Serum Vitamin D Among Children with Inherited Metabolic Disorders

By

Katie Elizabeth Geiger

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CERTIFICATE OF APPROVAL

This is to certify I have read the Master's Thesis of

Katie Elizabeth Geiger

and approve the research presented here in

[redacted]

Melanie Gillingham, PhD, RD, LD

[redacted]

Cary Harding, MD

[redacted]

David Koeller, MD

[redacted]

Kathleen Huntington, MS, RD, LD

TABLE OF CONTENTS

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LIST OF ABBREVATIONS AND ACRONYMS

$1,25(OH)_2D$	1,25-dihydroxy-vitamin D
1-OHase	25-hydroxyvitamin D-1 α-hydroxylase
25(OH) D	25-hydroxy-vitamin D
BA	Bone Age
B-ALP	Bone Alkaline Phosphatase
BH_4	Tetrahydrobiopterin
BMD	Bone Mineral Density
CNS	Central Nervous System
DBP	D Binding Protein
DCH	Doernbecher Children's Hospital
DEXA	Dual-Energy X-ray Absorptiometry
DRI	Dietary Reference Intake
IEM	Inborn Errors of Metabolism
IOM	Institute of Medicine
IRB	Institutional Review Board
OC	Osteocalcin
OCTRI	Oregon Clinical & Translational Research Institute
PAH	Phenylalanine Hydroxylase
PHE	Phenylalanine
PKU	Phenylketonuria
PTH	Parathyroid Hormone
RDW	Research Data Warehouse
UVB	Ultraviolet B
VDR	Vitamin D Receptor
TYR	Tyrosine

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Background: Recent research in children with Phenylketonuria (PKU), the most common inherited disorder of amino acid metabolism, suggests they have decreased bone mineral density (BMD). Although the etiology of this problem is unknown, children with PKU and other inherited metabolic disorders consume specialized diets that often severely restrict vitamin D-containing food sources. The primary goals of this study were to evaluate the prevalence of vitamin D deficiency in a group of children with inherited metabolic disorders who are consuming a medical food diet, and determine whether BMD in children with PKU correlates with diet and/or biochemical markers of bone metabolism.

Objectives:

1) To compare serum 25-hydroxy-vitamin D concentrations in children with inborn errors of metabolism (IEM) that are consuming a medically modified diet to those in a group of control children without the diagnosis of an IEM who consume an unrestricted diet.
2) To determine whether the BMD in a group of patients with PKU correlates with diet and/or biochemical markers of bone metabolism.

Methods: The research described here utilized both a retrospective case-control chart review and a prospective observational study carried out at the OHSU CDRC Metabolic Clinic investigating vitamin D status and bone mineral density in children with inherited metabolic disorders, including PKU. The retrospective chart review compared vitamin D

status in children with IEM against other children without an IEM diagnosis. The EPIC electronic medical record was queried to obtain serum 25(OH)D concentrations in children treated at the metabolic clinic who consumed medical foods and the serum 25(OH)D concentrations of subjects treated in a separate clinic at Doernbecher Children's Hospital (DCH) who did not consume medical foods. Control subjects were from any DCH clinic except for the metabolic clinic, and data was excluded from any control who had the diagnosis of a disorder that could directly interfere with 25(OH)D status or bone metabolism. A total of 918 records were obtained from the initial query, and after rigorous evaluation of the data, statistical analysis was performed on data derived from 537 total case and control subjects. Subjects who were missing data on age, zip code, diagnosis, or serum 25(OH)D level were excluded from the chart review, as well as subjects who resided outside of Oregon and Washington. The results were statistically compared using unconditional logistic regression analysis, and adjusted for differences in mean age at draw date, sex, geographic region, and season of blood draw.

For specific aim 2 we used a prospective observational design. A total of twenty subjects with PKU 9-20 years of age were recruited and evaluated over a 6-month period. Variables evaluated included serum 25(OH)D, PTH, plasma calcium, alkaline phosphatase, and phenylalanine, which were measured during routine clinic visits. Bone mineral density was determined via DEXA in the body composition core of the Oregon Clinical and Translational Research Institute (OCTRI). Spearman's correlation analysis was used to examine the relationship between the biochemical and dietary markers of bone metabolism and bone mineral density.

Results:

Aim 1:

1) There were no significant differences in serum 25(OH)D concentrations between case and control subjects, even when we adjusted for mean age, sex, season of blood draw, and region of residence.

Aim 2:

1) All 19 subjects who provided blood samples were sufficient in serum 25(OH)D when we used 20 ng/mL as the lowest acceptable level to define sufficiency.
2) Sixteen out of 19 subjects who completed the hip and spine DEXA scan demonstrated hip and spine BMD within normal limits (z-score greater than -2). Three subjects demonstrated a low spine or hip BMD, suggesting compromised BMD. No subject presented with both a reduced spine and hip BMD. A significant association was found between mean dietary calcium intake and spine BMD.

Conclusion:

1) For both case and control subjects, serum 25(OH)D concentrations were within the recommended range, and the influence of both endogenous and exogenous vitamin D sources appears to be sufficient in this population.
2) Children with PKU living in the northwest demonstrated normal BMD associated with normal serum 25(OH)D concentrations.
3) Our results support that a diet with adequate intake of key nutrients play a primary role in maintaining normal bone mineralization among this population.

Chapter 1

Introduction

Vitamin D, calcium, and phosphorous are essential for proper bone growth and integrity in children, and deficiency of any of these nutrients can lead to rickets[1]. Children with inherited metabolic disorders consume specialized diets that restrict natural food sources of vitamin D, which might place them at an increased risk for micronutrient deficiencies and bone disease[2]. In 2010, the Institute of Medicine (IOM) released revised dietary reference intakes (DRIs) for calcium and vitamin D. The report acknowledges that there is a great degree of controversy and uncertainty surrounding adequate levels of serum 25(OH)D to promote proper bone mineralization. Currently, many laboratories use 25(OH) D concentrations of 30 ng/mL to reflect the lower limit of normal for adults consuming a regular diet. In this report the IOM proposes that plasma levels of 20 ng/mL are associated with normal bone mineralization in healthy adults. However, this data does not include recommendations for children or adults consuming special diets for aminoacidopathies[3].

There are several factors that could predispose patients with an inherited metabolic disorder to vitamin D deficiency. The proper treatment for most of these metabolic diseases involves strict dietary management. A majority of clinics recommend that children practice lifelong adherence to specialized diets. These specialized diets for inborn errors of metabolism often severely restrict or eliminate vitamin D-containing food sources, such as dairy and fish. In addition, children treated at DCH live in a

northern climate with limited sun exposure during winter months. This restricts endogenous vitamin D synthesis that results from skin exposure to ultraviolet light. Vitamin D is essential for proper bone growth and integrity among children, and depleted stores can lead to osteomalacia. Phenylketonuria (PKU) is an inherited disorder of amino acid metabolism that results from mutations in the gene for phenylalanine hydroxylase (PAH). PKU is the most common inherited disorder of metabolism that requires treatment with a medical diet that includes restriction of foods that supply vitamin D. Prior studies of bone mineral density in children with PKU have been inconclusive, but there is evidence suggesting that children with PKU have a compromised skeletal status or a decreased bone mineral content[4]. In addition to the low vitamin D content of the diet, it has also been postulated that mutations in the PAH gene inherently predispose patients to osteoporosis independent of vitamin D[5].

The Metabolic Clinic in the Child Development and Rehabilitation Center at Oregon Health & Science University treats children and adults with inherited metabolic disorders. In addition to the risk for vitamin D deficiency resulting from their diet therapy, patients treated at the metabolic clinic have an added risk factor because of the limited ultraviolet light during the winter in Northwestern Oregon and other northern latitudes, which reduces endogenous vitamin D synthesis in the skin. The goal of this study is to evaluate vitamin D status and bone health in patients treated in the metabolic clinic.

Specific Aims and Hypotheses

We hypothesize that children with inherited metabolic disorders treated with medical diets in the metabolic clinic at OHSU will have lower serum vitamin D concentrations than control children on unrestricted diets. Further, we hypothesize that some patients with serum 25(OH)D levels greater than 20 ng/mL will present with evidence of low bone mineralization or rickets. We will test this hypothesis by comparing serum vitamin D concentrations in children with inborn errors of metabolism on medical diets to a control group of children on unrestricted diets followed by other DCH clinics. We also hypothesize that a subset of the study population will have evidence of rickets or low bone mineral density that is correlated to plasma vitamin D concentrations.

1) Specific Aim 1: This aim is a retrospective chart review of the electronic medical record that will not require subject consent. We will query the Research Data Warehouse (RDW) of the Oregon Clinical and Translational Research Institute to compare serum vitamin D concentrations in children treated at the metabolic clinic to the serum vitamin D concentrations of age- and sex-matched subjects treated in a separate DCH clinic.

   Task: Utilize the RDW database to search for serum vitamin D concentrations of children treated at the metabolic clinic using ICD 9.0 diagnostic codes (ICD 9.0; International Statistical Classification of Diseases and Related Health Problems) and control children treated at other DCH clinics. Data obtained

from all subjects will include: serum 25(OH)D concentration, age (years), sex, and date of the vitamin D measurement or analysis. Data obtained from control subjects will be screened for ICD 9.0 codes that may impact serum 25(OH)D status. Subjects will be excluded from participation in the study if they have a diagnosis that could affect either sun exposure or bone mineralization. Results will be statistically compared using unconditional regression analysis. *Hypothesis:* We expect that children with inborn errors of metabolism on medical diets will have significantly lower serum 25(OH)D concentrations than controls.

2) Specific Aim 2: This aim necessitates informed consent from subjects and their parent and/or legal guardian. We will measure the bone mineral density by dual-energy x-ray absorptiometry (DEXA), serum 25(OH)D, parathyroid hormone (PTH), plasma calcium, phosphorous, and alkaline phosphatase concentrations in the subset of patients with PKU treated at the metabolic clinic.

   Task: Recruit 20 patients ages 9-20 followed at the metabolic clinic for management of their metabolic disorder to participate in this study. After receiving informed parent or guardian consent, we will measure bone mineral density, blood and urine parameters during their routine visits to the clinic. We will examine the relationships between bone mineral density, serum 25-OH vitamin D concentrations, PTH concentration and urine calcium excretion with correlation analysis. *Hypothesis:* We expect that some

children with PKU will present with low bone mineralization and low serum 25-OH vitamin D concentrations. We anticipate bone mineral density and serum vitamin D concentrations will be positively correlated.

Chapter 2

Background and Significance

Phenylketonuria (PKU) is a rare inborn error of phenylalanine (Phe) metabolism affecting approximately 1 in 15,000 children in the United States[6]. Untreated PKU leads to irreversible neurological impairment and growth retardation[7]. Microcephaly is common and about 25% of patients with untreated PKU have epilepsy[8]. It is an autosomal recessive disorder resulting from a mutation in the gene for phenylalanine hydroxylase (PAH)[9]. PAH catalyzes the conversion of Phe to tyrosine (Tyr), and requires iron, molecular oxygen and tetrahydrobiopterin (BH_4) for activity[6,10].The PAH gene is primarily expressed in the liver and kidney[10]. Most often, the loss of PAH enzyme function in PKU patients results from mutations that lead to protein misfolding and its consequent degradation[10]. Under normal metabolic circumstances, a small amount of dietary Phe is used for protein synthesis, and the remaining amount is converted to Tyr[11]. However, when this pathway is blocked, as in PKU, the Phe not used for protein synthesis accumulates in body fluids or is converted to other metabolites [11,12]. This results in serum and tissue elevations in Phe (hyperphenylalinemia) and reduced concentrations of Tyr[13]. In PAH deficiency, Tyr becomes an essential amino acid[11].

Discovery of PKU

PKU was one of the earliest described metabolic diseases, and is one of the most common inborn errors of metabolism [7,14]. In 1934, a Norwegian biochemist and physician named Asborn Følling first identified and described PKU in two young children.

For this reason, many people still refer to PKU as "Følling's Disease". Prior to this discovery, individuals with PKU were not differentiated from individuals in the general population who exhibited indiscriminate neurological and cognitive deficits[15]. Følling explained the pathophysiology behind PKU by performing urine analyses of two siblings exhibiting the characteristic symptoms. He found that their urine contained high levels of phenylpyruvic acid, which is responsible for the "musty" odor often described in individuals with untreated PKU. In all of the children that Følling investigated, he observed shared traits among them, such as fair complexions, eczema, broad shoulders, stooping shoulders, spastic gait, and severe intellectual impairment[15]. Other characteristics often seen in patients with severe PKU include microcephaly and seizures[7].

Disease Mechanism

Accumulation of Phe interferes with the normal development and function of the central nervous system (CNS). Neurological, neurocognitive, and neuropsychological outcomes in children are correlated with blood Phe levels[10]. There are several theories behind this causality relationship, but experts remain uncertain about the exact pathophysiology of PKU. A long-standing theory postulates that elevated Phe levels in the brain disturb axon myelination, resulting in neurological deficits. Other researchers have proposed that high Phe levels may decrease neurotransmitter receptor density, and consequently reduce cell connectivity. This hypothesis involves competition for transport across the blood brain barrier: the amino acids Phe, Tyr, and tryptophan all

share the same transport system. Elevated serum Phe competitively inhibits dopamine and serotonin synthesis, possibly limiting transport and availability of Tyr and tryptophan[16].

Diagnosis, Treatment and Compliance

Neonatal screening to identify infants with PKU and allow for early dietary intervention is done in the United States, Europe, and many other parts of the world, and has helped prevent the severe and irreversible brain damage associated with PKU[13]. PKU can be classified into three subtypes according to blood Phe levels of patients consuming a diet with unrestricted protein intake: *Classic* PKU (Phe>1200 µmol/L), *moderate* (Phe 600-1200 µmol/L), and *mild* (Phe 360-600 µmol/L)[17]. Plasma levels below 360 µmol/L are considered nontoxic and these patients are generally not treated. The severity of the disease directly corresponds with higher levels of plasma Phe. There are over 500 mutations identified in the PAH gene, contributing to a wide variation in disease severity[18]. Individuals with classical PKU demonstrate very little or no PAH activity, and if left untreated their plasma Phe concentrations rise above 1200 µmol/L[18]. Individuals with milder forms of PKU, also known as hyperphenylalanemia, exhibit some residual PAH activity and their plasma Phe concentrations do not exceed 1000 µmol/L when left untreated[18].

