ABSTRACT

Bone biomarkers may be useful for examining the bone response to interventions in children. The purpose of this study was to determine the relationship between osteocalcin (OC), pyridinoline (PYD), and deoxypyridinoline (DPD) with changes in bone area (BA), bone mineral content (BMC), and bone mineral density (BMD) of the total body, lumbar spine, proximal femur, and radius in 32 prepubertal females over three years. Anthropometrics, maturation, calcium intake, and physical activity were also assessed. Osteocalcin, BA, BMC, and BMD at all sites increased significantly over time (p<0.0001). Annual increments of OC were significantly predicted by weight (slope = 0.305, p = 0.002), PYD by BMI (slope = 27.2, p = 0.002) and height (slope = 17.5, p = 0.046), DPD by BMI (slope = 3.52, p = 0.019). Bone biomarkers were not highly correlated to changes in bone variables. These results suggest that OC, PYD, and DPD are highly variable in children. Assessment of additional markers may be necessary to elucidate relationships between markers and changes in bone mass.

INDEX WORDS: bone, bone turnover markers, osteocalcin, pyridinoline, deoxypyridinoline, growth, children
BONE BIOMARKERS IN PREPUBERTAL FEMALES, 4-8 YEARS OF AGE

by

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BONE BIOMARKERS IN PREPUBERTAL FEMALES 4-8 YEARS OF AGE

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Maureen Grasso
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The University of Georgia
August 2003
DEDICATION

I would like to dedicate this thesis to my Mimi, Eleanor Harris Hardy. Mimi was the most understanding and accepting person I have ever known -- I learned so much from her. She showed patience, comfort, and kindness to everyone and I only hope I can be half the lady she was. Mimi died two years ago, when I was just beginning my graduate studies. Although I have not been able to talk to her and hear her say, “You know how much I love you,” I know she has been here all along. She has guided, protected, and strengthened me.

Mimi had osteoporosis and I know she suffered from the disease. It is a difficult thing to watch a loved one in pain, and I wish I could have done something for her at the time. I feel that with my research and that of many others, this disease can be prevented and treated, and fewer and fewer people will have to suffer from osteoporosis.
ACKNOWLEDGEMENTS

All of the following people have been incredibly helpful and supportive to me over the last few years, and I would like to express my sincere gratitude to them.

Dr. Lewis, thank you for keeping me motivated and excited about my research. I would never have thought about doing research if it hadn’t been for you, and now I can’t imagine doing anything else. Thank you for the great experience and wonderful opportunities you have given me. I look forward to all the research to come.

Dr. Harris, Dr. Fischer, and Dr. Johnson -- thank you all for your suggestions, encouragement, and patience. Your input on my project was indispensable, and I truly appreciate it.

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Salli and Tonya, you both have been vital to keeping down the stress levels. Salli, you always make me laugh and run (!) when I really need it. Tonya, you know how to do everything. I can come to you with any question and you always know the answer - really! Thank you both for your help and confidence in me.

I don’t know anyone else willing to live with a grad student and put up with all her stress and stupid problems. Jessica, thank you so much! You are the truest friend,
and you are always there to lend an ear, a shoulder, or a hand. Thank you for your kindness, compassion, and friendship.

Mom, Dad, Carey, and Robby -- you are the most patient family! Thank you for supporting me for 26 (!) years, and for never complaining that I was still in school. What I appreciate the most is that you not only let me, but encouraged me to pursue my interests and dreams no matter the costs to you. I love you so much.

Seth, you are the most wonderful person and I know you will be an even more wonderful husband. Thank you for all your trips to Athens, breakfast at the lab, interest in my research, and for never complaining that you had to wait on me. Your patience with me is amazing! I could not ask for a better man to spend the rest of my life with, and I can’t wait for it to start!
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CHAPTER 1

INTRODUCTION

Bone biomarkers are proteins found in the blood and urine resulting from the formation and resorption of bone. During this process of bone turnover, biomarkers spill over into the circulation, and measurement of these proteins are thought to reflect changes in bone mineral density (BMD). Levels of biomarkers found in the circulation may fluctuate with age, maturation, hormones, physical activity, and many other factors known to affect bone mass.

Bone biomarkers were initially used in adult populations to assess the response to various pharmacological treatments for osteoporosis. For example, it is known that higher levels of bone resorption markers are present in postmenopausal osteoporosis due to reduced estrogen levels. Thus, treatment with an antiresorptive agent should ideally lead to decreases in bone resorption markers and eventually slow or stop the loss of bone mass. Reference values of bone biomarkers have been established for adult populations, and biomarkers are now commonly used as indicators of bone disease, and to evaluate and monitor bone responses to interventions and treatments.

Research interests in osteoporosis, in more recent years, have begun to look more closely into methods for prevention of the disease. Exercise and calcium interventions in children have been shown to increase the rate of gain in bone mineral during growth and development, allowing children to achieve a greater bone mass before adulthood. The current opinion among experts in the field is that osteoporosis prevention may be best
accomplished through such interventions during early childhood and adolescence.\(^{(7)}\) It is important that we understand how such interventions affect BMD and the potential mechanisms via bone formation and resorption. This knowledge is necessary for identifying effective interventions to enhance peak bone mass and hopefully prevent osteoporosis, and this knowledge may be available through assessment of bone biomarkers in children. Longitudinal assessment of bone biomarkers holds the potential to track, or predict, changes in BMD and help determine the bone response to interventions.

In children and adolescents, bone biomarkers are rarely used for monitoring interventions due to a paucity of data regarding normal values and changes in biomarkers and their relation to changes in BMD with age and maturation. They are, however, commonly used to assess responses to growth hormone (GH) treatment in GH-deficient children. In these children who are growing at a slower than normal rate, lower levels of both resorption and formation markers are found. Because of the lack of reference values, many of these studies have compiled their own set of norms through cross-sectional data collection from healthy children.\(^{(8-10)}\) Although these data contribute somewhat to the establishment of reference values, they lack adequate sample sizes for particular age groups and maturation levels, and do not assess the relationship between biomarkers and BMD. Cross-sectional data may also be limited in that they do not reflect true individual changes and variation. For example, in the Saskatchewan bone mineral accrual study, Bailey et al found that bone calcium accretion rates in children were approximately 30\% higher when determined from longitudinal rather than cross-sectional
data collection.(11) This obviates the need for longitudinal studies to establish reference values for bone biomarkers.

A small number of studies have measured biomarkers longitudinally in pubertal adolescents, but these are generally short-term and the biomarkers are a variable of secondary interest.(12-14) One of these studies found that biomarkers did not predict changes in BMD,(14) while the other two found that some, but not all, biomarkers were related to changes in bone mineral content (BMC) or BMD.(12, 13) In a review article, Watts points out the need to focus on bone biomarkers as the primary endpoint if we are to fully realize their potential(15) as tools for assessing changes in BMD. Additionally, if prepuberty is an optimal time for the bone response to interventions, longitudinal collection of biomarkers during this stage of development could allow researchers to gain a greater understanding of the mechanisms of specific interventions that alter BMD. Finally, with the establishment of bone biomarker norms in children, we will have a novel, non-invasive method for assessing skeletal health status in a young population.

The present investigation was conducted as part of a larger prospective study, the University of Georgia Childhood Bone Study that began in 1997 in order to investigate the effects of gymnastics participation on bone. This prospective study will allow for bone growth comparisons between female gymnasts and non-gymnasts participating in other or no activities. Because prepuberty may be the ideal time for exercise interventions to affect bone mass, the study recruited only females in Tanner stage 1, or prepuberty with no history of sports participation. In addition to measurements of bone mass, urine and blood samples have been collected from each subject at baseline and at subsequent yearly testing sessions.
Serum intact osteocalcin and urinary pyridinoline and deoxypyridinoline were selected as the biomarkers of interest. Serum osteocalcin is a marker of osteoblast activity, and is considered a specific indicator of bone formation.\textsuperscript{(15, 16)} Pyridinoline and deoxypyridinoline cross-links are also considered reliable markers of growth and bone resorption in children. These are ideal due to their relatively low day-to-day variability, and the highly specific assay method (high performance liquid chromatography).\textsuperscript{(1)} Deoxypyridinoline is also important to measure because it is specific to type 1 collagen found in bone.

**HYPOTHESIS AND SPECIFIC AIMS**

It was hypothesized that bone biomarkers are reliable measures for evaluating changes in bone mass over time in prepubertal girls. The specific aim of the experiment was to determine if changes in levels of bone biomarkers in girls four to eight years of age over a three-year period track changes in BMD. Objectives under the specific aim were as follows: 1) to assess bone biomarkers, BMD, physical activity, dietary calcium, and sexual maturation in prepubertal girls over a three-year period; 2) to determine normal levels and rates of change in biomarkers over three years in prepubertal girls; and 3) to determine if changes in bone biomarkers significantly relate to changes in the other determinants of bone growth.
CHAPTER 2

LITERATURE REVIEW

In the following literature review, existing scientific data are presented on the topics of bone biology and metabolism, bone mineral including its assessment and relationship to bone biomarkers, and bone biomarkers and factors believed to affect them including sexual maturation, physical activity, and calcium intake.

Bone Biology

The human skeleton is a living tissue that serves to support movement, protect organs, supply a reservoir of calcium, and act as a site for red blood cell synthesis. Bone is comprised of both a protein matrix and a mineral portion in the form of hydroxyapatite. The matrix is approximately 90% type I collagen and 10% non-collagenous protein. The most abundant non-collagenous protein is osteocalcin. The bone matrix and mineral are not inert, but are constantly turning over throughout the lifecycle. The two processes of bone turnover are termed modeling and remodeling.

Bone turnover occurs within defined areas in the skeleton termed the basic multicellular unit (BMU). The BMU consists of the bone-forming cells termed osteoblasts, bone-sensing cells termed osteocytes, bone-resorbing cells termed osteoclasts, and the bone-lining cells which communicate with other cells in order to initiate the modeling and remodeling processes. It is believed that osteocytes, which are embedded osteoblasts, sense deformation or bending of the bone during weight-bearing
activity, for example. Through the compression of the bone tissue, a fluid shear stress is generated and stimulates the osteocytes and bone-lining cells to release chemical signals, or some type of second messengers into the blood supply. These messengers travel to the osteoblasts and stimulate both gene expression and transformation of bone-lining cells to osteoblasts. This signal cascade results in collagen and mineral synthesis and deposition.

Bone formation begins when matrix proteins are synthesized and secreted by osteoblasts and are laid down as a foundation prior to mineralization. Then osteoblasts secrete alkaline phosphatase, which catalyzes mineralization of the matrix. Matrix mineralization gives bone its strength and provides a reservoir of calcium ions for metabolic homeostasis. Preceding, or simultaneous to, bone formation by osteoblasts, osteoclasts acidify the bone surface to cause demineralization. Bone demineralization, also called resorption, has several purposes: it creates a cavity which osteoblasts move into and form new bone in order to replace old bone; it releases minerals and ions into circulation for needs elsewhere in the body; and, during childhood and adolescence, it decreases cartilage to allow new bone to form and push the ends of bones further apart for skeletal growth (Figure 2.1). Bone formation and resorption occur continuously and simultaneously in different areas of the skeleton depending on the needs of the individual. For example, the combination of osteoclasts resorbing cartilage on the inner surface of old bone, and osteoblasts forming new bone on the outer surface is referred to as modeling. Modeling occurs during childhood and adolescence to lengthen, widen, and alter the shape of bones according to the daily
stressors placed on it such as body weight, diet, or physical activity. The process of modeling ends as one completes maturation and enters early adulthood.

The process of remodeling is characterized by osteoclast-initiated bone resorption and eventual apoptosis of osteoclasts, followed by osteoblast migration to the same site to begin new bone formation. Remodeling occurs throughout life and functions to replace old bone with new

Figure 2.1. Bone growth through modeling. Osteoblasts form new bone on the outer surfaces while osteoclasts resorb bone and cartilage on the inner surfaces to push the ends of bone further apart, thus allowing growth. Adapted from Baron, 1999.

