

VITAMIN D SUPPLEMENTATION AND INSULIN-LIKE GROWTH FACTOR I IN
ADOLESCENTS

by

EMILY MARIE ROLLINS

(Under the Direction of Richard D. Lewis)

ABSTRACT

This ancillary study aimed to examine the effects of vitamin D supplementation on serum IGF-I and vitamin D metabolites in healthy adolescents over 12 weeks. Relationships between IGF-I and 25(OH)D, 1,25(OH)₂D, and iPTH were examined using Pearson correlations and nonlinear regression analysis. There were no significant relationships between change in vitamin D metabolites and IGF-I. However, a significant interaction was observed between sex and change in serum 1,25(OH)₂D over 12 weeks on changes in serum IGF-I ($P=0.04$). When probed, the interaction effect of sex approached significance in opposite directions for males ($t=-1.9$, $P=0.05$); and females ($t=1.7$, $P=0.07$). Our data suggests that vitamin D supplementation does not effect changes in IGF-I in healthy children, but there is a sex-dependent link between 1,25(OH)₂D and IGF-I. The reason for the interaction is unclear, but additional studies are needed to explore the varied IGF-I-1,25(OH)₂D relationships in males and females with vitamin D supplementation.

INDEX WORDS: Insulin-like growth factor I (IGF-I), vitamin D supplementation, adolescence

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by

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B.S., The University of Georgia, 2016

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment
of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2018

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August 2018

DEDICATION

This work is dedicated to my parents, Anthony and Amy Rollins, for their unwavering love, support, and encouragement throughout all my personal and educational endeavors.

ACKNOWLEDGEMENTS

First and foremost, I must thank my Creator for so intricately designing the human body and for allowing me the privilege to learn so much more about His immaculate design. Thank you, Lewis Lab, for cultivating my addiction to that 2 o'clock cafe au lait and for being my family away from home. You all have provided unending support, guidance, and fun these past few years. Dr. Lewis, I am forever grateful for you seeing potential in me and for helping me develop myself as a student, researcher, and professional. You have been a wonderful mentor, a very patient teacher, and the best example of what working with others should look like. Emma, thank you for being a calming presence, a hand always willing help, and a constant reminder that everything will get done. Thank you, Hannah, for being the best office-mate and friend I could ask for! You were always willing to listen to my stories and always ready to meet me bright and early at the Farmer's Market on Saturdays! Thank you, Staci and Lauren, for your patience, for letting me ask you a million questions, and for your friendships. I have learned so much from both of you and couldn't have completed this work without you! Kason, you were always ready to listen to me talk about my day, tell you about research that you probably weren't interested in, and encourage me to finish strong. Finally, I must thank my family for always pushing me to be the best that I can be and for encouraging and supporting me throughout my entire life!

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CHAPTER 1

INTRODUCTION

Osteoporosis is a degenerative skeletal disease and causes approximately 9 million fractures annually.⁽¹⁾ Within the United States, about 54 million Americans have osteoporosis and low bone mass.⁽²⁾ Further, it is estimated that 1 in 2 women and 1 in 4 men ages 50 and older will break a bone as a result of osteoporosis.⁽²⁾ Nationally, this disease is responsible for 2 million broken bones and \$19 billion in related costs annually.⁽²⁾ Considering the health risks and the financial burden associated with osteoporosis, prevention is of utmost public health importance. One key strategy for osteoporosis prevention is to maximize bone gains during youth. The majority of bone acquisition occurs between 10-18 years of age, with 90% of bone mineral content accrued by age 18.⁽³⁾ The rate of bone gains levels off around the age of 25 years, with some variation depending on bone site, so that suboptimal bone mass accrual during childhood and adolescence may increase the risk for development of osteoporosis later in life. Therefore, it is critical to better understand the factors influencing this process of peak bone mass and intervene early.

Numerous factors including genetics, nutrition, environment, and hormones affect and can potentially optimize peak bone mass. For example, calcium, vitamin D and high impact activities positively influence mineral acquisition during pubertal and adolescent growth.⁽⁴⁾ The hormones insulin-like-growth factor I (IGF-I) and sex steroids have profound effects on skeletal strength and growth during puberty. What is not known is whether there is an interaction of nutrition and hormones that could potentially influence the bone accrual process. Specifically, of

current interest is how IGF-I might mediate the effects of vitamin D on bone, as these two have known independent, but possibly synergistic, effects on bone. However, current studies exploring these relationships are limited.

Vitamin D is critical for bone mineralization, partially through its function in calcium homeostasis. Vitamin D3 is hydroxylated twice, once to 25(OH)D and then again to the active form, 1,25(OH)₂D, which helps maintain plasma calcium concentrations so that bone can be mineralized and repaired instead of being resorbed and permanently damaged.⁽⁵⁾ Thus, in children, inadequate or deficient vitamin D status can negatively affect bone leading to rickets, muscle weakness, and poor muscle function.^(6,7) There is inconsistent, but moderate evidence, that vitamin D improves bone mineral density (BMD) at different skeletal sites. A review of nine RCTs provides evidence that vitamin D supplementation improves bone gains in female adolescents, but not males, and in those with poorer vitamin D status.⁽⁴⁾

IGF-I is a key determinant of bone mineral accrual during growth directly supporting the proliferation and differentiation of osteoblast-like cells and bone,^(8,9) and indirectly through its impact on muscle growth.^(10,11) In diseases with inadequate IGF-I levels, such as growth hormone deficiency (GHD), patients are often diagnosed with low bone mass. While IGF-I is an independent predictor of BMD,⁽¹²⁾ studies in adolescents support the hypothesis that IGF-I has an indirect effect on bone via lean mass.^(10,11) In prepubertal females, while controlling for fat-free soft tissue, age, and sex, IGF-I explains a considerable amount of the variance in bone mass accrual at the total body, proximal femur, lumbar spine, and forearm. In fact, in a nine-year prospective study from our lab, IGF-I was observed to be a stronger predictor of BMC accrual than serum 25(OH)D.⁽¹³⁾

While both vitamin D and IGF-I have been demonstrated to have independent roles with respect to bone mineralization,^(5,9,12) cell and animal studies provide evidence for interactions. The two have been found to upregulate one another: 1,25(OH)₂D promotes the action of IGF-I by increasing IGF-I receptors in clonal osteoblastic cells⁽¹⁴⁾ and IGF-I may elevate 1,25(OH)₂D concentrations by stimulating the hydroxylation of 25(OH)D to 1,25(OH)₂D.⁽¹⁵⁾ Moreover, a randomized crossover study of healthy adult men reported that vitamin D mediated a stimulatory effect on 25(OH)D-1 α -hydroxylase by activating bone turnover and increasing calcitriol.⁽¹⁵⁾ In IGF-I knock-out mice, serum 25(OH)D concentrations are 24% lower vs wild-type controls and renal vitamin D receptor expression, 70% lower.⁽¹⁶⁾ Other in vitro studies demonstrated that IGF-I significantly increased 1 α -hydroxylase expression and serum 1,25(OH)₂D production by acting on renal tubules to convert 25(OH)D to 1,25(OH)₂D, further supporting interrelationships between the two.^(17,18)

Despite the findings in cell culture and animal models, studies in adults and children report conflicting results. Part of the discrepancies in the human trials partially relate to initial vitamin D or IGF-I status, important factors to consider when examining the effects of vitamin D supplementation on IGF-I. In a vitamin D supplementation trial in adults with GHD, the group that observed the greatest increase in circulating IGF-I had a higher prevalence of vitamin D deficiency.⁽¹⁹⁾ In otherwise healthy Italian adults, a cross-sectional positive correlation was observed between IGF-I and 25(OH)D. When subjects were grouped according to 25(OH)D serum levels, those with severe vitamin D deficiency (VDD) had lower serum IGF-I than those with a mild-to-absent 25(OH)D deficit.⁽²⁰⁾

The findings from cross-sectional studies in children vary and likely depend on the health status of children. In healthy Lebanese boys and girls, 8-18 years old, in which 56.2% were

vitamin D insufficient (<20 ng/mL), no significant correlations were found between IGF-I and 25(OH)D after adjusting for age, BMI, and height.⁽²¹⁾ However, positive associations were observed between 25(OH)D and IGF-I in a study of children with GHD, regardless of vitamin D status.⁽²²⁾ In vitamin D deficient children with rickets and growth stunted, administration of a bolus dose of vitamin D (300,000 IU) during a six-month period, significantly increased 25(OH)D, IGF-I, and linear growth.⁽²³⁾ The only other trial in adolescents exploring this vitamin D/IGF-I relationship was in Indian girls, ages 6-18 years ($n = 184$), who were supplemented with 60,000 IU vitamin D once per month for six months.⁽²⁴⁾ Although described as healthy prior to the intervention, 95% of participants were classified as having vitamin D insufficiency, based on the IOM cutoffs (≤ 5 to ≤ 20 ng/mL). Serum 25(OH)D levels significantly increased and all girls achieved vitamin D sufficiency (>20 ng/mL) following the intervention. Furthermore, circulating IGF-I and IGFBP3 concentrations significantly increased, though the increase was greatest in girls with moderate-to-severe vitamin D deficiency.⁽²⁴⁾

There are no available data that have examined changes in $1,25(\text{OH})_2\text{D}$ or that have addressed race and sex differences in response to vitamin D supplementation. To address these gaps, the present study utilizes secondary data from a vitamin D RCT that includes measures of $1,25(\text{OH})_2\text{D}$ and iPTH in both male and female and black and white, children and adolescents. The primary aim of this project was to determine the effects of varying doses of vitamin D supplementation on changes in serum IGF-I and vitamin D metabolites in healthy, vitamin D sufficient children, entering the early stages of puberty. We hypothesized that vitamin D supplementation would significantly increase serum IGF-I and vitamin D metabolites [25(OH)D, $1,25(\text{OH})_2\text{D}$, iPTH] over 12 weeks. The findings from this project will increase our understanding of the interrelationships between vitamin D nutrition and growth factors during

growth, including addressing a potential mechanism for how vitamin D supplementation improves bone mineral accrual. It is possible that the effect of vitamin D supplementation on IGF-I is potentiated under deficiency conditions. Because the participants in the current study are vitamin D sufficient, the results should be generalizable to the majority of worldwide.

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CHAPTER 2

REVIEW OF THE LITERATURE

Vitamin D and insulin-like growth factor I (IGF-I) play important roles in bone development and growth in adolescents. There is some evidence that the improvement in bone mass in children with vitamin D supplementation may be related to effects on IGF-I. This literature review will explore bone growth and factors influencing peak bone mass and the metabolic properties of vitamin D metabolites, IGF-I, and the literature encompassing the interrelationship between the two. Throughout these topics, I will discuss how factors such as race, sex, and puberty influence serum concentrations of vitamin D and IGF-I.

BMC Accrual and Growth

Bone is a mineral reservoir, composed of an organic matrix, an inorganic matrix, and water, that is essential for structural support, movement, and protection. There are two types of bone, cortical and trabecular. Cortical bone, which makes up 80% of skeletal mass, is dense tissue that composes the outer layer of bone and the shaft of long bones, providing the skeleton with bending strength.⁽¹⁾ Trabecular bone is porous in structure and is located in flat bones, the ends of long bones, and the axial regions. The structure and location of trabecular bone allows for metabolic functions via contact with blood vessels, bone marrow, and connective tissue.⁽²⁾ Within bone, three different cell types exist: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are bone-building cells that synthesize and deposit bone matrix. The number of osteoblasts present determines the rate of bone formation. Osteoblasts evolve into osteocytes, which are

inactive cells that line bone surfaces. Osteoclasts are responsible for the resorption of bone through the attachment and secretion of lysosomal enzymes that break down bone.