The goal of PKU treatment is adequate control of blood Phe concentrations in order to avoid the adverse developmental outcomes associated with the disease[19]. The brain is most vulnerable to toxic Phe levels during the first year of life, further emphasizing

the need for early diagnosis and treatment[16]. Dietary intervention is the most effective method for treatment of PKU[17]. In the 1950s, Horst Bickel first introduced the concept of a low-Phe diet to treat PKU[6]. Successful dietary treatment of PKU entails sufficient maintenance of plasma Phe concentrations while providing adequate nutrition for proper growth and development[18]. Appropriate control of Phe levels is largely influenced by medical food intake, the amount of natural protein consumed, and adequate energy intake[20]. The most common Phe-containing foods include: milk, eggs, cheese, nuts, soybeans, beans, chicken, fish, beef, peas, chocolate, bread, rice, pasta, flour, cookies, and some fruits and vegetables[6]. Children with PKU who adhere to the recommended low-Phe diet, have restricted intake of vitamin D-containing food sources such as dairy products, fatty fish, and meats. It is possible that this micronutrient deficiency could contribute to bone abnormalities among children with PKU. Protein-rich foods contain the highest amounts of Phe. Low-protein foods such as fruits and vegetables are consumed to meet the required amount of Phe in the diet. Foods, beverages, and medications containing the artificial sweetener aspartame must also be avoided, because aspartame releases Phe when digested.

The average daily Phe intake among the general US population is 3.4 g/day (3400 mg/day)[18]. Classical PKU patients should restrict their Phe intake to 0.2 to 0.5 g/day (200-500 mg/day), while patients with hyperphenylalanemia can liberalize their intake to greater than 0.5 g/day[18]. Phe tolerance is the amount of Phe an individual patient can eat and keep blood Phe within the treatment range, and is expressed as mg Phe/kg body weight/day[21]. Phe tolerance can be determined through frequent blood draws to

assess blood Phe concentrations which are compared to average daily Phe intakes. Measurement of Phe tolerance can help clinicians establish individual dietary prescriptions for patients[21].

Dietary intervention necessitates a careful balancing act because over-restriction of Phe can ultimately lead to protein malnutrition and nutrient deficiencies, resulting in poor growth, osteopenia or osteoporosis[11,18]. In order to consume enough protein to support proper growth and development and promote energy balance, PKU patients must incorporate protein substitutes into their diet. These protein substitutes are mixtures of free amino acids without or low in Phe[2]. Protein substitutes generally constitute 52% to 80% of a patient's total dietary protein[18]. Nutritional treatment for a low-Phe diet consists of natural foods low in Phe in combination with medical foods[2]. Other than their low Phe content, the macronutrient and micronutrient composition can vary considerably between products[2]. Depending on the formula, lipid content can vary from 0-2% to 53% of the total calorie content of the food. This can potentially impact absorption of fat-soluble vitamins, such as vitamin D[2]. These formulas help patients achieve adequate intake of protein and other nutrients to sustain proper growth and development[5]. Phe-free protein substitutes are recommended for all patients with PKU. Formulas provide many benefits, including adequate essential amino acids other than Phe to promote nitrogen retention, and energy and micronutrients to promote anabolism[22].

Currently, over 20 different protein substitutes are available to patients, and this number is increasing with time[2]. The diversity of products on the market allows for patients to choose the product that best meets their individual needs[2]. Even so, over the years patients have demonstrated poor compliance with dietary protein substitutes, despite continued efforts to improve the taste and appearance of formulas[27]. Dietary compliance is a multifaceted issue influenced by cognitive, emotional, physiological, and cultural factors[22]. More specifically, the nature and nurture of the patient, as well as cost and convenience factors of dietary treatment play a significant role in compliance[23]. Historically, medical professionals recommended that PKU patients could relax dietary restrictions after 8-10 years of age. However, more recent evidence has demonstrated that individuals should maintain dietary treatment as long as possible[16]. Experts currently recommend individuals practice lifelong adherence to a Phe-restricted diet in order to prevent neurological damage[17]. Still, a high percentage of patients do not follow dietary recommendations. This is a common issue observed among adolescents and adults[23]. Currently, the most utilized method of assessing dietary compliance is through measurement of blood Phe concentrations. However, these readings can be misleading if they are not taken regularly because blood Phe levels vary daily based on dietary intake and time of blood draw[23].

Vitamin D and Bone Mineral Density

Vitamin D is an essential hormone that is important for overall health and well-being[24]. Vitamin D is not only crucial for proper bone growth and development, but it

plays a lifelong role in maintaining musculoskeletal integrity[24]. The major physiologic function of vitamin D is maintenance of normal extracellular concentrations of calcium and phosphorous[25]. More specifically, vitamin D increases the intestinal absorption efficiency of these minerals, and stimulates maturation of osteoclatic stem cells to enhance bone resorption of calcium and phosphorous[25].

Deemed the "sunshine vitamin", vitamin D can be obtained exogenously as well as endogenously[26]. Sun exposure, or more specifically, UVB radiation, is necessary for vitamin D synthesis in the skin (endogenous source). Seasonal variation and geographical distance from the equator impact vitamin D synthesis. Dietary sources of vitamin D include eggs, fish, and butter (exogenous sources)[27].

Vitamin D isn't directly absorbed from the sunshine, but exposure to UVB radiation initiates cutaneous synthesis of the biologically active form of vitamin D, $1,25(OH)_2D$[25]. A precursor to vitamin D, called provitamin D, ergosterol or 7-dehydrocholesterol, is a rigid 4-ringed structure found in epidermal keratinocytes and dermal fibroblasts[26,24]. When the skin is exposed to sunlight, UVB radiation (290-315 nm) penetrates into the epidermis and dermis. This energy is absorbed by the double bonds in provitamin D, causing the B-ring to open up, resulting in the formation of previtamin D_3[26,24]. Previtamin D_3 is trapped within the plasma lipid bilayer, and after formation it undergoes rapid transformation of its double bonds to form vitamin D_3, also known as cholecalciferol[26,28]. Vitamin D_3 is ejected from the plasma membrane into the extracellular space. By diffusion, it enters circulation and travels to the dermal capillary

bed where it is bound to vitamin D-binding protein (DBP)[26,24,28]. Vitamin D can be ingested in the forms of either D_3 or D_2. Vitamin D_3 is synthesized by humans in the skin when exposed to UVB rays; Vitamin D_2 is synthesized by plants and comes from the irradiation of ergosterol isolated from yeast. Both forms can be used in food fortification[24]. Both structures are absorbed and metabolized in the same manner[28]. Once bound to DBP, these fat-soluble vitamins are incorporated into chylomicrons and enter the lymphatic system, where they are subsequently transported to the liver[24,28]. In the liver, D_2 and D_3 undergo hydroxylation on carbon 25 to form 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3[24]. Both structures are generally termed 25-hydroxyvitamin D, or 25(OH)D[24]. 25(OH)D is the major circulating form of vitamin D and is the primary clinical marker of vitamin D status[24]. Currently, there are 2 main types of measurement used for routine detection of circulating 25(OH)D_3 and 25(OH)D_2. One type is competitive immunoassay, and the other uses methods based on chromatographic separation followed by non-immunological direct detection. Immunoassays may have issues with specificity, particularly concerning the proportion of 25(OH)D_2 quantified during the process. The non-immunological detection methods using a combination of high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) are able to measure 25(OH)D_3 and 25(OH)D_2 independently. LC-MS/MS has become increasingly popular in recent years[29].

25(OH)D is biologically inert and must travel from the liver to the kidneys for conversion to 1,25(OH)$_2$D[28]. In the renal tubules, 25(OH)D is converted to 1α, 25-hydroxyvitamin D [1,25(OH)$_2$D] by the mitochondrial enzyme 25-hydroxyvitamin D-1 α-

hydroxylase (1-OHase)[28]. 1,25$(OH)_2$D is the biologically active form of vitamin D that is responsible for maintaining calcium and phosphorous homeostasis[28]. 1,25$(OH)_2$D also regulates its own synthesis through negative feedback, and decreases synthesis and secretion of parathyroid hormone (PTH) in the parathyroid glands[28]. From the kidneys, 1,25$(OH)_2$D enters the circulation once again and travels to its target tissues. In the small intestine, 1,25$(OH)_2$D interacts with the nuclear vitamin D receptor (VDR), which enhances intestinal calcium absorption by increasing expression of a calcium channel in the epithelial lining[28]. 1,25$(OH)_2$D also interacts with VDR in osteoblasts, which essentially promotes osteoclast maturation. Osteoclasts remove calcium and phosphorous from bone to help maintain serum homeostasis of these minerals. Adequate levels of calcium and phosphorous promote bone mineralization and preserve neuromuscular function[28].

Anything that influences the amount of UVB radiation penetrating the skin, or changes the quantity of provitamin-D in the epidermis or dermis will impact vitamin D production[26]. The skin pigment melanin acts as a natural sunscreen by absorbing UVB photons[26]. Consequently, persons with darker skin pigmentation require longer bouts of sun exposure to make the same amount of vitamin D as a lighter skinned person[26]. Other factors greatly impacting cutaneous vitamin D synthesis include latitude, time of day, and season. During winter months sunlight enters the earth's atmosphere at a more oblique angle, and a large amount of radiation is absorbed by the ozone layer[26]. At latitudes above 37^0 a decreased amount of UVB radiation is able to reach the earth's surface from November through February. Consequently, persons residing at or above

37° latitude produce little, if any, vitamin D_3 during these months. For this reason, populations in the northwestern United States are at an increased risk of vitamin D deficiency in the wintertime. Vitamin D is fat-soluble and may be stored in adipose tissue and used during winter months when little vitamin D is produced in the skin[26].

Vitamin D: A Historical Perspective

As recently as 1970, researchers determined that vitamin D qualifies as a hormone[25]. However, the discovery of the importance of vitamin D in skeletal formation and maturation dates back to the 17th century. Several researchers noticed the high prevalence of rickets among children who lived in industrialized cities in Great Britain and northern Europe[25]. Rickets is a bone disease caused by inadequate mineralization of the growing skeleton, resulting from nutritional deficiencies in vitamin D, calcium, and/or phosphorous[1]. Scientists observed that these children who were exposed to minimal sunlight, exhibited growth retardation, skeletal deformities, and generalized muscle weakness[25], [26]. The incidence of rickets continued to increase, and by the turn of the 20th century, it had spread to industrialized cities of northern Europe, and the northeastern United States[25]. One study estimated that by 1900, more than 90% of children in Leiden, Netherlands, and 80% of children in Boston were plagued by this crippling disease[26]. In the early 20th century, research conducted by Edward Mellanby and then Elmer McCollum led to the discovery of vitamin D and the significant role it plays in bone health[25]. These innovative findings spurred other scientists to introduce the concept of irradiating foods with UV radiation to treat and prevent rickets[26]. Initially

milk was irradiated and fortified with ergosterol, and then manufacturers began fortifying milk with synthetically produced vitamin D_2. Countries that adopted this fortification process basically eliminated rickets, and by the 1930s vitamin D was considered the new "miracle vitamin". Companies began fortifying foods such as peanut butter, hot dogs, soda pop, bread, and dairy products with vitamin D_2[26].

Vitamin D Deficiency and Osteopenia

Chronically low 25(OH)D levels may cause roentgenologic changes that are consistent with rickets[30]. A national study assessing 25(OH)D status among U.S. children reported that children who drank milk daily and took vitamin D supplements were less likely to be deficient[30]. Further, lower 25(OH)D levels in children and adolescents were associated with lower serum calcium and elevated parathyroid hormone (PTH) levels, additional biomarkers of bone metabolism[30]. Consequences of vitamin D deficiency include inability to reach one's genetically programmed height and peak bone mass, as well as onset of rickets[26]. Metabolically this occurs because insufficient vitamin D levels cause poor intestinal calcium absorption and increased renal calcium excretion, which lead to a mineralization defect of the collagen matrix laid down by osteoblasts and subsequent osteopenia[26].

Onset of osteopenia occurs when bone density is less than normal but is not as low as osteoporosis[28]. More specifically, the World Health Organization defines osteopenia by a bone densitometry T score of -1 to 2.5, while a T score of -2.5 or below warrants a diagnosis of osteoporosis[28]. A T-score is defined as the SD score of the

observed BMD compared with that of a normal young adult[31]. Osteopenia is highly correlated with vitamin D deficiency, osteoporosis, and other metabolic diseases[78]. Key factors to consider when determining osteopenia risk include genetics, diet, maintenance of normal vitamin D levels, and consistent weight-bearing exercise. Sufficient dietary intakes of calcium, vitamin D, and protein are important determinants of bone mineral density. More importantly, nutrition status is a modifiable risk factor[28]. Childhood and adolescence are crucial years for bone growth and development. Optimal bone formation during this time period can help prevent the onset of osteoporosis in adulthood[28].

Vitamin D insufficiency is a worldwide public health issue[37]. Severe deficiency manifests as rickets in children or osteomalacia in adults. Mild to moderate deficiency is prevalent among the general population and is often asymptomatic in individuals. Any symptoms expressed in mild or moderate cases generally manifest as bone or muscle pain and tenderness[32]. Early detection of deficiency is important, because it is associated with an increased fracture risk and onset of osteoporosis[32]. Methods to improve vitamin D status include food fortification, injections, oral supplementation, and regular sunlight/UVB exposure[32].

Parathyroid hormone (PTH) also helps maintain homeostasis by increasing renal re-absorption of calcium. Low 25(OH)D levels stimulate the release of PTH, and therefore high PTH levels may also indicate a deficiency[32]. In other words, circulating 25(OH)D levels are inversely associated with PTH levels. Currently, most laboratories

agree that serum levels of 30 ng/mL reflect normal 25(OH)D concentrations[3]. Concentrations below this are associated with a clinical diagnosis of rickets[33]. However, current research questions whether 30 ng/mL is an adequate level for optimal bone health[33]. However, a recent report published by the Institute of Medicine (IOM) suggests that levels of 20 ng/mL may be sufficient to promote normal bone mineralization in healthy adults[3]. When assessing bone mineral density in patients it is important to evaluate the levels of bone turnover markers, which include osteocalcin and bone alkaline phosphatase[34]. Serum concentrations of 25(OH)D_3, calcium, and phosphorus play significant roles in bone metabolism and may be good indicators of bone accrual in children and adolescents[17].

