal growth and development, patients must consume synthetic medical food containing vitamins, minerals and all amino acids except phenylalanine. Following a protein-restricted diet and adhering to prescribed medical food reduces severe complications of PAH deficiency; however secondary effects including poor bone health have been reported in patients regardless of dietary treatment [136]. Cross-sectional studies suggest patients with PAH deficiency have bone mineral density (BMD) significantly lower than a normal reference population [29]. BMD is a measure of the density of bones, reflecting mineral content in grams divided by the area scanned in centimeters^2. Each standard deviation decrease in BMD from the population mean (expressed as a Z-score or T-score) increases the risk of fracture [59, 120]. In patients with PAH deficiency, osteopenia is reported in 40-50% of adults [31, 36, 137], and

among children, 33% are reported to have BMD at least two standard deviations below their peers [29].

The Genetic Metabolic Dietitians International (GMDI) and the Southeast Newborn Screening & Genetics Collaborative (SERC) guideline for nutrition management of PAH deficiency, published in 2014, recommends all patients receive a dual energy x-ray absorptiometry (DXA) scan every 3-5 years beginning at age 8, and every 5 years as adults [12]. DXA, the most common technique to measure BMD [138, 139], uses low energy x-rays to scan bones of the spine, proximal femur and total body and estimates areal BMD (g/cm^2) [122] and corresponding Z-scores and T-scores [139, 140]. Despite GMDI/SERC recommendations for DXA scans for all patients with PAH deficiency, an estimated 80% of patients with PAH deficiency report they have not had a DXA scan (NBS Connect, 2014). DXA scans are generally not performed in patients under 20 years of age without a clinical indication such as fracture [120, 121] due to radiation exposure and difficulty in interpreting scans limiting its accessibility to patients with PAH deficiency, particularly adolescents and young adults. It is also expensive to meet monitoring recommendations as each DXA scan costs approximately $132 [141].

To address the gap between evidence-based recommendations to perform regular DXA scans and actual clinical practice, an alternative cost-efficient method to assess BMD in patients with PAH deficiency would be useful. Since the etiology of low BMD is unknown in this population, identifying patient characteristics that are significantly associated with low BMD is important. During clinic visits, these characteristics could be evaluated by a clinician to assess at-risk patients who may need a DXA scan. Other tools such as the Fracture Risk Assessment Tool (FRAX) employ a similar method of using

patient characteristics (sex, smoking status, BMD measured through DXA and others) to predict an individual's 10-year risk of fracture [142]. Similarly, in body composition literature, DXA-measured fat mass can also be predicted by skinfold measures, body weight, and sex [143]. Adapting the concept of prediction could be useful to improve clinical monitoring of bone health in patients with PAH deficiency. The aims of this study were to (1) explore clinical and dietary characteristics associated with BMD Z-score measured by DXA in patients with PAH deficiency and (2) use patient characteristics to develop models predicting BMD Z-score to screen for patients with low BMD who need follow-up DXA scans.

5.3 Methods

Study Sample

Data were collected as part of two studies of patients with PAH deficiency including DXA scans. The first study included participants in a clinical trial evaluating a generic form of sapropterin (BioMarin Pharmaceuticals), a tetrahydrobiopterin analog, among patients with PAH deficiency four years of age and older, before exposure to sapropterin. The second study included female patients with PAH deficiency attending Emory University Metabolic Nutrition Program's Metabolic Camp. Exclusion criteria were current pregnancy (not previous pregnancy), inability to provide consent, or biopterin therapy within the past eight weeks (sample one only). All study visits were conducted at the Emory University Hospital Clinical Research Network in compliance with Health Insurance Portability and Accountability Act (HIPAA) guidelines. Study protocols were approved by the Emory Institutional Review Board.

Data Collected

All patients were instructed to fast for at least eight hours before study procedures. Demographic data were self-reported. Anthropometrics [height, weight, body mass index (BMI)] were measured by trained research staff. Body composition (percent fat mass, percent lean mass) and total body bone mineral density (TBMD), age- and gender-matched TBMD Z-scores, and total body bone mineral content (TBMC) were measured by DXA scan (GE Lunar Prodigy) at the Winship Cancer Institute by the same trained technician. Blood and urine samples were also collected during study visits. Plasma amino acids were analyzed by quantitative ion exchange chromatography and

standardized per liter of creatinine [144]. Bone-specific alkaline phosphatase (BALP) was measured using immunoenzymatic methods.

To determine PAH deficiency severity in Kuvan study participants, whole blood was collected and processed by Emory Genetics Laboratory utilizing polymerase chain reaction (PCR) to amplify the 13 exons and flanking regions of the PAH gene. For Metabolic Camp study participants, blood spots were collected on filter papers and PCR was also used to extract DNA. We used a previously published method to assign an Assigned Value (AV) score to each mutation identified to indicate severity [76]; a lower AV score indicates a more severe mutation. AV scores for each mutation were added to create an AV sum and dichotomized to severe PAH deficiency (AV sum=2) or mild/moderate PAH deficiency (AV sum>2).

All participants kept records of dietary intake for three days prior to study visits. Completed three-day food records were reviewed with a registered dietitian during study visits and analyzed using Nutrition Data System for Research (NDSR) to calculate average daily intake of all nutrients [145]. NDSR includes a measure of dietary glycemic load (GL), an estimate of the impact of overall diet on blood glucose, accounting for number and type of carbohydrates consumed. Compliance with medical food prescription was calculated by dividing actual medical food intake (grams of protein) as reported by patients in 3-day food records, by medical food prescription (grams of protein) and expressed as a percentage. *Statistical Analysis*

Statistical analyses were conducted in Statistical Analysis Software (Version 9.4, 2014, SAS Institute Inc., Cary, NC). Descriptive statistics included mean and standard

deviation of continuous variables and frequencies and 95% confidence intervals (95% CI) of categorical variables. BMD Z-scores were compared by age, gender, BMI status, menstrual status, season of study visit, and vitamin D categories using analysis of variance with post-hoc Tukey's tests to assess significant differences (p-value<0.05). Correlation coefficients were calculated between each variable (patient characteristics, clinical parameters, dietary intake, and plasma amino acids) and total BMD Z-score. Kolmogorov-Smirnov tests for normality were performed to determine distribution of each variable and Pearson's correlation coefficients (normal distribution) or Spearman's correlation coefficients (nonparametric distribution) were calculated accordingly. Partial correlations between each variable and total BMD Z-score were also calculated to adjust for BMI. Age and sex are already incorporated in Z-scores and were not included as covariates.

Variables that were associated with total BMD Z-score in partial correlation analyses at a p-value of ≤ 0.10 were candidates for inclusion in linear regression models predicting total BMD Z-score. For predictive analyses, selection of variables into the model is data-driven. The goal of this approach is to maximize the model fit and a decision on whether to retain a particular covariate of interest is based on statistical tests and goodness-of-fit without a specified exposure of interest [146]. Backwards elimination strategy was used to select variables into four models with a cutoff p-value of 0.10. Multiple models were developed since different subsets of data are collected at different clinics, and the available variables may vary by site. The first model included all variables associated with BMD Z-score (p-value<0.10) in Table 2 as candidate predictors for selection into models by SAS. Three additional models utilized combinations of

variables including (1) dietary intake predictors calculated from food records only (Diet Model), (2) diet and plasma amino acids (DPA Model), and (3) a Basic Model with variables assessed at all metabolic clinics (compliance with diet and plasma amino acids).

After predictors were selected by SAS by backward elimination, overall fit of final models was assessed by examining r-square and mean square error (MSE) parameters. Interaction terms that were significant (p-value<0.05) were also entered into models. Multicollinearity was assessed by examining variance inflation factor (VIF); greater than 10 indicated collinearity among variables.

As a preliminary assessment of the model performance, Z-scores estimated from each of the four models were compared to the clinically significant endpoint defined as a DXA-based Z-score of less than -1. The results of this comparison were expressed as sensitivities, specificities, positive and negative predictive values for each model-predicted Z-score cutoff. The receiver operating characteristic (ROC) curve was constructed and the corresponding area under the curve (AUC) was estimated for each of the models.

5.4 Results

Table 5-1 shows characteristics of the 88 participants included in this study. All subjects were Caucasian. Sixty-one percent were female and the mean age was 18.8 $\pm$ 11 years. All patients were diagnosed through newborn screening or within the first year of life. All were early-treated, though a subset reported discontinuing diet for several weeks to months over time. Fifteen percent of patients were on sapropterin or PEGylated recombinant phenylalanine ammonia lyase. We found 43% had mild/moderate PAH deficiency (AV sum>2) and 41% had classical PAH deficiency (AV sum=2); we were unable to identify or determine severity of one or both mutations in 13 (16%) and 7 did not have genetic data available. In our sample, most patients were prescribed a complete medical food which contained micro and macronutrients in addition to amino acids. Six patients (7%) were prescribed incomplete formulas including only amino acids, as defined in the SERC/GMDI nutrition management guidelines [12]. None reported taking large neutral amino acids or glycomacropeptide-based products.

A total of 18 participants (20.4%) had a DXA-measured BMD Z-score below -1. In the whole sample, mean BMD Z-score was -0.326 (0.112), within normal range for BMD for age (Z-score> -2). There were no significant differences ($p>0.05$) in BMD Z-score by age group, gender, BMI category, AV sum category, menstruation status, or blood vitamin D category. Blood vitamin D concentration was within the normal reference range for age and sex in 84% of subjects. TBMD Z-score was significantly higher in patients who completed study visits in the summer and fall compared to those with study visits in the winter (p-value=0.002). Season of study visit was included as a

candidate predictor in model building; however, BMI category, menstruation status and blood vitamin D category were not.

Adjusting for BMI, total BMD Z-score was significantly positively correlated with compliance with medical food prescription (correlation coefficient=0.36), actual medical food intake [grams of protein equivalents (correlation coefficient=0.23)], and dietary intake of vitamin D (correlation coefficient=0.28) and calcium (correlation coefficient=0.24). Adjusted total BMD Z-score was significantly negatively correlated with medical food prescription (correlation coefficient=-0.23), dietary carbohydrate intake (correlation coefficient=-0.25), dietary sugar intake (correlation coefficient=-0.32), glycemic load (GL) (correlation coefficient=-0.26), and caffeine intake (correlation coefficient=-0.37) (Table 5-2). Plasma phenylalanine concentration, serum vitamin D concentration, dietary phenylalanine and total protein intake were not correlated with BMD Z-score. All significant variables were included as candidate predictors in model building.

Table 5-3 presents results of the four multivariable regression models that included different combinations of independent variables. Medical food compliance (actual intake/prescription, expressed as percentage) was the strongest predictor and was significantly associated with Z-score in all models. The first model (all variables correlated with BMD Z-score) also included the amount of protein consumed from medical food, caffeine intake, and alkaline phosphatase, a marker of bone turnover (r-square=0.3637). The second model (dietary intake predictors only) also included dietary phenylalanine to tyrosine ratio, dietary vitamin D intake, and overall glycemic load (r-square=0.2493). The third model (diet and plasma amino acid predictors) included these

same predictors as the second model plus plasma phenylalanine:tyrosine ratio, with a slightly higher r-square value of 0.251. Phenylalanine:tyrosine was forced into this model since statistical regression did not include it as a final predictor. The fourth (Basic) model included variables assessed in all patients with PAH deficiency at every clinic visit, but only medical food compliance was significantly associated with the Z-score, and the model fit was the lowest (r-square=0.1327).

Of the four alternative models the full model appeared to perform the best. VIF was below 10 for all predictors, indicating no multicollinearity. The interaction between medical food protein intake and blood bone-specific alkaline phosphatase was significant (p-value=0.02); however when entered into model, r-square increased from 0.36 to 0.38, thus the interaction term was dropped. With first-order terms only, the final model predicted Z-scores with sensitivity of 66.7%, likelihood ratio of 14.7, and AUC of 0.83 when compared to the clinically significant DXA-based Z-score of less than -1 (Table 5-4). There were five false-negatives (6.2%), and in two of these cases, the estimated Z-score was within 1 standard deviation of DXA Z-score, though mean deviation was 1.17 standard deviations from DXA Z-score.

5.5 Conclusions

To our knowledge, this is the most comprehensive analysis of associations between clinical characteristics and BMD Z-score in patients with PAH deficiency through mathematical modeling. BMD Z-score was most strongly positively associated with compliance with medical food prescription and dietary vitamin D intake and most strongly negatively correlated with caffeine intake and total glycemic load (GL).

The study also shows a lower prevalence of low BMD Z-score in patients with PAH deficiency than previously reported. While most studies use incorrect clinical definitions [147], low BMD (Z-score below -1) has been reported in 45-50% [33, 36] of patients with PKU. In this study, we found 20% of patients had BMD Z-scores below -1, and only 4 (4.5%) met the criteria set by the International Society for Clinical Densitometry (ISCD) (ISCD 2012) for low BMD for chronological age (Z-score below -2). Our sample may have included a higher ratio of females to males and older patients; however, the prevalence of BMD Z-scores below -1 may indeed be decreasing over time. In particular, medical food now includes higher amounts of micronutrients related to bone metabolism including calcium and vitamin D, and new pharmaceuticals are available which can allow higher intake of intact protein from foods [148].

We found no difference in BMD Z-score by PAH deficiency severity, also reported by two newer studies [29, 77], in contrast to the previously held belief that more severe patients have lower BMD [31, 40]. In addition, there was no difference in BMD Z-score by age category, as reported by other studies in the literature [29, 31]. These findings suggest variation in BMD Z-scores in patients with PAH deficiency is independent of severity and patient age.

The positive association between medical food compliance and BMD Z-score was the strongest relationship in this study. Total protein intake and dietary phenylalanine intake were not associated with BMD Z-score, although protein intake from medical food only was negatively associated with Z-scores. Other studies suggest patients with PAH deficiency with osteopenia or osteoporosis have a lower intact protein intake than patients with PAH deficiency with normal BMD [77]. Increasing intact protein intake and decreasing reliance on medical food protein to improve BMD is not a feasible recommendation since dietary protein increases blood phenylalanine concentrations in patients with more severe forms of PAH deficiency.

Our results are similar to what has been reported for healthy populations including positive associations between BMD Z-score and vitamin D intake [149] and negative associations with glycemic load and caffeine intake [150]. This is however the first study showing associations between nutrient intake including vitamin D and measures of the composite diet including dietary glycemic load and BMD Z-score in patients with PAH deficiency. This is also the first study to examine caffeine intake in patients with PAH deficiency and suggests, like in the general population, higher consumption is associated with lower BMD Z-score, though the relationship between caffeine and bone health is controversial [151]. Caffeine increases urinary excretion of calcium which can lead to a reduction in BMD and studies have reported caffeine intake is inversely associated with bone density in pre and perimenopausal women [152]. Notably, caffeine intake was much lower in patients with PAH deficiency (37$\pm$49 mg/day) compared to the national average (165$\pm$1 mg/day); however we hypothesize underreporting of caffeine consumption in this

study based on clinical observations that patients with PAH deficiency may consume more soda and caffeine-containing beverages than reported.

As a novel finding, the negative impact of a high glycemic load (GL) diet on BMD Z-score needs additional exploration. There is no evidence on GL and bone health in the literature for patients with PAH deficiency or the general population. GL estimates the impact of an individual's carbohydrate intake on blood glucose response, accounting for quality and amount of carbohydrate consumed [153]. Though carbohydrate metabolism is normal in patients with PAH deficiency [154], diets are typically high in simple carbohydrates (glucose, sucrose, fructose, etc.) and low in fiber [155], characteristics of a high GL diet. High GL diets can lead to higher blood glucose concentrations, suggested to alter bone metabolism by decreasing the activation of bone-building osteoblasts and decreasing the quality of mineralization [156]. A high GL diet in patients with PAH deficiency could lead to higher peaks and sustained increases in blood glucose and possibly insulin, potentially affecting bone over time.

