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EXAMINATION OF DIET, PHYSICAL ACTIVITY, BIOMARKERS OF BONE MINERALIZATION, BONE MINERAL CONTENT AND BODY COMPOSITION IN CHILDREN BETWEEN 5 YEARS OF AGE AND PUBERTY

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EXAMINATION OF DIET, PHYSICAL ACTIVITY, BIOMARKERS OF BONE MINERALIZATION, BONE MINERAL CONTENT AND BODY COMPOSITION IN CHILDREN BETWEEN 5 YEARS OF AGE AND PUBERTY

By

Marcia Kelly Scott

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ABSTRACT

EXAMINATION OF DIET, PHYSICAL ACTIVITY, BIOMARKERS OF BONE MINERALIZATION, BONE MINERAL CONTENT AND BODY COMPOSITION IN CHILDREN BETWEEN 5 YEARS OF AGE AND PUBERTY

By

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Osteoporosis is predicted to become a disease of epidemic proportions worldwide. Prevention of osteoporosis begins in childhood, recognizing that attainment of higher bone mineral content during the first two decades of life decreases the risk of developing fractures later in life. Body composition, bone mineral density (BMD), bone mineral content (BMC), diet composition, bone metabolism biomarkers, and physical activity levels were measured in healthy, prepubertal children (n=52), mean age of 7.6 years. BMD, BMC and body composition were determined by dual energy x-ray absorptiometry (DXA). BMD Z-scores ranged from –1.2 to +1.8 with 31% of subjects having Z-scores below negative 0.2. BMD and BMC correlated positively with percent body fat within a healthy range (r = 0.535 for BMD; r = 0.646 for BMC) and with total daily energy expenditure (DTEE) above basal energy expenditure (BEE) (r = 0.459 for BMD; r = 0.591 for BMC). BMD and BMC correlated negatively with protein intake (r = -0.508 for BMD; r = -0.564 for BMC), energy intake (r = -0.510 for BMD; r = -0.578 for BMC), calcium intake (r = -0.277 for BMD), phosphorus intake (r = -0.378 for BMD; r = -0.348 for BMC) and with serum osteocalcin (OC) (r = -0.507 for BMD; r = -0.499 for BMC).

Four prediction models for BMC and BMD were developed. Total BMC can be predicted by percent body fat, fruit and vegetable intake, calcium, phosphorus, energy and magnesium intakes, along with DTEE above BEE. This model explains 72.9% of the

variability in BMC. Alternately, BMC can be predicted by serum OC, urinary deoxypyridinium (DPD), DTEE above BEE, and percent body fat, with this model explaining 64.9% of the variability in BMC. BMD can be predicted by percent body fat, fruit and vegetable intake, calcium, phosphorus, energy, and magnesium intakes. This model explains 58.8% of the variability in BMD. The second prediction model for BMD includes percent body fat, serum OC, urinary DPD, serum 25(OH) Vitamin D_3, and DTEE above BEE, explaining 58.9% of the variability in BMD.

This study is unique in looking at many modifiable environmental influences of bone mineralization in a group of exclusively pre-pubertal subjects. With one third of these children having negative BMD Z-scores before they reach puberty, these data suggest concerns about inadequate progress toward attainment of peak bone mass. The negative correlation of protein to bone mass warrants further examination given the protein rich diets consumed by children in the US, with these subjects consuming over 3 times the recommended amount. Physical activity and fruit and vegetable intake appear to be strongly, positively associated to bone mass, supporting public health efforts to increase both physical activity and intake of fruits and vegetables. Biomarkers of bone metabolism, serum OC (marker of bone formation and turnover), and urinary DPD (marker of bone resorption) may merit further consideration for their potential use in monitoring bone growth in young children. Diet and lifestyle habits are forming for a lifetime in these early childhood years so knowing where to focus suggestions and interventions toward bone-building lifestyles at this age may decrease the risk of developing osteoporosis later in life as well as decreasing fracture risk throughout life.

DEDICATION

This work is dedicated to Taylor, Robinson, Alexandra, and Christian Scott
My family and my best friends

And to Jack and Janet Kelly and R. Taylor Scott, IV
Parents and first teachers

And to Jenny Taylor Bond
Teacher, mentor, friend, sister

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KEY TO COMMONLY USED ABBREVIATIONS

BEE basal energy expenditure

BMC bone mineral content

BMD bone mineral density

BMI body mass index

Ca calcium

cm centimeters

d day

DEE daily total energy expenditure

DPD deoxypyridinoline

DRI Dietary Reference Intake

DXA dual energy x-ray absorptiometry

HKNS Healthy Kids Nutrition Study

K potassium

kcal kilocalories

kg kilograms

LBM lean body mass

Mg magnesium

mg milligrams

mL milliliters

mmol millimoles

ng nanograms

OC osteocalcin

P phosphorus

INTRODUCTION

The World Health Organization predicts that osteoporosis will eventually become a disease of epidemic proportions worldwide. In the U.S., according to the USDHHS Healthy People 2010 report, one in ten people in the U.S. have osteoporosis and at least one third of them will experience an osteoporotic-related fracture after 50 years of age (USDHHS, 2000). Twenty four percent of people over 50 years of age who suffer hip fractures die within one year of the fracture and a majority of the remaining people never returns to their pre-fracture level of function. Often considered a geriatric disease, osteoporosis may also be considered a pediatric disease with geriatric consequences. Prevention of osteoporosis should likely begin during childhood, recognizing that attainment of higher bone mineral content during the first two decades of life decreases the risk of developing fractures later in life (Faulkner and Bailey, 2007).

This study, the Healthy Kids Nutrition Study, examined the diet, physical activity, and body composition of a group of children in the U.S. Midwest and looked at the relationship of these variables to the children's bone mass with the goal of identifying lifestyle factors that impact bone health. Diet and lifestyle habits are forming for a lifetime in these early childhood years so knowing where to focus suggestions and interventions toward bone-building lifestyles at this age may decrease the risk of developing osteoporosis later in life as well as decrease fracture risk throughout life.

CHAPTER ONE

CHAPTER ONE

REVIEW OF THE LITERATURE

Background Information

Osteoporosis is increasingly recognized as one of the major public health problems facing aging individuals of both genders, worldwide. It is predicted to become a disease of epidemic proportions within the next several decades (Riggs and Melton, 1995; WHO, 2003). As a disease, osteoporosis is broadly defined as diminished bone mass and reduced bone mineral density (BMD) at the level of 2.5 standard deviations below the referent BMD of young adults (Riggs and Melton, 1995; USDHHS, 2000; WHO, 2003). Osteoporosis presents clinically as fractures, both traumatic and nontraumatic in nature, resulting in significant morbidity, mortality, and health care costs (WHO, 2003). The two most important ways to reduce risk of osteoporosis are to attain peak bone mass in early life and to have a low rate of bone loss in later life.

Peak bone mass has genetic, hormonal, nutritional, and behavioral determinants (Soyka et al., 2000). What processes and factors influence bone growth in the important first two decades of life? Research over the past 20 years has begun to clarify the genetic and environmental contributors to bone growth in children. Advances in the fields of bone imaging and biomarker assays have contributed to an understanding of the importance of attainment of maximal bone mass during the growth years. The role of physical activity in a bone building lifestyle is now well recognized (WHO, 2003). Recognition of the critical importance of appropriate intake of the bone building nutrients has inspired renewed efforts to determine optimal intake levels as well as optimal total

diet composition in order to attain maximum bone mineral density and decrease the risk of bone disease.

In considering ways to decrease both the incidence and the severity of osteoporosis, approaches to interventions for this disease target methods of prevention and abatement of osteoporosis not just in adulthood but also during infancy, childhood, and adolescence. There is a consensus in the literature that attainment of higher bone mineral content during these first two decades of life decreases the risk of developing fractures later in life (Heaney et al., 2000). Though osteoporosis is generally considered a geriatric disease, osteoporosis may actually be a pediatric disease with geriatric consequences. Investigation of the pediatric origins of osteoporosis is centered on the underlying processes and factors playing a role in the evolution of reduced bone mass and reduced bone mineral density.

Bone mass at all ages exists on a continuum, determined by genetic factors and modified in either direction by environmental factors (Matkovic et al., 1998; Heaney et al., 2000). From birth through young adulthood, bone mass increases at a steady rate (Glastre et al., 1990; Southard et al., 1991; Heaney et al., 2000). Bone mass also tends to track throughout life, meaning that infants, children and adolescents that have higher bone mass tend to be the adults who have high bone mass (Ferretti et al., 1998; Heaney et al., 2000). This association introduces the possibility that "at risk" individuals may be identified early in life such that risk can be minimized to the extent that is possible. Approximately 26% of adult calcium is laid down during the 2 years of peak skeletal growth that occurs in adolescence (Bailey et al., 2000). Statistics on lifetime fracture incidence show that between the ages of approximately 5 and 15 years for boys and girls

and then after the age of 50 for women, the incidence of limb fractures is highest (Heaney et al., 2000). Low bone mineral content (BMC) in children is considered to put children at increased risk of fracture (Whiting, 2002). Attainment of peak bone mass may be an important pediatric health goal in order to minimize bone fractures through all stages of life.

In addition to osteoporosis, other public health concerns in children are associated with bone health. Calcium intake, physical activity, and body composition appear to be linked to bone growth (Soyka et al., 2000; Heaney et al., 2000; Greer and Krebs, 2006). Current trends in diet intake in children show increased consumption of soft drinks replacing other traditional "kid drinks" such as juice, milk, and water, trends that are contributing to an increasing incidence of overweight children as well as increasing concerns about the intake of nutrients important to bone health (Greer and Krebs, 2006). The prevalence of pediatric overweight is increasing at an alarming rate in the U.S., having doubled over the past two decades (AAP/CON, 2003). The National Center for Health Statistics (NCHS) defines overweight in the pediatric population as body mass index (BMI) at or above the 95th percentile for age and gender with "at risk for overweight" being defined by a BMI between the 85^{th} and 95^{th} percentile (CDC/NCHS, 2003). Currently 15.3% of 6- to 11- year old children are at or above the 95th percentile for BMI on the standard growth charts as developed by the Centers for Disease Control and Prevention, National Center for Health Statistics (AAP/CON, 2003). This increase in percent overweight is accompanied by a decrease in physical activity along with an increase in the pediatric incidence of many of the comorbid conditions seen with adult obesity such as cardiovascular, endocrinologic, orthopedic, pulmonary and psychological

problems (AAP/CON, 2003). Because of the overlapping etiologies of chronic health conditions that have roots in childhood, decreasing the risk of these conditions in the pediatric population becomes even more important to pubic health in the long term.

Bone and Bone Growth

The human skeleton contains 206 individual bones. They are composed of two types, cortical and trabecular bone. Cortical bone is the dense, compact tissue on the outer surface of bones, includes the long bones, and makes up 75-80 % of bone mass. Trabecular bone is more mesh-like than cortical bone, is present in flat bones, ends of long bones, and vertebrae and makes up 20-25 % of bone mass. All bone tissue is in a constant state of flux, involved in one or more of three processes: growth, modeling and remodeling. Growth is under the control of the endocrine system and encompasses overall skeletal growth. Modeling involves the laying down of bone onto bone surfaces and results in a net gain of bone. Remodeling is the dominant process in adults and is basically a recycling process. Older bone tissue is resorbed followed by new bone formation at the same site, preserving bone's mechanical integrity (Bailey et al., 1996). Bone is largely mineral (70-90%); however the remaining matter is organic material, most of which is collagen. Collagen plays a critical role in the structure and function of bone tissue hence many of the biomarkers of bone metabolism are related to the structure and formation of collagen, also referred to as bone matrix proteins (Young, 2003).

The newest frontiers in bone growth and health are in the areas of determining patterns of bone growth and in identification of osteoporosis-susceptibility genes. The tempo of growth, direction of growth, region of growth and rate of growth in differing sites even on a single bone appear to vary with age and exposure to all of the many

modifiers of bone growth. Bass et al., 1999, determined that by seven years of age, bone had reached 80% of its maturational peak but only 40% of its peak mineral content. They suggest that because growth provides first a bigger skeleton and then second a denser skeleton, negative influences on bone growth in childhood are of particular importance in the eventual prevention of bone fragility with age. Many potentially important candidate genes for osteoporosis have been identified (Cusack and Cashman, 2003). Many of these genes involve the regulatory proteins that influence bone growth while others of these so-called osteoporosis-susceptibility genes interact with nutritional factors that influence bone health. As progress is made in identifying these genetic pathways, genotype-specific bone health recommendations may evolve (Cusack and Cashman, 2003).

Influence of Gender and Race on Bone

As adults, males tend to have greater bone mass than do women (Nieves et al., 2005) and blacks tend to have higher bone mass than whites or Asians (Pothiwala et al., 2006), but that is not necessarily the case for pre-pubertal children. Assessment of bone growth in children has progressed from the earlier work using bone age to single photon absorptiometry (SPA) to the currently used dual x-ray absorptiometry (DEXA) and quantitative computerized tomography (Gluer et al., 1998; Shore and Poznanski, 1996). Initially, normative data for BMD in children was determined, measuring BMC at several sites with SPA. Geusens et al. (1991) did not see gender differences in BMC or BMD in prepubertal children. Patel et al. (1992) investigated differences in BMC of black versus white boys and girls, 6 to 20 years of age. They found that after adjusting for height, there were no race or sex differences in BMC. Gilsanz et al. (1991) saw no difference in vertebral bone density in black versus white prepubertal girls, although their later

response to puberty did differ. Russell et al. (2001) saw increased bone age, considered a correlate of BMC, in African American children five to twelve years of age over the bone age of Caucasian children of the same chronological age but they attributed the difference to the greater adiposity of the African American children.

In contrast, Specker et al. (1987) reported detectable gender differences in BMC after 4 years of age in a cohort of predominantly Caucasian children. Li et al. (1989) observed higher BMC in black children and in males. Bell et al. (1991) also observed gender and racial differences in the BMD of prepubertal children. Nowack et al. (1995) compared Hawaiian, Filipino, Asian, and Caucasian children and found that Hawaiian children had a higher BMC than Asian or Caucasian children. Ferretti et al., (1998) report that BMC follows lean body mass (LBM), fat mass, and height in prepubertal, Argentine boys and girls. Several studies using dual energy x-ray absorptiometry (DXA) report that, in general, BMD increases with age, height, weight and Tanner stage (Glastre et al., 1990; Southard et al., 1991; Del-Rio et al., 1994; Zanchetta et al., 1995).

Maynard et al. (1998) used Fels Longitudinal Study data to summarize BMC and BMD in 8 to18 year old white children. They found no gender differences in the eight and nine year old children, a finding confirmed by Fassler and Bonjour (1995) and by Nguyen et al. (2001). In an attempt to better understand both gender and ethnic influences on bone in prepubertal children, Horlick et al. (2000) measured BMD and BMC in white, black, and Asian children. They found BMC differed by gender but not ethnicity and BMD differed for blacks versus non-blacks but not gender. They concluded that both measures vary primarily as a function of bone area and body size. Clearly, certain nonmodifiable factors such as race/ethnicity and gender do play a role in

determining a child's bone growth toward attainment of peak bone density; however it remains to be elucidated which of these factors, if any, influence bone growth leading to osteoporosis risk later in life.

Role of Biomarkers in Assessment of Bone Growth

In addition to direct measurement of BMD with DXA, biomarkers of bone growth may be useful in predicting bone activity. Much progress is being made with the use of biomarkers of bone turnover, bone resorption and bone formation in adult women receiving treatment for low BMD. Biomarkers shown to have a positive correlation to bone formation include oseteocalcin (OC), bone alkaline phosphatase (BALP) and procollagen type I C-terminal propeptide (PICP). Biomarkers shown to have a correlation with bone resorption include hydroxproline, pyridinium cross-links (pyridinium (PYD) and deoxypyridinium (DPD)), galactosyl-hydroxylysine, and cross-linked C-terminal telopeptides of type I collagen (ICTP) (Eyre, 1996). Use of biomarkers in children has been less extensively evaluated, but some studies indicate that there may be a use for biomarkers in monitoring growth and therapeutic intervention in some populations of children (Crofton, 1998). In cross-sectional studies of children over a wide range of ages, bone biomarker concentrations tend to mirror the growth curve, a reflection of the active bone formation and resorption process inherent to bone growth (Crofton, 1998). Validation of biomarkers, methods of assay of biomarkers, understanding of biological variability and potential clinical application of biomarkers in the pediatric population are areas of study that are in their infancy.

Osteocalcin, also known as serum bone gla protein, is a frequently measured biomarker, considered to represent bone turnover (Lester, 1995). Osteocalcin is a 49

amino acid protein found in bone and synthesized predominantly by osteoblasts (Delmas, 1995). Osteocalcin represents up to 25% of the noncollagenous protein in bone. Post-translational vitamin K-dependent carboxylations of its three glutamic acid residues give OC hydroxyapatite-binding properties (Price et al., 1980). The majority of secreted OC is incorporated into the bone matrix but a fraction of newly synthesized OC is released into circulation where it can be detected by immunoassay. Intact OC is susceptible to proteolytic degradation in serum prior to its renal clearance. Immunoreactivity of the degraded forms of de novo OC may be similar to fragments released during osteoclastic degradation of bone matrix (Noonan et al., 1997). Osteocalcin is generally regarded as a marker of bone formation (Delmas, 1995).

Increased serum concentrations of OC are observed among postmenopausal women relative to premenopausal women and are associated with rapid bone loss (Gomez et al.,1994; Johansen et al.,1988; Ross and Knowlton, 1998). Increased OC is also seen in a number of conditions characterized by excessive bone turnover including osteoporosis, Paget's disease, hyperparathyroidism, thyrotoxicosis, and metastatic cancer (Delmas, 1995; Price et al., 1980; Gomez et al., 1994; Clarke et al., 1995). Osteocalcin concentrations decrease following antiresorptive therapy in a dose-dependent manner (Johansen et al., 1988; Garnero et al.,1994). These short-term changes are inversely correlated with long-term changes in BMD (Garnero et al., 1994). Osteocalcin concentrations are decreased in hypothyroidism, hypoparathyroidism, and in Cushing's syndrome caused by pharmacological glucocorticoid excess (Delmas, 1995; Clarke et al., 1995).

Evaluation of the literature in the area of biomarkers in children is influenced by the need to recognize that much of the available data mix pre-pubertal and pubertal populations of children. Studies have measured OC in children with congenital adrenal hyperplasia (Lisa et al., 1995) and in normal children of increasing age (Tommasi et al., 1996), and serum bone gla protein in normal children that correlated weakly with BMD (Glastre et al., 1990). Hillman et al. (1996) reported decreases in OC in children with PKU. Slemenda et al. (1997) documented changes in OC concentrations in prepubertal and pubertal subjects with OC concentrations peaking at Tanner I then beginning to decline. Fares et al. (2003) noted an increase in OC in boys and girls that peaked at pubertal Tanner III then began a decline. On the other hand, Mora et al. (1999) observed an inverse association between OC and BMD in children at varying stages of puberty. Slemenda et al. (1997) also noticed an inverse relationship between OC and BMD in a group of Ca supplemented children. Once the Ca supplementation ended, BMD and OC concentrations returned to concentrations similar to their unsupplemented twin controls. Van Coeverden et al. (2002) documented significant positive correlations between OC and BMC at several sites in a group of peri-pubertal Dutch children. These observations support OC's potential role in the assessment of bone growth in children. In normal, healthy children, OC concentrations can be expected to increase with age and body size until puberty, then gradually decline to low adult levels (Tommasi et al., 1996; Bonofiglio et al., 2000).

Deoxypyridinoline is considered a biomarker of bone resorption. The organic matrix of bone consists of approximately 90% type I collagen (Seyedin and Rosen, 1990). Trifunctional pyridinium crosslinks, PYD or DPD, form between hydroxylysine

or lysine residues at the C- and N-telopeptide ends of one collagen molecule and the helical portion of a neighboring molecule during collagen maturation (Seibel et al., 1992). This cross-linking provides the flexibility necessary for structural integrity to the collagen fibril. Osteoclastic degradation of bone collagen releases the crosslinks into circulation, and they are excreted in urine. Though the crosslinks are present in a number of tissues, the molar ratio of PYD and DPD in urine is very similar to that in bone, indicating that urinary concentrations of both are derived mainly from bone. Deoxypyridimium in particular, with a more limited tissue distribution than PYD, is derived almost exclusively from bone (Seibel et al., 1992; Robins et al., 1994; Delmas, 1995; Robins, 1995; James et al., 1996). As products of collagen maturation, they cannot be reused in new collagen synthesis, nor are they further metabolized (Robins et al., 1994; Delmas, 1995; Robins, 1995). Of the total pool of urinary DPD, approximately 40-45% is free and the remainder is bound to peptides (Seibel et al., 1992; Robins et al., 1994; Robins, 1995).

Increased urinary concentrations of DPD are observed among postmenopausal women compared to premenopausal women and are associated with rapid bone loss (Hesley et al., 1998; Ross and Knowlton, 1998) and an increased risk of hip fracture (Garnero et al., 1996; Daele et al., 1996). Increased DPD excretion is seen in a number of conditions characterized by excessive bone resorption, including osteoporosis, Paget's disease, hyperparathyroidism, thyrotoxicosis, malignant hypercalcemia, and metastatic cancer (Seibel et al., 1992; Delmas, 1995; Robins et al., 1994; Robins, 1995). Deoxypyridimium concentrations decrease rapidly following antiresorptive therapy in a dose-dependent manner (Delmas, 1995; Ross and Knowlton, 1998; Garnero et al., 1994).

These short-term changes are inversely correlated with long-term changes in bone mineral density (Ross and Knowlton, 1998; Garnero et al., 1994).

A handful of studies have looked at DPD as a marker of bone mineralization in children. Initial efforts to establish reference concentrations have been reported (Lieuw-A-Fa et al., 1995; Rauch et al., 1994). Rauch et al. (1994) as well as Bollen and Eyre (1994) report that DPD concentrations are highly correlated with growth velocity in normal children. Mora et al. (1999) looked specifically at DPD in relation to Tanner stage of development and found DPD peaking at Tanner II then declining with a positive relationship to bone volume rather than to bone density per se. Van Coeverden et al. (2002) documented significantly positive correlations between DPD and BMC at several sites in a group of peri-pubertal Dutch children. In general, DPD concentrations will increase with age until puberty and then begin to decline to a sustained low adult level (Rauch et al., 1994; Acil et al., 1996).

Relationship of Body Composition to Bone

The relationship of body composition to bone growth in children has not been as extensively studied as it has been in adults. Based on descriptive studies primarily in adult populations, it is considered that body weight over all weight ranges and bone density are positively related, with body weight being one of the best predictors of BMD (Whiting, 2002). Ilich et al. (1998) found that both lean body mass and body fat predicted bone mass in premenarchal girls from eight and thirteen years age. Pietrobelli et al. (2002) found that lean mass was the best predictor of BMC in 133 children and adolescents studied. Other recent pediatric studies suggest that overweight children have lower than predicted bone mineral content (Goulding et al., 2000). It is important to note

that most of the studies of overweight children include children between four and twenty years of age. Children are often grouped by age with limited regard to pubertal status (Zamboni et al., 1988; Rauch et al., 1994; VandenBergh et al., 1995; Molgaard et al., 1997; Carter et al., 2001; Iuliano-Burns et al., 2003), which limits the ability to generalize the findings due to the significant role of pubertal hormones in bone growth as well as the variations in initiation and duration of puberty for children. Few studies look exclusively at prepubertal children (De Simone et al., 1995; Manzoni et al., 1996).

Over the past decade, a few studies have provided insight into BMD in children of varying body composition. DeSchepper et al. (1995) studied the BMD of the lumbar spine of 59 obese children (24 of whom were prepubertal). While they did not see differences in spine BMD, they did see trends in BMD when they compared children based on duration and severity of obesity once the data were corrected for age and pubertal status. Children who were obese for more than seven years had a higher mean BMD than children obese for less than four years. Children with severe obesity had higher BMDs than children with moderate or mild obesity. In this study, only the lumbar spine was measured. Though this site does include trabecular bone, which is more sensitive to metabolic change than cortical bone, this single site measurement is not ideal for following BMD since whole body scans are available. Manzoni et al. (1996) looked at total and regional bone mineral content in 65 obese, prepubertal Italian children as compared to 50 lean counterparts. Lean mass correlated best to BMC in this population. No difference in total body BMC was found when corrected for age, sex or body composition of these children. However they observed significantly different BMC in the arms, legs, and trunk, when comparing the obese and lean children, suggesting that

BMC must be considered relative to body size. Looking at the influence of weight, age, and puberty on bone size and BMC, Molgaard et al. (1997) found that skeletal size is determined by body size while BMD is determined by age and pubertal status as opposed to weight. These findings are important to note because BMC depends on both the size and density of bone.

In a rat study, Foldes et al. (1992), found significant differences in the bones of lean versus obese juvenile rats. The obese juvenile rats' bones were lighter and smaller than their lean counterparts. In an earlier human study with sixteen obese, prepubertal children, Zamboni et al. (1988) found that BMC and BMC/bone width at the radius were lower in obese children as compared to non-obese controls. Differences in diet and several hormone concentrations were also seen. De Simone et al. (1995) looked specifically at growth velocity and bone growth over a four-year period in 1250 obese children between four and eighteen years or age with careful attention to Tanner stage of development. Growth velocity and skeletal maturation was accelerated in prepubertal, obese children who had a less dramatic pubertal growth spurt as compared to normal children. The obese children made progress toward their adult size at a younger age. The differences seen in bone age and chronological age in these obese children raise the question of the appropriateness of the use of reference standards based solely on age in the assessment of bone growth as well as in the determination of the need for bone building nutrients.

Fischer et al. (2000) noted that obese children had larger bones hence more total body BMC than their lean counterparts. However, this study combined subjects, aged five to thirteen and at various Tanner stages, basing the comparative analysis solely on

whether the child was lean or obese. Hasanoglu et al. (2000) also found higher BMD in 37 obese children compared to non-obese children. However when they divided the children by Tanner staging instead of age, they found that BMD was predicted by Tanner stage rather than body composition. Goulding et al. (2000) also grouped obese children by chronological age but expressed BMC and bone area as a function of body weight. They reported a mismatch between body weight and bone growth in overweight children. Bone mass and bone area were lower relative to body weight, a finding supported in obese rats (Foldes et al., 1992). Goulding et al. (2001) followed fracture cases in a case-control study of boys between three and nineteen years of age and found that not only did boys with the highest BMI have lower BMD and BMC, they also had a significantly higher risk of distal forearm fracture, adding an orthopedic risk to the already acknowledged risks associated with poor bone mineralization.

Bone Growth in Special Populations

Investigation of special populations of children, having conditions such as inborn errors of metabolism or eating disorders, also lend insight into the factors influencing bone growth in children. Using wrist radiographs to look at differences in diet and bone status, Baer et al. (1997) compared ambulatory and nonambulatory children. A high percentage of the nonambulatory children had low Ca and Vitamin D intake and ambulatory children had significantly higher bone area. Tsukahara et al. (1992) observed reduced BMD, as measured by DXA, in a small population of Japanese children with a variety of chronic diseases. Both Allen et al. (1994) as well as Hillman et al. (1996) reported decreased bone mineralization in children with phenylketonuria (PKU) as determined by DXA. In the children with PKU studied by Allen et al. (1994), BMD was

lower than in the control subjects in spite of a higher intake of Ca for the PKU children. Chaturvedi et al. (1993) saw a significant reduction in BMD in malnourished Indian children. Significant deviations in BMD compared to normal, healthy children have been documented in children with bone diseases, renal disease, and endocrine disorders (Shore and Poznanski, 1996) as well as in premature infants (Steichen et al., 1988; Specker et al., 2001). Soyka et al., 1999, reported that anorectic, adolescent girls as young as twelve years of age had significantly decreased BMD. Poor mineral accrual persisted in these girls during the first year of their recovery (Soyka et al., 2002). Dibba et al. (2000) measured BMD and BMC in primarily pre-pubertal children in rural Gambia whose Ca intake was approximately 350 mg per day in a randomized, double-blind, placebo-control study. The Ca supplemented group consumed at least 700 mg Ca per day. Measures of bone mineralization increased in the supplemented group but the children remained lighter, shorter, and less mature than reference children of the same age. It is evident that optimal skeletal growth in this group of Gambian children needed more than calcium supplementation.