Vitamin D Recommendations

Experts have suggested that inadequate sun exposure increases daily vitamin D requirements[30]. A recent report published by the Institute of Medicine (IOM) provides evidence that serum 25(OH)D levels of 20 ng/mL (50 nmoL/liter) reflect an adequate vitamin D status to promote skeletal health[3]. A serum 25(OH)D level of 20 ng/mL reflects a Recommended Dietary Allowance (RDA) of 600 IU/day. This circulating serum concentration of 20 ng/mL is based on an RDA that covers the needs of greater than 97.5% of the general population[3]. However, this data does not consider children or adults with metabolic disorders who are consuming special diets.

Very few foods naturally contain vitamin D, and only a few foods are fortified with it[26]. After World War II the vitamin D fortification process was poorly monitored,

and excess amounts of vitamin D were added to certain milk products, causing an outbreak of vitamin D intoxication among infants and young children[26]. Following this outbreak, Europe banned the vitamin D fortification of most dairy products, and to this day, very few European foods are fortified with vitamin D[26]. In the United States, milk, orange juice, bread, and some yogurts and cheeses are fortified with vitamin D[26]. Natural sources of vitamin D include oily fish such as salmon, mackerel, and herring, cod liver oil, sun-dried mushrooms, and other fish oils. However, vitamin D content of these foods varies considerably between sources[24]. Today, supplements and fortified foods, namely dairy products, are the primary dietary sources of vitamin D in the United States[27].

PKU, Vitamin D and Bone Mineral Density

Adherence to a Phe-restricted diet can lead to other complications such as bone changes[35]. Many studies have suggested that children and adolescents with PKU have a lower bone mineral density than healthy age- and gender-matched controls. Early studies examined the bone mineral density and skeletal status in PKU patients. A study published in 1966 observed several bone discrepancies between PKU patients who followed a Phe-restricted diet and patients on unrestricted diets. PKU patients following a low-Phe diet demonstrated significant weight and height retardation. In addition, the researchers detected bone changes by x-ray in treated PKU patients under one year of age[35]. Another early study found that children undergoing treatment for PKU often presented with osteoporosis, abnormal bone growth or other bone changes.

Researchers in the 1960s typically attributed these radiographic irregularities to protein malnutrition and clinical manifestations of the disease itself[36]. Many recent studies report the existence of impaired bone mineralization among children with PKU, but provide differing reasons to explain this observation. Research performed by Ambroszkiewicz et al. investigated bone turnover markers in pre-adolescent children, and showed that serum concentrations of osteocalcin, collagen type 1 cross-linked C-telopeptide, and cytokine osteoprotegerin were significantly lower in PKU children when compared to healthy age-matched controls. They concluded that pre-pubertal children with PKU may experience a decreased bone turnover rate compared to healthy controls[37]. Roato and colleagues also studied the relationship between bone turnover markers and bone impairment in subjects with PKU, demonstrating that increased osteoclast activity, concurrent with disruption of the bone formation and resorption process[9]. Modan-Moses et al. examined peak bone mass in PKU patients and acknowledged that compromised BMD exists in the PKU population, but could draw no conclusions regarding the etiological mechanism[7]. Data from a study by Hillman and colleagues revealed significantly lower BMD in PKU children, in addition to lower levels of bone formation markers B-ALP and OC. This evidence suggests that decreased osteoblast activity may exist in PKU children, which is consistent with impaired bone mineralization. The authors concluded that decreased bone mineralization could be due to a primary bone mineralization problem or may be secondary to reduced mineral availability among PKU subjects[38]. Schwan et al. found that treated PKU patients exhibit significant alterations in trabecular bone, but they could not conclude whether the bone

abnormalities were architectural or compositional in nature[39]. Adamczyk and colleagues investigated bone metabolism in children with PKU compared to healthy controls. Biochemical measurements revealed that those who were noncompliant with the diet demonstrated higher levels of PTH and bone formation markers B-ALP and OC. DEXA scans revealed that the noncompliant group had lower BMD measurements. Researchers concluded that high Phe levels were inversely related to BMD, and suggested that a possible bone mineralization problem exists in PKU patients with elevated blood Phe[40]. Al-Qadreh and colleagues examined the bone mineral status of PKU children on diet and proposed that a problem with bone mineralization exists at all ages in PKU patients but the problem worsens in adolescence, likely owing to poor dietary compliance. Data also revealed that increased plasma Phe concentrations and urinary excretion of Phe metabolites correlates with increased mineral losses in urine. This evidence further suggests that high Phe levels may be inversely related to BMD[41]. Greeves et al. investigated fracture incidence among PKU subjects and found that fracture risk increased with age. They postulated that this was due to poor dietary compliance after 8 years of age or dietary relaxation with an increase in plasma Phe concentrations. Researchers also acknowledged the possibility that fracture risk could be due to a cumulative disease-related or diet-related reduction in bone mass[42]. McMurry et al. looked at bone mineral status in children with PKU and its relationship with serum Phe control and dietary intake. They also found a pattern of poor dietary compliance or dietary relaxation with an increase in plasma Phe concentrations in older children/adults (most notably after 8 years of age). The authors felt strongly that

compliance with dietary therapy for PKU is associated with normal bone mineral development in young children[43]. Mendes et al. reported similar results when they investigated the relationship between dietary adherence, bone age (BA) and BMD in children and adolescents with PKU. Analysis of dietary intake revealed that children and adolescents who were non-adherent to diet demonstrated BA values greater than chronological age and lower BMD values. They concluded that non-adherence to diet and the consequent imbalance in intake of nutrients involved with bone metabolism plays an influential role in reduced bone mineralization among this population[44]. Conversely, a study by Allen et al. acknowledged the existence of low BMD in children with PKU but could draw no conclusions regarding the etiology of this mechanism. They found no correlation between BMD and plasma Phe or between BMD and dietary intake[4].

Nagasaka et al. investigated adequacy of nutrient intakes among PKU subjects and found that average daily intakes of fat, calcium, and vitamin D were not significantly different in PKU individuals when compared to a healthy control group[45]. No differences were detected in calcium and vitamin D intake between PKU patients and controls. However, serum measurements revealed that PKU patients had significantly higher levels of 1,25(OH)D and lower levels of 25(OH)D when compared to controls. Further, analysis of bone turnover markers showed that PKU patients had significantly higher levels of bone resorption markers than controls. Researchers proposed that a decreased vitamin D status and higher proportion of bone resorption to formation may contribute to impaired bone accrual among PKU patients[45]. This study supports the hypothesis that

insufficient nutrient intake may not be the cause of abnormal bone mineralization in PKU patients[7,45].

Overall, a majority of studies suggest that PKU patients following a Phe-restricted diet have compromised bone mineral densities. However, the etiology behind this phenomenon remains uncertain.

Further Research Needed

Current research provides no conclusive evidence regarding an increased incidence of low BMD and/or reduced vitamin D status in children with PKU undergoing treatment. Further, the relationship between BMD and biochemical and dietary factors remains unclear in this population. However, numerous studies have suggested that this population subset is at elevated risk for low bone mineral density and rickets, which is correlated with a compromised vitamin D status. Low bone density places children and adolescents at a high risk for developing skeletal fractures and osteoporosis. If children with PKU are indeed found to have a compromised bone mineral content associated with low vitamin D stores, then future studies can investigate the efficacy of vitamin D supplementation in this population.

Chapter 3

Methods

General Design

The work presented in this thesis represents both a retrospective case-control chart review and a prospective observational study carried out at the Metabolic Clinic investigating vitamin D status and bone mineral density in children with inborn errors of metabolism including the aminoacidopathy Phenylketonuria (PKU). The retrospective chart review compared vitamin D status in children with inborn errors of metabolism (IEM) against other children without an IEM diagnosis. The EPIC electronic medical record was queried to obtain serum vitamin D concentrations in children treated at the metabolic clinic and the serum vitamin D concentrations of age- and sex-matched subjects treated in a separate DCH clinic. The results were statistically compared using unconditional regression analysis. In addition, twenty children 9-20 years of age were recruited into the prospective study. Measurements of bone mineral density, serum vitamin D, and parathyroid hormone (PTH) were taken during routine clinic visits. Bone mineral density was determined by DEXA. Correlation analysis was used to examine the relationship between these markers of bone metabolism and bone mineral density. Written informed parental and child consent was obtained for the prospective study.

Specific Aim 1: We queried the Research Data Warehouse (RDW) for all serum vitamin D concentrations of subjects with the diagnosis of a metabolic disorder utilizing the ICD 9.0 codes. The list of relevant ICD 9.0 codes is found in Table 1.

Table 1. ICD-9 Codes for Case Subjects with the Diagnosis of a Metabolic Disorder

Diagnosis	ICD-9
Hyperphenylalaninemia, PKU	270.1
Homocystinuria	270.4
Maple Syrup Urine Disease (MSUD)	270.3
Tyrosinemia, Type I/II	270.2
Disorders of Urea Cycle Metabolism	270.6
Lysinuric Protein Intolerance	270.0
Propionic Acidemia (PA)	270.6
Methylmal Acidemia/Cobalamin C & D	276.2
Multiple Carboxylase Def (MCD)	270.7
Isovaleric Acidemia (IVA)	276.2
Glutaric Aciduria, Type I	270.7
Galactosemia	271.1
Histidinuria	270.5

The data was carefully reviewed and a list of all subject's age, sex and date on which the blood sample for vitamin D was drawn was noted. We also queried the RDW to obtain data for age- and sex-matched controls with serum vitamin D measurements.. Data obtained from control subjects was screened for ICD 9.0 codes that might impact serum 25-OH vitamin D status. Control subjects presenting with a diagnosis that may impact vitamin D status such as an eating disorder were excluded from the chart review.

Specific Aim 2: We recruited children ages 9-20 with the diagnosis of PKU who were regularly followed at the metabolic clinic. Prior to their clinic visit, packets were mailed out to potential study subjects inviting them to participate in the research study.

Mailed packets contained an explanatory letter of invitation, consent form, and child assent form. The study coordinator made follow-up phone calls to recruit these potential subjects. On the day of their clinic visit, the study coordinator met with study subjects and collected consent and assent forms before data collection began. Table 2 shows the criteria followed for subject recruitment.

Table 2. Inclusion and Exclusion Criteria for Subject Recruitment

Inclusion Criteria	Exclusion Criteria
Diagnosis of Phenylketonuria	Pregnant
Aged 9-20	
Written parental consent and child assent obtained	
Currently receiving treatment at the metabolic clinic	

Blood Sample Collection and Analyses

Serum samples were obtained during routine clinic visits. Vitamin D concentration was measured as the biologically inactive form of $25(OH)D_2$ and $25(OH)D_3$. Intact PTH levels, and plasma calcium and alkaline phosphatase concentrations were also measured. Data obtained was compared to established normal ranges for each laboratory measurement.

Table 3. Markers of Bone Mineral Density

Blood and Urine Analysis	Lab Test	Pediatric Minimum required sample
25(OH)D	MS/MS	0.6 ml serum
Calcium	Automated	0.5 ml plasma
Intact PTH	Chemiluminescent Immunoassay	0.5 ml serum

4 mL of whole blood was collected into a serum tube and allowed to clot. A green top, lithium heparin tube was used to collect 2 mL of whole blood and placed on ice. Both tubes were spun at 4°C x 2500 g x 10 min. The serum and plasma were then removed and stored at -80°C until the time of analysis.

Vitamin D_2 and D_3 were measured in serum by using a high pressure liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method in the OHSU Bioanalytical Shared Resource/Pharmacokinetics Core Laboratory. Serum samples (200 µL) were treated with 0.7 ml of acetonitrile:methanol (95:5) that contained 10 ng/mL of the internal standard, ($[^2H_6]$-25-OH-D_3). Standards ranging in concentration from 1 ng/mL to 200 ng/mL were prepared in phosphate buffered saline containing bovine serum albumin and treated exactly as the serum samples. Fifty µL of the treated sample or standards were analyzed using a Shimadzu Prominence HPLC interfaced to an Applied Biosystems 4000 Q-TRAP hybrid triple quadropole/linear ion-trap mass spectrometer with an atmospheric pressure ionization source (APCI) operating in the positive mode. Optimal multiple reaction monitoring parameters were obtained for each analyte and

consisted of the following parent/product ion pairs: 25(OH) D_3 m/z = 383.3 → 257.2; 25(OH)D_2 m/z = 395.4 → 209.3; and [$^2H_6$]-25-OH-D_3 m/z = 389.4 → 263.3. Peak area ratios of analyte to internal standard were obtained and the resulting linear regression equation was used to calculate serum 25(OH)D_3 and 25(OH)D_2 concentrations. The laboratory used this assay in conjunction with the National Institute of Standards and Technology (NIST)/National Institutes of Health (NIH) Vitamin D Metabolites Quality Assurance Program (modified from Stephanie Miksa's Thesis).

Serum iPTH was measured by an automated chemiluminescent immunoassay run on the immulite platform from Siemens Diagnostics (Deerfield, IL 60015). We used the turbo version which produces faster results and recognizes intact iPTH. The analytical sensitivity is 4.0 pg/mL and the reportable range is 5-2,500 pg/mL.

Plasma calcium and alkaline phosphatase were measured in plasma by the OHSU hospital clinical chemistry laboratory by automated analysis.

Bone Mineral Density Measurements and Analysis

Dual-energy x-ray absorptiometry (DEXA) scans were performed during outpatient clinic appointments. DEXA appointments were scheduled at the Oregon Clinical and Translational Research Institute (OCTRI) after subject consent was acquired. No complications occurred with DEXA. The scan took ten to thirty minutes to complete for each subject. DEXA scans were performed on lower spine and hips to measure BMD in these areas. The study coordinator accompanied subjects to the DEXA scan. DEXA scan results were expressed as a *z*-score. The *z*-score is a value that compares the

amount of bone an individual has to age-and sex-matched controls. A score above -1 is considered normal; a score between -1 and -2.5 is classified as osteopenia; and a score below -2.5 is defined as osteoporosis.