A single study has examined blood glucose in PAH deficiency patients, reporting lower fasting blood glucose (80 vs 83mg/dL; p-value=0.005), but similar insulin and insulin resistance compared to controls, despite the PAH deficiency patients' carbohydrate-rich diet (59% of calories from carbohydrates) [111]. Amino acids also play a role on the release of insulin. Arginine increases insulin secretion [157] while alanine and glutamine may increase gene expression in pancreatic beta-cells resulting in an increased production and release of insulin [158] which could exacerbate hyperinsulinemia induced by a high GL diet. Mechanisms are vaguely defined, but potential interactions between dietary GL and amino acid intake on insulin secretion and

sensitivity should be considered in patients with PAH deficiency. Conclusions on the relationship between a high GL diet and bone health cannot be drawn from the limited evidence available in patients with PAH deficiency.

Based on the results of this study, promoting optimal medical food compliance may be a feasible strategy to improve BMD Z-score. Patients should be assessed for compliance with medical food prescription at every clinic visit, already practiced in most metabolic clinics. Studies examining longitudinal change in BMD with interventions to improve medical food compliance may also be needed. The relationship between glycemic load, sugar and caffeine intake and BMD and bone mineralization in patients with PAH deficiency also need high-quality studies in the future.

Predictive Models

Four multivariate regression models were developed with subsets of variables significantly associated with BMD Z-score. Previous investigations in the field of nutrition and body composition have also shown utility of variables to predict outcomes such as fat mass measured by DXA [143, 159]. In each of the four predictive models developed in this study, patients who consumed a higher proportion of prescribed medical food had higher BMD Z-scores. Compliance with medical food prescription independently explained the largest amount of variation in BMD Z-score (13.3%) of all predictors examined.

We found the model with the highest r-square (0.36) and lowest MSE (0.80) included protein from medical food (grams), compliance with medical food prescription, total caffeine intake, and serum bone-specific alkaline phosphatase concentration, a marker of bone turnover. Inclusion of a bone turnover marker in a model predicting Z-

score suggests that bone turnover markers may be useful to include in comprehensive bone health assessment in patients [70]. Interestingly, protein intake from medical food only (grams/day) was negatively associated with BMD Z-score when included with medical food compliance in the full model. This finding may suggest the higher the amount of protein from medical food, the lower the amount of protein from intact sources which may provide higher-quality protein and contribute to higher Z-scores. Alternatively, a higher medical food prescription is indicative of a patient with a more severe PAH deficiency phenotype, and more severe PAH deficiency could be more conducive to development or progression of low BMD than patients with moderate or milder phenotypes. We cannot, however, draw conclusions and recommend future studies examine type of protein and source when examining impact on bone health in patients with PAH deficiency.

Limitations

Assessment of model performance showed that the full model may have some utility in identifying PAH deficiency patients with low BMD. A sensitivity closer to 80% would have been preferred since the model is intended as a screening tool; however, a sensitivity of 66.7% does not completely eliminate the possibility of clinical utility. It is important to point out, however, that the model performance parameters (e.g., sensitivity and AUC) reported here are data-specific and require external validation in an independent study. Inclusion of other bone turnover markers particularly the gold-standard markers collagen type I cross-linked C-telopeptide (CTx) and procollagen type I N-terminal propeptide (PINP), physical activity, fracture history, and other indicators related to BMD Z-score may also be introduced and analyzed in future studies. A

limitation of this study is its small size (n=88) and low prevalence of the studied outcome as only 20% of patients had BMD Z-scores below -1. In a rare disorder such as PAH deficiency, this is still one of the largest studies available to date.

Unfortunately, we do not have data on historical dietary intake and instead relied on cross-sectional data for dietary intake and all biomarkers measured. Changes in dietary intake, including medical food composition, over time could have an impact on BMD and while three day food records are considered reliable tools to assess dietary intake, do not reflect diet over time. In addition, mean blood phenylalanine concentration was high [mean=731$\pm$397 µmol/L (12.1$\pm$6.6 mg/dL)] even though patients reported good compliance with medical food in three day food records (mean=84.4$\pm$39.0% of total prescription), suggesting a potential reporting bias. Mean dietary phenylalanine intake was, however, 189.4 $\pm$ 150.4% of phenylalanine prescription (mean intake=904 $\pm$ 792 mg/day), partially explaining high blood phenylalanine concentrations. Finally, physical activity was not assessed in either study sample and could not be included as a model predictor, though it is a known modulator of bone health. In future studies, it is important to include a sample of individuals with PAH deficiency with more probable cases of low BMD than the studied sample and historical dietary intake and physical activity data if possible.

Final Conclusion

In patients with PAH deficiency, total protein intake from medical food, compliance with medical food prescription, dietary caffeine intake, and blood alkaline phosphatase concentration explained a moderate proportion of variability in BMD Z-scores, beyond variables traditionally related to bone health such as age, sex and BMI.

Inclusion of these variables in a model predicting BMD Z-score allowed distinguishing patients with low BMD from those with normal BMD using the cutoff of -1. If the proposed model turns out to be reproducible in other populations of patients with PAH deficiency, it may be considered for use in clinical practice. BMD Z-score was also positively associated with dietary vitamin D intake and negatively associated with dietary glycemic load, but neither variable were selected into predictive models through statistical methods.

If successfully validated in the future, a model predicting BMD Z-score to identify patients who should receive DXA may save significant healthcare costs compared to performing DXA in all patients as currently recommended [12]. In the meantime, promoting patient compliance with medical food prescription may promote more than just optimal phenylalanine concentration given its association with higher BMD Z-score.

Table 5-1. Characteristics of patients with phenylketonuria (n=88)

Characteristic	N (%)	%	95% CI	BMD Z-score Mean (SD)	p-value
Age (years) (mean=18.8±1.2)					0.2935
4-9 years	19	21.6	12.8, 30.4	-0.179 (0.80)	
10-15 years	22	25.0	15.8, 34.2	-0.595 (0.96)	
16-19 years	20	22.7	13.8, 31.7	-0.035 (1.46)	
20-40 years	23	26.1	16.8, 35.5	-0.526 (0.951)	
≥40 years	4	4.5	0.1, 9.0	0.150 (0.265)	
Gender					0.0737
Male	34	38.6	28.3, 49.0	-0.579 (1.06)	
Female	54	61.4	51.0, 71.7	-0.167 (1.03)	
Body mass index (BMI)[1] (mean=24.3±8.2)					0.3535
Underweight	5	5.8	0.8, 10.9	-0.640 (1.96)	
Normal	45	52.3	41.5, 63.1	-0.453 (0.76)	
Overweight	20	23.3	14.1, 32.4	-0.070 (1.53)	
Obese	16	18.6	10.2, 27.0	-0.056 (0.611)	
AV Sum					0.2690
Severe/Classical (Sum=2)	33	40.7	29.8, 51.7	-0.542 (0.961)	
Mild/Moderate (Sum>2)	35	43.2	32.1, 54.2	-0.157 (0.965)	
Undefined (Sum≥2)	13	16.0	7.9, 24.2	-0.261 (1.104)	
BMD Z-score (mean=-0.326±0.112)					
Normal (≥-1)	70	79.5	70.9, 88.1	0.033 (0.83)	
At risk (-1 to -2)	14	15.9	8.1, 23.7	-1.493 (0.31)	
Low (≤-2)	4	4.5	0.1, 9.0	-2.53 (0.33)	
Menstrual Status (n=53)					0.3697
Pre-menstruation	11	20.8	9.4, 32.0	-0.563 (0.74)	
Menstruating	41	77.4	65.7, 89.0	-0.061 (1.10)	
Post-Menopausal	1	1.9	0.0, 5.7	-0.100	
Season of study visit					0.0021
Spring (March-June)	20	22.7	13.8, 31.7	-0.650 (0.78)	
Summer (June-September)	47	53.4	42.8, 64.0	-0.070 (1.10)[b]	
Fall (September-December)	6	6.8	1.4, 12.2	0.433 (0.58)[b]	
Winter (December-March)	15	17.0	9.0, 25.1	-1.000 (0.94)[a]	
Vitamin D (ng/mL or pg/mL)[2]					0.2918
Normal (≥30 or Quest ref[1])	70	84.3	76.4, 92.3	-0.397 (1.05)	
Insufficient (21-29)	7	8.4	2.3, 14.5	0.157 (1.03)	
Deficient (≤20 or Quest ref[1])	6	7.2	1.5, 12.9	0.050 (1.30)	

a<b (p-value<0.05; Tukey's test)

[1]For patients under 16 years of age, BMI Z-score calculated using CDC references and assigned: <5%ile underweight, 5-85%ile normal weight, 85-95%ile overweight, >95%ile obese; BMI Calculator: http://www.quesgen.com/BMIPedsCalc.php
[2]For Kuvan study, Quest Diagnostic reference ranges used to calculate subjects as normal 1,25 dihydroxy-vitaminD or below reference range

Table 5-2. Model parameters and their correlations with total bone mineral density (BMD) Z-score (n=88)

	n	Mean	SD	Co-efficient	p-value
Patient Characteristics					
Age (years)	86	19.0	10.9	-0.13	0.252
Body mass index (BMI)	90	24.9	8.7	0.10	0.367
Percent fat mass (%)	86	32.0	12.2	0.03	0.764
Percent lean mass (%)	86	65.6	11.9	-0.08	0.487
Clinical Parameters					
Medical food prescription (g protein/day)	**83**	**48.7**	**15.9**	**-0.23**	**0.034**
Dietary phenylalanine prescription (mg/day)	82	493.8	328.7	0.04	0.704
Medical food intake (g protein/day)	**84**	**39.6**	**23.1**	**0.23**	**0.039**
Medical food compliance [% (intake/rx)]	**82**	**84.0**	**39.4**	**0.36**	**0.001**
Dietary Intake					
Phenylalanine (mg)	84	904.1	792.0	-0.11	0.334
Tyrosine (mg)	**84**	**4690**	**2174**	**0.21**	**0.051**
Phenylalanine to tyrosine ratio	**84**	**0.3**	**0.5**	**-0.19**	**0.082**
Energy (kilocalories)	84	1700.0	508.5	-0.13	0.247
Total Fat (grams)	84	54.0	25.7	0.00	0.967
Carbohydrates (grams)	**84**	**253.0**	**76.5**	**-0.25**	**0.025**
Total Protein intake (grams)	84	60.8	21.3	0.10	0.378
Cholesterol (mg)	84	38.7	56.2	0.07	0.519
Saturated Fat (grams)	84	16.0	9.6	0.12	0.277
Vitamin D (mcg)	**84**	**11.7**	**18.2**	**0.28**	**0.012**
Calcium (mg)	**84**	**1250.0**	**597.8**	**0.24**	**0.029**
Sugars (grams)	**84**	**93.4**	**53.3**	**-0.32**	**0.004**
Fiber (grams)	84	14.3	8.1	-0.18	0.109
Caffeine (mg)	**84**	**37.7**	**49.1**	**-0.37**	**0.001**
Glycemic Index (glucose)	84	66.7	23.0	0.12	0.286
Glycemic Load (glucose)	**84**	**158.4**	**74.2**	**-0.26**	**0.020**
Sodium (mg)	84	2804.0	1101.0	0.13	0.246
Phosphorus (mg)	84	1340.0	695.2	0.12	0.297
Zinc (mg)	**84**	**16.6**	**8.9**	**0.21**	**0.053**
Vitamin K (mcg)	84	137.4	82.6	0.14	0.213
Vitamin A (IU)	84	5781.0	3525.0	0.08	0.460
Vitamin E (IU)	84	14.2	7.0	0.10	0.377
Selenium (mcg)	84	76.3	33.3	0.03	0.816
Metabolic Profile					

Phenylalanine (µmol/L)	**86**	**739.6**	**397.6**	**-0.21**	**0.057**
Tyrosine (µmol/L)	86	48.9	17.3	0.16	0.154
Phenylalanine:Tyrosine ratio	**86**	**15.2**	**12.6**	**-0.18**	**0.098**
Other					
25-hydroxyvitamin D, blood	81	43.4	15.3	-0.17	0.131
Bone-specific alkaline phosphatase	**83**	**115.6**	**99.0**	**-0.21**	**0.061**

[a]*Partial correlation coefficients, adjusting for body mass index (BMI), vitamin D concentration, season of study visit and menstruation status*

****All Bolded variables were candidates for inclusion in predictive models shown in Table 3 (p-value<0.10)**

Table 5-3. Results of linear regression and best-fit model characteristics

	Beta	Standard Error	p-value	r^2	Root MSE
All variables				**0.3637**	**0.80381**
Intercept	-0.31646	0.26467	0.2355		
Protein from medical food (grams)	-0.0144	0.00707	0.0451		
Medical food compliance (%)	1.52603	0.3952	0.0002		
Dietary caffeine (mg)	-0.00658	0.00205	0.002		
Alkaline phosphatase (mcg/L)	-0.00398	0.00093004	<.0001		
Dietary intake only				**0.2493**	**0.86835**
Intercept	-1.5818	0.52003	0.0032		
Medical food compliance (% rx)	1.53652	0.3977	0.0002		
Dietary phe:tyrosine ratio	0.71342	0.35885	0.0503		
Dietary vitamin D (mcg)	0.01064	0.00541	0.0528		
Dietary glycemic load	-0.00262	0.00129	0.0453		
Diet and plasma amino acids				**0.251**	**0.87286**
Intercept	-1.47928	0.57437	0.0119		
Medical food compliance (% rx)	1.48439	0.41768	0.0006		
Dietary phe:tyrosine ratio	0.68513	0.36664	0.0654		
Dietary vitamin D (mcg)	0.01057	0.00545	0.0558		
Dietary glycemic load	-0.00259	0.0013	0.0491		
Plasma phe:tyr ratio	-0.00347	0.00806	0.6679		
Basic model				**0.1327**	**0.91609**
Intercept	-1.1152	0.23954	<.0001		
Medical food compliance (% rx)	0.9136	0.2579	0.0007		

Table 5-4. Validation of four models predicting BMD Z-score in patients with PAH deficiency (n=88)

Models	Full Model (n=81)	Diet Model (n=84)	Amino acid Model (n=84)	Quick model (n=84)
Cut-off	-0.85	-1	-1	-1
Mean deviation (SD)	-0.00006 (0.78)	-0.00045 (0.85)	-0.00076 (0.85)	6.80654E-7 (0.911)
Range of deviation	-1.8 to 2.4	-2.5 to 2.3	-2.5 to 2.3	-2.7 to 2.3
Misclassified	8 (9.9%)	16 (19%)	16 (19%)	19 (22.6%)
False Positives	3 (3.7%)	5 (6.0%)	5 (6.0%)	6 (7.1%)
False Negatives	5 (6.2%)	11 (13.1%)	11 (13.1%)	13 (15.5%)
Sensitivity (95% CI)	66.7 (38.4, 88.1)	31.25 (11.1-58.6)	31.25 (11.1-58.6)	18.75 (4.3-45.7)
Specificity (95% CI)	95.5 (87.3, 99.0)	92.65 (83.7-97.5)	92.65 (83.7-97.5)	91.18 (81.8-96.7)
Positive Predictive Value (95% CI)	76.9 (46.2, 94.7)	50.0 (19.0-81.1)	50.0 (19.0-81.1)	33.33(7.9-69.9)
Negative Predictive Value (95% CI)	92.7 (83.7, 97.5)	85.14 (75.0-92.3)	85.14 (75.0-92.3)	82.67 (72.2-90.4)
Positive Likelihood Ratio	**14.7 (4.6, 46.9)**	**4.3 (1.4-12.9)**	**4.3 (1.4-12.9)**	**2.1 (0.6, 7.6)**
Total Test Efficiency	**90.1%**	**81.0%**	**81.0%**	**77.4%**
AUC	**0.8313**	**0.7031**	**0.6783**	**0.6236**