Influence of Physical Activity on Bone

Physical activity influences bone growth and turnover both mechanically and metabolically. Mechanical influence occurs when bone is exposed to force of varying magnitude and duration. Metabolic influence occurs when bone is exposed to a hormonal and nutritional milieu that is itself influenced by physical activity as well as many other factors. Bone responds to mechanical strain in an adaptive manner explained by the mechanostat theory (Bailey et al., 1996; Murphy and Carroll, 2003). This theory posits that there are four mechanical usage windows of varying and proportional effective strain

levels, each of which stimulates a different bone response, varying from remodeling to modeling to repair. Varying levels of physical activity change the strain on bone and result in bone responses that maximize bone strength during and beyond the growth years (Bailey et al., 1996; Murphy and Carroll, 2003). Animal models have confirmed that dynamic strain of abnormal distribution, in other words the on-again, off-again plus twisting and pounding action that reflect normal variations of movement, is more osteogenic than stress alone and more osteogenic than repetitive strain on bone (Murphy and Carroll, 2003).

Because bone responds to mechanical strain, the influence of physical activity on bone mineral accretion during childhood is of great interest. This response is confounded by the changing hormones of puberty. Therefore, it is important that data reflecting bone growth for prepubertal children must be separate from that of peripubertal or pubertal children. Slemenda et al. (1994) found that weight-bearing exercise is associated with increased BMD in prepubertal and peripubertal children, but the greatest influence of physical activity was in prepubertal children. Kroger et al. (1993) did not see a relationship between BMD and physical activity, monitored over a one year period in seven to twenty year old children, a subpopulation of which were prepubertal children. Bailey et al. (1999), following children for six years, a period which included some prepubertal years, documented much greater total body BMC for boys and girls who were physically active. Three exercise intervention studies of pre- and peri-pubertal children also showed a positive effect of weight bearing activity on bone growth (Morris, et al., 1997; Bradney et al., 1998; McKay et al., 2000).

Strong evidence that physical activity contributes to bone mineral accrual can be seen in the so-called unilateral control studies. Such studies compare limbs that receive different mechanical loading from physical activity. Any differences seen can be attributed to differences in the mechanical load because both limbs have the same genetic, metabolic, and nutritional influences. Though these studies did not look exclusively at prepubertal children, a consistent association between limb use and BMD prevails. The dominant arms of Little League baseball players (Watson, 1974), and of tennis and squash players (Haapasalo et al., 1994; Kannus et al., 1995) had greater BMD than the nondominant arm. Even comparison of dominant and nondominant arms of children involved in routine daily activities showed increased BMD in the dominant arm (Faulkner et al., 1993; Bailey et al., 1996).

Simple comparisons of active versus inactive populations support the positive association between physical activity and BMD. Iuliano-Burns et al. (2003) saw a 3% increase in BMC in their group of exercised versus non-exercised group of pre- and early-pubertal girls. The bone building effect of exercise was further enhanced by Ca supplementation. Specker and Binkley (2003) observed a positive effect of physical activity in children three to five years old only in children with high Ca intake (1354 gm/day versus the placebo of 940 gm/day). On the other hand, Molgaard et al. (2001) saw no significant association with physical activity level and BMC in their study of five to nineteen year old children, nor did VandenBergh et al. (1995) find a relationship between BMC and physical fitness in seven to eleven year old subjects. Zanker et al. (2003) compared two small groups of children seven to eight years old, comparing a group of gymnasts with non-gymnasts, finding significantly higher BMD in the female

gymnasts, but not in male gymnasts. Bailey et al. (1999) observed that in the year after attainment of peak BMC velocity, physically active boys and girls had 9% to 17% greater bone mass than their inactive peers.

Metabolic consequences of physical activity are suggested to be due, in part, to an endocrine effect. Physical exercise is known to stimulate growth hormone (GH) release from the pituitary with varying responses observed due to varying duration and intensity of physical activity (Murphy and Carroll, 2003). This subsequent stimulation of insulin-like growth factors from the liver further influences skeletal growth. In addition, assuming the development of exercise-induced metabolic acidosis, the subsequent decrease in serum Ca is sufficient to stimulate increased parathyroid hormone (PTH) secretion. Intermittent PTH administration has an anabolic effect on skeletal tissue (Murphy and Carroll, 2003).

Relationship of Diet to Bone

Calcium intake is known to be associated with development of peak bone mass (Weaver, 2000). During attainment of peak bone mass, intakes of less than 1 gm/d of calcium are associated with lower bone density (Weaver, 2000). When conditions related to children's Ca intakes have been examined, differences in the BMD relative to differences in Ca intake are reported. In reviewing the literature for the influence of calcium intake on bone mass, it is important to note that results are often reported for groups of children who are peri-pubertal instead of clearly delineating pubertal status. The onset of puberty initiates a hormonal milieu that dramatically changes bone growth, making pubertal status an important variable.

Over 99% of Ca in the body is found in teeth and bones which comprise up to 2 percent of adult body weight (FNB/IOM, 1997). In bone, Ca exists primarily as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and is absorbed across the intestinal mucosa by active transport (predominantly with lower intakes) as well as by passive diffusion (predominantly with higher intakes) (FNB/IOM, 1997). The active transport process is dependent on 1, 25 dihydroxy Vitamin D_3. Fractional Ca absorption varies inversely with dietary Ca intake and varies throughout the life span. In infancy, fractional absorption of Ca from an adequate diet is estimated at 60%, adjusting to 28% in pre-pubertal children, 34% in early puberty, 25% in late puberty and young adulthood and declining by 0.21% per year with aging (FNB/IOM, 1997). Data for children twelve months to nine years of age are limited. In addition to a handful of balance studies, the adequate intake (AI) was estimated by correlating Ca intake and with bone mineral. The AI for Ca for children four to eight years of age was set at 800 mg/day, but the need for more data on Ca balance, Ca accretion and bone mineralization specific to pre-pubertal children was acknowledged (FNB/IOM, 1997).

In a carefully controlled study with pre-pubertal subjects, Lee at al. (1993) reported a positive relationship between higher Ca intake and higher BMC in children of both genders who were followed from birth to five years of age. More specifically, Ca intake during the second year of life had the strongest correlation to BMC at the age of 5 years. Henderson and Hayes (1994) also saw increased BMD (measured by DXA) in children and adolescents of both genders with a milk allergy who consumed higher amounts of Ca. Ilich et al. (1998) reported that, in a large group of preadolescent females, BMD of the whole body and BMD of the radius shaft was positively influenced

by lean body mass, body fat, skeletal age and dietary Ca intake. Barr et al. (2001) estimated habitual Ca intake in nine to twelve year old girls and found that it was positively associated with total body BMC over a two year peripubertal period, the first measurement being premenstrual but not necessarily prepubertal. Calcium intake was found to explain 1.6% to 5.3% of the variance in a two-year change in BMC. In a longitudinal study, Fisher et al. (2004), showed that the Ca intake of girls between five and nine years of age positively predicted BMD at nine years of age. Chan (1991) established differences in BMD between peripubertal girls receiving more than 1000 mg of Ca/day compared to those receiving less than 1000 mg/day. He reported that BMD is associated with age, weight and height as well as Ca intake. In a retrospective study, Stracke et al. (1993) measured BMD in adult men and women and saw a relationship between low BMD and recollection of low intake of milk and milk products as a child and adolescent. Stallings et al. (1994) compared the BMD of nineteen children on a low lactose diet, matched in age to children from Chan's study, and found that a low lactose diet resulted in a low calcium intake and lower BMD scores. Cadogan et al. (1997), who looked at Ca intake as milk in schoolgirls, some of who were pre-pubertal and pooled relative to Tanner stages, found that for some of the analyses, the girls who received their Ca via milk had more significant gains in BMD. In a randomized, controlled trial of milk supplementation of male and female school children beginning when the children were seven to nine years of age, Fehily et al. (1992) followed the children for fourteen years. At 20 to 23 years of age, BMD and BMC tended to be higher in the milk-supplemented group with strong positive associations of BMC with adult body weight and sports activity during adolescence. Using National Health and Nutrition Examination Survey III

(NHANES III) data, Optowsky and Bilezikian (2003) looked for an association between childhood milk consumption and BMD in young adult women and postmenopausal women. Early milk consumption was positively related to BMD for white women but not for black women and was positively related to adult milk consumption in both groups.

In spite of evidence of the importance of Ca in attainment of bone mass in prepubertal children, the influence and long term benefit of supplemental Ca remains unclear. Johnston et al. (1992) using a co-twin study model, saw increased BMD in prepubertal children who received Ca supplements. A follow-up study on these children, however, indicated that this benefit was not sustained as the BMD of the supplemented and unsupplemented groups were similar six years later (Slemenda et al., 1997). Bonjour et al. (1997) supplemented prepubertal girls' diets with Ca for one year in a double blind, placebo controlled study to confirm that a Ca enriched diet increased bone mass accrual. A follow-up study showed this increased bone mass was sustained beyond the end of supplementation (Bonjour et al., 2001). On the other hand, Lee et al. (1996) saw the benefit of Ca supplementation disappear eighteen months after treatment was withdrawn from this group of Chinese children who had received 300 mg of elemental calcium for eighteen months. Overall, evidence related to the long-term effects of Ca supplementation in childhood indicates that gains are not generally sustained (Abrams, 2005) suggesting bone mineralization depends on a constellation of factors, only one of which is Ca intake.

In addition to Ca, several other dietary factors are considered of importance in adequate bone mineralization, including vitamin D, phosphorus (P), and protein.

Phosphorus is one of the most abundant elements on earth and has a close interrelationship with calcium in the human body. Most of the P in the body is found in bone where the constant Ca to P ratio of 2:1 is maintained (Anderson et al., 2006). Phosphorus homeostasis is maintained by PTH and 1,25 dihydroxy Vitamin D. Phosphorus is abundant in the U.S. diet, found in high levels in soft drinks as well as in meat and food additives (Anderson et al., 2006). The Estimated Average Requirement (EAR) for P in the four to eight year old child is 405 mg/day (FNB/IOM, 1997). The median intake is estimated at 420 mg with the upper 50% of children consuming 420 to 1100 mg (Anderson et al., 2006). These intake estimates are for P contributed to the diet by food sources with the exclusion of food additives, as the P in food additives is not considered in the nutrient databases used for dietary analysis. The concern with high P intake is that chronic high intake may impair the adaptive mechanism needed for adequate Ca absorption and optimal bone accretion through interruption of the 1, 25 dihydroxy Vitamin D and PTH homeostasis system (Anderson et al., 2006), resulting in an adverse effect on bone.

Vitamin D also plays a critical role in bone health. The active form of Vitamin D, 1, 25 dihydroxy Vitamin D_3, is considered to be a steroid hormone. Vitamin D is a regulator of Ca homeostasis and has roles in a wide variety of cell differentiation and proliferation processes (Norman and Henry, 2006). The current AI for Vitamin D for children is five micrograms/day (FNB/IOM, 1997). Measuring circulating concentrations of the precursor to active Vitamin D, serum 25-hydroxy Vitamin D_3, best assesses Vitamin D status (Molgaard and Michaelsen, 2003). Concentrations below 10 ng/mL are considered at risk for deficiency (Norman and Henry, 2006) though there is no general

consensus on how to define optimal Vitamin D status (Molgaard and Michaelsen, 2003). Fortified milk and sunlight are the primary sources of Vitamin D intake for U.S. children. Concern over Vitamin D status is increasing due to decreased exposure to sunlight and decreased consumption of fluid milk (Molgaard and Michaelsen, 2003; Norman and Henry, 2006).

Protein's relationship to bone health is paradoxical. Protein is considered to have a bone health promoting effect in the elderly, but at higher levels of intake, many consider protein to be deleterious to bone health (Ginty, 2003). The positive influences of protein on bone are related to observed decreases in fracture risk in the elderly as protein intake increases over a baseline deficient level (Ginty, 2003). The influence of protein on attainment of peak bone mass during growth is not as clear-cut. An adequate intake of protein is necessary for growth and insulin-like growth factor (IGF) is an acknowledged mediator of anabolic processes whose production is up regulated by dietary protein (Ginty, 2003). Calcium supplementation via one pint of milk daily increased plasma IGF-1 and bone mineralization in their group of peri-pubertal girls, hypothesizing an association between the increase in dietary protein and rise in IGF (Cadogan et al., 1997). Later, Ginty et al. (2004) saw an increase in IGF-I and bone mineral gain secondary to calcium supplementation rather than protein increase in their study, raising the possibility that IGF-I responds to Ca as well as protein. How the anabolic action of IGF-I is regulated is not clear, especially in the area of the interrelationship of level of protein and Ca intake and bone mineralization in prepubertal children.

Protein, especially that of animal origin, is hypothesized to have a negative relationship to bone due to the reduction in blood pH resulting from hepatic oxidation of the sulfur-containing amino acids methionine and cysteine to H_2SO_4 as well as ammonium ion production resulting in increased bone resorption and increased urinary Ca losses (Ginty, 2003; Massey, 2003). In addition to meat protein, dairy and many legume and grain products have high potential renal acid loads (Massey, 2003; New, 2003). The resulting chronic, diet-induced, low-grade, metabolic acidosis is thought to reduce bone mineral content over time as bone mineral is slowly used to buffer the net acid load of the diet (Frassetto et al., 1998; Remer et al., 2003). Recent work has established the likely protective relationship of fruit and vegetable and potassium (K) intake on indices of bone health in populations of children and adults (New, 2003). Fruits and vegetables contribute to a dietary alkali load (K, sodium, Ca, and Mg generate salts of bicarbonate and citrate), which contribute to neutralizing the pH-lowering effects of protein (Frassetto et al., 1998; Remer et al., 2003; Ginty, 2003). Because bone health is influenced by many other factors including, other nutrients, physical activity, and hormonal status, in addition to pH, the Food and Nutrition Board of the Institute of Medicine has concluded in the DRI for protein issued in 2002, relative to consideration of intake of protein on bone health, that "the potential for implications of high dietary protein are not sufficiently unambiguous at present to make recommendations" (FNB/IOM, 2002).

Other nutrients involved in bone health include magnesium (Mg), fluoride (F), and Vitamin K. Magnesium is a required cofactor for more than 300 enzymes and 50% to 60% of the Mg in the body resides in bone. Though Mg deficiency is considered a risk

factor for osteoporosis (Volpe, 2006), the role of Mg in attainment of peak bone mass in children remains to be determined. Fluoride's role in dental health is well known and 99 percent of the body's F is found in calcified tissues (FNB/IOM, 1997). Through the years there have been a few reports to link F to BMD (FNB/IOM, 1997) but, like Mg, the role of F in attainment of peak bone mass in children is unknown. The role of Vitamin K in blood coagulation is fairly well understood, but more recently Vitamin K is receiving more attention for its role in bone health (Bugel, 2003). In both of these roles, Vitamin K is essential for the post-translational conversion of glutamyl residues to γ-carboxyglutamyl (Gla) residues in a class of Vitamin K-dependent proteins, including prothrombin and osteocalcin. Initial intervention studies indicate that perhaps Vitamin K, when supplemented along with Vitamin D, may increase BMD in adults (Bugel, 2003). In pioneering work in children, Kalkwarf et al. (2004) measured Vitamin K intake and several markers of bone turnover in girls between three and sixteen years of age. They were able to establish an association between Vitamin K status and reduced bone turnover in these peri-pubertal children.

Background Information Summary

The literature on factors contributing to the attainment of peak bone mass in pre-pubertal children is growing but much remains to be explained relative to prevention of osteoporosis risk later in life. Clearly there are nutritional modifiers such as Ca and protein intake, body composition modifiers such as percent body fat and physical activity modifiers. It is very important to understand the role of these modifiable influences of bone growth independent of the influence of the hormones of puberty. Much of the literature to date groups pre-pubertal children with those who are in puberty for analysis,

confounding the conclusions and generalizations that can be made about bone growth in those populations. Investigations of pre-pubertal children cannot be based on age but must instead be based on Tanner staging to allow consideration of physiologic age. Diets need to be carefully considered using validated tools and trained assessors. In addition, physical activity and body composition must also be precisely and accurately measured. It is only in understanding the associations among all of these factors that an understanding of bone growth in children can evolve toward recommendations that will impact the incidence of osteoporosis in these children's later years.

Study Objective and Hypothesis

This study addresses the question: Is there an association between diet, biomarkers of bone turnover, physical activity, and body composition in their effect on bone mineralization in healthy, Caucasian children between the ages of five years and puberty?

Additional Study Hypotheses and Questions

Children who have a higher percent of lean tissue (relative to total body weight) will have higher measures of bone mineralization.

Children who are more physically active have higher measures of bone mineralization.

Children who consume 67% or more of the AI for calcium will have measures of bone mineralization within normal limits.

Identify how study variables compare to reference values for this population of children.

Describe the sources of bone building nutrients in this population of healthy children.

Is there an association between levels of OC and DPD and BMD in prepubertal children?

Identify values for serum OC and urinary DPD in a group of healthy children.

The levels of OC, considered to reflect bone formation, will increase as bone mineral status decreases.

The levels of DPD, considered a marker of bone resorption, will increase as bone mineralization increases.

CHAPTER TWO

CHAPTER TWO

METHODS FOR THE HEALTHY KIDS NUTRITION STUDY

Study Summary

The Healthy Kids Nutrition Study (HKNS) was an observational study of a convenience sampling of children between the ages of five years and puberty. Bone mineral density and body composition of the children was determined by dual energy x-ray absorptiometry (DXA). Diets of the children were analyzed using a food frequency questionnaire (FFQ) and a usual, 24-hour diet recall administered by a registered dietitian. Two biomarkers of bone metabolism, serum osteocalcin (OC) and urinary deoxypyridinoline (DPD) were determined. Serum Vitamin D was also determined. Heights and weights of the children were collected. Physical activity for three days was measured using accelerometer technology. This study measured modifiable determinants of bone mineralization to examine associations among those variables and bone mineral density and bone mineral content in prepubertal children.

Project Design

Subjects and subject recruitment

This study was approved by full review of the University Committee on Research Involving Human Subjects (UCRIHS) at Michigan State University (MSU), was assigned the Institution Review Board number 03-1019 (Appendix 1; Appendix 2), and was renewed annually. Subjects were recruited from the greater Lansing, MI, area through word of mouth and fliers (Appendix 3). The recruitment target was 50 subjects who met the study inclusion criteria; 52 subjects were enrolled. Subjects were generally well, free-living children with no chronic disease conditions or birth defects other than possible

allergies or food sensitivities. Pubertal status was determined by parent/child interview to ascertain Tanner Stage I status. Children with minor conditions, such as ADHD or mild allergies, which have no direct relationship to bone growth, were included after review by the study physician (R.T. Scott, DO). Due to the pre-pubertal age of these subjects and the relatively small sample size, all races and both genders were recruited. The following criteria were used for subject selection. Inclusion criteria were well children, both genders, weight by NCHS standards of $\geq 5^{th}$ percentile BMI, age five years to puberty (puberty being defined by >Tanner I) at the beginning of the study, at least 37 weeks gestational age at birth, and all races. Exclusion criteria were any chronic disease or birth defect, condition or medication with potential impact on bone growth, chronic steroid use, pubertal stage of Tanner Stage 2 to 5, weight by NCHS standards of $\leq 5^{th}$ percentile BMI. Data were collected between February 2005 and June 2006.

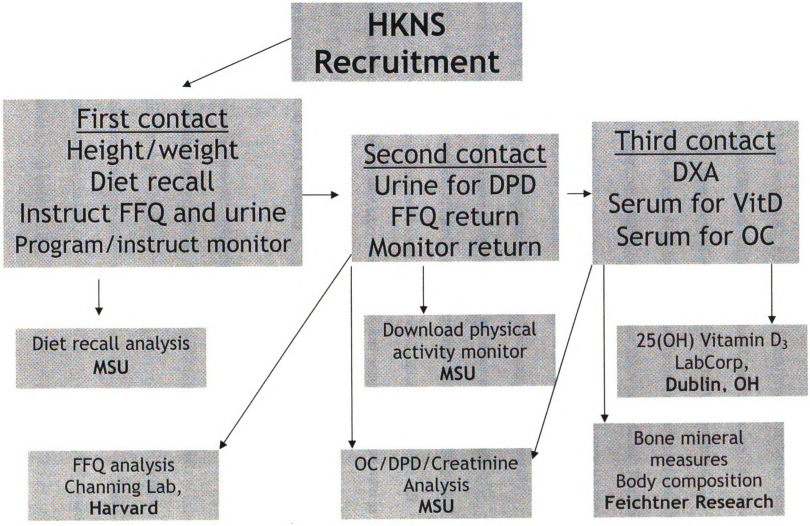
Client confidentiality and HIPAA compliance were considered (Appendix 4). Subjects were not informed of the exact nutrient or food group being investigated; rather the consent stressed investigation of overall nutrition and bone growth. A health questionnaire (Healthy Kids Nutrition Study Health Questionnaire) was administered to confirm each child's qualification to participate and to provide a general family history relative to bone health (Appendix 5). The health questionnaire covered inclusion and exclusion criteria as well as family history of osteoporosis and obesity, and the child's history of any illness or infection, which may have influenced growth. Once enrolled in the study, random numeric coding of all subjects' records and samples assured confidentiality.

Study procedures were scheduled at the caregiver's convenience, including evening and weekend contacts as necessary. Over a period of two to five weeks, subjects and parents came to three appointments for data collection, a process outlined in Figure 2.1 (with additional detail in Appendix 8). Parking vouchers were provided and study personnel assisted with pick up and delivery of study supplies and samples as needed. The total incentive for participation was $100.00, provided as a gift card for Target or Meijer. Each caregiver will be provided with a report of the study results for their child, per the study debriefing protocol (Appendix 6). Research personnel remain available for questions or concerns post-participation. The children's caregivers were instructed to report any abnormal findings, i.e. BMD Z-scores below –0.2, to their provider of choice for appropriate medical follow-up.

Study Staffing

A team of researchers and staff with the specific expertise required to complete all aspects of this study collaborated, developed and completed this study. The project manager, a Registered Dietitian, conducted all subject interviews and instructed the subjects and parents on study protocol and procedures. She obtained anthropometrics, conducted the diet recall interview, and provided instruction on completing the FFQ, wearing the activity monitor, and obtaining the first morning urine sample. She coded the FFQ for analysis and entered the 24-hour recall dietary information for analysis. Entries were double checked a second time by either a research assistant or the project manager. The nutrition database program within the MyPyramid Tracker (Center for Nutrition Policy and Promotion, 2005) was used for diet analysis of the recalls. The FFQ was sent to the Harvard Channing Laboratory for scanning. A phlebotomist who had

Figure 2.1
Healthy Kids Nutrition Study Protocol



experience in drawing blood from children collected one five ml blood sample from each subject for serum Vitamin D and serum OC. For Vitamin D analysis, the samples were sent to the Regional Laboratory of Ingham Regional Medical Center, Lansing, MI. The laboratory of Dr. Michael Orth, Department of Animal Science, MSU, conducted biomarker assays, OC and DPD. A radiology technician at Fiechtner Research under the supervision of Justus J. Fiechtner, MD, MPH, conducted the DXA procedures. The US Food and Drug Administration (FDA) requires a physician order for DXA measurement, thus a standing order from the study physician was placed on file in each subject's data file (Appendix 7). The study was coordinated out of the Michigan State University Department of Food Science and Human Nutrition with an office in the College of Osteopathic Medicine's Department of Family and Community Medicine. The project manager and two research assistants conducted data entry for statistical analysis. All entries were cross-checked by the data entry team. Statistical consultation was used as described in the data analysis section.

Measurement and Analysis of Variables

Diet analysis

Individual diet information was collected as a one day, usual diet recall and through a food frequency questionnaire (FFQ), the Youth and Adolescent Food Frequency Questionnaire developed by Harvard Channing Laboratory. The nutrient content of diets of the children was estimated from both the recall and the FFQ. The nutrition database program within the CNPP/USDA/DHHS MyPyramid Tracker, based on the USDA food database, was used for analysis of the diet recalls (CNPP, 2005). All

records, if submitted incompletely, received individual follow-up by study personnel with the parent or guardian.

The diet assessment tools and analysis program provide estimates for over sixteen nutrients. The recall analysis also estimated intake according to the 2005 US Dietary Guideline's food groups (CNPP, 2005). The FFQ chosen has been validated for use in estimating Ca intakes of young children (Stein et al., 1992; Rockett et al., 1997; Rockett and Colditz, 1997). Its validity in determining density of other nutrients in the diet is not as strong. Combining the FFQ with a careful diet recall improves the results to give a better estimate of intake. The food frequency used, the Harvard Semiquantitative Youth and Adolescent Food Frequency Questionnaire, was validated at Harvard University, School of Public Health, Boston, MA, USA (Thompson and Byers, 1994; Rockett et al., 1997). This food frequency can be customized via individual coding by the investigator to include specific foods of interest. Due to the prevalence of consumption of calcium-fortified juice, this single food was added to the FFQ. Supplement use was measured in the FFQ but not in the diet recall. The FFQ asks whether or not vitamins are used, how often, and for how long.

In order to explore the potential relationship of dietary protein on measures of bone mineralization, net endogenous acid production of the subjects' diets was estimated using the renal net acid excretion (RNAE) equation developed by Frassetto et al. (1998). This method of estimation uses dietary protein intake and dietary potassium intake such that RNAE = 62 (gm protein / mEq K) –17.9.

Anthropometrics

Height was measured to the nearest centimeter with a stadiometer (Invicta Plastics, Oadby, Leicester, England). Weight was determined to the nearest 0.1 kg on a digital scale (Seca, Model 882, Hanover, Maryland, USA). Standard pediatric measurement protocols to assure precision and accuracy were employed (Gibson, 1990; Lee and Nieman, 1996) and followed the National Center for Health Statistics guidelines for standard measurement (NCHS, 1996).

Measurement of Physical Activity

The Actical accelerometer (MiniMitter, Bend, OR) was used to measure physical activity. Actical is a compact, battery-operated physical activity monitor with physical characteristics similar to a small wristwatch. The monitor consists of the activity monitor itself and a waist or wrist/ankle band. For this study, the accelerometer was held at the waist via a belt, available in 2 sizes and adjustable with Velcro-type closures. Actical utilizes a piezoelectric accelerometer to monitor the occurrence and degree of motion. The sensor is an omni directional accelerometer, resulting in sensitivity to motion in all directions (MiniMitter Corp, 2003). It has been validated for use in estimating energy expenditure in young children (Pfeiffer et al., 2006). The sensor and associated signal processing considers both the degree and intensity of motion to produce an electrical current that varies in magnitude. An increased degree of speed and motion produces an increase in voltage. Actical stores this information as activity counts. The sampling frequency was 32 Hz and an epoch measurement of 15 seconds was chosen. Subjects wore the monitor on the waist continually for three, consecutive days (one weekend day and two weekdays).