Dietary Intake Analysis

Three-day diet records were given to each participant during their scheduled clinic visit. Subjects were asked to mail their completed 3-day diet records back to the study coordinator. The study coordinator mailed a gift card back to participants once completed 3-day diet records were received in the mail. Dietary analysis of each record was performed using the web-based nutrient analysis software program MetabolicPro. Food intake was analyzed for content of vitamin D, calcium, phosphorous, calories, protein, and phenylalanine. Dietary content of these nutrients was then compared to individual BMD scores.

Statistical Analysis

Specific Aim 1: We used unconditional regression analysis to compare serum 25(OH)D levels between the subjects with an inborn error of metabolism and control subjects consuming a diet not requiring medical formula. The RDW query yielded more than one control subject per subject with an inborn error of metabolism. The unconditional regression analysis allowed us to conduct an analysis comparing multiple control values for each subject. In this analysis, it was important to control for the seasonal variability in vitamin D status based on when the serum vitamin D concentration was measured. $P< 0.05$ was considered statistically significant.

Specific Aim 2: We used correlation analysis to investigate the relationships between bone mineral density and the markers of bone metabolism: serum 25(OH)D levels, and PTH concentration. Factors significantly associated with BMD were determined using a backwards stepwise multiple linear regression model. The dependent variable was BMD. Independent variables that were incorporated in the full model included vitamin D, PTH, alk phos, plasma Phe, and dietary intake of calories, protein, vitamin D, calcium, and Phe. $P < 0.05$ was considered statistically significant.

Statistical analysis was done using IBM SPSS Statistics Version 20.

Chapter 4

Results - Aim 1

Descriptive Statistics

Subject characteristics among those with a diagnosis of a metabolic disorder versus without the diagnosis of a metabolic disorder obtained from the retrospective RDW database query are given in Table 4. A total of 918 records were obtained with the query. Data was rigorously evaluated as outlined in Figure 1 based on our prior criteria. Subjects who were missing data on age, zip code, diagnosis, or serum 25(OH)D level were excluded from the chart review. If multiple serum 25(OH)D concentrations were provided for a subject, only the earliest draw date was considered. Subjects who resided outside of Oregon and Washington were excluded from the chart review. Control subjects were excluded if they had an ICD 9.0 code that could impact serum 25(OH)D status. The ICD 9.0 codes that we excluded from our data set are listed in table 5. Controls with other ICD 9.0 codes not believed to be directly associated with either altered bone and mineral metabolism or a diet that excluded vitamin D containing foods were included. These ICD 9.0 codes are listed in table 6. Case subjects were excluded if their diagnoses list did not include an ICD 9.0 code for a metabolic disorder.

Demographics: Statistical analysis was performed on data derived from 537 total subjects obtained from the database. Ninety-two of these subjects (17.1%) represented the population with the diagnosis of a metabolic disorder (cases). Four-hundred forty-

five of these subjects (82.9%) did not have the diagnosis of a metabolic disorder at the time of the database query (controls). Of those subjects in the case group, 47 (51.1%)

Table 4. Baseline characteristics and comparisons between cases and controls

Characteristic	Case (n=92)	Control (n=445)	p-value
Mean (±SD) age on draw date in years	12.5 ± 3.47	14.6 ± 3.39	<0.001
Sex, n (%)			0.264
Male	47 (51.1)	199 (44.7)	
Female	45 (48.9)	246 (55.3)	
Region^, n (%)			0.531
Northwest	60 (65.2)	318 (71.5)	
Southwest	24 (26.1)	101 (22.7)	
Northeast	4 (4.3)	10 (2.2)	
Southeast	4 (4.3)	16 (3.6)	
Season of blood draw, n (%)			0.331
Winter (10/1--5/31)	65 (70.6)	291 (65.4)	
Summer (6/1--9/30)	27 (29.4)	154 (34.6)	
Mean (±SD) [25(OH)D] in serum in ng/ml	27.1 ± 10.9	27.6 ± 11.2	0.672

^ N/S demarcation made relative to 45th parallel; E/W demarcation was the Cascade mountain range (determined via GIS).

Table 5. ICD 9.0 codes excluded from data set that could impact serum 25(OH)D status

ICD 9.0 Code	Diagnosis
242.00	Graves' Disease
245.2	Hashimoto Thyroiditis
244.9	Hypothyroid
733.90	Osteopenia
733.00	Osteopetrosis
246.9	Thyroid Disease
245.9	Thyroiditis

Table 6. ICD 9.0 codes among control subjects included in data set

ICD 9.0 Code	Diagnosis Name	ICD 9.0 Code	Diagnosis Name
170.9	Ewings Sarcoma	330.8	Rett Syndrome
190.5	Retinoblastoma	340	Multiple Sclerosis
194.0	Neuroblastoma	343.9	Cerebral Palsy
204.00	ALL (Acute Lymphoblastic Leukemia)	401.9	Hypertension
250.00	Diabetes Mellitus Type II, Uncontrolled	477.9	Allergic Rhinitis, cause unspecified
250.01	Type I Diabetes Mellitus	493.90	Asthma
253.2	Hypopituitarism	530.81	GERD (Gastroesophageal Reflux Disease)
256.4	PCOS (Polycystic Ovarian Syndrome)	555.2	Crohn's Disease of Small and Large Intestines
277.00	Cystic Fibrosis	556.9	Ulcerative Colitis
278.00	Obesity	729.1	Fibromyalgia
278.02	Overweight	756.17	Spina Bifida Occulta
279.11	DiGeorge Syndrome	758.0	Down Syndrome
280.9	Iron Deficiency Anemia	758.5	Trisomy 8
282.5	Sickle Cell Trait	760.71	Fetal Alcohol Syndrome
286.0	Moderate Hemophilia A	780.39	Seizures
286.4	VWD (Von Willebrand's Disease)	783.43	Short Stature
299.00	Autism	790.29	Insulin Resistance
300.4	Dysthymia	V08	HIV-1
311	Depression	V22.0	First Normal Pregnancy Supervision
314.00	ADD (Attention Deficit Disorder)	V42.7	Liver Transplant
314.01	ADHD (Attention Deficit Hyperactivity Disorder)	V69.2	High Risk Sexual Behavior
315.9	Unspecified Delay In Development		

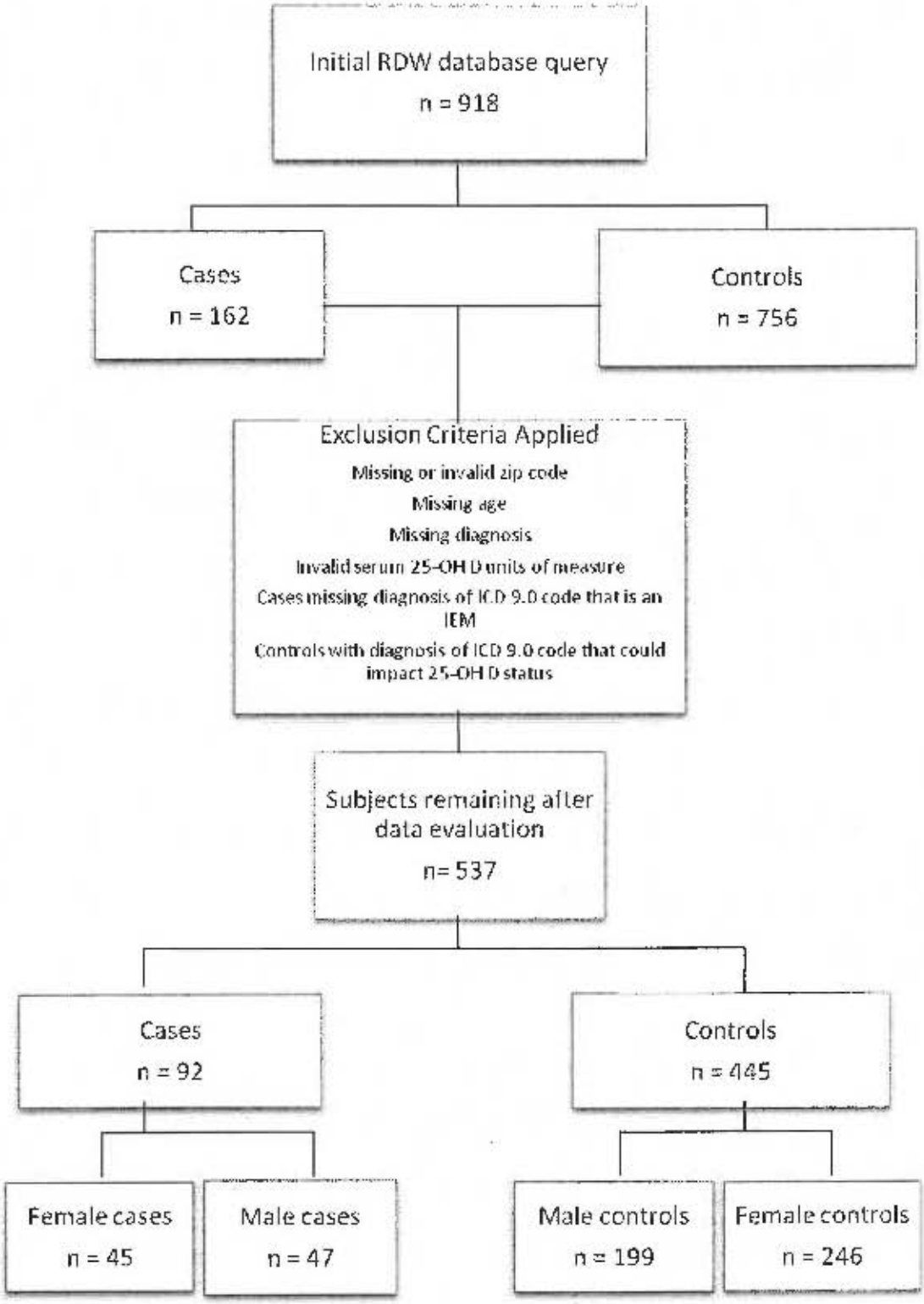


Figure 1. Distribution among the subjects with a diagnosis of a metabolic disorder (cases) vs. subjects without a diagnosis of a metabolic disorder (controls) with a serum 25(OH)D value form the retrospective RDW query.

were male and 45 (48.9%) were female, and of the control group 199 (44.7%) were male and 246 (55.3%) were female. The age range of subjects in both groups was 8 to 20 years of age. For cases, the average age at which serum 25(OH)D draw date occurred was 12.5 years ± 3.47, while the average age at draw date for controls was 14.6 years ± 3.39. Table 4 summarizes the distribution of subjects in the case and control groups.

Sun exposure: Subjects were assigned a geographic region in Oregon and Washington according to zip code. Regions were determined to account for disparities in annual sun exposure based on region of residence. The east/west division was separated by the Cascade mountain range. The 45th parallel was used as the north/south division. This resulted in 4 different geographic regions into which subjects were grouped: Northwest, Southwest, Northeast, and Southeast. A majority of subjects resided in the Northwest, with 60 cases (65.2%) and 318 controls (71.5%) grouped in that region. Twenty-four cases (26.1%) were from the Southwest, and 101 controls (22.7%) were from the Southwest. Four cases (4.3%) and 10 controls (2.2%) resided in the Northeast region. Four cases (4.3%) and 16 controls (3.6%) were grouped in the Southeast region.

Seasonality: In order to account for the seasonal variability of sun induced vitamin D synthesis, serum 25(OH)D draw dates were categorized by either a "winter" or "summer" date. A winter draw date was defined as a serum 25(OH)D that was measured between October 1 and May 31. A summer draw date was defined as a serum 25(OH)D that was measured between June 1 and September 30. Sixty-five cases (70.6%)

and 291 controls (65.4%) had a winter draw date, while 27 cases (29.4%) and 154 controls (34.6%) had a summer draw date.

Statistical Analysis

T-test: A two-sample t-test performed on mean age at draw date between cases and controls revealed a significant difference in age at draw date ($p<0.001$). We carried out a two-sample t-test to compare mean serum 25(OH)D levels between cases and controls ($p=0.672$). The average serum 25(OH)D concentration in cases was 27.1 ng/mL ± 10.9. The average serum 25(OH)D concentration in controls was 27.6 ng/mL ± 11.2. Serum 25(OH)D levels in cases ranged from 4.0-58.9 ng/mL. The range of serum 25(OH)D in controls was 5.0-87.0 ng/mL. There was no difference in the group means or range of serum 25(OH)D concentration between cases and controls.

Further tests were used to determine if there were differences by the season when the blood sample was obtained (seasonality) and if there were differences by region of residence between cases and controls. We used a chi-square 2 (case or control) x 2 (winter or summer) test with 1 degree of freedom to determine any significant differences between cases and controls in regard to season of blood draw ($p=0.331$). We used a chi-square 4 (regional quadrants) x 2 (case or control) test with 3 degrees of freedom to compare differences in geographical region between cases and control ($p=0.531$). There was no difference by season of blood draw or by regional quadrant of residence between cases and controls that could potentially influence our results. Mean 25(OH)D concentrations did not differ significantly by season of blood

draw in both case and control groups, demonstrating that seasonality did not impact vitamin D status (Figure 2). For cases, the mean 25(OH)D concentration for both a winter and summer draw was equivalent at 27.4 ng/mL. For controls, the mean 25(OH)D concentration for a winter draw was 27.5 ng/mL, and for a summer draw it was 27.4 ng/mL.

Unconditional Logistic Regression Analysis

To control for the many variables that may impact serum 25(OH)D concentration, we used unconditional logistic regression analysis to compare serum 25(OH)D between cases and controls. In the full model, we adjusted for mean age, sex, season of blood draw, and geographical region. There was no significant difference between serum 25(OH)D levels in cases and controls when data was adjusted for these five factors (p=0.549). Figure 3 uses a Whisker Box plot to demonstrate the range of serum 25(OH)D levels in cases and controls. Serum 25(OH)D concentrations were similar between our metabolic population consuming specialized diets and a control population who consume a normal diet.