Environmental factors, such as physical activity and dietary intake, influence the process of bone remodeling, which repairs the microstructure of bone. This coupled process occurs as the removal of bone initiates the formation of new bone, mediated by osteoclasts (Figure 2.1). Bone modeling takes place primarily during adolescence, though acute modeling may occur in the adult skeleton.⁽³⁾ Bone modeling changes the shape of the bone when new bone is formed on existing bone, which is directed by genetic determinants and responses to load. Unlike bone remodeling, this process is not dependent on the coupled process of osteoblasts and osteoclasts. Every surface of bone is remodeled in roughly a 2-yr period, and longitudinal bone growth occurs before the consolidation of bone mineral. During remodeling, the mediation process by osteoblasts is thought to be directed by the insulin-like growth factor system, as IGF-I works as a coupling factor to contribute to the recruitment and activation of osteoblasts to the resorption sites.⁽⁴⁾ As hormones influence osteoblast and osteoclast activity, a homeostatic imbalance of bone remodeling can lead to uncoupling, which may result in a net loss or gain of bone (Figure 2.1).⁽⁵⁾

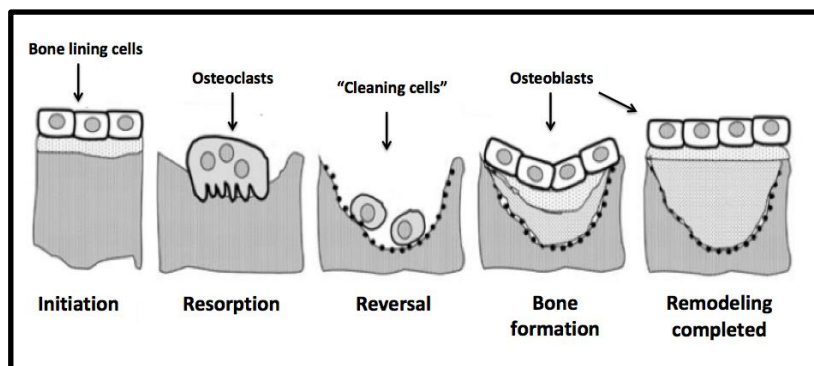


Figure 2.1. Bone Remodeling Process. Adapted from Lerner et al 2006⁽⁵⁾

Skeletal Development in Children

Skeletal development during childhood is a critical time for optimizing bone strength as the majority of bone mineral accrual occurs between 10-18 yr. Thus, any condition, disease, or deficiency that decreases bone formation or promotes resorption during this period may lead to suboptimal bone development and an increased risk of fractures later in life.

90% of adult bone mineral content (BMC) is acquired by age 18, with 28% of BMC gained the 2 years surrounding peak BMC velocity.⁽⁶⁾ Peak BMC velocity, a period of rapid bone modeling and remodeling where bone formation exceeds resorption, is achieved for specific skeletal sites at different times (i.e. total body at 22 yr, greater trochanter in females at 14.2 yr). Peak BMC accrual rates occur at approximately 12.5 yr in girls and 14.1 yr in boys and is followed by peak height velocity (Figure 2.2). The timing of peak BMC gains and onset of puberty varies according to many factors including genetics, race, sex, physical activity, and diet. 60-80% of peak BMC is attributed to genetics.⁽⁷⁾ The timing of puberty affects the total duration of skeletal growth. Further, the timing of peak BMC is more closely related to pubertal development than age. Blacks and Asians have earlier menarche than white and Hispanic females, and Chinese girls had earlier peak BMC gains than white girls in one study.⁽⁸⁾ In boys versus girls, peak BMC is greater and occurs later by about 1.5 yr. Further, the most rapid BMC accrual in girls occurs between 12-14 yr while this same heightened BMC accrual occurs between 13-15 yr in boys.

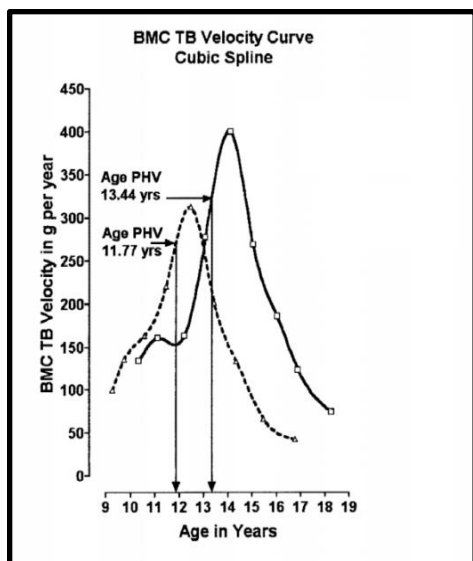


Figure 2.2. Chronological ages for boys and girls at total body (TB) peak bone mineral content (BMC) and peak height velocities (PHV). Adapted from Bailey et al.⁽⁶⁾

Diet and physical activity are responsible for 20-40% of bone development during growth, and these two factors also influence menarche. Diets low in total energy can result in amenorrhea and delayed menarche, which has been shown to have detrimental effects on bone.⁽⁹⁾ Diets low in specific nutrients, specifically calcium and vitamin D, can also be detrimental to bone. Calcium is essential for bone formation and suppression of bone resorption. On the other side of energy balance, more physically active adolescents had greater total hip and femoral neck BMC throughout puberty and into adulthood.⁽¹⁰⁾ Baxter-Jones et al. observed that adults who were more physically active during childhood had greater BMC than adults who were less physically active during childhood.⁽¹⁰⁾ Energy expenditure greater than energy intake can also delay menarche and growth, further negatively influencing bone.⁽¹¹⁾

Though the timing of peak bone mass varies amongst individuals, it is critical to attain the greatest possible bone mass during the pubertal years in order to protect against fractures and to prevent bone loss. There are various factors that influence bone development and growth,

including physical activity, body composition, and diet. Hormones such as vitamin D and IGF-I also play an important role in influencing bone development, which will next be explored.

Vitamin D

Production

Vitamin D, a fat-soluble vitamin and secosteroid hormone, is essential to many physiological functions. Humans metabolize vitamin D from the sun (endogenous) and/or the diet (exogenous). Vitamin D production occurs largely in the skin when 7-dehydrocholesterol pro-vitamin D₃ is exposed to ultraviolet radiation. Through photolytic processes, pro-vitamin D₃ becomes pre-vitamin D₃, which is then isomerized to become vitamin D₃, or cholecalciferol.⁽¹²⁾ This metabolic process can produce 80-100% of daily vitamin D requirements.⁽¹³⁾

Very few foods contain vitamin D or are fortified with vitamin D, so the majority of vitamin D humans get is via sun exposure or supplements. Though vitamin D exists naturally in the foods we eat, it is in minimal amounts. Vitamin D₂, ergocalciferol, is found in plants and yeast, while vitamin D₃ is in foods like egg yolks and oily fish. Thus, most dietary vitamin D comes from fortified foods, such as milk, juice, and cereal (Table 2.1).⁽¹⁴⁾ Data from NHANES 2005-2006 revealed that average vitamin D intake from food alone for males and females ranged from 204-288 IU/d and 144-276 IU/d, respectively,⁽¹⁵⁾ revealing that most adults fall short of the Recommended Dietary Allowances (RDAs) for vitamin D (Table 2.2). Additionally, only males ages 1-3 and 4-8 years, and females ages 1-3, 4-8, and 9-13 years met the adequate intake (AI) for vitamin D from food alone.⁽¹⁵⁾ Because AIs established for infants and children are only estimates based off adults AIs, it remains difficult to determine appropriate vitamin D intake requirements in adolescent populations. Thus, measuring serum biomarkers remains the best method of capturing vitamin D status. Both D₂ and D₃ are available in supplement form, though

D3 is preferred because it is more efficient at increasing serum 25(OH)D concentrations and is converted in the liver better than D2.⁽¹⁶⁾ Furthermore, once 1,24,25(OH)₃D₂ is formed, D2 is deactivated and irretrievable. D3, now 1,24,25(OH)₃D₂, is still able to bind to the vitamin D receptor (VDR) and requires an additional side-chain oxidation to become deactivated.⁽¹⁶⁾ This additional step provides an advantage for D3 to remain biologically active and subsequently maintain vitamin D status.⁽¹⁷⁾ A systematic review and meta-analysis of randomized controlled trials (RCTs) comparing the effects of D2 and D3 on serum 25(OH)D concentrations in humans reported that supplementation with D3 significantly increased serum 25(OH)D concentrations compared to supplementation with D2 ($P = 0.001$).⁽¹⁷⁾

Table 2.1. Selected Food Sources of Vitamin D

Food	IUs per serving*	Percent DV**
Cod liver oil, 1 tablespoon	1,360	340
Swordfish, cooked, 3 ounces	566	142
Salmon (sockeye), cooked, 3 ounces	447	112
Tuna fish, canned in water, drained, 3 ounces	154	39
Orange juice fortified with vitamin D, 1 cup	137	34
Milk, nonfat, reduced fat, and whole, vitamin D fortified, 1 cup	115-124	29-31
Yogurt, fortified with 20% of DV for vitamin D, 6 ounces	80	20
Margarine, fortified, 1 tablespoon	60	15

Sardines, canned in oil, drained, 2 sardines	46	12
Liver, beef, cooked, 3 ounces	42	11
Egg, 1 large (vitamin D found in yolk)	41	10
Ready-to-eat cereal, fortified with 10% of DV for vitamin D, 0.75-1 cup	40	10
Cheese, swiss, 1 ounce	6	2

Adapted from USDA 2011⁽¹⁸⁾

*IUs = International Units

**DV = Daily Value

Table 2.2. Recommended Dietary Allowances (RDAs) for Vitamin D

Age	Male	Female
0-12 months*	400 IU	400 IU
1-13 years	600 IU	600 IU
14-18 years	600 IU	600 IU
19-50 years	600 IU	600 IU
51-70 years	600 IU	600 IU
>70 years	800 IU	800 IU

Adapted from IOM 2010⁽¹⁹⁾

* Adequate Intake (AI)

Metabolism

Upon entering the body, vitamin D (either as cholecalciferol or ergocalciferol) is metabolically inactive until acted upon in the liver by 25-hydroxylase and metabolized to 25-hydroxyvitamin D₃ (25(OH)D), the major circulating metabolite.⁽²⁰⁾ Vitamin D binding protein (VDBP), synthesized by hepatic cells, largely affects the functionality of the vitamin. It binds to

vitamin D and its metabolites, transporting them to the liver.⁽²¹⁾ Once in the liver, two enzymes are responsible for the hydroxylation step: 25-hydroxylase and a cytochrome P-450, CYP27. After hydroxylation, 25(OH)D is once again bound to VDBP and is transported in circulation to the kidneys. Further hydroxylation via 25-hydroxyvitaminD-1 α -hydroxylase yields 1,25(OH)₂D, the active form responsible for the actions of the vitamin. This step is highly regulated via positive feedback by 1 α -hydroxylase and parathyroid hormone (PTH) via cAMP/phosphatidylinositol 4,5-bisphosphate-mediated signal transduction mechanism.⁽²²⁾ When PTH levels are high, 1 α -hydroxylase is upregulated which increases the rate of conversion from 25(OH)D to 1,25(OH)₂D. When 1,25(OH)₂D reaches a specific concentration, it decreases its own synthesis by decreasing the synthesis of PTH (negative feedback mechanism).⁽²³⁾