The three-day record of physical activity data from the monitor record was downloaded using the Actical Reader, companion hardware for use with the monitor, providing database-ready statistics on physical activity. The data were collected and reported as activity counts, energy expenditure, and duration of expenditure. The height and weight of each subject was uploaded to the Actical prior to its wearing. From this information, basal energy expenditure (BEE), using the Harris and Benedict equation (Harris and Benedict, 1919), was estimated by the Actical program so that daily total energy expenditure (DTEE) from physical activity was reported as kcals above estimated BEE. In addition, a brief physical activity questionnaire was completed that included two questions about physical activity from the CDC Youth Risk Behavior Surveillance Survey (CDC-MMWR. 2006) to allow comparison to national data. These data were collected for future use with the potential to validate the survey tool.

Biomarker Assays

Serum intact osteocalcin (OC) was measured with the Metra™ Osteocalcin assay (Quidel Corporation, Special Products Group, Santa Clara, CA, USA), an enzyme immunoassay in a microtiter stripwell format utilizing a murine monoclonal anti-OC antibody (Gomez et al., 1994). The OC in the sample competes for antibody binding sites with OC coated on the stripwell. A rabbit anti-mouse IgG antibody conjugated to alkaline phosphatase is added and the reaction is detected with the substrate, p-nitrophenyl phosphate. Color developed during the incubation of captured enzyme conjugate and substrate is measured at 405 nm in a microtiter plate reader. The OC values of unknown specimens are calculated from a calibration curve fit with a 4-

parameter logistic equation. Values are expressed in ng/mL. Twenty-five μL of serum per well (assayed in duplicate) were used for determination of OC.

The detection limit of the OC assay was 0.45 ng/mL. Samples up to 32 ng/mL could be read without dilution. Intra-assay precision coefficient of variations (CVs) were 4.8-10.0%. Interassay precision CVs were 4.8-9.8%. The manufacturer has established reference intervals for healthy men (3.4-9.1 ng/mL) and women (3.7-10.0 ng/mL). No reference standards were provided by the manufacturer for children. For this assay, a five ml venous blood sample was drawn via antiseptic techniques with universal biohazard precautions. Immediately after being drawn, the blood was allowed to clot. The serum was harvested after the sample was centrifuged for 20 minutes at 1500xG. All samples were immediately frozen at -20°C and then transferred to -80°C storage within 4 hours of being drawn. All stored samples were analyzed in a single batch within sixteen months of being drawn.

Urinary free deoxypyridinoline (DPD) was measured with the Metra™ DPD assay (Quidel Corporation). The assay is highly specific for the DPD molecule and does not cross-react significantly with pyridinoline, other collagen crosslinks, or collagen peptides (Robins et al., 1994). Metra DPD is a competitive enzyme immunoassay in a micro assay stripwell format utilizing a monoclonal anti-DPD antibody coated on the strip to capture DPD. The DPD in the sample competes with conjugated DPD-alkaline phosphatase for the antibody and the reaction is detected with the substrate, p-nitrophenyl phosphate. Color developed during the incubation of captured enzyme conjugate and substrate is measured at 405 nm in a 96-well micro assay plate reader. The DPD values of unknown specimens are calculated from a calibration curve fit with a 4-parameter

logistic equation. The DPD values are expressed in nmol/L. Urine (fifty μL) was diluted 1:10 in Assay Buffer per well (assayed in duplicate) as required for determination of DPD.

The detection limit of this assay is 1.1 nmol/L. Samples up to 300 nmol/L could be read without additional dilution. Intra-assay precision CVs were 4.3-8.4%. Interassay precision CVs are 3.1-4.8%. Due to the diurnal variation of DPD excretion, the reference and study samples were determined from first morning urine samples collected prior to 10 AM. Parents and subjects were instructed on the urine sample collection and given sterile supplies. The DPD concentrations were corrected for differences in urine concentration and output by dividing by creatinine as measured in each urine sample (Quidel Corporation). The final creatinine-corrected DPD results were expressed as nmol/mmol. The manufacturer has established reference intervals for healthy men (2.3-5.4 nmol/mmol) and women (3.0-7.4 nmol/mmol); none are provided for children.

First-morning urine samples were collected without preservative. Parents were instructed to put the urine specimen tubes in the home freezer until transport to the project office, after which the samples were transferred to -80°C storage and stored for less than 12 months. The DPD is stable for at least 21 months when stored at ≤-40°C. Modeling studies suggest DPD will be stable for at least 20 years when stored at -20°C (Gerrits et al., 1995). Samples may be frozen and thawed up to 5 times. Prolonged exposure to light, especially sunlight, should be avoided, but routine processing is not affected by normal, artificial laboratory lighting. All samples were analyzed within sixteen months of being stored.

Vitamin D measurement

Serum 25(OH) Vitamin D_3 was measured one time during the study, concurrent with the DXA measurement. The 5 ml sample was drawn in a nonfasting state, clotted, and then spun for serum. The serum was stored at -20°C and transported to the Regional Laboratory of Ingham Regional Medical Center (IRMC), Lansing, MI, within 24 hours of being drawn. Samples were stored at IRMC at $\leq$-20°C, batched, then sent to the accredited laboratory, LabCorp, Dublin, OH, for analysis of 25(OH) Vitamin D_3 via an immunochemiluminometric (ICMA) assay (personal communication, LabCorps Analytical Laboratories, Dublin, OH). All samples were analyzed within four months of being drawn using standardized storage and handling protocols. The reference standards provided by the analytical lab vary with age and season as 25(OH) Vitamin D_3 exhibits seasonal variation related to sun exposure although recommendations for optimal levels in children are not season-specific (Molgaard and Michaelson, 2003; Norman and Henry, 2006).

Dual Energy X-ray Absorptiometry

Whole and regional body BMC, BMD and body composition of lean mass and fat mass were determined by DXA. Whole body measures were taken using a GE LUNAR Prodigy machine at Fiechtner Research with pediatric software specific to the machine (software version 6.81; General Electric Lunar Corporation, Madison, WI). Measurement protocols specific to the GE-LUNAR Prodigy densitometer were employed, including daily calibration with a standard calibration block and calibration tri-weekly with a body phantom. The CV of this machine is reported to be 0.15% (personal communication, Fiechtner Research). The LUNAR densitometer is FDA

approved for investigational pediatric use in the USA and a physician's order is required for use (Appendix 7).

Bone mineral content as measured by DXA is the amount of mineral per length of the bone scanned, expressed in g/cm. Dividing the BMC by bone area gives bone mineral density in g/cm^2. Both measures are projectional area densities as opposed to true volumetric measures. Projectional measures, as opposed to cross-sectional measures, allow for the assessment of both trabecular as well as cortical components of the bone being measured. True volume measures of bone density are only measurable by using quantitative computed tomography (QCT), a technology not currently available to the investigators and which exposes children to significantly more radiation than does DXA (Shore and Poznanski, 1996). Because of growth, it is important to measure more than one site of the skeleton via DXA to get the best overall assessment of bone mineralization in children; thus a whole body scan was conducted. Results can be expressed as BMC, bone area and BMD. The GE Lunar Prodigy machine measured body composition (fat and lean) at the same time BMD was scanned, hence Lunar's definition of this measure as a "total body scan." The full body scan took approximately two minutes and exposed each child to 0.04 mRem of radiation, a very low dose exposure. In comparison, a television emits 10.0 mRem of radiation over the course of a year while a typical air flight in North America exposes the passenger to a 40.0 mRem dose of naturally occurring radiation. A standard medical x-ray carries a 40.0 mRem dose (J. Downs, GE Lunar, personal communication, 2004; Njeh et al., 1997).

Sample Size and Power Calculation

Reference data for the bone density of healthy, age-matched controls is included in the LUNAR pediatric software. These data are age and gender specific though at many age groups, data for prepubertal and pubertal children are pooled. The primary research question involves examination of the relationship of modifiable influences of bone growth within the study population rather than in comparison to the reference data, though the reference data is analogous to information that would be obtained by a control group of subjects. The BMD reference values from the GE Lunar DXA program were used in power calculations. According to Lunar's data (Wacker and Barden, unpublished monograph, 2001), the reference data are based on 1494 children between five and 19 years of age, only 471 of whom were between five and ten years of age. The average standard deviation (SD) for total body BMD in both males and females between the ages of 5 and 19 years is reported by Lunar to be 0.07 (Table 2.1). The SD within each age group is not reported. Given these data, the mean BMD for the seven year old male was used as the mean BMD of the population and a SD of 0.07 was used to calculate power, resulting in a minimum sample size of 16 to detect differences at a 95% Confidence interval. In consideration of realistic expectations of subject recruitment and retention over a reasonable time period as well as the limitations of the information available to estimate power, 52 subjects were recruited, allowing for subgroups by age or gender for post hoc categorization. Differences in measures of bone mineralization have been seen in studies of children with as few as fourteen to sixteen subjects in each group (Zamboni et al., 1988, Volek et al., 2003). The design of this study allowed for addition of subjects at any time during the study, addition of subjects after completion of this initial study,

addition of more study groups, and further follow-up of these same children over time. In the end, 52 subjects went though the study protocol with 51 subjects providing blood samples and all 52 participating in all other aspects of the study.

Data Analysis

Descriptive statistics were employed to describe the study population with data being presented as means ± SDs. The primary dependent variables are BMD, BMC, the Lunar DXA reported Z-score and BMC expressed relative to height in centimeters. The independent variables are select components of the dietary intake closely associated with bone growth as assessed by recall and by FFQ, diet as assessed by the USDA Dietary Guidelines, serum 25(OH) Vitamin D, serum OC, urinary DPD, gender, age, body composition expressed as percent fat and lean tissue, and physical activity expressed as energy expenditure. A two-tailed t-test was employed to determine significant differences between gender and age categories in all variables under study. Diets were expressed as nutrition adequacy ratios (NARs) for bone building nutrients. Relationships between variables were explored with 2-tailed Pearson's correlations. Multiple variable regression with stepwise enter method was used to determine variables that may be significant predictors of BMD and BMC. Data were also grouped to allow further analyses by cross tabulation. Data was analyzed using SPSS, version 13, Base System (SPSS, Inc., Chicago, IL, USA). The significance level for all tests was established as $p<0.05$ and all tests are bi-directional.

Table 2.1

Comparison of BMD and BMC averages by age group and gender, and average of within group standard deviation
Lunar[1] and HKNS[2] Data

Age by year	Females						Males					
	HKNS			Lunar			HKNS			Lunar		
	N	BMD (g/cm2)	BMC (g)	N	BMD (g/cm2)	BMC (g)	N	BMD (g/cm2)	BMC (g)	N	BMD (g/cm2)	BMC (g)
Five years old	4	0.816	670.75	17	0.781	701.00	4	0.810	737.75	17	0.772	677.00
Six years old	7	0.808	859.71	20	0.801	779.00	7	0.816	761.14	30	0.806	807.00
Seven years old	3	0.807	861.67	42	0.818	881.00	5	0.879	985.00	33	0.828	920.00
Eight years old	3	0.799	840.33	63	0.835	980.00	8	0.868	1113.38	58	0.845	1106.00
Nine years old	3	0.913	1261.33	47	0.854	1043.00	2	0.948	1327.50	39	0.868	1145.00
Ten years old	4	0.898	1152.75	52	0.880	1225.00	0			43	0.900	1357.00
Eleven years old	1	0.866	1119.00	62	0.910	1486.00	1	0.904	1158.00	56	0.923	1470.00
Total	25			303			27			276		
Average Std Dev		0.05	130.69		0.07	271.00		0.04	146.32		0.07	318.00

[1]Lunar: Mean values provided as reference data by GE Lunar (Wacker W, Barden HS. Pediatric reference data for male and female total body and spine BMD and BMC. Presented at the International Society of Clinical Densitometry: Dallas, TX; March 2001. (personal communication))
[2]HKNS; Mean values of subjects in the Healthy Kids Nutrition Study

CHAPTER THREE

ABSTRACT

BONE MINERALIZATION IN PREPUBERTAL CHILDREN: ASSOCIATION OF DIET, BODY COMPOSITION, AND PHYSICAL ACTIVITY

by

Marcia Kelly Scott

Background: Osteoporosis is predicted to become a disease of epidemic proportions within the next several decades. Attainment of higher bone mineral content during the first two decades of life decreases the risk of developing osteoporotic fractures later in life. Prevention of osteoporosis begins in childhood with attainment of peak bone mass.

Objective: To further clarify associations among determinants of bone growth toward attainment of peak bone mass including body composition, diet composition, and physical activity with bone mineral density (BMD) and bone mineral content (BMC) in prepubertal children, mean age of 7.6 years.

Design: BMD, BMC, and body fat were measured via DEXA in 52 subjects. Daily total energy expenditure (DTEE) was measured via accelerometer. Diet was assessed with a usual, one-day recall interview and a food frequency questionnaire. Relationships between variables were explored with two-tailed t-tests, two-tailed Pearson's correlations and stepwise multiple regression analysis ($p \leq 0.05$).

Results: In these children who were all within the normal, healthy range of body weight, bone mineralization correlates positively with percent body fat ($r=0.535$ for BMD and $r=0.646$ for BMC) and with DTEE ($r=0.460$ for BMD and $r=0.591$ for BMC). Protein intake had significant negative relationships to both BMD, $r=-0.508$, and BMC, $r=-0.564$. Energy intake also had significant negative relationships to both BMD, $r=-0.510$, and BMC, $r=-0.578$. A significant negative association was found between Ca intake and

BMD ($r=-0.277$, $p \leq 0.05$). In the final regression models, energy intake and P intake by height were significant negative predictors of BMD and BMC while DTEE, percent body fat, Ca intake by height, Mg intake, and fruit and vegetable intake were significant positive predictors. The relationships of these variables explain 58.8% of the variability in BMD and 72.9% of the variability in BMC.

Conclusion: This study supports the interrelationships of diet, physical activity, and body composition in contributing to development of peak bone mass. Increased bone mineralization was associated with higher body fat, more physical activity, and higher fruit and vegetable intake as well as moderate energy, Ca, Mg and P intake, supporting consideration of these variables in assessing bone health in children. These associations suggest that children at risk of poor bone mineralization can be identified in a noninvasive manner such that early intervention and prevention can be addressed at a young age.

Chapter Three formatting is consistent with the guidelines for authors for the American Journal of Clinical Nutrition.

CHAPTER THREE

BONE MINERALIZATION IN PREPUBERTAL CHILDREN: ASSOCIATION OF DIET, BODY COMPOSITION AND PHYSICAL ACTIVITY

INTRODUCTION

Osteoporosis, recognized as one of the major public health problems facing aging individuals of both genders, worldwide, is predicted to become a disease of epidemic proportions within the next several decades (Riggs and Melton, 1995; WHO, 2003). Broadly defined as diminished bone mass and reduced bone mineral density (BMD) at the level of 2.5 standard deviations below the referent BMD of young adults (Riggs and Melton, 1995; USDHHS, 2000; WHO, 2003), osteoporosis presents clinically as fractures, both traumatic and nontraumatic in nature, resulting in significant morbidity, mortality, and health care costs (WHO, 2003). The two most important ways to reduce risk of osteoporosis are to attain peak bone mass in early life and to have a low rate of bone loss in later life; hence, approaches to interventions for this disease target methods of prevention and abatement of osteoporosis not just in adulthood but also during infancy, childhood, and adolescence. Though osteoporosis is generally considered a geriatric disease, osteoporosis may actually be a pediatric disease with geriatric consequences (Faulkner and Bailey, 2007).

Many believe that attainment of higher bone mineral content during the first two decades of life decreases the risk of developing fractures later in life (Heaney et al., 2000; Faulkner and Bailey, 2007). Bone mass tends to track throughout life, meaning that infants, children and adolescents that have higher bone mass tend to be the adults who have high bone mass (Ferretti et al., 1998; Heaney et al, 2000). This association

introduces the possibility that "at risk" individuals may be identified early in life. Peak bone mass has genetic, hormonal, nutritional, and behavioral determinants (Soyka et al., 2000). Some of these determinants are modifiable and interventions targeted at individuals at risk should focus on these modifiable determinants of bone health.

Generalizing the findings of bone growth studies in children is challenging due to the variations in initiation and duration of puberty for children. Studies of bone growth in early childhood should consider pubertal status as well as modifiable influences on bone growth including body composition, physical activity, and diet due to the potential interaction of all of these determinants of bone health. Body composition may influence bone mineralization, especially in overweight children, who represent an increasing proportion of the pediatric population (Goulding et al., 2000; Goulding et al., 2001). Children with higher levels of physical activity have higher measures of bone mineral mineralization (Slemeda et al., 1994; Bailey et al., 1999; Iuliano-Burns et al., 2003; Specker and Binkley, 2003; Zanker et al., 2003). Calcium intake is acknowledged to be associated with development of peak bone mass (Weaver, 2000). During attainment of peak bone mass, intakes of less than 1 g/d of Ca are associated with lower bone density (Weaver, 2000). In spite of the importance of Ca in bone mineralization, evidence related to the long-term effects of Ca supplementation in childhood suggests that bone density gains associated with supplementation are not generally sustained (Abrams, 2005). Other diet components including phosphorus, Vitamin D and protein play important roles in bone mineralization (Ginty, 2003; Anderson et al., 2006; Norman and Henry, 2006). High protein intake is hypothesized to have a negative impact in bone mineralization (Frassetto et al., 1998). Thus, dietary intake of several key bone-building

nutrients should be considered when investigating the interrelationships of determinants of bone mineralization in early childhood.

This study, the Healthy Kids Nutrition Study (HKNS), was undertaken to further clarify associations among diet, physical activity, and body composition with bone growth and mineralization in a group of children between five-years of age and puberty. The HKNS is unique in its simultaneous consideration of key variables of bone mineralization. Understanding these associations early in life may contribute to the ability to identify children who are "at-risk" in terms of progress toward attaining peak bone mass. Early intervention could decrease their risk of developing osteoporosis later in life.

SUBJECTS AND METHODS

Subjects

Fifty-two subjects from the greater Lansing, MI, area were enrolled in the study. The children were between five-years of age and puberty (<Tanner 1), > the 5th percentile BMI by NCHS standards, and ≥ 37 weeks gestational age at birth. They were free living, included both genders and were generally well, without any chronic disease conditions or birth defects other than possible allergies or food sensitivities. Children with minor conditions that have no direct relationship to bone growth were included after review by the study physician. Pubertal status was determined by a thorough parent/child interview to ascertain Tanner Stage I status. Although recruitment by race was not targeted, all children were at least 50% Caucasian. The study was approved by full review of the University Committee on Research Involving Human Subjects at Michigan State University and met confidentiality and HIPAA requirements.

Diet analysis

Diet information was collected in two ways, with a diet recall and with a food frequency questionnaire (FFQ). An experienced Registered Dietitian obtained a usual, one-day diet recall via a consensus interview with a parent and the subject. The parent, along with the child, completed the Youth and Adolescent Food Frequency Questionnaire developed by Harvard Channing Laboratory, (Harvard University, School of Public Health, Boston, MA, US). Ca-fortified juice was added to this FFQ via individual coding by the investigator. The nutrient content of diets of the children was assessed by both the recall and the FFQ, providing estimates of over sixteen nutrients. The recall analysis also estimated intake according to the Dietary Guideline's food groups (CNPP, 2005). The same Registered Dietitian conducted the diet interviews, coded the FFQ for analysis, and entered the diet recall information for analysis using the nutrition database program within the MyPyramid Tracker, based on the USDA food database (CNPP, 2005). The coded FFQ was sent to Harvard Channing Laboratory for analysis. All records, if submitted incompletely, received individual follow-up by study personnel. Net endogenous acid production of the subjects' diets was estimated using the renal net acid excretion (RNAE) equation developed by Frassetto et al. (1998).

Anthropometrics

Height was measured to the nearest centimeter with a stadiometer (Invicta Plastics, Oadby, Leicester, England). Weight was determined to the nearest 0.1 kg on a digital scale (Seca, Model 882, Hanover, Maryland, USA). Standard pediatric measurement protocols to assure precision and accuracy were employed (Gibson, 1990;

Lee and Nieman, 1996) and followed the National Center for Health Statistics guidelines for standard measurement (NCHS, 1996).

Measurement of Physical Activity

The Actical accelerometer (MiniMitter, Bend, OR) was used to measure physical activity. Actical, a compact, battery-operated physical activity monitor with physical characteristics similar to a small wristwatch, was worn at the waist on a neoprene belt. This accelerometer has been validated for use in estimating energy expenditure in young children (Pfeiffer et al., 2006). The sampling frequency was 32 Hz and an epoch measurement of 15 seconds was chosen. Subjects wore the monitor continuously for three consecutive days (one weekend day and two weekdays). The Actical program provided the estimate of physical activity as kcals daily total energy expenditure (DTEE) above basal energy expenditure (BEE).

Serum Vitamin D measurement

Because of the critical role of Vitamin D in bone mineralization and Ca metabolism, serum Vitamin D was assessed in all subjects. A phlebotomist with experience in drawing blood from children collected one, nonfasting, five-ml blood sample for serum 25(OH) Vitamin D_3. This single serum 25(OH) Vitamin D_3 sample per subject was measured at the accredited analytical laboratory, LabCorp, (Dublin, OH) via an immunochemiluminometric assay (ICMA). The reference standards provided by the laboratory vary with age and season due to seasonal variability of sun exposure (range 7.0 ng/mL to 48.0 ng/mL).

DXA

Whole body measures were taken using a GE LUNAR Prodigy machine at Fiechtner Research (Lansing, MI) with pediatric software specific to the machine (software version 6.81; General Electric Lunar Corporation, Madison, WI). Measurement protocols specific to the GE-LUNAR Prodigy densitometer were employed, including daily calibration with a standard calibration block and calibration tri-weekly with a body phantom. The CV of this machine is reported to be < 1.0% (personal communication, Fiechtner Research). Measures obtained included body composition (kg fat mass and kg lean mass), BMD (gm/cm^2) and BMC (gm). The full body scan took approximately two minutes and exposed each child to 0.04 mRem of radiation. Motion artifacts were not a problem in this population.

Statistical Analysis

The BMD reference values from GE Lunar DXA program were used in power calculations (Wacker and Barden, 2001). The mean BMD for the seven-year-old male was used as the mean BMD of the population and a SD of 0.07 was used to calculate power. Recruitment of 52 subjects assured the sample size necessary to detect differences at a 95% Confidence Interval.

Descriptive statistics were employed to describe the study population with values reported as means ± SDs. The primary dependent variables are BMD, BMC, and the Lunar DXA reported Z-score. The independent variables are intake of nutrients in the diet closely associated with bone growth as assessed by recall and by FFQ, diet as assessed by the USDA Dietary Guidelines, serum 25(OH) Vitamin D_3, measures of body

composition, and physical activity expressed as DTEE above BEE. A two-tailed t-test was employed to determine significant differences between genders in all variables. Relationships between variables were explored with 2-tailed Pearson's correlations. Multiple regressions with stepwise variable entry were used to determine variables that may be significant predictors of BMD and BMC. Data were analyzed using SPSS, version 13, Base System (SPSS, Inc., Chicago, IL, USA). The significance level for all tests was established as $p<0.05$ and all tests are bi-directional.

RESULTS

Descriptive characteristics of the study subjects are summarized by age in Table 3.1 by gender and in total. The mean age of the subjects was 7.6 years (range 5.1 – 11.9). Twenty-seven of the subjects were male; twenty-five were female. BMIs ranged from 13.7 to 23.9 with a mean BMI of 16.8. Z-scores for the BMD by Lunar DXA ranged from negative 1.2 to positive 1.8. The mean Z-score was 0.14 with 30.8 percent of these subjects having Z-scores at or below negative 0.2. There were no gender differences in mean fat mass, lean mass, BMC or BMD (t-test, $p \leq 0.05$). Mean serum 25(OH) Vitamin D_3 was 30.6 ng/mL ± 10.2 (range 10.8 – 62.3). No individual had a Vitamin D_3 outside of the normal range.

Diet

Dietary intakes according to the 2005 US Dietary Guidelines are summarized in Table 3.2 by gender and for the total. Mean daily grain intake was 8.3 ± 3.7-ounce equivalents. Mean milk intake was 3.4 ± 1.8-cup equivalents. Mean meat and bean intake was 3.7 ± 1.9-ounce equivalents. Fruit and vegetable intakes were combined for analysis. Combined mean intake of fruits and vegetables was 2.2 ± 1.3 cup-equivalents.

Table 3.1

Physical characteristics, measures of bone mineralization, energy expenditure, and serum 25(OH) Vitamin D_3 by gender and in total

Variable		Gender		
		Male (n=27)	Female (n=25)	Total (n=52)
Age	Mean ± SD	7.4 ± 1.4	7.8 ± 1.9	7.6 ± 1.7
	Range	5.6 - 11.1	5.1 - 11.9	5.1 - 11.9
BMI	Mean ± SD	17.3 ± 2.5	16.3 ± 1.9	16.8 ± 2.3
	Range	14.7 - 23.9	13.7 - 20.6	13.7 - 23.9
Weight (kg)	Mean ± SD	26.3 ± 5.9	27.5 ± 7.6	26.9 ± 6.8
	Range	17.5 - 43.7	18.2 - 47.0	17.5 - 47.0
Height (cm)	Mean ± SD	128.4 ± 13.5	124.8 ± 9.9	126.5 ± 11.8
	Range	105.2 - 168.5	107.25 - 141.0	105.2 - 168.5
Fat mass (kg)	Mean ± SD	5.95 ± 4.78	6.00 ± 3.41	5.97 ± 4.14
	Range	2.09 - 18.77	2.17 - 16.42	2.09 - 18.77
DTEE above BEE (kcals) [2]	Mean ± SD	609 ± 225	512 ± 200	562 ± 217
	Range	323 – 1120	281 - 973	281 – 1120
BMD (g/cm^2)	Mean ± SD	0.885 ± 0.062	0.837 ± 0.060	0.847 ± 0.061
	Range	0.764 - 1.003	0.751 - 0.980	0.751 - 1.003
Z-score [1] (Lunar)	Mean ± SD	0.31 ± 0.64	-0.04 ± 0.59	0.14 ± 0.64
	Range	-0.60 - 1.80	-1.20 - 1.20	-1.20 - 1.80
BMC (g)	Mean ± SD	960 ± 262	933 ± 229	947 ± 245
	Range	618 – 1657	567 - 1514	567 – 1657
Serum 25-OH, Vit D (ng/mL) [3]	Mean ± SD	30.7 ± 9.4	30.3 ± 11.3	30.6 ± 10.2
	Range	13.8 - 57.5	10.8 - 62.3	10.8 - 62.3

[1] Significant difference between male and female subjects per t-test ($p \leq 0.05$)
[2] For this variable, n=26 (males), n=25 (females), n=51 (total)
[3] For this variable, n=26 (males), n=23 (females), n=49 (total)
BMI: Body Mass Index
DTEE above BEE: Daily Total Energy Expenditure above Basal Energy Expenditure
BMD: Bone Mineral Density
BMC: Bone Mineral Content

Table 3.2

Daily dietary intakes according to 2005 U.S. Dietary Guidelines by gender[1]

Variable		Gender		
		Male (n=27)	Female (n=25)	Total (n=52)
Grain intake[2]	Mean ± SD	8.9 ± 4.1	7.7 ± 3.1	8.3 ± 3.7
	Range	2.8 - 22.7	0.8 - 14.1	0.8 - 22.7
Milk intake[3]	Mean ± SD	3.2 ± 1.9	3.6 ± 1.7	3.4 ± 1.8
	Range	0.2 - 6.4	1.0 - 7.0	0.2 - 7.0
Meat and bean intake[2]	Mean ± SD	3.9 ± 2.0	3.4 ± 1.8	3.7 ± 1.9
	Range	0.7 - 9.4	0.1 - 6.4	0.1 - 9.4
Fruit and Vegetable intake[3]	Mean ± SD	2.3 ± 1.4	2.00 ± 1.3	2.2 ± 1.3
	Range	0.5 - 5.5	0.1 - 4.1	0.1 - 5.5

[1] 2005 U.S. Dietary Guidelines recommendations for children ages 2 to 8 years:
Grain, 6 oz.; Milk, 2 cups; Meat and beans, 5 oz.; Fruit, 1.5 cups; and Vegetable 2.5 cups.
[2] In oz. equivalents
[3] In cup equivalents

There were no differences in intake of fruits and vegetables by gender. Meat and bean intake, at 3.7-ounce equivalents, was lower than the recommended 5-ounce equivalents; however, there was a very broad range of intakes between 0.1-ounce equivalents and 9.4-ounce equivalents. Total intake of fruits and vegetables in this group of children, at 2.2 cup-equivalents for both fruits and vegetables combined, was lower than the recommended amount of 1.5 cups of fruits and 2.5 cups of vegetables (a total of 4 cup-equivalents of fruits and vegetables per day).