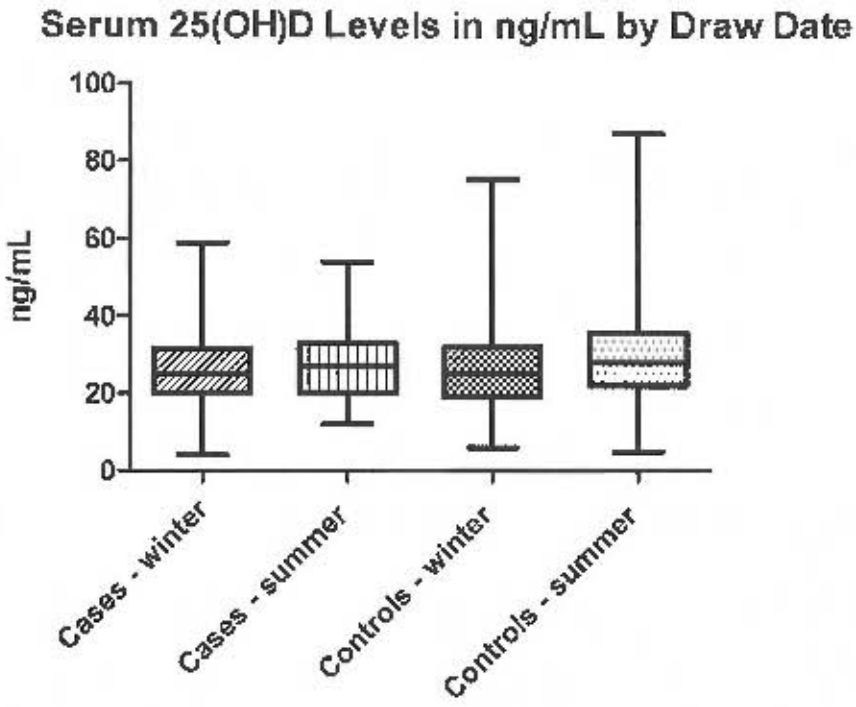


Figure 2. Serum 25(OH)D concentrations in cases and controls by season of blood draw. Box represents quartiles of data – Bottom of box indicates 25th percentile; Top of box indicates 75th percentile; Line in the middle is the median; Whiskers are the range of values for each group.

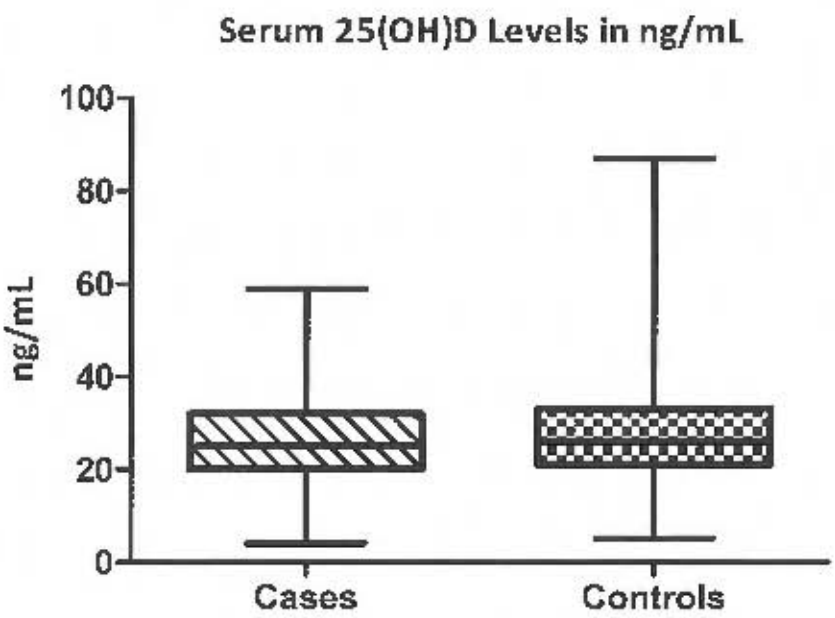


Figure 3. Serum 25(OH)D concentrations in cases and controls. Box represents quartiles of data – Bottom of box indicates 25th percentile; Top of box indicates 75th percentile; Line in the middle is the median; Whiskers are the range of values for each group.

Results – Aim 2

Descriptive Statistics

Figure 4 summarizes the distribution of subjects with PKU who were recruited from the metabolic clinic to participate in this research study. A total of 20 subjects were enrolled over a period of 6 months, from January 2012 through June 2012.

Demographics: Subject ages ranged from 9-19 years, with a mean of 12.6 ± 2.8 years. Eleven (55%) males and 9 (45%) females participated in this study. All subjects were diagnosed at newborn screening and treated with a low Phe diet from birth. Consent and assent were obtained from each subject prior to participation. This research study was approved by the IRB.

Biochemical Analysis

We were unable to do a blood draw on 1 participant, so serum analysis was performed on samples obtained from 19 of the 20 participants. Analysis of serum 25(OH)D concentrations found undetectable levels of serum $25(OH)D_2$ in 17 of the 19 subjects. Serum $25(OH)D_3$ levels were greater than 20 ng/mL in all 19 subjects. Consequently, $25(OH)D_3$ data was used for statistical analysis purposes. Table 7 summarizes the mean and range of $25(OH)D_3$ and iPTH levels. Mean serum $25(OH)D_3$ concentration was 34.8 ng/mL ± 8.5, with a range of 20.0-53.5 ng/mL. Mean iPTH level was 59.1 pg/mL ± 23.5. Plasma calcium and alkaline phosphatase concentrations are given in Table 7. The mean plasma calcium level was 9.48 mg/dL ± 0.271. Mean alkaline

phosphatase concentration was 202.89 IU/L ± 81.32. Plasma phenylalanine was measured in all 20 subjects during their same-day outpatient clinic appointment (Table 8). Plasma Phe levels ranged from 154-1204 µmol/L, with a mean level of 489.0 µmol/L ± 310.4.

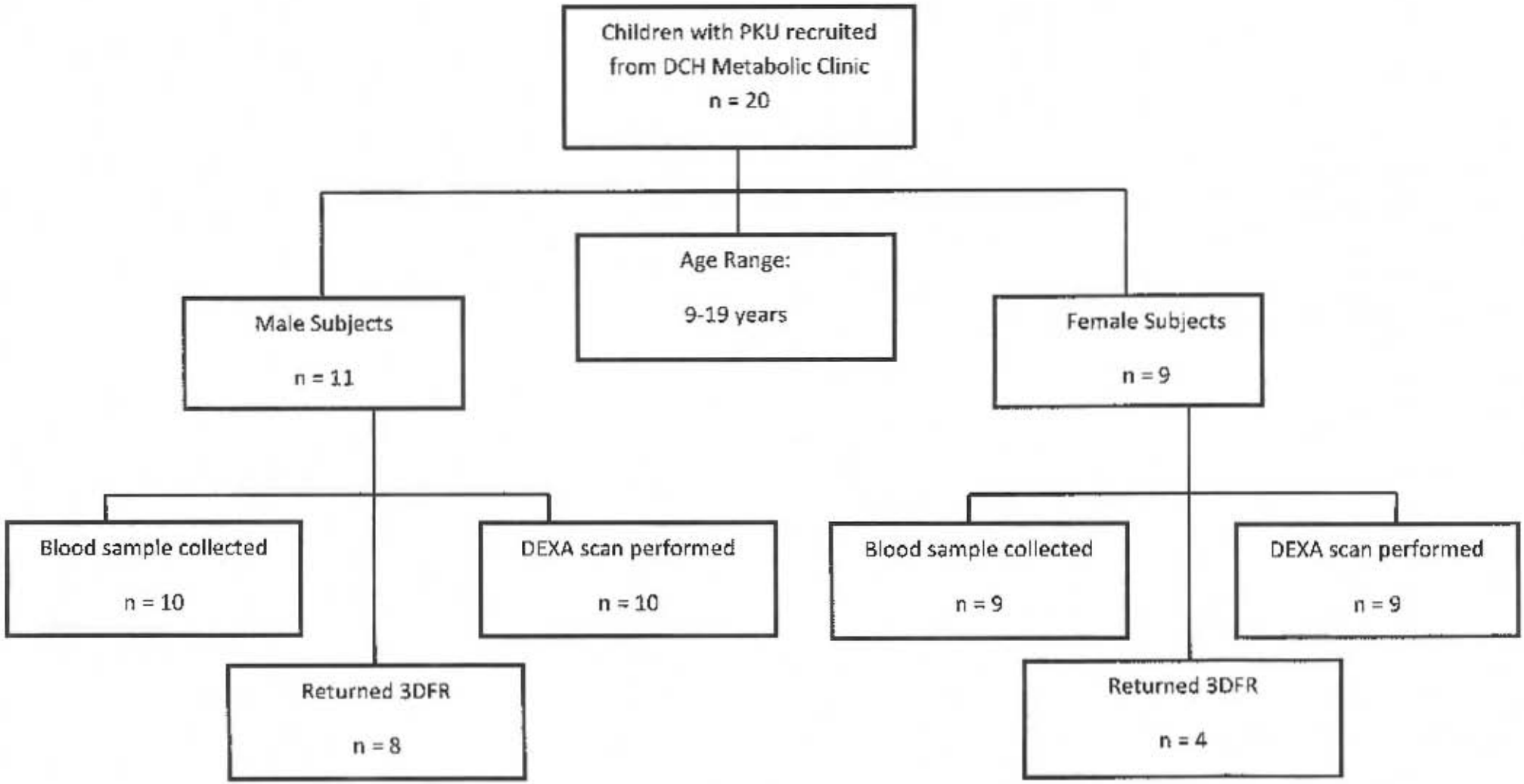


Figure 4. Subjects with Phenylketonuria Recruited from the Doernbecher Children's Hospital Metabolic Clinic over a 6-month period

Table 7. Mean serum and plasma levels among subjects

	25-OH D3 (ng/mL)	iPTH (pg/mL)	Calcium (mg/dL)	Alk phos (IU/L)	Phe (µmol/L)
Mean (±SD) level	34.8 ± 8.5	59.1 ± 23.5	9.48 ± .271	202.89 ± 81.32	489.0 ± 310.4
Range	20.0 – 53.5	28.6 – 125.0	8.70 – 9.90	69 – 321	154 – 1204
Number of subjects (n)	19	19	19	19	20

Table 8. Average 3-day nutrient intake among subjects (n=12)

	Energy (kcal)	Protein (gm)	25-OH D (µg)	Calcium (mg)	Phos (mg)	Phe (gm)
Mean (±SD) 3-day intake	2042.3 ± 844.8	48.9 ± 40.2	11.3 ± 9.35	1382.25 ± 1203.7	1368.7 ± 1126.7	0.61 ± .33
Range	1149.7 – 3601.2	6.8 – 158.5	0 – 30.0	82.0 – 4419.0	240 – 4458	.120 – 1.2

Dietary Intake

Three-day food records were returned for 12 out of the 20 study participants. The ages of the subjects who provided dietary intake data ranged from 11 to 17 years. We analyzed the average amount of calories, protein, vitamin D, calcium, phosphorus, and phenylalanine consumed by each individual over a 3-day period. Table 9 summarizes data on the range and mean of the 3-day intakes. Medical foods were the primary source of vitamin D, calcium, and protein.

Bone Mineral Density

DEXA scans were performed on all 20 subjects. However, the data from 1 subject was excluded during analysis because a full body scan was performed, rather than the hip-and-spine scan mandated by the research protocol. Statistical analysis was performed using information obtained from the remaining 19 participants. Z-score means and ranges for hip and spine DEXA scans are summarized in Table 10. Mean spine BMD *z*-scores were -0.4632 ± 1.12, and mean hip BMD *z*-scores were -0.4737 ± 1.22. Fifteen subjects have *z*-scores within normal limits. Four subjects demonstrated either an abnormal spine or hip BMD *z*-score. One subject had a high spine *z*-score at 3.1; another subject had a slightly low spine *z*-score at -2.1, suggesting osteopenia. Two other subjects had low hip *z*-scores at -3.6 and -2.4, suggesting compromised BMD.

Table 9. Z-scores from hip-and- spine DEXA analyses (n=19)

	z-score (spine)	z-score (hip)
Mean (±SD)	-.4632 ± 1.12	-.4737 ± 1.22
Range	-2.10 – 3.10	-3.60 – 1.60

Correlation Analysis

Spearman's Correlation analysis was performed to investigate the relationships between both spine and hip BMD and the markers of bone metabolism: serum 25(OH)D levels, iPTH concentration, plasma Phe levels, plasma calcium levels, plasma alkaline phosphatase, and average 3-day intake of calories, protein, calcium, vitamin D, phosphorus, and phenylalanine. Table 10 summarizes the results of the correlation analysis performed using all 19 subjects. There were no statistically significant associations between plasma or serum measurements when compared to both spine and hip *z*-scores. We ran this same correlation analysis with 11 subjects who had complete data sets, and this analysis yielded the same results as above. Therefore, we are presenting the statistics using all available data here (Table 10). Previous studies have suggested that BMD is related to metabolic control. We did not see a significant correlation between plasma Phe at this time point and BMD. A significant association was found between spine *z*-scores and mean dietary protein intake ($p=0.028$), dietary calcium intake ($p=0.007$), and dietary phosphorus intake ($p=0.048$). Subjects who had

higher intakes of dietary protein, calcium and phosphorous had higher BMD z-scores. No significant relationship was observed between hip *z*-scores and dietary intake variables.

After this initial analysis, we performed dietary normalization to adjust for the variability of intake with age. We normalized the data for dietary intakes of protein (gram/kilogram), calcium (mg/1000 kcal), and phosphorous (mg/1000 kcal). Correlation analysis using the adjusted variables revealed a significant relationship between spine BMD and mean calcium intake (p=0.007), but no significant relationship between spine BMD and mean protein intake (p=1.28) or mean phosphorus intake (p=0.120) (Table 11).