Figure 2.1. Bone growth through modeling. Osteoblasts form new bone on the outer surfaces while osteoclasts resorb bone and cartilage on the inner surfaces to push the ends of bone further apart, thus allowing growth. Adapted from Baron, 1999.

bone so that the skeleton may adapt to stresses, repair damaged bone, and supply the body with calcium to maintain homeostasis. A complete remodeling cycle (resorption and subsequent formation) can last from four to six months in adults; the remodeling cycle in children is more rapid. The remodeling cycle results in changes in bone mineral density over time. This is represented in Figure 2.2. Remodeling and modeling together cause bone mass to increase or decrease in response to stimuli such as exercise, hormones, and diet during childhood and adolescence.
Bone Biomarkers

Bone biomarkers are proteins secreted during modeling and remodeling that are measurable in blood and urine, and are used as indicators of the rate of bone turnover. There are markers of bone formation and resorption. Formation markers, measured in the serum, include type I collagen, osteopontin, osteonectin, alkaline phosphatase, and osteocalcin. Markers of resorption are measured primarily in the urine and include hydroxyproline, pyridinoline, deoxypyridinoline, N-telopeptides, and C-telopeptides.

Osteocalcin

Osteocalcin, a vitamin K-dependent protein, is the most abundant non-collagenous protein in bone and is specific to bone because it is synthesized only by osteoblasts\(^{15,16}\). Osteocalcin is believed to bind calcium in order to form hydroxyapatite during bone mineralization, and it most likely does so through use of

![Figure 2.2](image_url)

Figure 2.2. Illustration of the remodeling cycle of bone. 1. The bone-lining cells are resting. 2. The bone-lining cells are activated. 3. The bone-lining cells move away and allow the osteoclasts to begin bone resorption. 4. Osteoclasts undergo apoptosis and leave the bone site. 5. Osteoblasts move in and begin forming new bone. 6. Bone returns to its resting state. Adapted from Heaney.\(^{26}\)
its gamma-carboxyglutamate residues. Between 10% and 30% of osteocalcin secreted by osteoblasts spills into the circulation during bone formation, allowing intact osteocalcin to be measured easily in the serum. Osteocalcin is frequently measured by immunoassay in humans. Both radioimmunoassay (RIA) and two-site immunoradiometric assay are most commonly used. Intact osteocalcin was chosen as a measurement of bone formation over mid-fragment, or degraded, osteocalcin due to lack of a standard assay for mid-fragments at the start of the study. Osteocalcin is measured by radioimmunoassay in the laboratory of Dr. C.M. Gundberg (Yale University, New Haven, CT). The inter-and intra-assay CVs for intact osteocalcin measured by RIA in young children are less than 10% and 5%, respectively.

Cross-links

When osteoclasts demineralize bone, which is 90% type I collagen, they release type I collagen degradation products. Collagen molecules in bone are stabilized by forming a triple helix joined by cross-links of hydroxylysyl-pyridinolines (PYD) and lysyl-pyridinolines (deoxypyridinolines or DPD). The PYD and DPD cross-links occur at or near a link between aminotelopeptides. This is represented in Figure 2.3. The cross-links in collagen play a major role in maintaining the structure of the collagen network which gives bone its tensile strength. Upon degradation of bone, pyridinoline and deoxypyridinoline cross-links are released from collagen molecules into the circulation for eventual excretion in the urine. These cross-links are not metabolized or absorbed from the diet and thus are easily measured in urine by high performance liquid chromatography (HPLC; Dr. S. Shapses, Rutgers University). Use of HPLC gives highly specific results for bone resorption markers and is the most commonly used method for
measuring cross-links.\textsuperscript{(1,18)} The interassay CVs for PYD and DPD are 3.8% and 5.9%, respectively. Pyridinoline cross-links are found in tissues other than bone so they are not an exclusive measure of bone turnover.\textsuperscript{(31)} This is the rationale for including DPD in assessments of bone resorption.

![Figure 2.3. Pyridinium cross-links connect collagen molecules to stabilize the helical structure. During bone resorption collagen is cleaved and cross-links are released. Adapted from Christenson 1997\textsuperscript{(1)}](image)

The concentration of bone biomarkers in blood or urine changes throughout the lifecycle and with varying rates of bone formation and resorption. During childhood and adolescence, for example, the rate of growth and bone formation is much higher than during adulthood so bone biomarkers tend to be as much as five-fold higher in children than adults. Changes in biomarkers appear to follow changes in height velocity, which increase during puberty, a period of rapid bone turnover and growth. Biomarkers tend to peak around the time of early puberty or Tanner stage 2. The majority of data are from cross-sectional studies and show that both osteocalcin and the cross-links increase slightly during prepuberty (Tanner stage 1), increase dramatically during early puberty, peaking in Tanner stage 2, and then gradually decline to reach adult levels by post-puberty (Tanner stage 5). Typical values and changes in biomarkers observed by Mora
et al are presented in Table 2.1.\cite{32} There are ample longitudinal data that support these average values for pubertal children, but there are inadequate data (the majority are from cross-sectional studies) regarding normal values during prepuberty for norms to be established.

Table 2.1. Reference values for bone biomarkers throughout puberty. Values are means ± SD. Adapted from Mora et al.\cite{32}

<table>
<thead>
<tr>
<th>Tanner Stage</th>
<th>Osteocalcin (ng/mL)</th>
<th>Deoxypyridinoline (nmol/nmol Cr)</th>
<th>Pyridinoline (nmol/nmol Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.9 ± 1.3</td>
<td>69.8 ± 11.7</td>
<td>247.1 ± 38.5</td>
</tr>
<tr>
<td>2</td>
<td>18.3 ± 2.8</td>
<td>97.6 ± 11.4</td>
<td>337.7 ± 30.1</td>
</tr>
<tr>
<td>3</td>
<td>17.3 ± 2.1</td>
<td>68.5 ± 5.5</td>
<td>250.0 ± 17.1</td>
</tr>
<tr>
<td>4</td>
<td>16.2 ± 1.4</td>
<td>59.8 ± 4.9</td>
<td>213.2 ± 15.4</td>
</tr>
<tr>
<td>5</td>
<td>11.8 ± 0.9</td>
<td>33.8 ± 4.5</td>
<td>124.0 ± 1.2</td>
</tr>
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</table>

As bone biomarkers change with age, they also change according to a circadian rhythm. Osteocalcin tends to peak in the early morning, gradually decrease until afternoon, and then increase until late night-early morning; cross-links tend to peak during early morning and the trough is usually in late afternoon.\cite{18,33,34} Biomarkers may be influenced by food intake, so it is generally recommended that biomarker samples be collected following an overnight fast. Discrepancies between several studies also suggest that biomarkers can change with seasonal variations in sunrise and sunset.\cite{33} All sources of variability need to be accounted for when assessing bone biomarker status in individuals.

In order to observe systemic changes in bone turnover, it is appropriate to use bone turnover markers present in the urine and/or blood. Serum markers may be ideal for
such analyses because the blood volume is constant while urinary volume and osmolarity change throughout the day. However, some bone turnover markers exist only at very low concentrations in the serum and are below the level of assay detection. Such is the case for pyridinium cross-links.

Not all bone turnover markers are capable of passing through the basement membrane of the kidney, so urine does not contain all markers. Pyridinoline and DPD, on the other hand, are present at their highest concentrations in urine compared to any other body fluid. The cross-links exhibit 16-26% day-to-day variability, but this is believed to be true variability and not due to measurement errors resulting from their analysis in urine. Serum markers generally show less daily variability than urine markers, however, this may be due to the difficulty of consistently collecting urine samples at the same time of day. Furthermore, DPD excretion is not affected by food intake or renal function, and is not metabolized so its appearance in urine, aside from the daily variability, should be relatively stable in times of good health.

The current direction in assay development of bone turnover markers is towards greater use of serum markers, and one reason for this is that they do not require correction for creatinine excretion. Creatinine correction allows for some control over circadian rhythm and urinary dilution. At the same time, it corrects bone turnover markers for lean body mass since creatinine is reflective of muscle stores. Many researchers believe that correcting for creatinine excretion introduces another source of variability in urine markers. Additionally, correcting for lean body mass remains debatable among experts in the field, and when possible it is suggested to collect 24-hour or three-day average urine samples to achieve a total resorption assessment, similar to
that of serum markers. In large, prospective studies, however, it is recommended to use second morning void urine samples, corrected for creatinine, for testing efficiency.\(^{(35)}\)

The idea that serum markers more accurately reflect bone turnover is still debatable, however, as we do not completely understand the entire cycle of turnover markers of bone, including their degradation and metabolism. Furthermore, Fall et al found that urine and serum C-telopeptide and N-telopeptides in adult men and women were highly correlated (\(r = 0.87\) and \(r = 0.76\), respectively). These authors concluded that serum was no better than urine in reflecting bone resorption by telopeptides.\(^{(38)}\)

**Current Uses for Bone Biomarkers**

Bone biomarkers are commonly used in measuring the response to drug treatment or other interventions.\(^{(38, 39)}\) Changes in BMD measured by dual energy x-ray absorptiometry (DXA) typically cannot be detected in less than a one-year period due to the remodeling cycle, therefore, bone biomarkers are thought to be useful in monitoring short-term changes (two to three months) caused by anti-resorptive or other therapies.\(^{(40)}\) Studies in postmenopausal, osteoporotic patients using bisphosphonates and Hormone Replacement Therapy (HRT) have shown a decline in resorption markers including DPD (\(9.8 \pm 7.6\) vs. \(4.9 \pm 1.5\) nmol/mmolCr, \(p<.05\)) within a few months of treatment onset.\(^{(38, 39)}\) Studies involving healthy postmenopausal women have shown a general trend of reduced resorption markers corresponding to higher bone mass or decreased bone loss.\(^{(41, 43)}\) These data suggest that lower rates of resorption contribute to increases in BMD. Formation markers have not given consistent results across this age group\(^{(41)}\), but it is known that the increase in the remodeling cycle after menopause is generally
characterized by a small increase in formation and a larger increase in resorption of bone. This is depicted in Figure 2.4.

<table>
<thead>
<tr>
<th></th>
<th>Normal Adult</th>
<th>Postmenopausal Women</th>
<th>Postmenopausal on Bisphosphonates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation</td>
<td>↑↑</td>
<td>↑↑↑↑</td>
<td>↑↑↑↑</td>
</tr>
<tr>
<td>Resorption</td>
<td>↑↑</td>
<td>↑↑↑↑</td>
<td>↑↑↑↑</td>
</tr>
</tbody>
</table>

Figure 2.4. Changes in bone formation and resorption due to menopause. Changes result in net bone loss, but treatment can decrease resorption and bone loss.

Bone biomarkers also have been used to assess the response to growth hormone therapy in growth hormone-deficient (GH-d) children. Studies have found that over the treatment period, both formation and resorption markers positively correlate with changes in growth velocity,\(^9, 10, 44-46\) however, the researchers did not measure BMD or bone mass in these studies. Most of these studies include a cross-sectional analysis of biomarkers in a cohort of healthy, normal children for comparison with GH-d children. The results from cross-sectional cohorts give a general pattern of the change in levels of bone biomarkers across age groups, and show that biomarkers usually peak with height velocity, corresponding to Tanner stages 2 and 3.\(^{45-48}\)

Researchers are relatively confident in using biomarkers as part of specific disease diagnoses and assessments, however, due to the limited longitudinal data and variability in healthy subjects, biomarkers are not used for individual assessment of bone metabolism in non-disease states. There is a particularly limited database regarding prospective studies in healthy, prepubertal children; the majority of normative data in this
age group have been gathered from cross-sectional studies. In order to use biomarkers for individual screenings and assessments, reference values and normal fluctuations of biomarkers in prepubertal children must be better established through longitudinal measurement of multiple bone biomarkers during this maturational stage.

**Bone Mineral Assessment**

The amount of mineral in a given area of bone determines its strength and susceptibility to fracture, and is measured to diagnose osteoporosis and other bone diseases.\(^{40}\) Areal bone mineral density (aBMD) can be assessed at specific sites using DXA.\(^{40}\) Dual energy x-ray absorptiometry may be limited, however, in that the aBMD is a two-dimensional projection of a three-dimensional bone. Thus, the density is not a true volumetric density, and may under- or overestimate measurements. It is possible to assess the volumetric density, but DXA has a lower radiation exposure making it more appropriate for use in children. DXA gives an accurate measure of aBMD which can be compared to a normative database in order to diagnose osteoporosis and fracture risk.\(^{40}\) Lower values of aBMD correspond to increased risk of fractures. The World Health Organization has defined values below 2.5 standard deviations of the young adult norms as osteoporosis.\(^{49}\)

While measures of BMD are useful for diagnosing osteoporosis and monitoring long-term changes in bone mass, these values do not reflect the degree of bone turnover (resorption and formation) occurring in the skeleton. For example, if an individual's BMD increases over a one-year period, DXA does not show whether it increased due to higher rates of formation, lower rates of resorption, or a combination of the two. Additionally, short-term changes in BMD, such as during anti-resorptive therapy, are not
apparent from DXA measurements but may be observed through measuring biomarker levels. Bone biomarkers may be a useful tool for monitoring changes in rates of bone turnover in individuals. It remains unclear, however, if these bone biomarkers accurately reflect the degree of bone formation and resorption occurring in the skeleton, and thus changes in BMD, during different stages of the lifecycle.