Table 5-5. Mean BMD Z-scores in males and females with PAH Deficiency

	Males (n=34)		Females (n=54)		
Characteristic	**N (%)**	**BMD Z-score Mean (SD)**	**N (%)**	**BMD Z-score Mean (SD)**	**p-value**
Age category (years)					0.011
4-9 years	14 (41%)	0.057 (0.640)	5 (9%)	-0.840 (0.879)	0.026
10-15 years	7 (21%)	-1.314 (0.925)	15 (28%)	-0.260 (0.792)	0.012
16-19 years	4 (12%)	-1.050 (1.328)	16 (30%)	0.219 (1.412)	0.123
≥20 years	9 (26%)	-0.789 (1.144)	18 (33%)	-0.244 (0.745)	0.15
Body mass index (kg/m^2)[a]					0.30
Underweight (<18.5)	4 (12%)	-0.875 (2.179)	1 (2%)	0.300	0.66
Normal (18.5-24.9)	17 (50%)	-0.388 (0.857)	28 (54%)	-0.493 (0.699)	0.66
Overweight (25-29.9)	7 (21%)	-1.086 (1.032)	13 (25%)	0.477 (1.499)	0.02
Obese (>30)	6 (17%)	-0.333 (0.539)	10 (19%)	0.110 (0.615)	0.17
Plasma phenylalanine					0.61
Treatment range (120-360)	6 (18%)	0.267 (0.859)	12 (22%)	-0.412 (1.638)	0.67
Above range (>360)	28 (82%)	-0.761 (1.019)	42 (78%)	-0.202 (0.804)	0.013
Serum vitamin D (ng/mL)[b]					0.93
Normal (≥30/Quest ref[1])	30 (88%)	-0.617 (1.012)	40 (82%)	-0.233 (1.057)	0.41
Insufficient (20-30)	0 (0%)	--	7 (14%)	0.157 (1.026)	--
Deficient (≤20/Quest ref[1])	3 (12%)	-0.300 (1.517)	2 (4%)	0.750 (0.212)	0.13

[a]For patients under 16 years of age, BMI Z-score calculated using CDC references and assigned: <5%ile underweight, 5-85%ile normal weight, 85-95%ile overweight, >95%ile obese; BMI Calculator: http://www.quesgen.com/BMIPedsCalc.php

[b]For Kuvan study, Quest Diagnostic reference ranges used to calculate subjects as normal 1,25 dihydroxy-vitaminD or below reference range

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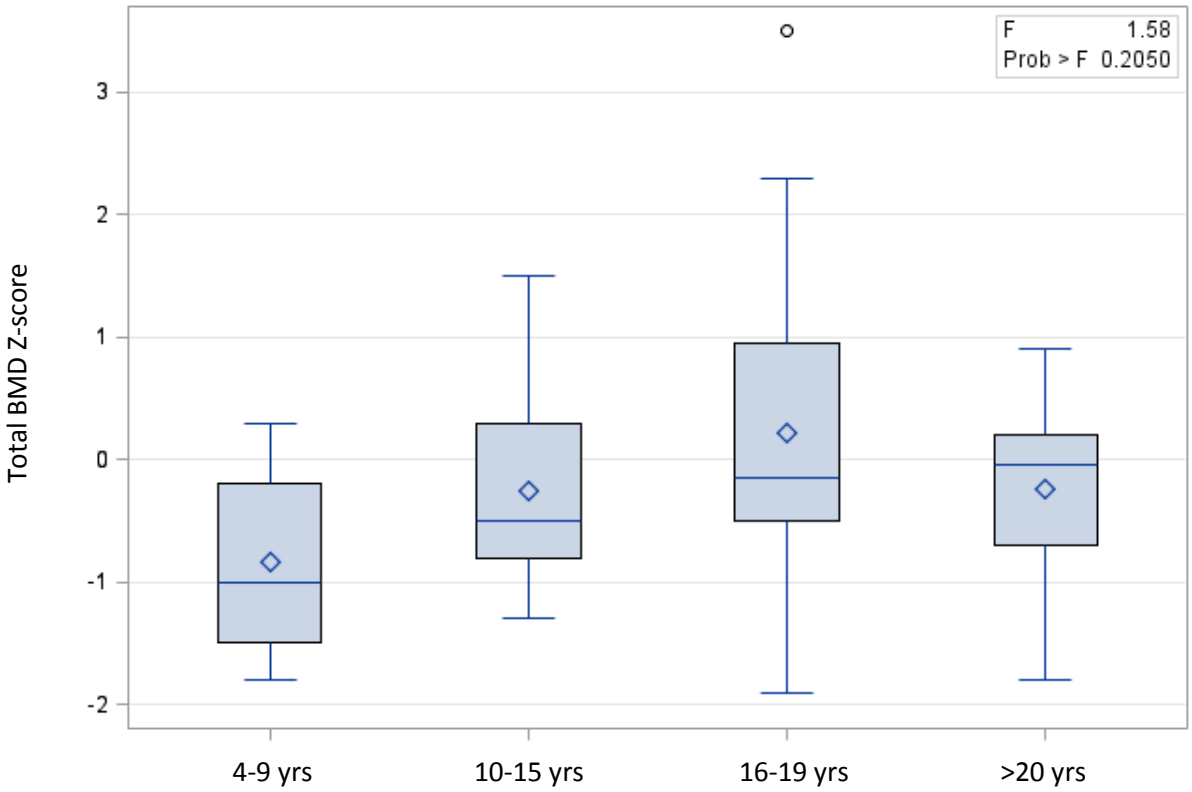
Chapter 5.7 Other Important Results

Chapter 5 resulted in the development of the largest cross-sectional dataset including bone health in patients with PKU (n=88) to our knowledge. This dataset is a tremendous resource to examine existing and future research questions. To confirm the hypothesis of low BMD in patients of both sexes and all ages with PKU, we examined differences in BMD Z-scores between males and females by age group. Table 5-5 examines mean BMD Z-score by gender and age category, BMI category, and serum vitamin D category.

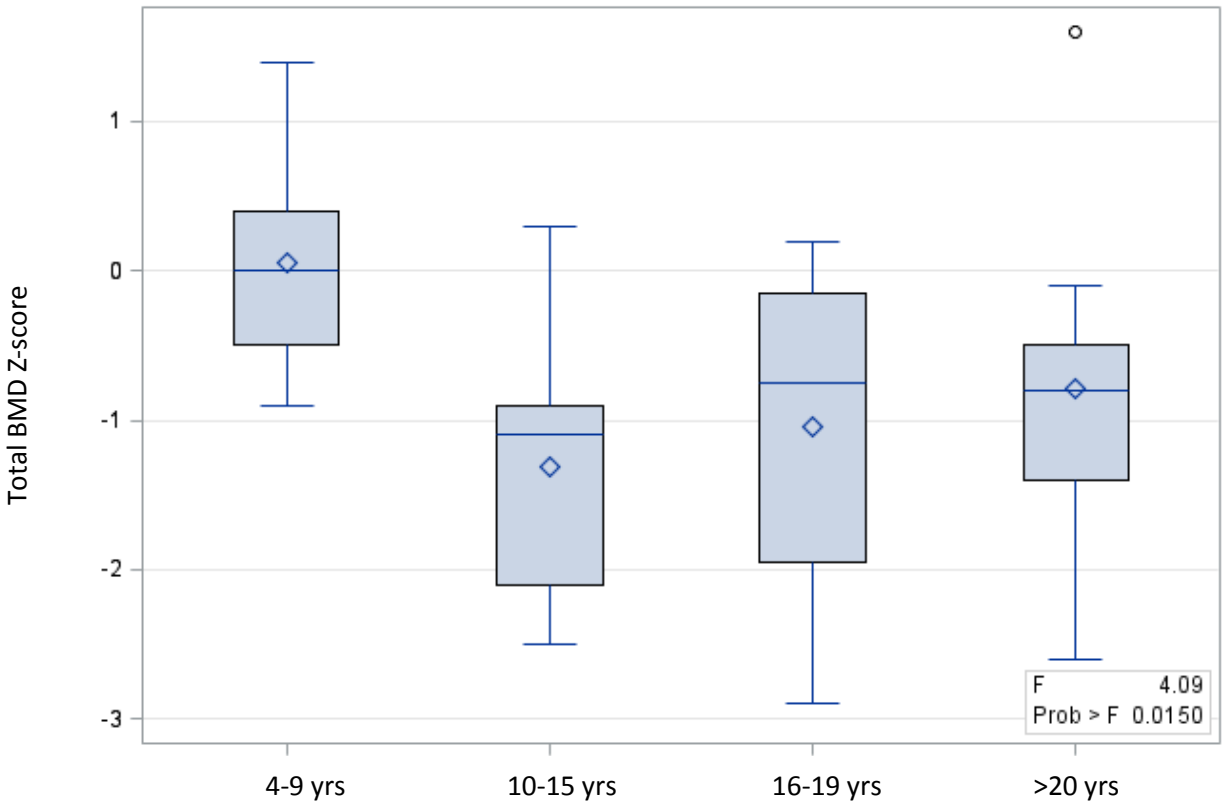
BMD Z-scores differed significantly by age category between males and females (p-value=0.011). Younger females 4-9 years of age had significantly lower BMD Z-scores than males (p-value=0.026); however, males 10-15 years of age had significantly lower BMD Z-scores than females (p-value=0.012). In fact, BMD Z-score of any age group was lowest for boys 10-15 years of age (mean=-1.314, standard deviation=1.328). BMD Z-score did not differ by BMI category between males and females; however, overweight males had significantly lower BMD Z-scores than overweight females. Moreover, males who had plasma phenylalanine above the treatment range (120-360 µmol/L) had lower mean BMD Z-scores than females. No differences were noted between males and females by serum vitamin D category.

Figure 5-1 shows the distribution of BMD Z-scores in our cohort of females by age group (4-9, 10-15, 16-19, and >20 years) and Figure 5-2 shows the distribution of BMD Z-scores in our cohort of males. Figure 5-3 shows the distribution of BMD Z-scores across age groups for males and females. The difference between genders over age groups is significant (p=0.016).



Mean:	-0.840	-0.260	0.219	-0.244
Std Dev:	0.879	0.792	1.412	0.745
Median:	-1.000	-0.500	-0.150	-0.050

Figure 5-1. Box and whisker plots of BMD Z-scores by age group in females with PKU (vertical bars represent 25-75%iles with maximum and minimum at end of whiskers; within vertical bars, horizontal line indicates median and diamond indicates mean)



Mean:	0. 057	-1.314	-1.050	-0.789
Std Dev:	0.641	0.925	1.328	1.144
Median:	0.000	-1.100	-0.750	-0.800

Figure 5-2. Box and whisker plots of BMD Z-scores by age group in males with PKU (vertical bars represent 25-75%iles with maximum and minimum at end of whiskers; within vertical bars, horizontal line indicates median and diamond indicates mean)

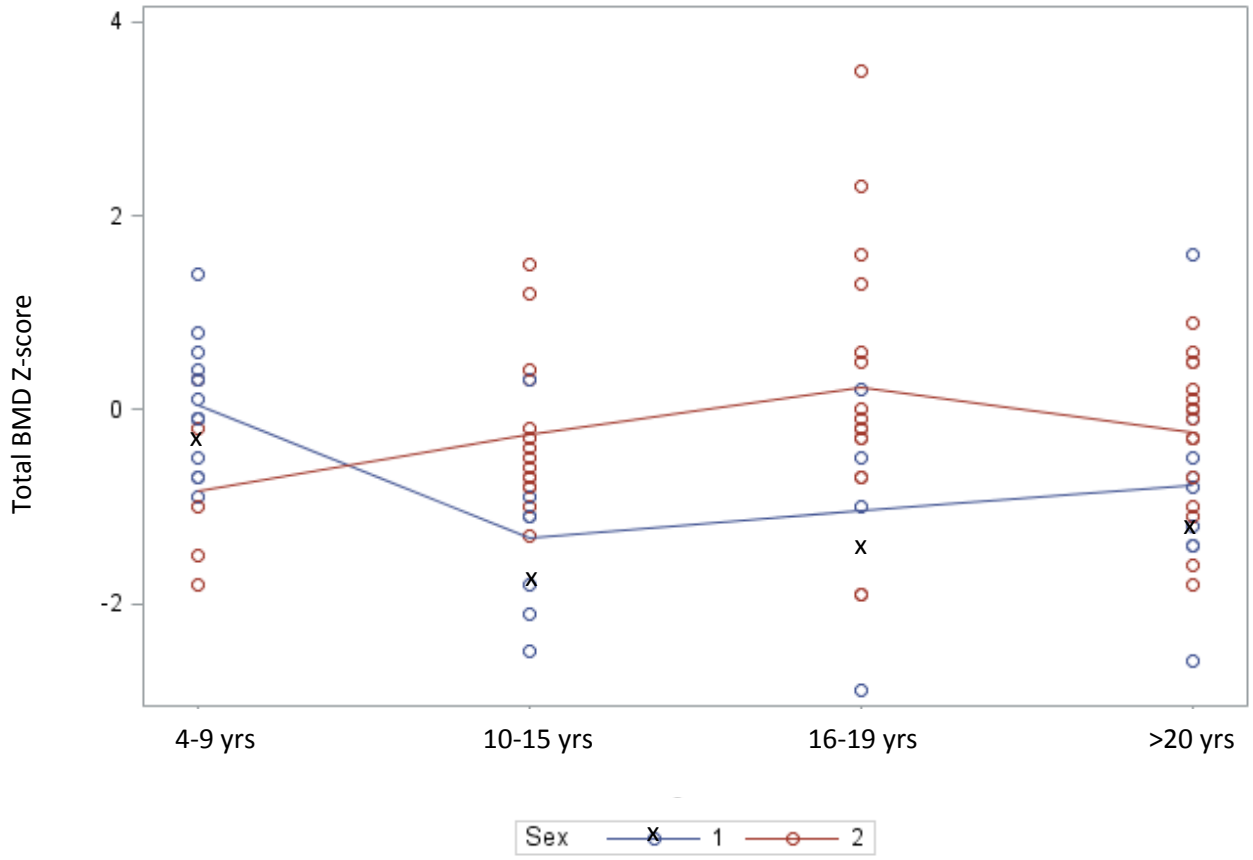


Figure 5-3. Distribution of BMD Z-scores across age groups by males (1, x) and females (2, o) with PKU

Interestingly, younger males started with BMD Z-scores closer to zero, the healthy population average for age and sex, but Z-scores decreased during puberty (10-15 years of age) while females started with lower BMD Z-scores but Z-scores increased at 16-19 years of age and then were maintained. The Fels Longitudinal Study, one of the largest prospective cohort studies of BMD in healthy boys and girls found BMD is not significantly different between genders before age 15, but is significantly higher in boys after [37]. In our study, pre-pubescent females (<9 years of age) had the lowest mean BMD Z-score of any age group in females, while pubertal males (10-15 and 16-19 years of age) had the lowest overall mean BMD Z-scores of males and females of any age.

Thus, the pattern observed in this study is different than the steady increase in BMD in healthy males from age 11-19 found in the Fels study [37]. BMD may therefore be a concern in males during puberty, a critical time of BMD accrual. This is evidenced by our finding that mean BMD Z-score was significantly lower in males 10-15 years of age compared to males 4-9 years of age. This is a cross-sectional observation, but offers more evidence that longitudinal studies are needed including DXA scans in females and males with PKU.