Nutrient intakes as measured by usual, one-day recall are summarized by gender and for the total in Table 3.3. Intakes according to recall do not include supplements. There was a significant difference between males and females only for fiber (t-test, $p \leq 0.05$) with males consuming more fiber. Mean caloric intake by recall was 2180 ± 545 (range 1123 – 3152). Mean protein intake by recall was 79 g ± 21 (range 37 – 124), representing a mean protein intake of 2.94 g/kg body weight. Total mean intake of Ca by recall was 1382.4 mg ± 580.9 (range 252.9 – 2715.0).

Nutrient intakes as measured by FFQ are summarized in Table 3.4. The FFQ reported intakes both with and without supplements. The FFQ asks whether or not vitamins are used, how often, and for how long. There were no significant differences in intake of any nutrient by gender as measured by FFQ. Fifty-six percent of the subjects reported supplement use. Of those 56%, 62 % report taking supplements for less than 4 years and fewer than 5 times a week. Mean caloric intake by FFQ was 2281 ± 474 (range 1265 - 3353). Mean protein intake by FFQ was 95 g ± 21 (range 37 - 137), representing a mean protein intake of 3.5 g/kg body weight. Total mean intake of Ca without

Table 3.3

Daily nutrient intake[2] as measured by usual, one-day dietary recall by gender and in total

Variable		Gender		
		Male (n=27)	Female (n=25)	Total (n=52)
Total kcals	Mean ± SD	2219 ± 507	2,138 ± 591	2,180 ± 545
	Range	1123 - 3152	1167 - 3115	1123 - 3152
Protein (g)	Mean ± SD	79 ± 22	79 ± 20	79 ± 21
	Range	37 - 124	48 - 113	37 - 124
Cholesterol (mg)	Mean ± SD	215 ± 136	201 ± 113	208 ± 124
	Range	25 - 607	64 - 539	25 - 607
Carbohydrate (g)	Mean ± SD	308 ± 72	294 ± 98	301 ± 85
	Range	132 - 432	102 - 511	102 - 511
Fiber (g)[1]	Mean ± SD	20 ± 8	15 ± 5	18 ± 7
	Range	6 - 41	2 - 28	2 - 41
Fat (g)	Mean ± SD	79.2 ± 28.2	75.9 ± 25.4	77.6 ± 267
	Range	31.5 - 171.3	34.3 - 127.4	31.5 - 171.3
Vit A (mcg RAE)	Mean ± SD	966 ± 491	1039 ± 790	1001 ± 647
	Range	271 - 1753	152 - 4359	152 - 4359
Vit C (mg)	Mean ± SD	115 ± 86	93 ± 62	104 ± 76
	Range	15 - 351	4 - 265	4 - 351
Vit E (mg)	Mean ± SD	7.0 ± 3.7	5.7 ± 3.3	6.4 ± 3.6
	Range	2.0 - 21.1	2.1 - 17.2	2 - 21.1
Ca (mg)	Mean ± SD	1344 ± 668	1424 ± 479	1382 ± 581
	Range	253 - 2715	790 - 2505	253 – 2715
P (mg)	Mean ± SD	1584 ± 505	1565 ± 442	1575 ± 471
	Range	645 - 2766	1001 - 2531	645 – 2766
Mg (mg)	Mean ± SD	327.4 ± 97.4	296.1 ± 85.0	312.4 ± 92.1
	Range	104.5 - 539.2	158.4 - 486.2	104.5 - 539.2
Fe (mg)	Mean ± SD	18.8 ± 8.9	16.7 ± 6.8	17.2 ± 8.0
	Range	5.8 - 48.6	5.1 - 32.1	5.1 - 48.6
Zn (mg)	Mean ± SD	13.1 ± 5.2	11.6 ± 3.3	12.4 ± 4.4
	Range	3.9 - 28.7	6.8 - 18.4	3.9 - 28.7
K (mg)	Mean ± SD	2865 ± 934	2,705 ± 911	2788 ± 917
	Range	1087 - 5199	1558 - 5083	1087 – 5199
Se (mcg)	Mean ± SD	105.5 ± 40.9	95.0 ± 30.6	100.4 ± 36.3
	Range	36.3 - 215.0	47.8 - 188.8	36.3 - 215.0

[1] Significant difference between male and female subjects per t-test ($p \leq 0.05$)
[2] Vitamin D not reported via one-day recall (not available in database)

Table 3.4

Daily nutrient intake[2] as measured by food frequency questionnaire[1] with and without supplements by gender and in total

Variable			Gender		
			Male (n=27)	Female (n=25)	Total (n=52)
Total kcals		Mean ± SD	2250 ± 484	2315 ± 472	2281 ± 474
		Range	1265 - 3353	1374 - 3327	1265 - 3353
Protein (g)		Mean ± SD	93 ± 22	97 ± 20	95 ± 21
		Range	37 - 126	45 - 137	37 - 137
Animal protein in diet (g)		Mean ± SD	62 ± 17	67 ± 17	64 ± 17
		Range	25 - 98	25 - 106	25 - 106
Non-animal protein in diet (g)		Mean ± SD	30 ± 11	30 ± 8	30 ± 10
		Range	12 - 64	14 - 47	12 - 64
Cholesterol (g)		Mean ± SD	240 ± 55	257 ± 85	248 ± 71
		Range	144 - 353	91 - 388	94 - 388
Carbohydrate (g)		Mean ± SD	309 ± 82	314 ± 68	311 ± 75
		Range	157 - 460	185 - 461	157 - 461
Fiber (g)		Mean ± SD	20.3 ± 7.3	19.5 ± 6.0	19.9 ± 6.6
		Range	7.7 - 36.9	9.5 - 35.5	7.7 - 36.9
Fat (g)		Mean ± SD	73.4 ± 15.8	78.2 ± 20.3	75.7 ± 18.0
		Range	44.0 - 118.8	44.7 - 110.2	44.0 - 118.8
K (mg)		Mean ± SD	3127 ± 927	3219 ± 701	3171 ± 819
		Range	1060 - 4785	1411 - 4864	1060 - 4864
Vit A (mcg RAE)	With supp	Mean ± SD	8399 ± 5179	7909 ± 3038	8163 ± 4251
		Range	2045 - 24575	3578 - 13120	2045 - 24575
	Without supp	Mean ± SD	1024 ± 465	1018 ± 353	1021 ± 411
		Range	246 - 2246	571 - 1960	246 - 2246
Vit C (mg)	With supp	Mean ± SD	130 ± 71	129 ± 48	129 ± 60
		Range	35 - 322	32 - 223	32 - 313
	Without supp	Mean ± SD	114 ± 60	113 ± 41	113 ± 51
		Range	35 - 262	26 - 183	26 - 262
Vit D (IU)	With supp	Mean ± SD	392 ± 189	413 ± 156	402 ± 172
		Range	45 - 708	48 - 863	45 - 863
	Without supp	Mean ± SD	310 ± 159	332 ± 116	320 ± 139
		Range	45 - 568	48 - 490	45 - 568
Vit E (mg)	With supp	Mean ± SD	8.4 ± 3.5	8.4 ± 2.5	8.4 ± 3.0
		Range	3.3 - 16.3	3.8 - 14.0	3.3 - 16.3
	Without supp	Mean ± SD	6.5 ± 1.7	6.6 ± 1.7	6.6 ± 1.7
		Range	3.3 - 10.0	3.2 - 10.0	3.2 - 10.0

Table continued - next page

Table 3.4 - continued

Ca (mg)	With supp	Mean ± SD	1497 ± 500	1503 ± 327	1500 ± 422
		Range	462 - 2577	790 - 1978	426 - 2577
	Without supp	Mean ± SD	1464 ± 496	1471 ± 325	1467 ± 419
		Range	426 - 2,532	722 - 1,978	426 - 2532
P (mg)	With supp	Mean ± SD	1742 ± 443	1823 ± 374	1781 ± 409
		Range	725 - 2559	829 - 2576	725 - 2576
	Without supp	Mean ± SD	1742 ± 443	1823 ± 374	1781 ± 409
		Range	725 - 2559	828 - 2576	725 - 2576
Mg (mg)	With supp	Mean ± SD	329.2 ± 91.3	338.2 ± 74.2	333.5 ± 82.8
		Range	125.4 - 476.1	186.6 - 531.1	125.4 - 531.1
	Without supp	Mean ± SD	321.0 ± 86.0	330.4 ± 73.4	325.5 ± 79.5
		Range	125.4 - 473.3	166.6 - 511.1	125.4 - 511.1
Fe (mg)	With supp	Mean ± SD	20.2 ± 8.3	20.7 ± 9.0	20.4 ± 8.5
		Range	7.2 - 38.3	10.2 - 54.7	7.2 - 54.7
	Without supp	Mean ± SD	16.1 ± 4.9	16.6 ± 6.5	16.3 ± 5.7
		Range	7.2 - 29.9	10.2 - 44.7	7.2 - 44.7
Zn (mg)	With supp	Mean ± SD	16.7 ± 6.1	16.8 ± 5.0	16.7 ± 5.5
		Range	6.7 - 30.0	8.2 - 29.9	6.7 - 30.0
	Without supp	Mean ± SD	13.4 ± 3.8	13.6 ± 2.7	13.5 ± 3.3
		Range	6.6 - 26.1	8.2 - 18.9	6.6 - 26.1

[1] Youth and Adolescent Food Frequency Questionnaire, Harvard Channing Laboratory, Harvard University, School of Public Health, Boston, MA, USA

[2] Vitamin K and selenium not reported (not available in database).

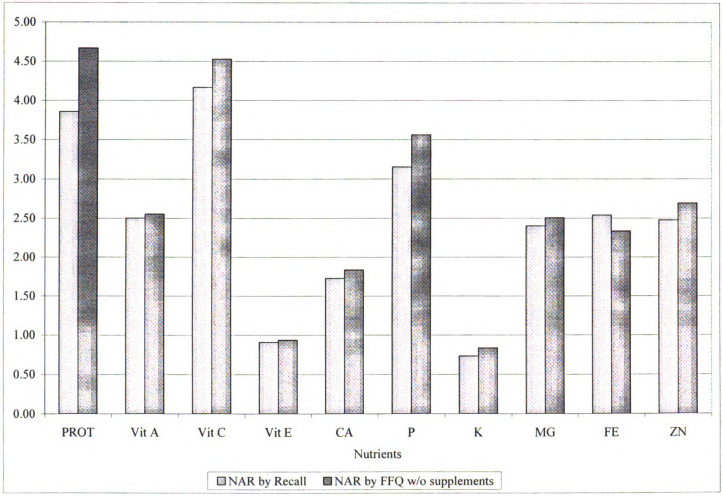
supplements by FFQ was 1467 mg ± 419 (range 426 – 2532). The Adequate Intake (AI) of Ca is 800 mg/day (FNB/IOM, 1997).

Analysis of dietary intake by FFQ also provides insight into the food sources of nutrients in the diets. Single item dairy foods (frozen dairy products, cheese, yogurt, fluid milks) made up 21 % of total caloric intake, 29.7 % of the total contribution to protein intake, 61.8 % of the contribution to Ca intake, 44.1 % of the contribution to P intake and 82.3 % of the contribution to Vitamin D intake (excluding supplement use). After dairy foods, the next highest contributors to caloric intake were peanut butter and jelly sandwiches (5.4%), chicken (4.4%), and cold cereal (3.9%). After dairy foods, the next highest contributors to protein intake were chicken (12.4%), peanut butter and jelly sandwiches (4.2%), and beef (3.9%). The next highest contributors to P intake, after dairy foods, were chicken (5.33%), peanut butter and jelly sandwiches (3.11%), and cold cereal (3.02%).

Nutrient intake can be expressed by nutrient adequacy ratios (NAR), a calculation which is an index of adequacy of a specific nutrient in the diet relative to an established standard need (Gibson, 1990). In this research, the most current US Dietary Reference Intakes (DRI) (FNB/IOM, 2005; FNB/IOM, 1997) were used as the standard. In order to evaluate dietary intakes as measured by recall and intakes as measured by FFQ without supplements, NAR values were calculated for nutrients that have been shown to have a strong association with bone growth (Figure 3.1). At a calculated NAR of greater than or equal to 1.0, intake can be considered to be adequate (Gibson, 1990). Both measures of diet assessment result in nutrient adequacy ratios of greater than 1.5 for protein, Vitamin A, Vitamin C, Ca, P, Mg, iron and zinc. The mean NAR for Vitamin E are 0.91 by recall

Figure 3.1

Mean nutrient adequacy ratios (NAR) for select nutrients as measured by recall and by FFQ without supplements



and 0.94 by FFQ. The mean NAR for potassium (K) are 0.73 per recall and 0.83 per FFQ. The US DRI for protein in early childhood (ages 4 to 13 years) is 0.95 g/kg body weight (FNB/IOM, 2005). Protein intake in this subject population was 3.1 times greater than the DRI per recall and 3.7 times greater than the DRI per FFQ. Food frequencies tend to result in higher estimates of intake than do recalls, with both methods tending to result in over reporting in populations with lower intakes, populations that would include children (Thompson and Byers, 1994). Given that NAR for 10 nutrients in Figure 3.1 were similar for both FFQ and recall, and given that both methods of assessment tend to over report intake, the intake as measured by diet recall was used in subsequent data analysis because the recall provided slightly lower estimates of intake.

Physical Activity

In exploring the relationships of the measured variables influencing bone mineralization with the dependent variables of BMD and BMC, several significant correlations were found as summarized in Table 3.5. Daily total energy expenditure above calculated basal energy expenditure (BEE) (Harris and Benedict, 1919) was 562 ± 217 kcals (range 281 – 1120) (Table 3.1). Daily total energy expenditure above BEE had a strong, significant positive relationship to both BMD ($r=0.460$, $p\leq0.01$) and BMC ($r=0.591$, $p\leq0.01$). Likewise, percent body fat had a strong, significant positive relationship to both BMD ($r=0.535$, $p\leq0.01$) and BMC ($r=0.646$, $p\leq0.01$). Both protein and kilocalories, expressed relative to body weight, had strong, significant negative relationships to both BMD and BMC (for protein and BMD, $r=-0.508$, $p\leq0.01$, 2-tailed, for protein and BMC $r=-0.564$, $p\leq0.01$, 2-tailed, for kcals and BMD, $r=-0.510$, $p\leq0.01$, 2-tailed, for kcals and BMC $r=-0.578$, $p\leq0.01$, 2-tailed). In order to evaluate the wide range

Table 3.5

Correlations of BMD and BMC with variables influencing bone mineralization

Pearson Correlation (n=52)	BMD (g/cm^2)	BMC (g)
% body fat	0.535**	0.646**
DTEE above BEE[1] (kcals)	0.460**	0.591**
Protein by weight[2] (g/kg)	-0.508**	-0.564**
kcals by weight[2] (kcals/kg)	-0.510**	-0.578**
Ca by height[2] [(mg/d)/cm]	-0.277*	-0.260
P by height[2] [(mg/d)/cm]	-0.378**	-0.348*
Mg $(mg)^2$	-0.135	-0.126
K $(mg)^2$	-0.137	-0.069
Fruit and Vegetable intake[2,3]	0.265	0.256
RNAE	0.196	0.247

** Pearson correlation significant at the 0.01 level (2-tailed).
*Pearson correlation significant at the 0.05 level (2-tailed).
[1] For this variable, n = 51
[2] All nutrient intakes as assessed by recall.
[3] Intake per 2005 US Dietary Guidelines in cup equivalents.
BMD: Bone Mineral Density
BMC: Bone Mineral Content
DTEE above BEE: Daily total energy expenditure above basal energy expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).
RNAE: Renal Net Acid Excretion as estimated by the method of Frassetto LA et al. Estimation of net endogenous noncarbonic acid production in humans from diet potassium and protein contents. AJCN 1998; 68:576-83.

of Ca intakes independent of age or body size, both of which also ranged widely, intake was expressed by height (mg Ca intake per day/cm height). A significant negative association was found between Ca by height and BMD ($r=-0.277$, $p\leq0.05$, 2-tailed). There was no correlation between Ca intake by height and BMC. Phosphorus intake by height (mg per day/cm height) was also negatively correlated to BMD ($r=-0.378$, $p\leq0.05$, 2-tailed) and to BMC ($r=-0.348$, $p\leq0.05$, 2-tailed). No significant correlations were found with other nutrients or food groups or with total Ca intake not expressed by height. Due to the strong negative correlation of protein to BMD and BMC, renal net acid excretion was estimated using the method of Frassetto et al. (1998). No significant correlation was found between RNAE, dietary potassium or fruit and vegetable consumption and BMD or BMC (Table 3.5).

Table 3.6 and Table 3.7 report final multiple regression analysis predicting BMD and BMC, respectively, from measured variables influencing bone growth in this population of prepubertal children. Independent variables found to have a significant correlation and those reported to have a strong association to bone growth in the literature (DTEE, % body fat, protein intake /kg body weight, kcals intake/kg body weight, Ca intake/cm height, P intake/cm height, Mg intake, K intake, fruit and vegetable intake) were entered in a stepwise manner. The final model for predicting BMD (Table 3.6) includes percent body fat, dietary intake of kcal by body weight, Ca and P by height, Mg intake and fruit and vegetable intakes. In this model, caloric intake relative to weight and P relative to height were the only significant negative predictors of BMD. The associated relationships of all of these variables explain 58.8 % of the variability in BMD. The final model for predicting BMC (Table 3.7) includes DTEE above BEE, percent body fat, kcal

Table 3.6

Multiple regression analysis of BMD by % body fat and intakes of kcals by weight, Ca by height, P by height, Mg, and fruits and vegetables

	BMD	
	B ± SEE	Sig.
Constant	0.824 ± 0.035	0.000
% body fat	0.003 ± 0.001	0.006
kcals by weight[1]	-0.001 ± 0.000	0.003
Ca by height (mg/cm)[1]	0.009 ± 0.003	0.006
P by height (g/cm)[1]	-0.017 ± 0.005	0.002
Mg (mg)[1]	0.000 ± 0.000	0.000
Fruit and vegetable intake[1,2]	0.008 ± 0.005	0.093
R^2 =	58.80%	
ANOVA	10,448	0.000

[1] All nutrient intakes as assessed by recall
[2] Intake per 2005 US Dietary Guidelines in cup equiv.
BMD: Bone Mineral Density

Variables entered into the regression model (stepwise): protein intake by weight, calcium intake by height, DTEE above BEE, % body fat, P intake by height, daily total Mg, fruit and vegetable intake, kcal intake by weight

Table 3.7

Multiple regression analysis of BMC by % body fat, DTEE above BEE, intakes of kcals by weight, Ca by height, P by height, Mg, and fruits and vegetables

	BMC	
	B ± SEE	Sig.
Constant	704.891 ± 125.332	0.000
% body fat	9.389 ± 3.104	0.004
DTEE above BEE	0.249 ± 0.109	0.027
kcals by weight[1]	-4.235 ± 1.306	0.002
Ca by height (mg/cm)[1]	26.973 ± 10.854	0.017
P by height (g/cm)[1]	-47.822 ± 17.744	0.010
Mg (mg)[1]	1.603 ± 0.412	0.000
Fruit and vegetable intake[1,2]	34.133 ± 15.675	0.035
R^2 =	72.90%	
ANOVA	16,521	0.000

[1] All nutrient intakes as assessed by recall
[2] Intake per 2005 US Dietary Guidelines in cup equiv.
BMC: Bone Mineral Content
DTEE above BEE: Daily Total Energy Expenditure above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

Variables entered into the regression model (stepwise): protein intake by weight, calcium intake by height, DTEE above BEE, % body fat, P intake by height, daily total Mg, fruit and vegetable intake, kcal intake by weight

intake by weight, Ca and P intake by height, Mg intake and fruit and vegetable intake, with the association among these variables explaining 72.9% of the variability in BMC. Caloric intake relative to weight and P intake relative to height were negative predictors of BMC. In that the B coefficient is indicative of the relative importance of an independent variable in contributing to the dependent variable in a given prediction model, Ca intake by height (B=0.009 ± 0.003, p=0.006), P intake by height (B=-0.017 ± 0.005, p=0.002), and fruit and vegetable intake (B=0.008 ± 0.005, p=0.093) are the strongest predictors of BMD. Calcium intake by height (B=26.973 ± 10.854, p=0.017), P intake by height (B=-47.822 ± 17.744, p=0.010), and fruit and vegetable intake (B=34.133 ± 15.675, p=0.035) are also the strongest predictors of BMC. The correlation between BMC and BMD and their unstandardized values as predicted by each model is illustrated in Figure 3.2 and Figure 3.3. Gender was entered into each model and did not influence the outcome of the final model.

DISCUSSION

Associations between diet, physical activity, body composition, and bone growth and mineralization are well documented for special populations such as the elderly and adolescents but studies looking exclusively at early childhood are limited. This study confirms these associations in a population of healthy, prepubertal, Caucasian children. Much of the variability in bone mineralization in this group of prepubertal children between 5 to 11 years of age can be explained by modifiable influences on bone mineralization. This group of children all had BMD Z-scores above minus 1.2. However nearly a third of the children had Z-scores below minus 0.2. By definition, BMD Z-

Figure 3.2

Correlation between actual BMD and predicted value of BMD based on % body fat, intakes of kcals by weight, Ca by height, P by height, Mg, and fruits and vegetables

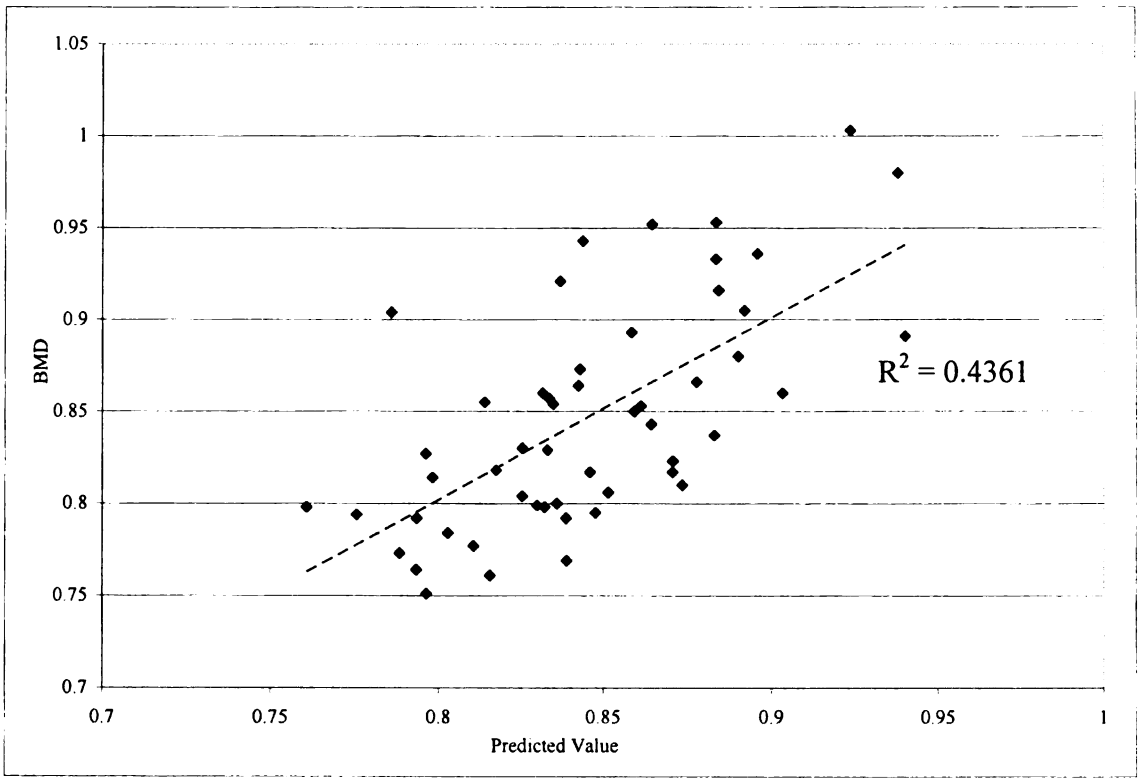
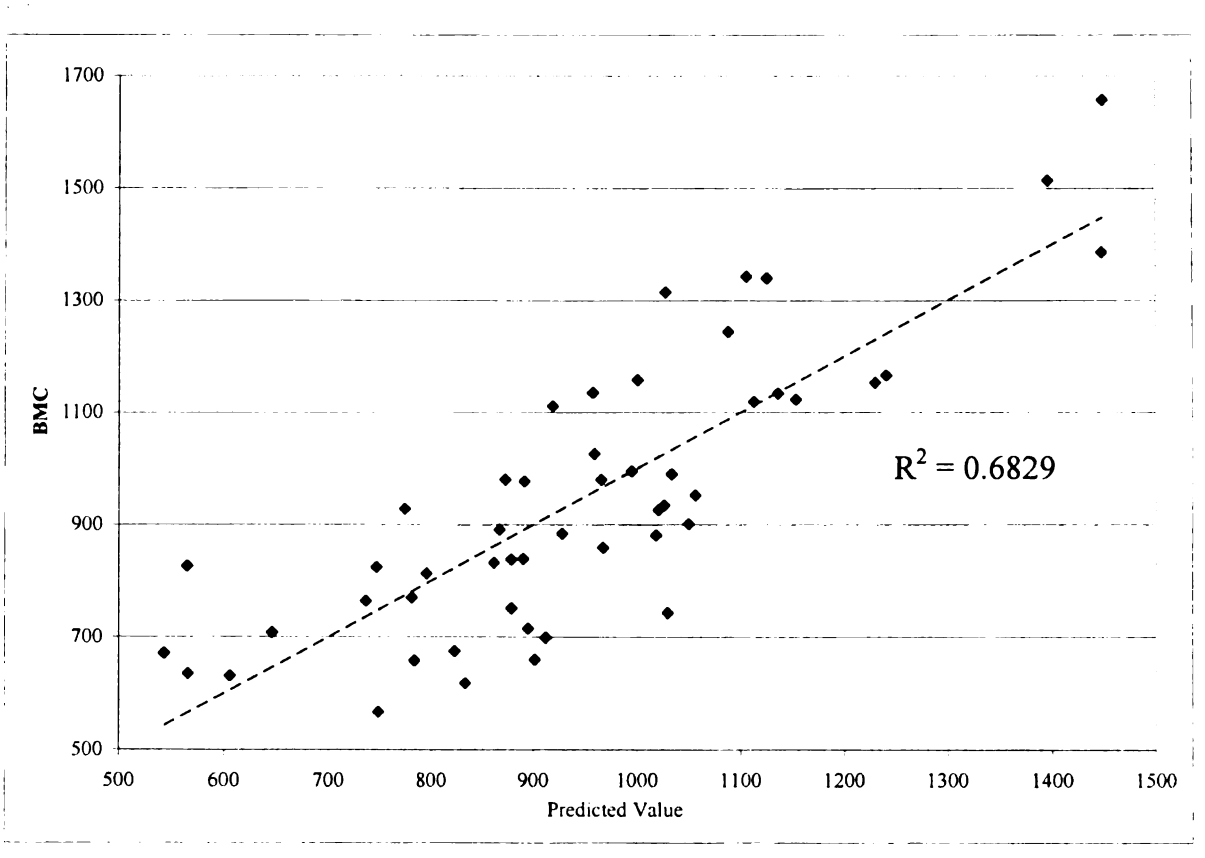


Figure 3.3

Correlation between actual BMC and predicted value of BMC based on % body fat, DTEE above BEE, intakes of kcals by weight, Ca by height, P by height, Mg, and fruits and vegetables



scores represent the central range of BMDs in a healthy reference population. The assumption is that, in the absence of disease, this central range is a desirable range. Because BMD tends to track throughout life (Ferretti et al, 1998; Heaney et al., 2000), the third of these children whose Z-scores are approaching negative 1.0 in early childhood, may already be at increased risk of fractures and osteoporosis later in life. In other words, progress toward accrual of peak bone mass may already be slowed in children with negative Z-scores for BMD during their early childhood years.