**Determinants of Bone Mass and Metabolism**

It is well known that bone growth and mass are affected by many factors throughout the lifecycle. Genetics may account for up to 80% of variability in BMD between individuals while environmental factors, such as physical activity and diet, may account for up to 35% of the variability. Other factors that have been shown to influence BMD and bone growth include hormonal fluctuations (sexual maturation) and growth factors (IGF-1). It has been shown that each of these variables may alter bone accumulation during childhood or bone loss during adulthood. The following review of the literature discusses each of these variables and how they may determine the rate of bone turnover and level of bone biomarkers in children.

**Sexual Maturation, Bone and Bone Biomarkers**

Sexual maturation is based on Tanner stages.\(^{(50)}\) Tanner stages range from stage 1 to 5 and are defined by physical development of secondary sex characteristics. Tanner stages are represented pictorially for research purposes, and these ratings provide an indication of hormonal status such as increasing levels of estrogen which appear as females enter and complete puberty. In females secondary sex characteristics include breast development and pubic hair with Tanner stage 1 representing no development of either characteristic, or prepuberty. Tanner stage 5 is represented by complete sexual
development and the onset of menarche. In relation to bone growth and development, it is known that the greatest rate of bone mass accrual occurs between Tanner stages 1 and 4 due to the increase in hormones including GH and estrogen.

Estrogen has been shown to affect bone mass throughout the lifecycle but the mechanisms remain unclear. Estrogen receptors exist on bone cells so many researchers believe that estrogen acts locally. It is known that estrogen directly affects bone at the cellular level by stimulating proliferation of osteoblasts. It may also function cellularly by stimulating apoptosis of osteoclasts and preventing apoptosis of osteocytes. Other evidence exists that suggests that estrogen may act indirectly by suppressing resorption factors such as prostaglandin E2 or interleukin-6, or by enhancing intestinal absorption of calcium.

Although the mechanisms are not known, it is clear that estrogen affects bone mass. For example, the drop in estrogen that occurs during menopause is associated with a gradual decline in bone mass due to increased bone turnover. Some have suggested that this increased turnover is accompanied by the uncoupling of bone formation from resorption and an increase in remodeling sites in bone which can lead to drastic bone loss in postmenopausal women. As low estrogen can affect bone mass in postmenopausal women, it can similarly affect bone mass in female adolescents. Several studies have found that amenorrhea and/or menstrual dysfunction resulting from low circulating estrogen are associated with a decreased bone mass accumulation. Additionally, studies of young women with anorexia nervosa have shown low levels of estradiol (24 ± 8 pg/ml), the active form of estrogen, while others have found a loss of bone mass associated with this low estrogen exposure in anorexics. Ito et al also found
that early menarche is associated with higher BMD when compared to age-matched controls.\textsuperscript{(65)} Such data suggest that greater exposure to estrogen, for example through attainment of menarche, may be associated with greater bone formation and lesser bone resorption. Moreover, graphic representations of changes in rates of bone mineral accrual show that rates change with pubertal stage and increasing hormonal levels.

There are several studies that have measured bone biomarkers in relation to pubertal stage in females. Mora et al found an independent effect of puberty on bone resorption markers in female subjects.\textsuperscript{(31)} N-telopeptides, PYD, and DPD all significantly increased in females between Tanner stages 1 and 2, or pre- and peripuberty [PYD \((\text{pmol/mmol Cr}) = 440.5 \pm 35.1 \text{ vs. } 510.6 \pm 42.1, p = .004\); DPD \((\text{pmol/mmol Cr}) = 75.4 \pm 6.8 \text{ vs. } 82.0 \pm 7.5, p = .02\)]. The authors were able to conclude that the onset of puberty causes changes in bone resorption.\textsuperscript{(31)} Blumsohn et al observed maximal levels of bone formation markers including osteocalcin between Tanner stages 2 and 3 in female subjects.\textsuperscript{(66)} Osteocalcin (OC), PYD, and DPD were all shown to increase through pre- and peripuberty (prepubertal OC = 16.9 \pm 1.3, DPD = 69.8 \pm 11.7, PYD = 247.1 \pm 38.5), peak between Tanner stages 2 and 3 (OC = 18.3 \pm 2.8 to 17.3 \pm 2.1, DPD = 97.6 \pm 11.4 to 68.5 \pm 5.5, PYD = 337.7 \pm 30.1 \text{ to } 250.0 \pm 17.1), and decrease to adult levels by Tanner stage 5 (OC = 11.8 \pm .9, DPD = 33.8 \pm 4.5, PYD = 124.0 \pm 1.2) in both male and female subjects in a later study by Mora et al.\textsuperscript{(32)} These cross-sectional data are supported by a longitudinal study by Cadogan et al which found that both bone formation (OC and bone-specific alkaline phosphatase) and resorption markers (DPD and N-telopeptides) peaked approximately 20 months before menarche, and followed the height velocity curve.\textsuperscript{(14)} Typical patterns of change in biomarkers are shown in Figures 2.5 and 2.6. These data
suggest that both bone formation and resorption increase with sexual maturation, providing the mechanisms for rapid bone growth and bone mineral accrual that occur during these stages of the lifecycle. Due to their variability during puberty, an evaluation of sexual maturation is necessary when determining normal values and rates of change in bone biomarkers in children and adolescents.

Figure 2.5. Reference values for osteocalcin through pubertal development in females. Values are based on cross-sectional data. (32)

Figure 2.6. Reference values for pyridinoline and deoxypyridinoline through pubertal development in females. Values are based on cross-sectional data. (32)
Physical Activity, Bone and Bone Biomarkers

The term mechanostat refers to bone’s ability to respond to stimuli or stressors. It is believed that through the process of mechanotransduction, bone responds to loading stimuli such as exercise by building new bone (modeling) and strengthening the existing bone (remodeling). It has also been suggested that exercise may alter bone due to its stimulation of GH which has been shown to influence bone mass and growth during childhood.

It is generally accepted that physical activity, particularly weight-bearing activity, provides stimuli that lead to greater bone mass accumulation in childhood and adolescence through the mechanostat theory. Several studies indicate that participation in certain sports confers higher bone mass to those individuals. For instance, gymnasts (both current college-age and adolescent, and retired gymnasts) in general have been shown to have higher BMD than controls or age-matched norms. Other sports including basketball, volleyball, soccer, and track have also been shown to promote greater BMD compared to non-athlete controls.

High impact sports such as those mentioned above have also been shown to contribute to greater increases in BMD during childhood and adolescence. In a study by Laing et al, gymnasts (n=7), 8-13 years of age, were found to have significantly higher BMD (g/cm²) at the total body (.90 vs.85), lumbar spine (.77 vs .67), and total proximal femur (.82 vs .70) compared to controls (n=10). Gymnasts also experienced higher rates of gain in bone mass at the total body and total proximal femur (p<.05) over three years when compared to non-gymnast controls. Additionally, a jumping program in children six to ten years of age, over a seven-month period, caused significant increases in BMD.
and BMC of jumpers (n=45) versus controls (n=44) at the lumbar spine (2.0% and 3.1%, respectively) and significant increases in BMC at the femoral neck (4.5%). Such studies provide convincing evidence for physical activity and the effects it may have on bone mass, however, the mechanisms for these changes in bone remain unclear. For example does physical activity increase bone formation, decrease bone resorption, or cause a combination of these two processes in order to increase bone mass? There are very few longitudinal studies that measure bone biomarkers over time and how they change in response to stimuli such as exercise. These are necessary to determine the bone-enhancing mechanisms that exercise stimulates.

In order to determine the mechanisms, Moyer-Mileur et al compared levels of procollagen type I carboxyterminal peptide (PICP) and bone-specific alkaline phosphatase (BSAP) (formation markers) and cross-links (resorption markers) in very low birth weight infants. Sixteen infants were put on a passive-resistance exercise program for 5-10 minutes per day for 25 days, and were compared to 16 other control infants. The exercising infants had significant increases in forearm bone area (p=.02) and BMC (p=.05) after the study period. Serum PICP remained relatively unchanged in the exercise group (-5ng/ml) while it decreased in controls (-94ng/ml; p=.03). Cross-links remained similar between groups for the study period. The biomarkers suggest that these changes were due to a higher level of bone formation in the exercising infants than in the controls.

A study by Lehtonen-Veromaa et al measured baseline OC, BSAP, PINP, and C-telopeptide (resorption) with BMD in peripubertal female gymnasts (n=51), runners (n=50), and non-athlete controls (n=54). Results were based on rates of change due to
differences between groups at baseline. Gymnasts were shown to have greater accumulation of bone mass at the hip sites. There were not any differences in biomarkers at baseline after adjustment for pubertal stage (biomarkers were higher in pre-puberty and early puberty then decreased). OC and PINP significantly correlated with the one year changes in BMD for the lumbar spine and femoral neck, accounting for 5-7% of the change at the femoral neck and 7-13% at the lumbar spine. C-telopeptide was not a predictor of changes in BMD in any groups at any sites.\(^{(76)}\)

In the cross-sectional study by Creighton et al, BMD differences were measured in high impact (basketball, volleyball, n=14), medium impact (soccer, track, n=13), non-impact (swimming, n=7), and sedentary controls (n=7).\(^{(73)}\) As noted earlier, BMD (g/cm) was higher in the high impact group compared to the non-impact and controls at the femoral neck (1.26 vs 1.04, p<.05) and trochanter (1.04 vs .86, p<.05), and greater at the trochanter in the medium impact group than the non-impact and controls (1.02 vs .86, p<.05). Total BMD (g/cm) was higher in the high impact group than all groups (4.9 vs 4.5, 4.2, 4.1). Serum OC and urinary N-telopeptides were also measured in this study. OC was significantly higher in both the high and medium impact groups compared to non-impact (30.6 and 32.9 vs 19.8 ng/ml, p<.05), and although not significant, there was a trend towards higher N-telopeptides in the non-impact group compared to other groups.\(^{(73)}\) The study suggests that it may be a combination of greater formation and lesser resorption that contribute to the higher BMD in athletes performing high load activities.

These results are further supported by a study by Fujimura et al in which young adult males were assigned to either an intense resistance exercise training group (n=8) or
the sedentary control group (n=7). The training program lasted for four months, and serum OC, PICP, BSAP, and DPD were measured. The observable period of the study was most likely too short to observe changes in BMD and no significant results were found. However, OC and BSAP, which were similar between groups at baseline, significantly increased after one month of training (OC = 19 to 24 ng/ml; BSAP = ~40 to 52 IU/l) and remained higher at all time points in the training versus the control group. Serum PICP remained constant in the training group while decreasing in the controls at two months and again at four months. There were no significant differences between groups or from baseline values for DPD.