PKU Severity Defined by Genotype and BMD

We also assessed mutations of the PAH gene for a subset of patients included in the dataset (n=81, 92%). Mutations are useful to determine disease severity and classify patients as having classical PKU, the most severe form of PKU with less residual PAH activity, higher phenylalanine concentrations, less tolerance of intact protein, and more reliance on medical food, versus mild or moderate PKU [3]. To examine mutations, blood was collected on a filter paper at the beginning of the Kuvan responsiveness trial, or on the first day of camp for Metabolic Camp participants. For the Kuvan responsiveness trial, DNA were exacted and mutations were analyzed by Emory Genetics Laboratory through amplification of the 13 exons and flanking regions of the PAH gene through polymerase chain reactions (PCR). For Metabolic Camp samples, DNA were extracted and analyzed from filter paper peripheral blood spots by PCR by the laboratory of Dr. Steven Dobrowolski, Department of Pathology at the University of Pittsburgh, a colleague of Dr. Rani Singh's.

We used a previously published method to assign an Assigned Value (AV) score for each mutation identified to measure the severity of PKU [76]; a lower AV score

indicates more severe mutations and thus more severe PKU phenotype. AV scores for each mutation were added to create an AV sum. Participants were considered classical if the AV sum was equal to 2 (n=33). Those who had an AV sum greater than 2 were considered to have mild/moderate PKU (n=35). In 13 patients, one mutation could not be identified, or had not been described with a particular PKU phenotype, thus the summary score was considered greater than or equal to 2.

Table 5-6. Differences in parameters reported in Chapter 5 by genotypes in patients with PKU

Variable	Classical PKU (n=33)[1]	Mild/Moderate PKU (n=35)[2]	AV Sum≥2 (n=13)[3]	p-value
	Mean (SD)	Mean (SD)	Mean (SD)	
Demographics				
Age (years)	19.4 (13.7)	17.9 (8.9)	20.5 (11.1)	0.75
BMI (kg/m^2)	23.6 (7.9)	24.7 (9.5)	26.8 (6.8)	0.53
Medical food prescription (g/day)	49.3 (11.6)	46.4 (20.4)	54.8 (11.8)	0.29
Phenylalanine prescription (mg/day)	358.9 (140.8)[a]	672.3 (426.1)[b]	315.4 (101.8)[a]	<0.01
Total BMD (g/cm^2)	0.989 (0.164)	1.039 (0.157)	1.056 (0.156)	0.31
BMD Z-score	-0.542 (0.961)	-0.157 (0.965)	-0.262 (1.04)	0.27
Percent fat (%)	30.2 (13.2)	32.0 (13.2)	36.4 (12.3)	0.35
Blood Biomarkers				
Phenylalanine (µmol/L)	829.8 (400.9)[b]	580.5 (351.4)[a]	890.5 (407.9)[b]	0.009
Phenylalanine to tyrosine	18.2 (14.1)	11.7 (9.5)	19.8 (13.6)	0.04
Bone-specific alkaline phosphatase (U/L)	135.6 (102.5)	123.4 (104.2)	103.4 (108.4)	0.64
Dietary Intake				
Calories (kcals)	1561 (342)[a]	1849 (539)[b]	1705 (636)	0.06
Fat (grams)	45.6 (15.9)[a]	65.0 (27.8)[b]	50.5 (30.5)	<0.01
Carbohydrates (grams)	244.1 (71.3)	260.4 (69.1)	253.8 (101.1)	0.68
Total protein (grams)	56.6 (16.2)[a]	62.5 (24.3)	73.0 (16.0)[b]	0.05
Medical food protein (grams)	43.1 (16.4)	35.9 (23.9)	48.3 (27.9)	0.17
Med food compliance (% of rx consumed)	90.2 (30.5)	80.2 (40.1)	98.2 (40.8)	0.28
Phenylalanine (mg)	590.1 (487.8)[a]	1177 (773.9)[b]	1082.5 (1239)	<0.01
Phe compliance (%)	165.7 (117.3)	208.8 (189.8)	191.1 (101.3)	0.51

[1]Classical PKU defined as an AV sum=2 a<b (Tukey's p-value<0.05)
[2]Mild/Moderate PKU defined as an AV sum>2
[3]Patients with an unidentified or unreported mutation were assigned an AV sum≥2

As shown in Table 5-6, classical PKU patients and those with an undefined mutation had lower dietary phenylalanine prescriptions and higher blood phenylalanine concentrations compared to those with mild or moderate PKU. Classical patients also consumed less calories, dietary fat and dietary phenylalanine than mild/moderate patients, and less dietary protein than undefined patients. Compliance with medical food prescription and dietary phenylalanine prescription did not differ between the three groups. These data also show total BMD, total BMD Z-score, and bone specific alkaline phosphatase did not differ by PKU severity, a commonly held theory throughout the PKU literature [20, 31, 40]. Two other recent studies, however, have also reported no difference in BMD by PKU severity. One study by Miras et al. in 2013 [77] utilized actual mutations to categorize patients while the other study by De Groot et al in 2012 [29] only included pre-treatment phenylalanine concentrations. Thus we are providing addition evidence that PKU genotype does not affect BMD.

Based on additional analyses of our 88-patient dataset, we proposed to measure not only BMD and BMD Z-score by DXA, but also gold-standard BTM, and to examine the impact of unreported variables including nutrient intake and source of nutrient, physical activity and inflammation on BMD, BMD Z-scores and BTM, described in Chapter 6. Unfortunately, Chapter 6 includes females with PKU only and we recommend future studies prioritize the inclusion of males with PKU. Particular emphasis should be placed on including adolescent males 10-15 years of age, the age group we found the lowest mean BMD Z-scores. In future investigations of bone health, baseline BMD should be measured and followed longitudinally, with detailed dietary intake and BTM.

Chapter 6. Associations between dietary intake physical activity and bone health in patients with PAH deficiency

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6.1 Abstract

Phenylalanine hydroxylase deficiency (PAH deficiency) is an autosomal recessive inborn error of metabolism characterized by deficiency of the phenylalanine hydroxylase enzyme. Patients cannot metabolize the amino acid, phenylalanine, and follow a low-protein diet to restrict dietary phenylalanine intake, with specialized medical food to supply a phenylalanine-free source of protein. This study assessed the impact of dietary intake and source of nutrition (food versus medical food) and self-reported physical activity on bone mineral density (BMD) and bone turnover markers (BTM) in patients with PAH deficiency. Blood from 44 fasted females ages 11-52 was analyzed for plasma phenylalanine, bone turnover markers [CTx (resorption) and P1NP (formation)], vitamin D, and parathyroid hormone (PTH). Dual energy x-ray absorptiometry measured body composition, bone mineral density (BMD), and BMD Z-scores to compare BMD to normal for age and sex. Three-day food records were analyzed and total nutrient intake, nutrient intake from food, and nutrient intake from medical food were calculated. Aerobic and strength-related physical activity was assessed by self-report using International Physical Activity Questionnaire-modified questions for leisure-time physical activity. Menstrual status, medications, co-morbidities, and previous fractures or breaks were self-reported through questionnaire. All participants had normal total body BMD (Z-scores>-2); however the majority of the sample (n=27; 64%), had high bone resorption (CTx) and normal bone formation (P1NP) indicating a possible uncoupling of bone turnover. Dietary vitamin D, calcium, protein and zinc were predominantly from medical food (>50% of total intake). Forty-four percent met CDC recommendations for aerobic physical activity and 46% reported a previous bone fracture or break. Bone turnover was positively associated with energy and zinc intake from food and negatively with fat, protein and magnesium intake from medical food and medical food compliance. The ratio of resorption to formation (CTx/P1NP) decreased significantly with older age and higher PTH, and nearly with higher medical food compliance (p-value=0.056). We found normal BMD in individuals with PKU, but BTM show uncoupling in favor of resorption. Optimal dietary intake, particularly medical food compliance and adequate micronutrient intake, likely impacts bone mineralization more than metabolic control in patients with PAH deficiency. Future research is needed to measure BMD and BTM longitudinally to examine changes over time and long-term risk of fracture, particularly in younger patients and those with lower PTH who may have uncoupled turnover.

6.2 Introduction

Phenylketonuria, also known as phenylalanine hydroxylase (PAH) deficiency, is an autosomal recessive inborn error of metabolism (IEM) diagnosed in infants through newborn screening (NBS) [6]. NBS involves a simple heel prick at birth to detect a number of treatable genetic disorders using a single blood spot. PAH deficiency was the first disorders included in the NBS panel. Patients with PAH deficiency lack sufficient quantities of the enzyme phenylalanine hydroxylase (PAH) due to mutations in the PAH gene. PAH functions to metabolize the amino acid phenylalanine to tyrosine, and in patients with PAH deficiency, blood phenylalanine can increase to dangerously high concentrations. The amino acid phenylalanine can cross the blood-brain barrier, affecting cognitive development and behavior and causing physical abnormalities. Deficiencies in tyrosine may also result, affecting downstream products of tyrosine metabolism including important neurotransmitters such as dopamine, norepinephrine and epinephrine [9].

To prevent the severe effects of high blood phenylalanine and low tyrosine, dietary treatment of patients with PAH deficiency begins early in life to restrict intake of phenylalanine. Foods containing protein generally include greater amounts of phenylalanine than patients may tolerate and natural protein, referred to as intact protein, is restricted to prevent increases in blood phenylalanine concentrations. Since protein is required for normal growth and development, patients must also consume a synthetic medical formula containing protein as elemental amino acids, but lacking phenylalanine. Medical food can supply up to 85-90% of total protein intake in patients with the most severe form of PAH deficiency [75]. Medical food often contains other nutrients, including macronutrients (fat and carbohydrates) and micronutrients (vitamin D, calcium,

and vitamin B12) that are difficult to consume in adequate amounts with protein restriction [160]. Protein restriction and medical food are recommended for life for all patients with PAH deficiency [12].

In patients who are compliant with dietary protein restriction and medical food, protein needs, defined as 120-140% of a patient's recommended dietary allowance (RDA), are met and often exceeded. Protein is provided in excess of the RDA due to the rapid absorption of elemental amino acids in medical food, but less efficient utilization compared to intact protein sources [13]. Few reports, however, show the efficacy of the PAH diet in meeting dietary recommendations for other macro- and micronutrients [103]. One study suggests that biochemical nutrient deficiencies are present, even in patients whose reported daily nutrient intake exceeds micronutrient recommendations by over 200% [161]. In a study of 156 patients, 81% had altered values in one or more marker of nutritional status including blood prealbumin, calcium, phosphorus, selenium, zinc, vitamin B12, folic acid, ferritin, or 25-hydroxyvitamin D, though 84% had adequate adherence to diet [162]. Absorption, efficacy and/or utilization of nutrients could differ by source (food versus medical food) and result in biochemical deficiencies despite adequate reported intake.

It is accepted that early and continuous dietary treatment of patients with PAH deficiency results in nearly normal cognitive outcomes [11, 163]. A systematic review, however, showed suboptimal outcomes in 140 of 150 included studies (93%) of at least one primary indicator of health status in early-treated patients, including low BMD [11]. Since the first report of compromised bone structure in neonates and infants with phenylketonuria in 1962, low BMD has been described in male and female patients of all

age groups [23]. Though definitions of low BMD have been inconsistently and often inaccurately applied to cohorts of patients to report osteopenia and osteoporosis, a pooled meta-analysis of BMD Z-scores in patients with PAH deficiency showed mean Z-scores at three sites are significantly lower than normal for age and sex but within normal range [164].

BMD and BMD Z-scores are good indicators of long-term bone status, but acute measures of bone health are also informative. Assessing bone turnover markers (BTM), the measurable byproducts of the bone turnover process, in blood or urine can provide insight into acute bone metabolism [71]. BTM reflect active bone resorption by osteoclasts and active bone formation by osteoblasts [68, 69], processes that are coupled under normal physiological circumstances. Based on a systematic review of evidence on all BTM currently available for measurement, the International Osteoporosis Foundation (IOF) and the International Federation for Clinical Chemistry (IFCC) recommend two specific bone turnover markers to be measured in serum, amino-terminal propeptides of type 1 collagen (P1NP) and carboxy-terminal cross-linked telopeptide of type 1 collagen (CTx) [71]. These markers of bone formation and bone resorption are currently the most reliable and valid indicators of bone turnover and are recommended for all studies on bone metabolism. Just one study in Poland has examined either gold-standard BTM and reported serum CTx is lower in pre-pubertal children with PAH deficiency compared to healthy controls [64]. Up until now, CTx and P1NP have not been concurrently reported in adolescents or young adults with PAH deficiency.

The cause of low BMD and potentially altered bone turnover in patients with PAH deficiency is unknown. Studies report inconsistent associations but generally agree

that plasma phenylalanine does not correlate with BMD or BTM concentrations [29, 57]. Studies have also examined the impact of a limited number of nutrients on BMD including protein, calcium, vitamin D and magnesium with no strong conclusions [136]. Protein, calcium and vitamin D are particularly important for acute regulation of bone turnover as well as cumulative measures such as BMD. Adequate intake of these nutrients is necessary to promote bone gain in early life and decrease bone loss in later life, promoting optimal and coupled bone turnover [165]. An imbalance of normal bone turnover is known as uncoupling and, over time, could lead to a reduction in BMD. This is of particular importance if uncoupling occurs before attaining peak bone mass. There is also a known link between physical activity, both aerobic and weight-bearing, and bone health [166], but physical activity has not been reported in patients with PAH deficiency [167].

The primary aims of this study were to assess dietary intake of all nutrients, including source, and physical activity to examine associations with gold-standard bone turnover markers (CTx and P1NP) and bone mineral density in a sample of females with PAH deficiency.

6.3 Patients and Methods

Participants

Females 11 years of age and older attending Emory University's Metabolic Camp, an annual summer camp for girls and women with phenylketonuria (PAH deficiency) from 2013-2015 were eligible for this study. Campers had the option to attend camp but not participate in this research study. 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.

Packets with IRB-approved research forms were sent to campers approximately two months before camp. Each packet contained consent and assent forms according to the camper's age. Consent from campers age 18 or older, or verbal assent for younger campers with written consent from parents or guardians were collected before camp over the phone with camp research coordinators. All components of the study, from blood draws to dual energy x-ray absorptiometry (DXA) scans were explained during the phone consent process. Participants also had time to ask questions about the study. Campers received and completed surveys on demographics, medical information, current dietary and medical food prescriptions, and insurance. All forms were returned to study coordinators before camp to expedite study visits.

Study Design

This study was a cross-sectional investigation of females with PAH deficiency. After confirmation of consent and assent, participants attended study visits on the first day of camp at the Emory University Hospital Clinical Research Network (18 years of age and older) or Children's Hospital of Atlanta Pediatric Research Center (under 18

years of age). All participants 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 before DXA scans were performed.

Dietary Intake

Three-day diet records were submitted by all campers and reviewed individually with a Metabolic Dietitian to ensure accuracy. If campers did not bring a completed 3-day food record to camp, the dietitian performed a 24-hour recall. All food records and 24-hour recalls were analyzed by a single dietitian using Nutrition Data System for Research (NDSR) [168]. All foods and medical formulas that were missing from the database were requested through the NDSR administrators at the University of Minnesota, Nutrition Coordinating Center. The average of the three days included in the record was calculated to estimate mean intake for each nutrient. Average nutrient intake from medical food (not including low-protein foods) over the three-day record was also calculated. Average nutrient intake from medical food was subtracted from total average nutrient intake to calculate average nutrient intake from food sources.

Total nutrient intakes were compared to age- and gender-specific recommendations included in the dietary reference intakes (DRI), a set of recommendations for healthy individuals [169]. For nutrients with an established Recommended Daily Allowance (RDA), total nutrient intake was compared to RDA and expressed as a percent of RDA. The RDA is the estimated requirement of a nutrient that meets the needs of at least 97% of the population, derived from the Estimated Average Requirement (EAR), set to meet the needs of approximately 50% of the population [169].

For nutrients without an established RDA, nutrient intake was compared to the Average Intake (AI) and expressed as a percent (%). For each nutrient, the percent of RDA from food sources and the percent of RDA from medical food was also calculated.