Factors affecting vitamin D status

Age and pubertal maturation status

Age and pubertal maturation affect vitamin D status. Serum vitamin D decreases with age, though the mechanism responsible for decreasing circulating 25(OH)D is unknown. Older and more pubertal mature adolescents have lower serum 25(OH)D than prepubertal. In a prospective study of adolescents, late pubertal children had significantly lower serum 25(OH)D averages than prepubertal children.⁽²⁴⁾ Cross-sectional data reports similar findings in which late pubertal adolescents have significantly lower mean values (75 nmol/L) compared to prepubertal children (95 nmol/L).⁽²⁵⁾ When compared to younger adults, individuals older than 70 yr produce 30% less vitamin D when exposed to the same dose of UV radiation.⁽²⁶⁾ This age-related exogenous vitamin D production decline seems to occur sooner in women (around 50 yr) compared to men (around 70 yr).⁽²⁷⁾

Sex

Sex differences in vitamin D status seem to vary according to maturational status. Data from NHANES III reveals that in children, lower serum 25(OH)D concentrations in females versus males could be due to differences in pubertal maturation. In a cross-sectional study of early pubertal children, no significant sex differences in 25(OH)D were found.⁽²⁸⁾ In a US RCT, mean values for 9-13 year old boys and girls were not different regardless of race (i.e., white 81 vs. 80 nmol/L and black 61 vs. 59 nmol/L).⁽²⁹⁾ In studies of older children in Ireland and the US, 25(OH)D was lower in girls versus boys at different latitudes.^(30,31)

Latitude/season

Vitamin D status varies with season and latitude, most apparently because of differences in sun exposure. Seasonal variations occur in every race. On average, serum 25(OH)D concentrations are higher during summer months when sun exposure is at its peak compared to winter months. Individuals in northern latitudes have less 25(OH)D compared to southern latitudes. In the present study, season was controlled for by taking samples from children during the winter months of October-December, when serum 25(OH)D levels were at their lowest. We controlled for latitude by taking samples from two different locations: 34°N and 40°N.

Race

The population in the present study are black and white, and because race is an important determinant of vitamin D synthesis, it is crucial to address the differences in vitamin D status among these races. In general, blacks have lower serum 25(OH)D concentrations than whites primarily because less vitamin D is synthesized in darker pigmented skin for the same UVB exposure.⁽³²⁾ According to NHANES data in children ages 12-19 yr, 70% of blacks and 40% of

Hispanics have serum 25(OH)D concentrations below the IOM threshold of 50 nmol/L, compared to only 7.7% of whites with values below the threshold.

Race cannot be addressed without considering the differences in VDBP between the groups. Total serum 25(OH)D is bound to VDBP, while free serum 25(OH)D is not attached to VDBP. Further, VDBP may act as a reservoir for serum 25(OH)D, limiting its availability and keeping it inactive. In postmenopausal women, though total serum 25(OH)D was lower in blacks, free 25(OH)D was the same.⁽³³⁾ Therefore, if free 25(OH)D levels are the same in blacks and whites, this may explain why low total serum 25(OH)D in blacks does not adversely affect their bone health. Data also suggest that while blacks have lower serum 25(OH)D concentrations, they have higher VDBP, possibly providing sufficient levels of the vitamin. It has been proposed that genetic polymorphisms or receptor fallacies are potential explanations for lower VDBP in blacks. Data presents conflicting results depending upon the antibody used when measuring vitamin D metabolites. For example, VDBP was lower in blacks when using a monoclonal antibody, but higher in blacks when using polyclonal.⁽³³⁾ In another study, free 25(OH)D concentrations reflected total 25(OH)D, and were both lower in blacks compared to whites. Conversely, total and free 25(OH)D concentrations were higher in Gambians than whites from the United Kingdom. These data cannot be explained by racial differences in VDBP or genotype, but are instead more likely due to differences in UV radiation and vitamin D synthesis within the skin.⁽³⁴⁾

Vitamin D and bone

Vitamin D is essential for bone mineralization, bone repair, and calcium homeostasis. 1,25(OH)₂D helps maintain constant plasma calcium concentrations in three distinct ways: 1) In the intestines, vitamin D stimulates calcium absorption from the diet or from supplements by

inducing proteins involved in intestinal calcium absorption. Environmental calcium, either from the diet or supplements, is used first under normal physiological conditions, but if calcium is absent, internal stores are used.⁽²²⁾ Even when animals are placed on a calcium-free diet, they are still able to maintain serum calcium concentrations. This ability to mobilize calcium in the absence of calcium, either from the diet or from supplements, is due to enterocytes.⁽²²⁾ In the absence of intestinal calcium, vitamin D stimulates osteoblasts to produce RANKL.⁽³⁵⁾ RANKL then stimulates osteoclastogenesis thereby causing osteoclast precursors to fuse together and create new osteoclasts, which ultimately enhances the bone resorption process. Thus, the calcium stored in bone is able to be released into the blood, even when calcium is absent from the diet. Vitamin D is unable to stimulate intestinal calcium absorption unless PTH binds to the osteoblasts before this process. 2) The primary role of vitamin D is to regulate the cells that are involved in bone modeling, specifically by stimulating calcium into the skeleton for storage. Vitamin D binds to VDR, increasing transcription of proteins involved in intestinal absorption of calcium and phosphate.⁽³⁶⁾ 3) In the kidneys, vitamin D works to recycle calcium that would otherwise be excreted by increasing renal reabsorption of calcium in the distal tubule. Approximately 7 grams of calcium is filtered daily in humans, demonstrating the importance of this recycling process for the maintenance of serum calcium concentrations. PTH acts on renal ducts to increase calcium and magnesium resorption while concurrently increasing phosphorous secretion. This results in a net decrease of phosphate and an increase in free calcium that is now available for circulation. As a result of these 3 functions, serum concentrations of calcium and phosphorous are supersaturated to levels required for proper bone mineralization.⁽³⁶⁾

Though vitamin D is essential to the three pathways, PTH is required for these vitamin D functions on bone. Calcium-sensing receptors located on the parathyroid gland detect any slight

changes in serum calcium concentrations. Sensing a decrease in calcium, the parathyroid gland will release PTH. If vitamin D levels are low, enterocytes are stimulated to absorb calcium and phosphate, and if calcium concentrations further fail to respond, PTH is secreted, which increases production of vitamin D to mobilize bone calcium. The most important function of PTH relating to vitamin D and bone is that PTH increases the conversion of 25(OH)D to 1,25(OH)₂D by acting on the renal enzyme 25-hydroxyvitamin D-1 α -hydroxylase. The resulting increase in 1,25(OH)₂D is then able to increase calcium levels by the three pathways described above.⁽²²⁾

Vitamin D supplementation and bone

While vitamin D is clearly important to the development of bone, evidence remains inconsistent regarding vitamin D supplementation improving bone gains in males and females. Further, few studies have been conducted in adolescents exploring the relationship between vitamin D status and BMC accrual over time. More specifically, there is inconsistent evidence for vitamin D and bone relationships among different skeletal sites. A study using data from the National Health and Nutrition Examination Survey (NHANES) III observed significant associations between vitamin D status and bone mineral density (BMD) in adults.⁽³⁷⁾ However, no large studies are available in children to observe significant associations between vitamin D and bone, leaving optimal vitamin D levels in this age group unknown.

The few studies that exist in children regarding the relationship between serum 25(OH)D levels and bone indices report inconsistent findings. A prospective study in adolescent females and a RCT in Lebanese adolescent females revealed inverse relationships between vitamin D and skeletal outcomes.^(38,39) A review of 9 RCTs present moderate evidence for vitamin D supplementation improving bone gains in female adolescents, but not males.⁽⁴⁰⁾ In a RCT of

Lebanese females ages 10-17 receiving vitamin D supplementation, both treatment groups were associated with greater total hip BMC ($p = 0.02$) and lean mass gains ($p = 0.05$) compared to the placebo group. Furthermore, premenarcheal girls receiving 200 IU dose vs. placebo had significant increases in trochanter BMC ($p = 0.05$) and marginally significant ($P = 0.08$) increases in total hip BMC.⁽³⁹⁾ Data from our laboratory revealed a significant negative relationship between 25(OH)D on BMC gains in adolescent females throughout pre- and early-puberty. In the same prospective study, subjects with lower levels of 25(OH)D showed greater BMC gains than those with higher levels of 25(OH)D over seven yr.⁽²⁴⁾

Biochemical marker of vitamin D status

25(OH)D is the widely accepted clinical marker of vitamin D status for many reasons. First, the 25-hydroxylation step in vitamin D metabolism is unregulated, reflecting substrate availability⁽⁴¹⁾. Second, because 25(OH)D is a summation of vitamin D intake and vitamin D produced from the sun⁽⁴²⁾, these levels more accurately reflect the body's stores of vitamin D.⁽²¹⁾ Third, 25(OH)D has a relatively long half-life (3 weeks) compared to the 4-6 hour half-life of 1,25(OH)₂D.⁽⁴¹⁾

Due to its short half-life and tight regulation of production, 1,25(OH)₂D provides limited information about the *nutritional status* of vitamin D.⁽⁴¹⁾ However, because 1,25(OH)₂D is the active form of the vitamin and is responsible for the actions of vitamin D, measuring serum 1,25(OH)₂D could provide a better understanding of the functional role of vitamin D and its relationship to other biochemicals, such as IGF-I. For the purposes of this study, we are not measuring 1,25(OH)₂D to assess vitamin D status. The metabolite is being measured to provide a clearer picture of the metabolic changes occurring and the entire process during adolescence.