There are short-term studies that also support the theory that it is greater bone formation which leads to greater BMD in athletes of weight-bearing activities. A study by Langberg et al looked at immediate responses of biomarkers to exercise sessions in trained adult male runners (N=17). PICP and ICTP (resorption) were measured pre-marathon and immediately post-marathon, and again for the following six days. The researchers found that following an immediate, transient decline in PICP (176 to 156 mcg/l), values actually rose (197mcg/l) and remained elevated for four days after the marathon. There were no changes in ICTP. These results agree w/ the studies by Fujimura, Creighton, and Lehtonen-Veromaa, and suggest that bone formation increases after exercise. Thus, with repeated stimuli or exercise sessions, there is the potential to retain elevated bone formation rates, which may lead to longer term increases in BMD over time. The response of biomarkers to physical activity, however, remains to be elucidated, particularly in prepubertal children.
Physical activity may be assessed using several methods in childhood. The most direct and objective measures of energy expenditure are doubly-labeled water, heart rate monitoring, and accelerometry. Each of these methods, however, has limitations in measuring bone loading from physical activity. Doubly-labeled water is considered the gold standard in measuring energy expenditure but it is expensive and does not provide information specific to bone loading.\(^{(19)}\) Suggestions for measuring physical activity in childhood bone research made by Khan et al include a measure of the forces generated by certain activities.\(^{(19, 79)}\) It would also be ideal to measure the bone loading aspects of activities, as suggested by Ainsworth et al,\(^{(80)}\) however a reliable tool for such an assessment has not been developed. In the current investigation, physical activity was assessed by accelerometry. (Accelerometers are small devices worn on the body that are able to sense movement and force. Inside the accelerometer is a lever which is displaced during activity. The lever is able to generate an electrical current that is proportional to the energy expended.\(^{(19)}\)

**Calcium, Bone and Bone Biomarkers**

Dietary intake has been shown to affect bone mass and growth during childhood. It is well recognized that adequate nutrition is necessary for achieving normal skeletal growth.\(^{(81, 82)}\) Several studies have shown that supplementing malnourished children with certain nutrients allows normal growth to resume. Supplemental calcium has been shown to increase bone mass and BMD in children, and is one of these nutrients that is vital for normal skeletal growth and is an independent determinant of bone mass in children.\(^{(83-86)}\)

The current adequate intake (AI) set by the Food and Nutrition Board for calcium in children ages 4-8 years is 800 mg per day and for children 9-13 years of age it is 1300
mg per day. Data from the Continuing Survey of Food Intake by Individuals (CSFII) shows that up to 50% of children between six and eight years of age and nearly all females older than nine years of age consume less than the current AI. Calcium intake below this recommended level, as is seen in many children in the United States, may be a risk factor for low peak bone mass.

Calcium is the most abundant mineral in the human skeleton accounting for approximately 60% of the total weight of bone in the form of calcium phosphate. Additionally, 99% of calcium in the human body is found in the skeleton. While peak bone mass is influenced strongly by genetics, environmental factors can account for up to 35% of the differences in bone mass. Calcium is one of the most important modifiable environmental factors. For example a study by Kalkwarf et al revealed that adults who consumed the lowest amounts of milk during childhood had lower BMD and greater fracture risks in adulthood. Similar results have been found in previous studies and it is well recognized, therefore, that dietary calcium deficiency during growth and adulthood contributes to a lower peak bone mass and bone mineral density. Furthermore, greater effects of dietary calcium on bone mass are usually more apparent at lower intakes and in pre- or post-pubertal children.

The rate of calcium utilization by the skeleton varies with age and sexual maturity. A balance study by Matkovic found that calcium retention and absorption are higher during infancy (0-12 months) and adolescence (9-17 years of age) and lower during childhood (2-8 years of age) and young adulthood (18-30 years of age). This pattern of calcium utilization closely resembles the rates of skeletal modeling/height velocity through the first three decades of life. Changes in bone biomarkers also closely
follow this curve, and provide additional support that calcium plays a large part in normal bone growth.

The mechanism through which calcium causes changes in bone remodeling is unclear, but extracellular calcium is recognized as a major factor in regulating bone remodeling. The calcistat theory offered by Pearce states that when blood calcium is above normal levels, bone resorption is decreased in order to decrease blood calcium levels.\(^{91,92}\) Furthermore, when blood calcium is low, calcium is then removed from bone by increased bone resorption. Several proteins are thought to be involved in the calcistat mechanism, but their roles have not been proven.\(^{92}\) If dietary calcium promotes bone growth by suppressing resorption, then it should follow that bone resorption markers would be lower in individuals with higher calcium intakes; however, researchers have not been able to prove this.

While it is clear that calcium supplementation increases bone mass in childhood, its effects on bone biomarkers remain unclear. A study by Abrams et al in 8-10 year old girls (n=34) found that by supplementing girls’ diets in order to achieve calcium intakes of 1200 mg per day, there was an increase in calcium retention and bone calcium deposition.\(^{93}\) This effect was greatest in girls between late pre-puberty (1 year before Tanner Stage 2)) and puberty (30.7 % vs. 36.6% absorption, p=.002; 1563 vs. 1952 mg/d calcium deposition, p<.01). The changes in calcium retention were similar to changes in IGF-1, alkaline phosphatase, and osteocalcin in these girls.\(^{93}\) This would indicate that calcium retention and bone deposition are increased by IGF-1 and greater bone formation, not decreased bone resorption.
Similar results were found by Cadogan et al in which adolescents were supplemented with milk. The milk group had greater gains in BMD (9.6% vs. 8.5%, p=.017) and greater IGF-1 levels (35% vs. 25%, p=.02) over an 18-month period compared to controls. In this study biomarkers were not different between the milk and control groups. One limitation to this study was that blood and urine samples were not fasting samples so biomarker values may have been influenced by previous intake, and these results should be interpreted carefully. Other studies by Dibba et al and Heer et al did find differences in biomarkers but their results are conflicting. In the study by Dibba et al, calcium supplements increased BMD in 8-12 year old children over a one-year period but actually decreased osteocalcin levels. On the other hand, Heer et al supplemented anorexic patients (mean age 14.2 years) with 2000mg of calcium per day for 11 weeks, and though BMD was not measured, formation markers significantly increased (129.89 vs. 234.55 mcg PICP/l, p=.018) while resorption markers significantly decreased (10496 vs. 7560.8 pmol CTX/l, p<.01). Conclusions from these studies do not agree and require further research to discern the effect of calcium on biomarkers.

Possibly the best indication of calcium's effect on bone biomarkers is shown in a study by Wastney et al in which girls, 11 to 14 years of age, were on a high (47.4mmol/d) or low (21.2mmol/d) calcium diet. Calcium kinetic studies were done to determine the path of calcium in the body. They found that girls on the high calcium diet retained more calcium (74% vs. 40%), had a 32% decrease in resorption markers, but no difference in formation markers. They concluded that the extra calcium is used for needs elsewhere, so less calcium is needed from the skeleton, thereby decreasing bone loss. Although this conclusion seems clear, there is much conflicting data regarding the effect of calcium
on bone biomarkers, particularly in young children. Additional studies are warranted to
determine the true effects of calcium on bone biomarkers.

Calcium intake may be measured by several methods including three-day diet
records, 24-hour recalls, and food frequency questionnaires (FFQ). In a study by Taylor
and Goulding, calcium intake was measured in children 3-6 years of age by using four-
day diet records and FFQs.\textsuperscript{(97)} The researchers found that FFQs tend to overestimate
calcium intake, and the diet records were more representative of actual calcium intake.\textsuperscript{(97)}
Similar studies in adults have also found that when comparing diet records to FFQs,
three-day diet records were shown to have less error (12-22\% error range compared to
30-30\% error range) and be useful for diet assessment of large groups.\textsuperscript{(98, 99)}

\textbf{Conclusion}

Bone biomarkers may provide an indication of bone growth and bone mineral
accrual in children, but available data are equivocal regarding the usefulness of
biomarkers for assessing bone changes over time. Little is known about bone turnover
and bone biomarkers in children and how determinants of bone mass affect them. Much
of the available literature focuses on GH-d children or adolescent and peripubertal
children. In addition, the majority of data regarding normal children are from cross-
sectional analyses. Because bone biomarkers provide some indication of rates of
remodeling, it is important that normal, healthy children are followed longitudinally to
monitor changes in rates of bone turnover and biomarkers. It is also important that we
understand how childhood interventions affect BMD and potential mechanisms via
formation and resorption. This knowledge is necessary for determining effective
interventions for enhancing peak bone mass attainment and preventing osteoporosis.
CHAPTER 3

LONGITUDINAL ASSESSMENT OF BONE TURNOVER MARKERS AND
BONE MASS IN HEALTHY, PREPUBERTAL FEMALES

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ABSTRACT

Assessment of bone turnover markers may be useful for examining the bone response to exercise or dietary interventions in prepubertal children. The purpose of this longitudinal study was to determine the relationship between serum osteocalcin (OC), urinary pyridinoline (PYD) and deoxypyrindinoline (DPD) with changes in bone area (BA), bone mineral content (BMC), and areal bone mineral density (aBMD) of the total body (TB), lumbar spine (LS), proximal femur (PF), and radius (R) measured by dual-energy x-ray absorptiometry (DXA) in 32 prepubertal females over three years. Height, weight, body mass index (BMI), sexual maturation, three-day dietary calcium intakes and physical activity measured by accelerometry, were also determined. Osteocalcin, BA, BMC, and aBMD increased significantly over time (p < .0001) at all sites. Using regression models with random subject effects, significant predictor variables for annual increments in bone turnover markers were annual increments of body weight for OC (slope = 0.305, p = 0.002), BMI and height for PYD (slope = 27.2, p = 0.002 and slope = 17.5, p = 0.046, respectively), and BMI for DPD (slope = 3.52, p = 0.019). Bone turnover markers were not highly correlated to changes in bone variables using canonical correlations (R = 0.35 for TB, R = 0.27 for LS, R = 0.36 for PF, R = 0.42 for R). These results suggest that the bone turnover markers, OC, PYD and DPD are highly variable in children and may not be useful in assessing the bone response to interventions. To our knowledge this is the first three-year prospective study assessing bone turnover markers in healthy, prepubertal children. Assessment of additional formation and resorption markers with multiple measures may be necessary to reduce variability and elucidate any relationships between bone turnover markers and changes in bone variables.
INTRODUCTION

Bone turnover markers have been used extensively in clinical trials to evaluate the efficacy of pharmacological treatments for osteoporosis.\(^{(1-3)}\) Osteocalcin (OC) and deoxypyridinoline (DPD) and pyridinoline (PYD) are proteins released with bone formation and resorption, respectively. Assessment of these proteins is thought to reflect the balance of bone remodeling and changes in areal bone mineral density (aBMD). Reference values of bone turnover markers have been established for adult populations, and they are now commonly used as indicators of bone disease, and to evaluate and monitor bone responses to interventions and treatments.\(^{(4)}\)

The assessment of bone turnover markers in pediatric populations is less pervasive, although monitoring of bone turnover with growth and maturation, especially in cases of growth-related disorders, has been a priority. The majority of available data on bone turnover markers in healthy children are the result of cross-sectional studies. Data from cross-sectional studies such as those by Mora et al and Marowska et al have contributed to reference values for several turnover markers and demonstrate general trends over maturation and age.\(^{(5-7)}\) Osteocalcin, PYD, and DPD appear to increase from prepuberty through early puberty (Tanner Stages 2 and 3), after which they decline through complete sexual maturation (Tanner Stage 5) and into adulthood. Although these data contribute somewhat to the establishment of reference values, they lack adequate sample sizes for particular age groups and maturation levels, and do not assess the relationship between changes in bone turnover markers and aBMD. Moreover, cross-sectional data such as these may be limited in that they do not reflect true individual variation and changes over time.
Few longitudinal studies exist that assess the relationship between bone turnover markers and aBMD during growth. A small number of studies have assessed turnover markers and aBMD prospectively, but these are limited to pubertal adolescents, have only six to 18 month durations, and report equivocal results with respect to the ability of the markers to predict changes in BMC and BMD.\(^{8-10}\) For example, Cadogan et al assessed serum OC and bone-specific alkaline phosphatase, and urinary N-telopeptides and DPD, and found that none of these markers predicted changes in total body aBMD over 18 months.\(^{10}\) These results, nevertheless, were limited in that the samples were collected in a non-fasting state. Conversely, the data of van Coeverden et al suggest that bone turnover markers can predict yearly changes in BMC in pubertal males and females.\(^{11}\) However, such highly variable results and the narrow age ranges used warrant the need for further long-term investigations of bone turnover markers during growth.

The current opinion among experts in the field is that osteoporosis prevention may be best accomplished through diet and activity interventions during early childhood and adolescence, although, definitive evidence through long-term randomized trials, is lacking.\(^{12}\) High load exercise and calcium supplementation trials in prepubertal children have demonstrated increased rates of bone mineral accrual,\(^{13-16}\) suggesting that the prepubertal years may be a time when bone responds optimally to dietary or exercise interventions. These findings have led to increased interest in the use of bone turnover markers for increasing our understanding of how such interventions influence the modeling and remodeling cycles, the balance of bone formation and resorption, and ultimately the aBMD response.
To our knowledge there are no longitudinal studies assessing bone turnover markers and their ability to predict changes in bone variables in prepubertal children. The objective of this investigation was to determine if changes in OC, PYD, and DPD are related to changes in bone variables over three years in prepubertal girls, four to eight years of age at baseline. Factors known to affect bone mass, including sexual maturation, height, weight, BMI, physical activity, and calcium intake, were also assessed and used as covariates in the analyses.