DXA Scans

All study participants had a dual energy x-ray absorptiometry (DXA) scan at the Winship Cancer Institute by the same technician on a GE Lunar Prodigy DXA machine. DXA measures bone mineral content (BMC, grams), bone mineral density (BMD, grams/cm^2) and body composition [fat mass (grams), % fat mass, lean mass (grams), % lean mass]. BMD Z-scores are calculated by DXA software to compare each participant's BMD to the average for a healthy female of the same age. ISCD standards recommend using Z-scores to assess low BMD in pediatric subjects, pre-menopausal women and men under 50 years of age [121]. In this study, participants were categorized as having normal BMD (Z-score > -2) or low BMD (Z-score $\leq$ -2) [170]. We also examined differences in variables examined in participants with a Z-score above -1 and participants with a Z-score of -1 or below.

Laboratory samples

A trained phlebotomist drew venous blood for all blood samples. Samples were transported to research labs and processed by trained laboratory technicians. Both research labs are affiliates of the Atlanta Clinical and Translational Science Institute (ACTSI), an entity funded by the National Institutes of Health (NIH) for clinical research. After preliminary processing, aliquots of plasma to measure amino acids were placed on ice and transported to the Emory Genetics Laboratory for analysis via ion exchange chromatography. A single aliquot of serum was sent to Maine Medical Center

Research Institute on dry ice to measure bone turnover markers [bone-specific alkaline phosphatase (BALP), C-terminal telopeptide of type 1 collagen (CTx) and pro-peptide of type 1 collagen (P1NP)], vitamin D (25-hydroxyvitamin D) and intact parathyroid hormone (PTH) using Immunodiagnostic System's 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 blood to measure all five markers using chemiluminiscence [171, 172]. CTx and P1NP are known to have a diurnal variation with concentrations peaking in the morning. Our participants were all fasting and blood samples were collected between 8:30 am and 12:00 pm in an attempt to minimize diurnal variation [173].

Other Variables

Participants completed a bone health history questionnaire which asked about previous fractures, broken bones, medications, concurrent conditions that could affect bone mineral density, vitamin D and multivitamin supplementation, and pubertal status [pre-menstrual, menstrual (regular or irregular), or post-menopausal]. A physical activity survey was also administered by study staff. The questionnaire used questions adapted from the International Physical Activity Questionnaire (IPAQ) and assessed moderate-intensity and vigorous-intensity aerobic physical activity, walking, sitting and strength training in minutes per week [174]. Examples of moderate and vigorous activities were included in questions. Total minutes of moderate-intensity equivalents per week was calculated by multiplying minutes of vigorous-intensity by two and adding to moderate-intensity physical activity. Total moderate-intensity equivalents was used to assess the prevalence of the sample meeting the Centers for Disease Control and Prevention's

(CDC) recommendations for aerobic physical activity for Americans, published in 2008 [175]. In those under age 18, >420 minutes of moderate-intensity equivalent aerobic physical activity is recommended per week and for adults, >150 minutes of moderate-intensity equivalent aerobic physical activity is recommended per week [175]. In addition, strength training in minutes per week was considered, since weight-bearing exercise impacts bone turnover and BMD. All study visits occurred during the summer in Atlanta, Georgia and seasonality was consistent for all participants.

Statistical Analysis

All statistics were performed using Statistical Analysis Software (SAS) version 9.4 [176]. Primary dependent variables were CTx, P1NP, BMD and BMD Z-score and primary independent variables were nutrient intake by source, dietary pattern defined by factor analysis, physical activity (aerobic and strength-related), and plasma phenylalanine (metabolic control). A BTM ratio (CTx/P1NP) was also calculated to assess the relative degree of resorption to formation [177]; a higher ratio indicated higher bone resorption compared to formation. The association of other variables related to bone health including menstrual status, fracture and bone break history, body composition, and blood vitamin D and PTH were also assessed. Normality of each variable was assessed prior to statistical analyses using the Shapiro-Wilk test. If outliers 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 results.

Descriptive statistics including mean, standard deviation, percent and 95% confidence interval (CI) were calculated for the following categories: age (11-15, 16-19, and >19 years of age), BMI [(kg/m^2) underweight <18.5, normal weight 18.5-24.9,

overweight 25-29.9, and obese $\geq$ 30], menstrual status (pre-menstrual, regular menstrual cycle, and irregular menstrual cycle), medical therapy [no medications, treatment with Kuvan, and treatment with pegylated ammonia-lyase (PEG-PAL)], meeting CDC recommendations for aerobic physical activity, reported fracture or break, and BMD Z-score category (normal or low). Mean BMD Z-score, CTx, P1NP and CTx/P1NP ratio were calculated for each category and compared using multivariate linear regression to control for age and BMI. To calculate significant differences between categories, Tukey adjustments for multiple comparisons were used (criterion p-value<0.01). Biomarkers (plasma phenylalanine, vitamin D, PTH, BALP, CTx, and P1NP) were described by mean and standard deviation and with respect to clinical reference ranges for age (low, normal or high). For biomarkers found to be higher or lower than the reference range, mean difference from the reference range and mean percent difference from the reference range were calculated using the upper or lower limit as appropriate.

For macronutrients (energy, fat, carbohydrates and protein) and bone-related micronutrients (Vitamin A, vitamin D, calcium, phosphorus, magnesium, iron, zinc, selenium, sodium and cholesterol), mean and standard deviation and percent (%) RDA or AI were calculated. Paired t-tests (or Wilcoxon signed rank tests for non-parametric data) were performed to determine if mean intake from food and mean intake from medical food were significantly different for each nutrient. Nutrients with greater than 50% of daily intake from medical food were noted. To assess associations between individual nutrients and BTM and BMD Z-score, Pearson's (or Spearman's for non-parametric data) partial correlation coefficients adjusting for age, BMI, total energy intake and plasma phenylalanine were calculated. Correlations between total nutrient intake, total nutrient

intake from medical food, and total nutrient intake from food were calculated separately. 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 Z-scores were also examined. A correlation matrix was also constructed to examine relationships between bone-related parameters included in this study (CTx, P1NP, CTx/P1NP ratio, BALP, PTH, Vitamin D, BMD and BMD Z-score), adjusting for age and BMI.

Development of Dietary Patterns

As a novel investigation, we also examined nutrient intake by constructing dietary patterns. To determine dietary patterns, a factor analysis was performed using 22 standard food groups, including medical food protein, adapted for individuals with PAH deficiency. Food groups were selected from previous studies on dietary pattern and BMD in healthy women [178], and adapted to the PKU population. Number of servings of each of the 22 food groups was calculated based on NDSR-generated food group consumption data for each individual. A principal component factor analysis with varimax rotation was used to calculate factor loadings for two categories of consumption (dietary patterns). The number of dietary patterns was selected based on feasibility in the 44-patient dataset. To characterize each pattern, only those components with a factor loading absolute value of >0.40 using PROC FACTOR in SAS 9.4 were included. Final dietary pattern scores were calculated based on the sum of the number of servings of each food group multiplied by factors for all food groups for each participant. Scores were kept continuous to calculate correlations with nutrients and bone outcomes.

6.4 Results

Forty-four females with PAH deficiency were included in the study, ranging from 11-54 years of age (Table 6-1). Fifteen (34%) were overweight or obese according to BMI. The majority of participants (77%) had phenylalanine concentrations above the therapeutic range, defined as 120-360 µmol/L. All participants had normal BMD for chronological age; nine (20%) had a BMD Z-score between -1 and -2. CTx, marker of bone resorption, was elevated compared to the reference range for age and sex in 81% of the sample while P1NP, marker of bone formation, was normal in 83%. On average, CTx was 0.79 ng/mL (342%) above the upper reference range for age and sex. The majority of the sample (n=27; 64%), had high CTx and normal P1NP indicating a possible uncoupling of bone turnover. CTx and P1NP were significantly different by age and BMI category. Younger and underweight participants had higher BTM concentrations. Thus, age and BMI were covariates in all analyses to control for effects on BTM.

Table 6-1. Characteristics of females with PAH deficiency (n=44)

	Mean (SD)	N	Percent	95% CI
Demographics				
Age (years)	20.1 (10.0)			
11-15		18	40.9%	25.8, 56.0
16-19		14	31.8%	17.5, 46.1
>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 ($\geq$30)		7	15.9%	4.7, 27.2
Menstrual status	--			
Premenstrual		3	7.1%	0, 15.3
Menstrual, regular cycle		21	50.0%	34.2, 65.8
Menstrual, irregular cycle		18	42.9%	27.2, 58.5
Postmenopausal		0	0%	--
Medical Therapy	--			
None		28	63.6%	48.8, 78.4
Kuvan (tetrahydrobiopterin)		14	31.8%	17.5, 46.1

PEG-PAL (Pegylated ammonia-lyase)		2	4.5%	0, 11.0
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%	--
Physical Activity (moderate-intensity aerobic equivalents, min/week)[1]	345.0 (102.7)			
Meets CDC Recommendations		19	44.2%	28.7, 59.7
Below CDC Recommendations		24	55.8%	40.3, 71.3
Fracture history				
Break and/or fracture		20	45.5	30.1, 60.8
No break or fracture		24	54.5	39.2, 69.9
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 Vitamin 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
Parathyroid Hormone (PTH) (pg/mL)	27.9 (11.6)			
Low for age		1	2.4%	0, 7.2
Normal for age		40	95.2%	88.5, 100.0
High for age		1	2.4%	0, 7.2
Bone specific alkaline phosphatase (BALP) (U/L)[2]	34.9 (32.2)			
Low for age		8	19.0%	6.6, 31.4
Normal for age		28	66.7%	51.8, 81.5
High for age		6	14.3%	3.2, 25.3
Carboxyterminal telopeptide of type I collagen (CTx) (ng/mL)[3]	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)[2]	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

[1]CDC Guidelines for aerobic physical activity for Americans (>420 minutes/week for children, >150 minutes/week for adults over age 18)
[2]BALP and P1NP indicate active bone formation
[3]CTx indicates active bone resorption

Differences across age categories

We also examined key variables in patients with PAH deficiency by age group (11-15, 16-19, and >19 years) (Table 6-2). BMI significantly increased over age categories (p-value<0.01) and by nearly 10 kg/m^2 from the youngest group to the oldest group. Ten BMI units can equate to two categories of adiposity, for example a BMI of 20 is normal while a BMI of 30 is obese. Participants in the middle age group (16-19 years of age) had the highest mean BMD and BMD Z-score compared to younger and older participants both of which approached significance (p-value=0.056, p-value=0.11).

Table 6-2. Differences in key variables by age group in females with PAH deficiency

	Age 11-15 (n=18)	Age 16-19 (n=14)	Age >19 (n=12)	
	Mean (SD)	Mean (SD)	Mean (SD)	p-value
Demographics				
Body mass index (kg/m^2)	22.6 (6.6)[a]	23.6 (3.8)[a]	32.2 (12.4)[b]	<0.01
Bone mineral density (g/cm^2)	1.076 (0.083)[a]	1.156 (0.074)[b]	1.102 (0.114)	0.056
BMD Z-score	-0.17 (0.9)	0.48 (1.02)	-0.13 (0.77)	0.11
Percent with fracture or break	50.0%	28.6%	58.3%	0.28
Physical Activity				
Moderate intensity physical activity (min/week)	335.6 (338.6)	288.6 (239.4)	466.3 (1220.6)	0.79
Percent meeting CDC PA recommendations	39%	64%	33%	0.22
Walking (min/week)	241.7 (474.5)	96.8 (79,0)	79.0 (134.1)	0.30
Sitting (min/week)	304.9 (151.4)	272.1 (173.9)	303.3 (192.5)	0.84
Strength training (min/week)	117.2 (253.6)	35.7 (69.5)	29.2 (57.8)	0.28
Biomarkers				
Phenylalanine (µmol/L)	713 (421)	873 (408)	762 (463)	0.58
Vitamin D (ng/mL)	32.1 (9.1)[a]	45.0 (16.7)[b]	36.5 (14.8)	0.038
Parathyroid hormone (pg/mL)	28.6 (11.0)	23.8 (8.4)	32.1 (14.9)	0.20
Bone-specific alkaline phosphatase (µg/L)	55.7 (42.7)[b]	20.9 (5.8)[a]	20.6 (7.6)[a]	<0.01
C-terminal cross-linking telopeptide of type I collagen (ng/mL)	1.48 (0.73)[b]	0.70 (0.30)[a]	0.43 (0.23)[a]	<0.01
Procollagen type I N propeptide (ng/mL)	318.4 (249.9)[b]	86.5 (38.8)[a]	52.7 (24.3)[a]	<0.01
CTx/P1NP ratio	0.06 (0.04)[b]	0.03 (0.02)	0.02 (0.01)[a]	0.002

a<b Tukey's post-hoc p-value <0.05

All BTM (BALP, CTx and P1NP) decreased over age categories (p-value<0.01) (Table 6-2), as expected based on the physiological decrease in bone turnover with aging [179]. CTx/P1NP ratio also decreased significantly with age (Figure 6-1; p-value=0.002) suggesting younger patients have a higher degree of uncoupling in the direction of higher resorption. CTx decreased by 71% from the youngest to the oldest age group while P1NP decreased by 83%, indicating P1NP decreases more over time than CTx and potentially explaining the decrease in CTx/P1NP ratio with age. Blood vitamin D was also significantly lower in the youngest age group compared to the middle age group (p=0.038). There were no differences in measures of physical activity (moderate aerobic activity, walking, sitting or strength training) across age groups.

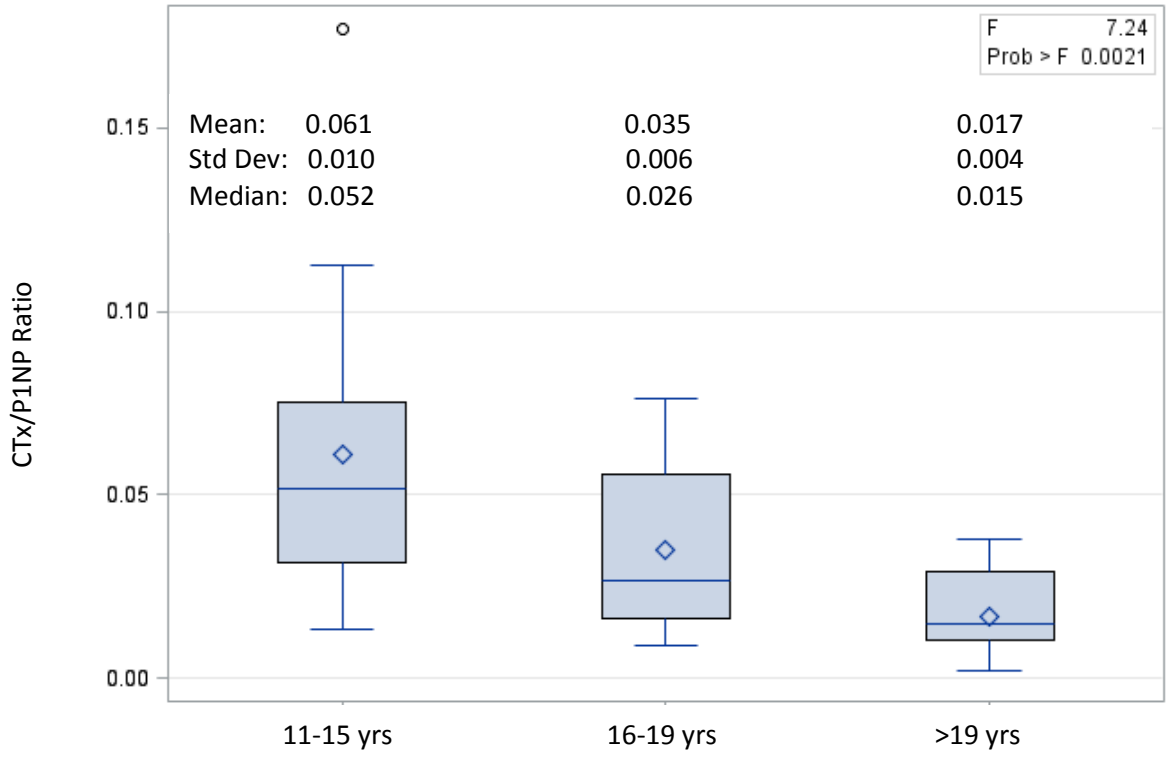

Figure 6-1. Box and whisker plot of CTx to P1NP ratio by age group in females with PAH deficiency (vertical bars represent 25-75%iles with maximum and minimum at end

of whiskers; within vertical bars, horizontal line indicates median and diamond indicates mean)

Differences compared to the U.S. average

We compared key variables reported in this study to the average U.S population or reference ranges. Fifty-three percent of participants over age 18 were overweight or obese compared to 69% of U.S adults [180]. Twenty percent of study participants under age 18 were overweight or obese, compared to the U.S. population prevalence of 31.8% [181]. Despite lower prevalence compared to the U.S. population, overweight and obesity are still issues in patients with PAH deficiency, just as in the normal U.S. population. There is an immediate need for studies examining risk factors for diabetes, hypertension, cardiovascular disease, and metabolic syndrome in the aging population of women and men with PAH deficiency.