US vitamin D intake

There is quite a bit of debate surrounding the definitions and classifications of vitamin D sufficiency, insufficiency, and deficiency. For the purposes of this project, these values will be defined according to the Institute of Medicine's (IOM) recommendations (Table 2.3). According to the IOM, 97% of US children will achieve optimal bone health with serum 25(OH)D levels >50 nmol/L. The IOM defines bone health specifically as rickets prevention, calcium absorption, and bone mineral accrual. In order to attain the recommendation of serum 25(OH)D >50 nmol/L, the IOM vitamin D recommended dietary intake was set at 600 IU/d.⁽¹⁹⁾ Vitamin D insufficiency is defined as having serum 25(OH)D levels between 30-50 nmol/L, leading to inadequate support of bone and overall health, while deficiency is defined as serum 25(OH)D <30 nmol/L. Vitamin D deficiency leads to rickets in children, characterized by reduced bone mineralization and a weaker skeleton.⁽⁴³⁾ Vitamin D deficiency also leads to muscle weakness and poor muscle function.⁽⁴⁴⁾ More recently, low serum vitamin D has been linked to diabetes mellitus, hypertension, dyslipidemia, asthma, and a multitude of other health issues.⁽⁴⁵⁾ Low levels of vitamin D associated with such adverse health outcomes may lead to suboptimal bone development.⁽³⁹⁾

Table 2.3. Vitamin D Cutoffs*

Health Status	Serum 25 (OH)D (nmol/L)
Vitamin D deficiency	< 30
Vitamin D insufficiency	30-50
Vitamin D sufficiency	> 50

*Levels based on Institute of Medicine 2011 report

The NHANES reveals the average serum 25(OH)D concentration of US children ages 6-11 and 12-19 yr to be 72.2 and 65.8 nmol/L, respectively. Though it appears that the majority of US children are achieving the vitamin D threshold, there are still a considerable number of US children falling below the threshold. The NHANES reports that 10% of children ages 1-11 yr and 25% of children ages 12-19 yr have serum 25(OH)D values <50 nmol/L, characterizing vitamin D insufficiency, and that 6.6% percent of children between 1-19 yr have 25(OH)D levels below 30 nmol/L.⁽²⁵⁾ Looking closer at individual states, the numbers of children characterized as insufficient and deficient appear to be more dramatic. For example, in a study of 307 black and white, male and female ages 11-18 yr, 42% had insufficient levels and 24.1% were deficient.⁽⁴⁶⁾ In Ohio, and study of 307 white and black females ages 12-18 revealed that 54% overall were insufficient and 17% were deficient.⁽⁴⁷⁾ In the US, the prevalence of vitamin D deficiency is greater in Hispanics and non-Hispanic black compared to whites. Within the population for the proposed project, we observed that 15% of children had serum 25(OH)D values <50 nmol/L and 1% had values <30 nmol/L.⁽⁴⁸⁾

Measurement of vitamin D metabolites

Diverse assay methodologies create a challenge for accurately ascertaining estimates of vitamin D status using serum 25(OH)D concentrations as a marker of status. Radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), liquid chromatography tandem mass spectrometry (LC-MS), high performance liquid chromatography (HPLC), and random access automated assays based on chemiluminescence (RAAA) are the primary assays currently used to assess 25(OH)D and 1,25(OH)₂D. Each assay method has its own strengths and weaknesses, including the capability to detect 25(OH)D₂ and D₃ metabolites equally, as well as being able to distinguish between other impertinent metabolites (e.g. 24,25(OH)₂D). Direct detection methodologies,

including HPLC, UV detection, and LC-MS, are considered the choice method by many. However, HPLC is step-heavy and the sample output is slow, and LC-MS equipment and maintenance are expensive and interpretations are complex.⁽⁴⁹⁾ The most desirable commercial assay kit for assessment of both 25(OH)D and 1,25(OH)₂D appears to be DiaSorin RIA as it detects D₂ and D₃ metabolites equally and received the highest accuracy score by the Vitamin D Quality Assessment Scheme (DEQAS).⁽⁵⁰⁾ When conducting vitamin D assays, it is important to participate in DEQAS quality assurance program to ensure accuracy and quality control of assays. The assay used for the current study employs a two-step competitive radioimmunoassay (RIA) procedure. First, 25(OH)D and other hydroxylated metabolites are rapidly extracted from serum with acetonitrile. Following extraction, the treated sample is assayed via an equilibrium RIA procedure based on an antibody specific to 25(OH)D. The sample, antibody, and tracer (contains ¹²⁵I) are incubated at 20-25°C for 90 minutes. Phase separation is completed following a 20-minute incubation at 20-25°C with a second antibody-precipitating complex. After this second incubation, a nonspecific binding (NSB)/Addition buffer is added, and samples are centrifuged to help reduce non-specific binding. The supernatants are decanted, and the radioactivity of the free antigen left in the supernatant is counted in each tube for at least one minute by the gamma counter. 1,25(OH)₂D was quantified using the same procedure as 25(OH)D, but with an antibody specific to 1,25(OH)₂D. Inter- and intra-CV from our lab for 25(OH)D ranged from 5.6-8.4% and 5.5-7.0%, respectively, and for 1,25(OH)₂D ranged from 12.4-17.6% and 11.6%, respectively.

An immunoradiometric assay (IRMA) was used to measure iPTH, an important regulator of calcium and subsequently vitamin D. The PTH-specific antibodies were labeled with radioisotopes (¹²⁵I) which bound to the free epitopes of antigens and formed an antigen-

antibody complex. Using a solid phase antigen, the unbound antibodies were removed. The remaining radioactive product was measured. The inter- and intra-assay CV were 4.8-6.9% and 2.3-5.7%, respectively.

IGF-I

Metabolism

IGF-I is a 70 amino acid polypeptide hormone involved in cell differentiation, proliferation, and apoptosis.⁽⁵¹⁾ It is a member of the IGF system which includes IGF-II, insulin, 3 cell surface receptors, 6 IGFBPs, and proteases. It is synthesized mostly in the liver where it acts as an endocrine hormone, but is also produced by various tissues, exerting both autocrine and paracrine functions. Because there are IGF-I receptors in nearly all tissues, most cell types are affected by this hormone. The majority of circulating IGF-I is bound within a ternary complex: IGF-I, insulin-like growth factor binding protein 3 (IGFBP3), and acid labile subunit (ALS). In fact, < 1% of total serum IGF-I circulates as a free hormone.⁽⁵²⁾ This ternary complex attenuates the biological activity of IGF-I by stabilizing IGF-I, reducing its clearance and prolonging its supply to target cells. IGF-I binds to insulin-like growth factor receptor (IGFIR), but insulin-like growth factor binding proteins (IGFBPs) have a higher affinity for IGF-I than to its receptor.⁽⁵¹⁾ These binding proteins moderate the binding of IGF-I to its receptor, mediating its availability.⁽⁵³⁾ Though there are 6 IGFBPs, IGFBP3 binds 75% of IGF-I in plasma and regulates the free active IGF-I concentration. Additionally, evidence supports the idea that free IGF-I is the only active form of the hormone. Furthermore, the molar ratio of IGF-I:IGFBP3 is considered to be an indirect reflection of free IGF-I.⁽⁵⁴⁾ IGF-I synthesis in extrahepatic tissues where it acts as a local growth factor is largely under the control of different hormones.⁽⁵⁵⁾ For

example, in fat and osseous tissue, it is proposed that IGF-I expression is stimulated by $1,25(\text{OH})_2\text{D}$.⁽⁵⁶⁾

IGF-I and bone metabolism

The skeleton is only second to the liver as the largest IGF-I reservoir, and is likely the most important determinant of bone, regulating muscle growth and supporting the proliferation and differentiation of osteoblast-like cells and bone.^(54,53) Both IGF-I and IGF-II are the most abundant growth factors present in skeletal tissue, though IGF-I is the more potent factor present. Their synthesis and activity are regulated by hormones like growth hormone (GH) and parathyroid hormone (PTH). IGF-I appears to have a dual role in enhancing bone formation and resorption. Its primary role regarding bone is to 1) stimulate differentiated osteoblastic function and bone formation, but it also works to 2) induce osteoclastogenesis by inducing RANKL synthesis.

1) Using osteoblast culture, studies have revealed that IGF-I stimulates osteoblast proliferation, speeds up their differentiation, and enhances bone matrix production.⁽⁵⁷⁾ The stimulation of type 1 IGF collagen expression in human marrow stromal cells (hMS) induces differentiation into osteoblasts. IGF-I induces osteoblast differentiation through this process as hMS cells are able to differentiate into either osteoblasts or adipocytes. Additionally, IGF enhances bone matrix production by binding to IGF-I receptors on osteoblast cells, thus stimulating collagen synthesis.⁽⁵⁸⁾ IGF-I reduces osteoblast apoptosis and promotes osteoblastogenesis by stabilizing β -catenin.⁽⁵⁹⁾ Zhang et al. selectively disrupted the IGF1R gene in mouse osteoblasts. This impaired signaling disrupted the rate of bone formation and slowed down mineralization of osteoids, which equated to decreased cancellous bone volume and altered trabecular architecture (decreased trabecular bone volume, thickness, and number). Impaired

IGFIR signaling did not appear to have an effect on cortical bone volume, highlighting that the marked effect on trabecular bone could be because cortical bone is subject to the highest rate of remodeling during the active period of pubertal growth.⁽⁶⁰⁾

Local secretion of IGF-I influences bone formation via osteoblast recruitment to fracture and bone resorbing sites.⁽⁶¹⁾ IGF-I has been shown to reduce osteoblast degradation by inhibiting collagen synthesis.⁽⁵⁸⁾

2) Osteoclasts express IGF-I receptors and IGF-I has been shown to have direct effects on their functions,⁽⁶²⁾ though the effect of IGF-I on osteoclasts is less clear than that of osteoblasts. In vitro, IGF-I induces RANKL synthesis and subsequently osteoclastogenesis, which may explain the stimulatory effects of IGF-I on bone resorption.⁽⁶³⁾ IGF-I may also mediate bone resorption directly or indirectly via osteoblasts as osteoblasts can stimulate the formation of osteoclasts.⁽⁶⁴⁾

Skeletal maturation is often regulated by mechanical forces, but GH and IGF-I exert anabolic forces on cortical and trabecular bone. These anabolic forces on cortical bone occur via periosteal bone apposition.⁽⁶⁵⁾ During the prepubertal stages, IGF-I, along with GH, are key determinants of longitudinal bone growth. IGF-I has been shown to stimulate longitudinal bone growth, dependent of GH, by stimulating the proliferation of chondrocytes, which produce and express IGFIR, in the growth plate.⁽⁶⁶⁾ This newly formed cartilage in the growth plate is infiltrated by blood vessels and becomes trabeculae. Though IGFBPs primarily bind to IGF-I and IGF-II to modulate the cellular effects of IGFs, they do exert IGF-independent effects on bone, having the ability to stimulate bone cell function independently of their interactions with IGFs.⁽⁵³⁾

IGF-I and bone studies

IGF-I secretion and bone mineral accrual follow corresponding patterns during growth. They both increase during puberty and peak in the years surrounding peak height velocity and then decline. The age-related decline in IGF-I is similar to that of bone.

IGF-I is an independent predictor of BMD,⁽⁶⁷⁾ and while IGF-I has been shown to have a direct effect on bone via osteoblasts, studies in adolescents support the hypothesis that IGF-I has an indirect effect on bone via lean mass.^(68,69) IGF-I is routinely a robust predictor of bone mass in early pubertal adolescents, with levels peaking during early puberty.⁽⁶⁸⁾ Matar et al. found positive associations between IGFI and bone parameters in early Tanner stages (1 & 2) in girls, but not boys, at the spine (BMC), femoral neck (area and BMC), and subtotal body (area, BMD, BMC). However, these associations between IGFI and bone mass parameters decreased after adjusting for lean mass, indicating that the effects of IGFI on bone mass may be partly mediated by changes in lean mass. When adjusting for fat mass, Matar et al. saw positive associations between IGF-I and bone parameters that persisted in early Tanner stages in both girls and boys.⁽⁶⁸⁾ In prepubertal females, IGF-I explains a considerable amount of the variance in bone mass accrual at the total body, proximal femur, lumbar spine, and forearm. In fact, IGF-I was a stronger predictor of BMC accrual than serum 25(OH)D.⁽³⁸⁾ Studies comparing IGF-I knockout mice with controls demonstrate that IGF-I is involved in bone mineral accrual.⁽⁷⁰⁾ Models of IGF-I deficient mice demonstrated skeletal malformations, delayed skeletal mineralization, and decreased chondrocyte proliferation and increased apoptosis.⁽⁷¹⁾ When IGF-I was absent from the liver, mice had decreased cortical bone.⁽⁷²⁾ A longitudinal study in prepubertal children revealed that the positive relationship between IGF-I and mid-tibia cortical BMC attenuated after controlling for muscle cross-sectional area.⁽⁷³⁾ In insulin resistant children, IGF-I was positively

associated with BMC/height, but after adjustment for FFST mass, the relationship between IGF-I and BMC/height was nullified.⁽⁶⁹⁾