Mean intakes of the 16 nutrients measured in this group of children met or exceeded the US DRI. The recommendations for intakes of nutrients in children are based on age rather than physiologic maturity or body size. Growth rates in children occur in four general stages, made apparent by the varying slopes of pediatric growth curves (CDC, 2003) and defined chronologically. Growth rates are highest in periods of birth to 3 years (infancy and toddlerhood) and then again in the period of puberty and adolescence (ages 9 to 18 years, where on an individual basis there is tremendous variation). The intermediate years, or early childhood years of 4 to 8 years, are a time of slower growth. On the other hand, physiologic growth and maturation in adolescence is followed through Tanner staging, a classification of stages of sexual development that is aligned with skeletal growth rates (Tanner, 1955). Nutrient intake recommendations have been set based on these general categories of pediatric growth as well (FNB/IOM, 1997; FNB/IOM, 2005). Likewise, standards for bone mineral measurements are based on chronological age (Wacker and Barden, 2001). As such, both nutrient requirements and bone growth standards have a chronological rather than a physiological basis. By limiting the HKNS subjects to Tanner stage 1, any associations confirmed in this study

are more likely due to physiologic processes other than puberty regardless of age or body size.

The subjects in this study ranged in age between 5 and 11 years of age, ranged in weight between 17 and 47 kg and ranged in height between 105 and 169 cm. These 52 children had great variation in body size, and hence bone size, at any given age. Given the large range in body sizes, variables related to bone mineralization could be expressed relative to height allowing for consideration of variability of body size in children at any given age. The Ca and P intake did not show a relationship to BMD or BMC until they were adjusted for height, after which significant correlations were found. Differences attributable to gender were not seen in this group of subjects though gender differences have been found in some studies of children in this age range (Specker et al., 1987; Bell et al., 1991; Bounds et al, 2005) but not in others (Fassler and Bonjour, 1995; Maynard et al., 1998; Horlick et al., 2000; Nguyen et al., 2001).

This group of children can be considered well-nourished with mean intakes of most nutrients having a NAR of greater than one. Calcium intake in this group was at or above the Adequate Intake (AI) of 800 mg/day for most of the subjects, with 69% of subjects consuming greater than 1050 mg Ca/day by recall or greater than 1300 mg Ca/day by FFQ. Only eight children consumed less than 800 mg Ca/day. Children in the HKNS differ from national samples that predict less than 40% of children in the US between 6 and 11 years of age achieve the AI for Ca (Greer and Krebs, 2006). Protein intake was 3 to 4 times higher than the DRI of 0.95 g/kg body weight, and Vitamin C was 4.5 times the DRI (FNB, IOM, 1997). The high protein intake assessed by recall was inconsistent with the low meat and bean group intake, assessed by the Dietary Guidelines

as lower than recommended (CNPP, USDA-USDHHS, 2007). This inconsistency could have been due to the large overall range of intakes and the contribution of grains and dairy foods to the total protein intake. Given the adequacy of these diets on a nutrient-by-nutrient basis, the influence that diet has on bone appears to relate to the relationship of these nutrients to each other when considered as a group as well as their subsequent interaction with bone mineralization and with other modifiable influences such as physical activity. On an individual basis, intake of protein, kcals, Ca, P, Mg, and K had an inverse relationship to BMD and BMC. However, when the association of these nutrients, was expressed relative to body and bone size, using multivariate regression analysis, only intakes of kcals, Ca, P, and Mg remained in the model as significant dietary predictors of BMD and BMC. In the prediction model, intakes of kcals/kg body weight, Ca/cm height, and P/cm height have an inverse relationship to BMD and BMC while Mg intake had a positive relationship.

Protein's negative correlation to bone mineralization has been hypothesized to be related to the effect of a chronic, low grade metabolic acidosis from the high levels of sulfur containing amino acids and ammonium from protein metabolism which place a hydrogen burden on the system and result in an increase in urinary Ca (Frassetto et al., 1998; Ginty, 2003; Massey, 2003; New, 2003). Grains have high concentrations of these amino acids as well and this group of subjects consumed an average of over 8 cups of grain foods per day. To explore this association with protein further, renal net acid excretion was estimated (Frassetto et al., 1998) but did not correlate to BMD or BMC in these subjects. Potassium intake (primarily from foods in the fruit and vegetable group) may offset the hypercalciuric effect of protein through contribution of base excess

(Massey, 2003; New, 2003). Though neither K intake per day nor fruit and vegetable intake per day correlated with BMD or BMC, fruit and vegetable intake did have a significant association with BMD and BMC in the final prediction models. This is especially interesting because protein was not a significant predictor in the final model, suggesting that other dietary factors may have had a mitigating influence on the negative effect attributed to protein (New, 2003; Tylavsky et al., 2004; Vatanparast et al., 2005).

In addition to interrelationships among nutrients, body composition as percent body fat was also associated with BMC and BMD, remaining in the final model as a significant predictor of BMD and BMC. Percent body fat draws a more meaningful relationship to measures of bone mineralization because some measures of lean tissue can include bone and are less modifiable, in a practical sense, than is body fat. A higher percent body fat predicts a higher BMD. However, the range of percent body fat within which this association was seen is, itself, within a normal, healthy range of body composition. In contrast, for this model, a low percent body fat contributes to predicting a lower BMD and BMC. It appears that in this group of young children, all of whom were well nourished, higher body fat within a normal range was beneficial to bone mineralization.

Physical activity is acknowledged to be beneficial to bone health (Murphy and Carroll, 2003). However the role physical activity plays in the accrual of peak bone mass and its interactions with the other factors influencing bone growth in the early childhood years is less clear. In general, children are less physically active now than in the past. This decrease is estimated as a decline of approximately 600 kcals/day over the past 50 years (Boreham and Riddoch, 2001). The mean DTEE above BEE of 562 kcals (Table

3.1) is 27 % of mean daily total caloric intake (Table 3.3) in this population and was significantly correlated to BMD and BMC (Table 3.5). In adults as well as in animal models, vigorous activity is an important contributor to bone mineralization (McMurray, 1995; Boreham and Riddoch, 2001). When energy expenditure was considered along with other factors in bone mineralization in the prediction model, DTEE remained significant as a positive predictor of BMC but not of BMD in these subjects. Physical activity must be considered in understanding the interrelationships among factors predicting bone mineralization of children of this age given the strong positive relationship between bone mineralization and energy expenditure through activity.

This group of children was selected to reflect a group of generally healthy children living in the US Midwest. Predicting their BMD or BMC would involve, in part, measurement of those modifiable influences of bone growth retained by the models developed. Thirty percent of these children had Z-scores that identify them as having BMD and BMC in what is technically defined as the lower range of normal and at risk of poor progress toward attainment of peak bone mass already in this early childhood period. Based on the increasing incidence and prevalence of osteoporosis in older adults, what is thought to be normal bone mineralization may actually be suboptimal. Bone growth and mineralization exist on a continuum which, when shifted, changes the end point of that continuum. In these early childhood years diet and lifestyle habits are forming for a lifetime so knowing where to focus suggestions and interventions toward bone-building lifestyles at this age may decrease the risk of developing osteoporosis later in life as well as decrease fracture risk throughout life.

This study confirmed several associations that support the importance of considering as many of the influences on bone mineralization as possible in order to assess adequacy of bone mineralization in prepubertal children. Characteristics of children who tend to have lower measures of bone mineralization include high protein intake, low percent body fat (within normal weight subjects), low levels of physical activity, high calcium intake, and low intake of fruits and vegetables. These associations suggest lifestyle adjustments that can influence BMD and BMC toward attainment of peak bone mass. Health care professionals can provide meaningful suggestions for identified "at risk" children that contribute to increasing bone mass and decreasing risk of osteoporosis later in life.

CHAPTER FOUR

CHAPTER FOUR

ABSTRACT

ASSOCIATION OF BODY COMPOSITION, DIET, PHYSICAL ACTIVITY, AND BONE BIOMARKERS WITH MEASURES OF BONE MINERALIZATION IN PREPUBERTAL CHILDREN

by

Marcia Kelly Scott

MICROABSTRACT: Prevention of osteoporosis begins in childhood with attainment of peak bone mass. Body composition, diet, activity, and biomarkers of bone metabolism were measured in 52 children. Better bone mass was associated with body fat, physical activity, serum OC and Vitamin D, and urinary DPD, supporting their consideration in assessing bone health in children.

INTRODUCTION: Osteoporosis is predicted to become a disease of epidemic proportions within the next several decades. Attainment of higher bone mineral content during the first two decades of life decreases the risk of developing fractures later in life. To further clarify associations among determinants of bone growth, body composition, bone mineral density (BMD) and bone mineral content (BMC), diet composition, bone metabolism biomarkers, and physical activity levels were measured in prepubertal children, mean age of 7.6 years.

METHODS: BMD, BMC, and body fat were measured via DEXA in 52 subjects. Daily total energy expenditure (DTEE) was measured via accelerometer. Diet was assessed with a usual, one-day recall interview. Serum OC, serum 25(OH) Vitamin D_3 and urinary DPD were measured. Relationships between variables were explored with

two-tailed t-tests, two-tailed Pearson's correlations and stepwise multiple regression analysis ($p \leq 0.01$).

RESULTS: Bone mineralization correlates positively with percent body fat ($r=0.535$ for BMD and $r=0.646$ for BMC) and with DTEE ($r=0.460$ for BMD and $r=0.591$ for BMC). Protein intake had significant negative relationships to both BMD, $r=-0.508$, and BMC, $r=-0.564$. A significant negative association was found between Ca intake and BMD ($r=-0.277$, $p \leq 0.05$). The final regression models for predicting BMD and BMC include percent body fat, serum OC, urinary DPD, serum 25(OH) Vitamin D_3, and DTEE above BEE. In these models, OC and DPD were significant negative predictors of BMD and BMC while DTEE and percent body fat were positive predictors. The relationships of these variables explain 58.9 % of the variability in BMD and 64.9% of the variability in BMC.

CONCLUSIONS: This study affirms the interrelationships of diet, physical activity and body composition in contributing to development of peak bone mass. These associations, along with biomarkers of bone metabolism, introduce the possibility that children at risk of poor bone mineralization may be identified and treated early in life.

EXERCISE
BONE TURNOVER MARKERS
BODY COMPOSITION
CLINICAL/PEDIATRICS
NUTRITION

CHAPTER FOUR

ASSOCIATION OF BODY COMPOSITION, DIET, PHYSICAL ACTIVITY, AND BONE BIOMARKERS WITH MEASURES OF BONE MINERALIZATION IN PREPUBERTAL CHILDREN

INTRODUCTION

Osteoporosis is increasingly recognized as one of the major public health problems facing aging individuals of both genders, worldwide. It is predicted to become a disease of epidemic proportions within the next several decades (Riggs and Melton, 1995; WHO, 2003). Osteoporosis is broadly defined as diminished bone mass and reduced bone mineral density (BMD) at the level of 2.5 standard deviations below the referent BMD of young adults (Riggs and Melton, 1995; USDHHS, 2000; WHO, 2003). Osteoporosis presents clinically as fractures, both traumatic and nontraumatic in nature, resulting in significant morbidity, mortality, and health care costs (WHO, 2003). The two most important ways to reduce risk of osteoporosis are to optimize bone mass while maturing and minimize bone loss while aging.

In considering ways to decrease both the incidence and the severity of osteoporosis, interventions for this disease target methods of prevention, not just in adulthood but also during infancy, childhood, and adolescence. Though osteoporosis is generally considered a geriatric disease, osteoporosis may actually be a pediatric disease with geriatric consequences (Faulkner and Bailey, 2007). Attainment of higher bone mineral content (BMC) during the first two decades of life decreases the risk of developing fractures later in life (Heaney et al., 2000). From infancy through to adulthood, bone mass tends to track throughout life, meaning that infants, children, and adolescents who have higher bone mass tend to be the adults who have high bone mass

(Ferretti et al., 1998; Heaney et al, 2000). This association introduces the possibility that "at risk" individuals may be identified early in life. Peak bone mass has genetic, hormonal, nutritional, and behavioral determinants (Soyka et al., 2000). Some of these determinants can be modified in "at risk" individuals.

In many studies of bone growth, children are grouped primarily by age with secondary consideration of pubertal status, if at all. Grouping by age limits the ability to generalize the results due to variation in initiation and duration of puberty, which accelerates bone development. In addition, physical activity must also be considered. Children with higher levels of physical activity have higher measures of bone mineral mineralization (Bailey et al., 1999; Iuliano-Burns et al., 2003; Slemeda et al., 1994; Specker and Binkley, 2003; Zanker et al., 2003). Body composition itself may influence bone mineralization, especially in overweight children (Goulding et al., 2000, 2001). Dietary influences on bone mineralization in early childhood have been focused primarily on calcium (Ca) intake. Calcium intake is associated with development of peak bone mass (Weaver, 2000). During attainment of peak bone mass, Ca intakes of less than 1 g/day are associated with lower bone density (Weaver, 2000). However, the evidence related to the long term effects of Ca supplementation in childhood indicates that gains are not generally sustained (Abrams, 2005), indicating bone mineralization depends on multiple factors, only one of which is Ca intake. Thus, intake of Ca as well as other key bone-building nutrients should be considered in conjunction with other factors when investigating determinants of bone mineralization in early childhood.

This study was undertaken to further clarify associations among diet, physical activity, and body composition with bone growth and mineralization in a group of

children between 5-years of age and puberty. In addition, the relationships of biomarkers of bone mineralization to prepubertal bone growth were explored. Understanding these associations early in life may contribute toward an eventual ability to identify children who are "at-risk" in terms of progress in attaining peak bone mass such that early intervention can decrease their risk of developing osteoporosis later in life.

SUBJECTS AND METHODS

The Healthy Kids Nutrition Study (HKNS) enrolled 52 subjects of both genders from the greater Lansing, Michigan area. The children were between 5-years of age and puberty (<Tanner 1), greater than the 5th percentile BMI by NCHS standards, and ≥ 37 weeks gestational age at birth. They were generally healthy, without any chronic disease conditions or birth defects other than possible allergies or food sensitivities. Children with minor conditions that have no direct relationship to bone growth were included after review by the study physician. Pubertal status was determined by a thorough parent/child interview to ascertain Tanner Stage I status. Although recruitment by race was not targeted, all children were at least 50% Caucasian. The study was approved by full review of the University Committee on Research Involving Human Subjects (UCRIHS) at Michigan State University (MSU) and met confidentiality and HIPAA requirements.

Diet information was collected as a usual, one day diet recall obtained via a consensus interview with a parent and the subject by a Registered Dietitian. The same interviewer entered the diets for analyses into the nutrition database program within the MyPyramid Tracker, based on the USDA food database (CNPP, 2005). This diet assessment tool and analysis program provided intake estimates for 28 nutrients (CNPP, 2005), five of which are reported in this paper.

Standard pediatric measurement protocols to assure precision and accuracy were employed (Gibson, 1990; Lee and Nieman, 1996) and followed the National Center for Health Statistics guidelines for standard measurement (NCHS, 1996). Height was measured to the nearest centimeter with a stadiometer (Invicta Plastics). Weight was determined to the nearest 0.1 kg on a digital scale (Seca, Model 882).

The Actical accelerometer (MiniMitter) was used to measure physical activity. The accelerometer was worn at the waist on a neoprene belt. This accelerometer has been validated for use in estimating energy expenditure in young children (Pfeiffer et al., 2006). The sampling frequency was 32 Hz and an epoch measurement of 15 seconds was chosen. Subjects wore the monitor continuously for three, consecutive days (one weekend day and two weekdays).

The height and weight of each subject were uploaded to the Actical from which basal energy expenditure (BEE), using the Harris and Benedict equation (Harris and Benedict, 1919), was estimated by the Actical program so that daily total energy expenditure (DTEE) from physical activity was reported as kcals above estimated BEE.

Serum intact osteocalcin (OC) was measured with the Metra™ Osteocalcin assay (Quidel Corporation, Special Products Group, Santa Clara, CA, USA), an enzyme immunoassay. Values are expressed in ng/mL. Twenty-five μL of serum per well (assayed in duplicate) were used for determination of OC. The manufacturer has established reference intervals for healthy men (3.4-9.1 ng/mL) and women (3.7-10.0 ng/mL). No reference standards were provided for children.

Urinary free deoxypyridinoline (DPD) was measured with the Metra™ DPD assay (Quidel Corporation), a competitive enzyme. DPD values, expressed in nmol/L,

were corrected for differences in urine concentration and output by dividing by creatinine (Quidel Corporation). The final creatinine-corrected results were expressed as nmol/mmol. The manufacturer has established reference intervals for healthy men (2.3-5.4 nmol/mmol) and women (3.0-7.4 nmol/mmol); none are provided for children. Due to the diurnal variation of DPD excretion, the first morning urine sample was used. Parents and subjects were instructed on the urine collection and given sterile supplies for the first morning urine sample collection.

Serum 25(OH) Vitamin D_3 was measured once during the study at the analytical lab of LabCorp (Dublin, OH) via an immunochemiluminometric (ICMA) assay. The reference standards provided by the lab vary with age and season due to the seasonal variability of sun exposure.

Whole body measures were made using a GE LUNAR Prodigy machine at Fiechtner Research (Lansing, MI) with pediatric software specific to the machine (software version 6.81; General Electric Lunar Corporation). Measurement protocols specific to the GE-LUNAR Prodigy densitometer were employed, including daily calibration with a standard calibration block and calibration tri-weekly with a body phantom. The CV of this machine is reported to be < 1.0% as in other papers) (personal communication, Fiechtner Research). Measures obtained included body composition (kg fat mass and kg lean mass), BMD (gm/cm^2) and BMC (gm). The full body scan took approximately 2 minutes and exposed each child to less than 0.04 mRem of radiation. Motion artifacts were not a problem in this population.

The BMD reference values from the GE Lunar DXA program were used in power calculations (Wacker and Barden, 2001). The mean BMD for the 7-year-old male was

used as the mean BMD of the population and a SD of 0.07 was used to calculate power. Recruitment of 52 subjects assured the sample size necessary to detect differences at a 95% Confidence Interval.

Descriptive statistics were employed to describe the study population with values reported as means ± SDs. The primary dependent variables are BMD, BMC, and the Lunar DXA-reported Z-score. The independent variables are select components of the dietary intake as assessed by recall, serum 25 (OH) Vitamin D_3, serum OC, urinary DPD, measures of body composition, and physical activity expressed as daily total energy expenditure in kilocalories. A two-tailed t-test was employed to determine significant differences between genders in all variables. Relationships between variables were explored with 2-tailed Pearson's correlations. Multiple regressions with stepwise variable entry were used to determine variables that may be significant predictors of BMD and BMC. Data were analyzed using SPSS, version 13, Base System (SPSS, Inc.). The significance level for all tests was established as $p<0.05$ and all tests were bi-directional.

RESULTS

Descriptive and body composition characteristics of the study subjects are summarized in Table 4.1, by gender and for all children. The mean age of the subjects was 7.6 years (range 5.1 to 11.9). Twenty-seven of the subjects were male; twenty-five were female. BMIs ranged from 13.7 to 23.9 with a mean BMI of 16.8. Z-scores for the BMDs by Lunar DXA ranged from negative 1.2 to positive 1.8. The mean Z-score was 0.14 with 30.8 percent of these subjects having Z-scores at or below negative 0.2. There were no differences in body composition measures between genders.

Biomarker measures are also summarized in Table 4.1. Mean serum 25(OH) Vitamin D_3 was 30.0 ng/mL ± 10.2 (range 10.8 to 62.3). No subject had a Vitamin D_3 outside of the normal range as defined by the analytical laboratory. Mean serum OC concentration was 25.4 ng/mL ± 4.65 (range 14.71 to 35.57). Mean urinary DPD concentration was 20.38 ± 5.96 nmol/mmol creatinine (range 9.02 to 41.80). There was a significant difference between males and females only for serum OC (t-test, $p \leq 0.05$) with females having higher serum OC concentrations.

Nutrient intakes are summarized by gender and for all children in Table 4.2. There were no differences by gender. Mean caloric intake was 2180 ± 545 (range 1123 to 3152). Mean protein intake was 79 g ± 21 (range 37 to 124), representing a mean protein intake of 2.94 g/kg body weight. Total mean intake of Ca was 1382 mg ± 581 (range 253 to 2715).

Based on the activity counts as measured by the activity monitor, energy expenditure above calculated basal energy expenditure (BEE) (Harris and Benedict, 1919) is estimated as summarized in Table 4.1. Total daily energy expenditure (DTEE) above BEE for these subjects was 562 ± 217 kcals (range 281 – 1120). There was no difference in DTEE above BEE by gender.

In exploring the relationships of the measured variables influencing bone mineralization with the dependent variables of BMD and BMC, several significant correlations were found as summarized in Table 4.3. Percent body fat had a strong, significant positive relationship to both BMD ($r=0.535$, $p\leq0.01$) and BMC ($r=0.646$, $p\leq0.01$). OC by height showed a significant negative correlation to BMD ($r=-0.507$, $p\leq0.01$) and to BMC($r=-0.499$, $p\leq0.01$). Neither serum 25(OH) Vitamin D_3 nor urinary

Table 4.1

Physical characteristics, measures of bone mineralization, and biomarkers

Variable		Gender		
		Male (n=27)	Female (n=25)	Total (n=52)
Age	Mean ± SD	7.4 ± 1.4	7.8 ± 1.9	7.6 ± 1.7
	Range	5.6 - 11.1	5.1 - 11.9	5.1 - 11.9
BMI	Mean ± SD	17.3 ± 2.5	16.3 ± 1.9	16.8 ± 2.3
	Range	14.7 - 23.9	13.7 - 20.6	13.7 - 23.9
Weight (kg)	Mean ± SD	26.3 ± 5.9	27.5 ± 7.6	26.9 ± 6.8
	Range	17.5 - 43.7	18.2 - 47.0	17.5 - 47.0
Height (cm)	Mean ± SD	128.4 ± 13.5	124.8 ± 9.9	126.5 ± 11.8
	Range	105.2 - 168.5	107.25 - 141.0	105.2 - 168.5
Fat mass (kg)	Mean ± SD	6.0 ± 4.8	6.0 ± 3.4	6.0 ± 4.1
	Range	2.1 - 18.8	2.8 - 16.4	2.1 - 18.8
DTEE above BEE (kcals) [1]	Mean ± SD	609 ± 225	512 ± 200	562 ± 217
	Range	323 - 1120	281 - 973	281 - 1120
BMD (g/cm^2)	Mean ± SD	0.885 ± 0.062	0.837 ± 0.060	0.847 ± 0.061
	Range	0.764 - 1.003	0.751 - 0.980	0.751 - 1.003
Z-score (Lunar) [2]	Mean ± SD	0.31 ± 0.64	-0.04 ± 0.59	0.14 ± 0.64
	Range	-0.60 - 1.80	-1.20 - 1.20	-1.20 - 1.80
BMC (g)	Mean ± SD	960 ± 262	933 ± 229	947 ± 245
	Range	618 - 1,657	567 - 1,514	567 - 1,657
Serum OC [2,3] (ng/mL)	Mean ± SD	23.82 ± 4.39	26.98 ± 4.43	25.40 ± 4.65
	Range	14.71 - 35.57	17.42 - 33.41	14.71 - 35.57
Urinary DPD (nmol/mmol creatinine)	Mean ± SD	20.00 ± 5.83	20.79 ± 6.20	20.38 ± 5.96
	Range	10.81 - 41.80	9.02 - 35.83	9.02 - 41.80
Serum 25(OH) Vitamin D_3 (ng/mL) [4]	Mean ± SD	30.4 ± 9.4	29.5 ± 11.3	30.0 ± 10.2
	Range	13.8 - 57.5	10.8 - 62.3	10.8 - 62.3

[1] For this variable, n=26 (males), n=25 (females), n=51 (total)
[2] Significant difference between male and female subjects per t-test ($p \leq 0.05$)
[3] For this variable, n=25 (males), n=25 (females), n=50 (total)
[4] For this variable, n=26 (males), n=23 (females), n=49 (total)
BMI: Body Mass Index BMD: Bone Mineral Density BMC: Bone Mineral Content
OC: Serum osteocalcin DPD: Urinary deoxypyridinoline
DTEE above BEE: Daily Total Energy Expenditure above Basal Energy Expenditure (BEE estimate per Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

Table 4.2

Intake of key bone-building nutrients per day measured by usual, one-day dietary recall[1]

Variable		Gender		
		Male (n=27)	Female (n=25)	Total (n=52)
Total kcals	Mean ± SD	2,219 ± 507	2,138 ± 591	2,180 ± 545
	Range	1,123 - 3,152	1,167 - 3,115	1,123 - 3,152
Protein (g)	Mean ± SD	79 ± 22	79 ± 20	79 ± 21
	Range	37 - 124	48 - 113	37 - 124
Ca (mg)	Mean ± SD	1,343 ± 669	1,424 ± 479	1,382 ± 581
	Range	253 - 2,715	790 - 2,505	253 - 2,715
P (mg)	Mean ± SD	1,584 ± 505	1,565 ± 442	1,575 ± 471
	Range	645 - 2,766	1,001 - 2,531	645 - 2,766
Mg (mg)	Mean ± SD	327.4 ± 97.4	296.1 ± 85.0	312.4 ± 92.1
	Range	104.5 - 539.2	158.4 - 486.2	104.5 - 539.2

[1] Intakes by recall do not include supplements.