In each of the three age categories examined, total body BMD was higher in our study compared to National Health and Nutrition Examination Survey (NHANES) references (data not shown) [182]. Median age at menarche is reported to be 12.4 years in American girls [183], but we did not assess age at menarche, only prevalence of those who had reached menarche. In those who had not begun to menstruate, the average age was 14.3 years, nearly two years older than the U.S. median. This could indicate a delay in puberty which is known to impact bone health, particularly peak bone mass; however, age of puberty has not been examined in a sample of females with PAH deficiency. In healthy girls, later puberty is associated with lower BMD and higher risk of fracture [184]. A case study in the PKU literature, however, reports early (precocious) puberty in one female with PKU at 7.5 years of age [185], but this is not likely representative of

puberty in all females with PAH deficiency. Of those who had reached menstruation in our sample, 46% reported irregular menstrual cycles. There is a critical need to assess puberty, age of puberty onset, menstruation and hormone levels in girls and women with PAH deficiency since hormonal impact on bone is critical [186].

Vitamin D insufficiency and deficiency have been widely reported in healthy children and adults. In U.S. children under age 18, the prevalence of vitamin D insufficiency is reported to be 42%. In this study, 42% of participants under age 18 were vitamin D insufficient and of all participants who were vitamin D insufficient, 83% were children or adolescents. In healthy U.S. adults, the prevalence of vitamin D insufficiency is reported to be 36.4-41.6%, compared to the prevalence in this study of 22.2%. Assessment of vitamin D status in younger children with PAH deficiency is of particular importance. While 95% of study participants had normal PTH concentrations, mean PTH of subjects under 18 (26.3 pg/mL) was much higher than reported for healthy girls ages 12-14 (15.6 pg/mL) and ages 14-16 (13.9 pg/mL), but nowhere near the upper limit of normal (52 pg/mL) [187, 188]. Though within the normal range, slightly elevated PTH, coupled with insufficient vitamin D levels could affect acute bone turnover and BMD.

In our sample, 44% met age-specific CDC guidelines for aerobic physical activity. We found the prevalence of meeting guidelines was almost double in the middle age group (16-19 years of age) compared to younger and older age groups. Measures of physical activity (moderate-intensity equivalents and strength training in minutes) did not correlate with BMI, lean mass or fat mass in subjects. The total sample prevalence of meeting guidelines was comparable to the U.S prevalence of adults meeting physical activity recommendations (49.2%). In the youngest group, however, the prevalence of

meeting recommendations (39%) was higher than the U.S. average (25%). It is possible that the instrument used to gauge physical activity in this study (modified IPAQ) is not appropriate and we recommend the development or adaptation of a simple survey to assess physical activity by age group in patients with PAH deficiency.

In our sample, 46% of participants reported a bone fracture, bone break, or both. We did not assess the type or cause of fractures or breaks. Though this was a cross-sectional analysis, this is the first report of fractures in patients with PAH deficiency since a study by Greeves et al. in 1997 reported an increased risk of fractures in patients with PKU eight years of age and older (risk ratio=2.6) compared to sibling controls [60]. In this study, there were no differences in BMD, BMD Z-score or any BTM between those who reported a fracture or break and those who did not. There were also no differences in any body composition parameter or physical activity measure we examined between those with fractures or breaks and those without. Longitudinal studies examining fracture risk and predictors of fracture risk in patients with PAH deficiency are needed.

Correlations between reported variables

Other variables relevant to BMD were also considered. Among subjects under age 18 (n=31), mean height-for-age percentile was 43.6 $\pm$ 28.0% (range 0-97%) and mean BMD-for-height percentile was 49.2 $\pm$ 32.6% (range 3-98%). BMD-for-height percentile was strongly positively correlated with BMD Z-score (r-square=0.55, p-value=0.0005), but not P1NP, CTx or CTx/P1NP ratio. Mean percent body fat was 34.4 $\pm$ 8.9% while mean percent lean mass was 62.8 $\pm$ 9.1%.

Adjusting for age, amount of lean mass was positively associated with BMD (r-square=0.47, p-value=0.003) while percent lean mass was marginally negatively

associated with BMD (r-square=-0.33, p-value=0.046) but not BMD Z-score, CTx, P1NP or CTx/P1NP. Amount of fat mass was positively associated with BMD (r-square=0.42, p-value=0.008) and BMD Z-score (r-square=0.34, p-value=0.03), but not CTx, P1NP or CTx/P1NP. Mean percent truncal fat was 35.8% $\pm$ 10.6% (range 11.4-56.9%), higher than the average for healthy women of 26.6% [189]. Android to gynoid fat ratio is another important measure of body composition and mean android/gynoid ratio was 0.94 $\pm$ 0.25 in this sample. In cross-sections of healthy women and children, the ratio is much lower, ranging from 0.45 [190] to 0.57 [191] to 0.60 [192]. A higher android fat mass is associated with an increased risk of cardiovascular disease and must be assessed further in patients with PAH deficiency [192].

Correlations between biomarkers showed CTx and P1NP were strong negatively associated with age (r-square=-0.72 and -0.84, p-value<0.001) and marginally with BMI (r-square=-0.31 and -0.32, p-value<0.05). Vitamin D was negatively correlated with PTH concentration (r-square=-0.40, p-value=0.01) as expected, but no other biomarkers. Bone turnover markers CTx, P1NP and BALP were strongly positively correlated; the strongest correlation was between CTx and P1NP (r-square=0.74, p-value<0.0001). The ratio of CTx/P1NP, an indicator of predominantly bone resorption, was strongly negatively associated with age (r-square=-0.59, p-value=<0.0001) and PTH (r-square=-0.56, p-value=0.0002). Blood phenylalanine and BMD Z-score were not correlated with any markers of bone metabolism considered. In addition, measures of physical activity (minutes per week of moderate-intensity aerobic activity, walking, and strength training) were not correlated with any biomarker or BMD or BMD Z-score.

Dietary Intake

Table 6-3 shows dietary intake of individual nutrients including source (food or medical food). Mean intakes of protein, Vitamin D, calcium and zinc were predominantly from medical food (>50% of total intake). Mean intake of most nutrients greatly exceeded the RDA/AI. In fact, only mean Vitamin D consumption fell below the RDA/AI. Though mean vitamin D intake was 90% of total requirements, 80% of participants' vitamin D intake fell below the RDA of 600 IU/day [193]. These data suggest that total intake of nutrients in patients with PAH deficiency meet and exceed recommendations, but key bone-related nutrients are coming predominantly from synthetic medical food, not intact foods. Differences in source of nutrients were compared between those under age 18 and adults. A higher percent of total vitamin D came from medical food in the younger age group (76%) compared to the older age group (52%; p-value=0.04); all other nutrients were comparable.

Table 6-3. Intake of individual bone-related nutrients and source from three day food records

	Total Intake		Food Intake		Medical Food Intake		
	Mean (SD)	% RDA/AI	Mean (SD)	% RDA/AI	Mean (SD)	% RDA/AI	p-value[1]
Macronutrients							
Total energy (kcals)	1521 (451)	--	1195 (414)	--	326 (283)	--	<0.01
Fat (grams)	51.2 (23.0)	--	41.0 (22.0)	--	10.2 (11.2)	--	<0.01
Carbohydrates (grams)	219.5 (64.5)	168.9	188.3 (61.1)	144.9	31.2 (29.8)	24.0	<0.01
Protein (grams)*	57.0 (27.3)	129.1	25.7 (23.8)	57.9	31.3 (23.6)	71.2	0.13
Micronutrients							
Vitamin A (IU)	5595 (3594)	247.8	3761 (3194)	167.2	1834 (1634)	80.7	<0.01
Vitamin D (mcg)*	13.5 (25.0)	90.1	2.5 (4.5)	16.6	11.0 (25.3)	73.5	<0.01
Vitamin B12 (mcg)*	5.2 (3.7)	228.7	1.3 (1.5)	57.0	3.9 (3.7)	171.7	<0.01

Calcium (mg)*	1195 (689)	100.2	395.5 (313.0)	33.5	799.2 (682.0)	66.8	<0.01
Phosphorus (mg)	1200 (574)	119	504 (288)	50.7	697 (593)	68.6	0.16
Magnesium (mg)	360.3 (183.0)	111.4	147.8 (70.8)	45.6	212.5 (183.7)	65.8	0.11
Iron (mg)	19.7 (9.9)	143.3	8.4 (4.9)	61.4	11.3 (9.9)	82.0	0.21
Zinc (mg)*	15.6 (9.5)	182.5	4,7 (3.8)	54.6	10.9 (9.8)	127.8	<0.01
Selenium (mcg)	73.8 (37.3)	141.5	39.6 (33.3)	76.0	34.2 (29.8)	65.5	0.74
Sodium (mg)	2840 (1290)	189.3	2174 (1211)	144.9	666 (654)	44.4	<0.01
Cholesterol (mg)[2]	41.3 (74.2)	13.8	41.3 (74.2)	13.8 (24.7)	0 (0)	0.0	<0.01

[1]Wilcoxon signed rank test, unadjusted differences between mean intake from food and medical food
[2]Cholesterol based on recommended intake of <300mg/day (no defined AI/RDA) [194]
*>50% of intake from medical food

Associations between nutrient intake and bone markers

Table 6-4 shows correlations between nutrient intake by source and bone indicators included in this study adjusted for age, BMI, plasma phenylalanine and total caloric intake. Energy and magnesium intake from food were positively correlated with P1NP (r-square=0.44, p-value=0.006) while magnesium intake from food was negatively correlated with P1NP (r-square=-0.43, p-value=0.008). Total protein and medical food protein intake were marginally negatively correlated with P1NP (both r-square=-0.39, p-value=0.02). Sodium from food was positively correlated with CTx/P1NP ratio (r-square=0.43, p-value=0.008) while medical food fat was negatively correlated with CTx/P1NP ratio (r-square=-0.44, p-value=0.008). Total BMD was negatively correlated with cholesterol from food (r-square=-0.42, p-value=0.008). BMD Z-score was only marginally correlated with sodium intake from food (r-square=0.39, p-value=0.014), but no other nutrients. Dietary phenylalanine intake was not associated with BTM, BMD or

BMD Z-score. Medical food compliance was negatively correlated to P1NP (r-square=-0.48, p-value=0.003).

Table 6-4. Source of nutrient intake and spearman's partial correlation coefficients* with bone turnover markers, BMD and BMD Z-score

	CTx (ng/mL)[a]		P1NP (ng/mL)[a]		CTx/P1NP		BMD		BMD Z-score[b]	
	r^2	p-value	**r^2**	p-value	**r^2**	p-value	**r^2**	p-value	**r^2**	p-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
Phenylalanine (mg)	0.31	0.07	0.29	0.08	0.39	0.02	-0.20	0.22	0.02	0.91
Medical Food	--	--	--	--	--	--	--	--	--	--
Food	0.31	0.06	0.30	0.08	0.39	0.017	-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

**Correlation coefficients adjusted for age, BMI, total calorie intake, and blood phenylalanine
Spearman's correlation coefficient p-value < 0.01 indicates significance

Dietary Patterns

Two dietary patterns were derived by factor analysis (Table 6-5). The first pattern was indicative of patients who consumed higher amounts of medical food but few nutrient-dense foods (high medical food intake, high consumption of butter and cream, dairy, and sweets) and labeled "Compliant, unhealthy". The second pattern was indicative or patients who consumed less medical food, more meat and few nutrient-dense foods (low medical food consumption, high in cakes and crackers, eggs and meat, and soft drinks) and labeled "Non-Compliant".

Table 6-5. Rotated factor patterns for food group for two dietary patterns in patients with PAH deficiency

	Compliant, unhealthy	Non-Compliant
Butter and Cream	0.79788	0.37022
Dairy	0.79559	0.39951
Sweets	0.5357	-0.01455
Protein from medical food	0.43456	-0.45666
Oil	0.13598	-0.39749
Cakes and Crackers	0.01912	0.70988
Eggs	-0.01723	0.5018
Meat	-0.27102	0.57052
Soft Drinks	-0.2732	0.38991

Factor loadings > |0.40| considered significant

After patterns were defined, correlations with nutrients were calculated (Table 6-6). Factor 1 was strongly positively associated with calories and protein and most bone-related micronutrients (p-value<0.01), except vitamin A and selenium. In addition, medical food compliance was positively associated with Factor 1 (r-square=0.53, p-value=0.0006). Factor 2 was not associated with macronutrients, but was significantly negatively associated with bone-related nutrients (p-value<0.01), except selenium. Factor 2 was strongly negatively associated with medical food compliance (r-square=-0.72, p-value=<0.0001), suggesting the intake of most micronutrients is dependent on the consumption of medical food.

Table 6-6. Spearman's correlations between factor scores and total intake of individual nutrients

	Compliant, unhealthy		Non-compliant	
	r^2	p-value	**r^2**	p-value
Macronutrients				
Calories (kcals)	0.43295	0.0042	0.04432	0.7805
Fat (grams)	0.3618	0.0186	0.06231	0.695
Carbohydrates (grams)	0.39324	0.01	-0.03654	0.8183
Protein (grams)	0.58204	<.0001	-0.32258	0.0372
Bone-related Micronutrients				
Vitamin A (IU)	0.21562	0.1702	-0.40686	0.0075
Vitamin D (mcg)	0.4673	0.0018	-0.52322	0.0004
Vitamin B12 (mcg)	0.51706	0.0005	-0.51122	0.0005

Calcium (mg)	0.61186	<.0001	-0.61899	<.0001
Iron (mg)	0.47784	0.0014	-0.60408	<.0001
Magnesium (mg)	0.60473	<.0001	-0.64152	<.0001
Phosphorus (mg)	0.58059	<.0001	-0.51171	0.0005
Selenium (mcg)	0.22259	0.1565	-0.22421	0.1534
Zinc (mg)	0.5365	0.0002	-0.58772	<.0001
Sodium (mg)	0.15728	0.3199	-0.10737	0.4986
Cholesterol (mg)	0.00510	0.9744	0.31335	0.0433
PAH Deficiency Diet				
Medical food prescription (grams protein/day)	0.0479	0.7721	0.28529	0.0783
Medical food compliance (%)	0.53175	0.0006	-0.72323	<.0001
Dietary phenylalanine prescription (mg/day)	0.04405	0.79	0.03735	0.8214
Dietary phenylalanine compliance (%)	-0.07915	0.632	0.31457	0.0511

In comparing dietary Pattern 1 and 2 to biomarkers, Pattern 1 (Compliant, unhealthy) was negatively correlated with both bone turnover markers P1NP (r-square=-0.43, p-value=0.006) and CTx (r-square=-0.43, p-value=0.006) but not BMD or BMD Z-score. Pattern 2 (Non-Compliant), on the other hand, was not correlated with any of the biomarkers or bone parameters examined, only marginally with BMD (r-square=-0.29, p-value=0.059). Medical food intake is the strongest determinant of the difference between the two patterns we developed and appears to promote the intake of nutrients associated with lower bone turnover.