Race and sex

Sex differences in serum IGF-I concentrations appear to vary according to race. According to NHANES III data in US adults, IGF-I is higher in males versus females. White females have lower IGF-I compared to black females. IGF-I levels are almost the same in white versus black men. In both sexes, blacks have the highest molar ratio of IGF:IGFBP3, or the highest IGF-I levels and lowest IGFBP3 levels. Mexican Americans have the lowest levels of IGF-I compared to whites and blacks.⁽⁷⁴⁾ In multiple studies of healthy children, IGF-I was higher in blacks versus whites.^(75,76) Higgins et al. observed that girls had higher IGF-I and IGFBP3 than boys. Multiple linear regression analysis revealed that the only significant independent predictors of IGF-I were IGFBP3 and African admixture. Neither diet, SES, physical activity, nor adiposity were related to higher IGF-I in blacks. It is proposed that a strong genetic component is responsible for this difference. Proposed explanations include polymorphisms of genes regulating GH/IGF-I system or maturational rate. The disparities could also be due to differences in maturational rate because black children generally enter puberty 1 yr earlier than whites do.⁽⁷⁶⁾

Puberty

IGF-I production increases from birth to puberty, with the highest rate of production occurring during puberty, and then slowly declining throughout adulthood.⁽⁵³⁾ In a study of English children, the timing of the rises in IGF-I during puberty were highly synchronized within individual children, suggesting that IGF-I may play a role in determining the timing of puberty.⁽⁷⁷⁾ In a longitudinal study, IGF-I peaks at Tanner stage 5 were associated with early

pubertal onset.⁽⁷⁸⁾ Throughout puberty, IGF-I measured cross-sectionally increased with age and pubertal stage throughout puberty.^(79,80) Table 2.4 provides reference values for IGF-I (ng/mL) formulated by the Mayo Clinic by age and Tanner Stage.

Table 2.4. Selected Reference Values for IGF-I (ng/mL)

Age	Male	Female
7 years	54-312	44-279
8 years	61-356	51-334
9 years	67-405	61-408
10 years	73-456	73-495
11 years	79-506	88-585
12 years	84-551	104-665
13 years	90-589	120-719
14 years	95-618	136-729
15 years	99-633	147-691
Tanner Stage		
Stage I	81-255	86-323
Stage II	106-432	118-451
Stage III	245-511	258-529
Stage IV	223-578	224-586
Stage V	227-518	188-512

Adapted from Mayo Clinic 2018⁽⁸¹⁾

Measurement

For the present study, serum IGF-I is quantified via human IGF-I Quantikine ELISA (R&D Systems) and has been validated.⁽⁸²⁾ The assay used employed the quantitative sandwich enzyme immunoassay technique. Each microplate was pre-coated with a monoclonal antibody specific for human IGF-I. Samples were pretreated with an acidic dissociation solution, vortexed and incubated at room temperature for 10 minutes, and mixed with a buffering protein with blue dye. Standards (recombinant human IGF-I) and pretreated samples were pipetted into plate wells and any IGF-I present was bound by the immobilized antibody. Unbound substances were washed away and then an enzyme-linked polyclonal antibody specific for human IGF-I was added to the wells. A wash solution of buffered surfactant was applied to remove any remaining unbound antibody-enzyme reagent. Substrate solution, a mix of hydrogen peroxide and tetramethylbenzidine, was then added to the wells and color changed in proportion to the amount of IGF-I bound in the initial step. Color development was stopped using sulfuric acid and the color intensity was read at 450 nm within 30 minutes and wavelength correction was set to 540 nm. A primary concern in IGF-I measurement is the interference of IGFBPs in the assay. Thus, direct measurements in untreated samples provide false values due to the slow dissociation of the IGF-I/IGFBP3 complexes during assay incubation. Different techniques can be applied to physically separate IGF-I from IGFBPs prior to measurement, though remaining binding proteins can still interfere in the assay.⁽⁸³⁾ Further, studies have revealed that IGF-I RIAs do not quantitatively measure serum IGF-I content.⁽⁸⁴⁾ Abellan et al. compared two commercially available RIA kits with different protocols with an ELISA kit (R&D Systems) for total serum IGF-I quantification. Precision and accuracy of all three methods were similar when comparing quality control samples. However, all serum samples for ELISA were above the limit of

quantification in which amounts for the precision and accuracy of quality control samples were adequate. With respect to inter-laboratory variability, the ELISA assay displayed the best global results. Though low efficacy in the dissociation process was observed, the ELISA technique presented the best characteristics of the three immunoassays studied.⁽⁸⁵⁾ Additionally, no specific facilities are needed when using ELISA and the use of hazardous solvents is significantly decreased. To account for the degree of variation in relation to the sample mean, coefficients of variation are reported. The inter-assay CV reflects precision between assays, and the intra-assay CV reflects precision within an assay. The inter- and intra-assay CV were measured for quality control and were 7.5-8.3% and 3.5-4.3%, respectively.

Interactions between IGF-I and 1,25(OH)₂D

Cell and animal studies provide evidence that 1,25(OH)₂D and IGF-I upregulate one another, though the interaction is not fully understood. Kurose et al. reported that 1,25(OH)₂D promotes the action of IGF-I by increasing IGF-I receptors,⁽⁸⁶⁾ and later Bianda et al. observed that IGF-I can also elevate 1,25(OH)₂D concentrations by stimulating the hydroxylation of 25(OH)D to 1,25(OH)₂D (Figure 2.3).⁽⁸⁷⁾ In 7 healthy men, treatment with subcutaneous IGF-I infusion for 5 days was followed by subcutaneous injection of GH. Free 1,25(OH)₂D concentrations increased significantly following IGF-I treatment ($2.2 \pm 5 \times 10^{-5}$ to $2.81 \pm 0.25 \times 10^{-5}$) while they were not significantly changed with the GH injection treatment. In IGF-I knock-out mice, there was a significant decrease in serum 1,25(OH)₂D (24%, $P < 0.05$) vs. wild type controls, and a 70% decrease in renal vitamin D receptor expression.⁽⁸⁸⁾ In another study, IGF-I significantly increased 1 α -hydroxylase expression and serum 1,25(OH)₂D production by acting on renal tubules to convert 25(OH)D to 1,25(OH)₂D, further supporting an interrelationship between the two. Further, the increases observed were dose-dependent. Large increases in

growth hormone were also found to stimulate the production of $1,25(\text{OH})_2\text{D}$ by increasing local IGF-I production in the kidneys.⁽⁸⁹⁾

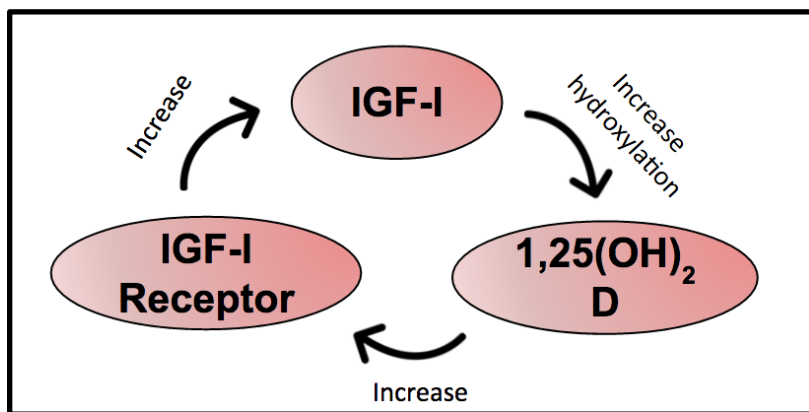


Figure 2.3. Interrelationship between IGF-I and $1,25(\text{OH})_2\text{D}$

Dietary phosphorous has been proposed to contribute to the effects of IGF-I on $1,25(\text{OH})_2\text{D}$ production. To determine whether IGF-I mediated the GH-dependent increase in serum $1,25(\text{OH})_2\text{D}$ induced by dietary phosphorous restriction, hypophysectomized rats were treated with either GH or IGF-I. The rats were fed a normal or low phosphorous diet, and serum $1,25(\text{OH})_2\text{D}$ concentrations were measured. Sham-hypophysectomized rats on restricted phosphorous diets demonstrated 159% increases in $1,25(\text{OH})_2\text{D}$ compared to hypophysectomized rats. In hypophysectomized rats with restriction of dietary phosphorous and GH injections, $1,25(\text{OH})_2\text{D}$ increased 156%. $1,25(\text{OH})_2\text{D}$ increased 185% in hypophysectomized rats with restriction of dietary phosphorous and IGF-I injections. These data suggest that IGF-I is able to restore the increase in serum $1,25(\text{OH})_2\text{D}$ induced by dietary restriction of phosphorous to the same level as GH, and that this GH-dependent increase in $1,25(\text{OH})_2\text{D}$ stimulated by dietary phosphorous restriction is mediated by IGF-I.⁽⁹⁰⁾

Interactions between IGF-I and 25(OH)D

Though data do not yet exist regarding how vitamin D supplementation affects IGF-I concentrations in healthy children, studies conducted in children with growth hormone deficiency (GHD) and rickets, as well as various adult populations, support an interrelationship between the two. In a retrospective study of children and adolescents ages 4-17 yr with GHD, vitamin D deficient subjects had lower IGF-I levels vs. non-deficient subjects, and there was a weak positive association between 25(OH)D and IGF-I in vitamin D deficient ($r = 0.21$, $p < 0.05$) and sufficient ($r = 0.17$, $p < 0.05$) groups.⁽⁹¹⁾ In a prospective study of children with rickets, positive correlations were found between serum 25(OH)D and IGF-I before ($r = 0.60$, $p < 0.001$) and after ($r = 0.59$, $p < 0.001$) vitamin D₃ treatment. Following treatment, the children's growth velocity standard deviation score correlated with the increase of 25(OH)D and IGF-I, which indicates that growth is mediated through the IGF system.⁽⁹²⁾ Likewise, in adults with GHD, vitamin D was associated with increased circulating IGF-I⁽⁹³⁾ and was also shown to mediate a stimulatory effect on 25(OH)D-1 α -hydroxylase by activating bone turnover and increasing calcitriol in healthy adult men.⁽⁸⁷⁾ In adults with metabolic syndrome, IGF-I levels increased with 25(OH)D supplementation (up to 75-85 nmol/L), although high doses of vitamin D supplementation had no effect on IGF-I.^(94,95) No positive association was found between 25(OH)D and IGF-I in severe obese subjects, but they did see that vitamin D supplementation decreased free IGF-I, which is measured by looking at IGF-I-IGFBP3 ratio. However, these findings may not be applicable to normal weight individuals.⁽⁵⁶⁾ Thus, there are inconclusive results on the clinical implications of vitamin D on IGF-I, especially in healthy children, displaying the need for further research on the subject.