DPD by height showed any correlation to BMD or BMC. Daily total energy expenditure had a strong, significant positive correlation to both BMD ($r=0.459$, $p\leq0.01$) and BMC ($r=0.591$, $p\leq0.01$). Protein, expressed relative to body weight, had strong, significant ($p\leq0.01$) negative relationships to both BMD, $r=-0.508$, and BMC, $r=-0.564$. Due to the wide range of subjects' body sizes and reported intakes, intake of Ca was expressed as mg/day/cm height. A significant negative association was found between Ca by height and BMD ($r=-0.277$, $p\leq0.05$). There was no correlation between Ca intake/cm height and BMC. Significant correlations ($p\leq0.01$) were also found for kcal intake per kg body weight (for BMD, $r=-0.510$, and for BMC $r=-0.578$) and for mg phosphorus intake per day by cm height (for BMD, $r=-0.378$, and for BMC, $r=-0.348$). No significant correlation to BMD or BMC was found with other nutrients (data not shown).

Table 4.4 and Table 4.5 report final multiple regression analyses predicting BMD and BMC from measured variables influencing bone growth in this population of prepubertal children. Independent variables found to have a significant correlation and those with a strong association to bone growth from the literature were entered in a stepwise manner (DTEE, % body fat, protein per kilogram body weight, Ca per centimeter height) as were the biomarkers serum OC, urinary DPD and serum 25(OH) Vitamin D_3. Though four nutrients had significant correlations to BMD and BMC, protein and kilocalories as well as Ca and phosphorus have collinear relationships that would influence the regression analyses given the limited nutrients included. Both protein and Ca have been shown previously in the literature to have a strong association to bone mineralization thus only protein and Ca were entered in the regression analyses.

Table 4.3

Correlations of BMD and BMC with variables influencing bone mineralization

Pearson Correlation	BMD (g/cm^2)	BMC (g/cm)
% body fat (N = 52)	0.535**	0.646**
Protein intake by weight[1] (g/kg) (N = 52)	-0.508**	-0.564**
Ca intake by height[1] [(mg/d)/cm] (N = 52)	-0.277*	-0.260
DTEE above BEE (N = 51)	0.459**	0.591**
OC by height ((ng/mL)/cm) (N = 50)	-0.507**	-0.499**
DPD by height ((nmol/mmol Cr)/cm) (N = 52)	-0.245	-0.204
Serum 25(OH) Vitamin D_3 (ng/mL) (N = 49)	0.028	-0.066

** Pearson correlation significant at the 0.01 level (2-tailed).
* Pearson correlation significant at the 0.05 level (2-tailed).
[1] All nutrient intakes as assessed by recall
BMD: Bone Mineral Density
BMC: Bone Mineral Content
OC: Serum osteocalcin
DPD: Urinary deoxypyridinoline
Cr: Creatinine
DTEE above BEE: daily total energy expenditure above basal energy expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

The final model for predicting BMD includes percent body fat, serum OC, urinary DPD, serum 25(OH) Vitamin D_3, and DTEE above BEE. In this model, the biomarkers OC and DPD relative to height were significant negative predictors of BMD. The associated relationships of all of these variables explain 58.9 % of the variability in BMD. The final model for predicting BMC includes percent body fat, serum OC, urinary DPD and DTEE above BEE, with the association among these variables explaining 64.9% of the variability in BMC. The B coefficient is indicative of the relative importance of an independent variable in contributing to the dependent variable in a given prediction model. Serum OC by height ($B=-0.435 \pm 0.165$, $p=0.012$), and urinary DPD by height ($B=-0.262 \pm 0.131$, $p=0.054$) are the strongest, albeit negative, predictors of BMD. Serum OC by height ($B=-1528.178 \pm 620.868$, $p=0.018$), and urinary DPD by height ($B=-936.158 \pm 492.864$, $p=0.064$) are also the strongest predictors of BMC. The correlation between actual BMC and actual BMD and their values as predicted by each model are illustrated in Figure 4.1 and Figure 4.2. The prediction models show good agreement with the actual data, R^2 for the BMD model being 0.5295 and R^2 for the BMC model being 0.6181.

DISCUSSION

Studies of bone growth in early childhood consider the combined effects of modifiable influences on bone growth including body composition, physical activity, and diet due to the potential interaction of all of these determinants of bone health. Much of the available literature investigating bone growth in childhood is difficult to interpret

Table 4.4

Multiple regression model predicting BMD as a function of % body fat, serum OC by height, urinary DPD by height, serum 25(OH) Vitamin D_3, and DTEE above BEE

Predictors	B
% body fat	0.003[1]
OC by height ((ng/mL)/cm)	-0.435[2]
DPD by height ((nmol/mmol Cr)/cm)	-0.262[3]
Serum 25(OH) Vitamin D_3 (ng/mL)	0.001[4]
DTEE above BEE (kcals)	0.00007134[2]
Constant	0.843[1]
R^2 =	58.9%

[1] $p < 0.01$
[2] $p < 0.05$
[3] $p = 0.054$
[4] $p = 0.072$
BMD: Bone Mineral Density
OC: Serum osteocalcin
DPD: Urinary deoxypyridinoline
Cr: Creatinine
DTEE above BEE: Daily Total Energy Expenditure above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

Variables entered into the regression model (stepwise): protein intake by weight, calcium intake by height, % body fat, serum OC by height, urinary DPD by height, serum 25-OH Vitamin D_3, DTEE above BEE.

Table 4.5

Multiple regression model predicting BMC as a function of % body fat, serum OC by height, urinary DPD by height, and DTEE above BEE

Predictors	B
% body fat	12.054[1]
OC by height ((ng/mL)/cm)	-1528.178[2]
DPD by height ((nmol/mmol Cr)/cm)	-936.158[3]
DTEE Above BEE (kcals)	0.377[1]
Constant	948.689[1]
R^2 =	64.9%

[1] $p < 0.01$
[2] $p < 0.05$
[3] $p = 0.064$
BMC: Bone Mineral Content
OC: Serum osteocalcin
DPD: Urinary deoxypyridinoline
Cr: Creatinine
DTEE above BEE: Daily Total Energy Expenditure above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

Variables entered into the regression model (stepwise): protein intake by weight, calcium intake by height, % body fat, serum OC by height, urinary DPD by height, serum 25(OH) Vitamin D_3, DTEE above BEE.

because study populations often include both prepubertal and pubertal children (Zamboni et al., 1988; Rauch et al., 1994; VandenBergh et al., 1995; Molgaard et al., 1997; Carter et al., 2001; Iuliano-Burns et al., 2003). The aim of the present study was to examine the association of BMD and BMC with protein and Ca intake, body composition, physical activity, and biomarkers of bone metabolism in order to better understand their relationship to and influence on bone growth during childhood, specifically the period between 5 years of age and puberty.

In these prepubertal subjects, gender differences were not apparent for any of the variables except serum OC by cm height and DXA Z-score. The literature reports inconsistencies related to gender differences in bone mineralization in prepubertal children. Some studies have seen gender differences (Specker et al., 1987; Bell et al., 1991; Horlick et al., 2000). Many studies found no gender differences (Geusens et al., 1991; Patal et al., 1992; Fassler and Bonjour, 1995; Maynard et al., 1998; Nguyen et al., 2001). As in this study, most of these studies adjusted for height or some other measure that accounted for body or bone size. Because of the similarities between the genders of the HKNS subjects for other bone-related variables measured, gender was not entered into the prediction models.

All of the HKNS children had Z-scores for BMD within one standard deviation of the mean. Reference norms for BMD and BMC contribute to determination of Z-scores specific to the equipment on which BMD is measured (Wacker and Barden, 2001). This measure places children in reference to other children in the population. The referent populations of children with whom these subjects are compared are drawn from the same larger population that is, itself, the population for whom osteoporosis is thought will

Figure 4.1

Correlation between actual BMD and predicted value of BMD based on % body fat, serum OC by height, urinary DPD by height, serum 25(OH) Vitamin D_3 and DTEE above BEE

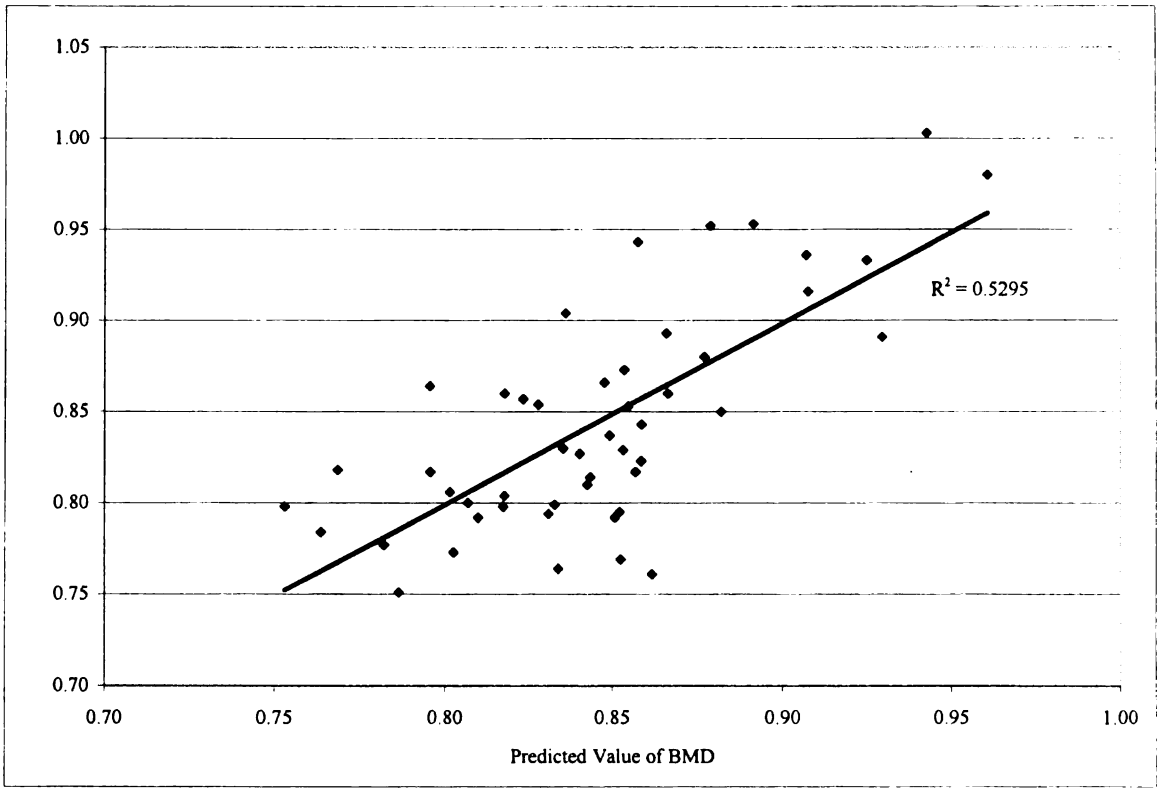
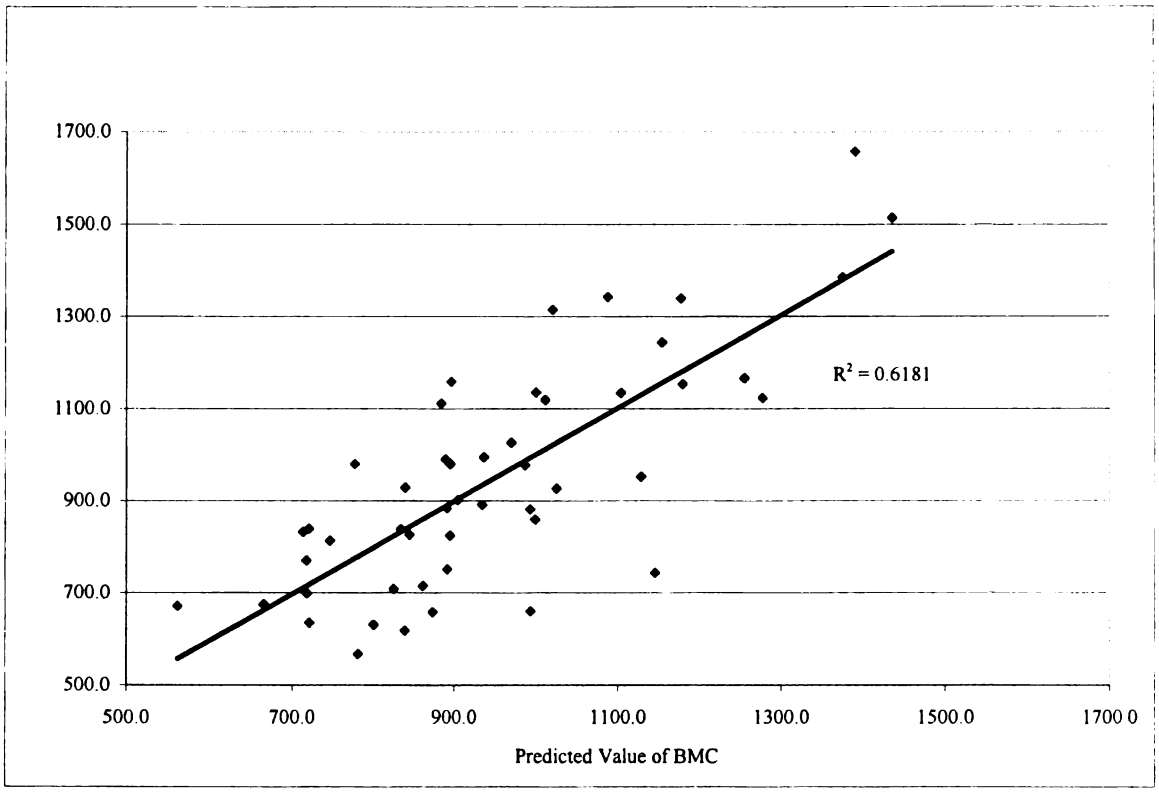


Figure 4.2

Correlation between actual BMC and predicted value of BMC based on % body fat, serum OC by height, urinary DPD by height, and DTEE above BEE



reach epidemic proportions. Thus, Z-scores do not necessarily indicate that optimal progress toward peak bone mass is occurring. If osteoporosis is a pediatric disease with geriatric consequences, lower Z-scores may indicate increased risk. Given that 31% of the children had Z-scores below negative 0.2, some children may not be making satisfactory progress toward attaining peak bone mass within their genetic potential.

Body composition in children has been positively related to measures of bone mineralization. Fehily et al. (1992) and Specker et al. (2001) saw a positive association with total body weight. Pietrobelli et al. (2002) and Ferretti et al. (1998) saw a positive relationship with lean mass. Ilich et al. (1998) found body fat to be a positive predictor of bone mass. In adults, in general, increased body weight predicts increased bone density (Pietrobelli et al, 2002). These 52 subjects had a wide range of heights and weights with some of the younger children being bigger than some of the older children; however, all subjects were within the range of normal growth for age falling within the 5^{th} and 95^{th} percentiles on the NCHS growth charts (NCHS, 1996). Lean mass could have been used to explore the relationship of body composition to bone mineralization as lean mass is considered to reflect muscle mass. Through the mechanical load of muscle's action on the skeleton, muscle can influence greater bone mineralization. Both muscle and organ tissues are included in the lean mass estimate provided via whole body DXA. Height, weight, and lean mass have strong collinear relationships to bone mass and to each other, as do weight and fat mass, complicating the choice of a single body composition measure to use to predict bone mineralization. Percent body fat had strong, positive correlations to BMD and BMC, was least complicated by collinear relationships, and, from a clinical perspective, can be estimated through simple anthropometric

measures and is modifiable through lifestyle habits. For these reasons, percent body fat was used in the models to predict BMD and BMC. Body fat appeared to have a beneficial effect on bone mineralization in prepubertal children in that a higher percent body fat, within the range of normal, predicts a higher BMD, whereas, in this model, a low percent body fat, one still within the normal range, contributes to predicting a lower BMD and BMC.

This group of children was a well-nourished group of subjects with mean intakes of most nutrients at or above the US RDA (FNB/IOM, 1997; 2005). Mean energy intake was 81-kcals/kg mean body weight, which is within the range of normal for healthy children of this age (FNB/IOM, 2005). The US RDA for protein in early childhood is 0.95 grams/kilogram body weight (FNB/IOM, 2005). Protein intake in this subject population was 3.1 times greater than the DRI recommendation. The strong negative correlation of this protein intake with BMD and BMC differs from findings of recent studies in children within this age range (Ilich et al., 1998; Bounds et al. 2005) and is, to our knowledge, the only such negative correlation seen to date in a group of children. Given the high protein intake of these children, the strong negative relationship of bone mineralization to dietary protein merits further exploration.

Ca intake in this group was at or above the US Adequate Intake (AI) of 800 mg/day for most of the subjects. Only 8 children consumed less than 800 mg Ca/day; none had excessive intake. These children differ from national samples that predict less than 40% of children in the US between 6 and 11 years of age achieve the AI for Ca (Greer and Krebs, 2006). The high mean Ca intake of the HKNS subjects, 1300 mg/day, is in the range of total Ca intake that is generally provided in supplementation trials that,

on the whole, do increase bone mass during the period of supplementation (Abrams, 2005). However, as with protein, Ca intake relative to height had a significant negative correlation with BMD, though not with BMC. This negative correlation was likely related to the collinear relationship of protein and Ca in that the highest source of protein and the highest source of Ca were single item dairy foods such as milk, chocolate milk, yogurt and cheese (unpublished data).

On an individual nutrient basis, protein and Ca were more than adequate in the diets but both protein and Ca had an inverse relationship to BMD and BMC. When, however, the association of these nutrients, expressed relative to body and bone size, was examined through the multivariate regression process, they did not remain in the model as significant dietary predictors of BMD and BMC.

Evaluation of the literature in the area of biomarkers in children is influenced by the need to recognize that much of the available data mixes pre-pubertal and pubertal population of children. OC concentrations in the HKNS subjects represent OC values for a healthy population of prepubertal children. In normal, healthy children, OC concentrations can be expected to increase with age and body size until puberty, then gradually decline to low adult levels (Tommasi et al., 1996; Bonofiglio et al., 2000). Several studies have measured OC concentrations in normal children of varying and increasing ages and found OC correlated weakly with BMD (Glastre et al., 1990; Tommasi et al., 1996). Slemenda et al. (1997) documented changes in OC concentrations in prepubertal and pubertal subjects with OC peaking at Tanner I then beginning a decline. Fares et al. (2003) noted an increase in OC in boys and girls that peaked at pubertal Tanner III then began a decline. Van Coeverden et al. (2002) documented

significant positive correlations between OC and BMC at several sites in a group of peripubertal Dutch children. Conversely, consistent with the prepubertal HKNS subjects, Mora et al. (1999) observed an inverse association between OC and BMD in children at varying stages of puberty. Slemenda et al. (1997) also noticed an inverse relationship between OC and BMD in a group of Ca-supplemented children. Collectively, though these observations are not always consistent, the potential role of OC in the assessment of bone growth in children as a marker for bone mineralization is supported.

In association with other independent variables, urinary DPD was a significant predictor of BMD and BMC. A few studies have looked at DPD as a marker of bone mineralization in children. Initial efforts to establish reference levels have been reported (Rauch et al., 1994; Lieuw-A-Fa et al., 1995). Rauch et al. (1994) as well as Bollen and Eyre (1994) report that DPD levels are highly correlated with growth velocity in normal children. Mora et al. (1999) looked specifically at DPD in relation to Tanner stage of development. They found DPD peaking at Tanner II then declining with a positive relationship to bone volume rather than to bone density per se. Van Coeverden et al. (2002) documented significantly positive correlations between DPD and BMC at several sites in a group of peripubertal Dutch children. In general, DPD concentrations will increase with age until puberty and then will begin to decline to a sustained low adult concentration (Rauch et al., 1994; Acil et al., 1996). In the HKNS subjects, urinary DPD, expressed per cm height, did not show a significant correlation to BMD or BMC on its own but was entered into the regression model to predict BMD and BMC. In association with other independent variables, urinary DPD per cm height was a significant predictor

of both BMD and BMC and may have potential as a biomarker for bone mineralization in children.

Public health concerns about adequate Vitamin D status have been raised in various populations including the elderly, postmenopausal women, and children in northern climes (Norman and Henry, 2006). Docio et al. (1998) and Lips (2004) suggest that a minimum desirable level of serum 25(OH) Vitamin D_3 in children should be 20 ng/mL or higher with slightly lower levels acceptable when Ca intake is high. The mean serum level of 25(OH) Vitamin D_3 in the HKNS subjects was 30.0 ng/mL. Seven subjects, or 16% of the group, had levels at or below 20 ng/mL. Like DPD, serum Vitamin D did not show a significant correlation to BMD or BMC on its own but was entered into the regression model due to its potential for an association through interactions with other variables as well as its overall importance in bone metabolism. In association with the other variables, 25(OH) Vitamin D_3 appears to have a significant positive relationship to BMD in these prepubertal children.

Physical activity is acknowledged to be beneficial to bone health. However, the role physical activity plays in the accrual of peak bone mass and its interactions with other factors influencing bone growth in the early childhood years is less clear. In general, children are less physically active now than in the past. This decrease is estimated as a decline of approximately 600 kcals/day over the past 50 years (Boreham and Riddoch, 2001). Previous studies in prepubertal children documented increases in bone mineralization in response to physical activity (Slemenda et al., 1994; VandenBergh et al., 1995). In a group of even younger children than these in the HKNS, children age 3- to 5- years, Specker and Binkley (2003) observed that physical activity was beneficial

to bone mineralization only at higher levels of Ca intake. Molgaard et al. (2001) and Iuliano-Burns et al. (2003) saw increased BMC in groups of girls who exercised and had higher Ca intakes, though only some of the girls were prepubertal. When energy expenditure was considered along with other factors in bone mineralization in the prediction model, DTEE remained significant as a positive predictor of BMC and BMD. Physical activity must be considered in understanding the interrelationships among factors predicting bone mineralization of children of this age. This relationship is especially pertinent given the downward trend in children's physical activity over the past 50 years.

Based on the relationships of measured variables to BMD and BMC, two prediction models were developed. Expressing the variables OC, DPD, and Ca relative to bone size is most appropriate in that the independent variables measured are being examined for their effect on bone metabolism. These prediction models, developed from a small cross-section of children in the Midwest, suggest that risk for lower BMD can be predicted from an anthropometric measure (percent body fat), two blood tests (OC and 25 (OH) Vitamin D_3), urinary DPD, and an estimate of physical activity. Low BMC can be predicted from serum OC, urinary DPD, an anthropometric measure of body fat, and an estimate of physical activity. DPD can also be measured in the serum so potentially a urine sample would not be needed.

Thirty percent of the HKNS children had Z-scores that identify them as having BMDs and BMCs in the lower range of normal and possibly already at risk of poor progress toward attainment of peak bone mass. Public health efforts with children currently promote healthy body weights through diet and physical activity in order to

decrease the risk of overweight and of weight-related chronic diseases. The physical activity focus will also promote bone health, which our study supports. Initial screening and assessment of bone status may be possible through an equation that considers key variables. Decreasing the risk of osteoporosis should begin in childhood with assessment and intervention that promotes optimal bone mass.

CHAPTER FIVE

CHAPTER FIVE

Strengths, Limitations, and Implications

The Healthy Kids Nutrition Study is unique in its simultaneous consideration of many of the variables that influence bone mineralization, joining a small body of research conducted exclusively on prepubertal children of elementary school age. Many factors are known to influence bone mineralization. Because of potential interactions among these factors it is important to study as many of them together as possible. This study was designed to measure these variables as accurately and precisely as possible. The subjects were carefully screened for health and prepubertal status. DXA determined body composition as well as bone mineral measures. Physical activity was measured over several days with a validated activity monitor. An experienced, registered dietitian assessed diets using careful interview methods and a validated FFQ. Experienced, qualified laboratory personnel conducted the serum and urinary measures. The design of the study allows for additional data to be gathered either with additional children or with these same children over time.

Though sufficient numbers of subjects were recruited to allow us to detect differences in bone mineralization, this was a convenience sample of Caucasian children from the U.S. Midwest thus limiting the generalizability of the results. As a cross sectional study, these data can drawn associations but does not suggest causality. Most of the children appeared to be of similar middle-class socioeconomic status and there were several groups who, through word of mouth recruitment, attended the same school and even the same class. Regional similarities in food markets, recreation opportunities, and

weather also contribute to the homogeneity of the group. Differences within the subjects could be detected when the continuous variables examined but once groups were divided through post hoc categorization, some of the groups were too small to allow for the groups to be compared, for example low versus high calcium intake.

This work contributed to the literature examining variables influencing bone mineralization. It supports the possibility that "at risk" children can be identified in a public health or clinical setting. Lifestyle factors that can be promoted to improve bone health have also been suggested. Protein intake in excess of the DRI appears to negatively impact bone in these children. Calcium intake, considered by height instead of chronological age, predicts bone mineralization differently than just total daily calcium intake, suggesting that height may need to be considered in assessing adequacy of calcium intake. Vitamin D concentrations were within the range of normal but higher levels did predict higher bone mass, and concentrations were lower than what some recent studies suggest is optimal, indicating a need to look further into Vitamin D status in Midwestern children. These children were all of normal weight. Even within this range of normal weights, controlling for height, the children with a higher percent body fat tended to have higher bone mass suggesting that body composition within the normal range can influence bone health. Finally, this study reports serum OC and urinary DPD levels for a group of healthy, prepubertal children. Osteocalcin showed a strong association with BMD raising the potential for its use as a biomarker to follow bone mineralization, especially in high-risk children.

The findings of this study related to relationships between diet and bone mineralization suggest that perhaps requirements for bone building nutrients in children

should be based body size rather than on age. If requirements are based solely on age, nutrition recommendations and interventions for conditions such as obesity may result in compromised linear growth or compromised bone mineralization. Caloric limitations usually suggested to treat overweight children could increase the risk of poor bone mineralization if there is a concurrent, proportional decrease in intake of bone building nutrients. When bone mineral content and BMD are calculated and then compared to age referenced normals, the larger body and bone size of the overweight child may not be properly assessed given that the reference values are age based. Likewise, children at the lower end of the growth curve may also be poorly assessed when compared to age referenced normals. In addition, the need to look at a variety of bone sites in order to get the best picture of bone growth in the preadolescent is borne out by the previous studies, each of which observed that use of overall BMC/age or BMD/age does not allow appropriate assessment of bone growth due to the differences in body composition and differences in the effect of physical activity relative to body composition on bone growth parameters (Fischer et al., 2000; Goulding et al., 2000; Hasanoglu et al., 2000; Molgaard et al., 2001.)

Future research in this area could include following this group of children in to puberty to see if their bone mineralization tracks into adolescence as has been suggested in the literature. The strong negative relationship to dietary protein merits further exploration. The low intake of fruits and vegetables along with the high protein intake in these children points the investigation in the area of dietary and metabolic acid base balance. In addition, this pilot group could be extended to include sufficient children to have at least 16 children in each post hoc group so additional relationships can be

explored. Similar studies with children of other races would be important as well as a comparison of groups with high and low intakes of protein, calcium and fruits and vegetables. Additional cross sectional as well as longitudinal studies are needed in this population in order to understand better how to keep children off the trajectory toward osteoporosis in their later years.

APPENDICES

MICHIGAN STATE
UNIVERSITY

Renewal Application Approval

January 18, 2008

To: Jenny BOND
204 Trout FSHN Bldg.