METHODS

Study Design

A three-year prospective, observational study of changes in bone turnover markers in young females was conducted. Prepubertal females, four to eight years of age at baseline, who have been enrolled in the University of Georgia Childhood Bone Study, investigating the influence of gymnastics training on bone and growth, were included in this study. Statistical power was calculated based on longitudinal measures of OC, PYD, and DPD over six months in females, ten years of age, from previously gathered data in our laboratory. Differences over time in these markers generated a large effect size of approximately 1.0. The selection criterion for inclusion in the current study was whether participants had completed all measurements over three years (n = 32). Setting alpha at the 0.05 level and based on a large effect size, our sample size was adequate to show significant main effects of time with bone turnover markers over three years with power $\geq 0.8$. 
Participant Characteristics

Participants were recruited from the Athens area through radio, newspaper, and flyer advertisements. At baseline, subjects were prepubertal (Tanner stage 1) and had participated in limited or no organized physical activity. Subjects and their parents self-selected to either participate in the gymnastics group or control group. Because the primary objective of the study was to assess changes in the biochemical markers over time, for purposes of the current study, gymnasts and controls were combined into one group, and physical activity controlled in the data analyses (n=6 gymnasts, n=26 controls). The gymnasts included in the sample participated in a recreational gymnastics program for approximately one hour per week.

Testing Protocol

All procedures were approved by the Institutional Review Board at the University of Georgia. Participant testing was conducted at baseline, one, two, and three years. Subjects and their parents were required to read and sign an assent and consent form, respectively, prior to annual testing sessions.

Anthropometry

Participants' weights were measured to the nearest 0.1kg using a calibrated double-beam balance scale (Fairbanks Scales, Kansas City, MO). Height was measured to the nearest 0.1cm using a wall-mounted stadiometer (SECA, Hanover, MD). Using a one-way random effects model, single measure intraclass correlations were calculated for anthropometric measures in girls six to ten years of age (n = 10) who were measured twice in a two-week period, and are calculated for body weight (r = 0.99) and standing height (r = 0.99). Body mass index (BMI) values were calculated by dividing weight in
kg by height in meters\(^2\). BMI values were individually plotted against the CDC Growth Charts, and results were presented to each participant's parent/guardian.\(^{17}\)

**Biochemical Markers**

Following an overnight fast, second void urine samples and blood samples were collected for analysis of bone turnover markers. Osteocalcin is synthesized by osteoblasts and is considered a specific indicator of bone formation.\(^{18,19}\) Intact serum OC was measured by radioimmunoassay in the laboratory of Dr. C.M. Gundberg (Yale University, New Haven, CT) according to the methods described by Gundberg et al.\(^{20}\) The inter-assay and intra-assay CVs are < 8.0\% and < 4.2\%, respectively. Bone resorption markers measured in this study included PYD and DPD. Pyridinoline and DPD are considered reliable markers of growth and bone resorption in young children.\(^{21}\) They are not metabolized or absorbed before excretion in the urine\(^{22}\) and do not significantly vary from day-to-day.\(^{21}\) Because these markers are muscle mass dependent, they are corrected for urinary creatinine excretion and reported in nmol/mmol creatinine. Urinary PYD and DPD were measured by high performance liquid chromatography in the laboratory of Dr. S. Shapses (Rutgers University, New Brunswick, NJ) according to the methods described by Pratt et al.\(^{23}\) The inter-assay CVs for PYD and DPD are 3.8\% and 5.9\%, respectively.

**Bone Mineral Density**

Areal bone mineral density (g/cm\(^2\)) was measured using dual-energy x-ray absorptiometry (DXA; QDR-1000W, Hologic, Inc., Waltham, MA). Total body (TB), lumbar spine (LS), total proximal femur (PF), and non-dominant radius (R) were assessed for bone area (BA, cm\(^2\)), bone mineral content (BMC, g), and aBMD. Dual-energy x-ray absorptiometry is considered an accurate method for evaluating bone mass in children.\(^{24}\)
Quality control was carried out through daily calibration with the calcium hydroxyapatite and epoxy lumbar spine phantom provided by the manufacturer. A laboratory CV of 0.27% was observed from 365 scans over five years. In our laboratory, test-retest measurements using DXA demonstrated the following CVs for the TB (0.58%) and LS (0.63%) in girls six to ten years of age (n=10) measured twice within a one-week period.

**Sexual Maturation**

Sexual maturation ratings were assessed annually by a physician using graphic representations of Tanner stages 1 through 5 which classifies female subjects by breast and pubic hair development. Tanner stage 1 represents no development or prepuberty; stage 2 represents the onset of puberty with initial development of breasts and pubic hair; stages 3 and 4 represent continued maturation of breasts and pubic hair; stage 5 represents achievement of menarche in females and the adult level of sexual development. Tanner stages provide an estimation of sexual maturation and reflect hormone levels that contribute to growth and development. Staging was based exclusively on breast rating in case of discrepancies between breast and pubic hair ratings. Tanner stage results were used for documenting that participants were prepubertal at baseline, and for statistically controlling for maturation when examining the dependent variables OC, PYD, and DPD.

**Physical Activity**

Physical activity was assessed annually by three-day accelerometry. Subjects wore accelerometers for two weekdays and one weekend day (CSA, Computer Science Applications, Model 7164). Accelerometers were worn above the iliac crest of the right
hip. Puyau et al suggest that this is the most effective placement for accelerometers in children.\(^{28}\) Accelerometers measure frequency and intensity of activity, and data are reported as counts/minute. Accelerometry has been shown to correlate well with energy expenditure in young children and adolescents, and is considered a reliable method for estimating physical activity.\(^ {29}\) Physical activity, reported in counts per minute, was used as a predictor variable in a regression model to help explain the change in bone turnover markers.

**Calcium Intake**

Three-day diet records, which have been shown to be valid and reliable for estimating calcium intake,\(^ {30-32}\) were used to determine mean calcium intakes annually. Parents received training with food models, pictures of serving sizes, and by completing a practice 24-hour recall with a trained lab technician to ensure accuracy of the records. Diet records included time of eating, type of food eaten, preparation method used, amount eaten, and specifically asked if any calcium-fortified foods were consumed. Diet records were analyzed by a trained lab technician using Food Processor II (version 7.5, ESHA Research, Salem, OR). Using a one-way random effects model, the intra-class correlation for the average measure of 3-days of dietary intake (n=10) completed twice in a two-week period for calcium was 0.71.

**Data Analyses**

Data were analyzed using Statistical Analaysis Software (SAS, Cary, NC, Version 8.2). Descriptive statistics were run for all variables. To determine whether variables increased with age, simple linear regressions were fit where each model included a random subject effect to account for within-subject correlation among repeated measures.
In addition, regression models were fit to explore the relationships between annual increments in bone turnover markers and the explanatory variables body weight, height, BMI, physical activity, and calcium intake. These models also included random subject effects to account for within-subject correlation. To investigate whether changes in bone turnover markers were reliable predictors of changes in bone parameters, canonical correlation analysis was used.\(^{(33)}\) Canonical correlations are used to determine relationships between two sets of grouped variables as opposed to multiple regression analysis, which assesses relationships between one dependent variable and a set of independent variables. Separate analyses were run to obtain canonical correlations between the annual increments in turnover markers, OC:DPD, and BA, BMC, and aBMD at all sites. A p-value of less than 0.05 was considered significant for all analyses.

**RESULTS**

Means ± SD for serum OC, urinary PYD and DPD, anthropometrics, sexual maturation, physical activity, and calcium intake at baseline, one, two, and three years are presented in Table 3.1. There was a significant time effect for weight, height, and BMI (p <0.05). Mean BMI (kg/m\(^2\)) at baseline was 16.5 for a mean age of six years, corresponding to values between the 75\(^{th}\) and 85\(^{th}\) percentiles for age. All subjects were classified as Tanner stage 1 for breast maturation at baseline. By 36 months, 19 subjects remained in Tanner stage 1, nine subjects were in Tanner Stage 2, and four subjects progressed to Tanner stage 3. There were no significant changes in physical activity or calcium intake over the three years. Physical activity (cts/min) at baseline was 626 ± 221 cts/min. Mean calcium intake at baseline approached the current Adequate Intake (AI; 800mg/d) for children 4-8 years of age (784 mg/d; ~98% of AI). Table 3.2 displays the
Mean values for body composition and bone measurements at baseline, one, two, and three years. Fat mass, fat-free mass, BA, BMC, and aBMD at all sites increased significantly with age (p < 0.05). Gains in aBMD at all sites were approximately 16%.

Mean OC, PYD, and DPD values are presented by age and Tanner stage in Table 3.3. The results are based on number of observations for each age group throughout the study. Osteocalcin and DPD were higher in Tanner Stages 2 to 3 (p > 0.05), while PYD was similar across all Tanner stages. There was a significant linear trend over time for OC which increased approximately 16% over three years (slope = 1.74, SE = 0.35, p < 0.0001), while no significant age effects were observed for PYD and DPD.

Mean values of bone variables by site and Tanner stage and age are presented in Table 3.4. The results are based on the number of observations available, similar to Table 3.3, in each Tanner stage and age throughout the three years. Significant increases occurred between Tanner stages 1 and 2 for BA and BMC at the TB; BA, BMC, and BMD at the LS; and PF BMC. Significant increases between Tanner stages 2 and 3 occurred for TB and LS BMC and BMD; PF BA, BMC, and BMD; and R BA and BMC.

Table 3.5 represents mean annual changes in bone turnover markers between chronological ages. There was a significant time effect for OC, as annual changes in OC increased with increasing age (slope = 0.67, SE = 0.33, p = 0.0484). No differences in annual changes were observed over time for PYD or DPD. During Tanner stage 1, OC (ng/mL; mean ± SE) changed an average of 1.1 ± 0.7 per year. Osteocalcin changed an average of 3.5 ±1.3 and 2.8 ± 2.6 during Tanner stages 2 and 3, respectively.

Pearson correlations were determined between the changes in all markers of bone turnover across the three-year period. Changes in OC were not related to the changes in
PYD or DPD (r = .239, p = .186; r = .0069, p = .970) over the three-year period. However, changes in PYD and DPD were positively and significantly correlated throughout the study (r = .832, p < .0001).

Changes in OC were predicted only by body weight (slope = 0.305, SE = 0.095, p = 0.002). Changes in PYD were significantly predicted by BMI and height (slope = 27.2, SE = 36.6, p = 0.002 and slope = 17.5, SE = 36.6, p < 0.05, respectively). Body mass index was the only significant predictor variable for changes in DPD (slope = 3.52, SE = 1.47, p = 0.019).

Table 3.6 presents estimates of the first canonical correlation coefficients between changes in bone turnover markers, the OC:DPD ratio, and changes in BA, BMC, and aBMD at all sites as well as Pearson correlations among individual variables. The correlations between the change in all three markers, OC:DPD, and changes over time in bone variables were low and non-significant for all bone sites.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>12 Months</th>
<th>24 Months</th>
<th>36 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropometrics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>6.0 ± 1.7</td>
<td>7.0</td>
<td>8.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Height (cm) (^a)</td>
<td>116.5 ± 13.2</td>
<td>123.2 ± 12.8</td>
<td>129.9 ± 12.9</td>
<td>136.6 ± 13.3</td>
</tr>
<tr>
<td>Weight (kg) (^a)</td>
<td>23.0 ± 7.0</td>
<td>27.0 ± 8.6</td>
<td>31.7 ± 10.4</td>
<td>36.4 ± 12.2</td>
</tr>
<tr>
<td>BMI (kg/m(^2)) (^a)</td>
<td>16.5 ± 1.9</td>
<td>17.3 ± 2.7</td>
<td>18.3 ± 3.2</td>
<td>19.0 ± 3.7</td>
</tr>
<tr>
<td><strong>Bone Turnover Markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin (ng/mL) (^a)</td>
<td>28.7 ± 13.7</td>
<td>29.9 ± 7.8</td>
<td>31.7 ± 8.4</td>
<td>33.4 ± 9.4</td>
</tr>
<tr>
<td>Pyridinoline (nmol/mmol Cr)</td>
<td>165.4 ± 53.2</td>
<td>170.3 ± 55.9</td>
<td>181.2 ± 57.7</td>
<td>176.7 ± 52.0</td>
</tr>
<tr>
<td>Deoxypyridinoline (nmol/mmol Cr)</td>
<td>51.2 ± 17.7</td>
<td>50.6 ± 19.8</td>
<td>55.0 ± 16.7</td>
<td>53.5 ± 12.8</td>
</tr>
<tr>
<td>Calcium Intake (mg/day)</td>
<td>784 ± 338</td>
<td>727 ± 283</td>
<td>872 ± 331</td>
<td>858 ± 384</td>
</tr>
<tr>
<td>Physical Activity (counts/minute)</td>
<td>626 ± 221</td>
<td>728 ± 403</td>
<td>745 ± 319</td>
<td>714 ± 358</td>
</tr>
<tr>
<td>Sexual Maturation Tanner Stage</td>
<td>1.0 ± 0.0</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± .4</td>
<td>1.5 ± .7</td>
</tr>
</tbody>
</table>