Summary

Vitamin D deficiency is prevalent among US adolescents and may affect BMC accrual and growth. There is moderate evidence to support vitamin D supplementation improving bone, while ample evidence suggests that IGF-I is the strongest predictor of BMC accrual. To date, there are only two trials in adolescent populations that addresses the relationship between vitamin D supplementation and IGF-I, one in children with rickets and the other with 95% of adolescent females being vitamin D deficient.^(92,96)

We hypothesized that vitamin D supplementation would significantly increase serum IGF-I and vitamin D metabolites [25(OH)D, 1,25(OH)₂D, iPTH] over 12 weeks. . The findings from this study will increase our knowledge of the interrelationships between vitamin D nutrition and growth factors during adolescents, including understandings of the potential mechanism by which vitamin D supplementation improves bone mineral density. The effect of vitamin D supplementation may be heightened under deficiency conditions. Because our study population is vitamin D sufficient, the results from this study will be more generalizable to healthy children worldwide.

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CHAPTER 3

VITAMIN D SUPPLEMENTATION AND INSULIN-LIKE GROWTH FACTOR I IN

ADOLESCENTS

Rollins EM, Laing EM, Cooper JA, Lewis RD. 2018. To be submitted to the Journal of Bone and Mineral Research.

ABSTRACT

Evidence suggests that vitamin D supplementation increases bone mineralization in youth, possibly indirectly through effects on muscle mass. Insulin-like growth factor I (IGF-I) is a key determinant of muscle and bone mineral accrual during growth, and one potential mechanism for vitamin D effects on bone is hypothesized to be through effects on IGF-I. Cell culture and animal studies provide evidence for an interaction between vitamin D and IGF-I, though studies in humans, specifically vitamin D sufficient populations, regarding the effects of vitamin D supplementation on IGF-I are lacking. Therefore, the aim of this study was to explore the effects of 12 weeks of vitamin D supplementation on serum IGF-I and relationships with serum vitamin D metabolites in healthy, vitamin D sufficient children. Participants included black and white boys and girls, 9-13 years of age at baseline from a former vitamin D dose response RCT. Serum 25(OH)D and 1,25(OH)₂D were measured using radioimmunoassay (RIA). Serum iPTH was measured via immunoradiometric (IRMA) assay. Serum IGF-I was measured at baseline and 12 weeks using quantitative sandwich immunoassay technique. Nonsignificant Pearson correlations between changes in IGF-I and changes in vitamin D metabolites (25(OH)D (P=0.48), 1,25(OH)₂D (P=0.81), iPTH (P=0.47)) were observed. The relationships between serum IGF-I, 25(OH)D, 1,25(OH)₂D, and iPTH were examined using nonlinear regression analysis while including age, race, sexual maturation, and sex as covariates. We observed a significant interaction between sex and change in serum 1,25(OH)₂D concentrations over 12 weeks on change in serum IGF-I concentrations (P=0.04). After probing the sex interaction, both males and females were non-significant (t=-1.9, P=0.05; t=1.7, P=0.07). Our data indicate that serum IGF-I is not significantly affected by vitamin D supplementation over 12 weeks in healthy,

vitamin D sufficient adolescents. However, the sex and $1,25(\text{OH})_2\text{D}$ interactions in response to vitamin D supplementation should be further explored.

KEY WORDS: INSULIN-LIKE GROWTH FACTOR I (IGF-I); VITAMIN D; GROWTH; CHILDREN

INTRODUCTION

In a recent evidence-based review, it was reported that there was moderate evidence for vitamin D supplementation to increase bone mineralization in youth,⁽¹⁾ possibly through vitamin D effects on muscle mass.⁽²⁾ One possible mechanism for vitamin D effects on bone is hypothesized to be through effects on insulin-like growth factor I (IGF-I). Serum IGF-I promotes increases in muscle mass and stimulates bone mineralization in adolescents, particularly specific to the periosteal surface of cortical bone.^(3,4) A recent cross-sectional study in children 9-13 years of age demonstrated a direct effect of IGF-I on cortical bone that was partially mediated by fat-free soft tissue (FFST).⁽⁵⁾

There has been increasing interest recently in the relationships between vitamin D and IGF-I. Cell and animal studies have provided evidence that 1,25(OH)₂D and IGF-I upregulate one another, such that 1,25(OH)₂D promotes the action of IGF-I by increasing IGF-I receptors and IGF-I may also elevate 1,25(OH)₂D concentrations by stimulating the hydroxylation of 25(OH)D to 1,25(OH)₂D.^(6,7,8) Additional in vitro studies reveal that IGF-I significantly increased 1 α -hydroxylase expression and serum 1,25(OH)₂D production by acting on renal tubules to convert 25(OH)D to 1,25(OH)₂D, further supporting interrelationships between the two.^(9,10)

Despite the findings in cell culture and animal models, studies in children and adults report conflicting results regarding the effects of vitamin D on IGF-I. Inconsistencies in human trials relate in part to the age of participants and vitamin D or IGF-I status,^(11,12) key factors to consider when evaluating the effects of vitamin D supplementation on IGF-I. Cross-sectional and vitamin D supplementation trials in adults report that those with the greatest increase in IGF-I had a higher prevalence of vitamin D deficiency.^(11,12) Results from cross-sectional studies in

children likely vary depending upon the health status of the children. In apparently healthy Lebanese boys and girls in which 56.2% were vitamin D insufficient, no significant correlations were found between 25(OH)D and IGF-I.⁽¹³⁾ In contrast, positive associations were observed between 25(OH)D and IGF-I in children with GHD⁽¹⁴⁾ and rickets.⁽¹⁵⁾ The only other trial exploring the vitamin D/IGF-I relationship was in Indian girls who, despite being described as apparently healthy, were classified as having mild-to-severe vitamin D deficiency prior to the intervention (≤ 5 to ≤ 20 ng/mL). Following the intervention, all girls achieved vitamin D sufficiency and serum IGF-I and IGFBP-3 levels significantly increased after supplementation, though the increase was greatest in girls with moderate-to-severe vitamin D deficiency.⁽¹⁶⁾

Despite these findings, race and sex differences regarding the effects of vitamin D supplementation on IGF-I have yet to be determined. For example, black children, on average, have higher levels of IGF-I compared to white children. Thus, vitamin D supplementation may have less of an effect on serum IGF-I in black children as IGF-I concentrations are already elevated. Lower serum 25(OH)D⁽¹⁷⁾ and higher IGF-I levels in black children may also allow for a greater opportunity for IGF-I to mediate the effects of vitamin D on bone, possibly by increasing the hydroxylation of 25(OH)D to 1,25(OH)₂D. Further, it is imperative to determine the effects of vitamin D supplementation on IGF-I in vitamin D sufficient children, as the significant correlations and increases in IGF-I with vitamin D supplementation shown in previous studies may be attributable to a greater room for increase in those who are vitamin D insufficient or deficient. In addition, the majority of studies only report 25(OH)D, not 1,25(OH)₂D, the active form of the vitamin.

Therefore, the aim of this project was to explore the effects of 12 weeks of vitamin D supplementation on serum IGF-I and relationships with serum vitamin D metabolites in

apparently healthy, vitamin D sufficient children, male and female, black and white, ages 9-13 years. We hypothesized that vitamin D supplementation would be positively associated with changes in serum IGF-I and vitamin D metabolites [25(OH)D, 1,25(OH)₂D, iPTH] over 12 weeks.

MATERIALS AND METHODS

Participants and Study Design

This ancillary study included baseline and 12-week data from black and white, male and female, adolescents ages 9-13 yr ($N = 323$) who participated in a 12-week RCT, the GAPI (University of Georgia, Purdue University, and Indiana University) trial.⁽¹⁸⁾ Children were enrolled during the winter months (October-December) of 2009-2011 at US northern latitudes 34° N and 40° N when vitamin D concentrations were at their nadir.

Participant inclusion criteria for the parent study included being non-Hispanic white and/or non-Hispanic black between the ages of 9-13 yr and in sexual maturation stages 2 and 3 for genitalia or breast development as described by Tanner.⁽¹⁹⁾ Exclusion criteria included menarche (in females), growth disorders, and diseases and/or medications known to influence bone metabolism.

In the parent study, participants were assigned via block randomization to receive vitamin D3 tablets containing either 0 (placebo), 400, 1000, 2000, or 4000 IU/d (Douglas Laboratories) daily for 12 weeks. Compliance was measured by pill count and defined as consuming $\geq 80\%$ pills given. Details related to compliance have been previously published.⁽¹⁸⁾ Adverse event information was gathered using an interviewer-administered questionnaire. Additional participant and study design details can be found in the original RCT.⁽¹⁸⁾

Power analysis was estimated using G*Power (Version 3.1.9.2; alpha = 0.05, power = 80%, effect size = 0.5, <http://www.gpower.hhu.de/en.html>). Data from a study that assessed changes in serum IGF-I with vitamin D supplementation in adult men was used for estimations.⁽¹¹⁾ A sample size of n=128 would allow for detection of changes in IGF-I with vitamin D supplementation over 12 weeks.

Anthropometry

Anthropometric measurements included weight (nearest 0.1 kg) using an electronic scale and height (nearest 0.1 cm) using a wall-mounted stadiometer at baseline and 12 weeks. Single-measure intraclass correlations and test-retest coefficients of variation (CV) were computed among females (n = 10) 6-10 yr and measured by the same individual twice for 2 wk, for height (0.99% and 0.4%) and weight (0.99% and 1.4%). Body mass index-for-age percentiles were also calculated.

Biochemical Assays

Fasting serum samples were collected at baseline and 12 weeks, which were stored at -80°C until analysis. Serum 25(OH)D was assessed using a two-step radioimmunoassay (RIA; Diasorin Labs). The inter- and intra-assay coefficients of variation (CV) ranged from 5.6-8.4% and 5.5-7.0%, respectively. Analyses were repeated when duplicate samples differed by $\geq 10\%$. Reliability of the assays was further monitored through the Vitamin D External Quality Assessment Scheme. Serum 1,25(OH)₂D was measured using a two-step radioimmunoassay (RIA; Diasorin Labs). The inter- and intra-assay CV were 12.4-17.6% and 11.6%, respectively. Serum iPTH was measured using an immunoradiometric assay (Diasorin). The inter- and intra-assay CV were 4.8-6.9% and 2.3-5.7%, respectively. Baseline and 12-week serum IGF-I samples were analyzed utilizing the same procedure: serum IGF-I samples were pretreated with an acidic

solution to stimulate dissociation, treated with a buffered protein containing blue dye and preservatives, and lyophilized to release IGF-I bound to IGF binding proteins. IGF-I concentrations were then quantified in duplicate using a quantitative sandwich immunoassay technique with recombinant human IGF-I (R&D Systems). The inter- and intra-assay CV were measured for quality control and were 7.5-8.3% and 3.5-4.3%, respectively. While it is preferable from a quality control standpoint to run the baseline and 12-week samples simultaneously, this did not occur. However, in a subsample of participants (n=40), baseline and 12-week samples were analyzed simultaneously. The baseline sample values were similar to previously run samples, while there was an average 22.7% increase in the 12-week IGF-I samples analyzed together with 20 baseline samples on the same kit. Therefore, all serum IGF-I at 12 weeks was adjusted upward by 22.7% based on the subsample analysis.