Table 11. Spearman's Correlation between spine and hip Bone Mineral Density z-scores and dietary factors after normalization

	Dietary PRO (gm/kg)	Dietary Ca (mg/1000 kcal)	Dietary Phos (mg/1000 kcal)
Spine BMD z-score Correlation Coefficient	.487	.756	.497
p-value	.128	.007	.120
Hip BMD z-score Correlation Coefficient	-.342	.219	.018
p-value	.304	.518	.958

Table 10. Spearman's Correlation between spine and hip Bone Mineral Density z-scores and serum, plasma, and dietary factors

	Plasma Ca	Plasma alk phos	Plasma Phe	Serum 25-OH D3	Serum iPTH	Dietary kcal	Dietary PRO	Dietary vit D	Dietary Ca	Dietary Phos	Dietary Phe
Spine BMD z-score Correlation Coefficient	.002	-.177	.121	.075	-.384	.387	.656	.563	.761	.606	.005
N	18	18	19	18	18	11	11	11	11	11	11
p-value	.993	.483	.621	.766	.116	.239	.028	.071	.007	.048	.989
Hip BMD z-score Correlation Coefficient	-.038	.039	-.027	.183	.020	-.323	-.050	-.165	-.041	-.123	-.273
N	18	18	19	18	18	11	11	11	11	11	11
p-value	.880	.877	.912	.467	.938	.332	.884	.628	.905	.719	.416

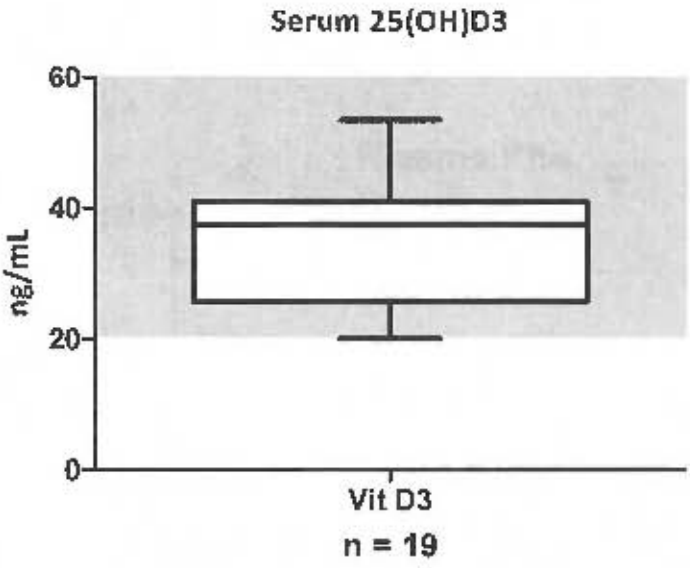


Figure 5. Serum 25(OH)D in Subset of Children with PKU Recruited from Metabolic Clinic. Gray shaded area represents the normal range for Serum 25(OH)D_3. Box represents quartiles of data – Bottom of box indicates 25th percentile; Top of box indicates 75th percentile; Line in the middle is the median; Whiskers are the range of values for each group.

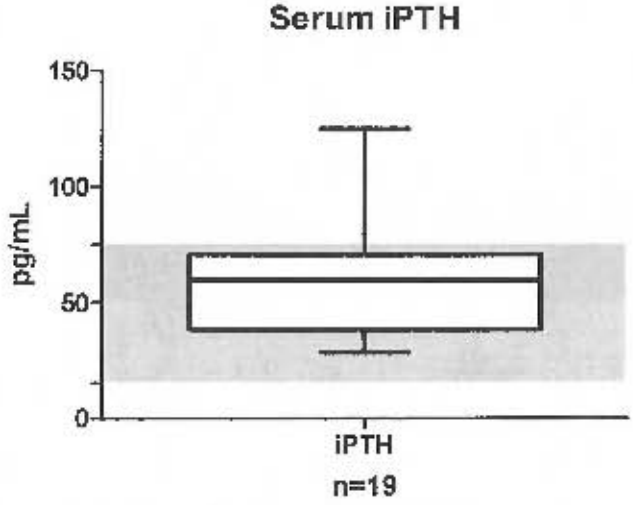
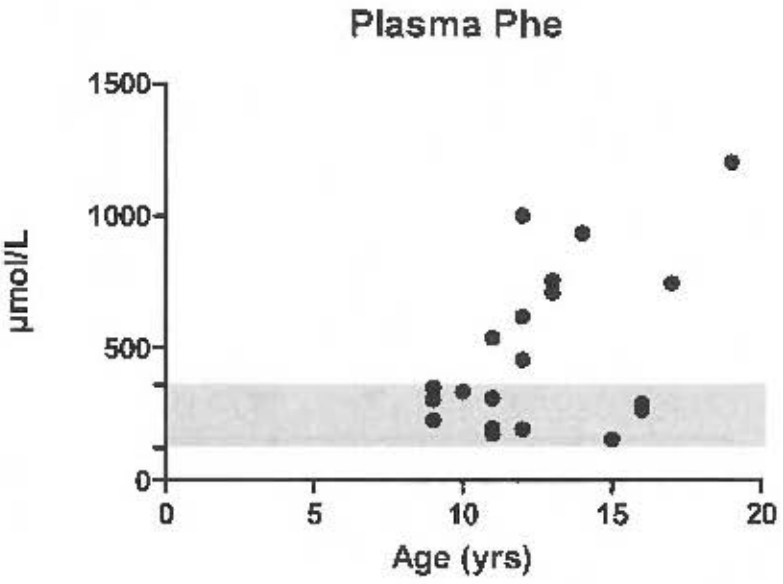


Figure 6. Serum iPTH in Subset of Children with PKU Recruited from Metabolic Clinic. Gray shaded area represents the normal range for serum iPTH. Box represents quartiles of data – Bottom of box indicates 25th percentile; Top of box indicates 75th percentile; Line in the middle is the median; Whiskers are the range of values for each group.

Figure 7. Plasma Phenylalanine vs. Age in Subset of Children with PKU. Shaded rectangle represents recommended treatment range of 120-360 µmol/L.



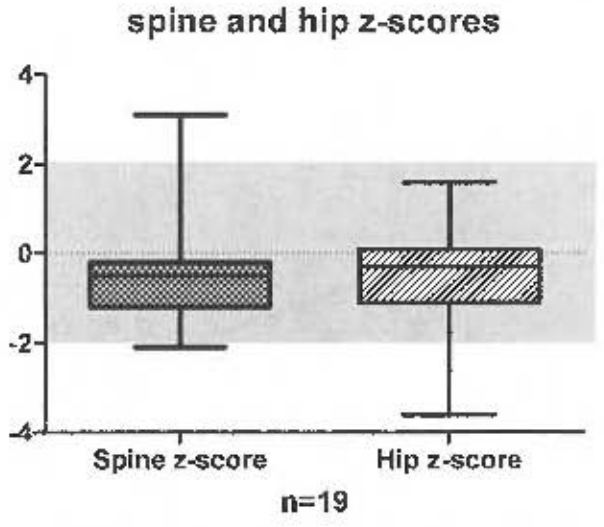


Figure 8. Range of spine and hip z-scores for BMD in subset of children with PKU (n=19). Gray shaded region represents normal range for z-scores (-2 to 2). Box represents quartiles of data – Bottom of box indicates 25th percentile; Top of box indicates 75th percentile; Line in the middle is the median; Whiskers are the range of values for each group.

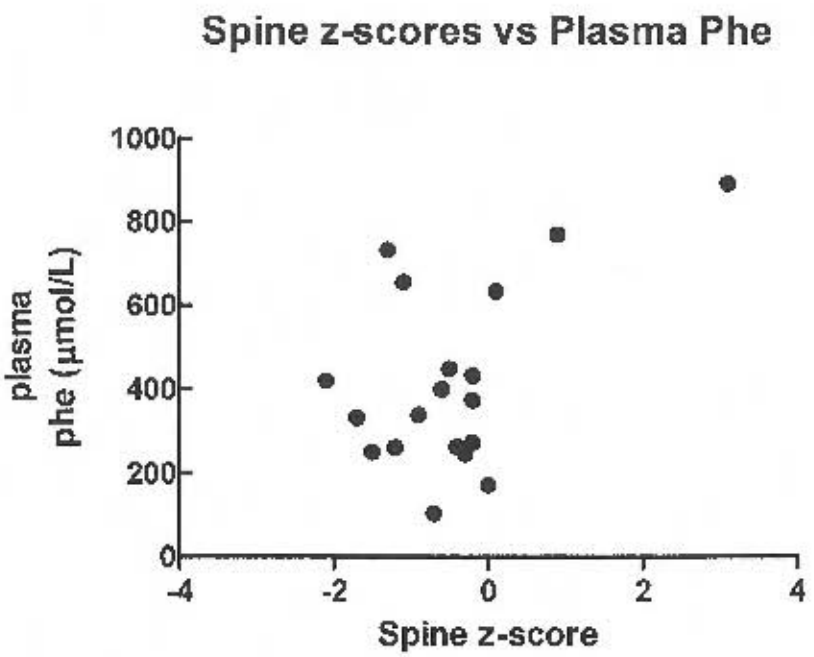


Figure 9. Spine z-scores for BMD vs. Plasma Phe (mean of 3 measurements over preceding year).

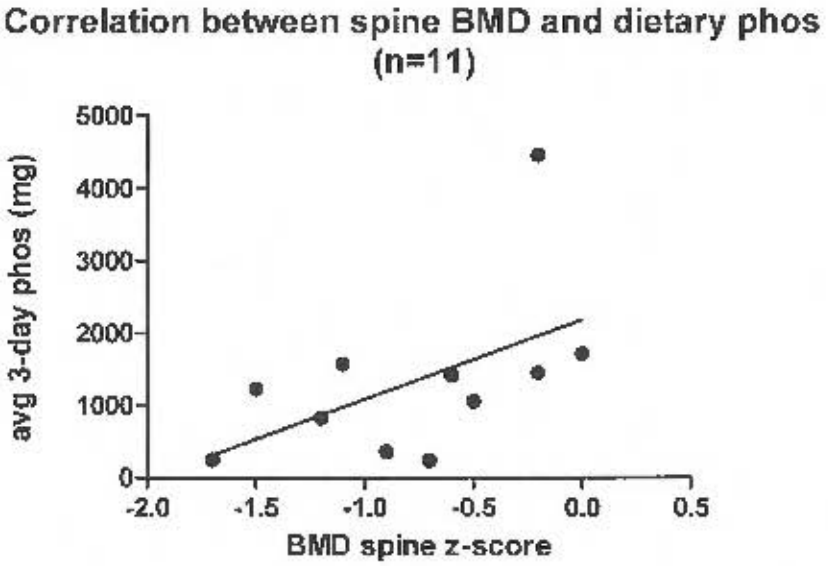


Figure 10. Correlation between spine z-scores for BMD and dietary phosphorous intake (n=11)

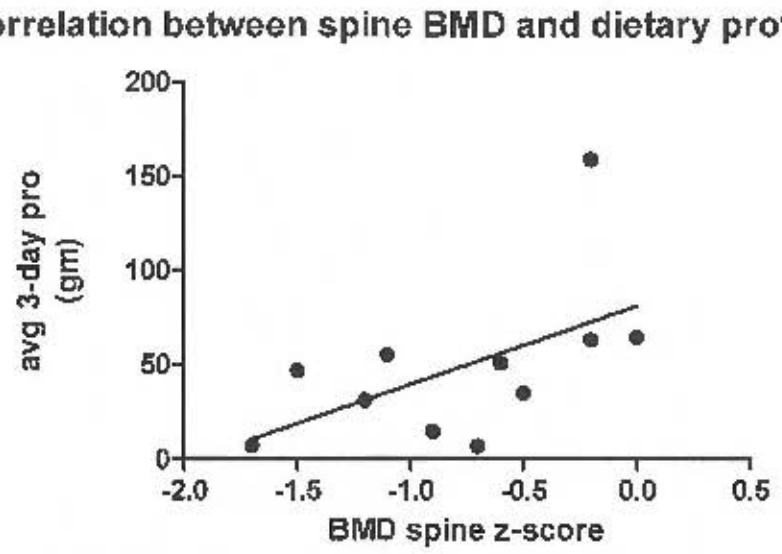


Figure 11. Correlation between spine z-scores for BMD and dietary protein intake (n=11)

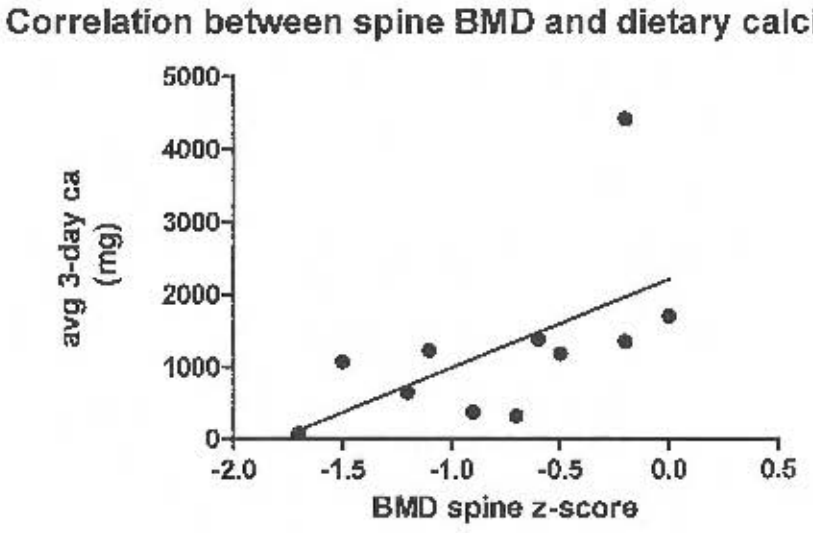


Figure 12. Correlation between spine z-scores for BMD and dietary calcium intake (n=11)

Chapter 5

Discussion

Summary

Aim 1

This retrospective study examined information extracted from a RDW database query about serum 25(OH)D levels on 537 children between the ages of 8 and 20. We investigated whether serum vitamin D status differed between children who consume vitamin D containing foods and children who consume a medically modified diet. We determined that in this population subset there was no significant difference in vitamin D status between these groups. This did not change when we adjusted data according to subject age, sex, geographic region, or season of blood draw. These results suggest that vitamin D, either from endogenous synthesis or from dietary sources, is similar between controls and children consuming medical foods.

This is a unique study in that it looked exclusively at serum 25(OH)D concentrations in a large retrospective database query of children consuming medical foods versus control children. It is noteworthy that the control children are not necessarily "healthy" children but they do not have diseases that could potentially impact serum 25(OH)D concentrations or bone mineralization. However, they do not have disorders which require them to consume specialized medical foods or avoid vitamin D containing foods. It is possible, though, that some of the control children had

conditions that could result in poor intake or malabsorption, such as Ulcerative Colitis or Crohn's Disease.