Values are means ± SD. \(^a\)Significant time effects from baseline to 36 months (p < .0001).
Table 3.2. Body Composition [Fat Tissue (g), Fat Free Tissue (g), Percent Body Fat], Bone Area (BA, cm²), Bone Mineral Content (BMC, g), and Bone Mineral Density (BMD, g/cm²) in Subjects Over Three Years

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>One Year</th>
<th>Two Years</th>
<th>Three Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat Tissue(g)a</td>
<td>6374 ± 3625</td>
<td>7671 ± 4786</td>
<td>9474 ± 6036</td>
<td>11165 ± 7050</td>
</tr>
<tr>
<td>Fat-free Tissue(g)a</td>
<td>16027 ± 4311</td>
<td>18338 ± 4596</td>
<td>21069 ± 5584</td>
<td>24019 ± 6822</td>
</tr>
<tr>
<td>Percent Body Fat</td>
<td>27.2 ± 8.1</td>
<td>27.6 ± 9.4</td>
<td>28.8 ± 10.3</td>
<td>29.5 ± 11.0</td>
</tr>
<tr>
<td>Total Body</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA a</td>
<td>1174 ± 267</td>
<td>1295 ± 282</td>
<td>1452 ± 317</td>
<td>1608 ± 351</td>
</tr>
<tr>
<td>BMC a</td>
<td>773 ± 246</td>
<td>896 ± 268</td>
<td>1055 ± 309</td>
<td>1224 ± 369</td>
</tr>
<tr>
<td>BMD a</td>
<td>0.646 ± .065</td>
<td>0.682 ± .066</td>
<td>0.716 ± .065</td>
<td>0.749 ± .072</td>
</tr>
<tr>
<td>Lumbar Spine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA a</td>
<td>29.2 ± 5.5</td>
<td>31.9 ± 5.7</td>
<td>35.0 ± 6.3</td>
<td>38.1 ± 7.1</td>
</tr>
<tr>
<td>BMC a</td>
<td>16.0 ± 5.0</td>
<td>18.1 ± 5.1</td>
<td>20.9 ± 6.2</td>
<td>24.3 ± 8.1</td>
</tr>
<tr>
<td>BMD a</td>
<td>0.539 ± .067</td>
<td>0.561 ± .067</td>
<td>0.586 ± .073</td>
<td>0.626 ± .095</td>
</tr>
<tr>
<td>Proximal Femur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA a</td>
<td>15.9 ± 4.2</td>
<td>17.7 ± 4.3</td>
<td>19.3 ± 4.8</td>
<td>21.6 ± 5.3</td>
</tr>
<tr>
<td>BMC a</td>
<td>9.8 ± 3.7</td>
<td>11.4 ± 3.9</td>
<td>13.0 ± 4.6</td>
<td>15.5 ± 5.7</td>
</tr>
<tr>
<td>BMD a</td>
<td>0.595 ± .082</td>
<td>0.630 ± .078</td>
<td>0.657 ± .082</td>
<td>0.700 ± .098</td>
</tr>
<tr>
<td>Radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA a</td>
<td>6.0 ± 1.4</td>
<td>6.7 ± 1.3</td>
<td>7.4 ± 1.6</td>
<td>8.4 ± 1.8</td>
</tr>
<tr>
<td>BMC a</td>
<td>2.1 ± 0.7</td>
<td>2.5 ± 0.8</td>
<td>2.9 ± 0.9</td>
<td>3.4 ± 1.1</td>
</tr>
<tr>
<td>BMD a</td>
<td>0.342 ± .047</td>
<td>0.365 ± .048</td>
<td>0.377 ± .048</td>
<td>0.396 ± .047</td>
</tr>
</tbody>
</table>

Values are means ± SD. aSignificant time effects from baseline to three years (p < .0001).
Table 3.3. Osteocalcin, Pyridinoline, and Deoxypyridinoline by Tanner Stage and Age

<table>
<thead>
<tr>
<th>Tanner Stage</th>
<th>Osteocalcin(^a) (ng/mL)</th>
<th>Pyridinoline (nmol/mmol Cr)</th>
<th>Deoxypyridinoline (nmol/mmol Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 106)</td>
<td>30.2 ± 1.2</td>
<td>171.7 ± 6.2</td>
<td>51.8 ± 2.4</td>
</tr>
<tr>
<td>2 (n = 18)</td>
<td>35.1 ± 1.8</td>
<td>172.0 ± 13.2</td>
<td>57.2 ± 4.0</td>
</tr>
<tr>
<td>3 (n = 4)</td>
<td>38.7 ± 2.8</td>
<td>225.4 ± 26.3</td>
<td>54.0 ± 7.2</td>
</tr>
</tbody>
</table>

Age

<table>
<thead>
<tr>
<th></th>
<th>Osteocalcin(^a) (ng/mL)</th>
<th>Pyridinoline (nmol/mmol Cr)</th>
<th>Deoxypyridinoline (nmol/mmol Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (n = 10)</td>
<td>29.7 ± 5.2</td>
<td>170.6 ± 15.1</td>
<td>52.7 ± 5.8</td>
</tr>
<tr>
<td>5 (n = 17)</td>
<td>26.7 ± 1.7(^a)</td>
<td>164.2 ± 14.5</td>
<td>46.8 ± 4.5</td>
</tr>
<tr>
<td>6 (n = 18)</td>
<td>30.5 ± 1.7(^a)</td>
<td>179.7 ± 17.6</td>
<td>52.2 ± 5.8</td>
</tr>
<tr>
<td>7 (n = 24)</td>
<td>30.0 ± 1.2</td>
<td>174.7 ± 7.4</td>
<td>52.6 ± 2.7</td>
</tr>
<tr>
<td>8 (n = 19)</td>
<td>29.7 ± 1.1</td>
<td>172.0 ± 11.6</td>
<td>54.4 ± 3.5</td>
</tr>
<tr>
<td>9 (n = 16)</td>
<td>30.0 ± 1.6</td>
<td>166.4 ± 12.2</td>
<td>51.4 ± 3.7</td>
</tr>
<tr>
<td>10 (n = 14)</td>
<td>34.8 ± 1.9</td>
<td>172.2 ± 15.1</td>
<td>52.9 ± 4.4</td>
</tr>
<tr>
<td>11 (n = 8)</td>
<td>38.4 ± 5.4</td>
<td>190.7 ± 27.9</td>
<td>61.0 ± 3.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. Tanner stage 1 represents prepuberty, Tanner stages 2 and 3 represent early-mid puberty. \(^a\) Significant increase over time (p < .05).
TABLE 3.4. AVERAGE BONE AREA (BA), BONE MINERAL CONTENT (BMC), AND AREAL BONE MINERAL DENSITY (ABMD) FOR TOTAL BODY, LUMBAR SPINE, PROXIMAL FEMUR, AND RADIUS BY TANNER STAGE AND AGE.

<table>
<thead>
<tr>
<th>Tanner stage</th>
<th>Total Body</th>
<th>Lumbar Spine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA</td>
<td>BMC</td>
</tr>
<tr>
<td>1</td>
<td>1312 ± 41</td>
<td>912 ± 39</td>
</tr>
<tr>
<td>2</td>
<td>1695 ± 57*</td>
<td>1302 ± 56*</td>
</tr>
<tr>
<td>3</td>
<td>1855 ± 92</td>
<td>1552 ± 93*</td>
</tr>
<tr>
<td>Age</td>
<td>4</td>
<td>917 ± 29</td>
</tr>
<tr>
<td>5</td>
<td>1046 ± 22</td>
<td>658 ± 21</td>
</tr>
<tr>
<td>6</td>
<td>1158 ± 32</td>
<td>768 ± 31</td>
</tr>
<tr>
<td>7</td>
<td>1300 ± 33</td>
<td>908 ± 33</td>
</tr>
<tr>
<td>8</td>
<td>1433 ± 44</td>
<td>1018 ± 40</td>
</tr>
<tr>
<td>9</td>
<td>1613 ± 42</td>
<td>1201 ± 48</td>
</tr>
<tr>
<td>10</td>
<td>1801 ± 49</td>
<td>1392 ± 54</td>
</tr>
<tr>
<td>11</td>
<td>1918 ± 69</td>
<td>1541 ± 86</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tanner stage</th>
<th>Proximal Femur</th>
<th>Radius</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA</td>
<td>BMC</td>
</tr>
<tr>
<td>1</td>
<td>17.7 ± 0.6</td>
<td>11.4 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>22.2 ± 0.9</td>
<td>16.1 ± 0.8*</td>
</tr>
<tr>
<td>3</td>
<td>25.8 ± 1.3*</td>
<td>21.3 ± 1.3*</td>
</tr>
<tr>
<td>Age</td>
<td>4</td>
<td>11.8 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>13.7 ± 0.5</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>6</td>
<td>15.4 ± 0.4</td>
<td>9.4 ± 0.4</td>
</tr>
<tr>
<td>7</td>
<td>17.4 ± 0.5</td>
<td>11.1 ± 0.6</td>
</tr>
<tr>
<td>8</td>
<td>19.1 ± 0.7</td>
<td>12.7 ± 0.7</td>
</tr>
<tr>
<td>9</td>
<td>22.2 ± 0.5</td>
<td>15.3 ± 0.6</td>
</tr>
<tr>
<td>10</td>
<td>24.2 ± 0.7</td>
<td>17.5 ± 0.9</td>
</tr>
<tr>
<td>11</td>
<td>26.4 ± 1.0</td>
<td>20.4 ± 1.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significantly different from previous age/tanner stage (p< 0.05).
**Table 3.5. Annual Change in Osteocalcin, Pyridinoline, and Deoxypyridinoline by Chronological Age**

<table>
<thead>
<tr>
<th>Age Range</th>
<th>Osteocalcin&lt;sup&gt;a&lt;/sup&gt; (ng/mL)</th>
<th>Pyridinoline (nmol/mmol Cr)</th>
<th>Deoxypyridinoline (nmol/mmol Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-5 (n = 10)</td>
<td>-3.6 ± 1.4</td>
<td>-6.4 ± 17.0</td>
<td>-6.8 ± 5.1</td>
</tr>
<tr>
<td>5-6 (n = 17)</td>
<td>2.7 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.3 ± 19.2</td>
<td>5.3 ± 4.5</td>
</tr>
<tr>
<td>6-7 (n = 18)</td>
<td>0.6 ± 1.0</td>
<td>7.3 ± 16.5</td>
<td>4.5 ± 5.2</td>
</tr>
<tr>
<td>7-8 (n = 12)</td>
<td>0.9 ± 1.3</td>
<td>-3.9 ± 16.1</td>
<td>-0.9 ± 5.2</td>
</tr>
<tr>
<td>8-9 (n = 14)</td>
<td>2.3 ± 0.9</td>
<td>-1.3 ± 14.4</td>
<td>-2.9 ± 3.5</td>
</tr>
<tr>
<td>9-10 (n = 15)</td>
<td>3.3 ± 1.8</td>
<td>9.0 ± 12.0</td>
<td>1.7 ± 3.8</td>
</tr>
<tr>
<td>10-11 (n = 8)</td>
<td>2.6 ± 2.8</td>
<td>-9.6 ± 33.5</td>
<td>-1.1 ± 5.4</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Significant time effects from youngest to oldest age group (p = 0.048). <sup>b</sup>Significant difference from previous age group (p = 0.025)

**Table 3.6. Canonical Correlations Between Changes in Osteocalcin, Pyridinoline, Deoxypyridinoline, OC:DPD and Bone Area (BA), Bone Mineral Content (BMC), and Bone Mineral Density (BMD).**