Re: **IRB# 03-1019** Category: EXPEDITED 2-8
Renewal Approval Date: January 14, 2008
Project Expiration Date: January 13, 2009

Title: EXAMINATION OF DIET, PHYSICAL ACTIVITY, BIOMARKERS OF BONE MINERALIZATION, BONE MINERAL CONTENT AND BODY COMPOSITION IN CHILDREN BETWEEN 5 YEARS OF AGE AND PUBERTY

The Institutional Review Board has completed their review of your project. I am pleased to advise you that **the renewal has been approved.**

This letter notes approval for data analysis only (contact with subjects and data collection is complete). Any further recruitment, data collection or contact with subjects will require IRB review and approval via a revision before implementation.

The review by the committee has found that your renewal is consistent with the continued protection of the rights and welfare of human subjects, and meets the requirements of MSU's Federal Wide Assurance and the Federal Guidelines (45 CFR 46 and 21 CFR Part 50). The protection of human subjects in research is a partnership between the IRB and the investigators. We look forward to working with you as we both fulfill our responsibilities.

Renewals: IRB approval is valid until the expiration date listed above. If you are continuing your project, you must submit an ***Application for Renewal*** application at least one month before expiration. If the project is completed, please submit an ***Application for Permanent Closure***.

Revisions: The IRB must review any changes in the project, prior to initiation of the change. Please submit an ***Application for Revision*** to have your changes reviewed. If changes are made at the time of renewal, please include an ***Application for Revision*** with the renewal application.

Problems: If issues should arise during the conduct of the research, such as unanticipated problems, adverse events, or any problem that may increase the risk to the human subjects, notify the IRB office promptly. Forms are available to report these issues.

Please use the IRB number listed above on any forms submitted which relate to this project, or on any correspondence with the IRB office.

Good luck in your research. If we can be of further assistance, please contact us at 517-355-2180 or via email at IRB@msu.edu. Thank you for your cooperation.

Sincerely,

Peter Vasilenko, Ph.D.
BIRB Chair

c: R. Taylor SCOTT
B209D W. Fee

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517-355-2180
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www.humanresearch.msu.edu
IRB@msu.edu

Appendix 2

Examination of diet, physical activity, biomarkers of bone mineralization, bone mineral content and body composition in children between 5 years of age and puberty

Consent to participate in a research study.

Please initial each section to indicate that you have read that section and have had your questions answered.

_____ This research is investigating the diet and nutritional needs of children relative to their body composition, bone development and physical activity patterns. Parents/legal guardians will be closely involved in all aspects of the study. This study is being conducted at Michigan State University in the Department of Food Science and Human Nutrition. The study experience will involve two (2) or three (3) appointments of 1-2 hours duration on the campus of Michigan State University and one appointment at Fiechtner Research on Jolly Road in the SE part of Lansing. Enrollment is for a six (6) month time frame.

Participants in this study must meet **three (3)** conditions.

1. Participants must be **5 years of age or older**.
2. Participants must be **prepubertal**
   (as determined by physician review of parental questionnaire).
3. Participants can be **any size or weight.**

You are being asked to involve your child in the following activities and procedures:

_____ **Complete a brief family health history.**
Health questions will be limited to issues related to the research question. Participants can decline to answer any question asked throughout the study on any topic.

_____ **Engage in an interview called a diet history with the study nutritionist.**

_____ **Complete a survey on usual food intake over time.**
This survey will be entered for computer analysis of nutrient intake and results will be given to the parent/guardian.

_____ **Complete a survey on usual physical activities.**
This survey will be entered for computer summary of usual physical activities.

_____ **Have height and weight measured.**
Equipment used is similar to that used in routine health care.

_____ **Wear a small physical activity monitor on a waist belt for 72 hours.**
The monitor is the size of a wristwatch and functions similarly to a car's acceleration detector and a pedometer that measures walking steps. It is worn under the clothes and the child will have the feeling of wearing a belt. The pedometer is water resistant and can be worn all 72 hours but may be removed for bathing if desired. The data recorded by the electronic monitor will be processed and decoded by computer. The results will be reported to the parent/guardian.

_____ **Provide one blood sample and one urine sample to measure Vitamin D and two compounds related to bone growth.**
Approximately one teaspoon of blood will be taken from the child's arm by a health care worker experienced in drawing blood from children using safe and sterile methods and equipment. The blood sample will be analyzed for Vitamin D and one measure of bone growth. The child will feel the discomfort of the needle when the blood is drawn. There may be some temporary bruising at the site of the blood draw. About 1/4 cup of a first morning urine sample, obtained by the child in the privacy of their home then kept cold until the study appointment, will be analyzed for the other marker of bone growth. The tests of bone growth are approved only for research use in children because they are newly developed and research is needed to begin to determine how to interpret these tests in children. Vitamin D levels will be reported to the parent/guardian.

_____ **Measurement of body fat, lean tissue and bone size on equipment known as a DEXA machine.**
The DEXA machine is familiar to many in its use to check the bone density of older women and men. This machine can measure bone and also fat tissue and lean tissue. Tests are done in a "child friendly" manner under the supervision of a specialist physician and a certified technician who check the equipment daily for proper functioning. The full body scan will take 2-4 minutes and will expose your child to 0.04 mRem of radiation, a very low dose exposure. In comparison, a television puts out 10.0 mRem of radiation over the course of a year while a typical air flight in North America exposes the passenger to a 40.0 mRem dose. A standard medical x-ray carries a 40.0 mRem dose. A full report of the body scan results will be provided to the parent/guardian.

_____ **Protection of your child's privacy**
All of the information collected will be confidential. To the maximum extent allowed by law, no information will be released without your consent. Once collected, the information about your child will be given an anonymous research code and then combined with the rest of the data. In case of an urgent need to contact the parent/guardian about any results, a coded ID card will be kept in a secure place, separate from the results and available only to the primary investigators and project manager.

_____ **Costs of participation**

Parents/guardians will need to transport children to two (2) study appointments and to one (1) appointment without the child. They may need to take time off of work to do so and may choose to hire a babysitter for siblings during that time. Every effort will be made to schedule appointments at the convenience of the family. Your child will receive a set of tests conducted at no cost to the family. Your participation in the study activities and procedures listed above will not involve any additional costs to you or your health care insurer.

_____ **Benefits and risks of participation to the parent/guardian and to the child**

Each child will receive:

A thorough diet analysis.
A summary of physical activity patterns.
Body composition measures (bone size, height, weight, % lean mass, % body fat)
Results of whole body scan, bone scan and Vitamin D blood test
Compensation of $100 divided to allow some compensation at each study visit
($25 at appointments one and two, $50 at appointment three).
Health information and/or referrals for area health programs.

We currently base diet recommendations on a child's age. It is possible that we should consider more than just age in determining the nutritional needs of children so that we can improve the health of this special group of children between 5 years and puberty. Your child's participation in this project will help us in determining these special needs.

As specified in the sections detailing activities and procedures, risks of participation include mild discomfort of the needle when blood is drawn and bruising at the site of the blood draw, very low dose radiation exposure with the DEXA procedure and inconvenience to the family for appointments, filling out surveys and collecting urine.

_____ **Your child's rights**

Participation in this study is entirely voluntary. Refusal to participate will involve no penalty or loss of benefits to which the child is otherwise entitled. The child may discontinue participation at any time without penalty or loss of benefits to which the child is otherwise entitled, including the partial compensation received through the time of withdrawal. No further compensation from the study will be awarded once participation is discontinued. Any results or information that are obtained which pose an immediate health concern will be brought to the parent/guardian's attention for referral to the child's primary care provider.

If your child is injured as a result of his/her participation in this research project, Michigan State University will assist you in obtaining emergency medical care, if necessary, for research related injuries. If your child has insurance for medical care, your child's insurance carrier will be billed in the ordinary manner. As with any medical insurance, any costs that are not covered or in excess of what are paid by your child's insurance, including deductibles, will be your responsibility. The University's policy is not to provide financial compensation for lost wages, disability, pain or comfort, unless required by law to do so. This does not mean that you are giving up any legal rights your child may have.

For questions or concerns about this study or to report a research-related injury, contact Jenny Bond, Ph.D., R.D., Primary Investigator, 517-676-2676, jbond@msu.edu. **For questions or concerns about research subjects' rights or a research-related injury**, contact Peter Vasilenko, Ph.D., Director of Human Research Protecions, 202 Olds Hall, MSU, East Lansing, Mi, 48824-1047, 517-355-2180, fax 517-432-4503, e-mail irb@msu.edu.

Your signature below indicates that you have read this informed consent and your questions, if any, have been answered by the researchers such that you agree to have your child participate in all described aspects of this research study.

_____

Signature of Parent/Guardian Date

_____

Signature of Child (if age appropriate) Date

_____

Child's verbal assent obtained - confirmation by parent

_____

Print Name of Child who is participating in the study

UCRIHS Approval

Appendix 3

Are you a parent of a child over 4 years of age?

Are you interested in helping your child keep his or her diet, physical activity and growth in a healthy balance?

Michigan State University
Department of Food Science and Human Nutrition
is conducting a study of healthy children, looking at relationships between nutrition, body composition, bone growth and physical activity.

WHO?
Children who are 5 years old and older and who have not yet entered puberty. Siblings welcome.

WHAT?
Measurements of the body such as height and weight will be taken. A whole body scan for bone growth and body composition will be done and there will be two blood samples taken. Physical activity patterns will be followed. All tests and assessments will be paid for by the study.

WHEN?
If qualified, children will be seen three or four times over 6 months for tests and data collection. Children can enter the study at any time during the enrollment period.

WHERE?
On and near the MSU campus.

BENEFITS?
Families will receive complete reports and explanations of all tests that will include physical activity details, results of the body scans and body measurements and complete diet nutrient analysis. Compensation of up to $100 is offered. All information is confidential..

INTERESTED?
If you have questions or would like to get more information:
Leave your name and number at (517)353-3037
OR
Send us an e-mail so we can contact you (msuhkns@hotmail.com)

Healthy Kids Nutrition Study
B 205 E West Fee Hall
Department of Family and Community Medicine
and Department of Food Science and Human Nutrition
Michigan State University
East Lansing, MI 48824
517-353-3037 leave a message
or e-mail msuhkns@hotmail.com

Appendix 4

PATIENT AUTHORIZATION FOR DISCLOSURE
OF HEALTH INFORMATION FOR RESEARCH

Patient Name: _____

Address: _____

Date of Birth: _____ **SS#:**___**not required**_____

I AUTHORIZE THE DISCLOSURE OF MY HEALTH INFORMATION

FROM:_Dr. Justus Fiechtner, MD___
Name of hospital or health care system or provider

Address
____3394 Jolly Road_____
___Lansing, MI 48910 _____

___517-272-9727_____
Phone/Fax Number

TO:_Healthy Kids Nutrition Study___
Name of researcher or research group

Address
____Dept of Food Science and Human Nutrition
__Michigan State University, East Lansing, MI 48824__

_____Study specific phone number added here__
Phone/Fax Number

DESCRIPTION OF INFORMATION TO BE DISCLOSED (select one):

___ALL information contained in my medical record.

OR

__X_ ONLY disclose the following information: Results of DEXA whole body scan

RESEARCH STUDY FOR THIS DISCLOSURE:

Title of Study: Examination of diet, physical activity, biomarkers of bone mineralization, bone mineral content and body composition in children between 5 years of age and puberty

Name of Research Leader:____Dr. Jenny T. Bond, PhD, RD and Dr. R. Taylor Scott, DO_____
Affiliation of Researcher:____Michigan State University_____
IRB#_____03-1019_____ Name of IRB_____same as project title_____

EXPIRATION (fill in one of the following):
Your Authorization to disclose the above information expires on___9 months after enrollment in the study (see consent date)__; or
Has no expiration date _____; or
Expires at the end of the research study _____; or
Expires six months from the date signed_____.

Appendix 4 continued

REVOCATION, REFUSAL, REDISCLOSURE:
You may revoke this Authorization in writing at any time by contacting the office of Dr. Fiechtner (see above) (e.g., the healthcare system or provider or hospital named above), but it will not affect any information already released to the researcher(s).

You may refuse to sign this authorization and your refusal will not affect your ability to obtain treatment, however, it may affect your ability to participate in this research study.

Your information that is disclosed to the researcher(s) may no longer be protected by Federal privacy regulations if the researcher(s) is not a health care provider covered by the regulations, however the researcher(s) agrees to protect your information as required by law.

_____

Signature of Patient or Personal Representative Date

_____

Name of Personal Representative and Relationship to Patient (or description of authority to act on behalf of the patient)

PROVIDE COPY TO PATIENT

Appendix 5

Healthy Kids Nutrition Study Health History Questionnaire

Child's Name:_____

Parent/Guardian providing this information:_____

A child's growth is determined by many factors including the child's health history as well as the health history of the child's family. Your answers to these questions about your child provide additional insight into your child's growth pattern.

The first page of questions confirm that your child is eligible to participate in this study based on his/her health history. The second set of questions provides information about your child's health and growth history as well as his/her family's health history. All information is confidential. Your child's name will be removed from this questionnaire and replaced with an anonymous identifier. Your answers to the first page of questions are required for participation in this study. Your answers to the second set of questions provide very important and helpful information but completion is not required for participation.

Child's Health History

Your child's date of birth:

_____

Your child's birth weight:

_____

How many weeks or days from the predicted birth due date was your child born?_____

Has your child been previously diagnosed with any medical condition?_____

Has your child been hospitalized for any medical condition?_____

Please describe and list approximate dates of diagnosis and treatment.

Has your child been seen by a doctor, nurse, nutritionist or any other health care professional for any kind of growth or nutritional problem?_____

Please describe and list the approximate dates of diagnosis and treatment.

Appendix 5 continued

Your child's pattern of growth
Circle the closest description for your child's growth from birth to today:

When your child was

0-12 months old	small for age	average for age	large for age
12-24 months old	small for age	average for age	large for age
2 - 3 years old	small for age	average for age	large for age
3 - 4 years old	small for age	average for age	large for age
4 - 5 years old	small for age	average for age	large for age
5 – 6 years old	small for age	average for age	large for age
6 – 7 years old	small for age	average for age	large for age
7 – 8 years old	small for age	average for age	large for age
9 – 10 years old	small for age	average for age	large for age
10 – 11 years old	small for age	average for age	large for age
11 – 12 years old	small for age	average for age	large for age

Family Health History - questions specifically related to growth and nutrition

Please list the child's family members who you consider to be overweight or underweight. You need not list names, rather list the relationship to the child, for example maternal grandmother/underweight.

Does anyone in the child's family have a history of weak bones? List their relationship and what you know about the bone problem. Also note the age of the family member when he/she developed the bone problem.

Does anyone in the child's family have a history of any kind of nutritional problems? List their relationship and what you know about the problem. Also note the age of the family member when he/she developed the nutritional problem.

Can you think of any other health issues related to hormones or metabolism, body size or bone growth in the child's family? Please list the family relationship and the health issue and age at which the health issue began, if known.

Appendix 6

Debriefing Protocol
Study Exit Interview Checklist

Study investigators will meet with each child and parent/guardian at the completion of the child's study enrollment. This meeting will be conducted in a confidential environment, allowing at least one-hour time. Written results and summaries will be provided in duplicate for the convenience of the parent/guardian in sharing with their child's primary health care provider. This interview will be conducted by a health care professional experienced in pediatric health counseling. At the meeting, the following topics will be covered:

1. Review of all dietary information obtained with summary and explanation of the nutrients assessed, giving special attention to inadequate and excessive intakes as compared to current dietary recommendations.

2. Review physical activity data, comparing activity records obtained with pediatric health guidelines for physical activity.

3. Review blood test results and interpret the results for the parent/guardian and child.

4. Review DEXA scan results and interpret the results for the parent/guardian and child.

5. Review and explain the anthropometric measurements obtained (height and weight). Measurements will be recorded on a pediatric growth chart provided for the parent/guardian's records.

6. Provide the parent/guardian with information on any health concerns and with referrals/contacts for health programs in which they may be interested. Special attention will be paid to addressing the parent/guardian and/or child's concerns, if any, related to any negative aspects of their body size, as well as concerns about growth, health or development and chronic disease risk or health concern of any kind.

7. Final study compensation payment will be dispersed as well as an additional small, age appropriate gift for the child.

SUBJECT ID _____

Standing Order

DEXA Total Body Scan (pediatric)

At the office of Justus Fiechtner, MD
3394 Jolly Road
Lansing, MI 48910
517-272-9727

Scan to be conducted in conjunction with the Healthy Kids Nutrition Study, MSU

Date:_____

Study PI: _____
R. Taylor Scott, DO

Appendix 8
Study Protocol Checklist

Date/initial each item as completed

Preliminary recruitment contact:

Initial qualifying contact:

Mail map/parking pass/health form:

First Study Appointment at Fee:

Subject contact information:

Review health questionnaire:

Review Tanner stages:

Child DOES meet study criteria

Child DOES NOT meet study criteria

Read/review/sign consent:

Read/review/sign HIPAA:

Obtain weight: _____ / _____

Obtain height: _____ /_____

Obtain usual food intake:

Provide parents to do list:

Instruct Food, Fitness, Fun time survey:

Instruct Eating survey (record #):

Instruct urine collection:

Make Fiechtner Research appointment:

Date/time:_____

Map to Fiechtner Research:

Activity monitor instructions:

Date for second appointment:

Date/time:_____

Give gift certificate #1 ($25):
Target or Meijer (circle)

Subject # _____

Second Study Appointment at Fee:

Drop off urine for DPD and creatinine:

Check cap seals and labeling of urine:

(Urine) To storage in Anthony:

Collect Eating survey:

Collect Fitness, Fun and Free time survey:

Review surveys for completeness:
FFQ:
Activity:

Confirm Fiechtner appointment:

Collect activity monitor:

Review monitor experience:

Give gift certificate #2 and #3 ($50):

After second appointment:

Download monitor data:

Sterilize monitor belt:

Third study appointment:
At Fiechtner Research

DEXA:

Serum for Vitamin D:

Serum for OC:

Gift certificate #3 ($25):

After third study appointment:

Vitamin D and OC to storage:

Fourth Study Appointment:
(OPTIONAL)

Debriefing per protocol:

Appendix 9
Nutrient intake as measured by usual, one-day dietary recall by age

Variable		Age		
		< 7.6 yrs (n=28)	> 7.6 yrs (n=24)	Total (n=52)
Total kcals	Mean ± SD	2,060 ± 595	2,319 ± 451	2108 ± 544
	Range	1,123 - 3,152	1,473 - 3,115	1,123 - 3,152
Protein (g)	Mean ± SD	74 ± 20	84 ± 19	79 ± 20
	Range	37 - 124	50 - 119	37 - 124
Cholesterol (mg)	Mean ± SD	189 ± 116	230 ± 131	208 ± 124
	Range	25 - 539	87 - 607	25 - 607
Carbohydrate (g)	Mean ± SD	289 ± 100	315 ± 60	301 ± 84
	Range	102 - 511	211 - 444	102 - 511
Fiber (g)	Mean ± SD	18.2 ± 8.7	17.3 ± 4.6	17. ± 7.1
	Range	2.0 - 41.0	10.0 - 28.0	2.0 - 41.0
Fat (g)	Mean ± SD	71.5 ± 26.3	84.7 ± 25.9	77.6 ± 26.7
	Range	31.5 - 171.3	34.3 - 131.5	31.5 - 171.3
Vit A (mcg RAE)	Mean ± SD	941 ± 458	1,070 ± 819	1,001 ± 646
	Range	271 - 1,753	152 - 4,359	152 - 4,359
Vit C[1] (mg)	Mean ± SD	85.0 ± 70.3	126.6 ± 76.8	104.2 ± 75.6
	Range	4.0 - 327.8	38.0 - 350.5	4.0 - 350.5
Vit E (mg)	Mean ± SD	6.7 ± 4.4	6.0 ± 2.3	6.4 ± 3.6
	Range	2.1 - 21.1	2.0 - 10.6	2.0 - 21.1
Ca (mg)	Mean ± SD	1,309.4 ± 555.7	1,467.5 ± 609.5	1,382.4 ± 580.9
	Range	259.9 - 2,505.0	661.0 - 2,715.0	252.9 - 2,715.0
P (mg)	Mean ± SD	1,509.7 ± 448.7	1,651.2 ± 493.9	1,575.0 ± 470.9
	Range	645.4 - 2,306.4	1,110.5 - 2,766.0	645.4 - 2,766.0
Mg (mg)	Mean ± SD	306.3± 104.3	319.5 ± 77.1	312.4 ± 92.1
	Range	104.5 - 539.2	219.6 - 486.2	104.5 - 539.2
Fe (mg)	Mean ± SD	16.2 ± 7.0	19.6 ± 8.8	17.7 ± 8.0
	Range	5.1 - 32.1	8.1 - 48.6	5.1 - 48.6
Zn (mg)	Mean ± SD	11.8 ± 3.4	13.1 ± 5.3	12.4 ± 4.4
	Range	3.9 - 17.0	6.4 - 28.7	3.9 - 28.7
K (mg)	Mean ± SD	2,702 ± 922	2,888 ± 920	2,788 ± 917
	Range	1,087 - 4,232	1,307 - 5,199	1,087 - 5,199
Se (mcg)	Mean ± SD	98.2 ± 41.1	103.1 ± 30.6	100.4 ± 36.3
	Range	36.3 - 215.0	52.7 - 188.8	36.3 - 215.0

[1] Significant difference between younger versus older subjects per t-test ($p \leq 0.05$)

Appendix 10
Nutrient intake as measured by food frequency questionnaire[1]
with and without supplements by age

Variable			Age		
			< 7.6 yrs (n=28)	> 7.6 yrs (n=24)	Total (n=52)
Total kcals		Mean ± SD	2313 ± 438	2244 ± 521	2281 ± 474
		Range	1385 - 3353	1265 - 3327	1265 - 3353
Protein (g)		Mean ± SD	97 ± 17	92 ± 24	95 ± 21
		Range	57 - 126	37 - 137	37 - 137
Animal protein in diet (g)		Mean ± SD	66 ± 16	62 ± 18	64 ± 17
		Range	38 - 98	25 - 106	25 - 106
Non-animal protein in diet (g)		Mean ± SD	31 ± 10	29.42 ± 9	30 ± 10
		Range	14 - 64	12 - 46	12 - 64
Cholesterol (g)		Mean ± SD	246 ± 62	251 ± 81	248 ± 71
		Range	94 - 325	111 - 388	94 - 388
Carbohydrate (g)		Mean ± SD	322 ± 72	299 ± 78	311 ± 75
		Range	165 - 460	157 - 461	157 - 461
Fiber (g)		Mean ± SD	20.5 ± 6.5	19.2 ± 6.9	19.9 ± 6.6
		Range	9.2 - 35.5	7.7 - 36.9	7.7 - 36.9
Fat (g)		Mean ± SD	74.5 ± 16.2	77.1 ± 20.3	75.72± 18.0
		Range	49.5 - 118.8	44.0 - 110.2	44.0 - 118.8
K (mg)		Mean ± SD	3287 ± 755	3036 ± 886	3171 ± 820
		Range	1299 - 4785	1060 - 4864	1060 - 4864
Vit A (mcg RAE)	With supp	Mean ± SD	8403 ± 4187	7883 ± 4398	8163 ± 4251
		Range	2194 - 24348	2045 - 24575	2045 - 24575
	Without supp	Mean ± SD	1009.2 ± 393.2	1035.7 ± 439.1	1021.4 ± 411.1
		Range	246.4 - 2204.2	317.2 - 2245.8	246.4 - 2245.8
Vit C (mg)	With supp	Mean ± SD	133.8 ± 54.6	124.5 ± 67.	129.5 ± 60.3
		Range	31.8 - 271.1	34.9 - 312.5	31.8 - 312.5
	Without supp	Mean ± SD	114.3 ± 46.8	111.9 ± 56.4	113.2 ± 50.9
		Range	26.2 - 210.7	34.9 - 262.1	26.2 - 262.1
Vit D (iu)	With supp	Mean ± SD	436 ± 168	363 ± 173	402 ± 172
		Range	45 - 863	48 - 604	45 - 863
	Without supp	Mean ± SD	337 ± 135	300 ± 143	320 ± 139
		Range	45 - 543	48 - 568	45 - 568
Vit E (mg)	With supp	Mean ± SD	8.6 ± 3.1	8.1 ± 3.0	8.4 ± 3.0
		Range	3.8 - 15.8	3.3 - 16.3	3.3 - 16.3
	Without supp	Mean ± SD	6.1 ± 1.4	6.7 ± 2.0	6.6 ± 1.7
		Range	3.2 - 10.0	3.3 - 10.0	3.2 - 10.0

Appendix 10 continued

Ca (mg)	With supp	Mean ± SD	1579.9 ± 433.0	1406.2 ± 396.5	1499.7 ± 421.7
		Range	426.2 - 2,577.2	610.1 - 1,964.0	426.2 - 2,577.2
	Without supp	Mean ± SD	1540.7 ± 429.1	1381.0 ± 397.6	1467.0 ± 418.6
		Range	426.2 - 2531.6	610.1 - 1,918.4	426.2 - 2.531.6
P (mg)	With supp	Mean ± SD	1842.0 ± 361.9	1709.6 ± 455.4	1780.9 ± 409.0
		Range	770.3 - 2559.2	725.2 - 2,576.2	725.2 - 2,576.2
	Without supp	Mean ± SD	1842.0 ± 361.9	1709.6 ± 455.4	1780.9 ± 409.0
		Range	770.3 - 2,559.2	725.2 - 2,576.2	725.2 - 2,576.2
Mg (mg)	With supp	Mean ± SD	345.7 ± 77.3	319.3 ± 88.4	333.5 ± 82.8
		Range	139.6 - 531.1	125.4 - 481.0	125.4 - 531.1
	Without supp	Mean ± SD	336.2 ± 73.6	313.0 ± 85.8	325.5 ± 79.5
		Range	139.6 - 511.1	125.4 - 469.6	125.4 - 511.1
Fe (mg)	With supp	Mean ± SD	21.96 ± 9.8	18.8 ± 6.7	20.4 ± 8.5
		Range	8.6 - 54.7	7.2 - 38.3	7.2 - 54.7
	Without supp	Mean ± SD	17.0 ± 6.9	15.6 ± 3.9	16.3 ± 5.7
		Range	8.6 - 44.7	7.2 - 23.7	7.2 - 44.7
Zn (mg)	With supp	Mean ± SD	17.7 ± 5.9	15.6 ± 5.0	16.72 ± 5.5
		Range	8.1 - 29.9	6.7 - 30.0	6.7 - 30.0
	Without supp	Mean ± SD	13.8 ± 3.6	13.0 ± 2.9	13.5 ± 3.3
		Range	8.1 - 26.1	6.6 - 18.9	6.6 - 26.1

[1] Youth and Adolescent Food Frequency Questionnaire, Harvard Channing Laboratory, Harvard University, School of Public Health, Boston, MA, USA

Appendix 11
Dietary intakes according to 2005 U.S. Dietary Guidelines by age[1]

Variable		Age		
		< 7.6 yrs (n=28)	> 7.6 yrs (n=24)	Total (n=52)
Grain intake[2]	Mean ± SD	7.6 ± 4.2	9.2 ± 2.8	8.3 ± 3.7
	Range	0.8 - 22.7	3.3 - 14.9	0.8 - 22.7
Milk intake[3]	Mean ± SD	3.2 ± 1.8	3.5 ± 1.8	3.4 ± 1.8
	Range	0.2 - 7.0	0.8 - 7.0	0.2 - 7.0
Meat and bean intake[2]	Mean ± SD	3.5 ± 2.1	3.9 ± 1.8	3.7 ± 1.9
	Range	0.1 - 9.4	0.9 - 6.2	0.1 - 9.4
Fruit and Vegetable intake[3]	Mean ± SD	1.9 ± 1.3	2.4 ± 1.3	2.2 ± 1.3
	Range	0.1 - 5.5	0.5 - 4.9	0.1 - 5.5