Statistical Analyses

Data were examined for outliers and non-normal distributions. Descriptive statistics were expressed as mean, standard deviation, and percent change from baseline to 12 weeks. Participants were excluded from analyses if they were missing 12-week serum IGF-I values. Paired t-tests were conducted to compare variables at baseline and 12 weeks. Because the aim of this study was to determine the relationship between serum vitamin D metabolites and serum IGF-I, the effect of dose of vitamin D supplementation on IGF-I was not examined. Pearson correlations were used to examine a subgroup analysis of 30 subjects with the greatest (n= 15) and least (n= 15) changes in 25(OH)D with changes in IGF-I while adjusting for race, sex, and sexual maturation rating stage. The relationships between changes in IGF-I and changes in vitamin D metabolites [25(OH)D, 1,25(OH)₂D, iPTH] were examined using bivariate Pearson correlations and nonlinear regression analysis while including age, race, sexual maturation, and

sex as covariates. After observing a significant interaction between sex and change in serum 1,25(OH)₂D concentrations over 12 weeks on change in serum IGF-I concentrations, the interaction was probed using multiple regression within each sex. These analyses included race, age, and sexual maturation as covariates. Statistical analyses were performed using SPSS (see above). P values <0.05 were considered statistically significant.

RESULTS

Descriptive participant characteristics are presented in Table 3.1. There were significant increases from baseline to 12 weeks for all variables except body fat percent ($P = 0.31$). Serum 25(OH)D, 1,25(OH)₂D, and IGF-I increased over 12 weeks (all $P < 0.01$), while iPTH did not change ($P = 0.98$).

A subgroup analysis was conducted to compare changes in IGF-I in those with the greatest changes in 25(OH)D ($n = 30$) versus those with the least changes in 25(OH)D ($n = 30$). Changes in IGF-I between groups were not significant. Correlations between changes in IGF-I and changes in 25(OH)D, controlling for race, sex, and sexual maturation rating stage, were not significant for those participants with large changes in 25(OH)D ($r = 0.31$, $P = 0.33$) or those with little changes ($r = 0.02$, $P = 0.95$).

Bivariate Pearson correlations between the changes in IGF-I and changes in vitamin D metabolites (25(OH)D, 1,25(OH)₂D, iPTH) over 12 weeks were not significant, even when controlling for race, sex, and sexual maturation rating stage [25(OH)D ($r = -0.04$, $P = 0.48$), 1,25(OH)₂D ($r = -0.02$, $P = 0.81$), iPTH ($r = -0.040$, $P = 0.47$)].

In the regression analysis, changes in 25(OH)D were not related with changes in IGF-I when controlling for race, sexual maturation rating stage, and age ($P = 0.32$; Table 3.2). There was a significant interaction between sex and change in serum 1,25(OH)₂D concentrations over 12

weeks on changes in serum IGF-I concentrations when controlling for race, sexual maturation rating stage, and age ($P=0.04$; Table 3.3). In females ($P=0.075$), change in $1,25(\text{OH})_2\text{D}$ was positively associated with change in IGF-I, whereas in males ($P=0.051$), change in $1,25(\text{OH})_2\text{D}$ was negatively associated with change in IGF-I. (Figure 3.1). No significant changes in iPTH with changes in IGF-I were observed ($P=0.78$; Table 3.4).

DISCUSSION

A recent evidenced-based review reported moderate evidence existed that vitamin D supplementation increases bone mineral accrual in children and adolescents.⁽¹⁾ Though it is unclear as to the mechanism behind the effects of vitamin D on adolescent bone, one RCT suggests the vitamin D effect could be moderated by fat-free soft tissue,⁽²⁾ and possibly linked to increases in insulin-like growth factor I (IGF-I).⁽²⁰⁾ The purpose of this ancillary analysis was to determine the effects of 12 weeks of vitamin D supplementation on serum IGF-I and relationships with serum vitamin D metabolites in children entering the early stages of puberty. The main finding of this study was that vitamin D supplementation did not alter serum IGF-I concentrations despite significant increases in $25(\text{OH})\text{D}$. A significant interaction existed, however, between sex and changes in serum $1,25(\text{OH})_2\text{D}$ concentrations on change in serum IGF-I concentrations over 12 weeks when controlling for age, race, and sexual maturation rating stage.

The parent study⁽¹⁸⁾ demonstrated large increases in $25(\text{OH})\text{D}$, the clinical marker of vitamin D status, with vitamin D supplementation ranging from 400 to 4000 IU/day. Contrary to the hypothesis, no significant correlations or interactions were observed between changes in $25(\text{OH})\text{D}$ and changes in IGF-I. Intervention studies exploring the effects of vitamin D supplementation on serum IGF-I in children and adults report conflicting results. Following

vitamin D supplementation, changes in IGF-I were found to be positively associated with 25(OH)D changes in children with GHD,⁽¹⁴⁾ rickets,^(15,21) and in adults with vitamin D deficiency,⁽¹²⁾ GHD,⁽¹¹⁾ and metabolic syndrome.⁽²²⁾ In contrast, no significant findings were reported in trials in children with vitamin D deficiency,^(16,13) hypertensive adults with vitamin D insufficiency,⁽²³⁾ prediabetes and hypovitaminosis D,⁽²⁴⁾ or adults who were overweight and obese.⁽²⁵⁾ Various study designs and population characteristics, specifically vitamin D status at baseline, may account for these differences. One explanation for our null findings with 25(OH)D and IGF-I is likely due to baseline vitamin D status. The majority of patients in studies reporting significant correlations between 25(OH)D and IGF-I were in vitamin D insufficient, deficient, or growth stunted populations, while our study participants had sufficient vitamin D status and had normal anthropometric measures. In general, vitamin D supplementation trials have demonstrated that when vitamin D status is lower at baseline, a greater response to supplementation is observed.⁽¹⁾

With respect to IGF-I, one hypothesis is that VDD creates an environment for GH/IGF resistance, leading to an increase in serum IGF-I in individuals with severe VDD following vitamin D supplementation.^(13,16) As the ligand activated vitamin D receptor is known to negatively regulate growth factors in the presence of insulin-like growth factor binding protein 3 (IGFBP3), low serum 25(OH)D may up-regulate the production of growth factors in the presence of IGFBP3.⁽²⁶⁾ Again, our subjects were healthy and had serum 25(OH)D levels approaching 70nmol/l, much higher than the Institute of Medicine (IOM) cutoff of 50 nmol/l.

This study was the first to report changes in 1,25(OH)₂D, the active form of the vitamin, when assessing the effects of vitamin D supplementation on IGF-I in adolescents. The most notable finding in the current study was the significant interaction between changes in serum

1,25(OH)₂D and sex predicting IGF-I concentrations over 12 weeks. When probed, the significant interaction approached significance for each group and interestingly, in females, as 1,25(OH)₂D increased, IGF-I increased ($t = 1.79$, $P = 0.075$), while the opposite trend was observed in males ($t = -1.97$, $P = 0.051$). The strength of the interaction was likely attenuated by the smaller sample sizes in each sex group. One possible explanation for the sex differences are sex-related differences in intestinal calcium transport mediated by vitamin D,⁽²⁷⁾ but in the parent study, we did not see any sex-related differences in calcium absorption.⁽²⁸⁾ Potential sex differences may be more likely due to estrogen potentiating the effect of IGF-I. Prepubescent girls have higher levels of estrogen than prepubescent boys, heightening during puberty. In animal models, estrogen has been shown to upregulate IGF-I gene expression,⁽²⁹⁾ though the exact relationships, specifically in children, between estrogen and IGF-I are still unclear.

It has been hypothesized that 1,25(OH)₂D up-regulates IGF-I translation and synthesis and IGF-I in turn can increase the hydroxylation of 25(OH)D to 1,25(OH)₂D.^(6,7) However, this hypothesis is not compatible with the inverse relationship between IGF-I and 25(OH)D we observed in males. It is also possible that IGF-I upregulates renal 1- α -hydroxylase, but has no effect on 25(OH)D.^(9,23,30) Trummer et al. observed a positive correlation between IGF-I and 1,25(OH)₂D, the product of 1- α -hydroxylase, but no significant correlation between 25(OH)D and IGF-I.⁽²³⁾ Thus, because the participants in the current study were vitamin D sufficient, they may not have had a physiological need for increased IGF-I production with vitamin D supplementation although 25(OH)D and 1,25(OH)₂D increased over 12 weeks.

Only one vitamin D intervention in adult males reported 1,25(OH)₂D and like the current study, they reported no significant effects of vitamin D supplementation on IGF-I concentrations, despite significant increases in 1,25(OH)₂D.⁽²³⁾ However, only males were studied and the

investigators were unable to explore sex differences. There are no other studies that have examined the effects of vitamin D supplementation on IGF-I in children and adolescents accounting for differences in sex. When evaluating iPTH, an important regulator of vitamin D metabolism, we did not observe significant correlations or interactions between changes in iPTH and changes in IGF-I, suggesting no significant relationship between iPTH and IGF-I after 12 weeks of vitamin D supplementation.

There are some limitations to this study. First of all, our baseline IGF-I samples were assayed separately from 12-week samples. While we not did conduct participant assays for each time-point at the same time, quality control assays were conducted on a subset of participants. We did not assess serum IGFBP3. In circulation, 75% of IGF-I is bound to IGFBP3, and the molar ratio of IGFI:IGFBP3 is thought to reflect the free, active form of IGF-I.⁽⁵⁴⁾ Additionally, IGF-I concentrations increase dramatically during pubertal growth and because this was an ancillary study, we only utilized sexual maturation ratings (SMR) as our measure of sexual maturation. Although we controlled for SMR stages and utilized a validated self-administered questionnaire, the SMR stages are categorical and do not provide information with respect to when a child is entering or leaving a particular stage. The use of skeletal age measures using hand-wrist radiographs would have been a preferable methodology to account for sexual maturation. Increases in serum IGF-I, while significant, approached only 1%. Inclusion of participants closer to their peak height growth may provide a sample that is likely to experience greater changes in IGF-I. Nonetheless, unlike other trials, this ancillary study was the first vitamin D supplementation trial to explore the relationships between vitamin D metabolites and IGF-I in apparently healthy, male and female and black and white, vitamin D sufficient children.

In conclusion, no significant correlations were observed between vitamin D metabolites (25(OH)D, 1,25(OH)₂D, and iPTH) and IGF-I in response to supplementation. However, a significant interaction between sex and changes in serum 1,25(OH)₂D concentrations on changes in serum IGF-I concentrations over 12 weeks was noted. It is unclear why the sex by 1,25(OH)₂D interactions occurred and their importance to pubertal growth and bone strength, but primary studies that account for biological age measures, examine sex steroids in both males and females, and include measures of IGFBPs in response to vitamin D supplementation should help clarify these interactions. Moreover, inclusion of both vitamin D sufficient and deficient children and adolescents may improve the ability to detect the responses to vitamin D supplementation based on physiological needs.