<table>
<thead>
<tr>
<th>Variable</th>
<th>OC</th>
<th>PYD</th>
<th>DPD</th>
<th>OC:DPD</th>
<th>Canonical Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ Total Body</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>BA</td>
<td>0.20</td>
<td>0.23</td>
<td>0.20</td>
<td>-0.13</td>
<td></td>
</tr>
<tr>
<td>BMC</td>
<td>0.15</td>
<td>0.15</td>
<td>0.07</td>
<td>-0.13</td>
<td></td>
</tr>
<tr>
<td>BMD</td>
<td>-0.16</td>
<td>0.02</td>
<td>-0.04</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>□ Lumbar Spine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.27</td>
</tr>
<tr>
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<td>-0.10</td>
<td>-0.03</td>
<td>-0.13</td>
<td>-0.17</td>
<td></td>
</tr>
<tr>
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<td>-0.10</td>
<td>-0.16</td>
<td>-0.12</td>
<td></td>
</tr>
<tr>
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<td>0.04</td>
<td>-0.11</td>
<td>-0.15</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>□ Proximal Femur</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>BA</td>
<td>-0.06</td>
<td>-0.01</td>
<td>0.01</td>
<td>-0.02</td>
<td></td>
</tr>
<tr>
<td>BMC</td>
<td>-0.02</td>
<td>0.06</td>
<td>0.03</td>
<td>-0.04</td>
<td></td>
</tr>
<tr>
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<td>0.21</td>
<td>0.13</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>□ Radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>BA</td>
<td>-0.06</td>
<td>-0.02</td>
<td>-0.20</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td>BMC</td>
<td>-0.10</td>
<td>-0.08</td>
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<td>-0.11</td>
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</tr>
<tr>
<td>BMD</td>
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<td>-0.20</td>
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<td>-0.12</td>
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</tbody>
</table>

Values presented are R values.
DISCUSSION

Bone turnover markers have typically been used in pediatric populations to assess the response to treatments in children with growth disorders and impairments. With the increasing interest in diet and exercise interventions aimed at maximizing peak bone mass in children, bone turnover markers are being employed to help facilitate our understanding of the bone remodeling response during such interventions. The objective of this investigation was to examine the changes in OC, PYD, and DPD in relation to changes in site-specific BA, BMC, and aBMD over three years in healthy, prepubertal females. Our primary finding was that bone turnover markers did not predict changes in bone for the TB, LS, PF, or R, despite significant increases in bone variables and OC over time. An equally important finding was that annual changes in bone turnover markers were significantly related to anthropometrics including body weight with OC, BMI and height with PYD, and BMI with DPD.

Data from cross-sectional studies suggest, similar to our findings, that OC remains relatively constant during Tanner stage 1, increases during stages 2 and 3 and then decreases through Tanner stage 5 and into adulthood. Mean OC values (ng/mL) for females in our study were 30.2 in Tanner stage 1 (n = 106), 35.1 in stage 2 (n = 18), and 38.7 in stage 3 (n = 4). These values and trends are similar to those observed by Heuck et al and Seydewitz et al but the majority of subjects in our sample were in Tanner stage 1 with a limited number in Tanner stages 2 and 3.\(^{34, 35}\)

While OC is a valid and reliable marker of bone formation in both adults and children, use of additional markers of bone formation may have been advantageous. During remodeling and modeling, bone is formed in a specific order with mineralization
as the last phase. It is believed that OC is involved in the mineralization process, whereas other markers, such as bone-specific alkaline phosphatase (BSAP) may be involved in matrix formation or initiation of mineralization. During growth the process of bone mineralization may not be able to keep up with the rapid volume expansion, and bone matrix laid down on one surface may be resorbed before it has the opportunity for complete mineralization. Therefore, bone turnover markers that reflect a different aspect of bone formation might more strongly correlate with changes in bone mass during prepuberty.

In spite of the fact that our mean values for PYD and DPD were similar to those presented by Mora et al for Tanner stages 1 and 2, there was high variability in levels and rates of change between individual subjects for PYD and DPD.\(^{(5)}\) Although subjects were tested at the same time of day and year to control for circadian rhythm, we employed staggered enrollment such that groups of subjects were tested in one particular season throughout the study. Reports from Rapuri et al and Heuck et al suggest that some markers of bone turnover change by season, with higher values during the winter months and lower values during the summer.\(^{(34,36)}\) Discrepancies between bone turnover markers in our investigation may have been due to the staggered recruitment and testing. However, the primary objective of our study assessed changes in markers, rather than absolute marker values, to predict changes in bone mass so this methodology should not have contributed to the lack of relationship between markers and bone properties.

The steadiness over time in PYD and DPD has also been observed in cross-sectional studies,\(^{(8,37)}\) and may be the result of several other factors. During the prepubertal years, growth is relatively slow, however, in Tanner stages 2 and 3, the rate
of height velocity and bone mineral accrual increases dramatically due to a combination of growth factors and sex hormones including insulin-like growth factor-1 and estrogen, respectively, in females. These effects were observed in our subjects in that significant increases in bone properties were found between Tanner stages 1 and 2 at the total body and lumbar spine, and between 2 and 3 at all sites. The majority of subjects in our study remained in Tanner stage 1, likely contributing to the little change in PYD and DPD over time, and possibly limiting any relationships between markers and bone properties. Had additional subjects entered Tanner stages 2 and 3, we may have seen significant relationships between variables.

Increases in fat-free mass accompany pubertal growth, contributing to increased creatinine excretion. Accordingly, significant increases in fat-free mass occurred over time in our subjects \((p < 0.0001)\). Urinary markers of bone turnover such as PYD and DPD are corrected for creatinine excretion, and if this is not reflective of increased creatinine due to gains in fat-free mass, values of PYD and DPD over time could be misleading. Future investigations should consider the use of serum markers of resorption which are independent of creatinine excretion and fat-free mass.

Interventions in children of a similar maturation as the majority of our subjects have shown that prepuberty may be an optimal time for bone to respond to various stimuli. The results from Slemenda et al and Bass et al show that prepubertal subjects significantly increased BMD in response to a calcium supplementation trial and exercise, respectively.\(^{14, 38}\) Neither study, however, included assessment of bone turnover markers during the intervention. In a follow-up to the calcium supplementation trial, Slemenda et al found that twins who had received calcium supplements and had significantly greater
gains in BMD also had significantly lower levels of OC which seemed to explain the BMD changes. This relationship was only found in prepubertal twins. These results may suggest that the ability of bone turnover markers to predict changes in bone mass during prepuberty, as the majority of our subjects were, occurs only in intervention trials where aBMD is altered beyond normal growth. In the present study, subjects' average calcium intakes were very similar to the AI for 4-8 year old children (98%). Additionally, their average level of physical activity was similar to levels found by those from the Iowa Bone Development Study which are considered normal. Perhaps the high between-subject variability in bone turnover markers requires a significant change in bone metabolism to statistically alter levels of markers, and with the average calcium intakes and physical activity levels of our subjects, bone turnover probably remained steady over the course of the study.

Bone growth during childhood and adolescence is site-specific and proceeds at various rates throughout the maturation process. During the prepubertal years, growth of the appendicular skeleton is more rapid than in the axial skeleton, which increases during puberty. For example, Sundberg et al found that by Tanner stage 2, females gained only 84% of aBMD at the femoral neck and 86% at the LS. Additionally, gains at the femoral neck preceded those in the spine by approximately one year, showing that growth of the legs is more rapid than trunk growth during prepuberty. Bass et al found similar growth trends in prepubertal females where percent of peak values for the radius BMC and length were slightly higher than those of the femur and spine during prepubertal growth. Considering that the majority of subjects in our sample remained in prepuberty, it is possible that low gains in aBMD of the sites measured contributed to the
lack of relationship between bone turnover markers and aBMD. Based on the R-values of the canonical correlations (R = 0.42 for radial bone measurements), there was a stronger, albeit non-significant, relationship between turnover markers and bone properties at the radius compared to other sites measured. This agrees with the rate of growth for the radius as part of the appendicular skeleton, and it is possible that measurement of other sites in the appendicular skeleton would have shown significant correlations with turnover markers.

In addition to fluctuating rates of growth throughout specific sites in the skeleton, the structural properties of bone also change according to maturation stage. Bass et al found that gains in cortical area of the humerus in prepubertal tennis players were primarily due to periosteal expansion, while in late puberty they were due to medullary contraction, both of which resulted in greater bone strength. These structural changes are not measurable through DXA, and may not be apparent in changes in aBMD. Thus, it remains possible that bone turnover markers in prepubertal children may better predict changes in structural properties rather than aBMD of bone.

In this investigation, changes in serum OC and urinary PYD and DPD were not related to changes in bone variables over three years but were related to indices of growth including weight, height, and BMI. Similarly, cross-sectional and longitudinal data in pubertal females have shown that bone turnover markers change similarly to height velocity, suggesting that they may be useful in monitoring growth but not changes in bone mineral during pre- and early puberty in females.

To our knowledge, long-term individual changes in bone turnover markers previously have not been assessed in healthy, prepubertal children. This study was
strengthened by the fact that all subjects were of the same maturation at baseline, had no
prior sports participation, and mean calcium intakes were adequate, based on the AI,
throughout the study.

Further prospective investigations into bone turnover markers in children are
warranted, however, in prepubertal females, markers may be best suited for use in studies
of interventions. Researchers should consider the use of multiple markers of bone
turnover in order to identify which markers, if any, are capable of predicting changes in
bone variables. Researchers should also choose assays available in the serum whenever
possible. Finally, assessment of all aspects of bone mineral, including density and
structural properties, in both the appendicular and axial skeleton may be necessary to
determine a relationship between changes in bone and bone turnover markers.


14. Slemenda CW, Peacock M, Hui S, Zhou L, and Johnston CC, 1997 Reduced rates of skeletal remodeling are associated with increased bone mineral density during


17. CDC, CDC Growth Charts: United States., in National Center for Health Statistics in collaboration with the National Center for Chronic Disease Prevention and Health Promotion.


44. Kubo T, Tanaka H, Inoue M, Kanzaki S, and Seino Y, 1995 Serum levels of carboxyterminal propeptide of type I procollagen and pyridinoline crosslinked
CHAPTER 4  
SUMMARY AND CONCLUSIONS

In this three-year prospective study, 32 healthy prepubertal females were followed to examine the relationship between the bone turnover markers, serum osteocalcin (OC) and urinary pyridinoline (PYD) and deoxypyridinoline (DPD), and anthropometrics, sexual maturation, calcium intake, physical activity, and changes in BA, BMC, and BMD. We observed significant time effects for OC, BA, BMC, and BMD at the TB, LS, PF, and R (p < 0.0001), all of which increased approximately 16% from baseline to three-year measurements. There were not significant time effects for PYD and DPD. Significant positive relationships were found between annual changes in OC and body weight, PYD and BMI and height, and DPD and BMI. Canonical correlations between changes in all bone turnover markers and changes in BA, BMC, and BMD at all sites were low and non-significant.

The findings of this study agree with cross-sectional data in prepubertal children in that bone turnover markers may not be useful in predicting changes in bone mass but may be most useful in monitoring changes in height and weight.\(^{(32)}\) Similarly, the one 18 month longitudinal study in pubertal females found that bone turnover markers were related only to gains in height.\(^{(14)}\)

The reliability of our results is strengthened by our consideration of other confounding factors related to changes in bone mass, such as calcium intake and physical activity. Additionally, circadian and potential seasonal variations were controlled for by
consistent methodology in annual testing sessions, and by focusing on changes rather than absolute values of markers in relation to changes in bone variables.

The majority of subjects remained in Tanner stage 1 for the duration of the study, and the limited range of marker values may have contributed to the lack of relationship between markers and bone properties. Tanner stage 1, or prepuberty, is characterized by relatively slow growth and bone turnover compared to puberty. The highest correlation ($R = 0.42$) observed between the markers and bone measurements occurred in the radius, which is part of the appendicular skeleton. Growth that does occur during prepuberty is focused more in the appendicular, rather than axial skeleton, which may also be a contributing factor to the lower correlations at other measured sites. Additionally, limitations in DXA may have contributed to our findings in that DXA is not able to measure the three-dimensional structural properties of bone that change during growth. Cross-sectional data from Mora et al show that PYD and DPD correlated with cortical area of bone but this relationship was observed only in pubertal subjects. It remains possible that bone turnover markers are capable of predicting changes in these bone properties but only during puberty. Further longer-term monitoring of our subjects as they enter puberty may allow for such relationships to emerge.