Differences in all variables by Z-score category

We compared variables between subjects with BMD Z-scores above -1 (n=35) to subjects with BMD Z-scores of -1 or below (n=9) (Table 6-7). There were no differences in nutrient intake, but women with BMD $\leq$-1 had higher BALP, CTx and P1NP suggesting higher bone turnover in those with lower BMD. This is a key finding supporting the potential utility of BTM in clinical practice to differentiate between those with normal and those with low BMD.

Table 6-7. Differences in key variables between subjects with normal BMD Z-score (>-1) and subjects with BMD Z-score ≤ -1

	Normal BMD (n=35)	BMD Z-score ≤ -1 (n=9)	
Demographics	Mean (SD)	Mean (SD)	p-value[1]
Age (year)	21.4 (10.8)	14.9 (2.8)	0.12
Body mass index (kg/m^2)	26.0 (9.0)	23.6 (8.6)	0.86
Bone mineral density (g/cm^2)	1.116 (0.092)	1.006 (0.114)	0.01
BMD Z-Score	0.214 (0.939)	-1.400 (0.343)	<0.0001
Anthropometrics*			
Percent fat mass (%)	35.6 (8.5)	28.4 (8.6)	0.09
Percent lean mass (%)	61.5 (8.8)	69.1 (8.5)	0.08
Android fat (%)	40.8 (11.5)	38.8 (14.3)	0.86
Gynoid fat (%)	42.3 (6.6)	42.9 (11.5)	0.89
Android/gynoid ratio	0.956 (0.267)	0.876 (0.142)	0.65
Trunkal fat (%)	36.2 (9.9)	34.1 (13.8)	0.74
Physical Activity			
Total moderate-equivalents aerobic activity (min/week)	371.7 (739.2)	296.1 (291.4	0.57
Sitting (min/week)	303.7 (175.9)	256.6 (122.7)	0.37
Walking (min/week)	152.4 (354.3)	146.7 (105.4)	0.81
Strength training (min/week)	74.0 (188.4)	41.1 (85.5)	0.37
Biomarkers			
Phenylalanine (µmol/L)	748.7 (427.7)	888.3 (417.8)	0.26
Vitamin D (ng/mL)	37.5 (13.1)	37.9 (19.1)	0.86
Parathyroid hormone (pg/mL)	27.8 (11.6)	28.2 (12.2)	0.34
Bone-specific alkaline phosphatase (µg/L)	26.1 (12.9)	67.2 (56.2)	0.0014
C-terminal cross-linking telopeptide of type I collagen (ng/mL)	0.804 (0.529)	1.473 (0.914)	0.03
Procollagen type I N propeptide (ng/mL)	117.3 (98.9)	370.3 (332.1)	0.002
CTx/P1NP ratio	0.036 (0.035)	0.057 (0.035)	0.37
Dietary Intake			
Calories (kcal)	1552 (443)	1408 (491)	0.69
Fat (grams)	53.2 (24.6)	43.5 (16.6)	0.48
Total protein (grams)	59.2 (28.4)	48.6 (22.1)	0.60
Medical food protein (grams)	32.3 (24.0)	27.6 (23.1)	0.83
Carbohydrates (grams)	220.6 (60.7)	215.6 (80.7)	0.99
Vitamin D (mcg)	14.1 (28.0)	11.3 (7.8)	0.74
Calcium (mg)	1184 (709)	1234 (647)	0.49
Medical food prescription (grams)	43.6 (16.6)	49.2 (16.8)	0.48
Medical food compliance (%)	101.8 (90.5)	78.4 (71.9)	0.52
Phenylalanine prescription (mg)	610 (507)	394 (220)	0.20

Phenylalanine (mg)	940 (1012)	702 (483)	0.78
Phenylalanine compliance (%)	213.1 (233.3)	193.8 (136.7)	0.94
Dietary Pattern			
Factor 1 (Compliant, unhealthy)	0.07 (1.02)	-0.26 (0.48)	0.40
Factor 2 (Non-compliant)	0.07 (0.99)	-0.26 (0.31)	0.53

[1]Adjusted for age and BMI *Adjusted for age only
Tukey's p-value<0.01 indicates significance

As an exploratory analysis, we utilized stepwise multivariate linear regression to test for associations between BMD and BMD Z-score and variables significantly different by BMD Z-score category in Table 6-7. We also considered key variables in three domains: body composition (fat mass and percent body fat, lean mass and percent lean, BMI), biochemical markers (bone specific alkaline phosphatase, C-terminal cross-lining telopeptide of type I collagen, and procollagen type 1 N propeptide) and dietary intake (calcium, vitamin D, total protein and medical food protein). We ran stepwise regression models with BMD and BMD Z-score expressed as continuous measures as the dependent variable and all of the independent variables also expressed as continuous measures.

Results showed the highest amount of variation in BMD Z-score was explained by blood P1NP and the amount of fat mass (r-square=0.24, p-value=0.0078). The highest amount of variation in BMD was explained by the amount of lean body mass and the percent lean mass (r-square=0.55, p-value=<0.0001). Percent lean mass was negatively associated with BMD while amount of fat mass was positively associated with BMD Z-score.

6.5 Conclusions

We examined comprehensive dietary intake and physical activity in relation to bone health in a sample of adolescent and adult females with PAH deficiency. All had normal BMD and mean intake of individual nutrients met or exceeded recommendations for age and sex except for cholesterol (41% of AI) and vitamin D (90% of RDA). Nutrients related to bone health including protein, vitamin D, calcium, zinc and vitamin B12 were consumed predominantly through medical food (>50% of total intake). The majority of participants (64%) had normal or low concentrations of P1NP (formation) but high concentrations of CTx (resorption) indicating an uncoupling of the natural bone remodeling process. The ratio of resorption to formation decreased significantly with age and PTH, and nearly medical food compliance (p-value=0.056), but not plasma phenylalanine concentrations, suggesting dietary intake has more of an impact on altered bone turnover than metabolic control. Forty-six percent of the sample reported a history of a fracture or break and 44% of the sample met CDC's 2008 Physical Activity Guidelines for aerobic activity for age.

Despite the finding of high bone resorption in this sample of females, DXA scans showed normal BMD for age in all participants and a mean BMD Z-score of 0.045 (range -1.9 to 2.3) contrary to reports of low BMD in previous studies of patients with PAH deficiency [21, 31, 33, 40]. Nine patients (20%) had a BMD Z-score below -1 and compared to those with Z-scores greater than -1, these participants had significantly higher BTM. They were also younger (mean=15 years vs 21 years; p-value=0.12), though the difference was not significantly significant.

Multivariate analyses showed the higher the percent lean body mass of participants, the lower the BMD. Actual amount of lean body mass was, however, positively associated with BMD (β=0.000007; p-value=<0.0001), consistent with other studies in healthy children and adolescents [195]. Those with a higher percent of lean body mass had a lower BMI (r-square=-0.47; p-value=0.0013). Thus, a low BMI may be driving the negative relationship between percent lean mass and BMD and BMD Z-score. Lower BMI is a known risk factor for lower BMD [196]. In addition, those with higher fat mass had higher BMD Z-scores. P1NP was negatively associated with BMD Z-score in multivariate analyses suggesting higher turnover may lead to lower BMD Z-score. This result is in agreement with our finding of significantly higher BTM in those with BMD Z-scores below -1.

The low prevalence of low BMD found in this study compared to other studies could be due to several factors. While this study included females only, we defined low BMD by ISCD criteria (Z-score < -2 for adolescents and pre-menopausal women) instead of alternative definitions used in previous studies [164]. We also hypothesize BMD is improving in patients over time as knowledge of dietary requirements and best management practices improves. In addition, 35% of participants were taking Kuvan or PEG-PAL, pharmaceuticals that allow liberalization of the diet to include more intact protein. As pharmaceuticals are used in more patients with PAH deficiency, bone health may be improving in the population on the whole. BMD Z-score did not, however, differ between those treated with Kuvan or PEG-PAL and patients on dietary restriction and medical food alone.

Dietary Intake and Bone Outcomes

We found higher bone turnover was associated with energy and zinc intake from food, and lower bone turnover was associated with fat, protein and magnesium intake from medical food, as well as compliance with medical food prescription. These findings suggest that medical food, including nutrients other than protein, significantly impact bone metabolism. BMD and BMD Z-scores were not associated with any nutrient except cholesterol intake from food (r-square=-0.48, p-value=0.003; r-square=-0.33, p-value=0.04). The lack of association between nutrients and BMD is likely due to the fact that three-day food records do not capture dietary intake over time which impacts BMD more than recent intake only. Other studies have examined the impact of single nutrients on a variety of bone indicators with mixed results. Most find no correlations between dietary calcium, protein, calories and other nutrients and BMD, BMD Z-scores, BMC or BTM in patients with PAH deficiency of all ages [21, 36, 57, 67, 197]. Only one study found positive correlations between calcium intake and phosphorus intake and BMD Z-score [33]. Unfortunately, none of these investigations controlled for total energy intake, an essential strategy in assessing the impact of diet on health outcomes in epidemiologic studies [198]. Examining dietary intake over time may provide more insight into relationships between nutrient intake and BMD in patients with PAH deficiency.

To examine dietary intake beyond single nutrients, we used factor analysis to develop dietary patterns in our sample. Factor analysis showed two distinct dietary patterns: a compliant but generally unhealthy diet (Pattern 1, Compliant, unhealthy), and a non-compliant and equally unhealthy diet (Pattern 2, Non-Compliant). Though the first pattern can be considered more compliant with medical food, the diet is high in butter,

cream, dairy and sweets and low in fruits and vegetables, representing an unhealthy overall diet. This is an important confirmation that the overall quality of the PAH deficiency diet has much room for improvement, even in those considered compliant with medical food. The compliant group had lower bone turnover markers, indicating better dietary compliance with medical food may have an impact on bone health, reducing potentially harmful elevated turnover. The two dietary patterns were associated with very different nutrient profiles, which we hypothesize is largely driven by medical food intake and were negatively correlated (r-square=-0.34, p-value=0.04), adjusting for age and total caloric intake. Pattern 1 was positively associated with age (r-square=0.38, p-value=0.014) and negatively with fat mass (r-square=-0.33, p-value=0.04) but not with blood phenylalanine. Pattern 2 was not correlated with age, body composition, or blood phenylalanine concentration.

We found all participants in this study had very low consumption of whole foods, including fruits and vegetables that are low in phenylalanine and recommended as staples in the PAH deficiency diet. Based on the results of our dietary pattern analysis, clinicians should encourage consumption of these low-phenylalanine foods instead of the current consumption patterns of snack foods, sweets, cakes, crackers and fats. Evidence suggests that patients can maintain an adequate degree of metabolic control when counting fruits and lower-protein vegetables as “free” foods [199]. Encouraging healthy, nutrient-dense foods such as vegetables and fruits could improve some of the negative outcomes reported in patients with PAH deficiency, including high bone turnover and low BMD.

Bone Turnover

In our sample, BTM concentrations indicated uncoupling of the natural bone remodeling process favoring bone breakdown, known as resorption. While CTx and P1NP were significantly positively correlated (r^2=0.74, p-value<0.0001), a completely coupled scenario would yield a correlation closer to 1.0. The ratio of resorption to formation decreased with age and PTH concentration (p-value<0.01) suggesting uncoupling could be more pronounced in younger patients and those with lower PTH. Individual BTM decreased over time [71], but the ratio of resorption to formation should be lowest in the youngest age group when bone formation is predominant. Our findings are opposite of the expected relationship between CTx/P1NP ratio and aging. A small sample size could limit the interpretation of the relationship, but we believe the potential higher degree of uncoupling in younger patients warrants further investigation.

The inverse relationship between PTH and CTx/P1NP ratio is more difficult to interpret. Normally, low PTH, also known as hypoparathyroidism leads to low blood calcium, high blood phosphorus concentrations, and reduction in bone turnover [200]. Conversely, high PTH causes increases in bone turnover. In our sample, PTH was not correlated to either BTM independently, only negatively with CTx/P1NP ratio and we hypothesize another factor may be at play. We hypothesize high PTH may increase overall bone turnover, but increase formation (P1NP) more than resorption (CTx) while low PTH may decrease overall bone turnover, but P1NP more than CTx, leading to the increased ratio of CTx/P1NP found in this study. Thus, the effects of PTH on bone turnover are largely driven by its impact on P1NP, not CTx. In fact, in multivariate regression analysis, the largest factor driving variation in CTx was age, followed by blood vitamin D (r^2=0.25; p-value=0.004), both of which were negatively associated.

Vitamin D likely plays a role in the relationship between PTH and CTx/P1NP ratio. Vitamin D concentration below 40 ng/mL is known to increase PTH and 64% of the sample had a vitamin D concentration below 40 ng/mL. Vitamin D and PTH were negatively correlated in this sample (r^2=-0.40, p-value=0.01) and blood vitamin D was positively correlated with BMD Z-score in multivariate analysis (β=0.02, p-value=0.026). Future studies may benefit from measuring not only 25-hydroxyvitamin D, a marker of vitamin D status, but also 1-25-dihydroxyvitamin D, the biologically active form of vitamin D. Medical food compositions may also need to be examined to ensure vitamin D needs are met in young patients, especially given 88% of children and adolescents did not meet the RDA for vitamin D and 83% of those included in this study with insufficient blood vitamin D levels were children or adolescents. Vitamin D supplements may be considered in younger patients with PAH deficiency with lower vitamin D levels, even if sufficient amounts of vitamin D are provided in medical food to ensure RDA is met. This may improve the higher CTx/P1NP ratio found in younger patients.

Blood phenylalanine was not significantly correlated with any blood biomarker, but moderately positively correlated with CTx (r-square=0.27, p-value=0.09). A single in vitro study has examined the impact of phenylalanine on bone resorption in patients with PAH deficiency, finding higher phenylalanine concentrations were associated with higher circulating osteoclast precursor cells [201]. In cultured cells from PAH deficiency patients, T cells were responsible for the activation of osteoclast precursor cells into mature osteoclasts which carry out the process of bone resorption [202]. T cells, an indicator of immune activation, may be an intermediate underlying the potential

relationship between higher phenylalanine concentrations and higher bone resorption found in this study [203].

The RANK system is another important consideration. RANK-ligand (RANKL) is expressed by activated T cells and ultimately induces maturation of osteoclast precursor cells by binding to RANK receptors [203]. Conversely, osteoprotegerin (OPG) also binds to the RANK receptor to block the binding of RANK and prevent the maturation of osteoclasts, thus downregulating bone resorption [204]. Future studies could assess T cell activation, RANKL expression, binding of RANKL to RANK receptors, osteoprotegerin and bone turnover marker concentrations in patients with PAH deficiency. Including the entire osteoclast activation system could help to elucidate the mechanism leading to an increased activation of osteoclasts with higher phenylalanine concentrations as reported by Roato et al [201] and the uncoupling of bone turnover to favor bone resorption found in this study.

Limitations and Future Directions

Our sample of patients included a cross-section of females only with PAH deficiency. These females were attending a camp for patients of Emory Genetics or clinics familiar with Metabolic Camp and many have returned for multiple years over time. These campers may be a special group of patients with PAH deficiency who are more invested in managing their disease or at least more open to following recommendation than other patients who rarely or never attend clinic visits. Campers may also have supportive families with more financial, educational and social resources than patients with PAH deficiency who have never attended camp. Thus, the specialized nature of this sample may have produced better compliance rates with medical food and

protein restriction and potentially better bone health. In addition, cross-sectional studies can never show causality, only associations between variables.