There are several prospective studies that have provided research on serum 25(OH)D concentrations and other markers of bone metabolism in children with metabolic disorders versus healthy controls. Rubio-Gozalbo and colleagues investigated vitamin D metabolites in patients with galactosemia and found them to have normal serum 25(OH)D concentrations[46]. Hillman et al compared BMD and biochemical parameters in healthy children versus children with PKU. They found similar concentrations of serum 25(OH)D in the case and control groups[38]. Modan-Moses et al measured serum 25(OH)D in a subset of patients with PKU and found normal concentrations in groups of subjects who reported to be diet-adherent as well as non-adherent[7]. Al-Qadreh et al measured serum 25(OH)D in children with PKU versus healthy controls, and observed that there was no significant difference in serum 25(OH)D concentrations when cases and controls were compared, and that serum 25(OH)D was within normal ranges[41]. Nagasaka and colleagues investigated serum 25(OH)D concentrations in adults with PKU. They observed that both males and females with PKU had significantly lower serum 25(OH)D levels when compared to controls[45]. McMurry and colleagues looked at bone mineral status and laboratory parameters of preschoolers, grade-schoolers, and older children with PKU compared to healthy controls. Investigators found no significant differences between serum 25(OH)D concentrations between grade-school age and older children with PKU when compared to healthy children. However, they did find that pre-school aged healthy controls had

significantly higher levels of serum 25(OH)D compared to pre-school aged PKU subjects[13].

Of the 6 studies discussed here, 2 of them found lower concentrations of serum 25(OH)D in a subset of subjects with PKU when compared to healthy controls. All of these prospective research studies examined a small population of subjects with a specific inborn error of metabolism. Our study differs from previous research because our results from this retrospective database query provide serum 25(OH)D data on a large group of both case and control subjects residing in the Northwest. Further, case subjects in this study included all children with an ICD 9.0 diagnostic code for a metabolic disorder that requires a modified diet for treatment in the RDW database.

Our study found that for both case and control subjects, serum 25(OH)D concentrations were within the recommended range (Figure 2). These results indicate that children in this population subset demonstrate adequate serum 25(OH)D levels. One statistically significant difference was that case subjects had a significantly lower mean age at draw date ($p<0.001$). A likely explanation for this distinction is that children with inborn errors or metabolism are usually diagnosed at birth or early on in life, and subsequently receive regular medical attention beginning at a young age. We tested if age was correlated with serum 25(OH)D concentration but there was no significant relationship between age and serum 25(OH)D concentration (Figure 13). This difference in age does not appear to be a factor that influences our results.

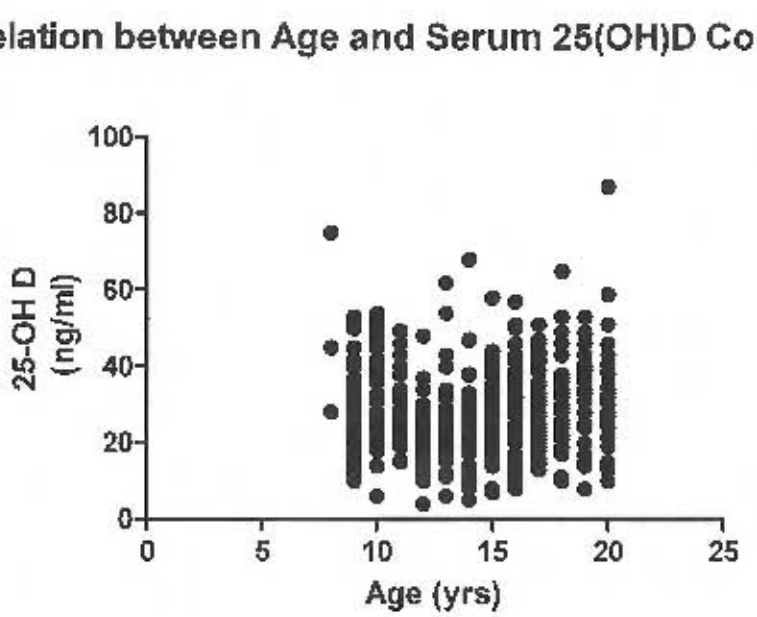


Figure 13. Correlation between age at draw date and serum 25(OH)D concentration.

Future research could investigate the dietary intakes of healthy children versus children with metabolic disease, and specifically examine intakes of vitamin D. Children with metabolic disorders are prescribed medical foods that contain vitamin D_3, and theoretically should receive an adequate amount of exogenous vitamin D if they consume the medical food. According to the findings presented here, the influence of both endogenous and exogenous vitamin D sources appears to be sufficient in our population.

Summary

Aim 2

This study investigated bone mineral density, dietary intake, and biochemical parameters in 20 children with PKU recruited from the metabolic clinic. Our findings

indicated that this subset of children with PKU do not have low BMD. This is contrary to other studies which have found that a majority of the children with PKU have low BMD. There are several possible reasons for the discrepancy between our findings and previous research.

The etiology for low BMD in this population remains unknown, but previous studies have suggested the following causes: 1) poor Phe control causes demineralization of bone; 2) a disruption in the bone turnover process; 3) inadequate diet of key nutrients prevents peak BMD from being reached; and 4) a decreased serum vitamin D status despite sufficient dietary intake.

Many studies have supported the postulation that bone demineralization among this population is related to poor Phe control. Further, several researchers have observed relaxed dietary adherence as children enter pre-adolescence and adolescence, and a concurrent decrease in BMD[40-43]. Bone demineralization was not a pervasive problem among our study population. Nine children had average plasma Phe levels on the day of their clinic visit that fell above the recommended treatment range (Figure 6). However, our analysis revealed that there was no significant correlation between plasma Phe levels and BMD. In addition, age seemed to have no reflection on BMD status. Older subjects in our study did not have higher plasma Phe levels than younger subjects. Therefore we cannot support the hypothesis that bone demineralization is related to poor Phe control.

A few researchers investigated markers of bone formation and resorption among children with PKU and observed a decreased rate of bone mineralization[9,37,38]. Our biochemical data suggested no abnormal trends in markers of bone metabolism in this population when we looked at serum markers of $25(OH)D_3$ and iPTH, as well as plasma markers of alkaline phosphatase and plasma calcium. However, we were not able to look at other markers of bone metabolism such as osteocalcin, osteoprotegrin, bone alkaline phosphatase, and collagen type 1 cross-linked C-telopeptide that other studies have investigated[9,37]. It is possible that if we had measured additional markers of bone resorption and formation we could have found abnormal concentrations but this is unlikely because the BMD was normal in our population.

Several researchers have suggested that an inadequate intake of key nutrients related to bone health prevent children from reaching their peak bone mass[13-15]. Our data suggest a significant relationship between BMD and dietary intake of nutrients related to bone health – specifically calcium. We did not find a significant correlation between BMD and vitamin D intake. When we normalized nutrient intake to body weight (protein) or total energy (phosphorous), the relationship to BMD became non-significant. The earlier correlation that we observed was most likely influenced by the relationship between increased nutrient intake, increasing bone mineral density and age. The p-values were trending towards significance and with only 11 diet records in our data, this is also influenced by a small n. However, after adjusting for this confounder, our analysis maintained a significant relationship between spine BMD and dietary calcium intake. These findings suggest that dietary intake of key nutrients plays a

crucial role in bone development among this population. The relationship we observed between BMD and calcium intake warrants further investigation into overall dietary intake and bone health among children with PKU.

Nagasaka et al. proposed that a sufficient nutrient intake might not necessarily result in normal bone mineralization among adults with PKU. They observed that average intakes of fat, calcium, and vitamin D met the RDAs and did not differ significantly between the PKU and control populations. However, serum measurements revealed that $1,25(OH)_2D$ levels were significantly higher and 25(OH)D levels were significantly lower among subjects with PKU when compared to healthy controls. They attributed low BMD in this population to a decreased vitamin D status and increased bone resorption[45]. Our data cannot sufficiently support the hypothesis that patients with PKU who are adherent to diet present with a decreased vitamin D status and low BMD because our study subjects had normal serum $25(OH)D_3$ concentrations. However, we compared vitamin D status in our prospective study population (aim 2) to that of the case population in our retrospective study (aim 1), and found a difference between the average serum 25(OH)D concentrations between the 2 populations. Our PKU subset of 19 participants had a higher mean serum $25(OH)D_3$ of 34.8 ng/mL ± 8.5, while our 92 case subjects had a mean serum 25(OH)D concentration of 27.1 ± 10.9 ng/mL. This suggests a selection bias in our prospective study such that healthy, more compliant subjects were more likely to volunteer to participate. Subjects in the prospective portion participated on a voluntary basis, while we did not require subject consent for the retrospective portion. Consequently, these serum 25(OH)D levels may reflect

differences in dietary compliance between the larger metabolic population and our smaller volunteer sub-set of subjects with PKU.

In this study we used one plasma Phe measurement, taken on the same day as the DEXA scan, to perform our analysis. Many other studies also use the plasma Phe level taken on the same day as the BMD measurement(s) for analysis purposes. However, one study took the average of 12 plasma Phe levels taken monthly over the preceding year[41]. Another researcher used all plasma Phe measurements taken from the 1st month after birth up until the day of the DEXA scan[47]. Hillman and colleagues took an average of 3 plasma Phe values prior to the study[38]. It is suggested that calculating an average of plasma Phe levels for a year is more reflective of dietary adherence. Taking this into account, we calculated the mean of 3 plasma Phe levels for each study subject over the preceding year. On average, these children are seen at the metabolic clinic 3 times per calendar year. Figure 9 demonstrates the BMD of each subject compared to their mean plasma Phe level for the preceding year. It is noteworthy that two of the subjects with the highest mean plasma Phe levels had the best spine BMD z-scores, perhaps further suggesting that control of plasma Phe concentrations does not significantly impact bone mineralization.

Future Research

The research presented here suggests that dietary intake of key nutrients such as calcium plays a crucial role in optimal bone development in this population. Future research may benefit by further investigating dietary intake in children with PKU as it relates to bone health. Further studies may also benefit by looking at other biochemical

markers, such as Osteocalcin and Magnesium. A 24-hour urine analysis could also be useful in examining calcium and protein turnover in this population. A case-control prospective study could compare BMD, dietary and biochemical markers among children with PKU and a matched healthy control population.

Conclusion

Children with PKU living in the Northwest had normal BMD associated with normal serum vitamin D. The only significant association we found occurred between BMD and dietary intake of protein, calcium, and phosphorous. Our data support a primary role of diet and adequate intake of key nutrients in maintaining bone health in this population.

Appendix 1 – Evidence Table Summarizing Association Between Bone Mineral Density and Children with Phenylketonuria.

Study Identification	Participants	Interventions	Outcomes	Proposed Mechanism
Adamczyk, Piotr; Morawiec-Knysak, Aurelia; Pludowski, Pawel; et. al. (2010) Bone metabolism and the muscle-bone relationship in children, adolescents and young adults with phenylketonuria *Journal Bone Mineral Metabolism*, August	45 children (25 boys, 20 girls) ages 8-19 years with PKU on diet since 1st month of life; 3 subgroups of patients: 1) *subgroup 1* - 15 children who had not yet reached sexual maturity, with recommended blood Phe levels; 2) *subgroup 2a* - 18 subjects in Tanner 5 stage with recommended blood Phe levels; 3) *subgroup 2b* - 12 subjects in Tanner 5 stage with blood Phe values above recommended level 562 control healthy children ages 5-18 years (278 girls, 284 males) all caucasian	BH, BW assessed on same day as DEXA scan, BMI was calculated; *DEXA* - BMC and BMD assessed in whole skeleton and lumbar spine, and LBM and FM were evaluated; *Laboratory Parameters* - serum carboxyterminal telopeptide of type 1 collagen (ICTP), bone alkaline phosphatase (bALP), osteocalcin, parathormone, calcitonin, total and ionized calcium; *z-scores* used to assess difference between PKU subgroups and between PKU subjects and references *Stepwise multiple regression analysis* - age, sex, BMI and Phe level were independent factors	Mean ICTP and bALP were higher in *1* vs *2*, but higher levels of bALP, osteocalcin and PTH were observed in the noncompliant *2b* subgroup;*DEXA*- *2b* had significantly lower TBBMD and SBMD compared to 2a, and no significant differences were found between *1* and *2a*; *2b* had significantly higher TBBMC, spine BMC, TBBMC/LBM and spine BMC/LBM than *2b;*	Stepwise regression analysis revealed that the most common observed factor with a significant negative impact on BMD values was Phe blood level Possible bone tissue impairment
Allen, Jane R; Humphries, Ian RJ; Waters, Donna L; et. al (1994) Decreased bone mineral density in children with phenylketonuria *American Journal of Clinical Nutrition*, 59: 419-22	32 prepubertal children (girls < 10 years, boys < 12 years) with PKU on diet since early childhood; 95 age-matched healthy control subjects	BH and BW measured on all subjects ; DEXA - TBMD was measured in all subjects, but due to time constraints SBMD was only taken on 24 of 32 PKU children and 55 of 95 control subjects Dietary intake was assessed by a 4-day food record - analysis was performed on food records of 30 PKU children and 12 healthy siblings; Plasma Phe levels were measured by HPLC; *t-test* used to analyze differences between PKU and controls	No significant difference in mean age and SD BH or BW between the PKU and control children; TBMD was significantly lower in PKU group than control SBMD was found to be lower in PKU subjects n the subset of children who it measured; The PKU group had similar intakes of energy, protein, and fiber but higher intakes of calcium, phosphorus, and magnesium. However, no correlation was found between TBMD or SBMD and dietary variables.	The etiology of low BMD in PKU children remains obscure. This study rules out inactivity or lack of sunlight exposure because PKU children had equivalent participation in outdoor sports to their peers. This study found no correlation between BMD and plasma Phe nor between BMD and dietary intake. One significant finding of this study was that children with PKU demonstrated higher dietary intakes of calcium, magnesium, and phosphorus (in excess of the RDI) compared to the control children.