Research has shown that bone of prepubertal children responds more readily than pubertal children to interventions such as calcium supplementation or exercise. Slemenda et al found that gains in BMD, due to calcium supplementation in prepubertal subjects, were accompanied by significantly lower OC levels immediately following the calcium intervention. Our study was a prospective observational design. The subjects in our study had mean calcium intakes similar to the AI for four to eight year old
children. Additionally, average physical activity levels were similar to those reported in the Iowa Bone Development Study.\(^{(101)}\) It is possible that a significant change in bone metabolism results only from intervention during prepuberty, and with average calcium intake and physical activity in our subjects, there were not adequate stimuli to up- or down-regulate bone remodeling as with the subjects of Slemenda et al.\(^{(4)}\)

In conclusion, serum OC and urinary PYD and DPD did not predict changes in bone properties despite similar increases in OC and aBMD at all sites. Bone turnover markers were related to changes in height and weight over three years in healthy female children and thus, may be useful in monitoring growth. Suggestions for future research include the assessment of additional markers of bone turnover and use of bone turnover markers during intervention trials or in larger samples of pubertal adolescents. Finally, the assessment of three-dimensional structural properties of bone concomitantly with bone turnover markers may provide important information regarding prepubertal bone growth.


APPENDIX A

Subject ID#: ______________________
Interviewer: ______________________
Date of Interview: _________________

Demographic Data:

I am going to ask you some questions about your age, family, and education. Your
mother or father can help you answer.

1. What is your date of birth? Month ________ Day ________ Year _______

2. What is your age? Years ________________ Months ________________

3. Gender: (Circle One) Female Male

4. What is your race? (Circle One) Caucasian

Black
Asian
Hispanic
American Indian
Other ________________________

5. Do you live with your parents? (Circle One) YES NO

5a. If no, with whom do you live? ________________________________

6. Do you have any brothers or sisters? (Circle One) YES NO

6a. If yes, list ages of: _______Years (Brother) _______Years (Sister)

_______Years (Brother) _______Years (Sister)

_______Years (Brother) _______Years (Sister)

6b. If yes, do they participate in gymnastics or others sports? (Circle One)

YES NO

6c. If yes, list the sport and gender of sibling. Sport____(Brother or Sister)

Sport____(Brother or Sister)
Sport____(Brother or Sister)
Sport____(Brother or Sister)

7. Do you have a twin sister? (Circle One) YES NO

8. At what age did you start gymnastics? _______Years _______Months
9. Was your mother a gymnast? (Circle One) YES NO

Subject ID#: ___________________
Interviewer: ___________________
Date of Interview: ______________

10. What is your parents income? (Circle One) Less than $9,999

$10,000 - $19,999
$20,000 - $29,999
$30,000 - $39,999
$40,000 - $49,999
$50,000 - $59,999
$60,000 - $69,999
$70,000 - $79,999
$80,000 - $89,999
$90,000 - $99,999
More than $100,000

11. What grade are you in school? (Circle One) Kindergarten 1st 2nd 3rd

12. What is your mother’s occupation? ______________________________

13. What is your father’s occupation? ______________________________

Subject ID#: ___________________
Interviewer: ___________________
Date of Interview: ______________

Health Data

Now, I am going to ask you to respond to a few questions about your health. I am the only one that will know how you answer these questions, so please be honest with your answers.

1. How much do you weigh? ___________ pounds (Actual scale weight _____ pounds)

2. How tall are you? ___________ feet ___________ inches

3. BMI=___________ (Interviewer to complete later)
4. Have you gained or lost any weight (≥ 10 pounds) in the past 3 months? (Circle One) YES NO
   4a. If yes, how much? +_______ pounds OR -_______ pounds
5. Have you had any height changes in the past 3 months? (Circle One)
   YES NO
   5a. If yes, how much? ___________ feet ___________ inches
6. How much would you like to weigh? ______________ pounds
7. How tall would you like to be? ___________ feet ___________ inches
8. How would you rate your present health? (Circle One)
   Poor Fair Good Excellent
9. Have you started your menstrual cycles? (Circle One) YES NO
   If so, what date?
10. Do you have any diseases or illnesses? (Circle One) YES NO
    10a. If yes, what diseases? __________________________
         __________________________
         __________________________
11. Are you taking any medications either prescribed by a doctor or over-the-counter (self-prescribed)? (Circle One) YES NO
    11a. If yes, what medications? ________________ Amount per day _____
         ________________ Amount per day _____
         ________________ Amount per day _____

Those were some difficult questions to answer because the questions were so private. I want to assure you again that I am the only person who knows how you answered these questions. Thank you for being so honest with your answers.

Subject ID#: ______________________
Interviewer: ______________________
Date of Interview: ________________

**Nutrition Data:**

These next questions are about your diet and eating habits. Try to think about how you eat.
1. Do you eat three meals per day? (Circle One) YES  NO
   1a. If no, why not? __________________________________________

2. Do you eat snacks during the day? (Circle One)  YES  NO
   2a. If yes, how many snacks per day do you eat? _____ snacks per day

3. Are you following a special kind of diet? (Circle One) YES  NO
   3a. If yes, what kind of diet? __________________________________

4. Do you take any vitamin or mineral supplements or any “nutrition pills”? (Circle One) YES  NO
   4a. If yes, what kind? ____________________ Amount per day ________
       ____________________ Amount per day ________
       ____________________ Amount per day ________

5. Have you ever been on a diet to lose weight? (Circle One) YES  NO
   5a. If yes, what kind of diet was it? _____________________________
   5b. How old were you when you were on this diet? ____ years ____ months
       ____ years ____ months

6. Have you ever eaten a large amount of food and then vomited to get rid of the food? (Circle One) YES  NO
   6a. If yes, how old were you? __________ years _______ months
       __________ years _______ months

7. Have you ever starved yourself for more than three days? (Circle One) YES  NO
   7a. If yes, how old were you? ___________ years _______ months

Thank you for answering all of those questions. You did really well, and I appreciate your being so truthful with your answers. Next, I am going to ask you about your physical activity during the past 7 days. Try to think back on last week and the activities that you may have done.
Bone Health Data:
The next questions have to do with your bones and your family’s bones.
1. Does anyone in your family (including your parent’s, grandparents, aunts, uncles, cousins) have osteoporosis or “humpback”? (Circle One)  
   YES  NO
   1a. If yes, who is it? ________________________________
2. Has anyone in your family (including your parents, grandparents, aunts, uncles, cousins) had a hip or wrist fracture? (Circle One)  
   YES  NO
   2a. If yes, who is it? ________________________________
3. Have you ever had a bone fracture or broken bone? (Circle One)  
   YES  NO
   3a. If yes, what bone(s)? ________________________________
   3b. If yes, how old were you? ________ years ________ months
4. Have you ever been told by a doctor that you have bone disease? (Circle One)  
   YES  NO
   4a. If yes, what disease? ________________________________
   4b. If yes, how old were you? ________ years ________ months

Physical Activity
The next questions that I will ask you are about your physical activity such as P.E., recess, and exercise. There are no right or wrong answers, so please answer these questions the best that you can.
1. How would you rate your physical activity level? (Circle One)  
   Inactive  
   Below average  
   Average  
   Above average  
   Very high

2. Do you have any health problems that limit your activity? (Circle One)  YES  NO
   2a. If yes, what health problem? __________________________

3. Do you exercise regularly (not including P.E. class)? (Circle One)  YES  NO
   3a. If yes, how often? ________ hours per day/week/month (Circle One)

4. Do you participate in P.E. at school? (Circle One)  YES  NO
   4a. If yes, how often? ________ hours per day/week/month (Circle One)

5. Do you play games during recess? (Circle One) YES  NO
   5a. If yes, what games or activities do you play?________________________
       __________________________
   5b. If yes, how many hours per day do you play during recess? ________ hours per day

6. Do you play games after school? (Circle One) YES  NO
   6a. If yes, what games or activities do you play?________________________
       __________________________
   6b. If yes, how many hours per day do you play after school? ________ hours per day
APPENDIX B

24 HOUR RECALL

Date of Record ________________________ Subject Code No. ____________________

DAY OF WEEK TAKE:  M  T  W  TH  F  S  SUN  (CIRCLE)

<table>
<thead>
<tr>
<th>Food and Beverage Consumed</th>
<th>WHAT DID YOU EAT?</th>
<th>AMOUNT</th>
<th>COOKING METHOD</th>
<th>TIME OF DAY</th>
<th>ACTIVITY WHILE EATING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example</td>
<td>EGGS</td>
<td>2 med.</td>
<td>fried</td>
<td>7:30 a.m.</td>
<td>talking with family</td>
</tr>
</tbody>
</table>

BREAKFAST


SNACK


LUNCH


SNACK


DINNER


SNACK


ANY OTHER TIME


APPENDIX C

DIRECTIONS FOR KEEPING A 3-DAY DIET DIARY

Please write down everything you eat (meals, snacks, beverages) for three days on these forms. Please select **TWO WEEKDAYS AND ONE WEEKEND DAY**. Use as much space as you need.

1. **Write down the date and day at the top of the form.**

2. **Write down the first foods you ate for that day. Write down:**
   
   The time of day you ate the food(s).
   
   Each food that you ate.
   
   How the food was prepared (baked, boiled, fried, microwaved).
   
   How much you ate (cup, 1/2 cup, pieces, tablespoons, teaspoons).

3. **It is important to describe each food you eat in detail.**
   
   **For example:**
   
   Write down brand names for each food you ate if you know them.
   
   Write down the type of milk (whole, 2%, or skim) and bread (white, wheat, etc).
   
   Write down if the food was fresh, frozen, or canned.
   
   If you ate a casserole or a salad, write down the foods there were in it and amounts.
   
   If you add things like butter, jelly, sugar, honey, or cream to foods or beverages, please write them down with the amounts used.

4. **Do you drink whole ________, 2% ________, 1% ________, or skim ___ milk?**

5. **Do you use white _________ or whole-wheat _________ bread?**

6. **What is the complete name and brand name of bread that you eat most often?**

   ----------------------------------------------------------------------

7. **About how many glasses of water do you drink each day? ________________**
DAY 1 OF THE DIET DIARY

ID: ___________________________ CHECKED BY: ________________________

DATE: ________________________ DAY OF THE WEEK: ____________

Did you drink a calcium-fortified beverage today (e.g. Calcium-fortified orange juice) or eat a calcium-fortified food (e.g. Total breakfast cereal)?  Yes  No

If yes, list all the calcium-fortified beverages/foods, with the BRAND name, and how much:

________________________________________________

________________________________________________

Write down everything you eat, beginning with the first thing you have for breakfast. Be sure to include very detailed information such as how the food was prepared, how much you ate, and the brand names.

<table>
<thead>
<tr>
<th>Time Eaten</th>
<th>Foods Eaten</th>
<th>Preparation Methods</th>
<th>Amount (cup, 1/2 cup, piece, etc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Tanner Staging</td>
<td>Breast</td>
<td>Pubic Hair</td>
<td></td>
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<tr>
<td>---------------</td>
<td>--------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Stage 1 (prepubertal)</td>
<td>Elevation of papilla only</td>
<td>No pubic hair</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>Elevation of breast and papilla as small mound, areola diameter enlarged, mean age: 11.4 years</td>
<td>Sparse, fine, pigmented hair slightly along pubic region. Mean age: 11.4 years</td>
<td></td>
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<tr>
<td>Stage 3</td>
<td>Further enlargement without separation of breast and areola. Mean age: 11.7 years</td>
<td>Dark, course, curled hair sparsely spread over mons. Mean age: 11.4 years</td>
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<tr>
<td>Stage 4</td>
<td>Secondary mound of areola and papilla above the breast. Mean age: 12.1 years</td>
<td>Adult-type hair, abundant but limited to the mons. Mean age: 12.0 years</td>
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<tr>
<td>Stage 5</td>
<td>Regression of areola to contour of breast. Mean age: 14.6 years</td>
<td>Adult type spread in quantity and distribution. Mean age: 13.7 years</td>
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</tbody>
</table>

APPENDIX D
APPENDIX E

2 to 20 years: Girls
Body mass index-for-age percentiles

<table>
<thead>
<tr>
<th>Date</th>
<th>Age</th>
<th>Weight</th>
<th>Stature</th>
<th>BMI*</th>
<th>Comments</th>
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</table>

*To Calculate BMI: Weight (kg) ÷ Stature (cm) ÷ Stature (cm) x 10,000
or Weight (lbs) ÷ Stature (in) ÷ Stature (in) x 703

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*Published May 30, 2000 (modified 10/15/00).
SOURCE: Developed by the National Center for Health Statistics in collaboration with the National Center for Chronic Disease Prevention and Health Promotion (2000).
http://www.cdc.gov/growthcharts

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