Bias could also exist in three day food records since all participants included knew they were coming to camp and would be submitting dietary intake. Participants may have changed their usual diet to appear more compliant or recorded false food items and false compliance with medical food. This is a limitation for dietary intake research in any population. More studies are required with larger groups of patients with PAH deficiency, including males, to fully understand different dietary patterns in this population. Though mean intake of most nutrients met recommendations in this study, many nutrients came primarily from medical food. Absorption of nutrients from medical food sources versus food sources has not been examined. It would be useful to examine absorption of individual nutrients from medical food compared to absorption rates from food sources to ensure dietary intake correlates to biologic availability.

In addition, we recommend investigating other methods of measuring bone status such as a quantitative heel ultrasound (QUS) as a potential alternative to DXA [205]. We also recommend assessing menstrual status and hormone levels in association with bone health, and the development and validation of a tool to assess physical activity levels in women with PAH deficiency. Finally, prospective cohort studies are need to assess the relationship between bone turnover markers and long-term risk of developing low BMD, fractures, and/or osteoporosis.

Clinical Applications

Adhering to the Genetic Metabolic Dietitians International (GMDI) recommendations to monitor BMD by DXA every 3-5 years in adolescents and every 5

years in adult patients with PAH deficiency is recommended [12]. Though we did not find any patients with low BMD according to ISCD criteria (BMD Z-score $\leq$ -2), 20% had a Z-score $\leq$ -1, of which long-term clinical implications are unknown. Adding measurement of P1NP and CTx to assess the ratio of resorption to formation in regular clinical practice could be useful if additional studies show correlations between BTM and BMD and long-term fracture risk. In addition, patients who are not taking medical food, or taking less than prescribed, may be at-risk for deficiencies in nutrients related to bone metabolism. Patients should be encouraged to consume recommended amounts of medical food to promote normal bone turnover and minimize potentially detrimental increases in turnover associated with uncoupling and excess resorption. In addition, promoting fruit and vegetable intake in lieu of unhealthy snack foods, fats and carbohydrates should be included in treatment recommendations for all patients with PAH deficiency given the unhealthy diets shown in our dietary pattern analysis.

Final Conclusion

In conclusion, patients with PAH deficiency have normal BMD but potentially uncoupled bone turnover with high bone resorption and normal bone formation. Patients are meeting recommended intakes for bone-related nutrients, largely through medical food intake except for vitamin D. Optimal dietary intake, particularly medical food compliance and adequate micronutrient intake, likely impacts bone mineralization more than metabolic control in patients with PAH deficiency.

Patients must be encouraged to comply with their medical food prescription, not only to meet protein requirements, but also to ensure intake of nutrients associated with BMD including vitamin D, calcium and zinc. A tool to accurately measure aerobic and

strength physical activity and its impact on bone health should also be a priority. Finally, future research must measure BMD and bone turnover markers longitudinally in patients with PAH deficiency to examine changes in BMD over time and long-term risk of fracture. We believe that the findings reported in this study, combined with results of Chapter 4, suggest bone health is not as poor in patients with PAH deficiency as previously hypothesized. We also suggest tools such as the predictive model developed in Chapter 5 could be useful in screening for the small subset of patients with clinically low BMD.

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Chapter 7. Discussion of Results and Conclusions

Low BMD is not a pressing concern for patients with PKU, also known as PAH deficiency. While BMD is lower than expected for age and sex, pooled data of all available high-quality evidence show mean BMD Z-scores are well within the range of normal at all sites examined (total body, lumbar spine, femoral neck). Our finding that incorrectly applied definitions have been used to quantify the prevalence of osteopenia and osteoporosis is critical. Moving forward, the appropriate definition of low BMD (Z-score $\leq$ -2) in children, premenopausal women and men under age 50 should be used [121]. Osteopenia should not be used to diagnose pediatric patients and the diagnosis of osteoporosis requires the presence of a fracture. When patients receive DXA scans, it is very important to interpret results using the correct definitions [120].

While we do not believe low BMD is a significant problem in the PKU population, especially given all subjects reported in Chapter 6 had normal BMD, a subset of patients with risk factors may benefit from screening and monitoring to prevent the development of osteoporosis and fractures. In addition, males 10-15 years of age have the lowest mean BMD Z-score of any sex or age, representing a group that may warrant additional investigation. Ideally, recommendations for regular DXA scans should be followed [12], though this is not feasible in many clinics. With validation, the model developed in Chapter 5 could be used to screen for patients with low BMD using routine patient parameters collected at clinic visits. In the future, measuring serum CTx and P1NP as recommended by the IOF and IFCC could also help to identify patients with abnormal bone turnover and potentially low BMD. Those with abnormal turnover should receive DXA scans, detailed dietary assessment and a follow-up measurement of serum

vitamin D. In most individuals with PKU who comply with dietary treatment and strive to maintain metabolic control, however, low BMD is not a concern.

Age, body composition and dietary intake likely contribute most to overall bone health in patients with PKU compared to other factors we measured including metabolic control. While blood phenylalanine may exert some effect on osteoclast differentiation, this has been shown in isolated cell studies only [201]. Our meta-analysis showed that the impact of phenylalanine on BMD in human studies is inconclusive and our primary data collection suggests phenylalanine is not associated with BMD or bone turnover markers. Instead, in our cross-section of 88 patients, we found higher dietary intake of vitamin D, calcium and medical food are associated with higher BMD Z-score, while higher dietary intake of carbohydrate, sugar, caffeine, and glycemic load are associated with lower BMD Z-score. These nutrients are important to bone metabolism in different ways and in Chapter 6, we show a dietary pattern characterized by higher medical food consumption is associated with lower bone turnover. Dietary intake and BMD should be carefully assessed, particularly in those with poor compliance to medical food.

The story of bone health in patients with PKU is a prime example of the grey area created between clinical research and patient care. A problem is identified and examined repeatedly by individual, poorly-powered studies; however, results are heterogeneous, defined using different criteria, and do not provide an evidence base strong enough to draw conclusions. This dissertation serves as a translational model to identify a problem, evaluate existing evidence, identify gaps, and develop an investigation to fill gaps. The final goal is to disseminate findings by creating evidence and consensus-based

recommendations for clinicians and a toolkit for patients and families outlining prevention and treatment strategies for low BMD in patients with PKU.

7.1 Assumptions and Limitations

As in any study, there are limitations. Chapter 4 included early-treated patients only, thus the conclusion that BMD is within normal range in most patients with PKU may not be applicable to late-diagnosed or late-treated patients. The review was also a collaboration between two research teams and was driven by two sets of initially different aims.

Chapter 5 and Chapter 6 involved primary data collection and present with a different set of limitations. Patients included in these studies were attending clinic visits at Emory Genetics Clinic or participating in Metabolic Camp at Emory University. These patients may have been more invested in PKU management and healthier overall compared to patients who do not attend regular clinic visits. Moreover, study participants knew they would submit diet records to the research team, thus there might be bias in recording. To examine relationships between dietary phenylalanine and dietary vitamin D and blood phenylalanine and blood vitamin D, Spearman's correlation coefficients were calculated. Neither were correlated [dietary and blood phenylalanine r-square=-0.06, p-value=0.70; dietary and blood vitamin D r-square=-0.13, p-value=0.45 (adjusted for age and BMI)], suggesting diet records may not provide the most accurate estimation of nutrient intake, at least for phenylalanine and vitamin D. Dietary assessment methods should be improved, especially in conditions and disorders where dietary intake is an essential component of overall care. Results of correlation analyses including dietary

intake should be interpreted with caution until findings are repeated in a separate sample of patients, ideally including males.

In Chapter 5, we did not validate predictive models in a separate set of patients with PKU. Validation is needed before models can be recommended for clinical use. The best model also included a BTM measured in blood, bone specific alkaline phosphatase. While this may be measured in PKU patients by some clinics, other clinics may need to add this marker to regular patient labs. BALP is, however, widely available and relatively inexpensive compared to other BTM included in Chapter 6. In addition, caffeine intake, included in the predictive model may be difficult to assess using the popular dietary analysis program used in patients with PKU, Metabolic Pro. Currently, Metabolic Pro does not calculate caffeine intake when analyzing diet records. Caffeine intake may need to be hand calculated by asking patients if they consume soda, coffee, energy drinks and other caffeine-containing foods. Tools exist to assess caffeine intake in healthy populations that could also be used in patients with PKU [206]. Alternatively, GMDI, the owners of Metabolic Pro, could add caffeine to the nutrients included in output reports. Metabolic Pro links to the National Nutrient Database for Standard References, coordinated by the USDA which does contain caffeine. Regardless, it is important for clinics to implement methods to collect all variables included in the predictive model before validation can occur.

While Chapter 5 attempted to account for a variety of unmeasured factors affecting BMD in patients with PKU, every possible variable could not be included. We did, however, include genetic data (mutations in PAH gene). Severity of mutations in the PAH gene has been hypothesized to impact BMD, but we found no difference in BMD Z-

scores between patients of different clinical severities (classical versus mild/moderate vs unidentified severity) as categorized by an AV sum [29]. A limitation to this analysis was the subgroup of patients (n=13) with an unidentified mutation or a mutation of undefined severity whom we could not classify using the AV sum methodology. If those patients had been definitively classified as classical or mild/moderate, differences in bone health may have emerged. We hypothesize, however, that our results would remain consistent and that genotype does not have an independent effect on BMD. Future studies should, however, include an assessment of the impact of genotype on BTM.

In healthy individuals, single nucleotide polymorphisms (SNP) may have a significant influence on BMD [207] and GWAS studies have attempted to identify relevant SNP [208]. The estrogen receptor gene and a gene related to the NF-kB signaling pathway appear to be significantly associated with BMD in premenopausal women [208]. In addition, the RANKL-OPG pathway and several other loci not traditionally associated with bone metabolism are associated with fracture risk [209]. In future studies on bone health in patients with PKU, genetic mutations in the PAH gene and whole genome sequencing would be very important to include in studies on fracture risk since 64-67% of variation in total body BMD is explained by additive genetic effects [207]. Unfortunately, we did not have whole-genome sequencing on any of our cohort of patients with PKU.

Finally, Chapter 6 includes females only, a limitation to generalizability, but a strength in understanding physiology of bone health in females with PKU specifically. Based on our finding that male patients with PKU 10-15 years of age have the lowest mean BMD Z-score, we recommend future studies include males whenever possible. All

data are, however, cross-sectional and highlight the need to establish a longitudinal cohort of patients with PKU to assess long-term outcomes and treatment strategies to optimize health outcomes.

7.2 External Validation of Predictive Models

Chapter 5 describes the development of screening tools to estimate BMD Z-score in patients with PKU. The results are promising, with a sensitivity of 66.7% compared to DXA Z-scores. The false-negative rate is low (6.2%) indicating most patients with low BMD, defined for screening purposes as < -1, had low BMD estimated by the model. A low false-negative rate is important in developing screening tools to make sure patients with the outcome of interest are not missed.

To validate the best model which included medical food compliance, medical food intake, caffeine intake and serum bone-specific alkaline phosphatase as predictors of BMD Z-score, these same data are needed from a separate population. Once collected, predictors should be entered into the model to predict BMD Z-score and compared to DXA Z-scores. Sensitivity, specificity, false positives, false negatives, and a final area under the ROC curve (AUC) with various Z-score cut-offs to distinguish normal BMD from low BMD should be calculated. If the model predicts BMD Z-score category with an acceptable degree of accuracy in another sample of patients with PKU, we can recommend its use in metabolic clinics. We will also examine if the model's BMD Z-score predictions improve with the addition of one or more BTM presented in Chapter 6 (CTx, P1NP, BALP) by using linear regression to develop a new predictive models using SAS. If the r-square value (variation in BMD Z-score explained by the selected combination of predictors) increases significantly with the addition of one or any

combination of BTM, models may be expanded to include BTM. If models are significantly improved with the addition of BTM, it is also an indication that BTM could be useful as regular clinical measure in patients with PKU to indicate those that may have lower BMD Z-scores who would benefit from a DXA scan.

7.3 Clinical Implications for Patients with PKU

While osteopenia and osteoporosis are not pressing issues in patients with PKU, we still recommend following GMDI-SERC guidelines to assess bone health through DXA regularly [12]. Clinicians may wish to educate their PKU patients and families that bone health is not a worry unless the patient presents with other risk factors, such as low consumption of medical food, low BMI or high BTM if assessed. Clinicians must stress the importance of medical food and complying with medical food prescription to meet not only protein requirements, but also requirements for bone-related micronutrients such as vitamin D, calcium and zinc.

Results of Chapter 4 have already been presented at the 2015 SERC-SERGG regional genetics meeting attended by metabolic clinicians [210]. The message that 90% of patients have normal BMD for age is an important conclusion and should be disseminated not only to clinicians, but also to patients and families. We plan to present the results of this dissertation to patients and families through the National PKU Alliance via webinar. An announcement will be sent through the National PKU Alliance listserv that new results regarding bone health are available and we encourage participation in the webinar from the entire PKU community.

7.4 Future Research

To provide a definitive conclusion on the state of bone health in patients with PKU, future research must include correct definitions of low BMD and osteoporosis. WHO criteria can be applied to post-menopausal patients and men over 50 years of age, but the majority of patients should be evaluated using the ISCD criteria. In addition, prospective fracture incidence must be assessed and correlated to baseline BMD, CTx and P1NP. In patients with BMD Z-scores between -1 and -2, it is unknown if risk of fracture is increased and this is an important group of patients to follow longitudinally. In longitudinal assessments of patients with PKU, validation of the predictive model presented in Chapter 5 should also be included by measuring dietary intake, medical food compliance and prescription, and the bone turnover marker bone-specific alkaline phosphatase. We also recommend including genetic mutations and whole genome sequencing if possible to identify how genetics impact BMD and fracture risk.

Future research must also examine absorption of nutrients from medical food versus intact food sources in patients with PKU. Since medical food is elemental, absorption may be increased or decreased depending on the nutrient and its absorption mechanism in the gastrointestinal tract. Intake of most nutrients meets requirements for most patients; however, when considering source, several nutrients come primarily from synthetic medical food, particularly bone-related nutrients vitamin D and calcium. Normal absorption is assumed, however, it has not been examined and should be in future research studies. It will be important to consider the source of nutrients and absorption when evaluating nutritional intake in patients with PKU moving forward.

7.5 Next Steps

To disseminate this work, the author plans to develop evidence and consensus-based recommendations to manage bone health in PKU. Guidelines for practitioners will be developed using the GMDI Delphi-Nominal-Delphi-Field Testing model (DNDF) as a framework and published online in open-access format. A toolkit will also be developed including treatment and management algorithms for patients presenting with normal, at-risk, or low BMD. Finally, a website and complementary smartphone application for consumers including patients with PKU and their families will be created to empower patients to take charge of their own health. The application will include the predictive equation developed in Chapter 5 so patients may enter clinical data, calculate BMD Z-score, and notify practitioners with any concerns. Moving forward, it is essential to interface more advanced technology with patient care. Bone health is an excellent preliminary model to highlight the potential utilities of predictive health.

7.6 Final Conclusion

Based on the results of a limited number of studies, the conclusion that patients with PKU have a high prevalence of osteoporosis and osteopenia has been based on incorrectly applied definitions. Our meta-analysis shows mean BMD Z-scores are lower than average, but well within the range of normal. BMD Z-scores are most correlated with dietary intake rather than metabolic control. Primary data collection shows evidence of high bone resorption and uncoupling of bone turnover, especially in younger patients and those with lower PTH. Uncoupling of bone turnover may underlie the slight decrease in BMD as evidenced by mean Z-scores below 0 in the PKU population on the whole. Medical food intake is beneficial to bone health and better compliance with medical food

prescription is associated with higher nutrient intake and lower bone turnover. Despite our finding that 90% of patients with PKU likely have normal BMD, regular assessment of bone health is still important to meet recommendations published by GMDI-SERC and to collect data to understand the relationship between BMD and BTM and long-term risk of fractures and osteoporosis.

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