Al-Qadreh, A; Schulpis, KH; Athanasopoulou, H; et. Al (1998) Bone mineral status in children with phenylketonuria under treatment Acta Paediatrics, 87: 1162-6	48 children (20 boys, 28 girls) ages 2.5-17 years with PKU on diet since infancy; 50 controls (22 boys, 28 girls) ages 3-15 years	Adequacy of dietary compliance was evaluated by calculating the average of serum Phe levels over the preceding 12 months (12 measurements), and the ratio of artificial:natural protein was calculated for every patient; Venous blood samples and 3-hour urine collections were obtained during the morning of their visit following a 12 hour fast - blood calcium and ionized calcium, magnesium, phosphorus, creatinine, alkaline phsophatase (ALP), parathyroid hormone (c-PTH), and 25(OH)D were measured; Urinary Ca/Cr, UP/PCr, UMg/UCr and hydroxyproline/UCr were calculated; Biochemical measurements obtained from the PKU subjects were compared to the control group	PKU children had reduced bone mineralization and severe osteopenia was found in 46% of patients; When PKU subjects were divided in 2 subgroups: <8 yrs and >8 yrs, the older group had greater bone loss and higher mean serum Phe levels; Girls had significantly lower BD than boys; There was a significant negative correlation of BD with age and Phe levels; and a positive correlation of BD to the ratio of artificial:natural protein intake was revealed; serum Ca and Mg were higher in PKU children than controls, ALP was lower in PKU patients, and no significant differences observed in PTH or 25(OH)D; PKU children had an increased UCa/UCr ratio, increased reabsorption of P; Serum P was higher in PKU patients <8yrs compared to the older subgroup; Mean serum Phe values significantly higher in PKU patients >8yrs; Positive correlation was observed between age and Phe concentration, and negatively with BD and with artificial:natural protein intake.	A problem with bone mineralization exists at all ages but the problem worsens in adolescense, likely owing to poor dietary compliance. Increased plasma Phe concentration and urinary excretion of Phe metabolites leads to increased mineral loss in urine.
Ambroszkiewicz, J; Gajewska, J; Laskowska-Klita, T (2004) A study of bone turnover markers in prepubertal children with phenylketonuria	37 children with PKU, divided into two groups: Group A - 12 children, ages 3-10 years, strict dietary adherence with mean serum Phe concentrations close to reference range; Group B - 25 children, ages 3-10 years, poor dietary adherence with increased Phe concentrations Reference population of 27 healthy children, ages 4-9 years "Ethnically homogenous" study population	Mean Phe concentration calculated for last 3 years of each patient's life; PKU children were fed with an AA mixture PAM, Milupa-PKU 2 or Phenylfree PKU children were found to have normal Ca and Phos levels; Venous blood samples were collected after an overnight fast, centrifuged and serum levels of Phe, Ca, and P were determined; the remaining serum samples were frozen and used for measurement of bone turnover - BALP (bone alkaline phosphatase), OC (Osteocalcin), CTX (Collagen type 1 cross-linked C-telopeptide), OPG (Cytokine osteoprotegerin)	Lower levels of bone formation/bone resorption markers in Group A (dietary adherence, normal Phe) than Group B (off-diet, elevated Phe) but the differences were not statistically significant OC, CTX, OPG concentrations were significantly lower in both groups of PKU children when compared to the healthy age-matched controls	Pre-pubertal children with PKU may experience a decreased bone turnover rate compared to healthy controls

Greeves, LG; Carson, DJ; Magee, A; Patterson, CC (1997) Fractures and phenylketonuria Acta Paediatrics, 86: 242-4	85 patients (44 males, 41 females) with PKU on diet, 98 controls (50 males, 48 females) from a total of 66 families. Patient ages ranged from 0.3 to 33.6 years (mean 12.1 years)	Parents of young PKU patients and older PKU patients were asked to complete a survey that asked questions about the subject's age, history of other illnesses which might affect fracture risk, dietary treatment, history, date, age and sit of fracture and any problems with fracture healing. The risk of fracture was than compared between PKU patients and their siblings.	21 PKU patients and 18 controls had a history of fracture, and the mean reported age of first fracture was higher in PKU patients than controls (9.4 yrs, SD 4.2 vs 6.5 yrs, SD 4.4). There was a significantly greater risk of fracture in PKU patients 8yrs of age (former policy was to relax diet at 8yrs old) whose fracture rate was 2.6 times higher than the rate in controls. All PKU patients followed some sort of dietary restriction, but compliance ranged from poor to strict.	Poor dietary compliance after 8 years of age or dietary relaxation with an increase in plasma Phe concentrations. OR this could be due to a cumulative disease related or diet related reduction in bone mass.
Hillman, L; Schlotzhauer, C; Lee, D; Grasela, J; Witer, S; Allen, S; Hillman, R (1996) Decreased bone mineralization in children with phenylketonuria under treatment	11 children with PKU (5M/6F, 10.9±4.2 yrs) compared to 64 controls (32M/32F, 11.4±4.2 yrs) and 11 controls were age- and sex-matched to PKU kids	Collected mean daily intakes of protein, calcium, phosphorus; Averaged 3 serum Phe values; Body composition and BMD measured using DEXA; Measured status of : Ca, Cu, Sn, Phos, albumin, creatinine, PTH, 25(OH)D, 1,25(OH)2D, B-ALP, PICP, OC, TRAP	Serum calcium and magnesium were significantly lower in PKU children; urine phos/creatinine low and percentage tubular reabsorption of phos was significantly higher for PKU children; No statistically significant differences between PKU and control children for: serum albumin and creatinine, weight, lean body weight, fat weight, % body fat, serum phos, copper, zinc and iron, urine calcium/creatinine and magnesuim/creatinine, 25(OH)D, 1,25(OH)2D and PTH; bone formation markers B-ALP and OC significantly low in PKU children, but bone resorption markers TRAP and urine Ca/Cr were similar; DEXA revealed that lumbar spine BMD and total lower extremity BMD were significantly lower in PKU children; Only significant correlations found: serum Phe positively correlated with age, protein intake negatively correlated with age; B-ALP highly correlated with alk phosphatase	Increased resorption was not seen in this study population, but the data may suggest decreased osteoblast activity, reflective of decreased bone mineralization. This decreased bone mineralization may be due to a primary bone mineralization problem or may be secondary to reduced mineral availability

McMurry, MP; Chan, GM; Leonard, CO; Ernst, SL (1992) Bone mineral status in children with phenylketonuria - relationship to nutritional intake and phenylalanine control	26 patients with PKU ages 1.9-25.5 years, most of whom had been diagnosed and treated since infancy Control population of 164 healthy, normal children ages 3-16 years (88 boys, 76 girls) Subjects were divided into groups: ages 1-5 years - preschoolers (9 PKU, 28 control); ages 6-11 years grade-schoolers (9PKU, 94 control); ages ≥12years - older subjects (8 PKU, 42 control)	Bone mineral status was determined using single-photon absorptiometry Postprandial blood was collected for plasma mineral analysis - Ca, Phos, Mg, alk phos, 25(OH)D, Cu, and Zn; serum was taken to determine albumin, Phe, and PTH Normal range for Ca was considered to be 30-150 nmol/L Clinic charts were used to average the Phe concentrations for each subject over the previous 6 mos 2-3 day food records were gathered and subjects were asked to complete a Dietary Compliance Questionnaire evaluates usual formula intake, avoidance of high-Phe foods, use of food equivalents, and the use of special low-protein foods	Up until 8 years of age, the PKU and control had comparable increases in BMC, but from 8 years on the BMC in PKU children fell ll below that of the controls, with bone mineralization progressing at a slower rate. After 8 years of age, PKU subjects were below the normal curve for bone mineral development Phos in gradeschooler, Mg in all PKU subjects, 25(OH)D in preschoolers, and ALP in all PKU subjects was lower; Plasma Ca concentrations were not different between PKU and controls; Albumin was within normal range for age and sex; Mean PTH concentration in PKU population was significantly lower than controls; PTH was not correlated with BMC, Ca, Phos, or Mg; Blood Phe concentrations were significantly higher in the older group than either yougar group - age was positively correlate with mean Phe concentration; Dietary compliance scores were negatively correlated with age and mean blood Phe concentrations	Poor dietary compliance or dietary relaxation with an increase in plasma Phe concentrations in older children/adults; Compliance with dietary therapy for PKU is associated with normal bone mineral development in young children
Mendes, AB; Martins, FF; Cruz, WMS; da Silva, LE; Abadesso, CBM; Boaventura, GT (2011) Bone development in children and adolescents with PKU	13 patients with PKU (ages 8-16 yrs) divided into 2 groups : 1) Children Group (CG) 4F/1M, 2) Adolescents Group (AG) 5F/3M. All diagnosed with PKU through neonatal screening	Nutritional assessment - collected data every 2 mos for period of 6 mos; quantified energy, protein, Phe, calcium, and phos intake; calculated Phe consumption based on observation that 1g of protein contains 50mg Phe. Bone Age - took hand and fist X-rays to determine bone age. Bone Densitometry - DXA scan to determine spinal bone mineral density (SBMD) - Plasma Phe - fluorometry.	In CG, subjects non-adherent to diet (NAD) consumed lower amounts of calcium and energy, had higher rates of plasma Phe and higher PRO intake from free foods; BA values higher than chronological age and lower BMD values. In AG-NAD, values were lower for energy intake, calcium, phosphorus, food PRO, and Phe intake than recommended; also demonstrated higher plasma Phe levels, BA greater than chronological age, and lower BMD values.	The non-adherence to diet in both CG and AG groups, and the consequent imbalance of key nutrients involved in bone metabolism suggests that these factors influence the failure to thrive in children and reduced bone mineralization in adolescents.

Modan-Moses, D; Vered, I; Schwartz, G; Anikster, Y; Abraham, S; Segev, R; Efrati, O (2007) Peak bone mass in patients with phenylketonuria	31 adult patients with classical PKU (18 female, 13 male) ages 19-41 (Phe 20 mg/dL)	3 day food diaries collected to assess Phe, protein, fat, Ca, vit D and energy intake; BMD was measured by DEXA scan; Laboratory studies to assess plasma Phe, 25(OH)D, calcium, phos, alk phos, total pro, albumin, estradiol, FSH, LH, cortisol, TSH, FT4, TT3, prolactin, IGF-1, PTH	No statistically significant differences for Phe levels between the diet-adherent and non-adherent groups; mean caloric, protein and calcium intakes were significantly higher in the diet-adherent group; 25(OH)D normal in all patients; Osteopenia detected in 11 patients, osteoporosis in 2; No correlation was found between BMD and age, serum minerals, blood Phe concentrations, 25(OH)D, alk phos, Ca and protein intake, BMI and body fat percentage	Compromised BMD appears to exist in the PKU population and several potential contributing factors can be considered, although this data does not favor any one in particular
Nagasaka, H; Tsukahara, H; Takatani, T; et al. (2011) Cross-sectional study of bone metabolism with nutrition in adult classical phenylketonuric patients diagnosed by neonatal screening. Journal of Bone Mineral Metabolism, April	34 classical PKU patients (21 women, 13 men) ages 20-35 years; 36 healthy contols ages 19-40 years	Collected 3-day food records to measure daily energy, protein, fat, calcium, and vitamin D intake; Measured blood levels of intact PTH, 25(OH)D, 1,25(OH)D; bone turnover markers of B-ALP, OC, type I collagen (ICTP), urinary deoxypyridinoline (D-Pyr), urinary N-telopeptide of type I collagen, and OPG; and urinary Ca/creatinine and P/Cr.	Average daily energy and protein intakes were significantly lower inPKU patients compared to controls, but no significant differences were seen in other macro- or micronutrient intakes; Intact PTH levels and bone resorption markers were significanty higher in female PKU patients; male patients had comparable intact PTH levels compared to controls but higher levels of bone resorption markers; Male and female PKU patients also had significantly higher 1,25(OH)D and lower 25(OH)D levels than controls; no differences in levels of bone formation markers; Serum levels of OPG were significantly lower in female and male PKU patients, and they had a higher urinary Ca excretion but similar P excretion; no significant correlation was observed between serum Phe levels and any bone parameter, but in both male and female PKU patients, a significant correlation was found between two bone resorption markers, D-Pyr and ICTP	Sufficient nutrient intake might not necessarily result in normal bone mineralization among PKU patients; Serum Phe levels do not appear to correlate with bone status; lower vitamin D status among PKU patients might be associated with a possible decrease in cholesterol production; A decreased vitamin D status and higher proportion of bone resorption to formation may contribute to impaired bone metabolism among PKU patients.

Roato, I; Porta, F; Mussa, A; D'Amico, L; Fiore, L; Garelli, D; Spada, M; Ferracini, R (2010) Bone Impairment in Phenylketonuria is Characterized by Circulating Osteoclast Precusors and Activated T Cell Increase	40 PKU patients (18 males, 22 females) 14.6±8.1 years and 40 age- and sex-matched controls	In vivo: PKU subjects were put on a Phe-restricted diet and received a Phe-free AA mixture 3x during the day to meet their nutrient needs; Controls received a normo-caloric regular diet meeting the daily nutrient recommendations Monthly Phe levels were collected for each patients one year prior to the study All subjects underwent phalangeal QUS measurement	Osteoclastogenesis in PKU patients was significantly higher than in the controls; the bone resorbing activity was higher in unstimulated PKU patients' cultures than in healthy controls; Circulating Osteoclast Precursors (OCPs) were higher in PKU patients; RANLK/OPG ratio was significantly higher in PKU patients than in healthy controls; In PKU subjects, spontangeous osteoclastogenesis was directly correlated with both age and blood Phe concentration, and these correlations were not observed in healthy controls; QUS assessment inPKU patients revealed an overall normal bone conditions compared to healthy population	Increased osteoclast activity, with disruption of bone formation/resorption process. Possible immune connection to T cells?
Schwan, R; Mokov, E; Scheidhauer, K; Lettgen, B; Schonau, E (1998) Decreased trabecular bone mineral density in patients with phenylketonuria measured by peripheral quantitative computed tomography	14 patients with PKU compared to 14 age-, gender-, height-, and weight-matched controls; no differences in physical activity levels between two groups; Divided into 3 groups: children (5-8 yrs), adolescents (13-16 yrs), and adults (19-28 yrs)	Measured plasma Phe levels and took 5 mean levels prior to study; osteodensitometry measured by pQCT of non-dominant radius	Spongy bone mineral density (SBMD) decreased for each subgroup compared to controls, with more pronounced differences in older subjects; When all 14 patients were considered, there was a significant SBMD reduction in PKU patients; Total bone mineral density (TBMD) slightly decreased in adolescents and adults, but reached no statistical significance	Treated PKU patients exhibit significant alterations in trabecular bone, but it is undetermined whether these abnormalities stem from architecture or composition issues.

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