[1] 2005 U.S. Dietary Guidelines recommendations for children ages 2 to 8 years: Grain, 6 oz.; Milk, 2 cups; Meat and beans, 5 oz.; Fruit, 1.5 cups; and Vegetable 2.5 cups.
[2] In oz. equivalents
[3] In cup equivalents

Appendix 12
Site-specific measures of bone mineralization by gender

Variable		Gender		
		Male	Female	Total
BMD (g/cm^2)	Mean ± SD	0.885 ± 0.062	0.837 ± 0.060	0.847 ± 0.061
	Range	0.764 - 1.003	0.751 - 0.980	0.751 - 1.003
	N	27	25	52
Z-score[1] (Lunar)	Mean ± SD	0.31 ± 0.64	-0.04 ± 0.59	0.14 ± 0.64
	Range	-0.60 - 1.80	-1.20 - 1.20	-1.20 - 1.80
	N	27	25	52
BMC (g)	Mean ± SD	960 ± 262	933 ± 229	947 ± 245
	Range	618 - 1,657	567 - 1,514	567 - 1,657
	N	27	25	52
Arm BMD (g/cm^2)	Mean ± SD	0.601 ± 0.058	0.610 ± 0.049	0.605 ± 0.054
	Range	0.486 - 0.711	0.533 - 0.734	0.486 - 0.734
	N	27	24	51
Arm BMC (g)	Mean ± SD	84 ± 28	89 ± 32	86 ± 30
	Range	46 - 154	38 - 190	38 - 192
	N	27	24	51
Leg BMD (g/cm^2)	Mean ± SD	0.780 ± 0.117	0.785 ± 0.107	0.783 ± 0.111
	Range	0.571 - 1.052	0.639 - 1.067	0.571 - 1.067
	N	27	24	51
Leg BMC (g)	Mean ± SD	301 ± 127	302 ± 113	301 ± 119
	Range	136 - 593	134 - 598	134 - 598
	N	27	24	51
Pelvis BMD (g/cm^2)	Mean ± SD	0.754 ± 0.097	0.750 ± 0.081	0.752 ± 0.089
	Range	0.599 - 1.028	0.600 - 0.932	0.599 - 1.028
	N	27	24	51
Pelvis BMC (g)	Mean ± SD	97 ± 34	95 ± 28	96 ± 31
	Range	51 - 192	46 - 155	46 - 192
	N	27	24	51

[1] Significant difference between male and female subjects per t-test ($p \leq 0.05$)
BMD: Bone Mineral Density
BMC: Bone Mineral Content

Appendix 12 continued

Arm BMC (g)	Mean ± SD	84 ± 28	89 ± 32	86 ± 30
	Range	46 - 154	38 - 190	38 - 192
	N	27	24	51
Leg BMD (g/cm^2)	Mean ± SD	0.780 ± 0.117	0.785 ± 0.107	0.783 ± 0.111
	Range	0.571 - 1.052	0.639 - 1.067	0.571 - 1.067
	N	27	24	51
Leg BMC (g)	Mean ± SD	301 ± 127	302 ± 113	301 ± 119
	Range	136 - 593	134 - 598	134 - 598
	N	27	24	51
Pelvis BMD (g/cm^2)	Mean ± SD	0.754 ± 0.097	0.750 ± 0.081	0.752 ± 0.089
	Range	0.599 - 1.028	0.600 - 0.932	0.599 - 1.028
	N	27	24	51
Pelvis BMC (g)	Mean ± SD	97 ± 34	95 ± 28	96 ± 31
	Range	51 - 192	46 - 155	46 - 192
	N	27	24	51
OC[1] (ng/mL)	Mean ± SD	23.82 ± 4.39	26.98 ± 4.43	25.40 ± 4.65
	Range	14.71 - 35.57	17.42 - 33.41	14.71 - 35.57
	N	25	25	49
DPD (nmol/mmol Creatinine)	Mean ± SD	20.00 ± 5.83	20.79 ± 6.20	20.38 ± 5.96
	Range	10.81 - 41.80	9.02 - 35.83	9.02 - 41.80
	N	27	25	52
Serum 25-OH, Vit D (ng/mL)	Mean ± SD	30.40 ± 9.35	29.47 ± 11.29	29.98 ± 10.17
	Range	13.80 - 57.50	10.80 - 62.30	10.80 - 62.30
	N	25	21	46

[1] Significant difference between male and female subjects per t-test ($p \leq 0.05$)
BMI: Body Mass Index
BMD: Bone Mineral Density
BMC: Bone Mineral Content
OC: Serum osteocalcin
DPD: Urinary deoxypyridinoline

Appendix 13

Energy expenditure above BEE and activity counts at 3 levels of intensity[1] by age

Variable			Age		
			< 7.6 yrs (n=28)	> 7.6 yrs (n=24)	Total (n=52)
Daily energy expenditure	Total[2]	Mean ± SD	493 ± 172	639 ± 238	562 ± 217
		Range	281 - 973	328 - 1,120	281 - 1,120
	Light[2]	Mean ± SD	106 ± 26	145 ± 38	124 ± 37
		Range	71 - 164	94 - 234	71 – 243
	Moderate	Mean ± SD	202 ± 51	237 ± 110	218 ± 84
		Range	119 - 292	131 - 549	119 – 549
	Vigorous	Mean ± SD	189 ± 142	235 ± 119	210 ± 133
		Range	51 - 576	48 - 525	48 - 576
Daily total activity counts	Total	Mean ± SD	502914 ± 270079	449995 ± 137417	478572 ± 218843
		Range	194342 - 1120443	192744 - 698463	192744 - 1120443
	Sedentary	Mean ± SD	4165 ± 1028	4411 ± 880	4278 ± 961
		Range	2945 - 5892	1452 - 5617	1452 - 5892
	Light	Mean ± SD	44813 ± 30491	37450 ± 7388	41426 ± 23056
		Range	25024 - 189367	21841 - 51880	21841 - 189367
	Moderate	Mean ± SD	140410 ± 36704	120627 ± 35616	131310 ± 37196
		Range	70565 - 235209	86386 - 206384	70565 - 235209
	Vigorous	Mean ± SD	318256 ± 256267	278737 ± 125181	300078 ± 205617
		Range	71705 - 904875	58684 - 517570	56684 - 904875

[1] Measured over 72 hours with an Actical accelerometer (MiniMitter Corp, Bend, OR)

[2] Significant difference between younger versus older subjects per t-test ($p \leq 0.05$)

BEE: Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

Appendix 14

Energy expenditure above BEE and activity counts at 3 levels of intensity[1] by gender

Variable			Gender		
			Male (n=27)	Female (n=25)	Total (n=52)
Daily energy expenditure	Total	Mean ± SD	609 ± 225	512 ± 200	561 ± 217
		Range	323 - 1120	281 - 973	281 - 1120
	Light	Mean ± SD	124 ± 39	124 ± 36	124 ± 37
		Range	77 - 243	71 - 209	71 - 243
	Moderate	Mean ± SD	237 ± 101	199 ± 61	218 ± 84
		Range	119 - 549	129 - 351	119 - 549
	Vigorous	Mean ± SD	226 ± 127	195 ± 139	210 ± 133
		Range	73 - 252	48 - 576	48 - 576
Daily total activity counts	Total	Mean ± SD	502530 ± 197950	454618 ± 239583	478572 ± 218843
		Range	275700 - 1071662	192744 - 1120443	192744 - 1120443
	Sedentary	Mean ± SD	4207 ± 1019	4,349 ± 914	4,278 ± 961
		Range	1452 - 5790	2,495 - 5,892	1,452 - 5,892
	Light	Mean ± SD	37213 ± 6888	45639 ± 31636	41426 ± 23056
		Range	21841 - 47808	25024 - 189367	21841 - 189367
	Moderate	Mean ± SD	141381 ± 38866	121239 ± 33209	131310 ± 37196
		Range	87126 - 235209	70565 - 188864	70565 - 235209
	Vigorous	Mean ± SD	318685 ± 201836	281471 ± 211800	300077 ± 205617
		Range	91863 - 904875	58684 - 893740	58684 - 904875

[1] Measured over 72 hours with an Actical accelerometer (MiniMitter Corp, Bend, OR)

BEE: Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

Appendix 15
Correlations of BMD and BMC with variables influencing bone mineralization by gender

By Gender		BMD (g/cm^2)		BMC (g)	
		r	Sig.	r	Sig.
% body fat	Total (n=51)	0.535	0.000**	0.646	0.000**
	Male (n=27)	0.582	0.001**	0.714	0.000**
	Female (n=25)	0.545	0.005**	0.578	0.002**
DTEE above BEE (kcals)	Total (n=51)	0.459	0.000**	0.591	0.000**
	Male (n=26)	0.465	0.017*	0.645	0.000**
	Female (n=25)	0.423	0.035*	0.527	0.007**
Protein by weight (g/kg)[1]	Total (n=52)	-0.508	0.000**	-0.564	0.000**
	Male (n=27)	-0.476	0.012*	-0.523	0.005**
	Female (n=25)	-0.542	0.005**	-0.616	0.001**
kcals by weight (kcals/kg)[1]	Total (n=52)	-0.509	0.000**	-0.578	0.000**
	Male (n=27)	-0.670	0.000**	-0.680	0.000**
	Female (n=25)	-0.349	0.087	-0.460	0.021*
Ca by height [(mg/d)/cm][1]	Total (n=51)	-0.277	0.047*	-0.260	0.062
	Male (n=27)	-0.015	0.940	-0.009	0.963
	Female (n=25)	-0.614	0.001**	-0.625	0.001**
P by height (g/cm)[1]	Total (n=52)	-0.378	0.006**	-0.348	0.011*
	Male (n=27)	-0.206	0.301	-0.182	0.365
	Female (n=25)	-0.604	0.001**	-0.573	0.003**
Mg (mg)[1]	Total (n=52)	-0.135	0.341	-0.126	0.372
	Male (n=27)	-0.259	0.192	-0.233	0.241
	Female (n=25)	-0.042	0.8412	-0.003	0.990
K (mg)[1]	Total (n=52)	-0.137	0.331	-0.069	0.625
	Male (n=27)	-0.055	0.786	-0.018	0.930
	Female (n=25)	-0.265	0.200	-0.148	0.481
Fruit and Vegetable[2]	Total (n=52)	0.265	0.058	0.256	0.067
	Male (n=27)	0.131	0.514	0.161	0.422
	Female (n=25)	0.402	0.047*	0.376	0.064
RNAE	Total (n=52)	0.196	0.163	0.247	0.078
	Male (n=27)	0.174	0.387	0.308	0.118
	Female (n=25)	0.275	0.183	0.153	0.464

** Pearson correlation significant at the 0.01 level (2-tailed).
* Pearson correlation significant at the 0.05 level (2-tailed).
[1] All nutrient intakes as assessed by recall
[2] Intake per 2005 US Dietary Guidelines in cup equivalents
BMD: Bone Mineral Density
BMC: Bone Mineral Content

Appendix 15 continued

DTEE above BEE: Daily Total Energy Expenditure above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).
RNAE: Renal Net Acid Excretion as estimated by the method of Frassetto LA et al. Estimation of net endogenous noncarbonic acid production in humans from diet potassium and protein contents. AJCN 1998; 68:576-83.

Appendix 16

Correlations of BMD and BMC with variables influencing bone mineralization by age

By Age		BMD (g/cm^2)		BMC (g)	
		r	Sig.	r	Sig.
% body fat	Total (n=52)	0.535	0.000**	0.646	0.000**
	< 7.6 yrs (n=28)	0.373	0.050*	0.540	0.003**
	> 7.6 yrs (n=24)	0.594	0.002**	0.748	0.000**
DTEE above BEE (kcals)	Total (n=51)	0.459	0.000**	0.591	0.000**
	< 7.6 yrs (n=27)	-0.033	0.869	0.231	0.246
	> 7.6 yrs (n=24)	0.591	0.002**	0.670	0.000**
Protein by weight (g/kg)[1]	Total (n=52)	-0.508	0.000**	-0.564	0.000**
	< 7.6 yrs (n=28)	-0.440	0.019*	-0.572	0.001**
	> 7.6 yrs (n=24)	-0.541	0.006**	-0.607	0.002**
kcals by weight (kcals/kg)	Total (n=52)	-0.509	0.000**	-0.578	0.000**
	< 7.6 yrs (n=28)	-0.440	0.019*	-0.579	0.001**
	> 7.6 yrs (n=24)	-0.587	0.003**	-0.663	0.000**
Ca by height [(mg/d)/cm][1]	Total (n=51)	-0.277	0.047*	-0.260	0.062
	< 7.6 yrs (n=28)	-0.303	0.117	-0.329	0.087
	> 7.6 yrs (n=24)	-0.292	0.166	-0.307	0.144
P by height (g/cm)[1]	Total (n=52)	-0.378	0.006**	-0.348	0.011*
	< 7.6 yrs (n=28)	-0.400	0.035*	-0.428	0.023*
	> 7.6 yrs (n=24)	-0.383	0.065	-0.366	0.079
Mg (mg)[1]	Total (n=52)	-0.135	0.341	-0.126	0.372
	< 7.6 yrs (n=27)	-0.284	0.143	-0.348	0.07
	> 7.6 yrs (n=24)	-0.074	0.73	-0.084	0.697
K (mg)[1]	Total (n=52)	-0.137	0.331	-0.069	0.625
	< 7.6 yrs (n=28)	-0.076	0.701	-0.134	0.497
	> 7.6 yrs (n=24)	-0.285	0.177	-0.185	0.386
Fruit and Vegetable[2]	Total (n=52)	0.265	0.058	0.256	0.067
	< 7.6 yrs (n=28)	0.354	0.065	0.203	0.301
	> 7.6 yrs (n=24)	0.099	0.647	0.164	0.444
RNAE	Total (n=52)	0.196	0.163	0.247	0.078
	< 7.6 yrs (n=28)	0.125	0.525	0.282	0.145
	> 7.6 yrs (n=24)	0.185	0.386	0.137	0.525

** Pearson correlation significant at the 0.01 level (2-tailed).

* Pearson correlation significant at the 0.05 level (2-tailed).

[1] All nutrient intakes as assesed by recall

[2] Intake per 2005 US Dietary Guidelines in cup equivs

BMD: Bone Mineral Density

BMC: Bone Mineral Content

Appendix 16 continued

DTEE above BEE: Daily Total Energy Expenditure above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).
RNAE: Renal Net Acid Excretion as estimated by the method of Frassetto LA et al. Estimation of net endogenous noncarbonic acid production in humans from diet potassium and protein contents. AJCN 1998; 68:576-83.

Appendix 17
Correlations of BMD and BMC by height with variables influencing bone mineralization by gender

By Gender		BMD (g/cm^2)		BMC/ht (g/cm)	
		Pearson Correlation	Sig.	Pearson Correlation	Sig.
% body fat	Total (n=51)	0.535	0.000**	0.646	0.000**
	Male (n=27)	0.582	0.001**	0.729	0.000**
	Female (n=25)	0.545	0.005**	0.591	0.002**
Protein by weight (g/kg)	Total (n=52)	-0.508	0.000**	-0.603	0.000**
	Male (n=27)	-0.476	0.012*	-0.553	0.003**
	Female (n=25)	-0.542	0.005**	-0.685	0.000**
Ca by height[1] [(mg/d)/cm]	Total (n=52)	-0.277	0.047*	-0.265	0.058
	Male (n=27)	-0.015	0.94	-0.041	0.838
	Female (n=25)	-0.614	0.001**	-0.624	0.001**
DEE light above BEE (kcals)	Total (n=50)	0.626	0.000**	0.762	0.000**
	Male (n=25)	0.711	0.000**	0.855	0.000**
	Female (n=25)	0.541	0.005**	0.659	0.000**
DEE moderate above BEE (kcals)	Total (n=50)	0.567	0.000**	0.694	0.000**
	Male (n=25)	0.605	0.001**	0.738	0.000**
	Female (n=25)	0.514	0.009**	0.583	0.002**
DEE vigorous above BEE (kcals)	Total (n=50)	0.191	0.183	0.271	0.057
	Male (n=25)	0.138	0.51	0.286	0.165
	Female (n=25)	0.220	0.291	0.237	0.254
OC by height ((ng/mL)/cm)	Total (n=50)	-0.507	0.000**	-0.480	0.000**
	Male (n=25)	-0.419	0.037*	-0.313	0.128
	Female (n=25)	-0.568	0.003**	-0.653	0.000**
DPD by height ((nmol/mmol Cr)/cm)	Total (n=52)	-0.245	0.079	-0.180	0.201
	Male (n=27)	0.006	0.978	0.121	0.548
	Female (n=25)	-0.450	0.024*	-0.493	0.012*
Serum 25-OH, Vit D (ng/mL)	Total (n=49)	0.028	0.848	-0.028	0.849
	Male (n=26)	-0.116	0.573	-0.008	0.969
	Female (n=23)	0.165	0.452	-0.063	0.774
RNAE	Total (n=52)	0.196	0.163	0.247	0.078
	Male (n=27)	0.174	0.387	0.287	0.147
	Female (n=25)	0.275	0.183	0.240	0.249

** Pearson correlation significant at the 0.01 level (2-tailed).
* Pearson correlation significant at the 0.05 level (2-tailed).
[1] All nutrient intakes as assessed by recall
BMD: Bone Mineral Density
BMC: Bone Mineral Content

DEE Light/Moderate/Vigorous above BEE: Daily Energy Expenditure Light/Moderate/Vigorous above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).
OC: Serum osteocalcin
DPD: Urinary deoxypyridinoline
Cr: Creatinine
RNAE: Renal Net Acid Excretion as estimated by the method of Frassetto LA et al. Estimation of net endogenous noncarbonic acid production in humans from diet potassium and protein contents. AJCN 1998; 68:576-83.

Appendix 18
Correlations of BMD and BMC by height with variables influencing bone mineralization by age

By Age		BMD (g/cm^2) Pearson Correlation	BMD (g/cm^2) Sig.	BMC/ht (g/cm) Pearson Correlation	BMC/ht (g/cm) Sig.
% body fat	Total (n=52)	0.535	0.000**	0.646	0.000**
	< 7.6 yrs (n=28)	0.373	0.050*	0.508	0.006**
	> 7.6 yrs (n=24)	0.594	0.002**	0.726	0.000**
Protein by weight (g/kg)	Total (n=52)	-0.508	0.000**	-0.603	0.000**
	< 7.6 yrs (n=28)	-0.440	0.019*	-0.594	0.001**
	> 7.6 yrs (n=24)	-0.541	0.006**	-0.623	0.001**
Ca by height[1] [(mg/d)/cm]	Total (n=52)	-0.277	0.047*	-0.265	0.058
	< 7.6 yrs (n=28)	-0.303	0.117	-0.333	0.084
	> 7.6 yrs (n=24)	-0.292	0.166	-0.267	0.207
DEE light above BEE (kcals)	Total (n=52)	0.626	0.000**	0.762	0.000**
	< 7.6 yrs (n=27)	0.462	0.015*	0.647	0.000**
	> 7.6 yrs (n=23)	0.575	0.004**	0.702	0.000**
DEE moderate above BEE (kcals)	Total (n=52)	0.567	0.000**	0.694	0.000**
	< 7.6 yrs (n=27)	0.242	0.223	0.385	0.047*
	> 7.6 yrs (n=23)	0.647	0.001**	0.822	0.000**
DEE vigorous above BEE (kcals)	Total (n=52)	0.191	0.183	0.271	0.057
	< 7.6 yrs (n=27)	-0.230	0.248	-0.083	0.679
	> 7.6 yrs (n=23)	0.466	0.025*	0.517	0.011*
OC by height ((ng/mL)/cm)	Total (n=52)	-0.507	0.000**	-0.480	0.000**
	< 7.6 yrs (n=26)	-0.381	0.054	-0.419	0.033*
	> 7.6 yrs (n=24)	-0.561	0.004**	-0.447	0.029*
DPD by height ((nmol/mmol Cr)/cm)	Total (n=52)	-0.245	0.079	-0.180	0.201
	< 7.6 yrs (n=28)	-0.261	0.18	-0.239	0.22
	> 7.6 yrs (n=24)	-0.112	0.602	0.049	0.821
Serum 25-OH, Vit D (ng/mL)	Total (n=49)	0.028	0.848	-0.028	0.849
	< 7.6 yrs (n=27)	-0.062	0.759	-0.215	0.281
	> 7.6 yrs (n=22)	0.143	0.527	0.183	0.415
RNAE	Total (n=52)	0.196	0.163	0.247	0.078
	< 7.6 yrs (n=27)	0.125	0.525	0.255	0.191
	> 7.6 yrs (n=23)	0.185	0.386	0.186	0.384

** Pearson correlation significant at the 0.01 level (2-tailed).
* Pearson correlation significant at the 0.05 level (2-tailed).
[1] All nutrient intakes as assesed by recall
BMD: Bone Mineral Density
BMC: Bone Mineral Content

Appendix 18 continued

DEE Light/Moderate/Vigorous above BEE: Daily Light/Moderate/Vigorous Energy Expenditure above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).
OC: Serum osteocalcin
DPD: Urinary deoxypyridinoline
Cr: Creatinine
RNAE: Renal Net Acid Excretion as estimated by the method of Frassetto LA et al. Estimation of net endogenous noncarbonic acid production in humans from diet potassium and protein contents. AJCN 1998; 68:576-83.

Appendix 19

Multiple regression model predicting BMC by height as a function of serum OC by height, DEE at light intensity above BEE, and DEE at moderate intensity above BEE

Predictors		B
OC by height ((ng/mL)/cm)		-9.057[1]
DEE Light Above BEE		0.017[1]
DEE Moderate Above BEE		0.006[1]
	R^2 =	73.40%

[1] $p < 0.01$

OC: Serum osteocalcin

DEE Light/Moderate above BEE: Daily Energy Expenditure at light/moderate intensity level above Basal Energy Expenditure per Harris-Benedict equation estimate (Harris JA, Benedict FG. A biometric study of basal metabolism in man. (Publication No. 279) Washington, DC: Carnegie Institute of Washington; 1919).

Variables entered into the regression model (stepwise): protein intake by weight, calcium intake by weight, % body fat, serum OC by height, urinary DPD by height, serum 25-OH Vitamin D, DEE above BEE at light intensity, DEE above BEE at moderate intensity, DEE above BEE at vigorous intensity.

Appendix 20
Crosstabs of dependent variable categories with independent variable categories

20a: Crosstabs of bone mineral density categories with variables influencing bone mineralization

% Fat mass by percentile	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Less than the 15th percentile	5	0	2	7
Between 15th percentile to less than the 85th percentile	11	11	15	37
Equal to or greater than the 85th percentile	0	1	7	8
Total	16	12	24	52

Ca by height by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low Ca by height	4	3	9	16
Medium Ca by height	3	4	5	12
High Ca by height	9	5	10	24
Total	16	12	24	52

P by height by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low P by height	2	4	10	16
Medium P by height	3	3	6	12
High P by height	11	5	8	24
Total	16	12	24	52

Mg by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low Mg	4	6	6	16
Medium Mg	2	1	9	12
High Mg	10	5	9	24
Total	16	12	24	52

Fruit and Vegetable intake by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Less than 2 cup equiv.	11	7	10	28
Between 2 and 3 cup equiv.	3	3	4	10
More than 3 cup equiv.	2	2	10	14
Total	16	12	24	52

Appendix 20a continued

Kcals by weight by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low kcals by weight	2	4	10	16
Medium kcals by weight	2	3	7	12
High kcals by weight	12	5	7	24
Total	16	12	24	52

20b: Crosstabs of bone mineral content categories with variables influencing bone mineralization

% Fat mass by percentile	BMC by categories			Total
	Low BMC	Medium BMC	High BMC	
Less than the 15th percentile	4	2	1	7
Between 15th percentile to less than the 85th percentile	12	9	16	37
Equal to or greater than the 85th percentile	0	1	7	8
Total	16	12	24	52

Ca by height by categories	BMC by categories			Total
	Low BMC	Medium BMC	High BMC	
Low Ca by height	3	3	10	16
Medium Ca by height	1	5	6	12
High Ca by height	12	4	8	24
Total	16	12	24	52

P by height by categories	BMC by categories			Total
	Low BMC	Medium BMC	High BMC	
Low P by height	1	5	10	16
Medium P by height	2	4	6	12
High P by height	13	3	8	24
Total	16	12	24	52

Mg by categories	BMC by categories			Total
	Low BMC	Medium BMC	High BMC	
Low Mg	3	6	7	16
Medium Mg	3	1	8	12
High Mg	10	5	9	24
Total	16	12	24	52

Fruit and Vegetable intake by categories	BMC by categories			Total
	Low BMC	Medium BMC	High BMC	
Less than 2 cup equiv.	10	7	11	28
Between 2 and 3 cup equiv.	3	3	4	10
More than 3 cup equiv.	3	2	9	14
Total	16	12	24	52

Appendix 20b continued

DTEE above BEE by categories	BMC by categories			Total
	Low BMC	Medium BMC	High BMC	
Low daily energy expenditure	8	7	2	17
Medium daily energy expenditure	5	3	8	16
High daily energy expenditure	3	2	13	18
Total	16	12	23	51

20c: Crosstabs of bone mineral density categories with variables related to bone mineralization

% Fat mass by percentile	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Less than the 15th percentile	5	0	2	7
Between 15th percentile to less than the 85th percentile	11	11	15	37
Equal to or greater than the 85th percentile	0	1	7	8
Total	16	12	24	52

OC by height by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low OC by height	3	1	11	15
Medium OC by height	2	4	4	10
High OC by height	11	7	7	25
Total	16	12	22	50

Urinary DPD by height by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low Urinary DPD by height	4	5	7	16
Medium Urinary DPD by height	1	3	8	12
High Urinary DPD by height	11	4	9	24
Total	16	12	24	52

Serum Vitamin D, 25-OH by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low Serum Vitamin D	3	6	5	14
Medium Serum Vitamin D	4	1	5	10
High Serum Vitamin D	8	5	12	25
Total	15	12	22	49

Appendix 20c continued

DEE at moderate level above BEE by categories	BMD by categories			Total
	Low BMD	Medium BMD	High BMD	
Low DEE at moderate activity level	7	6	2	15
Medium DEE at moderate activity level	3	5	8	16
High DEE at moderate activity level	6	1	12	19
Total	16	12	22	50

20d: Crosstabs of bone mineral content by height categories with variables related to bone mineralization

OC by height by categories	BMC by height by categories			Total
	Low BMC per cm	Medium BMC per cm	High BMC per cm	
Low OC by height	3	3	9	15
Medium OC by height	2	2	6	10
High OC by height	11	6	8	25
Total	16	11	23	50

DEE at light activity level above BEE by categories	BMC by height by categories			Total
	Low BMC per cm	Medium BMC per cm	High BMC per cm	
Low DEE at light activity level	11	4	1	16
Medium DEE at light activity level	5	4	6	15
High DEE at light activity level	0	4	15	19
Total	16	12	22	50

DEE at moderate level above BEE by categories	BMC by height by categories			Total
	Low BMC per cm	Medium BMC per cm	High BMC per cm	
Low DEE at moderate activity level	7	4	4	15
Medium DEE at moderate activity level	3	7	6	16
High DEE at moderate activity level	6	1	12	19
Total	16	12	22	50

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