Table 3.1. Descriptive Participant Characteristics

	Baseline			12 weeks				
	N*	Mean	SD	N*	Mean	SD	%Change	Sig.
Age, years	272	11.3	1.2					
Race (white/black), n	272	126/146	-					
Sex (male/female), n	272	135/137	-					
Sexual maturation stage (2/3), n	272	149/123	-					
Body Composition								
Lean mass, kg	270	31.2	6.7	269	32.3	6.9	3.4%	<0.01
Fat mass, kg	270	14.7	7.8	269	15.3	7.9	3.80%	<0.01
% body fat	270	29.8	9.6	269	29.9	9.6	0.3%	0.13
Anthropometry								
Weight, kg	272	47.6	12.1	272	49.2	12.3	3.0%	<0.01
Height, cm	272	150.7	9.1	272	152.3	9.3	1.0%	<0.01
BMI, kg/m ²	272	20.8	4.4	272	21.1	4.3	1.0%	0.02
BMI for age, %	272	68.9	28.7	272	71.1	27.0	4.9%	<0.01
Biochemical								
25(OH)D, nmol/L	272	68.8	18.2	272	92.9	40.1	17.1%	<0.01
1,25(OH) ₂ D, pmol/L	272	133.7	40.2	272	156.9	45.9	11.3%	<0.01
iPTH, pg/mL	272	27.8	10.9	272	27.7	10.6	-5.4%	0.88
IGF-I, ng/mL	272	234.0	99.5	272	242.1	90.9	1.0%	0.04

*Only participants with 12-week IGF-I values were included in analysis

BMI, body mass index; IGF-I, Insulin-like growth factor I; iPTH, intact parathyroid hormone; Body composition measures assessed by DXA

Table 3.2. Predictors of change in 25(OH)D

	<i>B</i> ¹	<i>SE B</i>	β	<i>p</i>
(Constant)	43.79	44.31	-	0.98
Δ 25(OH)D	0.84	0.83	-0.19	0.31
Race	-3.93	8.12	-0.03	0.63
Sexual maturation rating stage	-6.61	8.38	-0.05	0.43
Baseline age	-2.59	3.90	-0.05	0.51
Sex ² x Δ 25(OH)D	-0.53	0.53	-0.19	0.32
Sex ²	10.58	10.64	0.08	0.32

¹Unstandardized *B*²Males were coded as 2, and females were coded as 1

Table 3.3. Predictors of change in 1,25(OH)₂D

	<i>B</i> ¹	<i>SE B</i>	β	<i>p</i>
(Constant)	35.23	43.99	-	0.42
Δ 1,25(OH) ₂ D	1.41	0.71	0.39	0.049
Race	-4.09	8.07	-0.031	0.61
Sexual maturation rating stage	-5.89	8.31	-0.45	0.48
Baseline age	-2.29	3.91	-0.43	0.56
Sex ² x Δ 1,25(OH) ₂ D	-0.89	0.44	-0.41	0.045
Sex ²	13.48	10.04	0.10	0.18

¹Unstandardized *B*²Males were coded as 2, and females were coded as 1

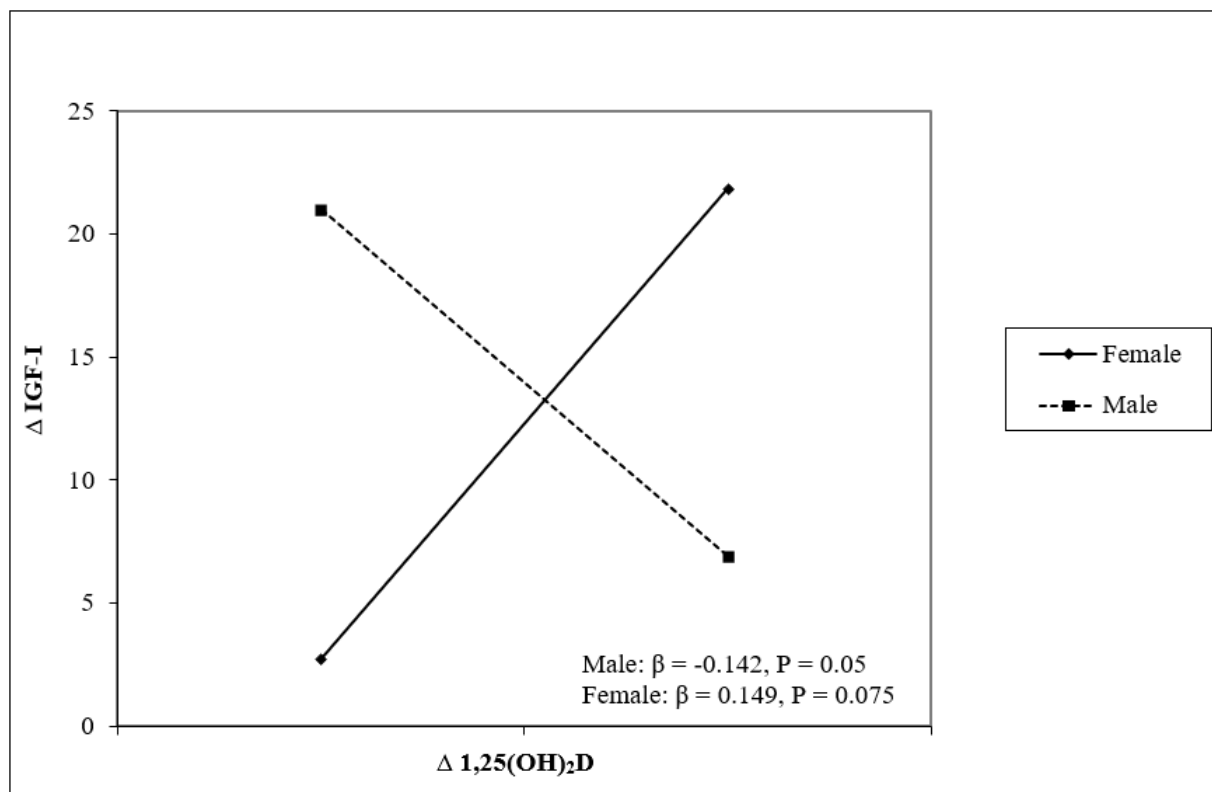


Figure 3.1. Nonlinear regression analysis shows significant interaction between sex and changes in serum 1,25(OH)₂D concentrations over 12 weeks on change in serum IGF-I concentrations.

Table 3.4. Predictors of change in iPTH

	<i>B</i> ¹	<i>SE B</i>	β	<i>p</i>
(Constant)	50.17	43.59	-	0.25
Δ iPTH	-0.34	1.24	-0.05	0.79
Race	-3.58	8.12	-0.03	0.66
Sexual maturation rating stage	-6.69	8.35	-0.05	0.42
Baseline age	-2.44	3.91	-0.05	0.53
Sex ² x Δ iPTH	0.14	0.81	0.03	0.87
Sex ²	5.34	9.22	0.04	0.56

¹Unstandardized *B*²Males were coded as 2, and females were coded as 1

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Adolescence is characterized as a period of rapid bone mineral accrual⁽¹⁾ though it remains unknown whether there is an interaction of nutrition and hormones that could potentially influence the bone accrual process. Moderate evidence provides support for vitamin D supplementation increasing bone mineralization in youth,⁽²⁾ perhaps indirectly by effects on muscle mass.⁽³⁾ IGF-I is an important determinant of bone mineral accrual, both directly^(4,5) and indirectly via skeletal muscle,^(6,7) during adolescent growth. Of current interest is how IGF-I might mediate the effects of vitamin D on bone, as these two have known independent, but possibly synergistic, effects on bone. Though cell culture and animal studies provide evidence for an interaction between vitamin D and IGF-I,^(8,9) studies in humans, specifically vitamin D sufficient populations, regarding the effects of vitamin D supplementation on IGF-I are lacking.

This ancillary project sought to determine the effects of 12 weeks of vitamin D supplementation on serum IGF-I and relationships with serum vitamin D metabolites in children entering the early stages of puberty. In our study population of healthy, vitamin D sufficient adolescents ages 9-13 years of age, vitamin D supplementation did not alter serum IGF-I concentrations, despite significant increases in 25(OH)D. Contrary to previous studies,⁽¹⁰⁻¹⁵⁾ our results do not suggest that vitamin D supplementation significantly increases IGF-I. Our contrasting results are likely due to the fact that the majority of patients in studies reporting significant correlations between 25(OH)D and IGF-I were in vitamin D insufficient, deficient, or

growth stunted populations.^(10,16) In general, vitamin D supplementation trials have demonstrated that lower vitamin D status at baseline lends to a greater response to supplementation.⁽²⁾

This study was the first to include measurements of 1,25(OH)₂D, the active form responsible for the actions of vitamin D, when evaluating the effects of vitamin D supplementation on IGF-I in adolescents. Despite initial null findings, a significant interaction existed between sex and changes in serum 1,25(OH)₂D concentrations on change in serum IGF-I concentrations over 12 weeks when controlling for age, race, and sexual maturation rating stage. Interestingly, in females, as 1,25(OH)₂D increased, IGF-I increased ($t=1.79$, $P=0.075$), while the opposite trend was observed in males ($t=-1.97$, $P=.051$). The interaction would have likely been stronger if the sample size in each sex group was larger. Only one vitamin D supplementation trial reported 1,25(OH)₂D.⁽¹⁷⁾ Like the current study, they reported no significant effects of vitamin D supplementation on IGF-I concentrations, but only males were included in the study, excluding any potential for evaluating sex interactions in the 1,25(OH)₂D-IGF-I responses to vitamin D supplementation and IGF-I. The sex by 1,25(OH)₂D interactions that occurred in the current study may be due to circulating concentrations of estrogen in females compared to males. Prepubescent girls have higher levels of estrogen than prepubescent boys, heightening during puberty. In animal models, estrogen has been shown to upregulate IGF-I gene expression,⁽¹⁸⁾ though the exact relationships, specifically in children, between estrogen and IGF-I are still unclear.

When evaluating iPTH, an important regulator of vitamin D metabolism, we did not observe significant correlations or interactions between changes in iPTH and changes in IGF-I, suggesting no significant relationship between iPTH and IGF-I after 12 weeks of vitamin D supplementation.

Overall, the findings from this thesis provide evidence that vitamin D supplementation does not significantly increase IGF-I in 12 weeks in a healthy, vitamin D sufficient adolescent population. Further, our data does not support a significant relationship between IGF-I and vitamin D metabolites (25(OH)D, 1,25(OH)₂D, iPTH) in response to vitamin D supplementation. Finally, our findings do not provide evidence supporting the hypothesis that effects of IGF-I on bone are moderated by vitamin D in healthy, vitamin D sufficient adolescents. However, our data do support a significant interaction between sex and changes in 1,25(OH)₂D on changes in IGF-I over 12 weeks, though it is unclear as to why these sex by 1,25(OH)₂D interactions occurred and their subsequent importance to pubertal growth and bone strength.

As with any secondary analysis, the available data was not initially collected to address the current research question. Perhaps if the trial was longer than 12 weeks, we might have observed greater changes in IGF-I in relation to vitamin D metabolites. Utilizing skeletal age measures, such as hand-wrist radiographs, would provide a more accurate depiction of sexual maturation and would have been a preferred methodology. Additionally, our baseline IGF-I samples were assayed separately from 12-week samples. While we not did conduct participant assays for each time-point at the same time, quality control assays were conducted on a subset of participants. In the future, it would be important to include IGFBP3 measurement, as the ratio of IGFI:IGFBP3 is thought to best reflect free IGF-I.⁽¹⁹⁾

In conclusion, this ancillary study was the first vitamin D trial to assess vitamin D metabolites and IGF-I in apparently healthy, vitamin D sufficient children. This study included both females and males, blacks and whites, unlike previous trials. Though no significant correlations between vitamin D metabolites [25(OH)D, 1,25(OH)₂D, and iPTH] and IGF-I in response to vitamin D supplementation were observed, we did see a significant interaction

between sex and changes in serum $1,25(\text{OH})_2\text{D}$ concentrations on changes in serum IGF-I concentrations over 12 weeks. Why the sex by $1,25(\text{OH})_2\text{D}$ interactions occurred and their importance to pubertal growth and bone strength is uncertain, but primary studies that account for biological age measures, examine sex steroids in both males and females, and include measures of IGFBPs in response to vitamin D supplementation should help clarify these interactions.

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